CORRELATION OF IN-FIELD SURVIVAL OF *ESCHERICHIA COLI* O157:H7 WITH RAINFALL, RELATIVE HUMIDITY AND SOIL MOISTURE

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Bachelor of Science in Food Science

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Stillwater, Oklahoma

2008

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2011

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CHAPTER I

INTRODUCTION

Salad greens have been increasingly reported to be contaminated with *Escherichia coli* O157:H7, which has been associated with numerous serious foodborne illness outbreaks that have had major economic impacts, not to mention the negative effect on the reputation of the food industry. Although the food industry is putting outstanding efforts into controlling and reducing the risk of microbial contamination, foodborne illness is still a threat in fresh produce such as fresh-cut prepackaged spinach. Bagged fresh raw spinach was recalled in 2006 after 3 people were reported dead and 196 people were hospitalized after consuming spinach which was believed to have been contaminated with *E.coli* 0157:H7 at the preharvest stage (Xicohtencatl-Cortes et al., 2009). Manure applied as organic fertilizer is suspected to be the cause of many *E.coli* 0157:H7 outbreaks and recalls (CDC, 2010) involving leafy crops planted very closely to the soil surface. The survival of this pathogen in the soil may be influenced by environmental factors such as rainfall, low soil temperature and relative humidity (Kudva et al., 1998).

Bovine species are natural reservoirs for *E.coli* O157:H7 and the bacteria can often be found in feces shed from the animals. If the manure is used as soil amendment, it substantially increases the risk of contaminating fresh produce planted in manure-

fertilized soils (Wang et al., 1996). Although most pathogenic bacteria shed in animal manure die over a short period of time, studies have shown that *E.coli* O157:H7 can persist for up to two months in bovine feces at temperatures ranging from 5°C to 22°C at different inoculums concentrations (Vidovic et al., 2007). Kudva et al. (1998) indicated that *E.coli* O157:H7 can survive in cow manure for up to two years even when left under unstable environmental conditions.

Survival of *E.coli* O157:H7 is very dependent on environmental conditions such as rain fall, soil temperature and humidity. For example, low rainfall has been shown to have a lethal effect on *E.coli* O157:H7 due to soil dryness and higher soil temperatures (Thordal-Christensen, 2003; Zhang et al., 2009[B]). *E.coli* O157:H7 is said to have the ability to leach through soil together with rainfall, which again suggests that drought conditions in the soil would decrease the survival rate of the pathogen (Gagliardi et al., 2000). In addition, studies have shown that younger spinach plants were prone to contamination by *E.coli* O157:H7 as the plants did not have thicker cuticles that help older plants avoid the invasion of the pathogens and also have underdeveloped internal defense systems that help older plants kill invading cells (Hora et al., 2005; Thordal-Christensen, 2003; Warriener et al., 2003). These findings therefore suggest spinach plants should be sampled before mature stage in order to investigate the internalization of enteric bacteria.

Recent studies have aimed at investigating the potential internalization of *E.coli* O157:H7 through the leaves or roots of spinach plants. Several studies indicated that *E.coli* O157:H7 survived at the sites of inoculation (leaves and roots), but there was not conclusive evidence showing the pathogen migrating to any other location within the

whole plant (Hora et al., 2005; Mitra et al., 2009; Zhang et al., 2009[A]). Another study investigated the internalization of *E.coli* O157:H7 by spraying inoculated irrigation water onto plants (Erickson et al., 2010[B]). In that study, surface contamination and internalization were only observed after high concentrations of inoculums were applied, and *E.coli* O157:H7 persisted only for short periods of time. Although most of these papers presented the results of studies on the interactions of *E.coli* O157:H7 with spinach either on the plant surface or internalized into the plant interior, the understanding on the effects of fluctuating environmental conditions on the survival rate of *E. coli* O157:H7 in or on leafy greens is still poorly understood.

Up to now, several studies have reported that increasing temperatures would cause higher mortality rates in *E.coli* O157:H7. In order to achieve more consistent results, the experiments were mostly conducted in climate-controlled labs or greenhouses in order to maximize control of temperate and overall climatic conditions. Therefore, the data might not best reflect the survival rate of pathogenic bacterial strains under the fluctuating weather conditions that would be seen in an on open field. Also, in many of these studies the data were collected right after the application of treatments. For example, the survival rate of pathogens in soil samples may have been studied shortly after the application of inoculated manure following by several simulated rainfall events, but intermittent sampling and observation over time has usually not been done (Stoddard et al., 1998). In the present study, the effects of environmental factors (relative humidity and rain fall) on the survival of Escherichia coli O157:H7 on spinach and in soils were evaluated periodically over the course of 5 months.

The overall objective of the present study was to determine the effects of environmental factors (rainfall, relative humidity, and soil moisture) on the survival non-pathogenic of *E.coli* O157:H7 on spinach cultivated in soils amended with inoculated cattle manure. Baby spinach was analyzed during the growing season (from week 3 to week 7) and only soil was examined after that to study the recovery of *E.coli* O157:H7. We expect that the results obtained could be useful for minimizing the risk of bacterial contamination of fresh greens, which is crucially important towards safer foods.

CHAPTER II

REVIEW OF LITERATURE

Background information of *E.coli* O157:H7

Escherichia coli O157:H7 is naturally found in the intestinal tract of normal healthy warm blooded ruminant animals, but it was only identified in 1982 after foodborne outbreaks involving hemorrhagic colitis were reported (Phillip, 1995). It is a short, Gram negative rod-shaped, facultative anaerobic and motile bacterium. The optimum temperature for *E.coli* O157:H7 growth is 37°C. While it is sensitive to high temperatures, it is able to survive at very low temperature for a long period of time (Doyle, 2008). *E.coli* O157:H7 is very tough and able to survive up to several weeks on exposed surfaces and can be found in composted animal manure after years of storage (Kudva et al., 1998; Jiang, et al., 2002; Wang et al., 1996; Topp et al., 2003; Rivas et al., 2007). The bacterium's biological characteristic as a facultative anaerobe enables it to survive and multiply outside the host for a short period of time and this actually facilitates the fecal-oral route of transmission. *E.coli* O157:H7 is one of the few strains

among hundreds serotypes of *Escherichia coli* that can cause serious foodborne illness in humans. (Fratamico et al., 2002)

The serotype E.*coli* O157:H7 is well known among the general public in the United States as this strain has been publicly identified as the cause of severe foodborne illness. The capital "O" following the name indicates the cell wall antigen, while "H" indicates the presence of the flagella antigen. The O antigen is a polysaccharide side chain which comprises the lipopolysaccharide (LPS) that is part of the outer membrane of *E.coli*. The LPS sugar units build the specific characteristic for this pathogen by expressing antigenic variation and serological specificity (Sheng et al., 2008). Therefore, several analyses to distinguish *E.coli* O157:H7 from other similar serotypes rely on differences in the presence and/or composition of various cell wall proteins. For example, *E.coli* O157:H7 is unable to ferment sorbitol under anaerobic condition in red MacConkey agar compare to most of other strains due to the lack of glucoronidase (a common enzyme produce by E.coli strains). The McConkey plate will appears pinkish in color due to the colorless colonies formed from the incomplete digestion of sorbitol. Additionally, due to the absent of glucoronidase in this strain, the O157:H7 serotype is negative for the rapid test for *E.coli* called fluorogenic assay as it does not hydrolyze the compound 4-methylum-belliferone glucuronide (MUG) (Doyle, 1984). In another analysis, the H-specific antigen is regularly determined by the agglutination method (Ratiner et al., 2003).

Pathogenic *E.coli* are differentiated into classes by their toxin producing capabilities and are commonly grouped as EEC (enterovirulent *E.*coli) as they infect and caused destruction of the entero-environment of the host. The EEC can be divided into 6

subgroups such as Enteroinvasive *E.coli* (EIEC) which causes severe diarrhea when infecting the intestinal wall, Enterotoxigenic *E.coli* (ETEC) which invade the intestinal lining by producing toxin, Enteropathogenic *E.coli* (EPEC) which commonly infect newborn babies, Enterroaggregative (EaggEC), which forms cell aggregation when infected to human intestinal cell linings, Verotoxigenic *E.coli* (VTEC) which could lead to Haemolytic Uremic Syndrome (HUS) and Enterohemorrhagic *E.coli* (EHEC) which is well known because of its pathogenic strain, *E.coli* O157:H7 could cause bloody diarrhea or known as hemorrhagic colitis (IFT, 1997).

Colonization of pathogenic *E.coli* O157:H7 is a complicated event through the interaction between adhesins (usually involve the bacterial flagella) and host cells. Once the EHEC adhere to the host, they start to produce shiga-like toxins (also referred as verotoxin). The Shiga toxin (Stx) producing *E.coli* O157:H7 (aka STEC) are usually the strains that cause foodborne illness outbreaks in United States. STEC has two different toxins which are Shiga toxin 1 (Stx 1) and Shiga toxin 2 (Stx 2) where Stx 2 is found to have increased risk to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Erickson and Doyle, 2007). Cergole-Novella et al. (2006) reported that Stx 1 was detected in human origin and this probably could explain the Stx 2 has higher virulence effect on human. Stx has been identified as potential bioterrorist agent by the Centers for Disease Control and Prevention (CDC) because the gene producing Stx can be isolated from Stx producing *E.coli* and then can be transferred to harmless *E.coli* strain through bacteriophages. Eventually, direct transmission causes the newly integrated nonproducing Stx strain to become dangerous. Stx toxins consisted one A subunit and five B subunits. A subunit is able to cause cytotoxicity by inactivating ribosomal subunits in eukaryotic

cells, which halts the synthesis of protein and eventually brings the cell to death. Through adherence and toxin productions, the intestinal lining develops lesions and these cause the characteristic bloody diarrhea. Shiga toxins are very selective in adhering to the receptor of the intestinal lining of their host. The B subunits of the toxin protein help to bind the toxin to specific cells known as globotriaosylceramide (Gb3) receptors found primarily on the surface of intestinal tracts (Asakura et al., 2001).

Stx alone is not able to cause full virulence in human until the bacterial cells were mediated with specific virulence markers. When a person is infected with stx positive *E.coli* O157:H7, the pathogens adheres to the host tissues through the *E.coli* common pilus (ECP), an adhesins which facilitate colonization by *E.coli* O157:H7. This colonization may lead to HC and HUS (Mora et al., 2007). Researches also studied that the presence of eae gene found in most of the EHEC strains is necessary to cause severe cell lesion in human such as HUS (Donnenberg et al., 1993). Moreover, Saldana et al. found that the presence of ECP in the bacterial cells could enhance the intimate attachment of eae gene to the intestinal surface (Saldana et al., 2009).

Asakura et al. found that pathogenic STEC strains that were isolated from human infection may be harmless to farm animals, probably due to the absence of specific receptors in the digestive tracts of farm animals (Asakura et al., 2001). Some potentially-pathogenic STEC strains might be transmitted to humans through animal shedding or via contaminated carcasses. Common symptoms of *E.coli* O157:H7 infections are usually severe or acute hemorrhagic diarrhea, abdominal cramp, bloating, with or without a fever which can last for 5-10 days. A very low percentage of those infected, mainly young children or the elderly will go on to develop HUS (Harris et al., 2003).

According to USDA food safety conference, regular *E.coli* such as Enteroinvasive *E.coli* (EIEC), Enterotoxigenic *E.coli* (ETEC), Enteropathogenic *E.coli* (EPEC) can only cause illness in infected patient when they are present in large numbers (~10^6-10^8colony-forming unit/g) (Greig et al., 2010). However, based on outbreak data, the EHEC strains of *E.coli* were found to cause infections in relatively low doses at around 10-100 CFU/g. The hypothesis is that this virulence is due to the ability of the Stx toxins to be transmitted to non-pathogenic or commensal strains of *E.coli*, which then develop the ability to produce Stx toxins after an appropriate incubation time and temperature. Also, most of cases of pathogenic *E. coli* transmission were found to involve the contamination of food products through surface contact with pathogen sources such as contaminated foods and direct or indirect contact with farm animals (Erickson and Doyle, 2007).

The economic impact of increasing *E.coli* O157:H7 related outbreaks in America have been huge, causing an estimated loss of approximately $405 million annually due to premature deaths, medical care, and lost productivity (Doyle, 2008). The increasing number of outbreaks caused by the pathogen has drawn a great deal of public attention. Thus, efforts are underway to seek solutions and avoid the negative impacts that have affected the good reputation of the food industry in the United States. Following is more information about the outbreaks' epidemiology, sources of contamination, and factors leading to the outbreaks of *E.coli* O157:H7.

Incidence of *E.coli* O157:H7 outbreaks in the US

In the United States, *E.coli* O157:H7 was first isolated from human feces in 1975, before it was recognized as a food pathogen. That recognition occurred seven years later

in 1982 when the bacterium was identified as the causative agent in two outbreaks of hemorrhagic colitis linked to undercooked hamburger patties. Foods are commonly known as the main vehicle for *E.coli* O157:H7 infections, particularly foods of bovine origin. Foods such as ground beef, hamburger patties, and unpasteurized milk are at higher risk of direct or indirect exposure to cattle manure, which may contain significant amounts of shed *E.coli* O157:H7. One study sampled fecal isolates collected from the fairgrounds at 32 agricultural fairs and showed that the pathogen was more predominate in bovine species: 13.8% of beef cattle isolates and 9.6% of dairy cattle isolates contained *E.coli* O157:H7 compared to 3.7% of pig isolates, 6.8% of sheep isolates, and 2.7% of goat isolates (Keen et al., 2006). Clearly then direct exposure to animal manure is a risk, especially cattle manure. In addition, indirect exposure is also a risk as *E.coli* O157:H7 can survive and grow without a host thus setting up the possibility of cross contamination when food products are accidentally exposed to livestock products such as carcasses, hides, or even unpasteurized milk, all of which can be contaminated by STEC shed in the animals' manure (Farrell et al., 1998; Ak et al., 1994).

