CORRELATION OF IN-FIELD SURVIVAL OF ESCHERICHIA COLI O157:H7 WITH AIR TEMPERATURE, SOIL TEMPERATURE, AND SOLAR RADIATION

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CHAPTER I

Introduction

Since the first recognized outbreaks of foodborne illness caused by *E. coli* O157:H7 in 1982, the pathogen has become increasingly prominent as a cause of serious illness in many countries around the world (Mclure, 2000). The sudden emergence of *E. coli* O157:H7 and the association of this bacterium with an increasingly wide range of foods have resulted in the Shiga-toxin producing group of *E. coli* bacteria (STEC) being a major focus for the food industry.

Foodborne diseases have a major public health impact in the U.S. According to the Center for Disease Control (CDC) foodborne diseases affect 76 million persons, cause 325,000 people to be hospitalized, and cause 5,000 deaths per year (Klonsky 2006). In this estimate 73,480 illness, 2,168 hospitalization, and 61 deaths annually are due to *E. coli* O157:H7 (Rangel et al., 2005). The factors contributing to the emergence of foodborne illness include: changes in human demographics and behavior; changes in technology and industry; increased international travel and commerce; microbial adaptation; and economic development and land use (Altekruse and Cohen, 1997).

Changes in food consumption have helped increase the number of foodborne outbreaks. Fresh fruit and vegetable consumption has increased nearly 50% from 1970 to

1994 (Altekruse and Cohen, 1997). This increase is likely due to the fact that consumers are more aware of what they eat now and they tend to want to be healthier. This trend has led to the increased consumption of minimally processed fruits and vegetables. Also the percentage of people eating away from home has increased a great deal. Fast-food restaurant and salad bars were rare in the 50s, now they are the primary sites for food consumption (Altekruse and Cohen, 1997). Outbreaks outside the home account for almost 80% of reported outbreaks in the U.S. in the 1990s (Altekruse and Cohen, 1997). International travel has increased dramatically during the 20th century. About 5 million international tourist arrivals were reported worldwide in 1950, and the number is expected to reach 937 million by 2010. Such travel facilitates the spread of foodborne illness because these tourists can become infected with a foodborne pathogen unknown in their home country during their journey, and by the time the symptoms start to appear they will have returned home (Altekruse and Cohen, 1997).

Many of the original *E. coli* O157:H7 outbreaks were related to undercooked or raw foods of bovine origin. However, the past decade has seen an increase in the number of outbreaks of human illness due to the consumption of minimally processed, ready to eat fresh produce (Warriner et al., 2003). Produce associated outbreaks were first reported in 1991, and have since gained in prominence (Rangel and Al., 2005). In any given year 39,000 hospitalizations and about 60 related deaths are expected due to foodborne illness related to produce (Klonsky, 2006). Since 1995, there have been 16 outbreaks of illness caused by *E. coli* O157:H7associated with spinach or lettuce (Gourabathini and Al., 2008). One particularly noteworthy outbreak occurred in August and September of 2006 and involved pre-bagged spinach contaminated with *E. coli*

O157:H7. This outbreak drew national attention due to the geographic dispersion of the reported illness across 26 states, causing 205 confirmed illnesses (Klonsky, 2006).

Clearly, the contamination of fresh produce, especially leafy greens such as spinach, with pathogenic bacteria such as *E. coli* O157:H7 is a serious public health issue. In this study we seek to help quantify the risk of *E. coli* O157:H7 contamination of the edible parts of spinach plants via inoculated soil. We will also look at the effect of environmental conditions such as soil temperature, air temperature, and solar radiation on the survival of the pathogen in the soil.

CHAPTER II

REVIEW OF LITERATURE

I. Epidemiology:

According to the CDC *E. coli* was recognized has a foodborne pathogen in 1982, after the investigation of two outbreaks involving hemorrhagic colitis in Oregon and Michigan (CDC, 1982). It was not until 1993 that *E. coli* was broadly recognized as an important and threatening pathogen, after a large multistate outbreak linked to undercooked hamburger patties from a fast food chain were identified as the vehicle of infection (Rangel et al., 2005). More than 700 persons in four states where infected; there were 51 cases of hemolytic uremic syndrome (HUS), which often results in kidney failure, in four states. Since that outbreak the reported incidence of serotype o157:H7 infections has risen, mainly due to better surveillance systems that have been implemented and increased awareness among physicians, clinical microbiologists, and consumers (Feng 1995). In the United States, there are an estimated 73,480 illnesses caused annually by*E. coli* O157:H7, leading to an estimated 2,168 hospitalizations and 61 deaths. After the first outbreak, human infection with *E. coli* O157:H7has been reported from over 30 countries on six continents (Mead and Griffin, 1998).

II. Background and microbiology of *Escherichia coli* O157:H7:

During the past decade Escherichia coli O157:H7 has evolved from a clinical novelty to a global public health concern (Mead and Griffin, 1998). *Escherichia Coli*, usually known as *E. coli*, are a large and diverse group of bacteria. The bacterium was discovered by German bacteriologist Theodore von Escherich in 1885. The bacterium belongs to the family Enterobacteriaceae and is a short, Gram-negative bacillus. It is a non spore-forming, rod shaped, facultative anaerobe, and is usually motile. Its optimum temperature for growth is 37 °C and colonies are usually non-pigmented, circular with smooth edges. One of the most important control measures for *E. coli* O157:H7 is heating. Numerous studies have shown that *E. coli* O157:H7is not unusually heat-tolerant, compared to other *E. coli* and other members of the Enterobacteriaceae. Temperature regimes like 70°C for 2 min that are usually used to control other infectious pathogens are more adequate for eliminating large number (10^6 cells/g) of *E. coli* O157:H7(Mclure, 2000).

E. coli is normally found in the lower intestines of warm blooded organism. Cattle have been known to be the main reservoir of the pathogen. *E. coli* is one of the dominant microflora in human and animal feces, and hence it has historically been used as an indicator of fecal contamination.

E. coli strains are divided into six groups based on virulence properties. They are enteropathogenic *E. coli* (EPEC), enterohemorragic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggreative *E. coli* (EAEC), and

diffusely adherent *E. coli* (DAEC) (Fratamico et al., 2005). The one type that people are more concerned with is within the EHEC *E. coli* group due to the severity of the illness that they can cause and their infectious dose, which is reportedly as low as 50 organisms. The most problematic organism within this group has been *E. coli* O157:H7 (Duffy, 2003).

E. coli O157:H7 is so named because it expresses the 157th somatic (O) antigen and the 7th flagella (H) antigen (Mead and Griffin, 1998). *E. coli* causes diseases in human by making toxins, known as shiga-toxin. Among the most important virulence characteristics of *E. coli* O157:H7 is its ability to produce one or more shiga-toxins. Shiga-toxin (Stx), also called verocytotoxins, and formerly known as shiga-like toxin, are a family of bacterial cytotoxins produce by shigella dysenteriae type 1 and shiga-toxin producing *E. coli*. The most common shiga-toxins produced by *E. coli* O157:H7 are Stx1 and Stx2 (Mead and Griffin, 1998).

The symptoms due to the infection generally differ by person but often include severe abdominal cramps, often bloody diarrhea, and vomiting with little or no fever. Production of shiga-toxins is not in itself sufficient to cause disease. There are other factors thought to contribute to the virulence of *E. coli* 0157; these include a 60 MDa virulence plasmid and the locus of enterocyte effacement (Mead and Griffin, 1998).

III. Clinical Symptoms and illness:

The clinical manifestations of *E. coli* O157:H7infection vary from symptom-free carriage to non-bloody diarrhea, hemorrhagic colitis, and abdominal cramps. In elderly, infants, and children *E. coli* O157:H7 infections can cause hemolytic uremic syndrome

(HUS) which results most often in kidney failure. Survivors of HUS, even after the clinical treatment, sometimes suffer a range of permanent disabilities including chronic renal insufficiency, hypertension and neurological deficits. The average interval between exposure and illness is 3days. Illness typically begins with abdominal cramps and non bloody diarrhea. Bloody diarrhea starts after 1 or 2 days. About 70% of the people that reported their illness report that they had bloody diarrhea, 30% had fever (Mead and Griffin, 1998). Usually, people infected with *E. coli* O157:H7 can recover from hemorrhagic colitis without using antibiotics for 5 to 10 days. It is not recommended to treat the disease with antibiotics, because some antibiotics may precipitate kidney complication without improving the course of the disease (Mead and Griffin, 1998). The severity of the disease it causes in combination with the low infectious does make *E. coli* O157:H7 a serious foodborne pathogen (Armstrong et al., 1996).

IV. Vehicles of transmission:

It is known that *E. coli* O157:H7 are mainly carried by cattle. Contamination of the beef carcass with the pathogen can be dangerous to the humans, because they can be infected by consuming undercooked beef products. Also, direct contact with cattle or their environment could be considered as another transmission vehicle. The first report of outbreak due to animal contact was in 1996. From 1996 to 2002, direct or indirect cow or calf exposure was noted in all 11 outbreaks: 5 on farms, 2 at county fairs, 2 at petting zoos, 1 at a barn dance and 1 at a camp (Rangel et al., 2005). Aside from farm animals, wild animals, particularly birds and reptiles, can also act as a vehicle for pathogens to contaminate soil and produce that may come in contact into contact with soil (Beuchat 2006).

While *E. coli* O157:H7 are mainly carried by cattle, hey can be present in other animals as well as in humans. Thus, public facilities can become a venue for person- to-person transmission. Indeed, person-to-person fecal-oral contamination has been implicated in at least fifty outbreaks to date. Child daycare centers, individual residences, communities, school, and residential facilities were all locations for those outbreaks (Rangel et al., 2005).

Water is also a source of transmission. Several incidents have shown that both drinking water and recreational water can serve as vehicles for transmitting *E. coli* O157:H7 infections (Feng, 1995). The first and largest waterborne outbreak associated with the pathogen occurred in Missouri in 1989, when people were drinking from a contaminated drinking water supply. Another outbreak caused by *E. coli* O157:H7 in 1991 may have involved recreational lake water in Oregon. A survey showed that those that became ill had swum in the lake during the previous 3-weeks. Transmission occurred when the swimmers swallowed lake water that was fecally contaminated by other bathers (Feng, 1995).

While consumption of contaminated, undercooked ground beef product has accounted for many outbreaks; several different vehicles of infection have been implicated in a number of foodborne *E. coli* O157:H7 outbreaks; among these are salad vegetables, acidic foods, yogurt, and water (Feng, 1995; Mclure, 2000). Outbreaks were associated with fermented sausage in 1995 and pre-sliced pepperoni in 1994. Milk has also been implicated as the vehicle of infection in both raw and pasteurized milk, yogurt (Amstrong et al., 1996). *E coli* o157:H7 infections have been associated with apple cider

also, these products were commonly regarded to be safe because of the low pH (3.6-4.2) (Mclure, 2000).

