Survival of Shiga Toxin-producing *Escherichia coli* and *Salmonella* serotypes in the feces of five animal species.

**THESIS**

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Abstract

Foodborne pathogens result in over 47 million cases of disease, 127,000 hospitalizations and 3,000 deaths annually in the United States. *Salmonella* and Shiga-Toxin producing *Escherichia coli* (STEC) are two important foodborne pathogens. In addition to the disease burden, the economic burden of foodborne disease can also be quite profound with estimates reaching almost 35 billion dollars. Historically, most foodborne disease outbreaks have been associated with animal products, however the incidence of foodborne outbreaks and disease cases associated with vegetable produce has shown an upward trend. Animals are natural reservoirs for *Salmonella* and STEC and they shed the organisms when they defecate. These animals act as a source of vegetable produce contamination via direct deposition of feces into vegetable fields or when improperly treated manure is applied or via water contamination. In an effort to reduce the incidence of vegetable-borne disease, producers are developing guidelines regulating the time required between a fecal contamination event and the harvesting of produce.

The first aim of our study was to identify a suitable mechanism for labeling *Escherichia coli* O157:H7 for use in long-time survival studies. Two labeling mechanisms were evaluated in this study (1) plasmid encoded green fluorescent proteins (GFP) and (2) chromosomal labeling. The use of plasmid encoded green fluorescent protein was not suitable for survival studies persisting over 9 days since the fluorescing cell population did not accurately reflect the actual cell population. Chromosomal labeling of *Escherichia coli* O157:H7 was more suited for these studies.

The second aim of our study was to determine the survival of foodborne disease associated serotypes of *Salmonella* and Shiga Toxin producing *Escherichia coli* in the feces of cattle, deer, feral pig, raccoon and water fowl. These animals were selected since they can be found in close proximity to many farms in Ohio. The STEC and *Salmonella* inocula had the shortest Decimal Reduction Time (DRT) in raccoon feces. The longest DRT was for the *Salmonella* inoculated water fowl feces. The suggested no-harvest interval of 194 days for cattle feces inoculated with STEC was the longest of the animals’ feces.
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Chapter 1 – Literature Review

Animal Reservoirs of Shiga-Toxin producing *Escherichia coli*
The role of Animal Reservoirs of STEC in Foodborne Disease

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Abstract

Shiga Toxigenic *Escherichia coli* (STEC) are zoonotic pathogens which reside as part of the normal intestinal flora of many warm blooded animals. Humans are infected via the fecal-oral route, with foodborne and waterborne transmission being the main sources of infection. People can also be infected via direct animal contact or contact with other infected individuals. Most human infections are foodborne, with contaminated meat and vegetables being the main source of infection. Though asymptomatic in most animals, STEC presents a wide spectrum of clinical signs in humans ranging from asymptomatic infection, to hemorrhagic colitis, hemolytic uremic syndrome and death. The natural reservoir for STEC is ruminants, with cattle recognized as being the main reservoir; however depending on geographical location the species of importance may vary. Many other livestock species including horses, pigs, bison and water buffalo have also been implicated as reservoirs of STEC. Wild birds and animals pose a unique risk due to their ability to traverse large distances, thus disseminating STEC in various environments. Domestic pets have also tested positive for STEC, and given the close interaction between humans and their pets, it is possible that pathogen transfer occurs between the species. This review discusses the main animal reservoirs implicated in the transmission of STEC and highlights the various sources of human infection. Identification of these reservoirs as well as understanding the dynamics of human infection is essential for the development of control strategies to reduce STEC infection in the human population.
**Introduction**

In the United States, Shiga Toxin-producing *Escherichia coli* (STEC) is estimated to cause in excess of 265,000 cases of illness per year resulting in over 3,600 hospitalizations and at least 30 deaths annually [1]. STEC was first identified as a foodborne pathogen in 1982 when it was associated with an outbreak of hemolytic uremic syndrome following consumption of undercooked hamburger meat [2]. STEC is part of the gastrointestinal flora of many animals. Ruminants are identified as the natural reservoir for STEC with cattle being the primary animal reservoir identified [3]. However many other animal species have been implicated as shedders or vectors including horses, pigs, dogs, cats, rabbits, wildlife, fish and flies [4-7]. Most of these animals can be asymptotically infected with STEC, and thus are able to shed the organism without detection.

There are over 400 different STEC serotypes [8], but most have not been associated with human disease. The most pathogenic serotype for humans is O157:H7 and accounts for 75% of all STEC infections worldwide; however, due to increased awareness and improved diagnostic techniques, there is an increasing incidence of non-O157 STEC infections. The Center for Disease Control (CDC) in 2011, estimated that two-thirds of the STEC cases in the United States were caused by non-O157 STEC serotypes. In the United States, the main non-O157 STEC serotypes implicated in human disease are O111, O26, O121, O103, O145 and O45. Globally, the serotype of importance may vary according to climatic and other environmental factors.
Infections with STEC result in a wide spectrum of clinical signs ranging from asymptomatic infections to severe clinical syndromes such as hemorrhagic colitis and possibly hemolytic uremic syndrome and and/or thrombotic thrombocytopenic purpura in approximately seven percent of the cases. Old persons, young children and immune-compromised persons are at greatest risk. STEC serotypes associated with human disease are classified as Enterohemorrhagic *Escherichia coli* (EHEC) based on the clinical signs they cause, thus STEC infections in humans are also termed EHEC [9].

**STEC Transmission to Humans**

STEC transmission is via the fecal-oral route and most infections are a result of consumption of contaminated products. Humans are infected with EHEC via three methods: (a) direct contact with animals and/or their environment, (b) consumption of contaminated food or water and (c) from person to person [3] (Figure 1). The infectious dose for humans is estimated to be less than 100 cells [10]. Contaminated food and water sources account for the majority of human STEC infections [11]. Investigation of 90 confirmed STEC O157 outbreaks which occurred between 1982 and 2006 revealed that food products were identified as the origin for 54% of the infections. Water and environmental sources accounted for approximately 10%, while direct animal contact only accounted for 8% of infections. Person to person transmission was reported to account for 20% of the outbreaks. [12]

Food is contaminated when it comes in contact with the feces of animals shedding the bacteria. In the United States, historically, undercooked hamburgers have been the major source of foodborne STEC human infections. During the period 1982 – 2002,
there were 182 STEC outbreaks attributed to food sources. Ground Beef was estimated to cause 41% of the cases and vegetable produce 21% [13]. From 1998 – 2008, there were 106 foodborne outbreaks of STEC where beef was identified as the origin of infection [14]. There has however been a decreasing trend in the number of outbreaks associated with ground beef due to increased abattoir sanitation, improved slaughter practices and increased consumer education. Other food products implicated as sources of STEC outbreaks are dairy products, ready to eat sausages, unpasteurized apple cider and fruit juices.

Interestingly though, the second most common source of foodborne STEC outbreaks is not of animal origin but rather through leafy vegetables. During the period 1973 to 2006 there were 502 foodborne disease outbreaks associated with consumption of leafy greens. This resulted in over 18,000 cases of illness and at least 15 deaths. [15]. In the United States, there were 59 STEC outbreaks associated with the consumption of leafy vegetables from 1998 – 2008, and another 34 outbreaks attributed to consumption of vine-stalked produce. In 2011, consumption of fenugreek sprouts were associated with over 2000 cases of acute gastroenteritis caused by STEC O104:H4 in Germany [16]. STEC infections from fruit and vegetable sources are estimated to cost over US$400 million dollars loss due to production loss and healthcare costs. [17].

Vegetable produce contamination also occurs as a result of direct or indirect contact with animal feces containing STEC serotypes. Direct contamination of produce may result from animals defecating in the fields, poor worker sanitation, use of untreated manure, or contaminated irrigation water. Irrigation water can be contaminated via fecal deposition by animals or via run-off of untreated effluent from animal farms or
residential areas. Research has demonstrated that STEC O157 can persist on the surface of produce for up to 177 days post inoculation. [18]. In addition to being surface contaminated, vegetables are capable of internalizing bacteria [19] when grown in a contaminated environment. Bacterial internalization renders most post-harvest treatments ineffective since they are primarily focused on surface decontamination. In an attempt to reduce STEC transmission via vegetables, many producers are developing Good Agriculture Practice contracts for farmers, e.g. the Leafy Greens Marketing Agreement (LGMA) in California, which specifically seek to reduce pre-harvest contamination of produce.

The Center for Disease Control has estimated that approximately 16,000 STEC cases in 2011 were attributed to direct animal contact which resulted in at least 200 hospitalizations and 2 deaths [20]. STEC has been recovered from the feces of many ruminant, non-ruminant livestock, pets, birds, wildlife and even fish species. Most of these animals are asymptomatic shedders of STEC. Direct transmission from animals to humans has been recorded to occur on numerous instances especially at petting zoos and animal fairs. Data from one outbreak of STEC at a Texas animal fair in 2003 showed that for each day someone visited the fair, the chances of contracting STEC increased by 51% [21]. Ruminants are the natural reservoirs for STEC. The main animal reservoir for STEC in the United States is cattle, however this may vary in the different geographic locations and climates. For example the main animal reservoir for STEC in Australia is sheep [3]. The risk of contracting STEC exists even if someone visits an animal housing facility without having direct contact with an animal. This was demonstrated by
Henderson (2008), who showed that persons visiting a cattle farm were 10 times more likely of contracting STEC compared to someone who did not visit such a farm [22].

STEC, due its wide host range, can be shed on many surfaces which humans come in contact with. STEC serotypes can persist in the environment for long periods and have been shown to survive up to 266 days in manure amended soil [23]. This implies that recreation fields fertilized with animal manure or contaminated with animal feces can remain a potential source for human infection for a prolonged period. STEC deposited in fields are also capable of being leached during heavy rain fall and could enter surface and underground water sources [24]. STEC is also capable of surviving in oligotrophic water sources for extended periods. This is of particular importance since some of these water sources are used for recreational activities or for drinking purposes. During the period 1992 – 2002 there were 31 STEC outbreaks which were caused by water sources, 21 of these outbreaks were associated with recreational water sources and ten were from consumption of contaminated drinking water. [13]

Person to person secondary transmission is also possible, and can account for 15% to 20% of cases within outbreaks [12, 13]. Of particular risk of disease due to secondary transmission are children (1 – 6 years of age) due to close contact, their immature immune systems, reduced personal hygiene and prolonged shedding time [12]. An analysis of STEC outbreaks occurring between 1982 – 2006 as a result of person to person transmission, showed that 45% of these outbreaks occurred due to transmission at home, 11% at nurseries and 10% at recreational water sources [25].
A common feature of most primary sources of STEC human infection is animals. STEC can be shed by a large number of asymptomatic animals for an extended period of time. This coupled with its remarkable environmental persistence and low human infectious dose (<100 cells) which renders STEC a serious threat to human health and well-being. To ensure this threat is mitigated, one must thus understand the important role animals play as reservoirs for STEC.