From the early 1990s to 2007, about 10 outbreaks were reported related to the foodborne Stx producing *E.coli* caused illness following the consumption of contaminated foods of bovine origin in US alone. But foods of bovine origin are not the only foods at risk. The ability of the pathogen to survive and reproduce in poor conditions outside a host such as plastic and stainless steel enables it to contaminate many other food products such as fresh produce (Ak et al., 1994, Farrel et al., 1998). For example, apple cider and orange juice containing organic acid, a weak acid, were contaminated by the acid tolerant *E.coli* O157:H7 in 1996 and 1999 respectively. In addition, fresh leafy

greens including lettuce, spinach, alfalfa and some other salads greens have increasingly been contaminated and caused substantial severe outbreaks due to the greater per capita consumption of raw, minimally processed fresh produce (Doyle, 2008). In addition, outbreaks associated with this pathogen have expanded to include carbohydrate rich processed foods: refrigerated cookie dough was recalled because of contamination with *E.coli* O157:H7 in 2009 (FoodNet, 2009).

Association of *E.coli* O157:H7 outbreaks with consumption of bovine origin food products

As noted previously, one of the characteristics of E. coli O157:H7 is its ability to survive for an extended period of time outside a host. This characteristic allows the surface of non living substances to function as another possible route of transmission for *E.coli* O157:H7 to foods. This presents particular challenges in foods of bovine origin as cattle are known to harbor the bacterium and the environment in processing facilities that handle cattle or bovine food products or ingredients may become contaminated. If this occurs, the pathogen may survive and multiply from slaughter all the way to the end product. Contamination may occur via contact with contaminated surfaces, processing equipment, or when products are cross-contaminated with other contaminated products or ingredients (Pennsylvania, 2009). Before food processors learned from the many outbreaks associated with beef products about managing the risks, food industries lacked the necessary food safety interventions in the production plant. Carcasses were often exposed to contact with the animal's intestines and feces during slaughter, both of which are known reservoirs of *E.coli* O157:H7. Fortunately, the chances of infection by enteric pathogens found on the meat surface are low. Because the pathogens on the surface are

readily destroyed by cooking, beef products such as steak have rarely been reported as the vector of foodborne illness (Bolten et al. 2009). However, if a contaminated carcass is ground, it can contaminate the grinding equipment and potentially create an environment for the transmission of *E.coli* from one batch of ground meat to the next that is processed using the same equipment (Flores and Stewart, 2004; Budzby et al., 1996). Hence, hamburger patties, which have a higher risk of having undercooked interiors, have been reported as the most predominant vehicle for E.coli outbreaks (Bolten et al., 2009).

Association of *E.coli* O157:H7 outbreaks with consumption of fruits and vegetables

Harvesting of fresh produce indirectly provides foodborne pathogens with chances to contaminate the crop due to the changing of the physiological state or the mechanical disruption of the surface that occurs with some crops. Ready-to-serve fresh-cut leafy greens that require only minimal handling and lack a processing step that have potential for foodborne outbreaks of *E.coli* O157:H7. Soil treated with animal manure as fertilizer, especially manure from bovine species, has been considered as the main agent to cross contaminate field crops, particularly those grown near to the ground (Doyle, 2007). Another risk factor is that ready-to-serve vegetables such as salad greens (e.g. spinach and lettuce), shredded vegetables (e.g. carrots and cabbage), and fruits including watermelon, cantaloupe, and cucumber have a larger surface area after they have been shredded or cut into cubes. Unpasteurized fruit juices, such as apple cider – once commonly sold in US – are other fruit products that can be contaminated by *E.coli* O157:H7 (Beuchat, 2002). Fresh culinary herbs have been reported to support viable *E.coli* O157:H7 even under chilled storage at 4°C (Warriener et al., 2005). However, apart from the emerging problem of contaminated leafy greens, including lettuce and

spinach, fruits and vegetables are less consistently reported as the source of foodborne illness outbreaks than are animal-based products. (Bjornsdottier et al., 2006).

E.coli O157:H7 contamination of spinach

In 2006, CDC (Centers for Disease Control) received reports from Wisconsin and Oregon health officials on people suffering from symptoms of bloody diarrhea after consumption of fresh bagged spinach. Thereafter, about 100 cases of *E.coli* infection were reported to the CDC from several states in the following two days. The contamination source was then verified after a series of trace back procedures conducted by the epidemiologic investigation department. They found that the contaminated spinach was produced in same processing plant during a single shift. The FDA later declared that all bagged spinach needed to be removed from the market and a warning was given to the public to avoid consuming any spinach (FDA, 2006[B]). The CDC speculated that the bagged spinach was from fields that were contaminated with irrigation water that had been contaminated with ruminant manure. However, investigators could not confirm the main source of the contamination. As a result of this outbreak, the FDA recommended that farmers observe Good Agricultural Practices (GAPs) and that food processing companies perform appropriate food safety practices to reduce the risk of contamination. Fresh food processing companies and spinach producers learned the lessons from the outbreak and recognized that four sources comprised the main contamination risks: animal manure, animal carcasses, transient wildlife, and water. Apart from knowing the causal factors, an understanding of the microbial ecology of this pathogen in connection with contaminated spinach is crucial in order to trace back the origins of the outbreak occurrences. The chances of new food product contamination incidents could be further

reduced once the origins of previous contamination were detected. In summary, associated industries were advised to establish standardized, verifiable, scientifically-tested interventions to reduce the risk of contamination in their processing plants. At the same time, on-farm GAP systems should be implemented and followed at all times (Brackett, 2009).

Structure of the spinach

Further studies of the diverse and complex associations between *E.coli* O157:H7 and spinach all the way from the field to finished product are important if we are to manage to control and reduce the possible risks of contamination. Firstly, understanding the relationship between naturally occurring microflora and pathogens is crucial. Mitra et al. (2009) showed that background microorganisms and *E.coli* O157:H7 were able to grow together in a wide range of diverse ecological niches, and that these niches could actually be provided by the great variations in surface morphology, internal tissue composition, and metabolic activities in the different parts of the spinach plant. Broadleaf plants – including spinach, lettuce, and parsley – should have similar microbial communities because of their comparable morphology (Mitra, 2009).

Mitra et al. (2009) also determined that the leaf surface microenvironment can affect the route used by *E.coli* O157:H7 to infect spinach. Different cultivars of spinach have different leaf surface morphologies. The study investigated the bacterial population density, location of the bacterial cells, and movement of *E.coli* cells inoculated into and onto 3 different cultivars. They used leaf drop (placing a drop of inoculums on each spot labeled on the leaf surface) as a method of microbial inoculation to simulate the splashing

of manure on the aerial surface of the leaf during rainfall. Viable cells were observed to survive for 2 weeks in the leaves and colonies were found clustered near stomata. Stomata were thus believed to provide convenient entry for pathogens into the spinach plant. One of the cultivars used in this study, the Savoy leaf cv.Tyee was observed to provide a higher numbers of niches compared to other cultivars used in this study. The researchers concluded that this was because niches formed between the ridges and valleys found on the leaf surface acted to protect colonies from sanitization. The study also suggested that leaves offered more suitable environments for pathogens growth compared to the rhizhosphere roots or the stem. In addition, different nutrients provided by the various cultivars were believed to result in different rates of pathogen survival (Mitra et al., 2009).

After some period of colonization of the pathogens at the wound surface, tremendous accumulation of the pathogens builds up a slime-like matrix known as a biofilm (Rivas et al., 2007). Biofilms consist of a matrix of bound cellular capsule material, which is made up primarily of the exopolysaccharide excreted by the colonizing bacteria. Bacterial cells may be present in large numbers in a biofilm; the population in one biofilm can comprise 10-40% of the whole bacterial population found on the leaf surface once they react with the cuticle or cell walls of the fresh produce tissue (Morris et al., 1998). Biofilms are believed to acts as a protective layer for pathogens by effectively shielding them from surface sanitizing agents, detergents, or inhibitory agents (Norwood et al., 2000; Uhlich et al., 2006). Biofilms have frequently been found to form in niches where sanitizing agents can hardly reach during post harvest washing (Carmichael et al., 1999). Some studies have shown that biofilms may act to isolate and protect bacterial

cells on the surface of leafy greens, including spinach, as the morphology of leaf surface provided possible niches for biofilm formation (Beuchat et al., 2002).

Fate of E.coli O157:H7 in rhizhosphere and soil

Plants of different species have different rhizhosphere microbial communities in the vicinity of their root exudates and they also have different root morphologies. In addition, as the plants mature, the rhizhosphere community of the plants will change dramatically due to the growth of naturally occurring microflora. These variations in microflora communities may explain the lower number of pathogens observed in mature plant tissues compared to the numbers observed in contaminated seedlings. The study further investigated the effects of soil temperature and moisture on the pathogen's survival rate by collecting data on the average daily temperature and total rainfall. They found that low temperatures weaken the plant's defensive activities and thus posed a higher risk for the pathogens to become internalized in the plant (Erickson et al., 2010[A]).

The study also showed that the rate at which roots of spinach were contaminated by *E. coli* increased only if the roots were exposed to very high doses of inocula (7 to 8 log CFU/g) in manure (Sharma et al., 2009). At lower inoculum concentrations, contamination could only be observed in hydroponic systems or when the soil directly around the roots was amended by inoculums. The researchers hypothesized that this may be explained by the fact that both methods of inoculation actually reduced the need for the pathogens to compete with other microbiota (Erickson et al., 2010[A]).

Microorganisms found in manure preferred to retain in upper layer of soils because the preferable pore size between soil particles, pH levels, temperatures, soluble

organic materials, and available water favor their growth. Movement of pathogens from contaminated manure through the soil profile depends on the type of soil, manure physiochemical composition, and the climate. Both timing and frequency of rainfalls after the application of manure greatly influenced the movement of pathogens through the soil. According to Saini et al. (2003), they found that *E.coli* leached into the soil from inoculated manure for substantial periods of time even though the manure was left for an extended time (16 days) on the soil before they conducted the simulated first rainfall. The researchers also believed that *E.coli* may excrete toxins that were able to retard the growth of other indigenous microbiota and eventually increased their rates of survival in the rhizhophere.

Possible routes taken by pathogenic *E.coli* O157:H7 to fresh produce

Avenues of extrinsic contamination by *E.coli* O157:H7

Sources such as soil, feces, irrigation water, inadequately composted manure, domestic animals, and rain fall have been described as extrinsic microbial contamination factors for fresh produce. Land used to cultivate spinach is recognized as the main source of contamination, especially if the land has been treated with cattle manure as a nitrogen source. Soil treated with manure has been shown to provide a nutrient-rich environment for the growth of pathogenic bacteria (Sharma et al., 2009). Cattle manure that is non-composted or improperly composted is a particular source of risk for soil contamination. According to several studies, *E.coli* O157:H7 can survive up to months or even years in the soil as long as the pathogens are provided with sufficient nutrients and other environmental factors in the soil habitat remain favorable (Sharma et al., 2009; Kudva et

al., 1998). Another study showed that *E.coli* O157:H7 found in shed bovine manure can survive as long as 120 days if the manure is aerated or may even survive for up to 21 months in non-aerated manure (Kudva et al., 1998).

Manures applied to the soil are frequently handled and treated using poor management techniques and may be stored for long periods of time before being applied. For example, contaminated cattle manure used as fertilizer was one suspected source of the 2006 E. coli outbreak linked to fresh spinach. Manure from the suspected ranches in close proximity to the spinach farms were found to contain a similar *E.coli* strain that was found in the contaminated spinach. Also, the investigators listed several possible reasons that caused the outbreaks. Reasons included water on the farm coming from lake or river sources and having multiple uses such as irrigation, animal watering and worker hygiene (Lisa, 2006).

Other possible contaminants would be the hooves of livestock that are allowed to graze fields that are subsequently used to grow produce. Animal hooves may be tainted with pathogens that are shed by the animals as they move around on the land. Rainfall or untreated irrigation water have also been identified as a possible contaminant. Contaminated water may cause widespread microbial contamination on a farm (Doyle, 2008).

Regardless of how pathogens contaminate fresh produce, these pathogens may become internalized in the plant tissues or else closely associated with the surface tissue, for example in biofilms. When this occurs, we can describe these bacterial cells as

intrinsic contaminants. This intrinsic contamination is particularly risky because these bacterial cells are often highly resistant to cleaning and sanitation treatments.

Internalization into plants

Several studies have established that pathogenic bacteria can be internalized into plant seeds at the germination stage, increasing in number in the rhizosphere of seedlings and within the growing plants. Some studies even showed that the pathogens can be recovered in aquatic plantation without involving soil after an extended period. However, one study conducted on inoculated spinach seeds stated that when bacteria were internalized via the vascular system, higher numbers of microorganisms were found at the seedling stage than in the mature plants (Jablasone et al., 2005). This suggests that the vascular system is not a common route for *E.coli* O157:H7 to be internalized into fresh produce. According to the researchers, *E.coli* was observed more frequently in seedling roots; this was presumed to be the result of higher temperatures during germination that were more favorable to the growth of the bacteria. In addition, the researchers speculated that the release of the nutrient by the roots at the seedling stage accelerated bacterial growth (Jablasone et al., 2005). This conclusion was supported when *E.coli* O157:H7 bacteria were found to exist in higher numbers near the sites of epidermal root junctions, which release exudates into the soil and offer pores that may allow the bacteria to become internalized (Franz et al., 2007).