Additionally, produce including fruits and vegetables have been frequently involved in *E. coli* O157:H7 outbreaks. Reported outbreaks of foodborne illness associated with fresh fruits and vegetables in the USA have nearly tripled since 1973 (Islam et al., 2004). Produce-associated outbreaks were first reported in 1991 and these products remain a prominent food vehicle (Rangel et al., 2005). The contamination event may occur either preharvest or postharvest and with or without subsequent growth of the pathogen eventually responsible for infection or intoxication (Beuchat, 2006). A number of recent outbreaks involved fresh produce such as lettuce, spinach, onion, carrots, radish etc. The *E. coli* O157:H7 outbreak of 2006 associated with the consumption of fresh spinach and shredded lettuce affected 276 people. Of those sickened, 148 people were hospitalized, 29 suffered from hemolytic uremic syndrome, and 3 people died (Sharma et al., 2009).

V. Spinach Horticultural Information:

Spinach (*Spinacia oleracea*), originally from central and southwestern Asia, is a flowering plant in the family of Amaranthaceae that is used as a vegetable. It is an annual herb, grown in fall to spring in temperate region as it is a cool-season plant. Consumers in the United States are eating up to 90% more spinach since 1992 and prefer fresh, which is the highest form with regard to microbial contamination. Fresh spinach consumption accounted for 60% of domestic consumption in 2000-2002 which is more than 7 times greater than in 1970 (Lucier et al., 2004). In 2004 in the U.S. the per capita consumption

of fresh spinach was 1.8 pounds, per capita servings per year was 10, and this rate of consumption translates into 180 servings of spinach per person per year (Klonsky 2006). Fresh spinach sales totaled $157 million in 2005 and accounted for only seven percent of the 2.1 billion in sales of the leafy greens (Klonsky 2006). Spinach is commercially sold loose, canned, bunched, in prepackaged bags, or frozen. Spinach is usually consumed as a salad green or a vegetable plate because of its reputation of a functional food. Spinach is rich in vitamins and minerals (folate and carotenoid content) that are beneficial to human health (Lucier et al., 2004).

VI. Source of microbial contamination of fresh product:

The sources of contamination of leafy greens are diverse; contamination may occur anywhere from the field to the consumers' houses. Fruits and vegetables can become contaminated with pathogenic microorganism while growing in fields or orchards, or during harvesting, postharvest handling, processing, and distribution. Many factors can contribute to microbial contamination throughout production and packaging of fresh produce. These include contaminated irrigation or process water, the use of biosolids or manure for fertilization, poor worker hygiene, and poor equipment sanitation (Johnston et al., 2005).

Pathogens capable of causing human diseases include viruses, bacteria and parasites that may be present in the soil or in the water used for irrigation where the product is grown. Bacteria are still the greatest concern in terms of serious illness and numbers of persons at risk of infection on an international scale (Beuchat, 1995). Several

factors play a role in whether or not pathogens are detected on produce at harvest. Proximity of the edible portion of the plant to the soil is one such factor, root crops or crops that are in proximity to the ground (spinach, lettuce) are more likely to be contaminated compared to products that grow higher. Also the numbers of pathogens in contaminated soil has an impact on the pathogen detection. Higher soil populations increase the risk that produce will be contaminated at harvest (Doyle and Erickson 2008). Ready-to-eat fresh vegetables, fruit, and prepared salads have a high potential risk of contamination because they are generally grown in a natural environment, and are often consumed without cooking or other treatments that could eliminate pathogens if they are present.

1. **Pre-harvest:**

Source of microbial pathogens on fresh produce at the preharvest stage include feces, irrigation water, inadequately composted manure, soil, air, animals and human handlers (Islam et al., 2004). A number of recent outbreaks have been linked to contaminated water (Solomon et al., 2002). Water can be a particularly troublesome vehicle of bacterial contamination. Flooding is a special concern because floodwaters can be contaminated with animal manure, which is then carried into production fields. The source of water used to irrigate the field also influences the potential contamination of spinach and other fresh produce. Wastewater and effluents are sometimes used as water sources in areas that are short on water; if those waters are not properly treated they become a source of contamination. The other source of contamination is animal contact. This occurs when fields are contaminated by animals that have grazed and shed their manure in the field, which may contain enteric pathogens.

2. **Harvesting:**

Human and mechanical contact also played a role in the contamination of the spinach. If spinach is handled by hands that have not been washed after defecation, bacteria can contaminate the product. Fresh market spinach is field packed. The entire plant is harvested at some point during the time from when it has five to six leaves to just before seed stalk formation. Plants are hand cut and tied into bunches of 8 to 12 plants (Lestrange et al., 2009). This hand harvesting and post-harvest handling increases the risk of contaminated hands transferring bacteria from fecal material to the spinach. Pathogens may also be transferred from unsanitary equipment and can contaminate the spinach that comes in contact with the equipment. Thus, equipment sanitation as well as workers' personal hygiene can play major roles in controlling and preventing contamination of spinach by pathogens during harvest and packing. Farm workers should be trained and practice good personal hygiene and farming equipment and machinery used for harvesting should be properly maintained, cleaned and sanitized after each use.

3. **Post-harvest:**

After harvesting, contamination of the produce may occur at different stage of the packing and processing. At commercial plants, spinach processing involves washing, drying, and packing (Ilic et al., 2008). Processing steps for leafy greens typically include direct human contact, immersion in water, as well as cutting or slicing, all of which have potential for contamination (Brackett 1999). Thus, proper sanitation practices for employee cleanliness and for equipment cleaning need to be followed in the processing environment as well.

Studies have demonstrated that *E. coli* O157:H7 attaches preferentially to the cut edges of lettuce leaves as well as to distinct features on the leaf surface such as trichomes, stomata, and cracks in the cuticle (Brandl 2008). *E. coli* can multiply on cut lettuce over a prolonged time periods during storage at temperatures ranging between 10 and 15°C, particularly when pretreated with warm chlorinated water (Brandl 2008). Therefore, proper temperature control is also critical in slowing or preventing the growth of undesirable microorganisms in a processing facility.

During distribution, fresh produce is often exposed to critical conditions; an example of this is when the delivery truck is not cooled properly. As noted above, temperature abuse may promote the growth of pathogenic microorganisms. Another way that contamination can occur during post-harvest transportation is through cross-contamination: fresh produce may be transported with raw meat, for example or in unwashed trucks. We know that raw meat is a potential source of pathogenic bacteria, which can contaminate the fresh produce.

VII. Post-harvest treatment and their effectiveness

Studies have shown that the earlier the microbial contamination occurs during production/processing, the more difficult it is to remove the pathogen. Fresh produce is usually washed and sanitized soon after harvest in order to reduce microbial load and kill pathogens that may be present. Various types of washers are available for fresh fruit and Vegetables. These include brush washers, reel washers, pressure washers, hydro air agitation wash tanks, and immersion pipeline washers. These units were designed to

remove soils from produce, but questions still remain about their ability to remove or inactivate bacterial contaminants (Gerald Sapers 2001).

A lot of studies have shown that the common practice of washing leafy greens is relatively ineffective in drastically reducing or eliminating microbial contamination. Chemical sanitizers are limited in their effectiveness due partly to the formation of microbial biofilms and also the physical structure of the plants that limit the accessibility of sanitizers to the sites where microorganisms reside. With respect to biofilms, the various microbial populations naturally present on leaf surfaces may have different susceptibilities to chlorine treatment. Thus, chlorine treatment may create conditions for growth of certain bacterial populations that in turn favor or limit pathogen survival.

With respect to plant structure, cells of *E. coli* O157:H7 have been shown to penetrate into the stomata and junction zones of cut lettuce leaves, becoming entrapped below the cut. Those cells located at the subsurface location were protected from sanitation with chlorine (Solomon et al., 2002).

Assuming that the internal tissues of spinach or other produce do become contaminated, relatively rigorous procedures are required to disinfect the plant tissues; methods such as irradiation that can penetrate the plant tissue may be effective. Niemira compared the method of sodium hypochlorite wash with irradiation to inactivate *E. coli* O157:H7 internalized in leaves of romaine lettuce and baby spinach (Niemira 2007). Their results showed radiation was more effective than chemical sanitizers against *E. coli* O157:H7 cells internalized in leafy green vegetables. The US Food and Drug Administration (FDA) authorized the use of irradiation in the food industry to kill *E. coli* in fresh produce like spinach. They have ruled that irradiation is safe, does not diminish

the nutritional value of the spinach, and it does not make the product radioactive. Even so, irradiation has not yet been commonly used for commercial post-harvest fresh produce in part because of the consumer concerns.

Application of anti-microbial agents in the vapor phase might be another means of reaching microbial contaminants attached in inaccessible sites. For example, acetic acid vapor treatment of cabbage, mung bean seeds, and grapes has been shown to reduce microbial populations and delay decay (Sapers 2001).

VIII. Interaction between fresh produces and the pathogen

Attachment of *E. coli* to leafy greens may be influenced by several different mechanisms, including bacterial surface charge (Dickinson and Koohmaraie 1989), hydrophobicity (Dickinson and Koohmaraie 1989), and extracellular polymeric substances and the presence of fimbriae (Wells et al., 2005). Also there are several factors, such as produce type, cultivar, and physiological state of the plant and that has an impact on the colonization of foodborne pathogen on produce (Critzer and Doyle 2010). Bacterial cells tend to locate in pores, indentations or other natural irregularities on the intact surface where there are protected binding sites. *E coli* O157:H7 also attach at cut surfaces, or in punctures and cracks in the plant tissue surface (Sapers 2001).

According to one study, once attached to the tissue of a fruit or vegetable, bacteria might become incorporated into a biofilm, which is an extracellular polysaccharide matrix that holds the cells together and glues them to the plant tissue surface (Gerald Sapers 2001). *E coli* O157:H7 in particular has been shown to be capable of producing a biofilm on the surface of the spinach leaves (Wells et al., 2008). Attachment to

biological and abiotic surfaces is the first critical step in colonization and is mediated by bacterial adhesins. In some cases, adhesion results in the proliferation of bacterial cells into more complex biofilm structures (Wells et al., 2008). Biofilm formation is associated with enhanced resistance to antimicrobials and hydrodynamic shear forces and thus may contribute to outbreaks of *E. coli* O157:H7 infection. Biofilm formation by *E. coli* O157:H7 has been demonstrated on plant surfaces and abiotic surfaces such as stainless steel, glass and plastic (Wells et al., 2008).

IX. Internalization of the pathogen:

Internalization of *E. coli* is when the bacteria enters the plant and become entrapped within it. In general, plants are not considered as hosts for enteric pathogens; however recent foodborne outbreaks associated with leafy greens have raised a great concern among scientific researchers about the possibility of internalization. The presence of pathogens in the soil is likely to be one of the sources for contamination which enhances the risk of transmitting pathogens to the surface of the plants before harvest, which may then potentially be internalized into the edible portion of the plants during germination and growth (Solomon et al., 2002).