**Role of Animals in STEC transmission.**

STEC is part of the gastrointestinal flora of many animals. Ruminants are identified as a primary reservoir for STEC, however many other animal species have been implicated as shedders or vectors. Most of these animals are asymptotically infected with STEC, and thus are able to shed the organism without being noticed. The infection rate in animals may vary due to a number of factors including age of animals, housing, diet, climate and sanitation.

One other reason which may account for the variation in herd prevalence at different locations and investigations is difference in the testing techniques employed. Studies using detection of Shiga toxin genes tend to have a higher infection rate than studies using bacterial isolation for diagnosis. This higher prevalence may not be an accurate assessment of the risk of disease since many nonpathogenic organisms may contain stx genes. Another factor which may cause different prevalence rates is that many bacterial studies do not include an enrichment step in the screening procedure. Addition of an enrichment step increases the sensitivity of the testing procedure allowing for detection of organisms whose initial population in the feces is below the detection limit.
Non-selective enrichment also allows injured cells or stressed bacterial cells to recover, thus increasing the sensitivity of STEC detection [8].

**Important Reservoirs of STEC**

**Cattle:**

Cattle are recognized as the natural reservoir for STEC, especially the serogroup O157. Similar to humans, cattle are exposed to STEC via contaminated food and water, or exposure to the feces of other animals shedding the organism. The infectious dose in cattle is estimated to be as low as 300 CFU [27]. STEC infections in cattle are usually asymptomatic due to the absence of vascular receptors for shiga toxins [28]. The absence of these globotriaosylceramide-3 (Gb3) vascular receptors, especially in the intestinal vasculature means Shiga toxins cannot be endocytosed and transported to other organs which may be sensitive to Shiga toxins [29]. The terminal part of the large intestine, the Recto-Anal Junction (RAJ) is the main site of STEC colonization in cattle. Most other bacteria do not target the RAJ as a site of colonization, thus there is little competition for this location [29]. This has been postulated as a reason for the long term persistence of infection in cattle. The bovine isolates of STEC O157 are also more resistant to adverse weather conditions than human isolates [30]. The low infectious dose and STEC’s ability to persist in the environment may account for the high prevalence seen in dairy and beef feedlot facilities.

Due to the ability of STEC to persist in the environment, contaminated pastures pose a risk for infection [31]. Poor silage production practices can facilitate STEC serotypes from contaminated forage to survive the silage making process. Improper feed
storage facilities or poorly designed feeding troughs can result in feed being contaminated with the feces of animals shedding organism. Once an animal is infected, they can exponentially increase the number of organisms in the environment when they defecate, thus increasing the risk of infection to other animals.

Water contamination can occur either at its source or at the farm. Surface water and ground water sources may be contaminated from effluent run-off from farms and urban areas. Leaching from pastures may also result in ground water contamination [24, 32]. At the farm, improperly designed water troughs can be contaminated by animal feces. LeJeune et al (2001) [33] showed that 1.3 % of 473 water troughs sampled in three USA states were positive of STEC O157. STEC has also been demonstrated to be able to persist for over four months in contaminated water troughs [34].

Other management practices may also affect the incidence of STEC in animal populations. Flushing alleyways with water increased the incidence of STEC in animals compared to other manure removal strategies [35]. Animals housed on sawdust were also found to have a higher incidence of STEC than animals housed on sand based bedding [36]. Movement of animals to and from farms also increases the risk of STEC infections. Animals carried to animal exhibitions have a greater likelihood of contracting STEC than animals not carried to shows [37]. These animals, on returning the farm, can then shed STEC, thus exposing other animals to infection.

In the United States, STEC O157 is found on almost all cattle farms with the organism being shed intermittently by most animals [38]. STEC is shed mainly via the feces of infected animals, however Shiga toxin genes have been detected from
*Escherichia coli* strains isolated from the milk of mastitic cows [39]. Milk from animals thus a potential source of STEC infection to the human population.

Globally the incidence of STEC O157 infection in cattle has been reported to range from 0% to 71% [40] and the herd infection rate has been reported to be up to 100% in some studies [8]. In the United States, the herd prevalence of STEC may range between 10 to 20% [41]. The global prevalence of STEC O157 in dairy cattle has been reported to range from 0.2% to 48.8% and 0.2 to 27.8% in beef animals while the global prevalence of non-O157 STEC infections in dairy cattle may range from 0.4% to 74% and 2.1% to 70.1% for beef animals respectively for two independent studies [42, 43].

Infected cattle can shed STEC O157 at levels as high as $1.1 \times 10^5$ CFU/gram feces [44] and for as long as 10 weeks [45]. Animals which continually shed STEC at high levels ($>10^4$ CFU/gram) for an extended period are termed ‘Super-shedders’. ‘Super-shedders’ although only representing less than 20% of the herd, have been shown to be responsible for up 96% of infections in the herd [46, 47]. Identification and removal of these ‘super-shedders’ could thus result in a reduction of the herd prevalence of STEC.

Calves tend to shed STEC at the lowest levels prior to weaning, however the highest shedding is exhibited in period immediately post-weaning. The shedding of STEC also tends to be higher in the warmer months with peak prevalence being in summer and early fall with a drastic decrease in prevalence during the winter months. [38]
**Small Ruminants**

Small ruminants, particularly sheep and goats are important reservoirs of STEC O157 [7]. There has been considerable research focused on the role of sheep in the epidemiology of STEC, however there is limited published research on the role of goats [7]. While cattle have been identified as the major reservoir for STEC in the United States, small ruminants play a greater role in the epidemiology of STEC infections in other countries. For example, in Australia, sheep have been identified as the host of significance [3] and has also been recognized as an important reservoir of O26 in Norway [48]. In addition to STEC serogroups O157 and O26, sheep have been cited as reservoirs for over 100 other serotypes of STEC including O115, O128 and O130 [7, 48]

Small ruminants are infected with STEC via the same route as cattle. The site of STEC colonization however is different. Unlike cattle where the site of colonization is the RAJ, there is no such tissue tropism exhibited in small ruminants [7]. Following infection with STEC O157, few attachment and effacement lesions are visible on the intestinal mucosa and the entire distal intestine is colonized apart from the not only the RAJ [7]. Further studies have also provided corroborative evidence of this lack of tissue tropism, with STEC O157 being found distributed throughout the entire gastro-intestinal tract with no preference for any anatomical location [49]. STEC O157 shedding patterns between rectally and orally inoculated sheep were found to be similar, thus indicating that STEC O157 may be unable to effectively colonize the terminal rectum [50]. This may account for the reduced shedding period compared to cattle.
Similar to cattle, small ruminants tend to be asymptomatic shedders of STEC. This trait was demonstrated when the screening of ‘healthy animals’ in Berlin reported that 66.6% of the sheep and 56.1% of the goats tested were found to be STEC carriers [5]. Similar results were obtained in Spain where 47% of healthy goats tested positive for shedding STEC [51]. The asymptomatic feature of STEC infections is possibly due to the lack of Shiga-toxin vascular receptors in small ruminants also.

Many direct contact human infections are attributed to contact with sheep and goats at petting zoos and open farms. One study investigating the prevalence of zoonotic agents on small city farms in southern Germany has found that 100% of the sheep and 89.3% of the goats tested positive for STEC [52]. Small ruminants, especially goats, tend to be more inquisitive, thus may have greater contact with humans increasing the potential for fecal oral transmission to humans [7]. Human infections have also been linked to the consumption of unpasteurized milk and cheese made from contaminated goat or sheep milk [51, 53]

Sheep are the primary reservoir for STEC in Australia with the serotype of importance being O26 however the risk of human infection was deemed insignificant due to low infection rates [3, 54]. Although the within herd prevalence was low, previous research reported that 90% of Australian sheep farms had animals testing positive for STEC [55]. In Norway however, the risk of human infection from sheep was much more significant since almost 50% of the sheep O26 isolates had similar MLVA profiles to that found in human clinical cases [48].
The STEC O157 flock prevalence in Spain was reported to be 8.7% and an individual prevalence of 7.8% [56]. A similarly low STEC O157 prevalence of 5.8% was also reported in Scotland [57]. Low STEC O157 prevalences were also reported in United Kingdom and Holland with the prevalence being 0.1% and 4.0%, respectively [58, 59]. Lesser developed countries have also reported the presence of STEC in their small ruminant population. In Vietnam, 100% of the goat farms surveyed had animals shedding STEC, and the within herd infection was dramatically higher than that reported elsewhere with up to 65% of animals being infected [60]. In Bangladesh, almost 10% of the small ruminants being slaughtered tested positive for STEC O157 [61]. The detection of STEC in animals from less developed countries is a serious threat to food safety, since in these countries there may not be strict hygienic slaughter practices, thus contaminated meat could easily enter the food chain.