Infiltration as other entry way to internalize plant

Studies have demonstrated that pathogenic bacteria could also infiltrate into plant micro-niches due to the fact that external water pressure may be higher than internal gas

pressure and because the hydrophobic nature of plant surfaces may help drive micro-droplets of water into cracks and fissures in the tissue. This may occur in the field or during processing as part of washing, cooling, or in transport flumes. Flagella, which allow the bacterial cells to move under their own power, have been observed to promote internalization of bacterial cells (Mitra et al., 2009). Detergents, which act to reduce water surface tension, may also facilitate the infiltration of water into plant tissues through wounds or bruises. However it occurs, water infiltration can lead to the internalization of pathogenic bacteria if these bacteria are present in the water.

Factors affecting intrinsic contamination by *E.coli* O157:H7

Intrinsic factors that affect the growth of specific rhizosphere microflora, such as *E. coli*, exist in and around a plant's outer surface tissue and cuticle. These include pH and the amount and composition of fluids present, especially in damaged plant tissue. Even though wounds in tissue surfaces have been proven to provide nutrients for pathogens, tissue fluids also function as antimicrobials that prevent the possible growth of pathogens and other microflora through the production of phytoalexins. Thus, the relationship between the plant and the rhizoshpere microflora may be complex. For example, nutritive plant tissue fluids have been shown to help *E.coli* O157:H7 survive and grow better when other naturally occurring microorganisms were inhibited, leading to fewer competitors for the pathogens (Saini et al., 2003).

In any case, plants with wounds and/or cut surfaces are believed to have a higher risk of microbial contamination. Wounds or cut surface tissue provide openings for the

pathogens to enter the plants more readily. Moreover, nutritious fluids that enhance the growth of pathogens have been excreted at the surface openings (Beuchat, 2002).

One study has observed that the effects of antimicrobials added to live stock feeds or water for pharmaceutical effect or as a weight-gain enhancer used could actually be lethal to humans as well as bacteria because, if the dosage applied is improper or the animals are treated continuously, bacteria that are naturally found in the animal's intestinal tract may develop drug resistance. The effects of drug resistance may be problematic in both pathogenic and non-pathogenic bacteria because both types have the potential to mutate, and non-pathogenic bacteria may ultimately become pathogenic when transferred into the human body. This is supported by the fact that most foodborne outbreaks are associated with contamination by non-typhoid bacteria that are a natural part of the microflora found in intestine, for example *E.coli* (Bicudo and Goyal, 2003).

Environmental factors affecting contamination

Rainfall

Rainfall has also been considered another environmental factor that increases the possible risk of in-field contamination of crops by providing another avenue of pathogen access. Splashing of tainted manure from the ground during rainfall events could contaminate the plant leaf surface. In addition, falling rain may lead to spreading pathogens in the field as running water may carry pathogenic bacteria to previously uncontaminated areas and thereby eventually cause contamination of plants cultivated in those areas. In addition to surface spreading, Saini et al. (2003) applied simulated rainfall events with controlled timing and frequency to soil samples that had been inoculated with

E. *coli* and found that *E.coli* could leach through the soil profile. In addition, the researchers found that rainfall carried the pathogens down into the core of the soil horizon where the pathogens became redistributed and were able to establish new colonies (Saini et al., 2003).

Aside from being a transmission vehicle, rainfall also supplies essential moisture to the enteric microorganisms, which allows them to survive better. The moisture in soils helped to distributing the organic matter content and substrates needed for bacteria growth. Higher clay content in soil after rainfall provided protection to the bacteria against predators (Brenna et al., 2010).

Soil Moisture

Soil moisture has been reported to affect the microbial population on fresh leafy greens in the field, and has been observed to significantly affect the growth rate of *E.coli* O157:H7 found on vegetables grown in an open field (Ibenyassine et al., 2006). Although rainfall does not pose the same risk of causing foodborne illness as some other water sources, such as untreated irrigation water, moisture from rainfall nevertheless favors the proliferation of microorganisms. One study found that moisture in the soil helps maintain microbial populations in field crops when they performed late irrigation termination (IT) on experimentally grown lettuce. Late IT was shown to reduce the microbial quality of lettuce by creating conditions that favored condensation on the leaf surface as moisture was lost from the soil. This raised the water activity of the leaf surface environment and thereby facilitated the growth of pathogens (Fonseca, 2006).

Another study found that subjecting vegetables to water stress could reduce the growth of bacteria because the stress conditions accelerated the plant's production of metabolic waste materials, which were believed to inhibit microbial population growth (Sudha and Ravishankar, 2002). Thus, low soil moisture may help to inhibit the growth and survival of E. coli O157:H7 in a number of ways. However, E.coli reportedly can survive in a low water activity environment, such as under chilled storage, for an extended period of time. Apart from that, soils could serve to protect bacteria, particularly those found near the roots of plants, by protecting bacterial cells from UV radiation from sunlight. Additionally, the soil environment immediately around the roots may retain moisture compared to other soil regions and this help protect the bacteria from desiccation.

The role of soil moisture in facilitating or inhibiting the contamination of produce in the field is not completely clear. However, on balance increased moisture may well increase the possible risk of contaminating the edible leaves of plants such as spinach. Higher moisture levels likely help pathogenic bacteria to persist in the soil and also help provide a means for enteric bacteria to gain access into the plant and become internalized.

Ways of controlling the *E.coli* carried in manure used as fertilizer

Shed animal manure is often collected by the farmers in slurry form and kept in various kinds of storage such as lagoons, above ground tanks, or earth basins, until field conditions are ready for manure application. Lagoon systems and anaerobic digesters have sometimes been used for better manure management and more efficient manure usage. These systems may also aid in retarding the growth of pathogenic bacteria.

Coliform bacteria are typically able to grow in anaerobic conditions over a long period of time, but most of them will diminish in about 30 days .Therefore, anaerobic lagoons are frequently used in temperate climate regions and have frequently been recommended to reduce the numbers of pathogens in manure storage. One study showed that the population of *E. coli* was reduced by up to 4 log units using a two-stage anaerobic lagoon treatment in which the anaerobic digester was equipped with a heater. The heater created a thermophilic environment in the system, which led to substantial reductions in bacterial numbers (Bicudo and Goyal, 2003).

Composting is often used as an on-farm manure treatment; the heat generated by the microbial action during composting may be sufficient to kill any pathogens that may be present in the non-composted manure. However, the temperature attained during composting and storage is important in determining the degree of efficient killing of the pathogens (Islam et al., 2005). Manure that reached higher temperatures during composting showed a decrease in the number of pathogens; several studies were conducted that showed that the population of pathogens decreased dramatically at 50°C and higher (Whitman et al., 2008). Duration of the composing operation is also critical as raw or improperly composted manure can contaminate clean compost during storage, even after one year of storage (Jiang et al., 2002; Kudva et al., 1998).

We know that rainfall and runoff of agricultural lands can play important roles as possible sources of microbial contamination when that water comes in contact with contaminated manure. Therefore, contamination of the water and subsurface water should be avoided by farmers. Some agricultural practices such as the use of vegetative filter strips may help to filter and direct the flow of runoff water. These may help limit erosion

and can help to contain runoff water in a designated collection area. This can prevent runoff water from contaminating streams as well as uncontaminated fields. One study showed that vegetative filter strips can help to remove 75-91% of fecal coliforms and 68-74% of fecal streptococci from the runoff collected from manure-amended fields (Beuchat, 2002).

Another method to decrease the risk from contaminated manure is to decrease the risk that the animals harbor pathogenic bacteria in their gastrointestinal tracts. Study results have shown that a grain-based diet may result in acidic stomach conditions in cattle, which are favorable for the growth of *E.coli* (Gilbert et al., 2005). This study showed that changing from grain diet to hay feeding led to a significant decrease in viable cells in the shed manure. Decreases of as much as 10^6 CFU/g were observed after five days in some cases. The authors speculated that grain feeding not only enhanced the growth of the pathogens by providing high quality nutrients, but also increased the capability of the pathogens to adapt to the acidic conditions. Thus, one way to decrease the presence of pathogens in shed cattle manure may be to change from a grain-base diet to silage.

Adding anti-microbial chemical compounds directly to shed manure have also been observed to help to diminish the possible number of viable pathogens found in that manure. The chemical agents tested included ammonia, formaldehyde, sodium hydroxide, and lime (Bicudo and Goyal, 2006). Lime has received more attention as a treatment for reducing pathogens in shed animal manure as it is less hazardous, easier to manage, and more economical. Ozone was shown to be another agent able to reduce the

bacteria found in animal waste. In one study ozone was able to reduce viable cells numbers up to 3 logs units (about 99.9%) at an addition rate of 1g (Zhang[C] et al., 2009).

According to Avery et al. (2005), *E.coli* O157:H7 was remained viable in manure for as long as 48 days under regular management of field condition. They also found that extended periods of storage, such as over winter storage, will eventually lower the number of viable cells, although complete elimination was difficult to guarantee. The higher the numbers of pathogens that persist in the manure prior to in-field application, the higher the risk that injured or viable cells may survive or even grow to numbers sufficient to cause infection. Therefore, researchers advised that crops should be harvested at least 8 months after the application of raw manure (Avery et al., 2005).

Studies have shown that plants grown above the soil surface are at lower risk to be contaminated compared to produce such as carrots or onions that are grown under or near the ground surface (Bicudo and Goyal, 2003). Research suggests that this is because soil amended with contaminated manure can be more easily transmitted to the roots, tuber or leaves by various factors such as runoff or splashing when the produce is grown under or close to the soil. Hence, subsurface drip irrigation was recommended as a production method that can help reduce the risk of contamination compared to furrow or overhead irrigation (Song et al., 2006).

Proper management of land used for cultivation of fresh produce is important as it is critical for retarding possible pathogenic bacterial growth. It is recommended that buffer zones always be prepared and positioned between grazing areas and cultivation areas to avoid cross contamination (Bicudo and Goyal, 2003). The hooves of grazing

animals have been observed to cause cross contamination within fields used for both grazing and cultivation as contaminated shed-manure may stick to the animals' hooves. Thus, the impact of grazing animals on the same land used for growing crops is to increase the risk of contamination (Bicudo and Goyal, 2003).

Research has also found that covering contaminated soil with a layer of pasteurized soil reduced the number of bacteria contaminating the leaf surfaces of plants grown in contaminated soil (Monteith et al., 1986). Researchers hypothesized that there was less aerosolization of the manure in the air surrounding the plants, which decreased the chances for crops to become contaminated. Rain splashes of pathogen-containing manure from the ground to tissues above the soil were also reduced (Bicudo and Goyal, 2003).

Conclusions

In conclusion, we know that *E.coli* can survive for a long time in soils amended with animal manure, certainly for up to several months. We also know that the pathogen may be internalized into the plants cultivated in contaminated soil and that the internalization may be mediated by the extrinsic and intrinsic factors discussed earlier. Thus, the proper management of animal manure in the field is crucial as even a low infective dose of *E.coli* may be enough to cause a foodborne outbreak in the human population.

The overall objective of the present study was to determine the effects of environmental factors (rainfall, relative humidity, and soil moisture) on the survival of *E.coli* O157:H7 on spinach cultivated in soils amended with inoculated cattle manure.

The numbers of cells used in this research were higher than the actual numbers of viable cells that are likely to exist in actual agricultural field conditions. This was done in hopes of being able to better quantify in-field contamination risks and therefore to suggest better methods to reduce the possible risks of in-field contamination.

CHAPTER III

METHODOLOGY

Bacterial strain and inoculums preparation

A non-pathogenic human feces strain, *E.coli* O157:H7 ATCC 43888 was obtained from the culture collection provided by the Robert M.Kerr Food and Agricultural Products Center (FAPC) at Oklahoma State University, Oklahoma. Although the strain does not produce any shiga-like toxins (stx-1 and stx-2), it is otherwise not different from Shiga-like toxin positive *E.coli* O157:H7 (Kudva et al., 1998).

The strain was subcultured 3 times at an inoculation rate of 1% (0.1ml of pure culture to 9.9ml of tryptic soy broth) and incubated at 37°C for 6-8h (on a shaker plate in order to accelerate the growth). The third subculture was serially diluted with peptone by a factor of 1:10 (1ml of third culture to 9ml of peptone water) to yield a concentration of about 10^{-6}cfu/g. The resulting dilution was plated on tryptic soy agar (TSA) and incubated at 37°C for 18h. Single colonies (one small and one large colony was chosen) with same morphology were picked from the plate with a loop and tested using gram staining to examine the purity of the colony. After confirming the purity of the colony, cells from that selected colony were grown in 10mL of tryptic soy broth (TSB) and

incubated at 37°C overnight. The inoculum was then diluted in phosphate-buffered saline (PBS), pH 7.4, to make a final concentration of 10^7cfu/g. The purified strain was then stored in a freezer at (-20°C) and subcultured 3 times as described above immediately prior to preparing in-field inoculums. The viability of the each culture was checked by looking at the turbidity of the tubes before subculturing. Inoculums used for in-field inoculation were prepared by transferring 2.5ml of the third subculture into 250ml of TSB and incubating the resulting mix for 18h at 37°C. The inoculums were kept in an ice chest with ice at all times before they were used for inoculation to avoid any killing of the cultures from direct sunlight or surrounding heat. Inoculums were applied immediately after being removed from the ice chest to avoid contamination.