Internalization is considered as one potential route of contamination of leafy greens. Different routes of uptake of the bacteria by spinach were observed during a study by Mitra et al. (2009). In the study, three different spinach cultivars with different leaf surface morphologies were used. The objective of the study was to monitor bacterial location, density, and movement after introduction of bacterial cells via four different routes. Those different routes were intake through the leaf surface via drop inoculation,

uptake through the roots from the soil via soil inoculation, direct leaf inoculation by stabbing, and direct leaf surface inoculation with applied pressure. Those methods were designed to mimic potential avenues of in-field contamination. Leaf drop inoculation mimicked contamination by sprinkler irrigation, stab inoculation mimicked natural wounding. Plant inoculated with the bacterium by pressure infiltration, which forced bacteria into the intercellular spaces of the leaf interior, resulted in very high bacterial densities. This indicates that the bacterium can survive for a long period inside the surface. The bacterium was present and viable for up to 2 weeks after inoculation (Mitra et al., 2009). *E coli* were found in plate counts of all inoculation methods except leaf drop. There was little evidence that the bacterium were internalized in the spinach by any treatment other than pressure infiltration of the leaf surface.

X. Manure as a fertilizer:

Recent scrutiny of the role of agricultural practices in contamination of fresh vegetables with pathogenic microbes has led to concern about the safety of using animal manures as fertilizer in vegetable production (Ingham et al., 2004). Animal wastes and effluents from farming practice include feces, manure and slurries. Feces are regarded as freshly deposited feces, manure are feces that have undergone some period of storage, and slurries are mixture that include manure urine and leftover feed that is held in a tank or pit, generally under anaerobic conditions (Duffy 2003).

Many outbreaks or cases of *E. coli* O157:H7 infection have been associated with water or food directly or indirectly contaminated with animal manure (Jiang et al., 2001). In the North Central region of the United States there are an estimated 10.4 million dairy

and feedlot cattle, and they produce between 23.6 and 35.5kg of manure per day (Ingham et al., 2004). It has traditionally been thought that the best way to dispose of this large amount of manure is by using the manure as fertilizer. Adding bovine manure to the soil has agronomic benefits through the addition of plant nutrients and organic matter. Manure nutrients help build and maintain soil fertility. It has been shown that manure can also improve soil tilth, increase water holding capacity, lessen wind and water erosion, improve aeration and promote the growth and survival of beneficial organisms (Islam et al., 2004).

However, bovine manure also contains enteric pathogenic microorganisms, for example *E. coli* O157:H7. To prevent contamination of soil with the manure, proper composting should be done. Composting is a heat treatment of the raw manure that will eliminate most pathogenic bacteria. Only a limited amount of manure is composted and the process may not be done under strict control. Usually with controlled conditions of aeration, moisture, particle size and carbon-nitrogen ratio of combustible material, composting temperature of 55-65° C can be reached, which would be sufficient to inactivate pathogens including *E. coli* O157:H7 (Duffy 2003).

XI. Survival of *E. coli* O157:H7 in the soil:

Although there are no significant environmental sources of *E. coli* and other fecal bacteria that are unrelated to direct fecal contamination, there have been studies supporting the idea that fecal bacteria can survive and grow for some period of time in the environment (Meays and other 2005). *E. coli* O157:H7 appears to have unique growth and survival characteristics, being able to survive under a wide range of environmental

conditions (Jones 1999). Long periods of survival in the soil have also been documented. In a study by Rasmussen and Casey (2001), survival was greatest (130 days) on soil cores containing rooted grass. After inoculation of the pathogen to the pasture, the population of *E. coli* O157:H7 decreased 4 to 5 log units within 50 days, but the pathogen was still detectable in the soil 99 days after inoculation (Rasmussen and Casey, 2001). Studies have demonstrated that *E. coli* O157:H7 can multiply in the soil, move through soil profiles, and be released into drainage water if there is sufficient rainfall occurring within a few days after application. Bacterial movement through the soil was more restricted in intact clay loam soils, which are less permeable than sandy soil (Rasmussen and Casey 2001).

Sunlight has been reported to be one of the most detrimental factors to the survival of *E. coli* in water, and available water has been suggested as being the most critical factor for *E. coli* survival in soil (Meays and other 2005). The type, the temperature, and the pH of the soil also have been observed to have an impact on the survival of the pathogen in the soil. With respect to temperature, *E. coli* O157:H7 has shown the ability to survive at 4°C for 3 months and has been observed to grow at 6°C (Jones 1999)

Conclusion:

In conclusion, there are numerous ways for bacterial pathogens to contaminate spinach during its growing process. With the growing market for fresh leafy greens, studies need to be conducted to ensure that consumers have a product free of pathogens. To do so, scientists need to understand the characteristics of the pathogen, how it

interacts with the produce in the field, and if there is a possibility of internalization. Then, sanitation protocols need to be developed so that the pathogen is reduced in number and eradicated from the produce if possible. With these goals in mind, this project is designed to test the hypothesis that the edible part of the spinach plant can be contaminated while using bovine manure inoculated with *E. coli* O157:H7 as a fertilizer, and also to test the hypothesis that environmental effects such as soil temperature, air temperature, and solar radiation have an impact on the survival of the bacterium in the soil.

CHAPTER III

MATERIALS AND METHODS

Introduction

During the past decade there has been an increase in the number of foodborne outbreaks associated with the consumption of fresh produce. Changes in food consumption have helped drive the increase in those outbreaks. Consumption of minimally processed vegetables has grown in the last decade due to their convenience and the trend toward a healthier diet. Consumers are more aware of what they eat now, and they tend to want to be healthier by eating foods perceived to be health-promoting. Consumption of fresh spinach alone grew by 130% between 1999 and 2006 in the United States (Ilic et al., 2008).

Outbreaks of food borne illnesses associated with consumption of fresh produce have also increased -- from 1% in the 1970s to 12% during the 1990s. From 1990 to 2004, fresh produce was responsible for the largest number of foodborne illness, accounting for 21% according to the Center for Science in the Public Interest Database (Ilic et al., 2008). In any given year, 39,000 hospitalization and about 60 related deaths are expected due to food borne illness related to fresh produce (Klonsky 2006).

Since the first recognized outbreaks of foodborne disease associated with *E. coli* O157:H7 in 1982, the pathogen has become increasingly prominent as a cause of serious illness in many countries around the world (Mclure 2000). Although many of the outbreak of foodborne illness associated with *E. coli* O157:H7 were attributed to food of bovine origin, such as ground beef, it appears that there has been an increase in the number of outbreaks associated with the consumption of minimally processed, ready-to-eat fresh produces (Warriner et al., 2003).

Sources of microbial pathogens on fresh produce include feces, irrigation water, inadequately composted manure, soil, air, animals and human handling at the preharvest stage. During harvesting, produce may become contaminated by human and mechanical contact; the pathogen may be transferred from unsanitary equipment and contaminate the produce. Also, during postharvest handling, contamination can occur at different stages of processing, distribution and storage.

Cow manure is commonly used as a fertilizer in agricultural production. Recent scrutiny of the role of agricultural practices in the contamination of fresh vegetables with pathogenic microbes has led to concern about the safety of using animal manures as fertilizer in vegetable production (Ingham et al., 2004). Most of the outbreaks of *E. coli* O157:H7 infection have been linked with water or food directly or indirectly contaminated with animal manure. In September 2006, a multistate outbreak of *E. coli* O157:H7 was linked to bag of fresh baby spinach (CDC 2006). This incident resulted in over 200 illnesses and at least three deaths in parts of Canada and across 26 states in the United States. The contaminated spinach associated with this outbreak was traced to the Central Coast of California (Stuart 2008). Feral swine were implicated as possible vectors

for the contamination of the spinach as they might have carried cattle feces and indirectly contaminated surface waterways or soil. This outbreak served as a reminder that there is growing concern with fresh produce and foodborne illness.

Recently it has been demonstrated that *E. coli* O157:H7 can colonize the leaf tissue of lettuce endophytically during growth in contaminated manure amended soil. This may constitute a public health risk as the bacteria are unlikely to be removed during postharvest sanitation or washing by consumers (Franz et al., 2008). This study was designed to investigate the risk of in-field contamination of the edible part of the spinach when using bovine manure inoculated with *E. coli* O157:H7 as a fertilizer. The study was also intended to examine the impact of environmental weather conditions on the survival of the bacterium in the soil.

Materials and Methods

Microorganisms and preparation of inoculum

In this study, a non-pathogenic strain of *E. coli* O157:H7 (ATCC 43888) was used. The culture was obtained from the culture collection of a food microbiology laboratory in the Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University. The bacterial strain was subcultured by transferring 100µL of the culture into 9.9ml of Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, Michigan) and incubating at 37°C for 18h. The procedure was repeated three times daily just prior to the experiment, and the resulting culture was incubated at 37°C for 18h. A purity check of the *E. coli* O157:H7 was performed after the culture was subcultured three times. This

was done by adding 1ml of the *E. coli* O157:H7 mixture (3rd subculture) to 9ml of peptone water. Serial dilution of the mixture was done and the diluted culture was plated in duplicate using a direct plating method with Tryptic Soy Agar (TSA). Plates were incubated at 37°C for 24h. The following day one single colony from the TSA plate was picked and a gram stain was performed. Before the day of the experiment, 2.5ml of the inoculum was added to 250ml of TSB and incubated for 18h.

Experimental design

The field experiment was done at the Oklahoma State University Cimarron Research Station in Perkins, Oklahoma starting on November 6th, 2010. The soil characteristics of the field site were determined by analyses at the Oklahoma State University Soil, Water, and Forage Analytical Laboratory. Plots were arranged in a randomized block design with five replications of the four treatments. The different treatments were: control, which did not receive any amendment other than tilling; treatment two, which was the addition of non-inoculated manure to the plot; treatment three, which was the addition of manure inoculated with *E. coli* O157:H7; and treatment four, which was the application of *E. coli* O157:H7 inoculum directly to the soil of the plot. The individual plots were about 6 m (20 ft) long and 5.5 m (18 ft) wide with 5.5 m (18 ft) between plots within each block and 3 m (10 ft) of alleyway between the blocks of plots. The total width of the experimental field was about 43 m (140 ft) by 22 m (72 ft). There was also a 10 ft alleyway between the blocks of plots. The entire experimental area received 79 kg of actual nitrogen and 62 kg of phosphorus (P_2O_5) per hectare before planting, which was incorporated to a depth of 2.5- 5 cm using a tractor mounted rototiller.

