ASSESSING THE RISK OF IN-FIELD MICROBIAL CONTAMINATION OF LEAFY GREENS USING INOCULATED SOIL

By

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CHAPTER I

INTRODUCTION

Foodborne Illness: an Emerging Disease

On September 2008, the Food and Drug Administration (FDA) announced what was known to be one of the largest food recalls in history. The action came after federal officials discovered that one of the peanut companies, Peanut Corp. of America in Georgia had processed and marketed peanut butter contaminated with salmonella. The outbreak began in the late summer and had caused about 500 cases of illness and 8 deaths in 43 states. This incidence was the latest addition to the list of many outbreaks that occurred in the United States alone, following the outbreaks of *Escherichia coli* O157:H7 and *Salmonella* associated with lettuce, peppers, and spinach.

Foodborne outbreaks are the latest food scare in the U.S. The Centers for Disease Control and Prevention (CDC) has estimated that foodborne diseases causes approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the U.S. each year. Out of the known causative pathogens, an estimated 73,480 illnesses are due to *Escherichia coli* O157:H7 (Mead and others 2000). In addition, more than 250

different foodborne diseases have been described. Foodborne disease is caused by consuming contaminated foods or beverages. The causes of the disease include viruses, bacteria, parasites as well as toxins and poisonous chemicals. The most at risk populations for foodborne disease are the elderly, pregnant women, immune-compromised individuals, and children younger than five years of age (McCabe-Sellers and Beattie 2004). An outbreak of foodborne disease is defined as two or more reported cases of a group of people consume the same contaminated food and a significant portion of that population come down with the same illness with specific criteria for diagnosis (CDC 2005, McCabe-Sellers and Beattie 2004). Due to the increasing numbers of foodborne outbreaks, the safety of the U.S. food supply becomes questionable in regards to sources of contamination, causes, as well as how to prevent future outbreaks.

The economic impact of foodborne disease is believed to be between 2 – 4 billion dollars (McCabe-Sellers and Beattie 2004). Foods consumed in institutions and other food services are considered to be the leading causes for foodborne outbreaks. The epidemiology of foodborne disease is constantly changing over time. Several contributing factors including a change in food supply, social environment, food production practices, and the types of food people eat have led to the emergence of new pathogens in the United States, (Altekruse and Swerdlow 1996; CDC 2005). Demand for new foods has led to increased numbers of imported foods in the U.S. This has placed consumers in increased contact with unfamiliar pathogens that may be carried by foods from outside the country. New health trends have led consumers to be more aware of what they eat, which has driven minimally processed fruits and vegetables to gain popularity due to their potential health benefits and a growing trend with regards to

"freshness". Such trends may create higher possibilities for fresh produce to carry pathogens that will cause foodborne illness when consumed. In addition, fewer people eat at home, which increases the percentage of outbreaks caused from eating at fast-food restaurants or consuming leafy greens from salad bars.

Raw foods of bovine origin are the most likely to be contaminated, followed by dairy products. However, in the past few years, fruits and vegetables have accounted for and have become increasingly recognized as vehicles for foodborne pathogens. One particular example of emerging foodborne infection *E. coli* O157:H7 that has been linked to contaminated lettuce and spinach. In the U.S., as many as 20,000 cases and 250 deaths per year are thought to result from *E. coli* O157:H7 infection. The source of an outbreak pathogens that are introduced to foods either before, during or after they are processed. The biggest problem with *E. coli* O157:H7 in foods is that very small numbers of the organism are sufficient to cause infection.

There is certainly a need for eliminating or at least reducing the risk of contamination by pathogens in the U.S. food supply. In addition, control of foodborne disease is everyone's responsibility, including food processors, retailers, foodservice personnel, as well as consumers.

CHAPTER II

REVIEW OF LITERATURE

Background and Emergence of *Escherichia coli O157:H7*

Escherichia coli was first described by a Bavarian pediatrician, Theodor Escherich, in the late 19th century (Kaper 2005). The bacterium was first known as *Bacterium coli* and was a normal inhabitant of healthy individuals. It belongs to the family *Enterobacteriaceae* and is a short, Gram-negative bacillus, facultative anaerobic and is usually motile. Its optimum temperature for growth is 37 °C and colonies are usually non-pigmented, circular with smooth edges.

Though *E. coli* has a long history in the development of the field of molecular biology, it was not until numerous cases of illnesses caused by *E. coli* O157:H7 outbreaks that attracted the most interest and attention of the general public. Some *E. coli* strains are associated with diseases and thus can be divided into five virulence groups: enteroaggregative (EAggEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), and enterotoxigenic (ETEC) (Jay 2000). Cattle have been known as the main reservoir of the pathogen. The most significant group, which has gotten a lot of attention over the past few years is the EHEC group that is known to produce toxin, known as Shiga toxin.

According to CDC, the most commonly shiga-toxin producing *E. coli* is known as *E. coli* O157:H7. It was discovered in 1975 from a patient with bloody diarrhea (Jay 2000). Infection of *E. coli* O157:H7 causes hemorrhagic colitis and is usually characterized by bloody diarrhea, severe abdominal pain, with little or no fever. Very young children and elderly have higher risks to develop severe illness than others. The microorganism was first recognized as a pathogen when an investigation of hemorrhagic colitis outbreaks occurred in 1982 associated with consumption of undercooked hamburgers at a fast-food restaurant chain (Wells and others 1983).

Escherichia coli O157:H7 has emerged as a significant pathogen in the United States and has become a threat due to the increasing numbers of outbreaks in the recent years. The epidemiology of outbreaks caused by it in the U.S. is reviewed in the next section.

Outbreaks of *Escherichia* coli O157:H7 Infections in the United States.

Foods as a vehicle of *Escherichia coli* O157:H7 infections

Food was identified as the most common vehicle for this foodborne pathogen from 1982 to 2002, especially foods of bovine origin, these range from 0.1 to 54.2% in ground beef, 0.1 to 4.4% in sausage, 1.1 to 36.0% in unspecified retail cuts, and 0.01 to 43.4% in whole carcasses (Erickson and Doyle 2007). *Escherichia coli* O157:H7 outbreak was first linked to undercooked ground beef patties in 1993, followed by other types of beef, produce, and dairy products (Rangel and others 2005). Ruminants have been identified as a primary reservoir of *E. coli* O157:H7. Contamination of carcasses

with this pathogen usually occurs during slaughter and may lead to cross-contamination to equipments and products in the plant. For example, studies showed that *E. coli* O157:H7 can survive on stainless steel and plastic, which can serve as intermediate sources of contamination during food processing operations (Erickson and Doyle 2007). Foods of non-bovine origin such as fresh produce are usually contaminated via environmental exposure.

Although bovine origin foods, especially ground beef and other beef products, remain the most predominant vehicle for outbreaks of *E. coli* O157:H7 infection, outbreaks due to foods of non-bovine origin such as fresh produce have also become increasingly common.

Escherichia coli O157:H7 infections associated with consumption of leafy greens

Produce-associated outbreaks were first reported in 1991 and these products remain a prominent food vehicle (Rangel and others 2005). In addition, another case-control study found that the outbreak caused by *E. coli* O157:H7 associated with consumption of lettuce occurred in July 1995 in western Montana, where 70% of patients reported consuming purchased leaf lettuce and was strongly associated with illness and was a source of *E. coli* O157:H7 (Ackers and others 1998). This outbreak was soon followed by other outbreaks associated with *E. coli* O157:H7 and lettuce consumption. Two outbreaks of *E. coli* O157:H7 infections occurred simultaneously in Chicago and Illinois during May to June 1996, which both were associated with consumption of mesclun lettuce, a mixture of small and red green leaf lettuce (Hilborn 1999).

Although previous studies have found lettuce as one of the most predominant produce sources of *E. coli* O157:H7 for years, it was not until September 2006 when the U.S. Food and Drug Administration (FDA) announced that bagged spinach was no longer safe for consumption due to contamination with *E. coli* O157:H7. It has been reported by the USDA that since 1996, leafy greens have accounted for 34% of all outbreaks and 20 of the 24 leafy green outbreaks in the United States have been associated with *E. coli* O157:H7. One of the main factors influencing the increasing numbers of outbreaks related to fresh produce, especially spinach is the increase of consumption during the last decade. Consumers in the United States are eating up to 90% more spinach since 1992 and preferred fresh, which is the most risky form with regard to microbial contamination. In addition, it is estimated that 75-90% of fresh-market spinach is processed into fresh-cut salads or bagged spinach (Calvin 2007). The consumer demand for convenience has increased consumption of produce that has undergone minimal processing, such as ready-to-eat fruit and vegetables (Roever 1998). The increase can also be attributed to the consumer's desire to maintain a diet that promotes better health (Burnett and Beuchat 2001). Furthermore, changes in agronomic, processing, preservation, packaging, distribution, and marketing technologies may have introduced an increased risk for human illness associated with pathogenic microorganisms (Beuchat 2002, Gerba and Smith 2005).

Sources of microbial contamination of leafy greens

Contamination of fresh produce such as leafy greens may occur before, during and after the harvest stage of process and handling. Washing and sanitizing leafy greens soon after they are harvested are usually conducted to reduce microbial load and kill pathogens that may be present and though sanitizing is effective, it sometimes can affect the sensory quality of the leafy greens. Due to this reason, it is essential to control microbial loads in leafy green vegetables by preventing contamination at all stages of production, such as harvesting, processing, storage, and preparation of leafy greens (Beuchat 2002; Harris and others 2003). In the case of fresh produce, events occurring before the crop is even planted can affect bacteriological quality and safety of the final product (Beuchat 2002).

Pre-harvest

Land use history is often overlooked by producers when considering potential sources of crop contamination. Fields can be contaminated by animals that have grazed and shed their manure, which may contain enteric pathogens (Brackett 1999). Several studies, which will be further discussed in the next section, showed some pathogenic microorganisms such as *Escherichia coli* O157:H7 were able to survive in the soil for months or even years. Flooding is also another concern since floodwaters can be contaminated with animal manure, which are then carried away and flood over croplands. The source of water used to irrigate the cropland also influences the potential contamination of leafy greens. Wastewater and effluents are sometimes

used in many water-short areas of the United States for crop and landscape irrigation (Gerba and Smith, 2005). Consequently, poorly treated wastewater can contain parasites, bacteria, viruses, and potential pathogens. Other pre-harvest sources of contamination include air or dust, insects, inadequately composted manure and human handling (Harris and others 2003). Studies also showed evidence that pre-harvest contamination contributed a significant risk for outbreaks. For example, an investigation of the case of outbreak of *E. coli* O157:H7 in western Montana in 1995 associated with leaf lettuce consumption found that manure containing compost obtained from a local dairy was used to fertilize its leaf lettuce, ultimately being the source of contamination was (Ackers and others 1998). Properly treated manure used as compost is an effective and safe fertilizer, but improperly aged compost may contain pathogens and gain access to the fields and can contaminate the crops (Ackers and others, 1998; Gerba and Smith, 2005). Understanding initial contributing factors of pathogen contamination will aid to develop better agricultural practices and improve preventative measures to minimize foodborne outbreaks associated with leafy greens.

Harvesting

Harvesting often introduces human and mechanical contact that has an impact on the microbial safety of fresh produce (Brackett 1998). Equipment sanitation as well as worker's personal hygiene play major roles in controlling and preventing contamination of pathogens to produce being harvested. Leafy greens are raw commodities that originate in agricultural environment and because of this, cleanliness and sanitary

practices are often ignored by growers or workers since these commodities already soiled. In a 1996 outbreak of *E. coli* O157:H7 infections occurred in Connecticut and Illinois involving mesclun lettuce, one of the contributing factors for contaminated mesclun lettuce was the lack of sanitary performance by the workers. The field where the lettuce was grown only had one portable toilet, which provided for approximately 10 employees and hand-washing facilities were not available (Hilborn 1999). Agricultural equipment and machinery for harvesting should also be properly maintained, cleaned and sanitized after each use. Microorganisms, including pathogens may be harbored in unclean and unsanitary equipment and may be transmitted to produce that comes in contact with it.