Soils and manure

The field used for these experiments consisted primarily of Teller fine sandy loams with 1-2 slopes (NRCS, 2011) and was previously used to grow a variety of agronomic and horticultural crops. Teller fine sandy loams consisted of grayish to grayish-brown top soil and yellowish-brown in the middle layer (5 to 15 cm). It is good for cultivation during dry season as the soil is well drained and good in conserving soil moisture. The soil can be utilized for vegetable plantation in a small scale (USDA, 1915[A]). The soil was analyzed for physical and chemical properties by the Soil, Water and Forage analytical laboratory at Oklahoma State University. Soil samples were reported to have a pH of 6.6, P index of 42 and K index of 323. The plots were treated with about 79 kg of actual nitrogen and 62 kg of phosphorus (P_2O_5) per hectare. No potassium was added to the plots. Herbicide, Dual magnum: S-metolachlor (Syngenta Crop Protection, Canada Inc.) was applied to the field plots at a rate of about 1.2 kg per

hectare to the field one day after planting. Application of herbicide was aimed to avoid any previous crop growth affect the growth condition of the spinach and planting process.

Composted cow manure (Black Kow® composted cow manure, Black Gold Compost Co., Oxford FL) was obtained as a soil amendment. The well-composted cow manure was tested and proved to have no significant population of indigenous E. coli bacteria (data not shown).

In field experimental design

Field studies were conducted at the OSU Cimarron Valley Research Station located one mile north of Perkins at the intersection of highways 33 and 177 in Payne County, OK. This was about 19 kilometers from the laboratory at the FAPC where analyses were conducted. In-field experiments were begun in the spring of 2010 and finished in the spring of 2011. The overall dimensions of the field were 46 m (150 ft) east to west and 23 m (75 ft) north to south. However, the area of all the plots only took up about 43 m (140 ft) of the total length and about 22 m (72 ft) of the total field width. Each plot was about 6 m (20 ft) long and 5.5 m (18 ft) wide (~33 m^2 or 360 ft^2) with 5.5 m (18 ft) between plots within each block and 3 m (10 ft) of alleyway between the blocks. The boundaries of each plot were marked with flags.

The experimental design for this study was randomized complete block. A total of four treatments were applied and each treatment was replicated five times. Thus, 20 plots were used and each combination of treatment and replication was randomly assigned to a single plot. Each plot was identified in the field with a wooden stake marked with a code to signify treatment and replication number. The four treatments were: non-inoculated

control plots; plots amended with non-inoculated manure; plots amended with inoculated manure; and plots in which the inoculums were applied directly to the soil.

Soils and manure preparation and inoculation

All plots beds were lined out with a tractor. Manure amendment plots were treated with about 11.3 kg of manure per plot. Prior to application, the manure was diluted in a bucket with about 19 l of water, and then mixed vigorously with an electric mixer for approximately 1-2 minutes to make slurry. This slurry was applied evenly to a given treatment plot by a plastic bucket (500ml). Slurries were prepared and applied to the plots amended with non-inoculated manure prior to the preparation and application of inoculated manure. Inoculated slurries were prepared by creating the manure slurry as described above, then mixing in 250 ml of the bacterial culture, which contained ~5.3×10^8 CFU bacteria g^{-1}.

Inoculums for the inoculated-soil plots were prepared by diluting 250 ml of the bacterial culture, which contained ~5.3×10^8 CFU bacteria g^{-1} with about 800 ml of distilled water. This mixture was then sprayed evenly on the surface of the treatment plot using a 15 l hand pump sprayer until the plot surface was uniformly moist.

Germination and field growth conditions of spinach plants

Immediately after the applications of inoculums, all the plots were tilled to a depth of about 10-15cm with a tractor mounted rototiller. All non-inoculated plots were tilled prior to tilling inoculated plots. After tilling, all plots were direct seeded with Olympia spinach using a Hege model 1000 plot planter. To prevent cross-contamination

between plots, seeding was conducted in the following order: control plots; non-inoculated manure plots; inoculated manure plots; and then direct-inoculated soil plots. A side-roll overhead sprinkler system was used to irrigate the field for two weeks right after the planting. The irrigation system adopted was set up to run north to south.

Sampling of soils

Soils samples were first collected within 2 hours of the initial in-field inoculation event and then every week thereafter for a total of eight weeks. After that, the numbers of *E.coli* O157:H7 detected were below 10^1cfu/g and therefore sampling was conducted once a month for three months.

The soil samples were collected aseptically with a sterilized soil probe with a diameter of 2.5 cm. Soils samples were collected down to a depth of about 5 cm from the top layer of soil and about 10 sub-samples were collected from each plot and combined into a composite sample. A sterilized spoon was used to remove all soil material into a sterile Whirl-Pak (Nasco, Fort Atkinson, WI) bag. The soil probe and sampler's gloves were cleaned with de-ionized (DI) water followed by a 70% ethanol rinse and wiping with a paper towel. The soil probe was confirmed to be dry before subsequent soil sampling to avoid the possibility that bacteria in the soil samples might be killed by residual ethanol. Soil samples were kept in an ice chest with 6 packs of sealed Ziploc® bags (SC Johnson, Racine, WI) of ice to keep them cool and avoid desiccation from direct sunlight or heat. Soil samples were analyzed within 24 hours after sampling.

Sampling of spinach leaves

Spinach leaves were sampled on the fifth week after planting. The leaves were plucked by hand wearing gloves sterilized using 70% ethanol. Approximately 20 leaves were randomly picked from plants in each plot and collected into sterile bags. Gloves were sterilized with 70% ethanol after sampling from every plot of same treatment and changed between treatments. The harvested leaves were placed in an ice chest with 6 packs of ice bags (as ascribed above) as soon as sampling was complete. Plant samples were analyzed on the same day of sampling. Plant samples were taken 7 times continuously from 3 week postinoculation and once a month in January through March 2011 except for the month of February 2011 when sampling was precluded due to a severe snow storm.

Enumeration of bacteria in all bacterial treated soils and manure plots and spinach leaves

Both soil and plant samples were assessed for *E.coli* O157:H7 and total coliforms. After massaging the soil sample bags to distribute the sample, 100 g of soil was weighed into a 500ml Pyrex glass bottle under a fume hood. The soil was then diluted with peptone water at a ratio of 1:5. The mixture of soil and peptone water was shaken well for 2 minutes and this solution was used as the highest dilution for enumeration.

For the spinach leaves, 25 g of leaf tissue was weighed either directly into a petri dish when the plants were young and the leaves were small and tender or into a sterilized mortar and pestle when the leaves were mature. The sterilized mortar and pestle was used to macerate the mature leaf tissue. Peptone water was added into the petri dish or mortar at a ratio of 1:10 to make highest dilution.

Serial dilutions were made from the prepared samples in 10-fold steps. Necessary dilutions were estimated by referring back to the past preliminary results. Every dilution was plated in duplicate on petri dishes, 4 plates per treatment, using two media: Violet-Red-Bile Agar (VRBA) and CHROMagar™ *E.coli* O157. Colony counts were calculated as CFU/g.

VRBA was selected to determine the presence of total coliforms, which could include the inoculated *E.coli* O157:H7, generic E. coli, and other related bacteria. These were termed background microflora. Plates were incubated at 37°C overnight. Colonies of interest had a dark purple color and only colonies of suitable size and surrounded by a halo were counted.

In recent years, microbiologists have adopted CHROMagar ™ *E.coli* O157 (CHROMagar, Paris, France) to isolate the shiga-toxins producing *E.coli* O157:H7 due to its ability to reliably detect the strain even at very low volumes of testing samples. *E. coli* O157:H7 colonies on Chromagar can be distinguished by their mauve color while light blue colonies represent other background microflora by supplementing the medium with potassium tellurite. Pottasium tellurite also functions as a recovery medium for *E. coli* O157:H7. Other benefits of Chromagar include the stability of the color of the colonies over several days and the ability to identify the subset of *E. coli* O157:H7 that carry shiga-toxins genes. Accordingly, CHROMagar™ *E.coli* O157 agar was used in order to detect the presence of *E.coli* O157:H7 in both soil and leaf samples after incubation at 37°C for 18h.

Detection of *E.coli* O157:H7 after inoculation in soils and plants leaves by using BAX PCR assay

An enrichment medium consisting of GN Hagna broth (Difco, Becton Dickinson, Sparks, MD) supplemented with antibiotics was used to enrich the sample dilutions for 18h at 37°C. The antibiotics and their rates of use were as follows: 50ng/ml cefixine; 10mg/ml cefsulodine; and 8 mg/ml vancomycin. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO).

The enrichment broth was used in order to detect the presence of *E. coli* O157:H7 in samples where the numbers of bacteria present were too low to permit successful enumeration. In these cases, 1:10 dilutions of soil and plant samples were incubated in the enrichment broth overnight as 37°C. The BAX PCR *E.coli* O157 MP assay (Qualicon BAX ® System Q7 instrument, DuPont Corp., Wilmington, DE) was then used to confirm the presence of *E. coli* O157:H7. The procedures specified by the manufacturer's instructions were followed to perform the testing.

Statistical Analysis

All the microbial data (in CFU/g) were log transformed. Results were analyzed using PC SAS Version 9.2 (SAS Institute, Cary, NC). Analysis of variance procedures (PROC MIXED) were applied to a 2-factor factorial treatment design (Manure, no-manure, inoculated, non-inoculated) on a by-week basis (repeated measures). The intrasubject correlation structure was modeled by using an autoregressive model with a time period 1 covariance structure. Individual effects of the 2 factors were calculated using the SLICE option in an LSMEANS statement. Combination effects of the 2 factors

were also calculated using same method for each given week. In addition, linear effects of week for each of the combination of the 2 factors were also analyzed using planned contrasts. Degree of significance was set at $p<0.05$. Correlation coefficient was performed using Microsoft Excel 2007.

CHAPTER IV

RESULTS

Survival of *E.coli* O157:H7 over time

E.coli O157:H7 applied via inoculated manure

Average weekly population counts for *E. coli* O157:H7 applied via inoculated manure as detected on CHROMagar O157 are seen in Figure 1. Results for Week 0 (the time of inoculation) showed that high initial concentrations of bacterial cells were successfully applied. The total recovery of *E.coli* O157:H7 immediately inoculated with manure (manure slurry) was about 6 $\log_{10}$ CFU/g as determined by direct plating on antibiotic supplemented CHROMagar O157 (data in appendix Table 10). In the in-field conditions, we observed that the viable cell counts of *E.coli* O157:H7 dropped significantly (exceeded a 95% confidence interval) from Week 0 to Week 1. The drop was from 4.6 $\log_{10}$ CFU/g to 2.3 $\log_{10}$ CFU/g (Figure 1). Although no significant differences in population numbers were seen throughout the remainder of the sampling period, the numbers of surviving *E.coli* O157:H7 were observed to decrease gradually following Week 1, and the trend continued through week 20 of sampling

E.coli O157:H7 applied via direct spray

Average weekly population counts for *E. coli* O157:H7 applied via direct spray as detected on CHROMagar O157 are seen in Figure 2. For this treatment, the total recovery of *E.coli* O157:H7 in spray inoculums after added of distilled water was about 7 $\log_{10}$ CFU/g as determined by direct plating on antibiotic supplemented CHROMagar O157 (data in appendix Table 10). As seen in the populations of bacterial cells added via inoculated manure, the cell counts of *E.coli* O157:H7 dropped significantly week 0 to week 1 after treatment, in this case from 3.8 $\log_{10}$ CFU/g to 2.4 $\log_{10}$ CFU/g (Figure 2). The survival rate then remained fairly constant through week 3, whereupon another significant decrease was observed between week 3 and week 4. Following that, no significant differences were seen in the population numbers throughout the remainder of the sampling period.

Comparison of survival rates of *E.coli* O157:H7 among inoculation treatments

Although no statistically significant differences were seen between numbers of *E. coli* O157:H7 in manure-amended plots versus direct-spray plots from week to week (data in appendix Figure 6), the numbers of *E.coli* O157:H7 recovered were consistently greater in manure-amended soils during the course of experiment.

Using Least Square Means analysis, results from the week of inoculation revealed that *E.coli* O157:H7 in inoculated manure-amended plots and in plots inoculated via direct spray were significantly different from other treatments, while control and non-inoculated manure amended plots were not different from each other (Table 1). Counts observed on week 1 through 3 after inoculation indicated that both inoculated treatments

were not different from each other but were significantly different from both control and non-inoculated manure-amended treatments. On week 4 to 6, only counts of *E.coli* O157:H7 inoculated manure amended plots were significant different from other treatments. After week 6, counts of enteric bacteria in all treatments were not significantly different from each other for the remainder of the study.

Comparison of survival among treatments averaged over all weeks

Averaged over all weeks, counts of coliforms and *E.coli* O157:H7 were significantly different from each other during the course of study ($p<0.05$) for all treatments based on variance procedures (PROC MIXED). However, coliform counts in spinach leaves were found to be significantly different only between non-inoculated manure and inoculated manure treatments ($p< 0.05$). For *E.coli* O157:H7 counts in spinach leaves, no statistically significant differences were observed between inoculated manure plots ($p=0.1294$) and inoculated soil plots ($p=0.0583$).