Treatment of soils

Commercial bags of manure ("Black Kow" composted cow manure, Black Gold Compost Co., Oxford FL) were used to inoculate the plots that were assigned the manure treatment and the manure plus *E. coli* O157:H7 treatment. A slurry type of manure was made by adding about 19 l of water to about 11.3 kg of manure and mixing it vigorously with an electric mixer for approximately 1-2 minutes. This slurry was applied to a single plot and the process was repeated for all manure-treated plots. The slurry inoculated with *E. coli* O157:H7 was mixed with 250ml of the culture, which contained ~$5.3x10^8$ CFU g^{-1}. Manure slurry was prepared and applied to all non-inoculated plots prior the preparation and application of inoculated slurries. Slurries were spread uniformly on the plots and the plots were then tilled to a depth of about 15-20 cm with a tractor-mounted rototiller.

The plots that were assigned to treatment 4, direct-spray application of *E. coli* O157:H7 inoculum, were treated by adding 250ml of the culture at ~$5.3x10^8$ CFU g^{-1} to 3.79 l of water in a hand-pump sprayer, mixing, and then spraying the inoculum uniformly across the treatment plot using one flat-fan spray nozzle. After the inoculum was applied, the plots were then tilled to a depth of about 15-20cm with a tractor-mounted rototiller. All non-inoculated plots were tilled prior to tilling inoculated plots.

After application of the different treatments, all plots were directly seeded with spinach seed (Olympia variety) using a Hege model 1000 plot planter. All non-inoculated plots were seeded prior to seeding inoculated plots. Following treatments of the soil and planting of the spinach seeds, the entire experimental area received an herbicide

application: Dual Magnum (S-metolachlor) at a rate of 0.73kg active ingredient per hectare.

Sampling of soil and spinach leaves

At selected time intervals (week 0, 1, 2, 3, 4, 5, 6,7,8) (after week 8, sampling was done once a month for two more months, February and March) soil samples were aseptically collected from 10 locations selected randomly within each plot using a soil probe (7.85cm) to sample down to about 5-8 cm deep from the surface. Samples from each plot were combined in a sterile Whirl-Pak bag (Nasco, Fort Atkinson WI) for subsequent analyses.

Leaf samples were also collected on the third week of the experiment through the rest of the experiment (except on February due to several snow storms), by randomly selecting and pulling leaves from different areas within a plot and collecting sampled leaves in plastic bags.

In all cases, to prevent cross-contamination, samples were collected starting with the control first, proceeding to the non-inoculated manure, then sampling the inoculated manure plots, and ending with the plots treated with the direct application of *E. coli* O157:H7. Gloves were also worn and changed between different treatments during sample collection. Soil and plants samples were placed in a shaded insulated cooler, transported to the laboratory, and then refrigerated at 4°C until analysis.

Enumeration and Enrichment Procedure

For the laboratory experiments, each soil sample was weighed (100g) into 500ml sterile bottle and then 400ml of peptone water (Difco Laboratories, Detroit Michigan) was added to yield a 10^{-1} dilution. The diluted sample was then manually shaken for 2min. Plant samples were weighed (2g-3g), then macerated with sterile mortars and pestles, Peptone water was added to the samples according to the weight of the plant to yield a 10^{-1} dilution. Serial dilutions of each sample were prepared with 99ml peptone-water and sample dilutions were plated in duplicate using a direct plating method with Violet Bile Red Agar (VRBA), and using a spread technique with CHROMagar O157:H7supplemented with 50ng/ml cefixine, 10mg/ml cefsulodine, and 2.5µg of potassium tellurite. Plates were incubated at 37°C for 18h prior to enumeration of the bacteria.

By using the sample weight dilution factors, and the number of presumptive colonies, the log number of CFU per gram of soil or plant material was calculated for each treatment plot. When no colonies were detected for the least diluted sample plated, the log number of CFU per gram was recorded as being less than the smallest dilution factor. *Escherichia coli* O157:H7 present in the soil were detected by enrichment to maximize the chances of detecting bacteria present below the detection limit of enumeration. Approximately 10ml of the soil mixture and 1ml of the macerated spinach mixture was added to 90ml and 9ml of Gram Negative Hajna broth respectively (GN Broth, Difco, Becton Dickinson, Sparks, MD) supplemented with 50ng/ml cefixine, 10mg/ml cefsulodine, and 8mg/ml vancomycine, and incubated at 37°C for 18h. The resulting enrichment cultures were streaked onto CHROMagar O157 supplemented with 50ng/ml cefixine, 10mg/ml cefsulodine, 2.5µg of potassium tellurite. Typical mauve

colonies were enumerated as *E. coli* O157H7; blue colonies were enumerated as *E. coli* for the CHROMagar 0157. For VRBA, pink to red colonies with bile precipitate were enumerated as coliforms.

Detection of *E. coli* O157:H7 using the BAX PCR assay

The presence and the identity of *E. coli* O157:H7 in the enriched samples was confirmed by PCR assay using a Qualicon Bax ® System Q7 instrument (DuPont Corp., Wilmington, Delaware) programmed to optimize for specific detection of the bacteria, following the manufacturer's instructions.

Weather data

Weather data were retrieved from the Mesonet website (www.mesonet.org) for the entire length of the experiment. Soil temperature, air temperature, and solar radiation were the variables that we used for this experiment.

Statistical analysis

Plots were arranged in a randomized block design with five replications of the four treatments. Number of *E. coli* and *E. coli* O157:H7 were measured in colony forming units (CFU) per gram soil or leaf material. These populations were transformed using logarithms to achieve normal distributions. All statistical analyses were conducted using PC SAS Version 9.2 (SAS Institute, Cary, NC). Analysis of variance procedures (PROC MIXED) were conducted utilizing a two factor (manure and inoculum) factorial treatment design with repeated measures (weeks). An autoregressive covariance structure with a time period of 1was used to model the intrasubject correlation structure.

Simple effects of a factor (manure or inoculum) relative to the other factor and to week were calculated using the SLICE option in an LSMEANS statement; the combined effects of manure and inoculum in a given week were also calculated. The linear effects of week for each combination of manure and inoculum were calculated as well with planned contrasts. Significance was set at $p<0.05$.

CHAPTER IV

RESULTS

Spinach Leaf Assays:

During all experiments, *E. coli* was not detected in any spinach leaf samples collected. This indicates that bacteria from the inoculated soil did not contaminate the leaves during growth, or that contamination occurred as such a low level that the bacteria were not detected by our sampling methods.

Survival of bacteria over time in inoculated plots:

Generic *E. coli* bacteria:

Average populations of generic *E. coli* expressed as $\log_{10}$ CFU g^{-1} soil for the control and treatment plots are shown in Table 1. As predicted, the bacterial counts were high after the application of the different treatments. The generic *E. coli* counts declined dramatically over time, however. The average number of *E. coli* immediately after inoculation was 5.0 $\log_{10}$ CFU g^{-1} and 4.1 $\log_{10}$ CFU g^{-1} respectively for plots amended with inoculated manure (treatment 3) and plots treated with direct spray application of

bacterial inoculum (treatment 4). The final average numbers at the end of the experiment were 1.9 $\log_{10}$ CFU g^{-1} for treatment-3 plots and 1.3 $\log_{10}$ CFU g^{-1} for treatment-4 plots (Table 1). Overall, although statistically significant differences were not always observed, generic *E coli* survived better in treatment 3 than in treatment 4 when comparing simple averages.

When comparing the different treatments, significant differences were found between the different treatments for the weeks 0 and 1 for generic *E. coli* counts. Counts for the plots inoculated by direct spray application of bacterial inoculums were significantly different than the control plots and the plots amended with inoculated manure in week 2. After week 2, no significant differences in generic *E. coli* bacterial counts among treatment plots were observed

Table 1. Average number of generic *Escherichia coli* in soil samples by week.

	Mean[1] Log10 CFU g-1			
Weeks	Control (Treatment 1)	Soil amended with non-inoculated manure (Treatment 2)	Soil amended with inoculated manure (Treatment 3)	Soil treated by direct-spray application of inoculum (Treatment 4)
0	2.30a[2]	3.03b	4.13c	5.04d
1	2.21bc	2.67ab	1.70c	3.14a
2	3.16a	3.18a	2.94a	2.84a
3	3.02a	2.97a	3.13a	2.64a
4	2.73a	2.59a	2.69a	2.70a
5	2.75a	2.75a	2.68a	2.44a
6	2.73a	2.57a	2.50a	2.46a
7	2.48a	2.46a	2.17a	1.74a
11	1.94a	2.09a	2.42a	2.32a
15	1.80a	1.99a	2.22a	1.28a
20	1.26a	1.73a	1.93a	1.34a

1. n=20.
2. Means within the same row with the same letter are not significantly different ($p < 0.05$)

E. coli O157:H7 bacteria:

Average populations of *E. coli* O157:H7 expressed as $\log_{10}$ CFU g^{-1} soil for the control and three treatment plots are shown in Table 2. For the *E. coli* O157:H7 counts, the average number at the beginning of the experiment was 4.59 $\log_{10}$ CFU g^{-1} and 3.8 $\log_{10}$ CFU g^{-1} for treatments 3 and 4 respectively (Table 2). Overall, a rapid decline in numbers was seen from week 0 to week 1, and a more gradual decline thereafter. The numbers of *E coli* O157:H7 dropped below the limit of detection via direct plating after

six weeks in treatment 3 and after four weeks in treatment 4 (Table 2). Although statistically significant differences were not consistently observed between treatment 3 and 4 from week 0 to week 2, from week 3 to week 6 the numbers of surviving *E. coli* O157:H7 were significantly higher in treatment 3 than in treatment 4 (Table 2). Thus, the numbers inoculated bacteria remained higher for a longer period of time when added to the soil in manure slurry.

After week 7, we sampled on a monthly basis to test for the presence of bacteria in the soil. Although numbers were too low to enumerate, enrichment procedures were used to test for the presence or absence of bacteria in the soil. The BAX procedure was used to confirm the presence of the bacteria in the enriched samples. *E. coli* O157:H7 were detected by enrichment until the end of the experiment (week 20) in treatments 3 and 4.

Escherichia coli O157:H7survived in the soil for at least 20 weeks. There was a significant difference between the different treatments on a weekly basis from the first week through the last week of the experiment. This indicates that a week effect was observed for the average *E. coli* and *E. coli* O157:H7 populations for each treatment. After plotting the means ±2 standard errors of the mean, we can say with 95% confidence that there is a difference in the population of *E. coli* O157:H7 between week 1 and the rest of the weeks for both treatment 3 and treatment 4 because the standard error bars do not overlap (Figures 1&2). Also we can see a decline in number of *E. coli* O157:H7 over time for treatment 3 and 4 (Figures 1 & 2). The figures also show that weeks 1, 2, and 3 are different than subsequent weeks for spray-inoculated soil plots but not for inoculated manure plots (Figures 1 & 2).

Table 2. Average number of *Escherichia coli* O157:H7 in soil samples by week.