Post-harvest

After the commodities are harvested, the sources of microbial contamination may occur at different processing steps as well as during distribution and storage. Processing steps of leafy greens typically include direct human contact, immersion in water, as well as cutting or slicing, all of which have potential for contamination (Brackett 1999). There were many reasons for the cause of outbreak of *E. coli* O157:H7 infections in Connecticut and Illinois. Besides of the lack of sanitary performance by the workers, another reason for the outbreak included unsanitary handling and processing of lettuce and baby greens after they were harvested. Investigators found that gloves were not worn during the processing of lettuce and after processing, the harvested baby greens were placed in a previously used box, and delivered to the facility in unrefrigerated trucks (Hilborn 1999). Sanitizers are usually used in leafy greens washing water, which was

also another problem found with the case of outbreak above where the primary wash tank was filled with unchlorinated wash water. The way in which processing plants are designed and maintained will also affect the microbial safety of the products. For example, the temperature should be controlled suitably for the products in the processing line to prevent the growth of undesirable microorganisms. During the distribution, fresh produce is often exposed to critical conditions, such as temperature abuse which will promote the growth of pathogenic microorganisms. Livestock or raw meat can be sources of pathogenic microorganisms such as *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes*, thus transporting fresh produce combined with livestock or raw meats must be avoided to prevent cross-contamination. Trucks or other transporting vehicles must also be cleaned and sanitized after shipping livestock or raw meats. Though producers have sole responsibility to ensure the safety of leafy greens, consumers in fact have the same responsibility. Soon after fresh produce are purchased, exposure to warm temperature must be limited and leafy greens must be stored at refrigeration temperature at home and prevent cross-contamination with raw-meats.

Control of pathogens in leafy greens

Washing and sanitizing treatments are common procedures for leafy green vegetables and are critical since this type of food is often eaten raw. These procedures are performed to remove dirt, debris, soils, pesticide residues and unwanted materials from the surface of the commodities. In addition, sanitizing is performed to reduce microbial load to a safe level and eventually to prolong shelf-life of fresh commodities

during storage. Research shows that some pathogens were able to survive sanitation by forming biofilms as well as attaching to the surface of the commodities or even gaining entry to the inside of the plant tissues.

Inefficacy of sanitizing agents for leafy greens

Chlorine, at a concentration of 200 ppm, is currently the most common chemical sanitizer used in the produce industry. However, the efficacy of washing and sanitizing treatments are still being investigated because some studies suggested that sanitizing agents were incapable of reducing microbial populations to the desired level. One study indicated that treatment of harvested lettuce plants with 200 ppm chlorine failed to eliminate *E. coli* O157:H7, potentially because the organic material in the wash water limited chlorine activity (Solomon and others 2002). Physical and chemical factors and the behavior of bacteria affect the efficacy of sanitizing agents. Physical factors include exposure time, temperature, concentration and soil. Generally, the longer time a sanitizing agent is in contact with the produce, the more effective it is. This is the same for concentration, in which higher concentrations will have greater affect in reducing the numbers of bacteria on the products. Chemical factors include pH, water properties and inactivators. Sanitizing agents are dramatically affected by the pH of the solution, depending on their compositions. In addition, impurities in water can markedly affect the efficacy of sanitizing agents and therefore must be filtered before mixed with sanitizing agents. Organic and inorganic compounds may react chemically with sanitizers giving rise to inactive products. Other factors that limit the efficacy of sanitizing treatments are discussed in the next section.

Behaviors of pathogens

The efficacy of sanitizing agents can also be limited due to interaction they have between pathogenic microorganisms and the commodities. For example, microbial resistance to sanitizing agents e.g. chlorine usually depend on the location and the degree of attachment on the surface of the produce (Novak and others 2003). The effectiveness of sanitizing treatments may depend on the time interval between contamination and when the sanitizing is being performed. The longer the microorganisms stay on the surface of the products, the harder they are to remove as they become firmly attached to the surface. It has been shown that treatment of harvested lettuce plants with 200 ppm chlorine failed to eliminate *E. coli* O157:H7 (Solomon and others 2002). Organic materials such as plant tissues were found to be the likely cause for the inefficacy of chlorine treatment. Some pathogenic microorganisms have the ability to form biofilm. Biofilm is a glue-like substance, known as exopolysaccharides (EPS), excreted when bacteria adheres to conditioned surfaces (Trachoo 2003). Exopolysaccharides or biofilm enables bacteria to anchor to all kinds of surfaces, including metals, plastics, soil-particles, and even food surfaces. Different species of microorganisms possess different abilities to attach or form biofilm on different surfaces. Some pathogenic microorganisms that are capable of forming biofilm include *E. coli* O157:H7, *Listeria monocytogenes*, *Salomenalla* species, *Erwinia* species and *Pseudomonas fluorescens*. In the study of growing bean sprouts, when BacLight-stained hypocotyl sections were viewed under an epiflourescent microscope numerous biofilms were observed between the grooves of epidermal cells and across waxy cutical layer derived from beans inoculated with *E. coli* and *Salmonella* (Warriner and others 2003). Similar findings also

indicated that *E. coli* was capable of forming biofilms and formation was induced by environmental stresses (Schembri and others 2003; Ren and others 2004). One study found that *rpoS* is the gene that is responsible in the formation of biofilms by *E. coli* and deletion of that gene greatly reduced the ability of *E. coli* to grow in biofilms (Adams and McLean 1999). Biofilms are used by bacteria to protect them in adverse environments by altering the pH to enhance survival within the biofilm and to help with attachment to the surface of the plant. In this state, bacteria, especially pathogenic microorganisms can be very resistant to antimicrobial properties of sanitizing agents. In addition, other types of microorganisms can be introduced to existing biofilms on plant surfaces and thus biofilms may consist of different types of microorganisms other than bacteria, such as yeasts and fungi.

Structures of leafy greens

Higher numbers of pathogenic microorganisms are found in damaged fruits and vegetables than in intact ones (Aruscavage and others 2006). Damage to plant tissues can provide points of entry for pathogenic microorganisms to the plant interior. There are two ways in which damaged tissue can enhance microbial proliferation. First, because the tissue is already damaged, the wound may have been contaminated with pathogenic microorganisms. Secondly, when the tissue of the produce is damaged, nutrients and water are leak, thus favoring the growth of pathogens on the produce. Bacteria such as pathogens also tend to cluster in specific regions. The most common areas of bacterial aggregations are located at the base of trichomes, around the stomata, and along veins in the leaves. Using confocal scanning laser microscopy, it was discovered that *E. coli*

O157:H7 was found attached to the surface and trichomes, entrapped 20 to 100 μm below the surface of lettuce leaf in stomata and cut edges (Seo and Frank 2002). The reason for these specific binding sites is that these regions have greater wettability than other areas of the plant, in which the nutrients and water are more likely to leak out and available to favor the growth of pathogens (Jeter and Matthysse 2005). The attachment to specific sites on the surface of the produce as described above is one of the reasons pathogenic microorganisms are hard to reach with sanitizing agents. Punctures, cuts and splits present in pears, carrots, potatoes and zucchinis are also ideal harboring sites for microorganisms and once they are established at these sites, pathogenic microorganisms will be difficult to eliminate.

Motility seems to be the mechanism used by pathogens to be able to move from one site of the plant to the other. Pathogens may gain entry into the plant via cracks in the epidermis and fissures created during emergence of lateral roots (Warriner and others 2003). Other sites of entry for pathogens include stomata, wounds or rots caused by other plant pathogens. Moreover, the internalization of pathogenic microorganisms into fresh produce can occur in warm commodities with internal air spaces when they are placed in colder water, such as during washing by immersion in a dump or tank. As the fruit or vegetables cool, the internal gas contracts, which then create a partial vacuum that will draw in water through pores, channels, and punctures (Novak and others 2003).

Significance of In-Field Microbial Contamination

Since the outbreaks caused by *E. coli* O157:H7 infections associated with ground beef and other beef products, the survival characteristics of the organism in foods have been investigated in many studies. Once in foods, *E. coli* O157:H7 displays a remarkable ability to survive some of the conditions and processing procedures used in the food industry. Survival studies done in ground beef to determine rates of thermal inactivation found that *E. coli* O157:H7 was more sensitive to heat than *Salmonella* (Doyle and Schoeni 1984). It has also been shown that *E. coli* O157:H7 has an acid-adaptive response and the expression of this system enhances survival in acidic foods (Abdul-Raouf and others 1993; Leyer and others 1995). This is significant as fruit juice, such as apple cider, can be a vector for foodborne illness.

While the survival characteristics of *E. coli* O157:H7 in foods has been the main focus of numerous studies, less information is available on the organism's behavior and ability to survive in the environment. As it was discussed in the previous section, sources of most outbreaks associated with leafy greens are pre-harvest and occur in the fields where the crops are grown. Therefore, knowledge of the behavior of *E. coli* O157:H7 in soil amendments and upon pre-harvest contact with edible parts of the plants will hopefully lead to a reassessment of agricultural practices to improve the safety and quality of leafy greens.

Survival of *E. coli* O157:H7 in bovine manure and manure slurry

Animal wastes especially of the bovine-origin are known as an excellent organic fertilizer source. It is a routine and common practice in many countries, including in many agricultural practices in the Unites States. When properly treated, they provide nutrients, such as nitrogen, phosphorus and potassium that enhance growth and crop yields. However, this raises a concern that when using animal waste, they will spread enteric pathogens in soil and may eventually be transmitted to the crops grown on contaminated land, which when not properly washed and sanitized before consumption can cause foodborne illness.

Healthy cattle may harbor *E. coli* O157:H7 in their gastrointestinal tracts and shed the microorganism in their feces. Among dairy herds in the northwestern of USA, 75% were identified as fecal positive for *E. coli* O157:H7 (Jones 1999). Cattle, particularly calves and heifers colonized with *E. coli* O157:H7 may shed the organism at levels ranging from 10^2 to 10^5 colonies forming units (CFU) per gram (Himathongkham 1999). The survival of pathogens may be influenced by various environmental conditions. These conditions include temperature, solid content, pH, bacterial concentration, aeration, and the length of time that manure or slurry (a mixture of manure, urine, split feed, and water that is held without aeration) is held before it is applied to land (Kudva and others 1998; Himathongkham 1999; Avery and others 2003). A study where feces from sheep were inoculated with *E. coli* O157:H7 and exposed to the environment showed that the microorganism was able to survive for more than one year (Kudva and others 1998). In addition, this study is also a good example to compare laboratory data to on-farm conditions. When *E. coli* O157:H7 was inoculated into manure under a laboratory

setting, they found the microorganisms to survive for shorter periods of time than it survived in manure held in the environment. This highlights the significance of in-field experiments in contrast to laboratory settings.

The finding that *E. coli* O157:H7 can survive for a long period of time in animal manure shows the importance of properly aging the manure before being used as fertilizers. Study shows that before land application, a $\geq 10^5$-fold reduction of *E. coli* O157:H7 is required in order to make manure safe, then it should be held for 105 days at 4 °C or 45 days at 37 °C (Himathongkham 1999). In the past, animal waste was composted to reduce the number of viable pathogens. Though composting is ideal, due to advancement in mechanized farming have led to large numbers of animals per farm and a quicker and easier methods for disposal were then needed, hence the processing of slurry mixture to be used as fertilizer. It has been reported that *E. coli* O157:H7 survival was reduced by 1- to 2-log reduction in cattle slurry compared to cattle manure, with survival enhanced by low temperatures (Kudva 1998). Such reduction may be a result of changes in the chemical composition of slurry, including accumulation of NH_4^+ and organic acids, reduction in O_2 and available carbon that may have inhibitory effects on the pathogen (Kudva 1998; Jones 1999).