The shedding of STEC in small ruminants has been demonstrated to be age and season dependent. Younger animals tend to have a lower prevalence of STEC than older animals [51, 62-64]. A longitudinal study spanning six months in the United States demonstrated a peak in STEC prevalence during summer [65]. This trend was also observed in Italy, where animals screened during the warmer months of the year had a higher prevalence of STEC O157 [63]

Other Ruminants

In addition to cattle, sheep and goats, other ruminant species have also been identified as shedders of STEC such as water buffalo (*Bubalus bubalis*) have been identified as an important reservoir of STEC O157 in many countries [66]. The water
buffalo is reared in many countries due to its ability to serve as a dual purpose animal, capable of being both a milk and meat producer. Buffalo are also able to thrive much better on poor quality forages than *Bos taurus* species, thus making them suitable for subsistence farming. There are large commercial meat and milk water buffalo herds in Asia and South America, while in Europe it is primarily reared for milk production. In Bangladesh, STEC colonies were isolated from 38% of the buffaloes sampled prior to slaughter. Almost half of these isolates were identified as being O157 [61]. Galiero et al (2005) reported an almost a similar prevalence in Italy with 14.5% of the animals shedding O157. In Vietnam, 27% of the buffalos screened were found to be positive for STEC. Serotyping of the isolates however revealed that none of the isolates were O157 [60]

Another potentially important animal reservoir of STEC is the American Bison (*Bison bison*). In the United States there has been increasing consumption of bison meat [67]. Fecal samples from 342 bison screened at slaughter demonstrated that almost 50% were positive for STEC O157:H7 [68]. STEC O157 has also been isolated from the carcass of slaughtered bison at a prevalence of 1.13% [69]. Non O157 STEC serotypes including O45, O103, O111, O113, O121 and O145 have also been isolated from bison carcasses; however none of these isolates possessed *stx* genes[70]

**Horses**

There is a dearth of published data on the epidemiology of STEC infections in horses. There are also no published case reports describing the clinical features of STEC infection in horses. The published data on the prevalence of STEC in horses indicates that
they are not major reservoirs of STEC. Only one of 400 horse fecal samples screened in Germany was positive for STEC. The serotype isolated was O113: H21 [71]. STEC has also been detected in the equine population in the United States with a similarly low prevalence. Only one of 242 horse fecal samples from Ohio tested positive for STEC O157:H7. Interestingly this case shared housing accommodations with a goat which also shed STEC O157. The isolates from both animals had indistinguishable MLVA patterns [72]. Screening of fecal samples from horses located in the Sacramento Valley revealed a slightly higher prevalence than that recorded in Ohio. Four out of 156 samples (2.6%) tested positive for Shiga toxin 2 gene [73]. Notably, as was seen in Ohio all the infected horses were also housed on farms containing ruminants. Despite the low STEC prevalence in horses, there are reported human clinical cases associated with infection from horse contact [74], and as such one must be aware of this potential source of infection.

**Swine**

Swine are not recognized as a major reservoir of STEC due to the low prevalence reported in many studies [32, 75]. The prevalence of STEC O157:H7 in swine has been reported to range from 0% to up to 10%, with the prevalence in the United States usually being less than 2% [32, 75-77]. Unlike ruminants, pigs possess vascular stx receptors and thus STEC infections can be highly pathological for swine, with these animals exhibiting edema disease. Similar to humans, the most pathogenic serotype in pigs is STEC O157:H7 [75].
Though relatively a low prevalence of STEC O157:H7 has been reported, swine have been shown to be able to harbor and shed STEC for up to two months post infection [78]. Non-O157 STEC serotypes have also been isolated from pigs; however, many of these isolates lack the virulence factors required to cause human disease [3]. Despite a low prevalence of pathogenic STEC serotypes, the potential for human infection from swine still exists. This risk is exemplified by a recent Canadian outbreak of STEC O157:H7 associated with consumption of pork, with infected persons having the identical STEC O157:H7 isolate to that found in the pork meal served [79].

**Birds**

Birds are capable of harboring many bacterial organisms in their gastro-intestinal tract including STEC O157, which was first detected in the gull population in 1997. Since then STEC has been isolated from starlings, pigeons, sparrows and numerous other avian species [80, 81]. Many species of wild birds can be found in close proximity to livestock operations. These birds are attracted to farms since they can easily obtain a food source from animal feed. Nielsen et al (2004) identified that 2% of the wild bird fecal samples collected in close proximity to farms contained stx genes. Similar results were also obtained in England where 1.5% of wild bird samples had the stx1 gene, 7.9% stx 2 gene and 4.9% eae gene [82]. Similarly, low prevalence rates of STEC O157:H7 in the starling population in Ohio, Scotland and Japan have also been reported [83-85]. Though the STEC infection levels are reportedly low, the potential of these birds to infect other birds as well contaminate the environment is of serious risk. Studies have shown that once infected, a starling may shed STEC O157 at levels greater than 100 CFU/gram feces for up to 13 days post-infection [86].
The migratory pattern of birds and the fact they can traverse long distances in a single day means they can serve as a mode of transmission of STEC between and within farms. This was demonstrated by Williams et al (2011) [87], who reported that starlings and cattle on different farms had molecularly indistinguishable subtypes of STEC O157:H7, thus confirming that transmission via starlings is possible. Of particular importance is the fact that these migratory birds interact with peri-domestic birds such as pigeons thus potentiating the transmission of STEC. Pigeons and finches have been identified as two species which can potentially serve as a source of human infection since these birds inhabit buildings, parks and other recreational areas and are in close association with the human population [84, 88]. Fecal depositions by these birds increase human exposure to STEC.

STEC infection has also been reported in domestic poultry. The prevalence of STEC O157:H7 in domestic chicken is relatively low ranging from 0% to 1.5% depending on the geographic location sampled [75, 76, 89, 90]. Interestingly, the prevalence in turkeys was higher than in chickens, with up to 7.5% of fecal samples testing positive [75, 90]. Experimentally infected chickens have been reported to harbor and shed STEC O157:H7 in their feces for extended periods in excess of eleven months [91]. Pet birds such as canaries (Serinus canaria domestica) have also been reported to be capable of harboring and shedding STEC [92].

The fact that both wild and domestic birds are able to harbor, transmit and shed STEC is of serious concern since they are potentially major risk to human health and disease, and as such precautions should be taken to limit human or animal exposure to the excrement from these birds.
Pets

Pets, especially dogs and cats are capable of shedding a diverse range of STEC serotypes in their feces [93-96]. Dogs and cats have historically had close interaction with humans, with exchange of microbiota occurring resulting in the possible transmission of STEC between species. These animals can be asymptomatic shedders of STEC as demonstrated by Beutin et al (1999) [5] who reported that up to 12% of healthy dogs shed STEC in their feces. In addition to household dogs, farm dogs can act as a vector for the transmission of STEC. These animals are usually able to move freely between animals and humans thus potentiating the spread of EHEC [97]. Dogs have also been reported to shed non-O157 STEC serotypes in their feces [93]. Human infections due to canine exposure are also reported with the one outbreak in Sweden resulting in 50 human cases after attending a dog show.[98]

A highly virulent strain of STEC O146:NM has also been isolated from the feces of an asymptomatic cat in Argentina [99]. In Germany there is also evidence of a cat and its owner shedding the same STEC O146:NM serotype [100]. In this case, the source of the infection could not be determined, nor which animal was index case.

Wild Animals.

Similar to wild birds, wild terrestrial animals can disseminate STEC over large distances resulting in direct transmission to other animals and/or the contamination of pastures, vegetable produce fields, recreational areas and/or or water sources. This potential is magnified if these wild animals share the same environment with known ruminant reservoirs such as cattle, sheep or goats. Globally, wild animal species
identified as STEC reservoirs include deer, elk, feral pigs, rabbits and opossum. In the United States, deer and feral swine are two of the more important wild life species implicated in the transmission of STEC O157 to the human population.

(a) Deer

There are an estimated 30 million White Tailed Deer in the United States (AP 2005). The role of White Tailed Deer (Odocoileus virginianus) as a reservoir for STEC was first reported in 1999 when almost 2.4% of deer sampled tested positive [101]. The presence of STEC O157:H7 in deer feces was later confirmed by Renter et al (2001) who found the STEC prevalence in Nebraska White Tailed Deer to be 0.24%. Since then other species of deer including Red deer (Cervus elaphus), fallow deer (Dama dama) and roe deer (Capreolus capreolus) have also been identified as capable of shedding STEC serotypes [102, 103]. Almost 50% of Pennsylvanian White Tailed Deer fecal samples screened tested positive for stx genes; however, only 8% possessed the eae gene which is necessary for colonization of the human intestine. [104].

Feral deer are known to share pastures with cattle and can also be found in close proximity to many dairy farms. The close association between deer and livestock implies that deer can serve to maintain and disseminate STEC infections between and within cattle herds [105].

The risk to human population as a result of venison consumption is exemplified by an outbreak of non O157 STEC amongst high school students which was associated with consumption of venison they had caught and processed. Two STEC serotypes,
O103:H2 and O145:NM were isolated from the samples analyzed; however, the O145:NM serotype was found to be Shiga toxin negative [106].

(b) Feral swine

Feral swine is another wildlife species which has been associated with STEC disease in the human population. There are approximately five million feral swine in the United States and they can be found in over 35 states (USDA 2011). These animals are highly adaptable to varying environmental conditions and can act as a vector for disease between livestock farms and also as a source of contamination to vegetable production fields.

In the United States, feral swine was first identified as a reservoir for STEC O157:H7 in 2007 in California [107]. In that study, STEC O157:H7 was isolated from 14.9% of the swine specimens tested, and these isolates were found to be indistinguishable from STEC O157:H7 isolates obtained from an outbreak in the human population associated with the consumption of spinach. Interestingly cattle, feral swine and environmental samples from the region where the spinach was cultivated all had the same STEC isolate O157 [107]. STEC has also been detected in feral swine from Sweden, Switzerland and Spain. Approximately 9% of the tonsil samples screened (n=153) in Switzerland were positive for STEC O157, but none of the corresponding fecal samples were positive [108]. A similar prevalence of 8% was reported for Spanish feral swine fecal samples [109]. The isolates were serotyped and 3.3% of the animals were identified as shedding STEC O157:H7 and 5.2% of the animals as shedding non-O157 STEC.
The identification of STEC from feral swine samples indicate that they can play a role in the epidemiology of STEC infections. As such their ability to potentially contaminate vegetable production fields as well as serve as vectors for STEC transmission between livestock must be recognized, and measures employed to mitigate this risk.

**Conclusion**

STEC is a virulent pathogen, with most warm blooded animals capable of acting as asymptomatic reservoirs for infection. Most animals are infected via direct or indirect contact with the feces of an infected animal. The most prevalent and pathogenic serotype in the human population is STEC O157:H7 which has also been shown to be present within the gut flora of many animals. Although cattle are recognized as the main reservoir of STEC and many control strategies are aimed at reducing cattle infection, one must be conscious of the role other animal species can serve in the transmission of STEC. Depending on the geographic and climatic factors, the species of importance can vary. Livestock species including bison, horses, pigs and water buffalo have all been demonstrated to be capable of harboring these organisms. Wild birds and animals pose a unique risk in their ability to travel large distances increasing the dissemination of STEC in the environment and thus potentiating its spread. Domestic pets are also capable of harboring STEC, and thus serve as a source of contamination within the household. Recognition of the vast number of animal reservoirs is essential for developing proper control measures. Control measures aimed at reducing STEC infection in cattle will reduce the public health risk however it will not eliminate it.
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Figure 1. Transmission Cycle for STEC O157:H7 Animals are the reservoir of STEC O157:H7. Fecal deposition or run-off from livestock farms can contaminate vegetable fields and water sources. Vegetable fields can also be contaminated when if contaminated water is used for irrigation. Waterborne infections occur when people consume or bathe in water contaminated with animal feces. Foodborne disease occurs when persons consume animal products and vegetable produce contaminated with animal feces. Humans can also be infected via direct contact with shedding animals. Humans can also serve as a source of infection to other humans.
Chapter 2

Plasmid loss limits the use of GFP labeled *Escherichia coli* O157 in long-term survival studies
GFP Plasmid Stability

Plasmid loss limits the use of GFP labeled *Escherichia coli* O157 in long-term survival studies.