Background microflora detected in soils

Generic coliform bacteria were enumerated in all 4 treatments plots in order to account for the possible interaction between background microflora (e.g. generic E.coli) and *E.coli* O157:H7. Results from direct plating methods indicated that the coliforms counts were not substantially different in both control and non-inoculated manure treatment soil plots (figures shown in appendix), indicating that the composted manure used to amend the treatment plots did not contain high numbers of coliform bacteria. The populations of indigenous bacteria in both control and non-inoculated manure plots peaked at about 2.5 $\log_{10}$ CFU/g and decreased to about 1 $\log_{10}$ CFU/g over time.

Coliform bacteria populations were also enumerated in both inoculated manure and spray-inoculated soil plots. These population counts, which included the inoculated *E. coli* O157:H7 cells, followed the same pattern and generally did not differ significantly from the counts seen for E. coli O157:H7 proper (figures not shown) for either treatment.

Weekly effects were observed when Lease Square Means testing was used to analyze the bacterial cell counts. Coliforms counts were significantly different between treatments with inoculation and treatments without inoculation up until one week after soil inoculation (Table 2). After week one, the counts were not consistently significantly different.

Detection of *E.coli* O157:H7 in soil samples inoculated into enriched media

In order to detect the possible presence of viable *E. coli* O157:H7 cells that might not detected via direct plating, especially when the counts were below the detection limit of 1 $\log_{10}$ CFU/g, soil samples collected were enriched using GN broth with antibiotics. The enrichment broth samples were incubated for 18h at 37°C and were then plated on CHROMagar and analyzed by BAX PCR system. As expected, the BAX PCR provided more positive results for the presence of *E.coli* O157:H7 than the direct plating counts (data not shown). However, positive results, deemed to be false positives, were also observed to be present in control and non-inoculated manure soil samples (data not shown).

Survival of bacteria observed in comparison with in-field environmental conditions

Overall weather conditions

A summary of the weather data obtained from Mesonet weather station is shown in Table1. The data included average humidity and average rainfall over October 2010 to March 2011 in Perkins. During the time period of this experiment, relative humidity varied from about 50% to 75% and rainfall events from 0 mm to 0.5 mm (Table 3). During the course of the study, the relative humidity was generally in the mid to high range, near saturation. Total rainfall was low, due to the winter season.

Survival of bacterial cells compared with rainfall

The survival rates of generic coliform bacteria, generic *E. coli*, and *E.coli* O157:H7 in association with rainfall are shown in Figures 3a, 3b, and 3c respectively. When observing population trends, we saw that generic coliform bacteria in inoculated-manure plots recovered in numbers somewhat following higher rainfall events (Fig 3a). However, the numbers of generic *E. coli* were not notably affected by rainfall (Fig 3b).

A rainfall event may have exerted a positive effect on the survival rate of *E.coli* O157:H7 in inoculated manure as the counts of the bacteria increased at week 11, which was one week after a rainfall event (Fig 3c). Overall, a positive correlation was observed between rainfall and *E. coli* O157:H7 counts one week after rainfall. The Pearson correlation coefficient for this relationship in inoculated soils treatment was R= 0.63 (Table 3). By contrast, for plots inoculated via direct spray, the R-value for the week-to-week relationship was R= 0.35 (Table 3). Thus, rainfall may have had a lagging effect on bacterial survival.

Generally, population of coliforms, non O157 *E.coli*, and *E.coli* O157:H7 were correlated with rainfall as the Pearson correlation coefficients R-values were higher than the R-values for relative humidity (Table 3).

Survival of bacterial cells compared with relative humidity

The survival rates of generic coliform bacteria, generic *E. coli*, and *E.coli* O157:H7 in association with relative humidity are shown in Figures 4a, 4b, and 4c respectively. As noted above, the populations of enteric bacterial cells were observed to decline gradually following week 1 in control plots, non-inoculated-manure amended plots, inoculated-manure amended plots, and spray-inoculated soil plots. The numbers of background microflora, i.e. the generic coliforms found in control, manure amended soils (inoculated and non-inoculated), and inoculated soils samples, were directly correlated with relative humidity in the different direct plating media (Fig 4a). Coliforms in inoculated manure soil samples were seen to grow following an increase in relative humidity. A recovery in the numbers of non O157 *E.coli* was seen after the sharp drop post week 1 following an increase in the relative humidity (Fig 4b). Recovery in the numbers of *E.coli* O157:H7 was also seen in inoculated soils during the period of monthly sampling around week 11 (Fig 4c). However, inoculated manure experienced gradually decreasing cell counts throughout the study correlated to relative humidity. Also, the correlation between relative humidity and the survival rate of *E.coli* O157:H7 in inoculated manure-amended soil was weak and had a negative correlation, perhaps due to fluctuating relative humidity. The overall correlation results showed a low R-value of -0.26 (Table 3). Enteric bacteria counts in soil inoculated via direct spray treatment were seen to have an even weaker correlation with relative humidity (R= 0.07, Table 3).

Survival of bacterial cells compared with soil moisture

The survival rates of generic coliform bacteria, generic *E. coli*, and *E.coli* O157:H7 in association with soil moisture are shown in Figures 5a, 5b, and 5c respectively. A sharp drop in soil moisture and a sharp decrease in our target organism's bacterial counts were both observed between week 0 to week 1. The highest soil moisture (% water holding capacity) was then observed between week 1 and 2, after that initial drop. Coliform counts increased slightly following the highest soil moisture readings in both control and non-inoculated-manure-amended soil samples, but both inoculated plots went through inconsistent changes in the survival rate over time (Fig 5a).

As mentioned above, a sharp drop in soil moisture was associated with decreased counts of generic *E.coli* in all treatments from week 0 to week 1 (Fig 5b). However, counts were consistent for a period of time (no growth was observed) when the soil moisture did not substantially decrease following week 2. The counts of *E.coli* O157:H7 in soils inoculated via direct spray were observed to follow the trend of decreasing soil moisture during the 7 weeks after inoculation, and enteric bacterial counts were not stable throughout the period in inoculated-manure-amended plots (Fig 5c).

The correlation between bacterial counts in inoculated-manure-amended soils and average soil moisture from the previous week (1-week lagged effect) was fairly strong with r= 0.74 (Table 3). The same-week correlation was notably weaker with r= 0.19. Interestingly, the Pearson correlation coefficient for the one-week lagged effect comparing soil moisture and direct-spray inoculated soils was very weak with an r-value

of 0.08. The same-week comparison was actually stronger in this case with an r-value of 0.44 (Table 3).

Detection of *E. coli* O157:H7 spinach leaves

Sampling of spinach samples started on week 4 after soil inoculation and data were collected for 7 weeks total. All spinach samples analyzed in the control treatment were negative by direct plating (data not shown). Possible positive findings were observed in the inoculated plots, but the numbers were too low to enumerate via direct plating.

Detection of *E.coli* O157:H7 in spinach leaves after enrichment

Using the enrichment and PCR assay method described above, leaf samples were evaluated for the presence of *E. coli* O157:H7. Unfortunately, the results were inconsistent and again positive detections, taken to be false positives, were observed in the non-inoculated plots (data not shown). Because of the inconsistencies in the data, no firm conclusions could be drawn from these data.

Table 1. Cell counts of *E.coli* O157:H7 enumerated in direct plating of all treatments

Mean[1] log recovered (log_{10} CFU g^{-1})				
Weeks	Control	Non-inoculated manure	Inoculated manure	Inoculated soils
0	0.7c[2]	0.7c	4.6a	3.84b
1	0.7b	0.7b	2.33a	2.36a
2	0.7b	0.7b	1.7a	2.18a
3	0.7b	0.7b	2.17a	1.95a
4	0.7b	0.7b	1.7a	0.95b
5	0.7b	0.7b	2.01a	0.7b
6	0.7b	0.7b	1.56a	0.7b
7	0.7a	0.7a	0.99a	0.7a
11	0.7a	0.7a	0.7a	0.94a
15	0.7a	0.7a	0.7a	0.7a
20	0.7a	0.7a	0.7a	0.7a

1. n = 20.
2. Means with different letters (a, b, c) within the same row are significantly different ($p<0.05$).

Table 2. Cell counts of coliforms enumerated in direct plating of all treatments

Mean[1] log recovered (log_{10} CFU g^{-1})				
Weeks	Control	Non-inoculated manure	Inoculated manure	Inoculated soils
0	1.98b[2]	1.96b	4.7a	5.1a
1	1.7b	1.7b	2.63a	2.66a
2	2.37a	2.52a	1.63a	2.65a
3	2b	1.68b	2.88a	2.29ab
4	1.62a	1.48a	1.82a	1.3a
5	1.33ab	0.7b	1.86a	0.99b
6	1.29ab	0.99b	1.87a	0.7b
7	0.7a	1.16a	1.28a	0.99a
11	0.98b	0.7b	1b	1.85a
15	0.7a	0.96a	1.51a	0.7a
20	0.96a	1.26a	1a	1.11a

1. n = 20
2. Means with different letters (a, b, c) within the same row are significantly different ($p < 0.05$).

Table 3. Pearson correlation coefficients for coliform bacteria cell counts and average weekly relative humidity and average weekly rainfall.

	Pearson Correlation Coefficients	
	Inoculated manure amended soil	Direct Spray Inoculated soil
Average relative humidity	-0.26	0.07
Average rainfall	0.07	0.35
Average soil moisture	0.19	0.44
	One-week Lagged Pearson Correlation Coefficients	
	Inoculated manure amended soil	Direct spray Inoculated soil
Average relative humidity	-0.18	-0.01
Average rainfall	0.15	0.63
Average soil moisture	0.74	0.09

Table 4. Relative humidity and daily rainfall data achieved from the Mesonet weather site during the period from October 2010 to March 2011

Week	Date	Average Relative Humidity (%)	Average rainfall (mm)	Average soil moisture (%)
0	29 Oct-5 Nov	52.89	0.00	86.13
1	6 Nov-12 Nov	61.98	0.17	81.43
2	13 Nov-19 Nov	74.35	0.07	92.71
3	20 Nov-26 Nov	63.42	0.00	91.29
4	27 Nov-3 Dec	51.33	0.00	88.00
5	4 Dec-10 Dec	70.05	0.00	84.43
6	11Dec-17 Dec	53.25	0.00	81.00
7	18 Dec-24 Dec	70.30	0.03	78.71
11	14Jan- 21 Jan	72.72	0.01	NA
15	11 Feb-18 Feb	55.20	0.02	NA
20	18 Mar-25 Mar	55.72	0.00	NA

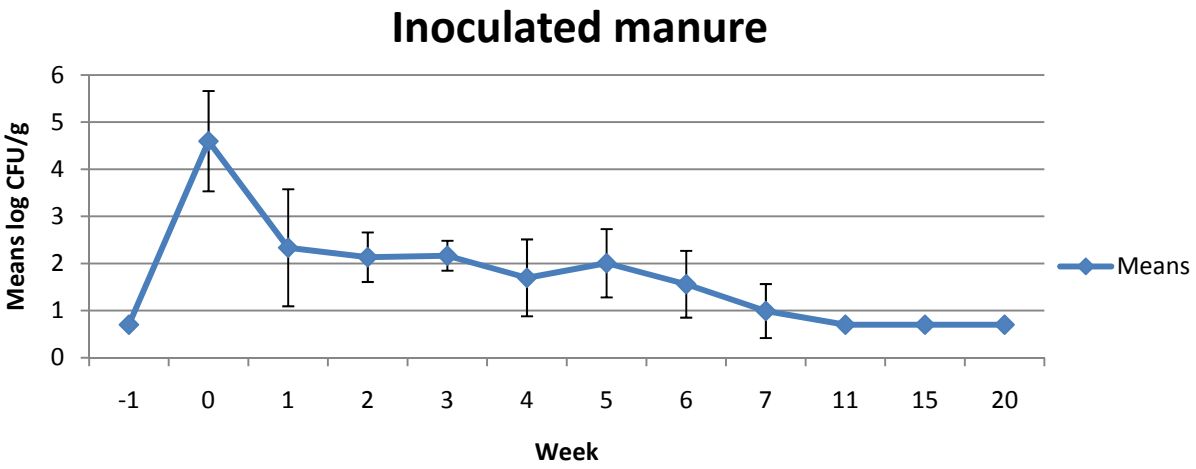

Figure 1. Means ±2 standard error of the survival rate of *E.coli* O157:H7 in soil amended with inoculated manure before (week -1) and after treatment (week 0 to week 20). Detection limit of direct plating was 1 $\log_{10}$ CFU/g.