Survival of *E. coli* O157:H7 in soils

Though survival of *E. coli* O157:H7 in animal manure has been investigated in many studies, unfortunately, only little information is available on the behavior of *E. coli* O157:H7 in different soil types and the influence of environmental conditions on the pathogen. One study conducted in the U.K. showed that *E. coli* from livestock (cattle,

sheep and pigs) had extensive survival times when they were deposited onto grassland (Avery and others 2003). After 14 days of grazing, populations of *E. coli* survived in the soil ranged from an average of 134 days to 162 days. In May 2000, twenty scouts aged between 8 and 20 who attended a scout camp at the New Deer Agricultural Showground were confirmed as having *E. coli* O157:H7. Investigators later revealed that *E. coli* O157:H7 was transmitted via hands contaminated with mud from the field that had been grazed by approximately 300 sheep prior to the camp and subsequent testing of 28 animals revealed 17 shedding of *E. coli* O157:H7 (Ogden and others 2001). The contamination was widespread within the field due to heavy rainfall during the camping period that caused localized flooding and mud.

The speed with which *E. coli* levels on land decline can be influenced by several factors. They include the soil type, rainfall, UV radiation, temperature, animal diets, and in-soil predation by other microorganisms. To confirm that sunlight is, in fact, one of the environmental factors found to be one of the most detrimental to the survival of *E. coli*, a fecal-pat experiment was conducted to investigate the survival of *E. coli* under 4 levels of solar exposure controlled by using shade cloth. The results showed that *E. coli* was able to survive for more than 45 days in the hot, dry summer weather, and shade enhanced the organism's survival (Meays and others 2005). In addition, fecal pats under 0% shade cloth had the lowest *E. coli* counts, followed by the 40%, 80%, and 100% treatments, with 0.018, 0.040, 0.11, and 0.44 x 10^6 CFU/g., respectively (Meays and others 2005). The reason for the low rate of survival of *E. coli* under 0% shade was because of the decline in percent moisture of the fecal pat and it is suggested that available moisture is

one of the most important factor affecting bacteria survival in the soil (Meays and others 2005, Habteselassie and others 2008).

Pathogens such as *E. coli* O157:H7 has showed ability to leach through the soil profile and the rate is especially influenced by rainfall, soil type and management (Gagliardi and Karns 1999). Movement of *E. coli* O157:H7 through different types of soil (a clay loam, a sandy loam and a silt loam soil) was investigated and compared by using intact (no-till) and disturbed (tilled) soil cores with simulation of a rainfall at the rate 25.4 mm per hour to determine the effects on the populations of *E. coli* O157:H7 in the leachate samples (Gagliardi and Karns 1999). Results showed that *E. coli* levels in daily leachate samples exceeded the initial inoculum levels for all treatments except the intact clay loam soil cores and the microorganism replicated better in disturbed soil in contrast to intact soil and levels of leaching were similar between no-till and tilled soil (Gagliardi and Karns 1999). The authors found that nutrients in the soil played a role as they leached out of the disturbed soil cores and *E. coli* and other organisms in the soil had to compete with other soil microflora for available nutrients. Nitrogen also has been shown to enhance the survival of pathogenic *E. coli* both in the soil and in a laboratory setting (Gagliardi and Karns 1999; Kauppi and others 1998).

The source of water to irrigate the crop should be also considered for overall quality and safety of the leafy greens. Transmission of *E. coli* O157:H7 through water is an emerging concern to which the pathogen can contaminate the soil and be a vehicle for transmission to the leafy greens. Fecal contamination of water can come from many sources, such as wildlife, livestock and even humans. Field studies showed that *E. coli* O157:H7 was detected in carrots and onions when they were irrigated with previously

inoculated irrigation water (Islam and others 2005). In addition, the authors found that the persistence of *E. coli* O157:H7 in soil is also dependent on the type of vegetables grown in the soil, with inactivation more rapid in soil in which onions were grown than in soil where carrots were grown, which might be due to the presence of high concentrations of antimicrobial phenolic compounds in onions compared to carrots (Islam and others 2005).

Furthermore, the ability for *E. coli* to grow and survive in agricultural soil may be dependent on strain variability of the microorganisms. By means of ERIC-PCR an *E. coli* community in swine manure slurry was fingerprinted and results showed *E. coli* strain C279 and strain C278 were able to survive in the soil, although strain C279 showed a distinct competitive advantage over C278 in a loam soil incubated at 30 °C (Topp and others 2003). According to the authors, it was likely that strain C279 was able to utilize nutrients in manure slurry and strain C278 was not. These findings also emphasize that cultures of *E. coli* used in the laboratory or cultures inoculated into either livestock manure and soil may yield different results (Topp and others 2003). However, there are several reasons for using non-pathogenic *E. coli* in experiments, especially when they are performed outside the laboratory. Besides for the safety purposes, although they may not always correlate well with the pathogens, non-pathogenic *E. coli* can still be used as good indicators of fecal contamination and potential pathogens, as well as easier to handle and less costly to detect and enumerate than are the actual pathogens (Meays and others 2005). As it has been discussed above, some studies did show evidence when using non-pathogenic *E. coli*, the organism was able to survive in the soil for a long period of time (Bogosian and others 1996; Bolton 1998; Meays and others 2005; Topp 2003).

Mode for transmission of *E. coli* O157:H7 onto and into edible parts of the plants during germination and growth

Traditionally, plants are not considered as hosts for enteric pathogens; however recent foodborne outbreaks associated with leafy greens have raised a great concern. The presence of pathogens in the soil is most likely to be one of the sources for contamination, in which it enhances the risk for transmitting pathogens from contaminated soil to the surface of the plants during preharvest and even potentially internalize into the edible portion of the plants during germination and growth.

Escherichia coli O157:H7 has been found on carrots and onions for up to 74 and 168 days, respectively after seedlings were planted on inoculated soil and irrigated with contaminated water (Islam and others 2005). Similar results were also found on lettuce plants when they were exposed to either spray or surface irrigation and *E. coli* O157:H7 persisted on growing lettuce plants for 20 days following spray irrigation with contaminated water (Solomon and others 2002). Additionally, several studies also have shown the potential for association of *E. coli* O157:H7 with internal plant tissues and structures. In 1996, hydrophonically grown radish sprouts were suspected to be the vehicle of a large outbreak of *E. coli* O157:H7 infections in Japan, which led to an investigation whether *E. coli* O157:H7 was able to proliferate during germination and growth of contaminated radish sprouts seeds and if they could be transmitted to the edible parts (Itoh and others 1998). Results showed that viable *E. coli* O157:H7 was able to detected on the outer surfaces as well as in the inner tissues and stomata of cotyledons of radish sprouts (Itoh and others 1998). Similar results were also found on lettuce in which

E. coli O157:H7 was found to have the ability to migrate to the internal location in lettuce plant tissue through the roots (Solomon and others 2002). Another study was conducted using suspension of bioluminescent *E. coli* P36 to investigate its interaction with mung bean during sprouting. At the end of the sprouting process (day 4), bioluminescent *E. coli* P36 was detected inside the harvested, surface-sterilized bean sprouts with an average concentration of 3.3 ± 0.6 CFU g^{-1} (Warriner and others 2003). These findings suggest that the mechanism which enables *E. coli* O157:H7 can be limiting factors to control pathogens in leafy greens.

Although transmission and presence of *E. coli* throughout plant tissues is well documented in the laboratory, the mechanisms by which the microorganism colonizes and spread within plants are still not fully understood. Adhesion of *E. coli* to leafy greens may be influenced by several different mechanisms, including bacterial surface charge (Dickinson and Koohmaraie 1989), hydrophobicity (Dickinson and Koohmaraie 1989; Hassan and Frank 2003), extracellular polymeric substances and the presence of fimbriae (Jeter and Matthysse 2005).

In conclusion, when soils are treated with composts containing *E. coli*, the organism can survive for at least several months depending on soil type, rainfall, temperature and agricultural practices. This raises a concern that pathogens may be transmitted from soil onto the surface of the plants and even into the internal tissues. Increasing popularity and demands for ready to eat vegetables has led to recognizing the significance of preventing preharvest contamination to enhance the safety of ready to eat vegetables. The goal of this study was to monitor field survival of non-pathogenic *E. coli*

in the soil using different inoculation methods and to determine if they would be transferred onto or into edible parts of the plants during germination and growth.

CHAPTER III

MICROBIAL CONTAMINATION OF SOIL AND TURNIP GREENS USING INOCULATED SPENT MUSHROOM COMPOST

Abstract

Composts made from animal waste, plant materials, or combinations of both have been widely used in the horticulture industry as a soil amendment for various types of vegetable crops. One of the concerns associated with using organic composts consisting of animal waste is that pathogenic microorganisms, such as *Escherichia coli* O157:H7 may present in improperly treated compost. Field studies were conducted beginning on May 23, 2007 to determine the survival rate of non-pathogenic *E. coli* 1472 in the soils. Plots were arranged in a randomized block design with four rows in each plot and four replications for each of the two treatments (non-inoculated control and *E. coli* inoculated), measuring 1.2m x 4.6m (5.6 m^2) per plot. Previously inoculated spent mushroom compost (SMC) was applied to all plots with a rate of 80 kg/plot of non-inoculated SMC, whereas treatment plots received SMC as a split application with 60 kg/plot of non-inoculated SMC, followed by 20 kg/plot of SMC inoculated with total yield of ~3.0 x10^4 colony forming unit (CFU) of *E. coli* per gram of compost. After applications of SMC, Top White Globe turnip seeds were planted. Samples for the

detection of *E. coli* were taken three weeks after the initial treatment for both soil and plant samples and continued to be taken in a weekly basis for the next four consecutive weeks. The results showed inconsistency in the survival rate of *E. coli* in both soil and turnip green leaf samples throughout the experiment. However, data indicated that *E. coli* was able to survive and persist in the soil for at least several weeks. *Escherichia coli* was detected in turnip green leaf samples and although site of attachment in the plants was unknown, data showed abilities for *E. coli* to survive in plant samples. Our findings highlight the needs for better agricultural practices to minimize pre-harvest cross-contamination of leafy greens.

Introduction

In the horticulture industry, the use of compost is a common practice that has been associated with desirable soil properties for farming. Compost can be made from animal waste, plant materials, or a combination of both. Studies have shown that organic composts were able to improve biological, chemical, and physical attributes of the soil compared with synthetic fertilizers (Bulluck and others 2002). Properly treated organic compost can be very safe and effective, however improperly treated compost may carry risks of harboring enteric pathogens, such as *Escherichia coli* O157:H7. This may create hazards when fresh produce is grown in contaminated soil. The risk for cross-contamination may be high and pathogens may be transmitted to humans. Several foodborne outbreaks caused by *E. coli* O157:H7 infections have been associated with consumption of leafy greens (Hilborn and others 1999; Burnett and Beuchat 2001; Calvin

2007). Higher risks are associated with consuming fresh or raw vegetables without adequate washing, which leads to greater potential for food-borne illness to occur (Burnett and Beuchat 2001; Harris and others 2003; Calvin 2007).

Spent mushroom compost (SMC) has been known as a soil amendment used for vegetables crops. The compost is usually derived from harvested mushroom beds, which is unsuitable for further mushroom cultivation and thus is used for vegetable crop production. This study was conducted using SMC that had been previously inoculated with non-pathogenic *E. coli* and then applied to the soil prior seeding of turnip greens.

The objective of this study was to initiate research efforts to lay the groundwork for future studies involving several crop groups. As a preliminary study, this was designed to identify the survival rate of non-pathogenic *E. coli* in soils when inoculated with contaminated organic compost and to determine whether the microorganism would be transmitted onto or into turnip greens grown in contaminated soil.