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Keywords: GFP Plasmid, Rifampicin, Survival studies, Marker stability *Escherichia coli* O157:H7

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Abstract

Plasmids encoding green fluorescent protein (GFP) are frequently used to label bacteria, allowing the identification and differentiation from background flora during experimental studies. Although these plasmids have been used in many survival studies, the long-term plasmid stability in bacteria has not been evaluated. In this study, the stability of a GFP plasmid in *Escherichia coli* O157 was assessed in an oligotrophic environment (Phosphate Buffered Saline) without antibiotic selective pressure. The GFP-labeled cells only reflected the actual total bacterial population for 8 days. There was a continual decrease in the percentage of plasmid bearing cells: On the 9th day, only 30% of cells maintained the plasmid. On day 14, less than 1% of the cells were fluorescing and from day 19 onward, no fluorescing cells were observed. The non-fluorescing cell population at this time was almost 5 log$_{10}$ CFU/ml. These results demonstrate that the use of GFP labeled *E.coli* O157:H7 in prolonged survival studies may result in the underestimation of survival time. Chromosomal labeling of *E.coli* O157:H7 may be thus more suitable for these studies.
Introduction

Foodborne pathogens accounts for over 48 million cases of disease in the United States annually [1, 2]. Shiga toxigenic *Escherichia coli* (STEC) O157:H7 is one of the major pathogens responsible for numerous outbreaks of foodborne disease causing over 90,000 cases annually within the United States [2]. Ruminants are recognized as the natural reservoir for STEC with cattle being the main reservoir in the United States [3] however many other animals can act as reservoirs and shed the bacteria when they defecate [4-6].

The majority of human STEC infections occur as a result of foodborne infections [7]. Historically, animal products were the primary sources for STEC transmission, but more recently indirect routes of transmission, via water, the environment or vegetable products have now been attributed as the major source of STEC infection to humans [8].

Understanding the survival of *E. coli* O157:H7 in the environment is useful to assess risks associated with environmental contamination. To determine accurately the survival of an organism in the environment one must be able to identify the bacteria of interest and accurately estimate its population size until it no longer persists in the environment. This can be achieved by identifying genotypic or phenotypic traits that allow differentiation from background organisms. In nature however, unique markers are not always naturally occurring. Genetic manipulation of organisms of interest either via transformation or chromosomal insertions, deletions or mutations is useful laboratory methods to tag bacteria. Two popular phenotypic characteristics which allow easy identification and differentiation include the use of antibiotic resistance and/or UV fluorescence.
The green fluorescent protein (GFP) genes which encode for the fluorescing protein were originally isolated from the jellyfish *Aequorea victoria*. These proteins fluoresce when exposed to UV light and can thus be used to identify bacteria containing the GFP plasmids. These plasmids also contain genes which encode for antibiotic resistance. The presence of these plasmids has been reported to have no effect on the biochemical, morphological or survival characteristics of the bacterial cells labeled with them [9]. The use of plasmids encoding GFP provide the advantage of allowing ease of detection with minimal additional processing and without the need for agar supplementation for metabolic selection [10], which can be detrimental to the recovery of sublethally injured organisms [11]. However, since plasmids are extrachromosomal DNA molecules, they can be lost from the cell via spontaneous segregation and deletion during replication, exposure to high temperatures, nutrient deprivation or the use of curing agents [12].

The purpose of this study was to evaluate the stability of GFP-labeled *E. coli* O157:H7 when removed from an antibiotic selective environment and placed in an oligotrophic environment similar to that which would be encountered during a prolonged environmental survival study. In addition, we compared the recovery of GFP-labeled *E. coli* O157:H7 with the stability of a chromosomally marked *E. coli* O157:H7 in a similar environment.

**Materials and Methods**

**Bacterial Strains and Labeling of *E. coli* O157:H7**

Two *E. coli* O157:H7 isolates were used in this study. One isolate was obtained from a research feedlot facility in Ohio (LeJ Lab# EC 811) and the other from a human clinical case associated with spinach consumption (MDD 321/LJH 1186 [13]). The parent human clinical
isolate as well as the rifampicin resistant transformed *E. coli* O157 mutant was kindly provided by Dr. Michelle Danyluk, University of Florida. The pGFPuv plasmid used in the plasmid stability component of this study was obtained from Clontech Laboratories Inc. (Mountain View, CA). Transformation was attained via electroporation using the Gene Pulser 2 (BioRad, CA.) in a traditional fashion [14].

The banked parent isolate (LeJ Lab# EC811) was allowed to thaw and one loopful streaked onto a SMACct agar plate and incubated overnight at 35°C. The next day one colony was selected and used to inoculate a 50 ml centrifuge tube containing 10 ml of lysogeny broth (LB Lennox Accumedia MI). This was then incubated overnight at 35°C for 24 hours. The next day one ml of this broth was used to inoculate a flask containing 30 ml LB broth. This flask was then placed on a shaker and incubated at 35°C until the OD$_{600}$ of 0.6 was attained. The contents of the flask were then transferred to a 50 ml centrifuge tube and contents centrifuged at 5000 rpm for 20 minutes at 4°C. The supernatant was then decanted and the pellet resuspended with 25 ml of sterile water. This procedure was repeated two more times. After the third washing, the pellet was resuspended in 10 ml of sterile water. Forty microliters of this washed cell culture was mixed with 1μl of plasmid DNA. The sample was placed in a 0.1cm cuvette and subjected to 1.5kV, 200Ω and 25 μF electric pulse. The cells were then resuscitated by incubation at 37°C for 2 hours. After incubation, the sample was plated on lysogeny broth agar (LB – Lennox, Acumedia, MI) supplemented with 50 μg/ml ampicillin (Fisher Scientific, NJ). Three fluorescing transformants (EC 1759, EC 1760 and EC 1761) were then selected visually based on their fluorescence and amplified in brain heart infusion broth (Acumedia, MI) and banked at -80°C with 30% buffered glycerol (VWR International, OH).
Chromosomal marking of *E. coli* O157:H7 (MD321) was achieved by selecting for naturally occurring resistance to rifampicin resistance. Briefly, the parent *E. coli* O157 isolate was propagated overnight at 37°C in tryptic soy broth (Acumedia, MI) and 100 µl of this broth was transferred daily to fresh media with increasing concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, 100µg/ml) of rifampicin (Fisher Scientific). The final broth culture was plated on LB agar with rifampicin (80µg/ml) and incubated overnight at 37°C. Three isolates recovered from the LB plates were amplified overnight in Brain Heart Infusion broth and banked at -80°C with 30% buffered glycerol for use at a later time.

**Growth kinetics of labeled bacteria and wild-type *E.coli 0157* parent:**

The growth kinetics of the labeled strains and the wild-type parent strains were compared for over a 12 hour period. The three transformed GFP labeled isolates (EC 1759, EC1760, and EC 1761) were recovered from frozen stock and streaked for isolation on LB-Miller agar supplemented with 50 µg/ml ampicillin (LBAP50). One colony from each culture was then used to inoculate separate 15 ml centrifuge tubes containing 5 ml of lysogeny broth (LB -Lennox Acumedia,MI) supplemented with 50 µg/ml ampicillin. These inoculated tubes were then placed on an orbital shaker (Bellco Biotechnology, NJ) at speed #3 and incubated at 37°C for 24 hours. The next day, 1 ml of each broth was used to inoculate separate flasks containing 500 ml of LB-Lennox broth supplemented with 50 µg/ml ampicillin. The flasks were then placed on a shaker (speed 3) and incubated at 37°C until time of sampling. One hundred microliters from each of the three broth cultures were sampled every hour. Ten-fold dilutions of each sample were made in Phosphate Buffered Solution (Amresco, OH) and spread plated using sterile glass beads on LBAP50. Plates were incubated for 24 hours at 37°C and the concentration of bacteria in the broth determined at each sample period. The growth rate of the parent strains were evaluated
using a similar method; however, without the use of ampicillin in the broth media or the LB agar plates. The growth rate of the rifampicin–resistant *E. coli* O157:H7 mutant was also compared to that of the parent using a similar method with the inclusion of rifampicin (concentration 80 µg/ml) in the media in lieu of ampicillin.

**Validation of labeled strains in long-term survival study**

(i) *Removal of Nutrient content and Antibiotic Selection Pressure*:

Starting cultures were prepared as described above: The three GFP labeled isolates were streaked from frozen stock for isolation on LBAP50. One colony from each isolate was then used to inoculate separate 15 ml centrifuge tubes containing 5 ml of LB Broth supplemented with 50 µg/ml ampicillin. These inoculated tubes were then placed on an orbital shaker (speed 3) and incubated at 37ºC for 24 hours. The next day 1ml of each broth was used to inoculate separate flasks containing 250 ml of LB-Lennox broth supplemented with 50 µg/ml ampicillin and incubated for 16 hours at 37ºC on an orbital shaker (Speed 3). Forty-five ml samples of each inoculum were then placed in 50ml centrifuge tubes and centrifuged at 5000xg for 20 minutes. The supernatant was then decanted and the bacterial pellet re-suspended in 45ml of 1x phosphate buffered saline. This wash procedure was repeated 2 more times to remove antibiotic and nutrient content from the inocula. After the third washing and re-suspension of the bacterial pellet in PBS, the washed inocula were aliquoted into 1 ml volumes in 2 ml micro-centrifuge tubes and stored at 37ºC until time of sampling.