	Mean[1] Log_{10} CFU g^{-1}			
Weeks	**Control (Treatment 1)**	**Soil amended with non-inoculated manure (Treatment 2)**	**Soil amended with inoculated manure (Treatment 3)**	**Soil treated by direct-spray application of inoculum (Treatment 4)**
0	0.70c[2,3]	0.70c	4.60a	3.84b
1	0.70b	0.70b	2.33a	2.36a
2	0.70a	0.70a	1.70a	2.18a
3	0.70a	0.70a	2.17a	1.95a
4	0.70b	0.70b	1.70a	0.95b
5	0.70b	0.70b	2.01a	0.70b
6	0.70b	0.70b	1.56a	0.70b
7	0.70a	0.70a	0.99a	0.70a
11	0.70a	0.70a	0.70a	0.94a
15	0.70a	0.70a	0.70a	0.70a
20	0.70a	0.70a	0.70a	0.70a

1. n = 20
2. Means within the same row with the same letter are not significantly different ($p < 0.05$)
3. Values below 1 are considered below the limit of detection.

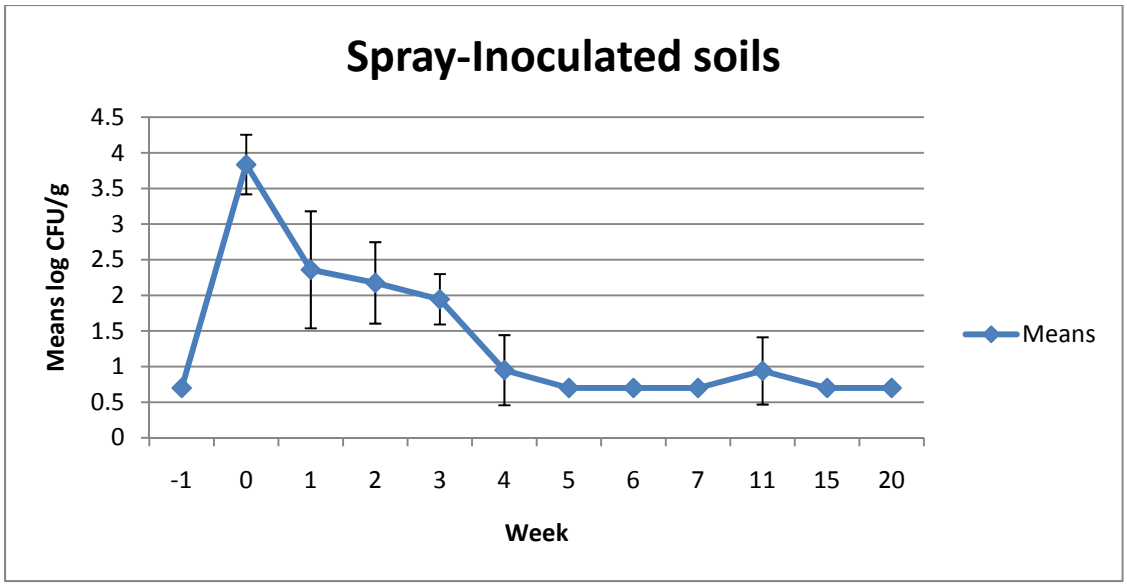

Figure 1. Mean (n=20) population of *E. coli* O157:H7 by week in plots inoculated by direct spray. Error bars represent ±2 standard errors of the mean (95% confidence interval).

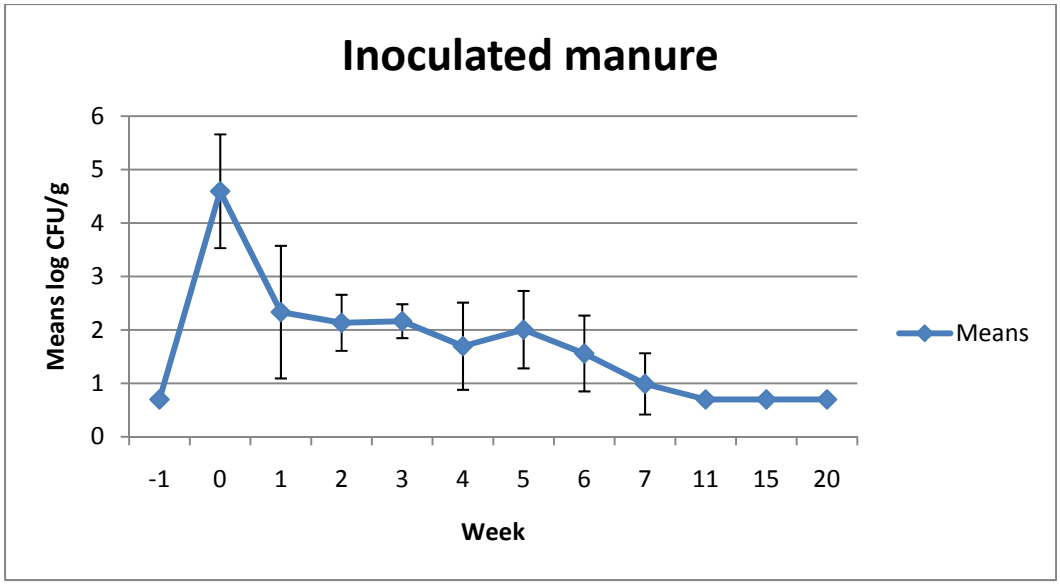

Figure 2. Mean (n=20) population of *E. coli* O157:H7 by week in plots amended with inoculated manure. Error bars represent ±2 standard errors of the mean (95% confidence interval).

Correlations among bacterial survival and air temperature, soil temperature, and solar radiation:

Range of weather factors over the course of the experiments:

The average weekly air temperature varied from -0.6 to 12.1°C (31.0 to 53.8°F) and the soil temperature averages varied from 3.7 to 13.4°C (38.7 to 56.1°F). The average total solar radiation varied from 14.5 to 4.9MJ/m^2 during the course of the experiment. Graphical representations of bacterial survival curves for generic E. coli and E. coli O157:H7 in the different treatment plots along with the measured weather factors are shown in figures 3 - 8. A casual examination of the graphical data shows that clear relationships between soil temperature, air temperature, and solar radiation and bacterial survival are difficult to discern. Thus, correlation coefficients were calculated to help elucidate any possible correlations.

Correlations between weather factors and bacterial survival:

Pearson's correlation coefficients were calculated for the relationships between average weekly air temperatures, soil temperatures, and total solar radiation and bacterial population numbers. In addition, to evaluate possible lagging effects between the measured environmental factors and the survival of the bacteria, Pearson's correlation coefficients were calculated for the average weather conditions such as soil temperature from the previous week and the average log unit of the bacteria of the following week.

Over the course of the experiment soil temperature had a strong positive correlation with coliform bacteria populations in treatments 1 (control) and 2 (non-inoculated manure) with r-values of 0.75 and 0.82 respectively (Table 3). Looking at the

lagged correlation, the r-values increased for treatment 1 to 0.83, but decreased for treatment 2 to 0.57 (Table 4). For treatments 3 (inoculated manure) and 4 (spray-inoculated soil), the r-values were weakly positive for treatment 3, more strongly positive for treatment 4 (Table 3). The same mixed effect of lagged correlations was seen for treatments 3 and 4 as for treatments 1 and 2: the r-value decreased for manure-treated plots but increased for bare soil plots. Correlations among air temperature and coliform bacterial populations showed a pattern very similar to those seen with soil temperatures (Tables 3 and 4). Comparing solar radiation and generic coliform bacterial populations, lagged correlations were generally stronger than non-lagged correlations, but no correlation with an absolute value greater than 0.64 was observed (Tables 3 and 4).

Examining the correlations among generic *E. coli* populations and the weather conditions measured, we saw generally weak, positive correlations for the same-week data (Table 5). The lagged correlations are notably higher for soil and air temperature, with similar patterns again seen for both (Table 6). Strong positive lagged correlations were seen for treatments 1, 2, and 4 with r-values of 0.74, 0.81, and 0.91 respectively. The exception was inoculated manure, which showed a relatively weak correlation coefficient of 0.51 (Table 6). As with the coliform populations, average total solar radiation generally correlated weakly with generic *E. coli* bacterial population numbers (Tables 5 and 6). The lagged correlation became stronger for treatment 4, but otherwise showed no clear pattern (Tables 5 and 6).

With respect to the correlations among *E. coli* O157:H7 populations and the weather conditions measured, we saw relatively weak (0.42, treatment 3 versus air temperature) to moderately strong (0.69, treatment 4 versus soil temperature) positive

correlations between air and soil temperatures and bacterial population counts (Table 7). When the lagged correlation coefficients were calculated, the r-values increased for all comparisons (Table 8), to a maximum of 0.84 (treatment 4 versus air temperature). In all cases the r-values were higher for treatment 4 than for treatment 3. As with the other bacterial counts, the correlations among total average weekly solar radiation and average weekly *E. coli* O157:H7 counts were relatively weak (Table 7). No clear effect of examining the lagged correlations was observed (Table 8).

Table 3 – Pearson's Correlation Coefficient for average weekly soil temperature, air temperature, and total solar radiation versus mean (n=20) weekly coliform populations (Log_{10} CFU g^{-1})

	soil temperature	air temperature	total solar radiation
Control	0.75	0.62	0.21
Non inoculated manure	0.82	0.70	0.37
Inoculated manure	0.48	0.45	0.52
Inoculated soil	0.61	0.52	0.38

Table 4 – Pearson's Correlation Coefficient for average weekly soil temperature, air temperature, and total solar radiation from the previous week versus mean (n=20) weekly coliform populations (Log_{10} CFU g^{-1})

	soil temperature	air temperature	total solar radiation
Control	0.83	0.85	0.67
Non inoculated manure	0.57	0.66	0.59
Inoculated manure	0.42	0.42	0.14
Inoculated soil	0.73	0.75	0.64

Table 5 – Pearson's Correlation Coefficient for average weekly soil temperature, air temperature, and total solar radiation versus mean (n=20) weekly generic *E. coli* populations (Log_{10} CFU g^{-1})

	soil temperature	air temperature	total solar radiation
Control	0.38	0.13	-0.26
Non inoculated manure	0.63	0.40	0.09
Inoculated manure	0.28	0.10	0.08
Inoculated soil	0.54	0.37	0.31

Table 6 – Pearson's Correlation Coefficient for average weekly soil temperature, air temperature, and mean total solar radiation from the previous week versus mean (n=20) weekly generic *E. coli* populations (Log_{10} CFU g^{-1})

	soil temperature	air temperature	total solar radiation
Control	0.74	0.85	0.22
Non inoculated manure	0.81	0.77	0.42
Inoculated manure	0.51	0.56	-0.09
Inoculated soil	0.91	0.88	0.69

Table 7 – Pearson's Correlation Coefficient for average weekly soil temperature, air temperature, and total solar radiation versus mean (n=20) weekly *E. coli* O157:H7 populations (Log_{10} CFU g^{-1})

	soil temperature	air temperature	total solar radiation
Inoculated manure	0.53	0.42	0.52
Inoculated soil	0.69	0.58	0.46