Materials and Methods

Microorganisms and preparation of inocula

Because the study was conducted outside the laboratory, a non-pathogenic strain of *Escherichia coli* was used. The culture, *Escherichia coli* 1472 was obtained from the culture collection of a food microbiology laboratory in the Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University. The bacterial strain was cultured by transferring 100 μL of thawed frozen culture into 10 ml of Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, Michigan) and incubating at 37°C for 18 h. The

culture (1%) was subcultured at least three times daily just prior to the experiment, and incubated at 37°C for 18 h.

Experimental design

The field experiment was initiated at the Oklahoma State University Vegetable Research station in Bixby, Oklahoma starting on May 23rd, 2007. Plots were arranged in a randomized block design with four replications for each of the two treatments with plots inoculated with generic *E. coli* and non-inoculated control plots. Each plot had a dimension of 1.2m x 4.6m (5.6 m^2). Prior to inoculation with *E. coli* and planting of turnip green seeds, the entire experimental area received approximately 0.2 kg a.i. per hectare (rate in weight active ingredient per area of land) of Treflan (trifluralin) incorporated to a depth of 2.5-5 cm using a tractor mounted rototiller.

Treatment of soils

All plots received approximately 80 kg of SMC with an application rate equivalent to about 146 metric tons/hectare. For inoculated SMC treated plots, each received 60 kg of non-inoculated SMC, followed by addition of 20 kg of SMC that was previously inoculated with *E. coli* at level of ~3.0 x 10^9 CFU (colony forming unit) per gram of compost . Total inoculation rate of *E. coli* was ~3.0 x 10^4 CFU per gram of applied SMC for each plot. Spent mushroom compost were applied uniformly onto the surface of each plot then incorporated to a depth of approximately 5 cm with one pass of a tractor mounted rototiller. Following SMC application, all plots were directly seeded with Purple Top White Globe turnip at a seeding rate of 1,240,000 seeds per hectare

using a research cone planter. To avoid cross-contamination, plots were planted in the order of non-inoculated plots first, followed by inoculated plots.

Sampling of soil and turnip green leaves

Three weeks after treatment and inoculation, soil samples were collected from each plot using a soil probe from three different areas 5-8 cm deep from the surface and collected in a plastic bag. Leaf samples also were collected at Week 3 from each plot by randomly selecting and pulling plants from different areas within a plot and collected in plastic bags. All samples were collected on a weekly basis starting from week 3 through week 9 of the experiment. The samples were packed on ice and were transported to the laboratory in the Food and Agricultural Products Center of Oklahoma where they were placed in a refrigerator at 4°C and analyzed within 24 h.

Microbial analysis of soil and turnip green leaf samples

For the laboratory experiments, 11 g of each soil sample was weighed into a sterile Whirl-Pak bag and 90 ml of peptone-water (Difco Laboratories, Detroit, Michigan) was added to yield 10^{-1} dilution. The sample was pummeled in a Stomacher for 1 minute. Serial dilutions of each sample (1:100, 1:1000, 1:10000) were prepared with 99 ml peptone-water and plated in duplicate using a direct plating method with CHROMagar *E.coli*. Plates were incubated at 37°C for 24 h prior to enumeration of colonies of *E. coli*.

Four grams of turnip green leaves samples from each plot was weighed for analysis without surface sterilization. Leaves were ground using sterile mortar and pestle prior to plating and 99 ml of peptone-water was added to yield 10^{-1} dilution. Series of dilutions (1:100, 1:1000, 1:10000) were prepared and plated in duplicate using a direct plating method with CHROMagar *E.coli*. All plates were incubated at 37°C for 24 h prior to enumeration of colonies of *E. coli*. Typical blue colonies were enumerated as *E. coli* for all soil and leaf samples.

Statistical analysis

The descriptive statistics were conducted using a Microsoft Excel 2003 spreadsheet.

Results

The results revealed inconsistency in the average counts (CFU g^{-1}) of *E. coli* obtained from both non-inoculated control plots and plots amended with previously inoculated SMC (Table 1). The low detection level of *E. coli* in non-inoculated control plots stayed consistent, except for week 7 (<2.8 $\log_{10}$), which was higher compared to any of the sampling weeks (<2.0 $\log_{10}$). The inoculated plots had inconsistent counts throughout the experiment and results did not show a decrease in the average *E. coli* counts (Table 1). In addition, no *E. coli* was detected in any of the soil samples collected at week 5, however an increase in the average count was observed the following week before it finally dropped again.

Similar results also were found in samples of turnip green leaf collected from the non-inoculated control and treated plots (Table 2). At the initial sampling week (week 3), the average counts of *E. coli* were similar for the non-inoculated control plots when compared to the experimental plots treated with inoculated SMC. The average counts of *E. coli* decreased significantly to a non-detectable level the following weeks.

Discussion

In this study, low counts of *E. coli* were recovered for all soil samples throughout the experiment. This suggests that insufficient *E. coli* cells were inoculated into the SMC prior to application. Another factor that may have contributed to the low observed counts in the treated plots is that SMC may possibly possess antimicrobial properties, which may have suppressed the survival of *E. coli* in the soils. One previous study suggests that SMC has a high soluble salts content that can be harmful to microorganisms that are sensitive to salinity (Wang and others 1984). Another study of salinity function in seawater on *E. coli* also found that a maximum loss in *E. coli* viability occurred at salinities of 30% (Anderson and others 1979). In addition, there was a record of heavy rainfall prior to the initial sampling that may have washed away the microorganisms and reduced their numbers significantly.

The unpredictable results observed from non-inoculated control plots could be due to several reasons. Heavy rainfall had also caused flooding over the experimental plots and, as a result, floodwater may have carried *E. coli* from the experimental plots and cross-contaminated the non-inoculated control plots. Another possible explanation for

our inconsistent results could involve temperature differences between the sampling weeks, which may have affected the average counts of *E. coli* from soil samples collected from inoculated plots. However, data collected were not adequate to verify any of these possible explanations.

Although the average numbers throughout the experiment was low and never exceeded 3.0 $\log_{10}$, *E. coli* still persisted in the soil and was recovered in some of the samples both from control and inoculated plots. Data indicated that *E. coli* recovered from inoculated and control plots can survive for at least 7 weeks. Rainfall may have affected these results as well. Gagliardi and Karns (2000) found that rainfall may provide essential moisture in the soil, moisture that can promote the survival and even growth of soil-borne *E. coli.*

Because surface sterilization of leaf samples was not conducted, it could not be determined from the data whether *E. coli* was found outside or inside the plants. Following sampling at Week 3, *E. coli* counts decreased by approximately 3.0 $\log_{10}$ CFU g^{-1} for all samples and stayed at the same level until the end of the experiment (Table 2). Though the mechanism of *E. coli* attachment to the plant is unknown, it is evident that the microorganism was able to survive in or on the plant surface once contamination occured. Our results are in accord with those of other studies that found that diarrheagenic *E. coli* were able to bind to plant surfaces, including alfalfa sprouts, tomato, and *Arabidopsis thaliana* seedlings incubated in water (Jeter and Matthysse 2005). In addition, *E. coli* O157:H7 also has been reported to attach to cracks in the cuticle, trichome, and stomata of the surface of leafy greens (Seo and Frank 1999). This raises the concern that plant

structures may serve to protect pathogens against sanitizing agents commonly used in fresh produce industry.

This preliminary experiment provided little information on the survival rate of *E. coli* in the soils when amended with contaminated compost. It also could not be concluded from this experiment that *E. coli* was in fact transmitted from the contaminated soils to the turnip green leaves when seeds were directly planted in contaminated soils. Therefore, further research was done by repeating the experiment with some modifications, such as using a different *E. coli* carrier medium, increasing *E. coli* inoculation concentration and providing gaps between plantings to prevent cross-contamination of non-inoculated control plots.

Table 1. Average numbers of *Escherichia coli* in soil samples (n=4) from plots amended with inoculated SMC compost and non-inoculated control.

	Log_{10} CFU g^{-1}	
Week	**Control Plots**	**Inoculated Plots**
3	<2.0*	2.7
4	<2.0*	<2.7***
5	<2.0*	<2.0†
6	<2.0**	<2.8**
7	<2.8***	<2.0**

†*E. coli* was not detected in any of the samples
Number of * indicates number of positive samples

Table 2. Average numbers of *Escherichia coli* in turnip green samples (n=4) grown in non-inoculated control soils and in soils amended with inoculated SMC compost.

	Log_{10} CFU g^{-1}	
Week	**Control Plots**	**Inoculated Plots**
3	5.4	5.1
4	<2.5*	<2.0†
5	<2.0*	<2.0**
6	<2.0†	<2.0†
7	<2.0†	<2.0†

†*E. coli* was not detected in any of the samples
Number of * indicates number of positive samples

CHAPTER IV

SURVIVAL OF *ESCHERICHIA COLI* IN SOIL TREATED WITH INOCULATED WOOD SHAVINGS AND ITS POTENTIAL TRANSFER ONTO AND/OR INTO SPINACH LEAVES

Abstract

Field studies were conducted on November 5, 2007 through February 20, 2008 to determine the survival of non-pathogenic *E. coli* in soil treated with inoculated wood shavings. A randomized block design was used for the experiment with four treatments and four replicates per treatment for a total of 8 plots, each measuring 1.2m x 4.6m (5.6 m^2). Wood shavings were inoculated with *E. coli* strain 1472 with a concentration of $2.5x10^9$ cfu/ml prior to treatment of soils. Control plots received non-inoculated wood shavings, while the treatment plots received *E. coli* inoculated wood shavings. Following application of wood shavings, all plots were direct seeded with the spinach variety Padre. Samples of soil were taken at week 1, 2, 4, 5, 6, 10, 14 and of spinach at week 5, 6, 10, 14. Results showed that *E. coli* in soils treated with inoculated wood-shavings survived for 14 weeks or longer; however *E. coli* was not detected from spinach leaves. The study was repeated on April 25, 2008 through June 12, 2008, using the same procedure and the same objective. Results indicated that *E. coli* survived for only 4 weeks compared to the 14 weeks

survival time in the previous study, however due to poor crop stands, spinach leaf samples were not collected. Differences in results between the two studies may be due to the seasons in which spinach plants were planted. Increase in temperature in the spring may also have a negative effect on the survival of *E. coli* in soils.

Introduction

According to the USDA, U.S. consumers today are eating more spinach due to a growing trend toward healthy lifestyle by eating more fresh fruits and vegetables. On the other hand, in recent years, the frequency of documented foodborne outbreak of *E. coli* O157:H7 infections associated with consuming leafy greens has also increased significantly. In September 2006, fresh, bagged baby spinach was identified as a source of a multistate outbreak of *E. coli* 0157:H7 (CDC 2006). The outbreak caused a total of 204 cases of illness and 3 deaths across 26 states and Canada. Investigators were able to trace the outbreak to *E. coli* O157:H7 isolated from cattle feces just one mile from an implicated spinach field on a ranch located on the central California coast. Feral swine were considered as vectors involved in the contamination of spinach plants as they might have carried cattle feces and indirectly contaminated surface waterways or soil (Jay and others 2007). Though mechanisms in which the pathogen was transmitted to the spinach plants was unclear, the investigation highlights the importance of in-field microbial contamination of leafy greens.

In the previous study, although the counts were inconsistent, data indicated that *E. coli* survived in soil amended with contaminated compost for at least several weeks. Thus, soil was shown to be a potential risk for in-field contamination of leafy greens. In addition, *E. coli* was also detected from the leaves of turnip greens and persisted for a period of time. Since spent mushroom compost (SMC) perhaps had antimicrobial properties that might have prevented the survival of *E. coli*, wood shavings were used in this study as an alternative inoculation media. The objective of this study was to refine the previous experiment using a different inoculation media and to determine the survival rate of *E. coli* in soils and whether *E. coli* could be transmitted to the spinach leaves.

Materials and Methods

Microorganisms and preparation of inocula

The same strain of non-pathogenic *E. coli* was used in this experiment and the culture was prepared using the same methods and conditions as described in the previous chapter.