A similar method to that described above was used to obtain a rifampicin resistant *E. coli* O157 inoculum devoid of nutrient and antibiotic selection pressure. The washed inocula was then also separated into 1ml aliquots and stored at 37ºC until time of sampling.
(ii) **Evaluation of Labeling Stability**

Plasmid Stability – Evaluation of plasmid stability was performed daily. Serial dilutions from each of the three replicate PBS 1 ml survival microcosms was diluted with PBS up to a dilution of $10^{-6}$. One hundred microliter samples of each dilution was spread plate on LB-Miller Agar using sterile glass beads. These plates were then incubated at 37°C for 24 hours. The number of total colonies on a plate were counted and aided by UV transillumination, the number of fluorescent colonies present were enumerated. Using plates yielding between 30 and 300 colonies, the numbers of fluorescing and non-fluorescing colonies present on each day were calculated. Each day, up to a ten (if present) fluorescent colonies were tested to confirm their identity as *E. coli* O157 using a latex agglutination test (Oxoid, Kent, UK) and cultured on LBAP50 to confirm their loss of resistance to ampicillin.

(iii) **Plasmid Screening**

One non-fluorescing colony was used to inoculate 45 milliliters of LB broth. This starter culture was incubated on an orbital shaker at speed#3 for 24 hours at 37°C. After incubation, 600 µl of the culture was transferred to a 1.5ml microcentrifuge tube. The tube was the centrifuged (Microfuge 2, Beckman Coulter, CA) at 14,000 rpm for two minutes at 4°C. The tube was carefully removed from the machine and 550 µl of the supernatant removed ensuring the bacterial pellet was not disturbed. The bacterial pellet was then resuspended and 0.5µl of RNaseA (20 mg/ml – Novagen, MA) and 40 µl of Phenol/Chloroform/Isoamyl alcohol (25:24:1 – Sigma, MO) were added to the tube and the contents thoroughly mixed. The contents were then centrifuged as described above and the supernatant transferred to a sterile 2ml microcentrifuge tube. The supernatant was then subjected to gel electrophoresis on a 0.7% agarose gel (AMRESCO, OH). The same procedure was repeated as outlined above for the transformed
fluorescing *E. coli* O157:H7. A 1 kb ladder (Promega Corporation, WI) was used to identify the approximate location where the pGFPuv plasmid would be found.

**(iv) Stability of rifampicin-resistant *E. coli* O157:H7**

For comparative purposes, an evaluation of the stability of a chromosomally marked *E. coli* O157 was conducted weekly. Serial dilutions from each the three replicate PBS 1 ml survival microcosms was diluted with PBS up to a dilution of 10^{-6}. One hundred µl samples of each dilution was spread plate using sterile glass beads on LB-Miller Agar and also on LB-Miller agar supplemented with 80 µg/ml rifampicin. The number of colonies present on each plate after 24 hr incubation at 37°C was counted. Ten colonies from the LB-Miller agar plate were selected weekly and subjected to an *E. coli* O157 latex agglutination test to confirm the presence of the O157 antigen.

**Statistical analysis**

A Repeated Measures ANOVA test (Minitab 16.0) was used to examine the difference in growth rate between mutant *E. coli* O157 and their respective the wild-type parent. Likewise, a Repeated Measures ANOVA was also used to evaluate the stability of the *E.coli* mutants. Growth data was fitted to a Gompertz model [15] and paired t tests were used to compare model coefficients (lag time, maximum growth rate, and maximum asymptote). Differences were considered to be statistically significant at P <0.05.

Gompertz model: y = a*exp (-exp(b-(c*time)))

Lag time = (b-1)/c

Maximum Growth rate = (ac)/e  [e=2.7183]
Maximum asymptote = a

Results

**Growth Kinetic Evaluation:**

The evaluation of the labeled strains and the parent strains revealed no significant differences in the growth kinetics: Lag periods for both the GFP plasmid labeled *E. coli* O157, and the chromosomally marked rifampicin resistant *E. coli* O157 were similar to their respective parent (Figure 2.1, Table 2.1). The Log phase or exponential growth phase was also indistinguishable between the parent and resistant strains.

**Validation of labeled strains in long-term survival study:**

*(i) GFP Plasmid Labeled* *E. coli* O157:H7.*

At the 9\textsuperscript{th} day, there was a notable drop in the number of fluorescent cells relative to the total cell count (Figure 2.2). At this point, the number of green fluorescing cells was $0.5 \log_{10}$ CFU/ml less than the total cell count (number of fluorescing cells + non fluorescing cells) and plasmid retention was calculated to be 30%. Another large difference between fluorescent and non-fluorescent counts was observed on day 14. This difference in cell counts was approximately $2.5 \log_{10}$ CFU/ml and plasmid retention had decreased to less than one percent. After day 19, no green fluorescing cells could be detected although the total cell count which was now only comprised of non-fluorescing cells was at approximately $5 \log_{10}$ CFU/ml. Using a Repeated Measures ANOVA test the difference between the total cell count and the number of fluorescing cells was found to be statistically significant (P<0.01). All of the randomly selected non-fluorescent cells agglutinated with the anti-O157 latex agglutination test. Non-fluorescent
colonies did not grow on media with ampicillin. The plasmid profiles of the representative fluorescent and non-fluorescent colonies were distinguishable: Notably, the predicted 3kB band in the fluorescent colony was absent in the ampicillin-susceptible, non-fluorescent colony (Figure 2.4).

(ii) Chromosomally Labeled E. coli O157:H7

The chromosomally labeled E.coli O157:H7 was detected in a non-selective, oligotrophic environment at the same concentration as the parent (rifampicin sensitive) strain ($P=0.67$) (Figure 2.3). The initial inoculation dose was approximately 8.0 log$_{10}$ CFU/ml. There was a steady decrease in the cell counts obtained on both the LB-Miller agar and the LB-Miller+ Rifampicin agar plates with the most dramatic decrease of 1.5 Log$_{10}$ counts observed in the first week. From day forty onward to end of the study (day 72), the CFU/ml count remained at approximately 5 log$_{10}$ CFU/ml.

Discussion

These experiments demonstrate that the use of the fluorescent phenotype in GFP-labeled E. coli O157:H7 does not accurately reflect total culturable E. coli O157:H7 cells under low nutrient conditions in the laboratory. The extent to which similar disparities in plasmid retention occur in other matrices and with other E. coli O157 strains could have important implications on the interpretation of experimental survival studies that have used this method of marking. In contrast, chromosomally labeled E.coli O157:H7 may provide a more suitable method of labeling E.coli O157:H7 for long-term survival studies since it maintained its phenotypic marker for the duration of the experiment.
The stability of the plasmid in the GFP labeled *E. coli* O157 population until day 9 is consistent with previously published data [9, 10, 16, 17], and highlights the fact that it may be suitable for use in short-term survival studies. The loss of the plasmid and consequently the visible fluorescent marker is of great concern, since many published studies extending beyond 9 days have used this labeling method for evaluation of bacterial persistence [18-20]. In contrast, the comparable populations of enumerated rifampicin-resistant and rifampicin sensitive *E. coli* O157:H7 cells indicate that this marker accurately represented the actual cell population for the duration of the study.

Genetic differences can result in differences in plasmid segregation and differences in the rate of emergence of plasmid free mutants [21]. Thus, rapid loss of plasmid from the tested *E. coli* O157:H7 may not be representative of all *E. coli*, but these experiments demonstrate that rapid loss occurs in some strains. If researchers choose to label *E. coli* O157 for experimental purposes, it would be prudent to assess the stability of the specific plasmid in the unique host prior to launching long-term experiments. Likewise, because of possible difference in the genomic backbone of the strains used for the GFP survival and the rifampicin-labeling, we refrained from making direct comparison between the utility of these markers.

In this study, the evaluation of the growth kinetics for the GFP-labeled *E. coli* O157 and the chromosomally labeled *E. coli* O157 indicate that both labeling procedures did not alter the short-term growth characteristics of the organisms under the laboratory condition assayed. The presence of the plasmid did not place a significant metabolic burden on the cell. This finding is consistent with previously published data which also demonstrated the presence the GFP plasmid did not affect the growth kinetics of the bacteria in optimal growth conditions [9, 22-24].
Plasmid loss can be caused by either structural or segregational instability [21]. Structural instability occurs as a result of loss of the DNA sequence during replication or recombination. Segregational instability results in the failure of the plasmids to be distributed between the two daughter cells thus resulting in the evolution of cells that are plasmid free. Segregational instability can be mediated by nutrient availability, growth rate, host and cellular genotype. This underscores the need to understand the stability of the plasmid in the environment to be tested prior to conducting survival studies. Variations in survival times in different matrices measured using GFP-labeled cells may be artifacts of plasmid stability instead of bacterial survival in general.

Spontaneous rifampicin-resistance is typically conferred by mutations in the RNA polymerase [25]. As such, it is possible that this mutation or compensatory mutations may affect bacterial growth and or survival. The results obtained for the rifampicin resistant *E. coli* O157 in this study demonstrated that the chromosomal mutation induced did not negatively alter the growth of the organism over the time period tested. The use of homologous recombination to insert antibiotic resistance genes into known locations in the genome may provide another alternative to confer a phenotypic marker to *E. coli* O157.

While GFP labeling of bacterial cells has many advantages, one has to be conscious of the potential instability of the plasmid maintenance in long-term survival experiments. This experiment demonstrated that once the selective pressure had been removed, plasmid loss and consequently loss of phenotypic labeling characteristics occurs in nutrient deficient matrices.
Acknowledgements

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References


Figure 2.1. Growth rate of labeled and parental *E. coli* O157:H7. (a) GFP Labeled and parent \( (P=0.76) \). (b) Chromosomally labeled and parental \( (P=0.26) \). Error bars represent standard error.
Figure 2.2. Stability of GFP-labeled *E.coli* O157 in an oligotrophic, non-selective media. Dilutions are cultured daily on LB agar. Values are expressed as the mean Log CFU/ml of the three isolates ± standard error. *p*<0.01
Figure 2.3. Stability of Chromosomally Marked Rifampicin Resistant *E. coli* O157 in an oligotrophic, non-selective media. Weekly dilutions are cultured on LB-agar and LB agar supplemented with 80μg/ml Rifampicin. Values are expressed as the mean Log CFU/ml of three isolates ± standard error (p=0.67)
Figure 2.4. Gel Electrophoresis demonstrating the location of the pGFPuv plasmid at the 3Kb region obtained from the GFP Labeled isolate (lane 3). In lane 2, the non-fluorescing cell has no DNA band in this region indicating loss of plasmid. Lanes 1 and 4 contain the 1kb DNA ladder.
Table 2.1. Growth Parameters for parental *E.coli* O157 and their respectively labeled mutants.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Lag time (hours)</th>
<th>Maximum Growth Rate</th>
<th>Asymptote Value ( \log_{10}(N/No) )</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC 811</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td>2.49±0.04</td>
<td>0.65±0.08</td>
<td>2.2</td>
<td>0.20</td>
</tr>
<tr>
<td>GFP Labeled mutant</td>
<td>2.27±0.34</td>
<td>0.64 ± 0.13</td>
<td>2.1 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><strong>MDD 321</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td>3.44 ± 0.14</td>
<td>0.54 ± 0.05</td>
<td>2.6 ± 0.14</td>
<td>0.40</td>
</tr>
<tr>
<td>Chrom. labeled mutant</td>
<td>3.13 ± 0.15</td>
<td>0.68 ± 0.05</td>
<td>2.3 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

There was no statistical difference between the lag times, maximum growth rate, or asymptote values for the parental *E.coli* strains and their respectively labeled mutants. Values shown are the average of three replicates ± standard error of the mean.
Chapter 3

The Survival of *Salmonella* and Shiga-toxin producing *Escherichia coli* serotypes in the feces of five different animal species.
Pre Harvest Food Safety.