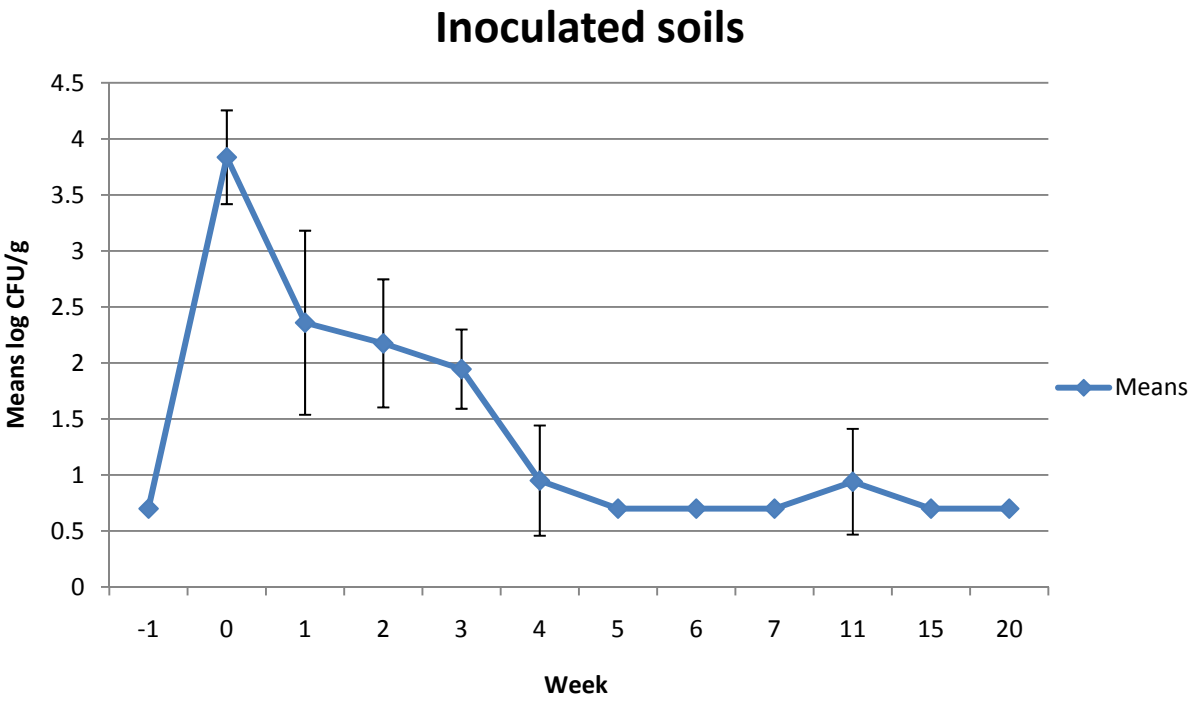

Figure 2. Standard error of the survival rate of *E.coli* O157:H7 in soil treated by direct spray application of bacterial inoculums before (week -1) and after treatment (week 0 – week 20). Detection limit of direct plating was 1 $\log_{10}$ CFU/g.

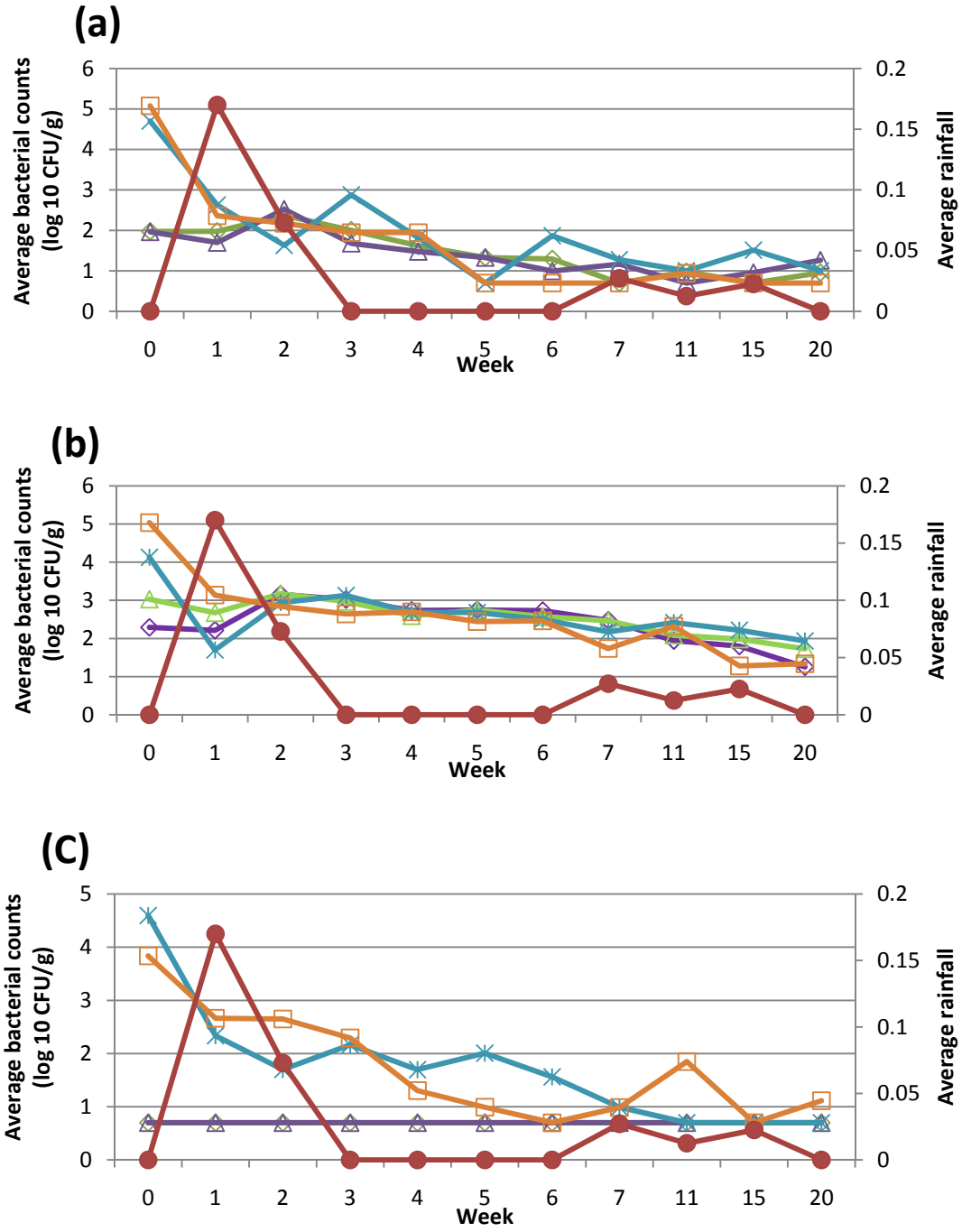

Figure 3. Average viable bacterial counts in soil correlated to rainfall over 20 weeks. (a) Average rainfall and in-field survival of generic coliforms. (b) Average rainfall and in-field survival of generic O157 *E.coli.* (c) Average rainfall and in-field survival of *E.coli* O157:H7. (◊) Control; (△) Non-inoculated manure; (✳) Inoculated manure; (▭) Inoculated soil; (▲) Average rainfall. Data points are the average $\log_{10}$ CFU/g of 5 replicates of 4 treatments.

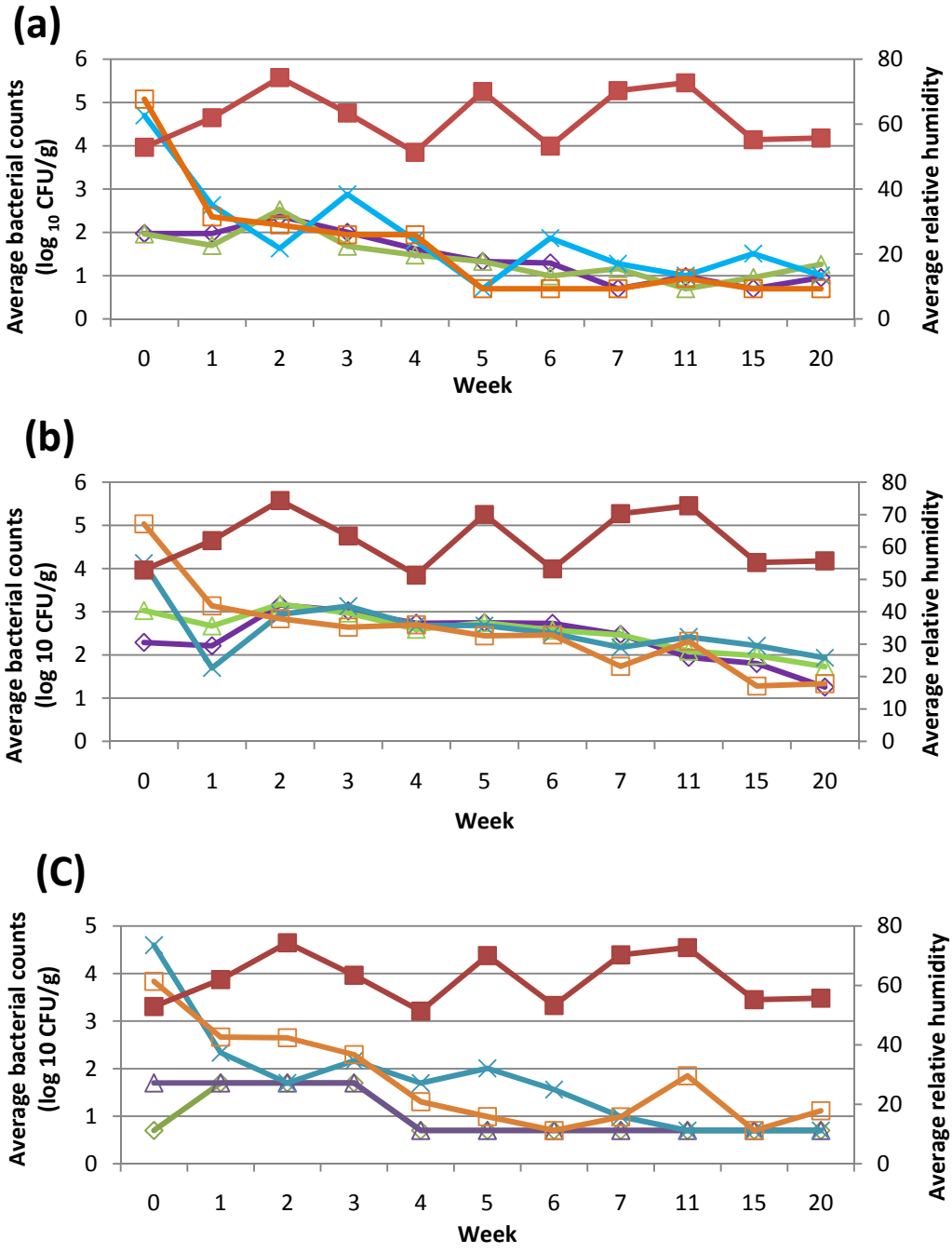

Figure 4. Average viable bacterial counts in soil correlated to relative humidity over 20 weeks. (a) Average relative humidity and in-field survival of generic coliforms. (b) Average relative humidity and in-field survival of generic *E.coli.* (c) Average relative humidity and in-field survival of *E.coli* O157:H7. (◊) Control; (△) Non-inoculated manure; (✳) Inoculated manure; (▭) Inoculated soil; (■) Average relative humidity. Data points are the average log CFU/g of 5 replicates of 4 treatments.

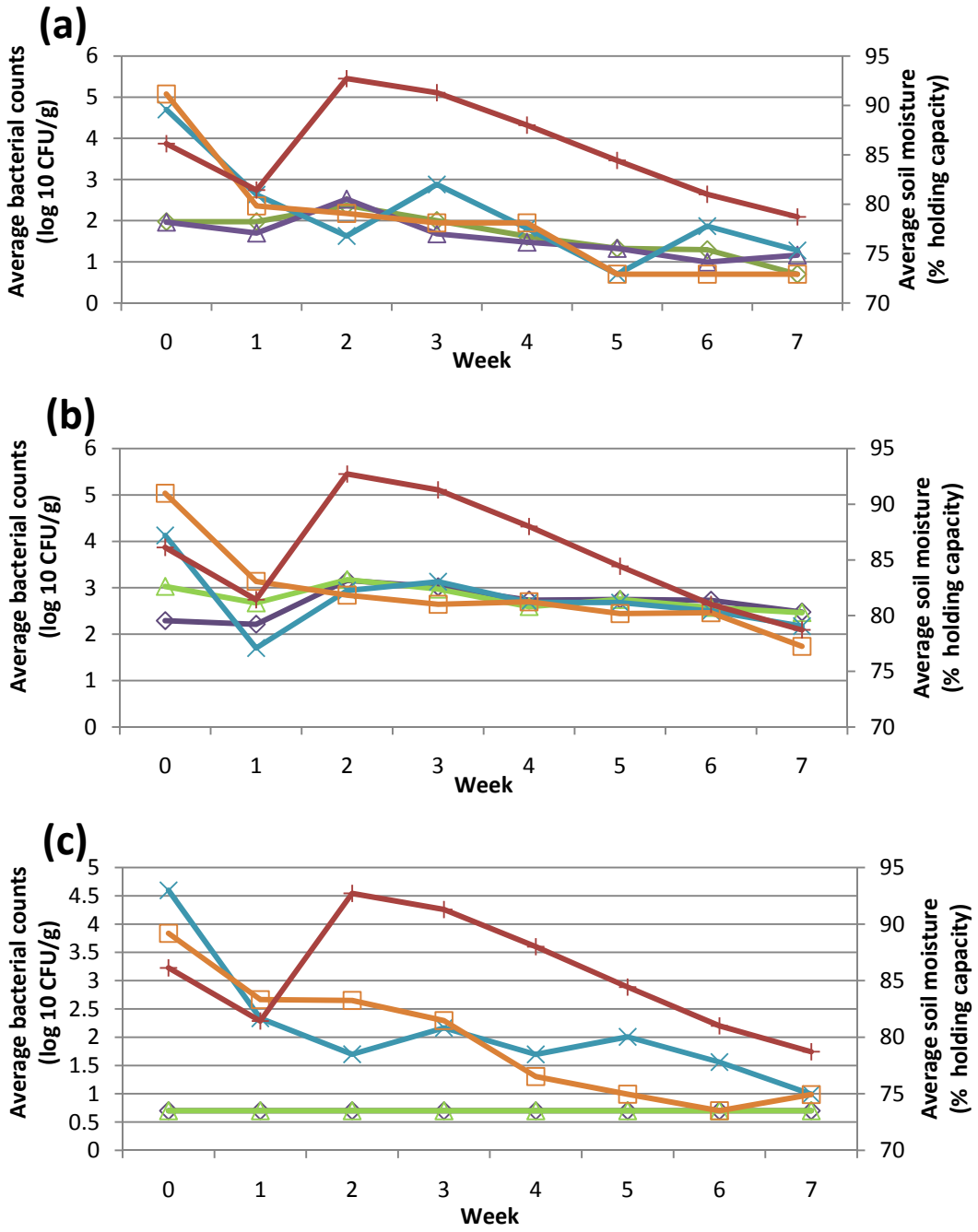

Figure 5. Average viable bacterial counts in soil correlated to soil moisture over 7 weeks. (a) Average soil moisture and in-field survival of generic coliforms. (b) Average soil moisture and in-field survival of generic O157 *E.coli.* (c) Average soil moisture and in-field survival of *E.coli* O157:H7. (◊) Control; (△) Non-inoculated manure; (x) Inoculated manure; (▭) Inoculated soil; (▲) Average soil moisture. Data points are the average log CFU/g of 5 replicates of 4 treatments.