Experimental design

The field experiment was conducted at the Oklahoma State University Vegetable Research station in Bixby, Oklahoma on November 5th, 2007. Plots were arranged in a randomized block design with four replications for each of the two treatments, *E. coli* inoculated plots and non-inoculated control plots. Each plot had a dimension of 1.2m x 4.6m (5.6 m^2).

Inoculation of wood shavings

Country Boy brand wood-shavings used as composts in this experiment were obtained from a local Atwood's store in Stillwater, Oklahoma. Prior to treatment of the soil, approximately half of a bale of wood-shavings (~5.5 kg) were placed in a cement mixer and were inoculated with *E. coli* by spraying of 250 ml of the culture at ~10^9 CFU g^{-1} diluted in 3.8 l of water using a 7.6 l hand-pump sprayer with one flat-fan spray nozzle while keeping the mixer rotating during inoculation.

Treatment of soils and seeding of spinach

Control plots received a total of 22 kg of non-inoculated wood shavings, whereas each treatment plot received ~16.5 kg of non-inoculated wood shavings with addition of ~5.5 kg of wood shavings previously inoculated with *E. coli*. All wood shavings application were made uniformly by spreading over the plot and then tilled with 1.4 m wide tractor mounted rototiller at a depth of 3.8 cm. After applications of wood shavings, all plots were direct seeded to the spinach variety Padre, which was obtained from Seminis Seed Company at a seeding rate of approximately 2.7 million seeds per hectare. Following treatment of soils and planting of spinach seeds, the entire experimental area received 0.73 kg per hectare of Dual Magnum (S-metolachor) followed by approximately 1 cm of water from overhead irrigation.

Sampling of soil and spinach leaves

At week 1, 2, 4, 5, 6, 10, 14, soil samples were collected from each plot using a soil probe from three different areas 5-8 cm deep from the surface and collected in a plastic bag. To prevent cross-contamination, samples were collected in the order of non-inoculated control plots first. Gloves were also worn during samples collection. Leaf samples were also collected starting from week 5 and on week 6, 10 and 14 from each plot by randomly selecting and pulling plants from different areas within a plot and collected in plastic bags. The samples were packed on ice and transported to the laboratory in the Food and Agricultural Products Center of Oklahoma and placed in a refrigerator at 4°C until analyzed.

Microbial analysis of soil and spinach leaf samples

Microbial analysis of soil and spinach leaf samples were conducted using the same procedure as described in the previous chapter.

Subsequent experiment conducted in the spring 2008

Following the fall 2007 spinach experiment, another experiment was conducted to repeat the study using the same experimental design and procedure. The experiment began on April 25, 2008 with inoculation of wood shavings and treatment of plots as described above. Unfortunately, due to heavy rain, the experimental site was tilled up and the entire process was repeated in respective plots on May 9, 2008. Sampling of soils was conducted at weekly intervals (0, 1, 2, 3, 4, and 5). Samples were collected, processed and analyzed using the same procedure described above.

Statistical Analysis

Escherichia coli counts for soil and spinach leaf samples were converted to $\log_{10}$ values in order to achieve a normal distribution of the data. Analysis of variance for each set of data was conducted using a randomized block design, with each replication as a block. Data were analyzed using the mixed models procedure of SAS (SAS Inst. Inc., Cary, N.C., U.S.A.) with weeks as repeated measure. The average *E. coli* counts from the fall 2007 data was analyzed using short-term (weekly) and long-term analysis (monthly). A LSD test was used to separate means with significant differences between treatments by week interaction were determined at the 95% confidence interval ($p < 0.05$).

Results

Soil Analysis

For the fall 2007 experiment, *E. coli* was not detected in any soil samples collected from the non-inoculated control plots and was recorded as <10 CFU g^{-1} (<1.0 $\log_{10}$ CFU g^{-1}) throughout the experiment. *Escherichia coli* survived in soil samples treated with inoculated wood shavings for at least 14 weeks. For the short term analysis, there was a week effect observed for the average numbers of *E. coli* concentration in soil samples ($p < 0.0062$). There was no difference in the average counts of *E. coli* observed from week 1 through week 5 of the experiment. However, a significant difference was

observed at week 6 at which the level of *E. coli* in treated soils significantly declined to 2.7 $\log_{10}$ from 4.4 $\log_{10}$ at week 1 (Figure 1). For the long term analysis, a week effect was also observed for the average numbers of *E. coli* in soil samples ($p < 0.0080$). A significant decrease was observed at week 10 or about 2 months after the initial experiment at which there was approximately 2.0 $\log_{10}$ CFU g^{-1} reduction compared to week 1 (Figure 2). However, no difference was observed between the average numbers in soils at week 10 and 14 ($p = 0.8463$).

The same experiment was repeated in the spring 2008, but the average concentration of *E. coli* in soils treated with inoculated wood-shavings declined to an undetectable level after only 3 weeks. The average numbers of *E. coli* at the initial experiment was 3.3 $\log_{10}$ CFU g^{-1} and the final detectable concentration at week 3 was 1.7 $\log_{10}$ CFU g^{-1}. Although the average numbers of *E. coli* at the initial experiment (week 0) was not measured in the fall 2008, the average numbers of *E. coli* for spring 2008 were lower for all of the weeks when compared to fall 2007 means (Table 1). There was season effect ($p < 0.0001$) when comparing the average numbers of *E. coli* in soils from fall 2007 and spring 2008 data. However, there was no significant season by week interaction ($p = 0.9371$).

Spinach Analysis

For the fall 2007 experiment, *E. coli* was not detected in any of the spinach leaf samples collected. For the spring 2008 experiment, samples of spinach leaves were not collected due to heavy rain which resulted in poor crop stands.

Discussion

Survival of enteric pathogens such as *E. coli* O157:H7 has been widely investigated with results showing that the microorganism is able to survive in soils for a long period of time (Bogosian and others 1996; Cools and others 2001; Islam and others 2005; Jiang and others 2002). These studies support our previous findings that *E. coli* was able to survive in soils amended with inoculated wood shavings for at least 7 weeks (49 days). On the other hand, the inconsistency found in the results emphasizes the needs to repeat the study with some modifications. We predicted that *E. coli* would be able to survive in soils longer when inoculated using a different inoculation media, such as wood shavings. In addition, findings from our previous study suggested that cross-contamination of leafy greens grown in contaminated soils could occur. Though the transmission of bacteria to plants was possibly due to rain and sites of bacteria attachment were not fully understood, results suggested that once contamination of leafy greens occurred, the microorganism could persist in the food supply for a period of time. The objective was to repeat the study to determine if a higher survival rate of *E. coli* in soils would be observed when using inoculated wood shavings as a media and to determine the potential mechanisms by which *E. coli* could be transmitted to spinach plants when grown in contaminated soils.

In this study results revealed that *E. coli* persists in soils for long periods of time (14 weeks) and at considerably higher average concentration compared to what was found in our previous study. Rapid decline in the average numbers of *E. coli* in soils amended with inoculated wood shavings was observed 6 weeks after the initial treatment

through week 10; the average counts then stayed consistent through week 14. Previous investigations have shown that soil type, rainfall, UV radiation, and temperature influence the die-off rate of *E. coli* under field conditions (Jiang and others 2002; Avery and others 2004). Investigations on solar radiation on the average numbers of *E. coli* in soils have shown evidence that it was effective in reducing the average numbers of *E. coli*. One study demonstrated that fecal pats under 0% shade using a cloth had the lowest average counts of E. coli compared to 40%, 80% and 100% shade (Meays and others 2005).

When the study was repeated in the late spring 2008, results showed that the average numbers of *E. coli* in soils amended with inoculated wood shavings was approximately 1.5 to 2.0 $\log_{10}$ CFU g^{-1} lower for all of the weeks compared to the fall 2007 average *E. coli* concentration. Since the inoculation method was the same for both experiments, unequal inoculation dosage between the fall 2007 and spring 2008 experiment is suspected to be one of the causes for this difference. In addition, a 1.0 $\log_{10}$ CFU g^{-1} reduction was observed after only a week. The differences in the average concentration of *E. coli* and the rates of decline also may be due to season and temperature variability between the two experiments. Survival rate and characteristics of *E. coli* at different temperatures have been widely studied. One study suggested that measured survival times of *E. coli* in agricultural soil could most likely be shorter in the summer due to higher average temperatures (Avery and others 2004). The results of our study conform to their observation in that we found the organism to persist in the soil for only about 3 weeks in the spring 2008 as opposed to 14 weeks in the fall 2008 experiment.

Furthermore, our findings are also consistent with the results of other studies that show that the survival rate of *E. coli* was higher at cooler temperatures. A study on survival of *E. coli* in manure-amended loam soils amended with manure showed that the microorganism of different strains declined more rapidly at 30 °C than at 4 °C (Topp and others 2003). Another study showed that *E. coli* was able to survive longer in Minnesota soils at 4, 15, and 25 °C than when incubated at 30 °C or 37 °C (Ishii and others 2006). Data from another study suggested that die-off rate doubled with a 10 °C rise in temperature (Reddy and others 1981).

In the fall 2007 study, according to Oklahoma Mesonet database, the average high air temperature during the experiment period at the test site was 11 °C in November and the average low was 3 °C in December and January. The spring 2008 experiment was conducted in early May and the air temperature was higher with an average of 18 °C to 25 °C for May and June, respectively (See Appendix). Thus, the average air temperature was about 14.5 °C higher for our spring experiment than for our fall experiment. This likely explains the more rapid decrease in microbial numbers in the spring experiment.

It is believed that the higher average numbers of *E. coli* at lower temperatures is due to less competition for available nutrition in soils or antagonistic activity from other indigenous soil organisms towards *E. coli* (Cools and others 2001; Gagliardi and Karns 2002). Aside from low temperature, wetter topsoil conditions are also common in the winter season, which have been shown to contribute to better survival of this microorganism (Cools and others 2001). The solar radiation study revealed that the highest loss in the average numbers of *E. coli* in fecal pats with 0% shade was associated with high loss of percent moisture (Meays and others 2005).

Although the survival rate of pathogens in agricultural soils is generally higher in the cold season, perhaps creating greater risks for contamination of leafy greens in that time of year, the behavior of pathogens in the warm season should not be ignored. One study revealed that *E. coli* O157:H7 could survive in manure-amended soil under dry conditions (less than 1% moisture) for extended periods of time (Jiang and others 2002).

Survival of *E. coli* in soils for an extended period of time raises major concerns that pathogens can be transmitted to edible parts of the plants when those plants are produced in agricultural soils amended with composts containing pathogens. In the fall 2007 study, *E. coli* was not recovered for any of the spinach samples collected for any of the weeks, although *E. coli* was recovered from the soils. This indicates that cross-contamination or the uptake of *E. coli* from contaminated soils did not occur. In contrast, other studies that have investigated the risks of using manure-based soil amendments for growing vegetables have found that transmission of *E. coli* O157:H7 may occur outside as well as inside of the plants (Itoh and others 1998; Solomon and others 2002).

For example, under laboratory conditions one previous study demonstrated that when lettuce seeds were directly planted in soil beds in which the soil had been fertilized with manure containing *E. coli* O157:H7, the microorganism was recovered from the seedlings (Solomon and others 2002). Another study revealed that *E. coli* O157:H7 was recovered not only from the surface but also from the inner tissues radish sprouts, in which seeds had been experimentally contaminated with the pathogen (Itoh and others 1998).