The Survival of *Salmonella* and Shiga toxin- producing *Escherichia coli* serotypes in the feces of five different animal feces.

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Abstract

Animal feces can harbor a plethora of foodborne pathogens which can contaminate vegetable produce directly or indirectly when shed by defecating animals. The main objective of this study was to determine the rate of decline of foodborne disease associated Shiga Toxin-producing *Escherichia coli* (STEC) and *Salmonella* serotypes in different animal fecal matrices. Five chromosomally labeled STEC and *Salmonella* serotypes were mixed to create the respective inoculant. Fecal samples were inoculated at a concentration of $10^4$ – $10^5$ CFU/gram of feces and stored at room temperature until sampled at days 0, 1, 3, 5, 7, 14 and monthly.

The *Salmonella* inoculated cattle and feral pig feces yielded growth (i.e >10 CFU/gram) on direct plating at 224 days post inoculation (dpi). The STEC inocula remained positive on direct plating in the cattle and waterfowl feces until 194 dpi. In the deer and water fowl feces, the STEC inocula yielded positive growth on direct plating until 82 dpi. Both the STEC and *Salmonella* inocula yielded growth on direct culturing the raccoon fecal matrix until 56 dpi. Positive growth of the *Salmonella* inocula in the deer feces was observed until day 194. On day 224, the *Salmonella* inoculated waterfowl feces yielded no growth on direct plating and also after enrichment. Although samples were negative on direct culturing, with the exception of the *Salmonella* inoculated water fowl feces, all other fecal inocula were positive on enrichment. The survival of these organisms were dependent on the fecal matrix, thus guidelines can be adjusted to providing the fecal type can be positively identified. A no harvest period of 194 days is recommended to ensure the microbial risk is mitigated if the fecal type cannot be identified.
Introduction

*Salmonella* and Shiga-toxin producing *Escherichia coli* (STEC) have been identified as two important foodborne pathogens associated with vegetable consumption [1, 2]. Within the United States, foodborne disease affects one in six American annually resulting in over 127,000 hospitalizations and 3,000 deaths. The economic burden is also quite profound and estimated to be close to $35 billion [3]. Historically consumption of animal products was the main source of foodborne disease however recent evidence has indicated that vegetable commodities now account for over 50% of these infections [2, 4].

*Salmonella* is the second leading cause of diarrheal disease in the United States causing in excess of one million cases annually, while STEC is estimated to cause 350,000 cases [1, 5]. These pathogens can be found in the gastrointestinal tract of many people and warm blooded animals [6]. In the case of STEC, most of the animals may be asymptptomatically infected and shed the organism. With *Salmonella*, animals may exhibit a chronic carrier state post infection and shed intermittently. Persistence of these pathogens in the environment is based on a number of factors including initial concentration, organic content, temperature, humidity, pH, moisture and interaction with other microorganisms [7].

The increasing incidence of vegetable associated foodborne disease has increased the focus on its microbial safety. Vegetable contamination can occur prior to harvest or during processing and preparation for consumption. Reduction of pre-harvest contamination is of utmost importance since post-harvest surface decontamination procedures are largely ineffective in removing or eliminating pathogens [8]. Pre-harvest contamination with STEC and *Salmonella*
may occur via direct deposition of contaminated fecal material on the fields by humans and animals and through the use of improperly treated manure. Indirectly this contamination may occur via flooding and use of contaminated irrigation water or equipment.

The objective of our current study was to determine (a) the rate of decline of STEC and *Salmonella* serotypes in the selected animal feces.

**Methodology**

**Experimental Design**

This study utilized a 5x2 factorial design. There were five fecal types and two bacterial cocktail inocula. The five fecal matrices evaluated in this study were: 1) Cattle, 2) Deer, 3) Raccoon, 4) Water Fowl and 5) Feral Pigs. Six serotypes of *Salmonella* and five STEC serotypes were mixed to create the *Salmonella* and STEC bacterial cocktails, respectively, as described below. Each fecal type was independently inoculated with both bacterial cocktails and there were three replicates of each inoculated feces.

**Fecal Collection**

Deer feces were collected from a semi-intensive deer production farm located in Central Ohio. The animals were allowed to graze on confined pastures and their diet supplemented with crushed corn. Feces were collected from both male and female animals of varying ages. The animals were observed in the fields and fresh feces collected from animal when they defecated.

The cattle feces were collected from a research beef feedlot facility in Ohio. These were mainly adult animals and were also semi-intensively reared. They were fed a diet consisting of
hay and processed corn. As with the deer, the animals were observed in the paddock and the fresh feces were collected from animals when they defecated.

Feces from Canadian geese and wild ducks were collected from two public locations in North East Ohio. These animals were not captive, however at one location the birds were seen feeding on grain provided by an individual. Fresh fecal samples were collected from the environment and the feces from both locations pooled. The raccoon feces were collected from an abandoned cabin located on the outskirts of a dairy farm in North East Ohio. Raccoon feces were also collected from a forested site identified by a trapper/hunter on the same dairy farm.

The feral pig feces were obtained from a captive herd reared at the National Wildlife Research Center in Fort Collins, Colorado. The feces were transported via refrigerated air-freight. All fecal samples were stored at 4°C until inoculation

The fecal samples from the five animals were screened to ensure they contained no microorganisms resistant to rifampicin (80 µg/ml). Briefly, one gram of each fecal material was weighed and placed into a separate 15 ml centrifuge tubes. Nine milliliters of buffered peptone water (Accumedia, MI) was then added to each tube, and agitated on a vortex for one minute. One milliliter samples from each tube were then spread plate using glass beads onto lysogeny broth (LB-Miller, Accumedia, MI) agar containing 80µg/ml rifampicin (Fisher Scientific, NJ) and 50µg/ml cycloheximide (Sigma-Aldrich, NJ). These plates were incubated for 24 hours at 37°C and observed for any growth.

Bacterial serotypes

Five Shiga toxin producing *Escherichia coli* (STEC) serotypes were used in this study. These serotypes O103, O104, O111, O145 and O157 were previously isolated from human
clinical disease (Table 3.1). Six *Salmonella* serotypes were used for the preparation of the *Salmonella* inoculum. The *Salmonella* serotypes had also been linked to outbreaks of disease in the human population.

The bacterial serotypes with the exception of *Escherichia coli* O103 were obtained from Dr. Michelle Danyluk, Citrus Research and Education Center, University of Florida. The *Escherichia coli* O103 isolate was obtained from the Minnesota Department of Public Health. The properties of the bacterial serotypes are listed in the table 3.1.

**Rifampicin resistant bacterial mutants**

To distinguish the bacterial inoculum from existing background microflora in the fecal samples, we chromosomally labeled the bacterial serotypes by inducing them to become rifampicin resistant.

Chromosomal marking of the STEC and *Salmonella* isolates was achieved by selecting for naturally occurring resistance to rifampicin. Briefly, each parent isolate was propagated independently overnight at 37°C in tryptic soy broth (Acumedia, MI) and 100 µl of each broth was transferred daily to fresh media with increasing concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml) of rifampicin (Fisher Scientific, NJ). The final broth cultures were plated on LB agar with rifampicin (80 µg/ml) and incubated overnight at 37°C. Three isolates from each broth were recovered from the LB plates were amplified overnight in in Brain Heart Infusion broth and banked at -80°C with 30% buffered glycerol (VWR International, OH) for use at a later time.

**Preparation of the inoculum**
The rifampicin transformed isolates were streaked individually onto labeled LB-Miller agar plates supplemented with 80 µg/ml rifampicin and incubated at 37°C for 24 hours.

One colony from each isolate was then used to inoculate separate 15 ml centrifuge tubes containing 5 ml of lysogeny broth (LB- Lennox, Acumedia, MI) supplemented with 80 µg/ml rifampicin. The contents of centrifuge tubes were then thoroughly mixed on a vortex and placed in the incubator on an orbital shaker (Bellco Biotechnology, NJ) at speed three and incubated for 24 hours at 37°C.

The next day, 1 ml of each broth was used to inoculate separate flasks containing 500 ml of LB-Lennox broth supplemented with 80 µg/ml rifampicin. These flasks were then incubated for 16 hours at 37°C on an orbital shaker (speed 3). Following incubation, 45 ml samples of each inoculum were then placed in 50 ml centrifuge tubes and centrifuged at 5000xg for 20 minutes. The supernatant was decanted and the bacterial pellet resuspended in 45 ml of 1x phosphate buffered saline (AMRESCO, OH). This procedure was repeated two more times to ensure removal of any nutrient content or antibiotic selective pressure. After the third washing and re-suspension of the bacterial pellet, the Optical Densities of the resuspended solutions were measured. Using data obtained from previously plotted growth curves, the solutions were further diluted to obtain an inoculation dose predicted to be approximately $10^4$ to $10^5$ CFU/gram feces.

(a) **Inoculation of feces.**

In triplicate, 30 grams of cattle feces was weighed into sterile WhirlPAK® bag. Approximately 0.5 ml of each of the five STEC inocula were then added to the feces in the WhirlPAK® bag and the contents were stirred using a sterile wooden tongue depressor. The contents of the Whirlpak® where then further mixed using a stomacher/masticator (IUL.
instruments) at high speed (8 strokes per second) for four minutes. The inoculated fecal samples were then measured into one gram aliquots and placed into labeled 15 ml centrifuge tubes and incubated at room temperature (22°C ±3°C) until day of testing. This inoculation procedure was repeated for the deer, water fowl, feral pig and raccoon fecal samples. This procedure was also repeated for the *Salmonella* inoculation for each of the fecal types.