CHAPTER V

DISCUSSION

The survival characteristics of *E.coli* O157:H7 in amended soils could be greatly affected by natural environmental factors. The ability of *E.coli* O157:H7 to survive for an extended period of time in agricultural soils amended with cattle manure poses a risk of contamination to plants grown in these soils. In this study, the spinach was grown under conditions simulating commercial spinach production and cattle manure was used as a soil amendment. Samples of untreated control soil, soil amended with non-inoculated composted cattle manure, soil amended with inoculated cattle manure, and soil inoculated by direct spray application of bacterial inoculum were tested for the presence and numbers of *E.coli* O157:H7 over time. The levels of the pathogen were then compared to the levels of background microorganisms, which in this study were identified as generic coliforms. We also determined how weather –related environmental data (relative humidity and rain fall) collected via a nearby Oklahoma Mesonet weather station were correlated with the bacteria cell counts.

Numbers of *E.coli* O157:H7 were reduced by as many as 1.5-2.0 $\log_{10}$ CFU/g within one week after inoculation in both inoculated manure and direct-spray inoculation soils. These results echo the findings of Franz et al. (2005) who also observed a

reduction of 1.5 log CFU/g with an initial inoculation level of 6.0 $\log_{10}$ CFU/g. Overall, the populations of *E.coli* O157:H7 in all the inoculated plots decreased sharply over the first 7 days. One explanation for this sharp decline could have been the shock induced by introducing enteric pathogens into a new, lower-temperature environment.

Another possible explanation may relate to the soil type used in this study. The soil in field used in this study was Teller fine sandy loam, which is a commonly-occurring soil found in Oklahoma. The parent material of the soil components was a loamy alluvium with a low runoff rate; the slope in our test field was 1-3%. This soil was suitable for crop production due to its high drainage, which provides good moisture infiltration capacity and facilitates the provision of sufficient moisture to the spinach plants. The soil is also well aerated characteristic and porous enough to hold nutrients. Accordingly, background microfloras have been reported to be high in fine sandy loam soils in previous studies (Sharma et al., 2009). Populations of indigenous bacteria in the soil may have acted as interspecies competitors to the inoculated pathogens for nutrients and water. Other studies have reported that microflora originating from soils exhibit antagonistic interactions with *E.coli* O157:H7 when the enteric pathogens were introduced into the soil as well as when they were introduced into manure-amended soils. (Jiang et al., 2002) Therefore, the relatively rapid reduction in the numbers of inoculated bacteria that we observed in this study could be due to the effects of biotic factors.

In addition, fine sandy loam soils have a lesser clay content than some other soil types. One study reported that the population of *E.coli* O157:H7 declined faster in sandy soils than in clay soil; the clay soils were believed to have more pore niches that served to protect enteric bacteria from natural predators in the soil (Natvig et al. 2010). In addition,

some studies suggest that enteric bacteria are better able to adhere to soil particle in clay soils, which also helps to preserve their numbers over time (Natvig et al., 2002). Although Ibekwe et al. (2010) reported that cell counts remained higher for a longer period of time in sandy soils as compared to clay soils, the experiment only looked at populations over a short time period.

It is important to note that the soil was not sterilized in this study because we attempted to simulate the conditions of in-field spinach production as closely as possible. Sterilized soil would have eliminated competing or predatory microflora and thus may have boosted the survival rates of our inoculated pathogens (Vidovic et al., 2007). In addition, nutrient degradation and the loss of a diverse community of natural soil microorganisms may act to reduce the quality of the soil and eventually halt the regular growth of spinach plants. These poor quality spinach plants might be more easily contaminated with enteric pathogens due to weakened defense systems. Thus, future studies may benefit by comparing pathogen survival and contamination rates in non-sterilized and sterilized soils.

This study's weekly sampling was changed to monthly sampling beginning in January 2011 due to the repeated detection of *E.coli* O157:H7 only at very low levels (below the detection limit of 1 log CFU/g). Given the fluctuating outdoor temperatures at that time, we can assume that the plots experienced repeated freeze/thaw cycles. Natvig et al. (2002) found that the cycle of freeze-thaw events caused high mortality among *E.coli* O157:H7 bacteria in manure-amended soil. The bacteria inoculated into our test plots may have experienced the same lethal effect, contributing to low numbers and a poor ability to recover when the temperatures rose and the spinach began to grow again

in the early spring. Our present study only investigated the in-field survival of *E. coli* O157:H7 only in winter-spring conditions. Further studies may need to be done in summer-fall to examine the pattern of bacterial growth under higher temperature, rainfall, and relative humidity.

In this study we did observe a trend toward enhanced survival of *E.coli* O157:H7 in manure-inoculated soils versus soils treated by direct spray application of bacterial inoculums. The population counts were significantly higher for two additional weeks, 6 weeks versus 4 weeks after inoculation for the manure-inoculated soils and the direct-spray inoculated soils respectively. This suggests that manure-amended soil provided more favorable conditions for the survival of the enteric bacteria, such as better nutrients and perhaps increased physical protection. Our results are similar to those reported by Ongeng et al. (2011). They found that the numbers of *E.coli* O157:H7-Rifr were higher in manure-amended soil as compared to non-amended soil and speculated that this may be due to the nutrients provided by cattle manure as well as a shading effect from having a manure layer on top of the soil.

The environmental factors examined, rainfall, relative humidity, and soil moisture showed weak correlations with the survival rate of both coliforms and *E.coli* O157:H7. A lagged effect of higher relative humidity on the survival rate was not observed in general, although it was seen in the inoculated manure plots. This may have been due to the higher water-holding capability of the organic material in those plots. O'Brian and Lindow (1988) showed that bacterial population sizes experienced higher decline when incubated under low humidity than wet conditions and the degree of decline was related to the bacterial strain, plant species and plant cultivars.

The correlations between average weekly soil moisture and bacterial counts evaluated in the present study suggested that coliforms and generic *E.coli* counts tended to correlate better with increased soil moisture than did the *E.coli* O157:H7 counts over the 7 weeks of observation. Our results support those of Berry and Miller (2005), who reported that the numbers of total coliforms declined or increased only slightly and tended to remain stable over time in cattle manure with fluctuating moisture levels. By contrast, they found that moisture content had a relatively large impact on the survival rate of *E. coli* O157:H7, as coliforms and generic *E.coli* were detected after the *E. coli* O157:H7 counts were below detection levels (Berry and Miller, 2005). Vidovic et al. (2007) also observed that dry soil condition can lead to higher mortality in *E.coli* O157:H7, especially on the surface of soil plots.

Interestingly, the numbers of pathogens were observed to be greater in weeks 3; 11 and 20 for both inoculated manure and direct-spray inoculated soil after the cell numbers declined sharply at 1 week post inoculation. The lagged effects of rainfall were seen to correlate moderately (Pearson's correlation r-value = 0.6263) to the numbers of enteric pathogens cells. This could be due to the lingering effect of increased moisture, which provided the inoculated *E.coli* O157:H7 with an improved environment for survival and possibly even some growth. This hypothesis is supported by Ongeng et al. (2011), who showed that the numbers of *E.coli* O157:H7-Rifr stayed high if inoculated soil and manure-amended soil in an open field maintained a high moisture level.

Although the numbers of inoculated *E. coli* O157:H7 dropped below the limits of detection via direct plating in all treatments after six weeks, enrichment and PCR testing showed that the pathogens did persist at some low level in the both inoculated manure

and direct-spray inoculated plots for at least 20 weeks. This shows the persistence of this microorganism, and the importance of preventing soil contamination by enteric pathogens in minimizing the risks of foodborne illness. This point is made even clearer when one considers that other studies have indicated that enteric pathogens in the soil may be able to adhere directly to plants roots (Islam et al., 2004).

In this study, spinach planted in the inoculated plots did not yield significant counts of *E.coli* O157:H7, perhaps due to the low probability of bacteria moving from the soil and adhering to any given plant. Mitra et al. (2009) reported that surviving bacterial cells did not move within the plant or multiply even after they inoculated the bacteria directly onto the plant leaves. Another study reported that *E.coli* O157:H7 was observed on the roots of spinach but did not translocate into the edible tissue (Hora et al., 2005). This suggests that there is a low probability that *E.coli* O157:H7 could contaminate the plants leaves without direct, persistent adherence. This may have occurred during our study, but at such low levels that our sampling methods were not able to detect it.

In conclusion, this study demonstrated that *E. coli* O157:H7 remained above the limit of detection by direct plating for 6 weeks versus 4 weeks in inoculated manure-amended soil versus direct-spray inoculated soil. The lagged effect of rainfall was also observed to have a moderately strong positive correlation with the survival rate *E.coli* O157:H7 in inoculated plots. No consistent correlation was seen between relative humidity and bacterial counts. We speculate that rainfall played an important role in maintaining the pathogen counts by keeping the soil moist, especially in manure-amended plots, where a higher correlation coefficient was seen.

CHAPTER VI

CONCLUSION

Contamination of *E.coli* O157:H7 on fresh produce remains a food safety concern for the food industry, especially in light of recent foodborne illness outbreaks in both the U.S. and in many parts of Europe. The present study aimed to evaluate the effects of natural, in-field conditions on the survival of *E.coli* O157:H7on spinach. The results were intended to be useful in providing information on the bacteria's survival ability in actual, real-world environmental conditions; this information should help producers and processors better manage the food safety risks associated with open-field cultivation.

In general, although there was a great deal of variability in the data and statistically significant differences were not consistently observed, *E.coli* O157:H7 was detectable via direct plating in the plots amended with inoculated manure for up to 49 days over the winter-spring season. Over the same time period, *E.coli* O157:H7 was detectable via direct plating for as long as 28 days in the plots inoculated by direct spray. Accordingly, it is possible that, when used as a soil amendment, manure may assist the survival of enteric bacteria introduced into the soil. This may occur as a result of additional nutrients and physical protection from harsh environmental conditions

supplied by the organic material in the manure. Further studies would be necessary to substantiate this possibility under our test conditions.

With respect to the rapid decline in the numbers of *E. coli* O157:H7 seen in our study, it is possible that the antagonistic activity of indigenous microflora found in both manure and soils might be the cause. Again, more study would be necessary to support this hypothesis.

Out of the three weather conditions that we observed in the present study – which were relative humidity, rainfall, and soil moisture – only rainfall was seen to have a moderately strong positive correlations (higher r-values) with the population counts of enteric pathogens during the course of the study in both inoculation treatments. Increased numbers of *E.coli* O157:H7 were seen in both inoculated treatment plots following higher weekly average rainfall. The lagged effect of rainfall suggests that higher numbers of *E.coli* O157:H7 may follow an increase in soil water content.

Because of the variability seen in the data, it is important to note that any results obtained are tentative. The bacterial counts we obtained might not representative of the real numbers of survivors due to insufficient replication of treatments or possibly due to sampling methods that did not yield truly representative samples. It may be beneficial to use larger sample sizes in future studies.

Another possible issue with the current study is that the manure used as an amendment was not fresh, but had been commercially composted. Fresh manure may have had a different effect on the persistence of *E.coli* O157:H7 in soils, for example the background microflora of the commercially-composted manure did not closely mimic the

real life in-field environment of a spinach planting accidentally contaminated by animal waste or intentionally fertilized using raw manure as an organic fertilizer.

Also, in the current study we used higher level of bacterial inoculum than the levels of contamination that have been reported in previous foodborne outbreaks. We did this in order to better quantify the survival of target pathogens. However, the increased numbers of bacteria may have skewed the results and therefore did not accurately reflect the actual ability of the pathogens survive and grow in the plots. Thus, a lower concentration of bacteria might be a more realistic method to assess the survival ability of *E.coli* O157:H7 in the open field.

Overall, it is important to note that survival rate of *E.coli* O157:H7 is strongly affected by a range of diverse environmental conditions including the background microflora – including antagonistic and even predatory microorganisms, available nutrients, soil composition, and weather. Further research into these conditions, for example examining different seasonal periods such as summer-fall, which would certainly have very different temperature, moisture, and UV radiation ranges, is needed to truly understand the best ways to reduce the risk of in-field contamination of leafy greens by enteric pathogens.