Differences in the experimental designs and methods used in other studies may directly contribute to the discrepancy between their findings and ours. For example, in

the present study the planting of spinach seeds was conducted under field conditions that were very different from the laboratory settings used in the previous studies. The discrepancy between the results in the present study and those of other studies may also be related to the different plants as well as strains of *E. coli* and their physiological state at the time of application, which may also have affected the survival rate. However, results collected from the present study concur with results from other field studies involving lettuce and spinach plants (Johannessen and others 2004; Hora and others 2005; Johannessen and others 2005). Although in those studies, *E. coli* O157:H7 was introduced at a time when the plant had reached the seedling stage of growth, the pathogen was not recovered from the edible parts of the plants. Unfortunately, very limited studies are available on the potential transmission of pathogens to plant tissues when seeds are directly planted in contaminated soils under field conditions or real agricultural practices.

In conclusion, results from this experiment provide useful information that may be helpful in devising ways to handle compost potentially contaminated with *E. coli* O157:H7 in such a way as to reduce the risk of in-field microbial contamination of leafy green vegetables. However, further studies need to be done, including investigations of different avenues by which agricultural soils can be contaminated and how those differences may affect the survival rate of *E. coli*.

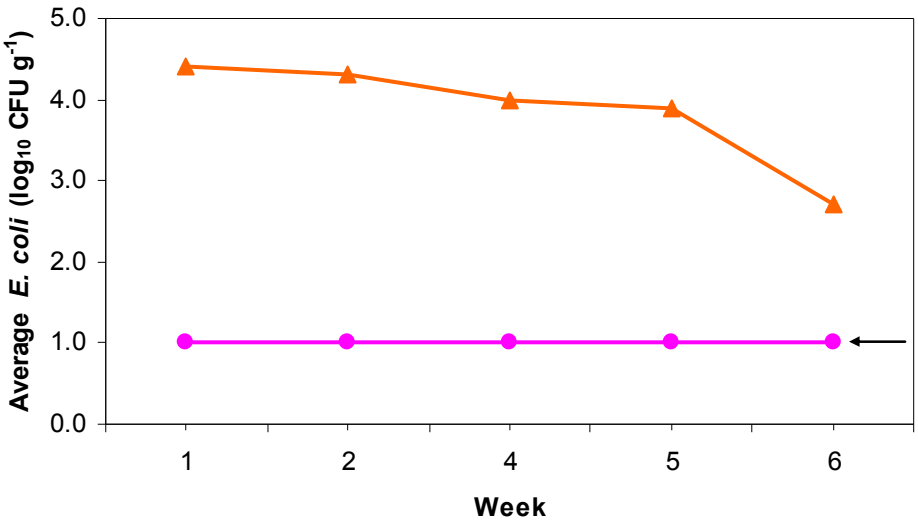

Figure 1. Short-term analysis on survival rate of *E. coli* in soils applied with inoculated wood shavings (▲) and in non-inoculated control soils (●) in fall 2007. Arrow (←) indicates no detectable *E. coli* in soil samples. $P < 0.05$

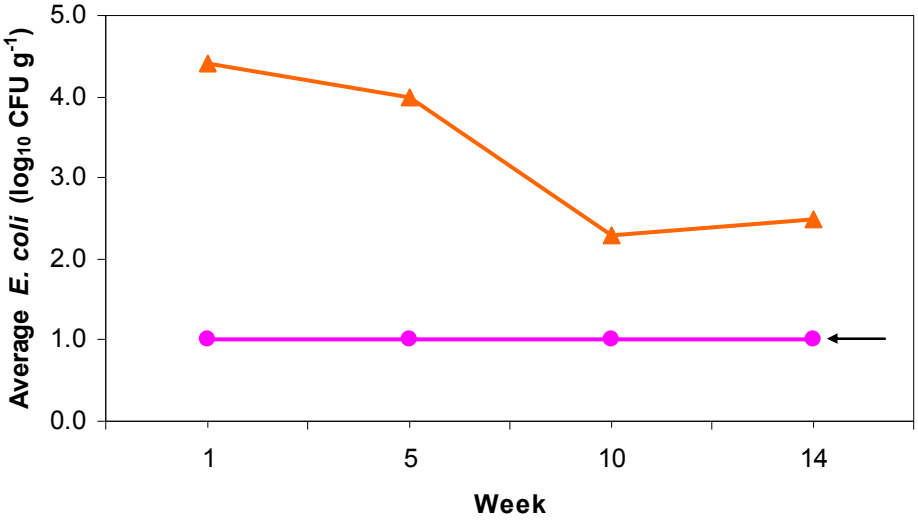

Figure 2. Long-term analysis on survival rate of *E. coli* in soils applied with inoculated wood shavings (▲) and in non-inoculated control soils (●) in fall 2007. Arrow (←) indicates no detectable *E. coli* in soil samples. $P < 0.05$

Table 3. Comparison of numbers of *E. coli* (mean ± SEM, n = 4) in inoculated soils during the first three weeks of experiments conducted in the fall 2007 and spring 2008. *E. coli* was undetectable after week 3 in soil samples collected in the spring 2008 experiment. *E. coli* counts were transformed to $\log_{10}$ CFU g^{-1}.

	Week			
	0	**1**	**2**	**3**
Fall 2007	NS	4.4 ± 0.2^{a}	4.3 ± 0.2^{a}	NS
Spring 2008	3.3 ± 0.2^{a}	2.6 ± 0.5^{ab}	2.5 ± 0.2^{b}	1.7 ± 0.3^{b}

NS = soil samples were not collected
[ab]Means in the same row with same superscript are not different ($P < 0.05$).

CHAPTER V

SURVIVAL OF *ESCHERICHIA COLI* IN SOILS USING DIFFERENT INOCULATION METHODS AND ITS POTENTIAL INTERNALIZATION INTO SPINACH LEAVES

Abstract

Spinach and other leafy greens have been associated with outbreaks of *Escherichia coli* O157:H7 infections. In-field microbial contamination has also become a significant source of foodborne illness outbreaks due to soil that is contaminated with animal manure and/or compost as well as from contaminated water runoff from adjacent fields or polluted irrigation water. Field studies were conducted on October 2, 2008 to determine the survival of non-pathogenic *E. coli* in soil treated with contaminated wood-shavings compost and water runoff. Wood-shavings compost and water were inoculated with *E. coli* strain 1472 with a concentration of $2.5x10^9$ CFU ml^{-1}. A randomized block design was used for the experiment with four treatments and four replicates per treatment for a total of 16 plots, with dimensions of 1.2m x 6.1 m (7.3 m^2). The four treatments included one with inoculated wood-shavings, one with inoculated water applied and tilled, one with inoculated water applied without subsequent tilling, and one non-inoculated control. Results showed a significant treatment by week interaction for soils

treated with inoculated wood-shavings and bacteria-water mixture with tilling ($p < 0.0001$). *Escherichia coli* was able to survive for at least 23 weeks when in soils treated with inoculated wood shavings though data showed a constant decrease in the average numbers of *E. coli*. *Escherichia coli* was not detected in spinach leaf samples collected from any of the treatment plots.

Introduction

Aside from contaminated compost, soils can also be contaminated via polluted water used to irrigate the crops or surface water runoff. Rainfall and snow melt can generate surface runoff, which may carry pathogenic microorganisms from adjacent field where livestock inhabit and shed their feces. This may result in the spreading of pathogens to other areas and into the soil by leaching through the soil profile. Movement and leaching of pathogens through the soil profile has been studied extensively using simulated rainfall (Gagliardi and Karns 2000). Their findings indicated that if *E. coli* O157:H7 from runoff water reached the soil; the microorganism could survive, replicate, and move vertically for some time.

Our previous studies indicated that there was a need for conducting another field experiment with different methods of inoculation to provide more information on the survival of *E. coli* under field conditions. We had seen that wood shavings could be used as an effective inoculation media. In addition, although our previous findings showed that the microorganism was not transferred onto or into the spinach plants, further

investigations needed to be carried out to test the hypothesis that internalization of *E. coli* may occur when spinach is grown on contaminated soil.

The objective of this experiment was to determine the survival rate of *E. coli* in the soil using different methods of inoculation under field conditions and to determine the presence of *E. coli* inside the plant tissues.

Materials and Methods

Microorganisms and preparation of inocula

The same strain of non-pathogenic *E. coli* was used in this experiment and the culture was prepared using the same methods and conditions as described in previous chapters. For this experiment, three liters of total inoculum was needed and divided into twelve bottles of 250 ml TSB containing 1% of culture of *E. coli* per bottle. The cultures were incubated at 37°C for 18 h prior to the experiment. The cultures were transferred to the experimental field in an ice-chest.

Experimental plot design

The initial field experiment was conducted on October 2nd, 2008 at the Oklahoma State University Vegetable Research station in Bixby, Oklahoma. Plots were arranged in a randomized block design with four replications for each of the four treatments, with each plot measuring 1.2m x 6.1 m (7.3 m^2) and a 7.6 m gap between plots. The study included three treatments inoculated with non-pathogenic *E. coli* and a non-inoculated control (Treatment D). The three treatment groups were described in Table 1, which

included plots treated with inoculated wood-shavings (Treatment A), plots sprayed with water-bacteria mixture, tilled and seeded (Treatment B), and plots tilled, seeded and sprayed with water-bacteria mixture (Treatment C).

Inoculation of compost and water-bacteria mixture

The same brand wood-shavings (Country Boy) used as composts in this experiment was obtained from a local Atwood's store in Stillwater, Oklahoma. Prior to treatments of the soil, approximately half of a bale of wood-shavings (~5.5 kg) were placed in a cement mixer and were inoculated with *E. coli* by spraying of 250 ml of the culture at ~10^9 CFU g^{-1} diluted in 3.8 l of water using a 7.6 l hand-pump sprayer with one flat-fan spray nozzle while keeping the mixer rotating during inoculation. Samples of inoculated wood-shavings were collected in plastic bags to be analyzed for determination of the average numbers of *E. coli* in the wood-shavings. Inoculated water was prepared by mixing 250 ml of *E. coli* culture at ~10^9 CFU g^{-1} with 3.8 l of water.

Treatment of soils and seeding of spinach

The four treatments included one with inoculated wood-shavings (treatment A), one with inoculated water applied and tilled (treatment B), one with inoculated water applied without subsequent tilling (treatment C), and one non-inoculated control (treatment D). Each of the treatment A plots received ~5.5 kg of previously inoculated wood shavings by spreading them uniformly over the plot and then tilled with 1.4 m wide tractor mounted rototiller at a depth of 7.5-10 cm. Each of the treatment B plots was sprayed uniformly with 3.8 l of water-bacteria mixture using a hand-pump sprayer with

one flat-fan spray nozzle at the rate of 3.8 l per plot prior to seedling. Prior to inoculation, each of the treatment C plots was tilled followed by spraying the water-bacteria mixture uniformly the surface of each plot using a hand-sprayer at the rate of 3.8 l per plot. After soil treatment applications, plots were direct seeded to the spinach variety Padre, which was obtained from Seminis Seed Company, at a seeding rate of approximately 2.7 million seeds per hectare. Following treatment of soils and planting of spinach seeds, the entire experimental area received 0.73 kg active ingredient per hectare of Dual Magnum (S-metolachor) followed by approximately 1 cm of water from overhead irrigation.

Sampling of soil and spinach leaves samples

Sampling of soils was carried out weekly on October 2nd, 2008 through December 9th, 2008 and followed by monthly sampling through March 26th, 2009. Soil samples from each plot for each treatment were collected in a plastic bag using a soil probe from three randomly selected areas 5-8 cm deep from the surface. Sampling of spinach plants began on October 21st, 2008, three weeks after the initial treatments and planting when the leaves were large enough for sampling. Spinach leaves were pulled from the soil but without pulling out the roots and were collected aseptically in sterile plastic bags. Sampling of spinach leaves was also carried out at the same time interval as the soils, except no leaf samples were collected for week 14. From each plot, spinach leaves were obtained from a randomly selected plant and was collected in plastic bags. The samples were packed in ice and transported to the laboratory in the Food and Agricultural Products Center of Oklahoma and placed in a refrigerator at 4°C until analyzed.