Table 8 – Pearson's Correlation Coefficient for average weekly soil temperature, air temperature, and total solar radiation from the previous week versus mean (n=20) weekly *E. coli* O157:H7 populations (Log_{10} CFU g^{-1})

	soil temperature	air temperature	total solar radiation
Inoculated manure	0.77	0.68	0.49
Inoculated soil	0.79	0.84	0.57

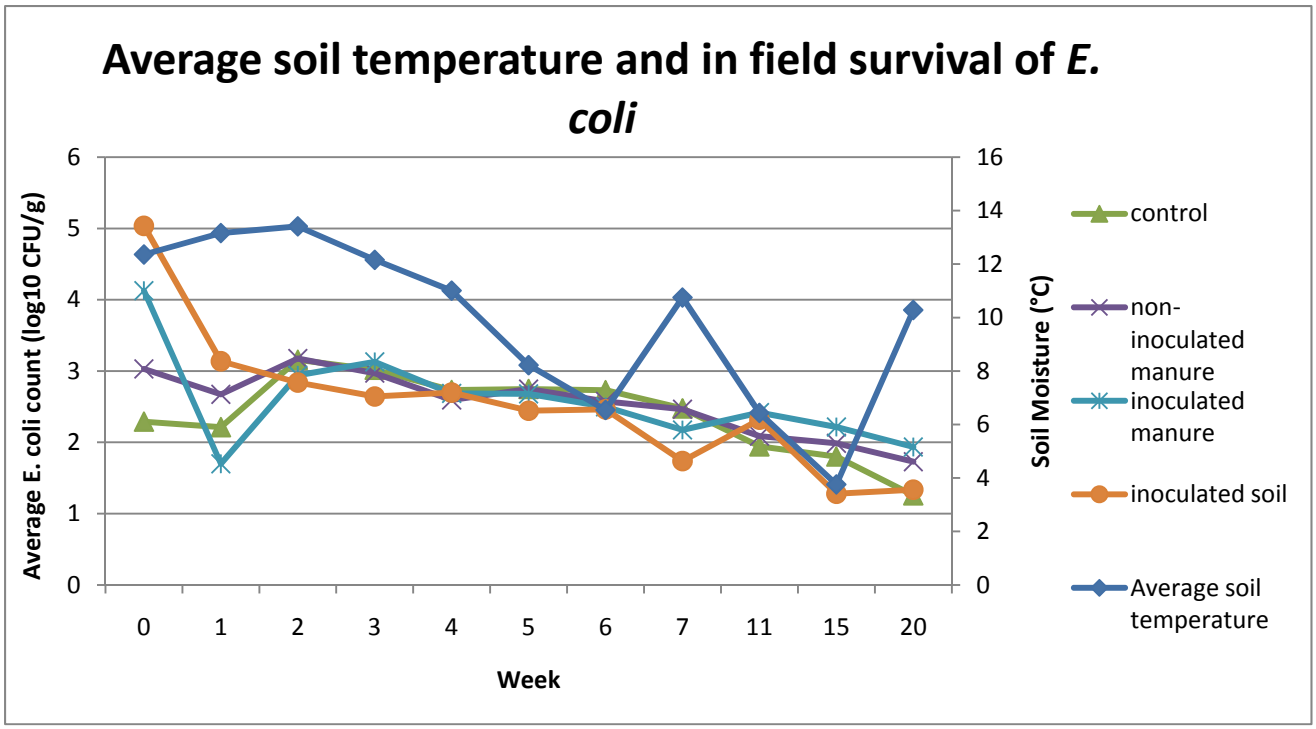

Figure 3. Average soil temperature and in-field survival of generic *E. coli* for the different treatments

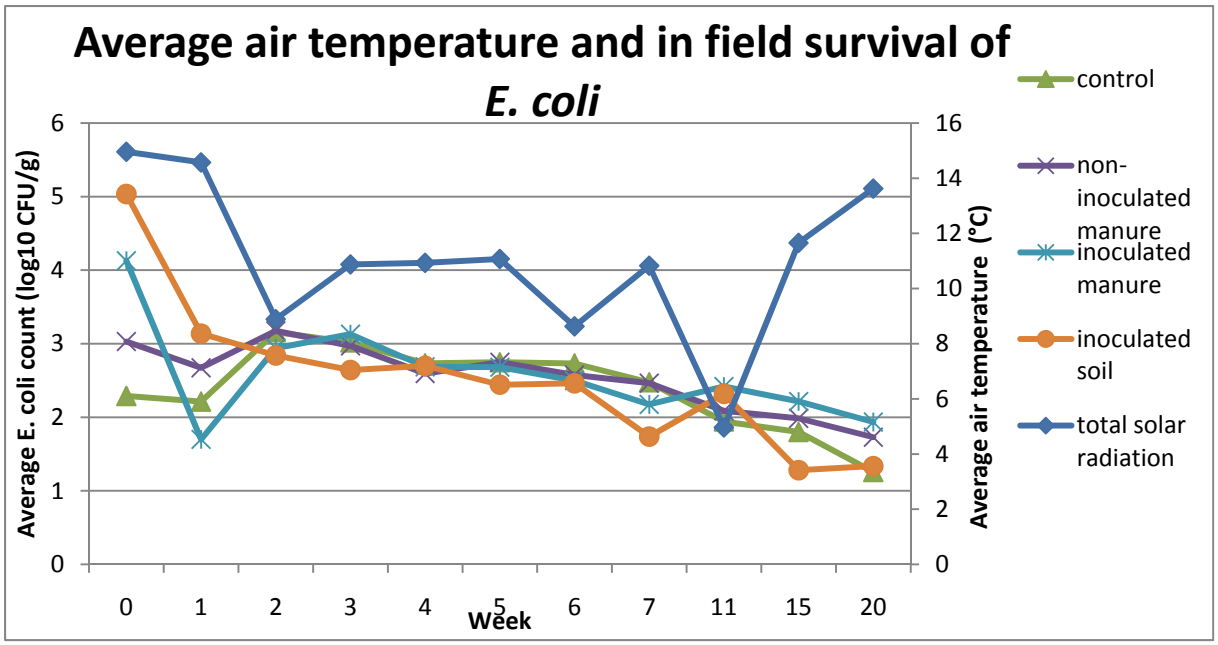

Figure 4. Average air temperature and in-field survival of generic *E. coli* for the different treatments

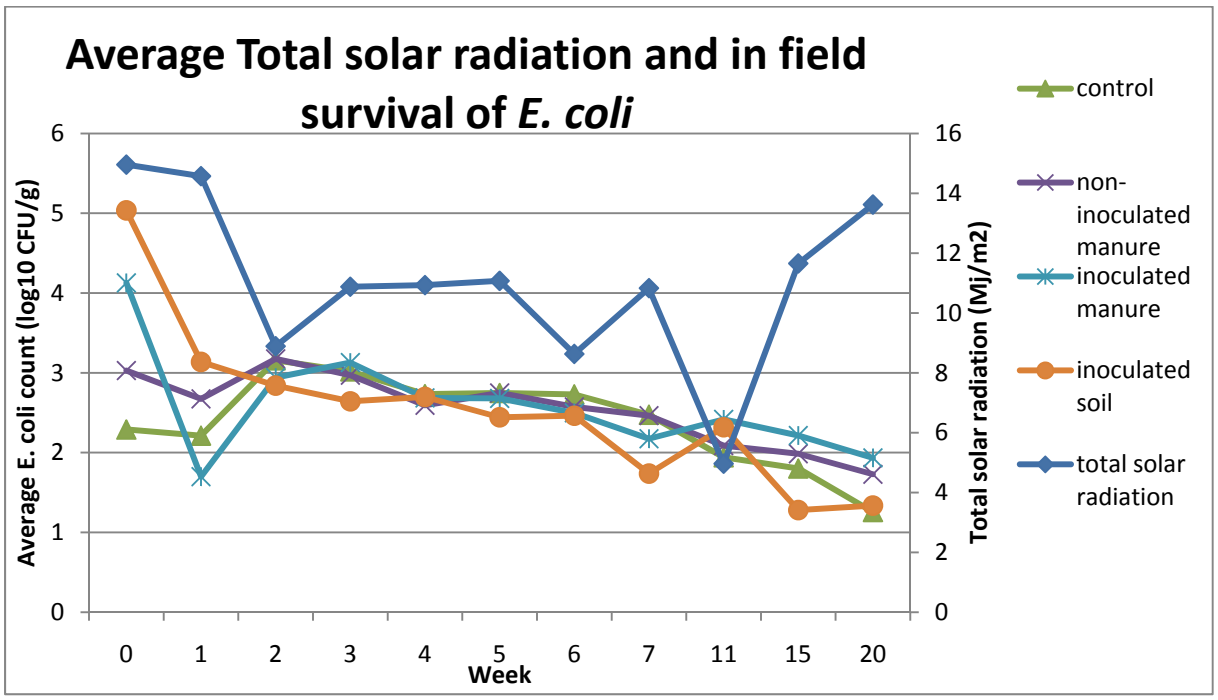

Figure 5. Average total solar radiation and in field survival of generic *E. coli* for the different treatments

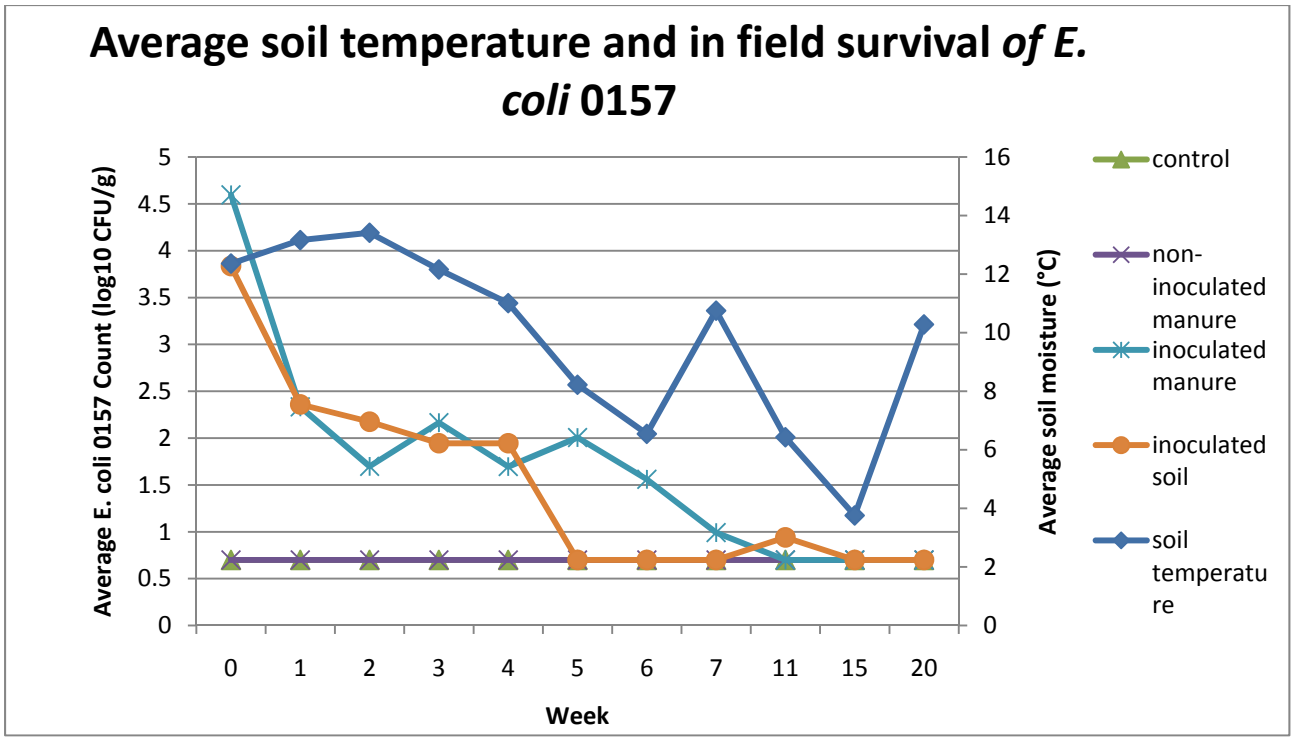

Figure 6. Average soil temperature and in field survival of *E. coli* O157:H7 for the different treatments