**Sampling and bacterial enumeration**

The bacterial counts were performed on days 0,1,3,5, 7, 14 and monthly post-inoculation. On each of the specified days, three of each of the one gram aliquots from each inoculated fecal types were sampled.

Nine milliliters of buffered peptone water (BPW, Acumedia,MI) were added to each tube and the contents agitated on a vortex for one minute to ensure thorough mixing of the fecal suspension. In some cases vortexing alone was not sufficient and the fecal pellet had to be crushed using sterile wooden sticks. The fecal suspension was then further diluted by adding 155 μl of the fecal suspension to a 2 ml microcentrifuge tube containing 1400 μl of BPW. The contents of this tube were then mixed using a vortex. The fecal suspensions were then serially diluted further by transferring 155 μl of each dilution to subsequent microcentrifuge tubes each containing 1400 μl of BPW.

One milliliter from each tube was then surface plated using sterile glass beads onto 150 ml LB-Miller agar plates supplemented with 80 μg/ml rifampicin and 50 μg/ml cycloheximide (Sigma-Aldrich, NJ). The plates were placed in the incubator for 24 hours and the number of colonies counted. The 15 ml centrifuge tube containing the 10⁻¹ dilution of the fecal suspension
was placed in the incubator for 24 hours at 37°C to allow for enrichment. The quantification limit was calculated to be 10 cells per gram of feces.

When no colony growth was detected via direct culturing on the LB-Miller agar plates after 24 hours, sampling of the enriched fecal suspensions were done. One milliliter of the incubated enriched fecal suspension was surface plated onto 150ml LB agar plates containing 80 µg/ml rifampicin and 50 µg/ml cycloheximide, to determine the presence/absence of colonies. Bacterial death in a fecal type was deemed to have occurred when zero colony counts were obtained from enriched fecal suspensions on three consecutive sampling dates.

Statistical analysis

Colony counts obtained on each day were converted to CFU/gram to determine the bacterial population per gram of feces. On the days where samples were negative on direct plating but positive on enrichment, those samples were randomly assigned a CFU count between 1 and 9. This is because the sensitivity of our methodology was 10 CFU/gram feces, thus cell counts between 1 and 9 would not be detected. Samples negative on direct plating and also negative on enrichment was assigned a zero value. The CFU/gram values were then log_{10} transformed (log_{10} CFU/gram). Using the Software GIaFit V1.6, the best deactivation model was selected. The survival data for the Salmonella and STEC inocula was standardized by using the formula:

\[
\text{Survival} = \frac{\log_{10}(CFU/g +1)_{Dx}}{\log_{10}(CFU/g+1)_{D0}}
\]

Where \(D_0\) = the day of inoculation

\(D_x\) = the day of sampling.
Using a Repeated Measures ANOVA design in Minitab (version 16.2, Minitab Inc), the data was analyzed to determine if there was significant difference in the survival of the STEC and *Salmonella* inocula in the different animal feces. Tukey Method for multiple pairwise comparisons was applied to identify the source of the variation. Statistical differences were deemed significant at $P<0.05$. 
Results

Survival of Shiga-toxin producing *Escherichia coli* in different animal feces (Figure 3.1)

**Cattle:**

The three replicates were inoculated with at an average dose of $4.5 \log_{10}$ CFU/gram feces. For the first three days post inoculation, the CFU/gram feces increased slightly reaching a peak of $4.6 \log_{10}$ CFU/gram feces on day three. After day three there was a gradual but consistent decline in the number of cells obtained on testing days. On day 194, all three bovine samples yielded no growth on direct plating. The first of three replicates were deemed negative by enrichment on day 224 when after three consecutive enriched samples tested (Day 224, 231 and Day 238) recorded no growth.

**Deer:**

The three deer fecal replicates received an average inoculation of $4.5 \log_{10}$ CFU/gram feces. The CFU/gram feces increased gradually until day seven and the peak recorded level was $\log_{10} 5.6$ CFU/gram feces. After day five, there was a gradual decline in the CFU/gram such that by the end of one month (28 days) the bacterial population was almost 58% of the original concentration. From day 82 onward, the fecal samples were negative for growth by direct plating but were however positive for STEC when enriched. On Day 138 the first of three deer replicates were deemed negative for STEC after three consecutive sampling dates (Days 138, 145 & 152) yielded no growth even after the enrichment step had been employed.
Raccoon:

The three raccoon fecal replicates received an average inoculation of $4.4 \log_{10} \text{CFU/gram}$ feces. Similar to cattle, the CFU/gram count increased until day three where the maximum count of $\log_{10} 4.9 \text{ CFU/gram}$ was obtained. However unlike the other animal fecal samples, there was drastic decline in the CFU/gram recorded at 7 dpi. By the 28th dpi the $\log_{10} \text{CFU/gram}$ feces had reached below 1.0 and by day 56 all samples tested negative for STEC by direct plating. Although all samples remained negative on direct plating, they still yielded positive cell counts when enriched.

Water Fowl:

The waterfowl fecal samples received an inoculation of $4.9 \log_{10} \text{CFU/gram}$ feces. The STEC population remained at fairly constant level until day 14. On day 28 post-inoculation, there was a decrease in the STEC population such that the STEC population decreased from $4.0 \log_{10} \text{CFU/gram}$ feces on day 14 to no growth on direct plating on day 28. However, the population increased to $1.3 \log_{10} \text{CFU/gram}$ feces on day 56. All three samples once again tested negative without enrichment on day 82 and continued to be positive on enrichment for the duration of the experiment.

Feral Pig:

The feral pig feces received an inoculation of $4.7 \log_{10} \text{CFU/gram}$ feces. The STEC population peaked on day three, and then gradually declined. All three STEC replicates tested negative on direct plating on day 82. All three samples however continued to test positive for STEC when enriched.
Survival of *Salmonella* Inocula in different animal feces (Figure 3.2)

**Cattle:**

The three replicates were inoculated with an average dose of $5.3 \log_{10}$ CFU/gram feces. No increase in the *Salmonella* population was observed, instead there was a gradual decrease until day 82. From day 82 to 224 there was a downward trend in the *Salmonella* counts. On day 194, all three Salmonella samples tested negative on direct plating. One *Salmonella* replicate was deemed negative by enrichment on day 194 since it yielded no growth even with enrichment on three consecutive sampling dates (day 194, 201 and 208).

**Deer:**

The three deer fecal replicates received an average inoculation of $4.9 \log_{10}$ CFU/gram feces. The CFU increased on day 1 to a peak level of $6.2 \log_{10}$ CFU/gram feces. After day one, there was a gradual downward trend in *Salmonella* population with the greatest decrease seen between days 56 to 82. All three deer replicates tested negative on direct plating on day 194.

**Raccoon:**

The three raccoon fecal replicates received an average inoculum of $4.7 \log_{10}$ CFU/gram feces. Similar to deer feces the CFU/gram count increased on day one to a maximum count of $\log_{10} 5.1$. On the next sampling date, a decrease in *Salmonella* population was observed similar to that observed with STEC. The magnitude of this reduction in the colony count was not seen in other animal feces. By day 28 post inoculation, the $\log_{10}$ CFU/gram feces had reached almost 1.0 and by day 56 all samples tested negative on direct plating (without enrichment) for *Salmonella*. 
Although all samples remained negative on direct plating, they still yielded positive cell counts when enriched.

**Water Fowl:**

The waterfowl fecal samples received an inoculation of $4.8 \log_{10}$ CFU/gram feces. The *Salmonella* population increased to $6.1 \log_{10}$ on day one. After day one there was a constant downward trend in the *Salmonella* population observed. On day 110 all three samples tested negative but were positive on enrichment. On day 138 there was increase of less than one $\log_{10}$ count, however by the next sampling day, day 166, all the samples were once again negative on standard culture. By day 224, all three replicates were deemed negative for *Salmonella*. Only in this fecal type did the inocula not persist in any of the replicates for the duration of the experiment.

**Feral Pig:**

The feral pig feces received an inoculation of $5.0 \log_{10}$ CFU/gram feces. The *Salmonella* population peaked on day one, and then gradually declined until day 194. On day 194 all three replicates tested negative for *Salmonella* on direct plating but were positive on enrichment. On day 224, the *Salmonella* population had increased to $0.91 \log_{10}$ CFU/gram feces.

**Survival Model for Bacterial inocula in different fecal matrices**

The survival data for both inocula in different animal feces were analyzed using GInaFiT V1.6 [9] and the most appropriate model selected based on the adjusted $R^2$ values obtained (Figures 3.3)
The Biphasic model [10] was used for reflecting the survival time of both inocula in different animal feces since it accurately fit the two phases exhibited in the survival curve.

**Decimal Reduction Time for the bacterial inocula in the animal fecal matrices**

The Decimal Reduction Time (DRT) for *Salmonella* and STEC was determined using the respective biphasic model. The adjusted R² values ranged from 0.91 to 0.99 (Table 3.2). The decimal reduction time of the stress sensitive portion of the *Salmonella* inocula was marginally lower than the STEC inocula in cattle and feral pig feces.

No-harvest intervals were based on the time taken to achieve a 4 log10 (4D) reduction in the population of the bacteria. A reduction of 4D represents a 99.99% decrease in the bacterial population. Given that most animals shed STEC and Salmonella at fecal concentrations less than $10^4$ CFU/gram feces [11-13], using the 4D value will reduce the risk of disease transmission. Extending the no-harvest periods beyond this time will not result in any further significant decrease in the bacterial population and thus minimal reduction in the risk of disease transmission.

**Effect of fecal matrix on inocula survival**

**Shiga toxin- producing *Escherichia coli* Inoculum**

Analysis of the bacterial survival data revealed that the fecal matrix did affect the survival of STEC inoculum ($P<0.05$). There was no statistical difference between the STEC survival in the cattle and deer feces, however they were statistically different from the survival in feral pig, raccoon and waterfowl fecal matrices.
**Salmonella Inoculum**

The fecal matrix was found to statistically significantly affect the survival of the *Salmonella* inoculum (*P*<0.05). The survival of the *Salmonella* inocula in the deer feces was statistically different from that of cattle and raccoon. There were no other statistical differences between the other values.