Soil analysis

Microbial analysis of soil samples was conducted using the same procedure described in the previous chapter. However, serial dilutions were prepared and plated in duplicate using a direct plating method with CHROMagar *E.coli* and violet red bile agar (VRBA; Difco^{TM}; Becton, Dickinson and Company; Sparks, MD). Violet red bile agar plates were overlaid before being incubated at 37°C for 24 h prior to enumeration of *E. coli* colonies. E. coli colonies were identified as blue colonies on the CHROMagar plates, whereas on the VRBA plates, *E. coli* appeared as pink colonies with a precipitation zone around. The final counts were calculated by averaging the CFU g^{-1} of CHROMagar and VRBA.

Spinach analysis

Four grams of spinach leaf samples from each treatment were obtained and washed with tap water to remove soil from the surface of the leaves, followed by surface sterilization by one minute immersion in 70% ethanol (95% Ethyl Alcohol; Pharmco; Brookfield, CT), followed by one minute immersion in 30% bleach (Ultra Bleach, Great Value; Wal-Mart; Stillwater, OK) and washing in sterile de-ionized water for three times. Spinach leaves were ground using mortar and pestle prior to plating and 36 ml of 0.1% peptone water was added. Series of dilutions (1:10, 1:100, 1:1000, 1:10000) of each sample were prepared with 0.1% peptone water and plated in duplicate using a direct plating method with CHROMagar *E.coli* and VRBA. All plates were incubated at 37°C for 24 h prior to enumeration of colonies of *E. coli*. When *E. coli* was not detected by direct plating, enrichment was performed by transferring 1 ml of the 1:10 dilution into a

10 ml of Gram-negative enrichment (GN; Difco™; Becton, Dickinson and Company; Sparks, MD) broth and incubated at 37°C for 24 h. Dilutions of cultures (10 μL) were then surface plated on CHROMagar *E. coli* plates.

Statistical Analysis

The average numbers of *E. coli* for soil and spinach leaf samples were converted to $\log_{10}$ values in order to achieve a normal distribution of the data. Analysis of *E. coli* survival in soil samples was performed in two parts, including comparison of the three inoculation treatments at week 0, short-term analysis (week 0 through 10), and long-term analysis by preserving as much possible the spacing between time events (week 0, 5, 10, 14, 18, 23). Data were analyzed using the mixed models procedure of SAS (SAS Inst. Inc., Cary, N.C., U.S.A.) with weeks as repeated measure. Least significant difference (LSD) was used to separate means with significant differences between treatments by week interaction were determined at the 95% confidence interval ($p<0.05$).

Results

Soil Analysis

The final concentration of *E. coli* in the inoculated wood-shavings prior to treatments of soils was at least 2.5×10^5 CFU g^{-1}. *Escherichia coli* was not detected in any soil samples collected from the non-inoculated control plots and was recorded as <10 CFU g^{-1} (<1.0 $\log_{10}$ CFU g^{-1}) throughout the experiment. Comparing between the three

treatments (A, B, and C) at the initial experiment (Week 0), there was no difference in the average numbers of *E. coli* in soil samples for any of the treatments or inoculation methods ($p = 0.1608$), indicating equal inoculation concentration for all three treatments (Table 2). However, starting from week 1 through the end of the experiment (week 23), *E. coli* was not detected in any of the soil samples collected from treatment C and thus recorded as <10 CFU g^{-1} (<1.0 $\log_{10}$ CFU g^{-1}).

Since *E. coli* was no longer detected in soil samples collected from treatment C plots and the non-inoculated control plots, thus analysis was performed only on treatment A and B. When comparing treatment A and B on a 0 to 10 weekly interval (Figure 1), there was a significant treatment by week interaction ($p = 0.0096$). At week 1, there was no difference in the average numbers of *E. coli* between treatment A and B ($p = 0.1620$). Similar results were also observed for week 3 through week 5 ($p \geq 0.1336$). However, starting from week 6, there was a significant difference between treatment A and B, in which higher means of *E. coli* concentration were observed in soil samples of treatment A plots. The average numbers of *E. coli* declined progressively on both treatment A and B over a 10-weeks period, but did so more rapidly for treatment B. Approximately 1.5 $\log_{10}$ and 2.5 $\log_{10}$ reduction in *E. coli* counts were observed by week 10 for treatment A and B, respectively.

Due to the decreasing in the average numbers of *E. coli* in soils, we decided to monitor its survival at monthly intervals. Thus, in addition to the short-term analysis, a long term analysis was conducted by preserving as much as possible the spacing between time events starting from week 0 to week 23 (Figure 2). In this long term analysis, a significant treatment by week interaction was highly evident for the two treatments ($p <$

0.0001). The graph shows that *E. coli* detected in soil samples from treatment A plots was able to survive longer with a higher concentration. At the end of the experiment (week 23), the average numbers of *E. coli* in treatment A soil samples were reduced by approximately 3.5 $\log_{10,}$ whereas the average numbers of *E. coli* in treatment B soil samples were reduced to a non-detectable level (<1.0 $\log_{10}$).

Spinach Analysis

E. coli was not detected in spinach leaf samples collected from any of the treatment plots. Enrichment of ground spinach leaf samples also provided negative results in 0.01 g of spinach.

Discussion

Recent outbreaks associated to the consumption of leafy green vegetables have made it necessary to examine the overall survival times and characteristics of *E. coli* O157:H7 populations in soil as a significant potential source of in-field microbial contamination. Our objective was to determine if different inoculation methods would have any effects on the survival rate of *E. coli* in soils. Different inoculation methods used in the study included the use of inoculated wood shavings compost and water-bacteria mixture by means to simulate contaminated soil amendments and polluted water runoff, respectively.

Our findings suggest that non-pathogenic *E. coli* can survive for at least 23 weeks in soils, which corresponds to other studies that found the ability of non-pathogenic *E.*

coli to survive in soils for a long period of time (Bogosian and others 1996; Bolton 1998; Meays and others 2005; Topp 2003). However, through this study, we learned that the survival rate likely also depends on methods of inoculation or, in other words, sources of contamination. When soil was directly inoculated with *E. coli* and water mixture but without tilling, and thus only sat on the soil surface, the concentration of *E. coli* in the soil samples decreased from an average of 3.4 $\log_{10}$ CFU g^{-1} at the initial sampling week to an undetectable level (<1.0 $\log_{10}$ CFU g^{-1}) by week 1. The rapid loss of *E. coli* in these soils may be due to high exposure of *E. coli* to adverse environmental conditions, such as weather, temperature, and sun exposure. In addition, a significant decrease may also have occurred as results of rain that washed the microorganism out of the inoculated plots. Others have observed such an effect (Bolton and others 1999).

When our plots were inoculated with water-bacteria mixture but tilled after application and thus incorporated into soil profile, *E. coli* was able to survive and persist in the soil for up to 10 weeks. Blending or tilling of soils has been suggested to increase soil aeration and leach out of nutrients, such as nitrogen, phosphorus and potassium, which could lead to a decrease in the survival of *E. coli,* whereas exposure to sunlight has a significant negative impact (Meays and others 2005).

These findings suggest that when soil is contaminated by polluted runoff or irrigation water, the survival rate of *E. coli* may depend on how deep the microorganisms leach into the soil layers. Tillage practices as well as available nitrogen has been known to enhance transport and survival of *E. coli* O157:H7 in soil (Gagliardi and Karns 2000).

An increase in the average numbers of *E. coli* observed at week 2 and 9 supported previous findings that *E. coli* not only shows persistence but the microorganism can also

replicate in soils (Gagliardi and Karns 2007). On the other hand, this observed variation may have occurred simply because *E. coli* was not uniformly distributed in the plots during applications.

Application of previously-inoculated wood shavings into soils gave the longest survival rate of *E. coli* throughout the 23-week period. Although data showed a constant decrease in average *E. coli* populations and there was an approximately 2.5 $\log_{10}$ reduction at the end of the experiment (23 weeks), it is possible for *E. coli* to survive and persist longer in soil. The highest survival rate of *E. coli* in soils amended with inoculated wood shavings may be due to attachment characteristics of the microorganisms to the surface of wood shavings. Characterization of *E. coli* attachment to surfaces has been widely investigated, but limited only to food and plant surfaces.

The speed at which the average numbers of *E. coli* are reduced over time in soil regardless of different methods of inoculation is likely influenced by several factors. However, the significant contributing factors in our study may have included rainfall, sunlight and temperature variability. Even though these factors were not measured or recorded in our study, they have been suggested to have negative impacts on the average rate of *E. coli* decline in animal feces and soils (Avery and others 2004).

Escherichia coli was not detected inside the spinach leaves samples obtained from any test plots whether or not enrichment media were used. However, the maximum amount of sample assayed (1:10 dilution) was only 0.1 g for direct plating and 0.1 for enrichment. This was necessary due to limitation of the method used. The enrichment should have been done using a much larger portion of spinach leaves. This experiment tends to confirm our previous findings that the microorganism did not penetrate into the

tissue of the plants, thus internalization of *E. coli* most likely did not happen. Our results on the other hand, could not determine whether or not the organism was on the surface of the leaves. However, if cross contamination of spinach did occur and *E. coli* was attached on the surface of the spinach plants, results suggest that surface sterilization was effective to eliminate the bacteria from the surface of the plants.

In conclusion, the long-term survival of *E. coli* in contaminated soil demonstrates the need for a better farm waste management to prevent pre-harvest contamination of leafy greens. It is also necessary to understand the risks involved with different types of contamination such as animal manure used as soil amendments or polluted water runoff. Our findings suggest that soils amended with organic material possess greater risks as *E. coli* were more persistent in this type of situation. Absence of *E. coli* inside spinach plants indicate that the uptake of *E. coli* from contaminated soils did not occur.

Table 4. Different treatments (inoculation methods) applied to soils used to plant spinach seeds.

Treatment	Media	Methods for Soil Inoculation
A	Wood shavings inoculated with 250 ml *E. coli culture* in 1 gal. H_2O	Applied with inoculated wood shavings→tilled→seeded
B	H_2O (1 gal.) mixed with 250 ml *E. coli*	Sprayed with contaminated water→tilled→seeded
C	H_2O (1 gal.) mixed with 250 ml *E. coli*	Tilled soil→seeded →contaminated water
D	None	Non-inoculated tilled soil

Table 2. Average counts of *Escherichia coli* in soils (mean ± SEM) treated using different inoculation methods: inoculated wood shavings (A), sprayed with contaminated water→tilled→seeded (B), and tilled soil→seeded→contaminated water (C) at week 0.

Treatment	Average *E. coli* (log10 CFU g-1)
A	4.3 ± 0.52^a
B	4.9 ± 0.52^a
C	3.4 ± 0.52^a

[a]Means in the same column with same superscript are not different ($P < 0.05$).

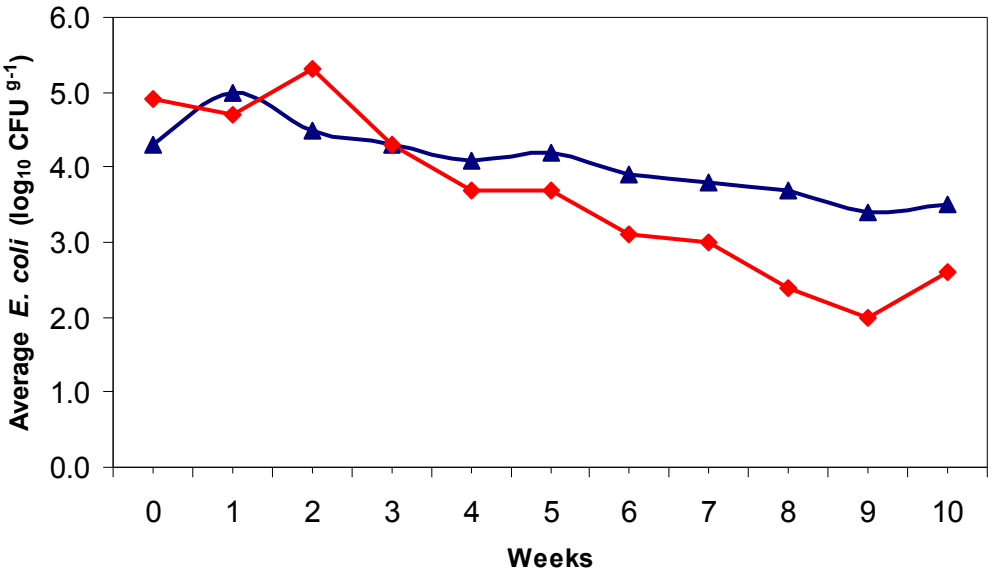

Figure 3 Short-term analysis on survival rate of *Escherichia coli* in soils applied with inoculated wood shavings (▲) and contaminated water with tilling after application (♦). Interaction between treatment and week was significant (p = 0.0096).