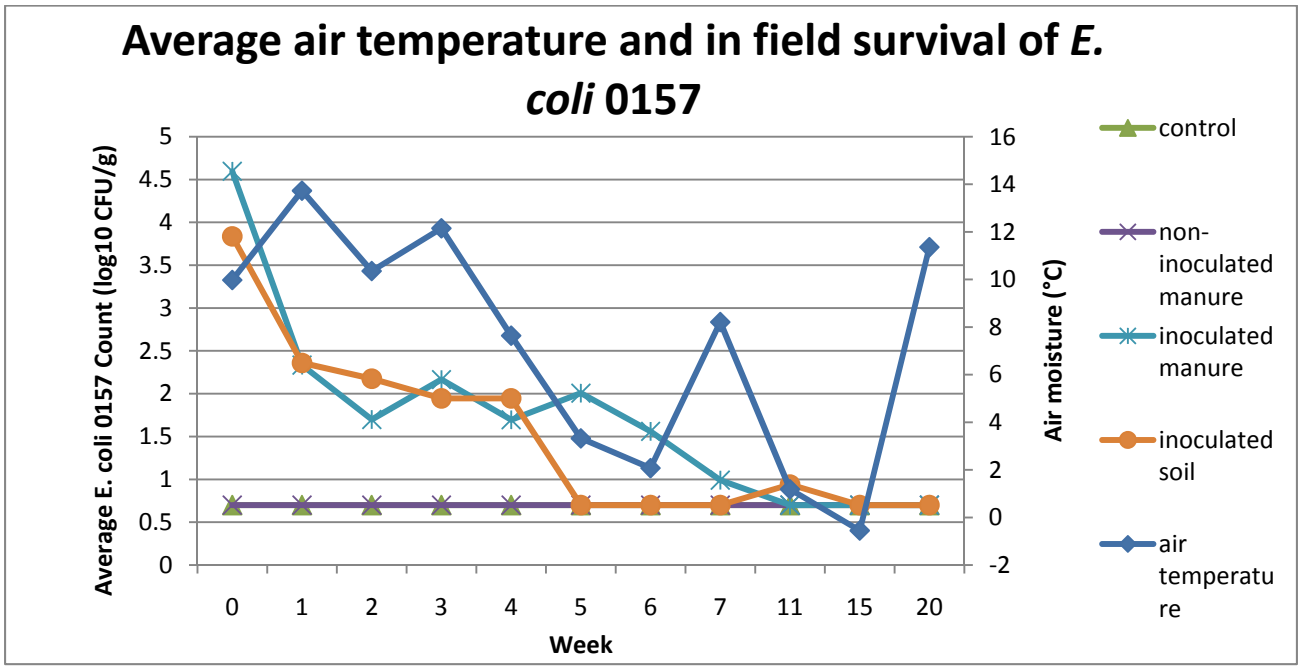

Figure 7. Average air temperature and in field survival of *E. coli* O157:H7for the different treatments

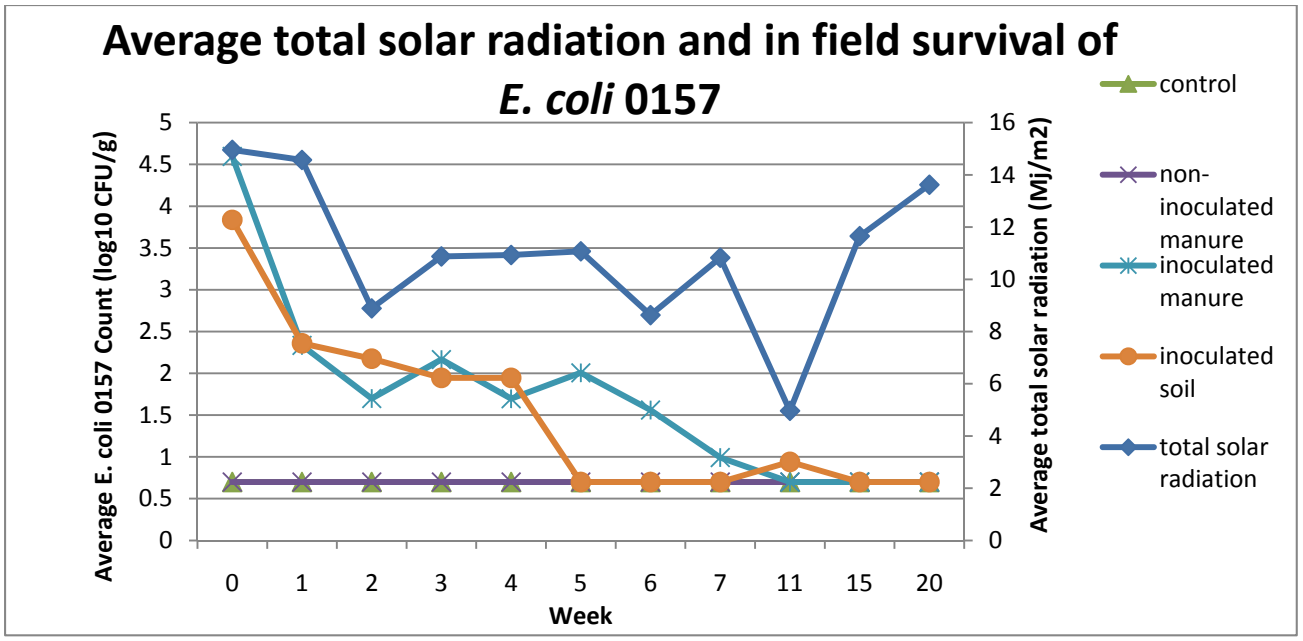

Figure 8. Average total solar radiation and in field survival of *E. coli* O157:H7for the different treatments

CHAPTER V

Discussion and Conclusions

The use of animal manure as a fertilizer has become a critical issue in agricultural production of fresh, ready-to-eat produce due to the fact that animal manure can provide an avenue for the entry of pathogens into the food chain. Because it is not cooked before being consumed, fresh produce represents a particular food safety risk. Risk of contamination is higher for crops grown in soils where manure is applied, especially for crops grown close to the ground (Islam et al., 2005). Numerous foodborne outbreaks have been associated with the consumption of leafy greens such as spinach or lettuce due to contamination with *E. coli* O157:H7. The sources of contamination for those outbreaks have been linked to fecal waste or improperly composted manure. The survival of enteric pathogens such as *E. coli* O157:H7 in the environment is of fundamental importance to predict accurately the risks associated with farm practices such as the applications of manure (Islam et al., 2005). It has been shown that *E. coli* O157:H7 can survive in the soil for at least 60 days at 25°C, and for at least 100 days at 4°C (Ingham et al., 2004).

In this study, the results indicate that *E. coli* and *E. coli* O157:H7 remained in soils for at least 20 weeks with air temperatures that varied from 0 to 12°C and soil temperatures that varied from 3.7 to 13°C. However, the results also showed that

numbers declined rapidly so that after about four to six weeks following soil inoculation, the population had declined to the point that viable cells could only be detected after an enrichment process. Although statistical significant differences were not consistently observed, overall the populations of *E. coli* O157:H7 declined more rapidly in plots inoculated by direct spray than in plots amended with inoculated manure. *E. coli* O157:H7 counts remained above the minimum level of detection about two weeks longer – six weeks versus four weeks – in the manure-amended plots than in the direct-spray inoculated plots. This could potentially have significance for leafy greens that are harvested and sold as "baby greens."

The trend toward higher average populations of *E. coli* O157:H7 in manure – amended soil plots may be due to the fact that the bacteria had more nutrients necessary for survival in the manure. Zhang et al. (2009) noted that irrigation water containing cow manure may prolong the survival of *E. coli* O157:H7 on lettuce leaves because of the nutrients it provides (Zhang et al., 2009). The higher numbers may have also been due to the fact that the average soil temperature was 9.8°C and the average air temperature was 7.2°C for the entire length of the experiment (November through March), and that is still too cold for the microorganism antagonistic to *E. coli* O157:H7 to grow. A study concluded land application of manure in spring or fall was preferable to application in summer due to the fact that *E. coli* and total coliform populations were found to be significantly greater as summer air temperature increased to 27°C (Becker et al., 2010). It has also been showed that when the bacteria is introduced into the soil by manure, it mostly stay attached to manure particles instead of a homogenous distribution throughout the manure amended soil mass (Franz et al., 2008). This may imply that the *E. coli*

O157:H7 present in treatment 3 had more protection and therefore does not die off as quickly as the *E. coli* O157:H7 present in treatment 4.

The type of soil used in our experiment was teller fine sandy loam, and some studies have established that the survival rate of *E. coli* O157:H7 depends on the type of soil. The initial rate of decline of *E. coli* O157:H7 has been observed to be faster in sandy soil but that rate of decline slows over time compared to the more constant rate seen with loamy soil (Franz et al., 2008). In general studies have shown that finer textured (clayey) soils resulted in prolonged survival of introduced bacteria compared with coarser textured (sandy) soils because of the higher availability in clayey soils of pore spaces that offer protection against predation by soil-borne antagonistic microorganisms. Since our type of soil is a mixture of both types (Sandy and loamy), it might have promoted both the initially-observed rapid decline and the prolonged survival of the bacteria in small numbers.