**Discussion**

There are numerous published studies on the survival of STEC and *Salmonella* in cattle and other farm animal feces, however there is a dearth of published data on the survival of these organisms in other fecal matrices. This is the first study reporting the survival of these organisms in deer, feral pig, waterfowl and raccoon feces. The survival data obtained in this study demonstrates the ability of STEC and *Salmonella* serotypes to survive for a prolonged time in animal feces. However, it should be noted that although organisms may persist in the environment, they may not be in a sufficient quantity to cause disease since they will be below the required infectious dose.

The survival of bacteria in the environment depends on many factors including the chemical characteristics, pH, and moisture content of the feces. Survival can also be affected by interactions with other microorganisms in the environment. Predation of bacteria can also reduce its survival ability in the environment as demonstrated by Ravva et al. (2010), who identified a species of protozoa which consumed STEC O157:H7.[14]. Climatic factors such temperature, rainfall and solar radiation can also influence survival of the organism in the environment. The survival of bacteria in the environment can be described as being biphasic with the population
being comprised of a stress sensitive and a stress resistant subpopulation [15-17]. The stress resistant subpopulation refers to that section of the population that has entered the state of resistance/dormancy. The elimination of bacteria from the environment is thus almost impossible since when faced with adverse conditions bacteria may enter a state of dormancy (Gram-positive) or resistance (Gram-negative) [18].

A dairy cow can produce up to 70 kg of wet manure per day and in the United States it is estimated that 100 billion kilograms of cattle manure is produced annually with most of this being applied to fields as fertilizers [19, 20]. Cattle may shed *Salmonella* in their feces at rates of $10^2$ to $10^7$ CFU/gram feces [21]. To adequately reduce the risk of microbial contamination from an animal shedding *Salmonella* at high levels ($10^7$ CFU/gram feces) a no harvest or no cultivable period of 63 days should be instituted to mitigate the threat of microbial transmission. The shedding of STEC from cattle normally ranges from 10 to 100 CFU/gram feces [12, 13]. There are however animals which can act as super-shedders and shed STEC at rates of $10^4$ to $10^7$ CFU/gram feces; however they represent less than 8% of the cattle population. Taking the risk posed by these animals into account, a no harvest or non-cultivatable period of 194 days is therefore suggested. Extending withdrawal periods beyond this time will not significantly reduce the bacterial population since most of these bacteria would be from the stress resistant subpopulation, and represent only a small percentage of the population.

STEC was recovered from cattle feces via direct plating until day 166, while *Salmonella* was still being detected via direct plating at day 224. STEC was still being recovered from cattle feces at day 224 on enrichment. Other studies focused on O157:H7 have demonstrated that the survival time ranged from 49 days to greater than 220 days [21-25]. Non-O157 serotypes have also been demonstrated to persist for extended periods in bovine feces. Fukushima et al (1999)
demonstrated the survival of O26 and 0111 for up to 18 weeks under laboratory conditions with the inoculated feces being incubated at 15°C. This serotype was also demonstrated to survive for 90 days under field conditions in manure heaps left unturned [26]. Salmonella has been reported to persist for a greater time than STEC when exposed to similar conditions[27]. Salmonella serotypes have been reported to persist for up to two years in manure and manure amended soils [20, 28, 29]. The survival of Salmonella in the environment can differ between serotypes possibly due to their host adaptability, with host non-specific not required to persist in the environment as long as host specific serotypes [30].

Previous studies have indicated an incidence level of 3% for O157 STEC and 5% for non-O157 STEC in feral pigs [31]. The prevalence of Salmonella in feral swine has been reported to range between 12 to 22% [32, 33]. Although feral pig manure is not used for soil amendment, the intrusion and defecation of the animals on vegetable fields can result in contamination of soil and entry of foodborne pathogens into the food chain [34]. Similar to cattle feces, Salmonella could be recovered from feral pig feces up to 224 days post-inoculation, however STEC was only recovered until day 56 on direct plating. The Decimal Reduction Time for the stress sensitive portion of the Salmonella inocula was 22 days and it was 19 days for the STEC inocula. There are limited published reports reporting the concentration of STEC and Salmonella in the feces of these animals. Using a 4D value (99.99% of organism killed), will mean that 138 days and 52 days will be adequate no-harvest/non cultivatable guidelines post contamination with Salmonella or STEC contaminated feces.

There are an estimated 30 million wild deer in the United States. The prevalence of STEC in deer has been reported to be as high as 2.4% [35] and the estimated prevalence of Salmonella in deer has been reported to range from 1% to almost 8% [36, 37] Similar to feral pig, the main
risk of vegetable contamination from deer feces is their intrusion and defecation on vegetable
fields or water sources used for irrigation. The *Salmonella* inoculum was found to persist similar
to STEC in deer feces. *Salmonella* was recovered via direct plating up to day 166, but STEC was
only recovered until day 56. Following field contamination with deer feces, the implementation
of no harvest guidelines of 78 days (*Salmonella*) and 52 days (STEC) will result in 99.99% of the
stress sensitive bacterial population dying. Similar to cattle and feral pig, extending the
guidelines beyond this time period will not result in significant decrease in the bacterial
population.

Water fowl are identified as a source of surface water and pasture contamination [38-40].
They can also contaminate many recreational areas when they defecate. One goose is reportedly
capable of producing up to five pounds of feces per day and this can result in mass
contamination, since these birds are usually found in flocks [41]. Previous research has identified
geese as a source of numerous foodborne pathogens including *Salmonella, Campylobacter,*
*Listeria* and STEC O157:H7 [42-45]. These birds are also able to travel large distances per day
and thus disperse pathogens over a wide area. Geese are known to forage within vegetable crops
and also inhabit ponds and other surface water sources [46]. The birds can thus contaminate
produce when they defecate within vegetable fields and water sources used for irrigation. For
this study we pooled wild duck and Canadian geese feces. STEC was recovered until day 166,
whilst *Salmonella* was recovered until day 138. The mitigation of the threat of these foodborne
pathogens in water fowl feces will require no harvest guidelines of 32 and 74 days respectively
for *Salmonella* and STEC.

Raccoons can reside in a wide range of habitats including agricultural, forested and urban
areas. Raccoons have been identified as a reservoir for numerous pathogens including
Salmonella, Leptospira, and Campylobacter [47-49], however there is only one report of STEC being isolated from raccoons feces. This animal had been residing within the hay barn of dairy farm [50]. Despite an extensive literature search no other reports of STEC raccoon infection could be found. Neither Salmonella nor STEC was recovered after day 56 on direct culture, but samples remained positive on enrichment. Field contamination with raccoon feces requires a no-harvest or non-cultivable time guideline of 32 days to mitigate the transmission of STEC and Salmonella

**Conclusion**

The prolonged survival time of STEC and Salmonella serotypes under laboratory conditions demonstrate their ability to persist long periods. Contamination of fields with these pathogens can occur via the application of untreated manure or deposition of feces by infected animals. Producers adopting a no harvest period or non-cultivable of 194 days will sufficiently mitigate the possibility of transmission of STEC or Salmonella regardless of the animal fecal matrix it is shed in.
Acknowledgements

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References


### Table 3.1. The *Salmonella* and STEC isolates used in the Survival Study.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Reference code</th>
<th>Clinical Outbreak</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> Typhimurium LT-2</td>
<td>ATCC 700720</td>
<td>Environmental isolate</td>
<td><em>Salmonella</em> enterica subsp. Typhimurium (ATCC® 700720™)</td>
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<td><em>Salmonella</em> Montevideo</td>
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<td>Human- tomato linked</td>
<td>University of Florida</td>
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<td><em>Salmonella</em> Anatum</td>
<td>K2669</td>
<td>CDC clinical isolate – Tomato outbreak</td>
<td>University of Florida</td>
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<td><em>Salmonella</em> Javiana</td>
<td>ATCC BAA-1593</td>
<td>Pennsylvania tomato outbreak</td>
<td>[51]</td>
</tr>
<tr>
<td><em>Salmonella</em> Branderup</td>
<td>04E61556</td>
<td>Roma Tomato Outbreak</td>
<td>University of Florida</td>
</tr>
<tr>
<td><em>Salmonella</em> Newport</td>
<td></td>
<td>Environmental isolate from Tomato</td>
<td>University of Florida</td>
</tr>
<tr>
<td><em>E.coli</em> O111</td>
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<td>Apple – New York Clinical isolate</td>
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<td>Spinach Outbreak</td>
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<td><em>E.coli</em> O103:H2</td>
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<td>Minnesota – Venison processing</td>
<td>Minnesota Department of Public Health</td>
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Table 3.2. Decimal reduction time for *Salmonella* and STEC Serotypes in different animal feces.

<table>
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<tr>
<th>Inocula</th>
<th>Fecal sample</th>
<th>Adjusted R²</th>
<th>Decimal reduction time (days)</th>
<th>No-harvest interval (days)</th>
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<td>STEC</td>
<td>Cattle</td>
<td>0.98</td>
<td>22</td>
<td>194</td>
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<td></td>
<td>Deer</td>
<td>0.96</td>
<td>19</td>
<td>52</td>
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<td></td>
<td>Feral Pig</td>
<td>0.97</td>
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<td>52</td>
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<td></td>
<td>Waterfowl</td>
<td>0.95</td>
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<td></td>
<td>Raccoon</td>
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<tr>
<td><em>Salmonella</em></td>
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<tr>
<td></td>
<td>Deer</td>
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<td></td>
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<td>0.97</td>
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<tr>
<td></td>
<td>Raccoon</td>
<td>0.99</td>
<td>9</td>
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Figure 3.1. Survival of STEC inocula in different animal feces. Survival = \( \frac{\log(X_n) + 1}{\log(X_0)} \), where \( X_n \) is the CFU/g on day \( N \) and \( X_0 \) is the inoculation dose. Errors bars represent the standard error. Quantification limit (QL) is 0.08  DL- Detection Limit
Figure 3.2. Survival of *Salmonella* inocula in different animal feces. Survival = \([\log(Xn)+1]/[\log Xo]\), where \(Xn\) is the CFU/g on day \(N\) and \(Xo\) is the inoculation dose. Errors bars represent the standard error. Quantification limit (QL) is 0.08; DL – Detection limit.
Figure 3.3 Survival models for *Salmonella* and STEC inocula in different animal feces. Survival = \[
\log_{10}\left(\frac{(X_N+1)}{X_0}\right),
\]\ where $X_N$ is the CFU/g on day $N$ and $X_0$ is the inoculation dose.
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73. Larson, D.G., Prevalence of Shiga toxin-producing Escherichia coli in the Sacramento Valley equine population, in Biological Sciences (Molecular and Cellular Biology) 2009, California State University, Sacramento, 2009.