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APPPENDICES

Table 5. Chemistry and physical characteristics of soil and soil used in this study

Matrix	pH	Sand	Silt	Clay
Soil	6.6	58	23	19

Table 6. Fall 2010 -Spring 2011 Inoculated Spinach Planted Project treatment

Treatment	Treatment descriptions	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
1	Control-With no manure and inoculum applied	101	204	301	402	502
2	Manure amended plots (uninoculated)	102	202	303	404	504
3	Inoculated manure amended plots	103	203	302	403	503
4	Inoculated plots (spray)	104	201	304	401	501

Figure 6. Plot map for Fall 2010-Spring 2011 Experiment Plot Map

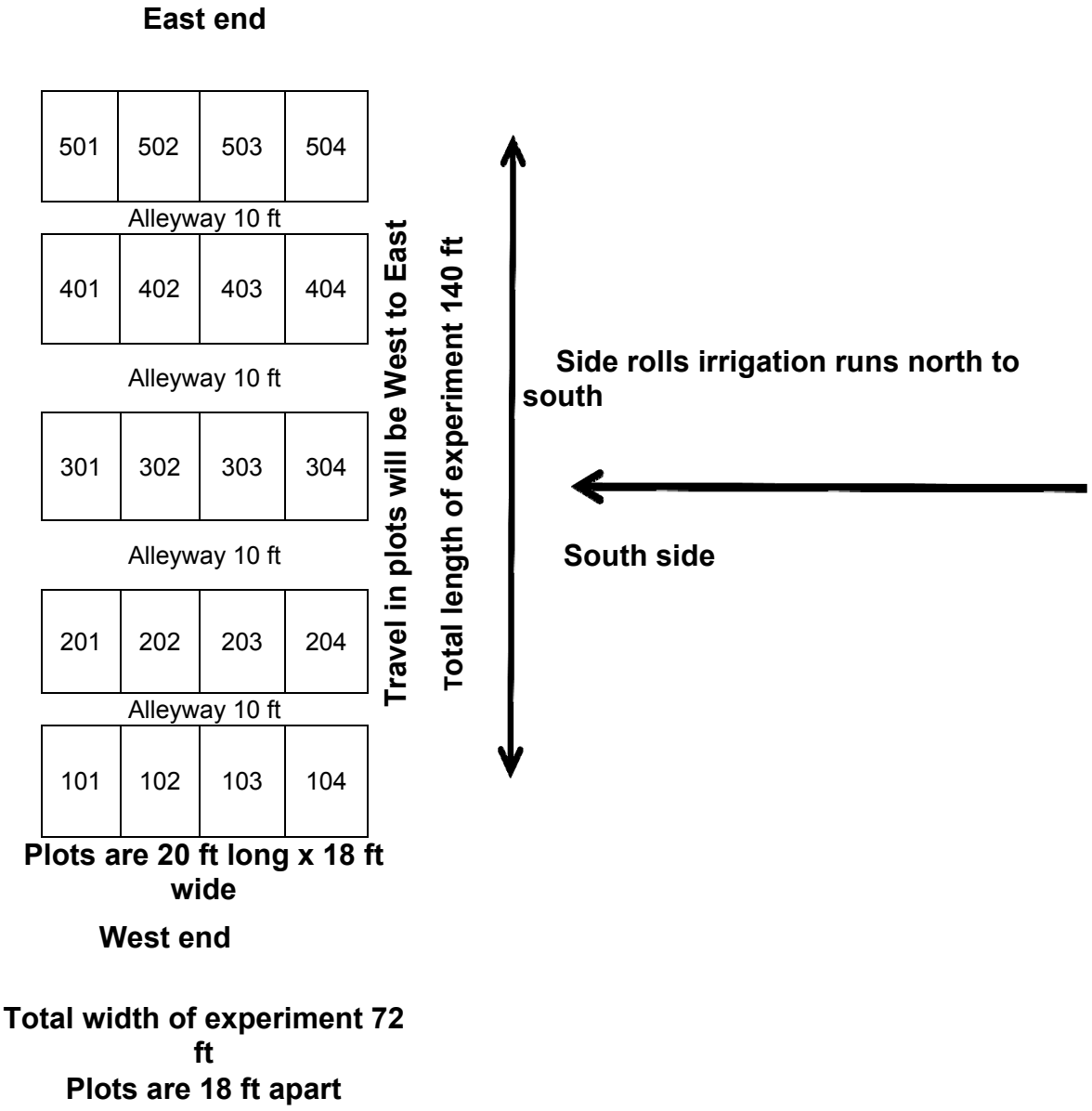

Table 7 Brief descriptions of methods applied to different treatments spinach planting plots.

Treatment	Treatment descriptions	Methods applied
1	Control-With no manure and inoculum applied	Non-inoculated tilled soils --> seeded
2	Manure amended plots (uninoculated)	Non-inoculated tilled soils --> manure amended --> seeded
3	Inoculated manure amended plots	Tilled soils --> inoculated manure amended --> seeded
4	Inoculated plots (spray)	Tilled soils --> spray with inoculums --> seeded

Table 8. Existence of E.coli O157:H7 confirmed by BAX PCR E.coli O157 MP assay in soil after enriched with antibiotics added GN broth.

	Number of positive/ total examined			
	Treatment			
Week	Control	Non-inoculated manure	Inoculated manure	Inoculated soil
-1*	0/5	0/5	0/5	0/5
0	0/5	0/5	5/5	5/5
1	0/5	4/5	4/5	4/5
2	0/5	0/5	5/5	5/5
3	0/5	0/5	1/5	1/5
4	0/5	0/5	4/5	4/5
5	0/5	0/5	4/5	5/5
6	0/5	2/5	5/5	2/5
7	0/5	1/5	3/5	5/5
11	0/5	0/5	5/5	1/5
15	0/5	1/5	5/5	1/5
20	0/5	0/5	3/5	0/5

Table 9. Existence of *E.coli* O157:H7 confirmed by BAX PCR *E.coli* O157 MP assay in spinach leaves after enriched with antibiotics added GN broth.

	Number of positive/ total examined			
	Treatment			
Week	Control	Non-inoculated manure	Inoculated manure	Inoculated soil
4†	0/5	0/5	1/5	1/5
5	0/5	0/5	1/5	0/5
6	0/5	1/5	0/5	0/5
7	0/5	0/5	0/5	0/5
11	0/5	1/5	0/5	0/5
15	0/5	0/5	0/5	0/5

†Sampling of spinach leaves started on week 4 postinoculation

Table10. Cell counts of inoculums recovered as plated in Chromagar O157

Description of enumeration	CFU/g
Subculture of inoculums (in TSB) applied	5.3×10^8
Spray inoculums after distilled water added	4.9×10^7
Inoculated manure slurry	1.5×10^6

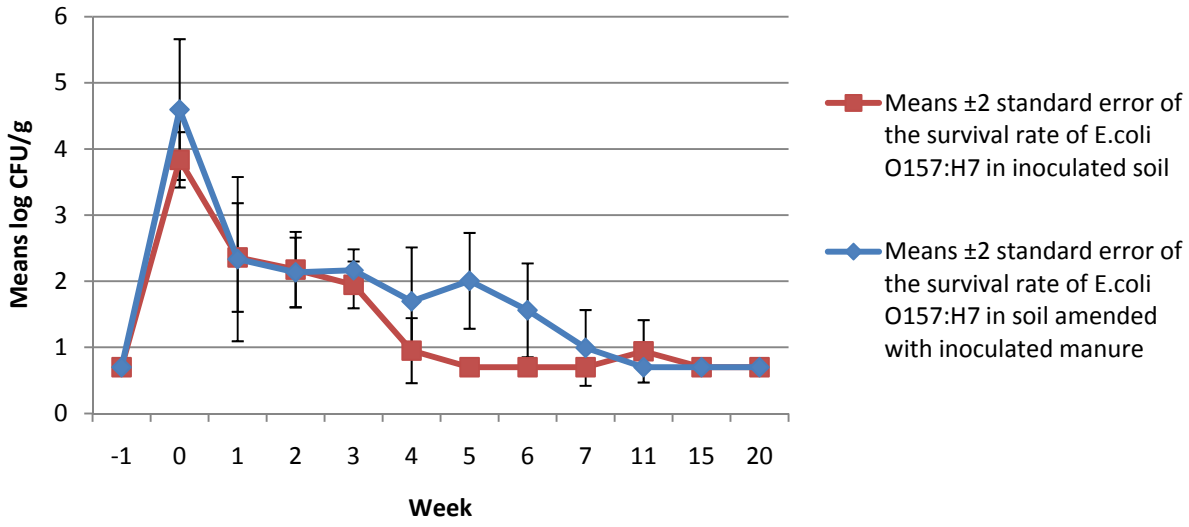

Figure 7. Comparison of survival rates of *E.coli* O157:H7 among inoculation treatments

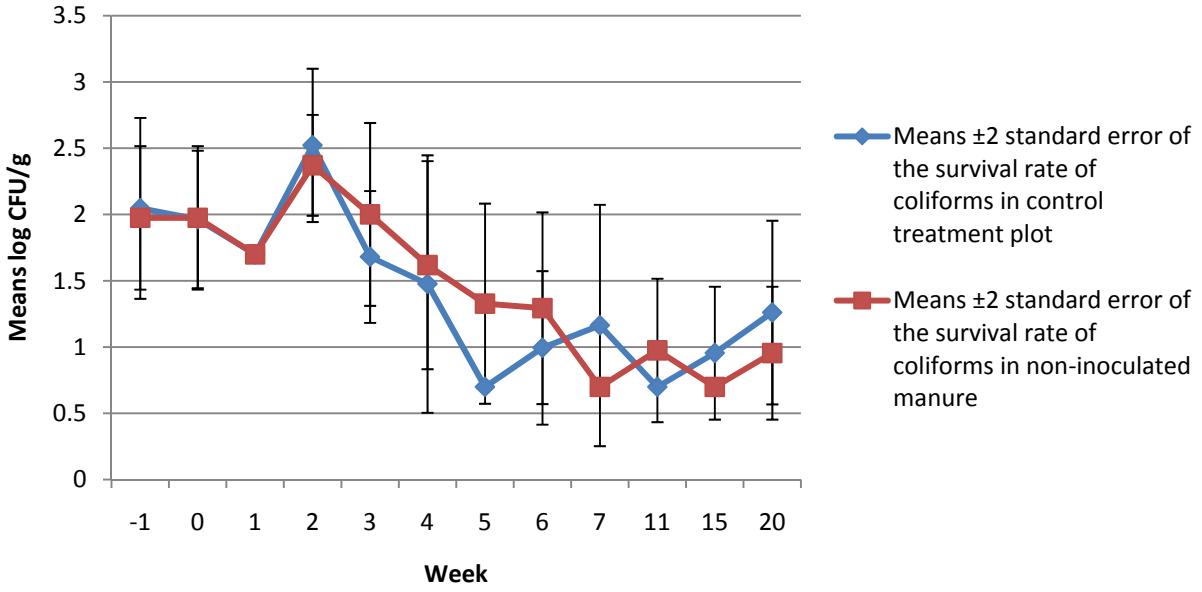

Figure 8. Background microflora detected in soils

VITA

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Candidate for the Degree of

Master of Science

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CORRELATION OF IN-FIELD SURVIVAL OF *ESCHERICHIA COLI* O157:H7 WITH RAINFALL, RELATIVE HUMIDITY AND SOIL MOISTURE.

Pages in Study: 75 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope and Method of Study:

The objective of the study was to determine possible correlations between selected environmental factors (rainfall, relative humidity, and soil moisture) and the survival of non-pathogenic *E.coli* O157:H7 in soils amended with inoculated cattle manure and in soils inoculated by direct-spray application of bacterial inoculums. Bacterial transfer onto and survival on spinach grown in inoculated plots was also examined. The in-field study was conducted at the Oklahoma State University Cimarron Valley Research Station, Payne County, Oklahoma. A total of 4 treatments were applied in 20 plots arranged in a randomized complete block design. The treatments employed were: non-inoculated control plots; plots amended with non-inoculated manure; plots amended with inoculated manure; and plots treated by direct-spray application of bacterial inoculums. Soil sampling was conducted on a weekly basis for 7 weeks, and then once in month in January, February, and March 2011. Soil samples were assayed for the presence and surviving numbers of inoculated *E.coli* O157:H7 cells. Immature spinach leaves were harvested during the growing season (from week 3 to week 7) and analyzed for the presence of *E.coli* O157:H7 in or on the leaf tissue.

Findings and Conclusions:

The population of inoculated *E.coli* O157:H7 were observed to decline rapidly in all inoculated plots. From week 0 to week 1, about a 2-log reduction was seen. 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. Thus, we conclude that manure as a soil amendment may assist in the survival of enteric bacteria. The inoculated bacteria were detectable by enrichment for at least 20 weeks in all inoculated plots. Rainfall was more highly correlated with the survival of the inoculated bacteria than the other weather conditions examined, and a one-week lagged effect was observed. No consistent, strong correlation was seen between soil moisture or relative humidity and the population counts of *E. coli* O157:H7. No *E. coli* O157:H7 cells were confirmed present in or on the spinach leaves harvested from the inoculated plots; this may been due to limited leaf sampling. Recommendations for future studies include evaluating additional types of inoculation and soil and leaf sampling techniques.

ADVISER'S APPROVAL: William McGlynn