Previous studies have shown that weather conditions, desiccation, soil type, predatory protozoan populations, and the degree of manure incorporation are all likely to have various impacts on the pathogen survival in manure fertilized soils (Ingham et al., 2004). The results of this present study have shown that soil temperature had an effect on the survival of *E. coli* and *E. coli* 0157. In general, the bacterial counts decreased at lower temperatures. Our results showed that when the previous week had higher temperatures, the average log unit population of *E. coli* O157:H7 increased for the following week. This was also true for the generic *E. coli.* These results are in contrast to those found by Williams et al. (2004) in which the persistence of *E. coli* O157 was notably decreased when they were incubated at 20°C relative to those held at 5°C

(Williams et al., 2004). This may be explained by the fact that *E. coli* might survive better at lower temperature because they have less competition from other microorganisms for available nutrition. At 5°C, microorganisms antagonistic to *E. coli* O157:H7 may be less competitive because of slow or no growth or failure to produce significant amount of antimicrobials against *E. coli* O157:H7 (Jiang et al., 2001). It is also worth noting that our maximum in-field temperatures were around 13°C, below the 20°C temperature examined in the above-cited study.

Previous studies have demonstrated the impact of solar radiation on the survival of *E. coli*, the average number of *E. coli* was significantly lower in fecal pats that were under 0% and 40% shade cloth compared to those under the 80% and 100% shade cloth (Meays et al., 2005). In a study by Zhang et al. (2009), after the inoculation of the surface of lettuce leaves, *E. coli* O157:H7 survived longer on the abaxial side of the leaves than on the adaxial side. This is due to the fact that adaxial side of the leaf is exposed to the sunlight during daytime hours and tends to be drier. The abaxial side is exposed to indirect light and may be at lower temperature (Zhang et al., 2009). Similarly Erickson et al. (2010) observed a greater survival of *E. coli* inoculated into the interior of the lettuce heads compared with cells inoculated onto the surface. Exposure to UV radiation has been identified as a factor contributing to the inactivation of bacteria (Erickson et al., 2010). Given these results, we might have expected to see a negative correlation between total solar radiation and bacterial counts. However, we did not see this for the most part. Overall, the relation between solar radiation and bacterial survival was not clearly demonstrated in this study.

Survival of *E. coli* in soils for a long period of time increases the risks of contamination of leafy greens, such as spinach, when those plants are cultivated in agricultural soils amended with pathogen-containing manure. In our case, we did not do a surface sterilization of the spinach leaves, and *Escherichia coli* O157:H7 was not detected in any spinach leaf samples. The lack of internalization of *E. coli* O157: H7 on fresh produce was also established in a study by Zhang et al. (2009). They used different methods of soil inoculation, but the bacteria were not found on the inside of the lettuce even though *E. coli* O157:H7 was present in the soil during the entire length of the experiment (Zhang et al., 2009). Even though *E .coli* O157:H7 was not detected on the spinach plant during our experiment, contamination has been shown to occur in other studies. Mitra et al. (2009) showed the potential for *E. coli* O157:H7 to colonize spinach plant. They confirmed the ability of *E. coli* O157:H7 to survive as an epiphyte on the spinach phylloplane, but they saw negligible evidence of natural entry into the plant interior. One study has demonstrated that the edible portion of lettuce can be contaminated through transport of the pathogen into the plant via the root system (Solomon et al., 2002). Contamination of soil-grown produce occurred in a study in which it was found that *E. coli* O157:H7 was detected for 168 days on carrots and for 74 days on onions post application of contaminated compost or water to the soil (Islam et al., 2005). This indicates that the persistence of *E. coli* O157:H7 in the soil or on vegetables may depend on the type of vegetable being grown in the contaminated soil. The discrepancy between those results and those of our present study may be due to differing planting and growing methods as well as differing environmental conditions.

As noted previously, we did not do a surface sterilization of the spinach leaves, and *Escherichia coli* O157:H7 was not detected in any leaf sample even though we used a fairly high initial concentration for our treatments (4.59 $\log_{10}$ CFU g^{-1} and 3.8 $\log_{10}$ CFU g^{-1} for treatment 3 and 4 respectively). For further studies, other methods of inoculation should be used, for example using inoculated irrigation water (irrigation water containing cow manure) to irrigate the field. Also, using inoculated seeds could be a good approach, as Warriner et al. (2003) used that technique and their results showed that *E. coli* O157:H7 become established in the roots of the plants. We only used one strain of *E. coli* O15:H7 in our study, but other studies made a mixture of several strains. This could have influenced our results as the particular strain we used may not adequately represent the colonization abilities of other *E. coli* O157:H7 strains in similar experimental procedures. Also, it is possible that the sample size used was too small (0.1 ml of leaf homogenate for direct plating and 0.1 ml for enrichment). In future studies a larger mass of spinach leaves could be used to create the homogenate for further testing.

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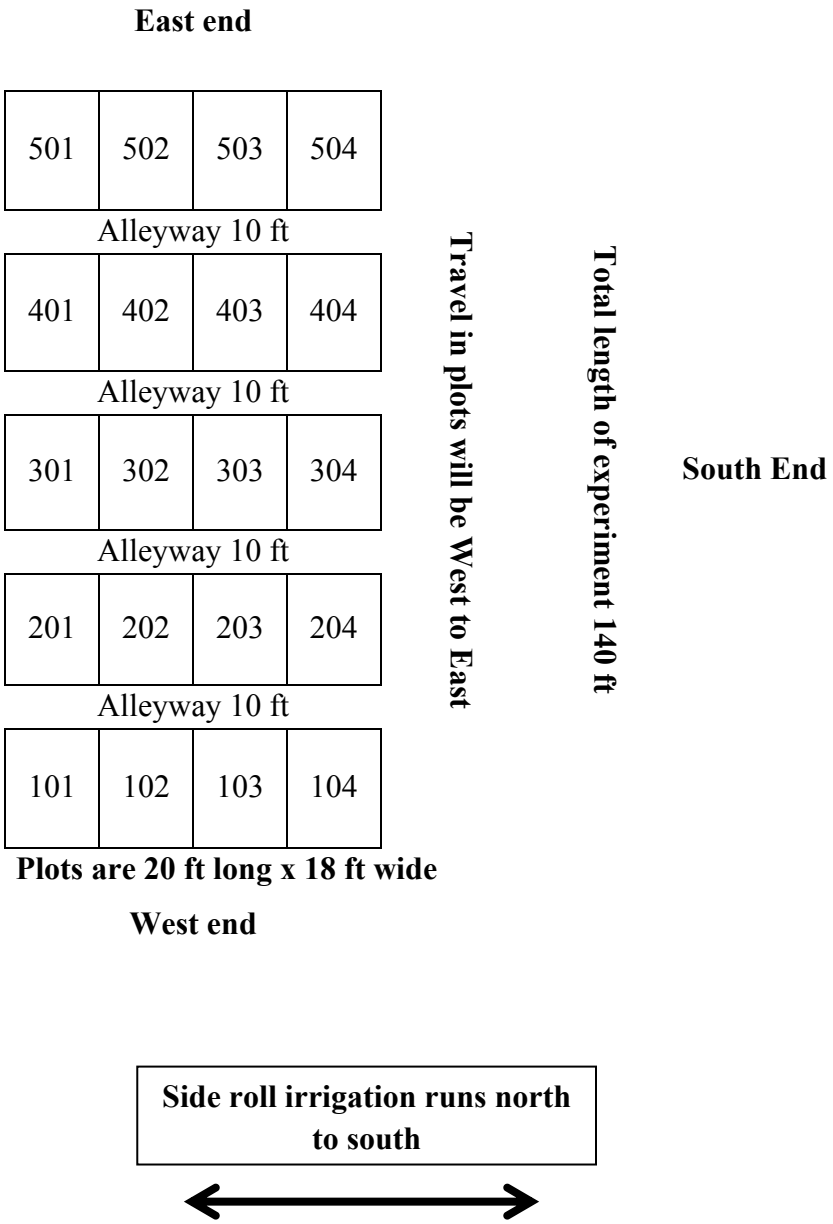
APPPENDICES

Fall 2010 and Spring 2011 Spinach Experiment Plot Map and Treatment Description



Treatments no.	Treatment description	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
1	Control	101	204	301	402	502
2	uninoculated Manure	102	202	303	404	504
3	Inoculated Manure	103	203	302	403	503
4	Spray inoculum	104	201	304	401	501

Table 2. Average log number of coliforms (CFU/g) in soil sample (n=20) from control plot, plot amended with non inoculated manure, plots amended with inoculated manure and plot amended with inoculum only.

weeks	Control	Non-inoculated manure	Inoculated manure	Inoculated soil
0	1.975012	1.963414	4.700157	5.077779
1	1.975012	1.69897	2.629226	2.664172
2	2.371744	2.522116	1.628373	2.649455
3	2.001239	1.68028	2.879782	2.29351
4	1.61875	1.476042	1.818824	1.303703
5	1.327823	1.327823	0.69897	0.99145
6	1.293596	0.994394	1.868184	0.69897
7	0.69897	1.163414	1.277054	0.985243
11	0.975012	0.69897	1	1.849576
15	0.69897	0.954721	1.510989	0.69897
20	0.954721	1.260747	1.002673	1.112607

VITA

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Scope and Method of Study:

This project was designed to test the hypothesis that the edible part of the spinach plant can be contaminated while using bovine manure inoculated with *E. coli* O157:H7 as a fertilizer, and also to test the hypothesis that environmental effects such as soil temperature, air temperature, and solar radiation have an impact on the survival of the bacterium in the soil. The field experiment was done at the Oklahoma State University Cimarron Research Station in Perkins, Oklahoma. Treatments included non-inoculated soil used as control, soil amended with manure, soil amended with inoculated manure, and soil treated by direct-spray application of bacterial inoculum. Spinach (variety Olympia) was planted in treated soils. Soil samples were collected weekly at first and later monthly and analyzed for *E. coli* O157:H7 using a direct plating method. Ground spinach leaves were also analyzed for presence of *E. coli* O157:H7. Weather data were obtained and correlated with the survival rate of the bacteria.

Findings and Conclusions:

We observed that the population of inoculated bacteria declined fairly rapidly; an approximately two-log reduction in numbers was seen from week 0 to week 1. The population of inoculated cells dropped below the limit of detection by direct plating after 4 weeks for the direct-spray inoculated plots and after 6 weeks for the inoculated-manure plots. This study further demonstrated that *E. coli* O157:H7 survived in the soil for at least 140 days in soils amended with inoculated manure and inoculum only. A moderately strong one-week lagged correlation between bacterial population and soil temperature was observed: r= 0.77 and 0.79 for inoculated manure and direct-spray inoculated plots respectively. We did not observe a clear effect from sun exposure. Contamination of the spinach leave did not occur during the entire length of the study. Recommendations for future studies include additional strains of *E coli* bacteria, other types of inoculation techniques, and improved sampling procedures.

ADVISER'S APPROVAL: William McGlynn