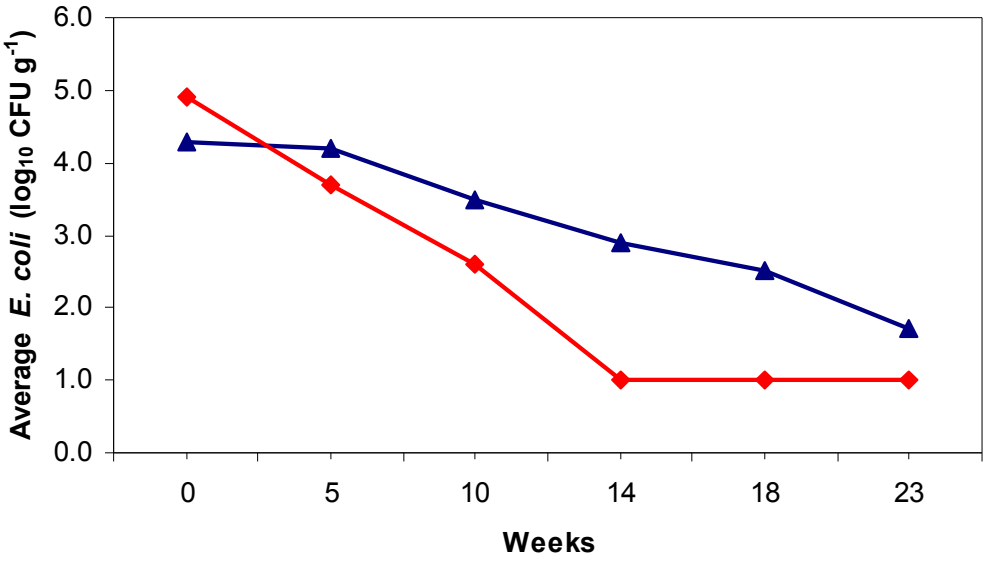

Figure 4 Long-term analysis on survival rate of *Escherichia coli* in soils applied with inoculated wood shavings (▲) and contaminated water with tilling after application (♦). Interaction between treatment and week was significant ($p < 0.0001$).

CHAPTER VI

OVERALL DISCUSSION

Recent outbreaks of infection caused by *E. coli* O157:H7 associated with the consumption of leafy green vegetables have raised concerns about the origins of contamination. On-farm microbial contamination prior to harvesting has been investigated as a significant source of the pathogens responsible for many of the outbreaks. Use of organic composts containing animal manure is a common practice in agricultural industry. However, animal manure may often carry enteric pathogens and when spread onto fields may create high risks for contaminating crops grown in those fields. Management of in-field microbial contamination requires an understanding of the sources or types of soil contamination, survival characteristics of the pathogens in soils, as well as environmental factors affecting their survival. Long-term survival of *E. coli* in agricultural soils emphasizes the importance of understanding its potential for transmission to leafy greens.

In the present study, survival of *E. coli* in soils was investigated via three different experiments using different inoculation media and methods. Potential transmission of the microorganism to turnip greens and spinach were also examined.

Results from the present study indicated that *E. coli* can survive in soils for at least 7 weeks and up to 23 weeks (161 days) when amended with spent mushroom compost (SMC) or wood shavings, respectively that had been inoculated prior to application. Several variables are believed to control the survival of enteric pathogens in soils, including the physical and chemical nature of soils, atmospheric conditions, such as sunlight, moisture and temperature, competition with naturally occurring soil microflora, and waste application methods and techniques (Crane and Moore 1985; Ogden and others 2001; Avery and others 2004). In addition to cell death, another relevant factor that may cause reduction of *E. coli* concentrations in soils may be rainfall events that wash the microorganism out of the inoculated soils (Bolton and others 1999).

Lower survival rate of *E. coli* observed in the first experiment suggests that SMC may contain antimicrobial properties that could speed up the die-off rate of pathogens, however further research is needed to evaluate this hypothesis. Highest survival rate of *E. coli* was observed in soils when amended with inoculated wood shavings. Although the survival characteristics were not fully understood, ability of *E. coli* to attach to wood shaving surfaces may influence its survival rate. However, limited studies are available on this matter and the hypothesis needs to be further investigated. The present study was limited to a period of 160 days at the most; however another study has indicated that *E. coli* can be detected in manure-amended soils in low concentration for up to 250 days (Cools and others 2001). This suggests that amending agricultural soils with animal manure carrying enteric pathogens agricultural soils may create persistent risks for microbial contamination of crops.

The pattern of recovery of *E. coli* cells from turnip green leaves in our first experiment suggested that cross-contamination occurred as results of the heavy rainfall and flooding over the crops, and not due to the uptake of the microorganism from the inoculated soils. These concur with results from other studies in which *E. coli* was recovered from the plants when they were irrigated with contaminated water (Solomon and others 2002; Islam and others 2005). Flooding events did not occur in our other two spinach experiments and *E. coli* was not recovered from any spinach leaf samples. This suggests that direct exposure or contact between the leaves and contaminated soils or some other contamination source is required for *E. coli* to be integrated into edible parts of the plants. However, one previous study has demonstrated that the transport of *E. coli* O157:H7 into lettuce plants was possible without direct exposure to a contamination source but instead via the roots system (Solomon and others 2002). The discrepancy between those results and those of our present studies may be due to differing types of plants, methods, and environmental conditions. Another spinach study was able to recover *E. coli* O157:H7 from the surface the roots but the microorganism did not become distributed to the aerial plant tissues (Hora and others 2005). Possibly *E. coli* could have been recovered from the roots of spinach plants in the present study, however since the objective of this study was to investigate the presence of *E. coli* on the surface or inside the edible portion of the plants, the roots were excluded from analysis.

Furthermore, although *E. coli* was not recovered from the spinach leaves, they could still be present in numbers below detection limit, hence the need to develop a better and more sensitive method to isolate the microorganisms. Another limitation of our microbial analysis method was that the maximum amount of sample assayed was only 0.1

g for direct plating and 0.1 g for enrichment. Therefore, in the future, studies should include assay of larger amount of spinach leaves especially in enrichment culture.

CHAPTER VII

CONCLUSION

In conclusion, the present study provided evidence that *E. coli* showed persistence for at least 161 days in soils amended with inoculated wood shavings under certain conditions. It is important to note that the microorganism may possibly survive for a longer period of time under different conditions than those we have investigated. When *E. coli* was directly incorporated into soil profiles, it survived for up to 98 days, whereas *E. coli* on the surface of soils could only survived for only a week. Several environmental factors that influence survival of *E. coli* include temperature, sunlight, and composition of soils, moisture and competition with indigenous soil microorganisms. Other relevant factors that can be controlled include strains of *E. coli*, microorganism physiological state at the time of application, methods of waste application, and how deep *E. coli* is able to leach into soil profile.

Our studies also indicated that internalization of *E. coli* into edible parts of plants did not occur in 0.01 g of spinach. This may be due to limitation of the method used in which the amount of spinach sample assayed for microbial analysis was very small. However, when leafy greens are directly in contact with the source of contamination, such as contaminated floodwater or irrigation water, the microorganism can attach to plants for a period of time. Further research under field conditions and real

agricultural practices are needed to confirm whether direct or indirect contamination of vegetable seeds will result in recovery of pathogens in the tissues of mature plants. In addition, a larger amount of spinach sample should

Concerns about the safety of U.S. food supply from pathogens, such as *E. coli* O157:H7 associated with leafy green vegetables will continue into foreseeable future. Assessment of in-field microbial contamination is necessary to improve agricultural practices and help insure that safe, high quality foods are delivered to U.S. consumers.

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APPENDICES

Fall 2007 and Spring 2008 Spinach Experiment Plots Map and Treatment Description

Treatments	Treatment description	rep 1	rep 2	rep 3	rep 4
1	No *E. coli*	101	202	302	401
2	Inoculated wood shaving	102	201	301	402

Plots Map

402
2
401
1
302
1
301
2
202
1
201
2
102
2
101
1

Fall 2008 Spinach Experiment Plots Map and Treatment Description

Treatments	Treatment description	rep 1	rep 2	rep 3	rep 4
1	No *E. coli*	101	202	304	403
2	inoculated wood shaving/tilled/plant	102	203	301	404
3	*E. coli* mixture/tilled/plant	103	204	302	401
4	tilled/plant/spray with *E. col*i mixture	104	201	303	402

Plots Map

204 3	404 2	
203 2	403 1	
202 1	402 4	N ↑
201 4	401 3	
104 4	304 1	
103 3	303 4	
102 2	302 3	
101 1	301 2	

Oklahoma Mesonet Climatological Data Summary for Bixby in December 2007

MESONET CLIMATOLOGICAL DATA SUMMARY — December 2007 — Time Zone: Midnight-Midnight CST
(BIXB) Bixby — Nearest City: 2.0 NE Bixby — County: Tulsa
Latitude: 35-57-46 — Longitude: 95-51-58 — Elevation: 604 feet

DAY	TEMPERATURE (F) MAX	MIN	AVG	DEWPT	DEG DAYS HDD	CDD	HUMIDITY (%) MAX	MIN	AVG	RAIN (in)	PRESSURE (in) STN	MSL	WIND SPEED (mph) DIR	AVG	MAX	SOLAR (MJ/m2)	4" SOIL TEMPERATURES SOD	BARE	MAX	MIN
1	68	44	57.0	49.2	9	0	94	67	75	0.00	29.28	29.93	S	15.9	32.9	2.57	52.3	50.0	55	47
2	70	31	53.4	42.3	15	0	90	41	67	0.01	29.45	30.10	NW	12.1	31.8	7.56	54.8	54.4	59	45
3	51	21	35.5	20.6	29	0	96	23	59	0.00	29.81	30.47	NA	4.3	13.3	12.45	51.0	43.5	49	40
4	65	32	45.6	27.1	16	0	91	25	52	0.00	29.32	29.96	NA	3.6	11.7	10.53	50.4	44.4	51	39
5	53	29	39.1	29.7	24	0	94	49	70	0.00	29.36	30.01	NA	10.1	31.3	10.61	49.9	44.3	48	41
6	45	29	36.3	26.8	28	0	82	50	69	0.00	29.39	30.04	SE	9.6	20.6	5.26	48.7	41.4	44	39
7	45	33	40.5	38.4	26	0	98	68	93	0.01	29.31	29.96	NE	6.0	13.2	1.16	48.9	43.1	45	41
8	42	32	39.9	39.3	28	0	99	95	97	0.07	29.44	30.10	N	9.4	21.8	1.88	49.3	45.0	46	43
9	31	29	29.7	28.4	35	0	98	92	95	NA	29.63	30.29	NA	NA	NA	1.13	47.6	39.8	43	38
10	32	29	31.1	30.6	34	0	100	95	98	NA	29.57	30.22	NA	NA	NA	1.02	44.8	36.9	38	36
11	37	32	34.4	34.0	30	0	100	93	98	NA	29.45	30.10	NA	NA	NA	1.07	43.3	39.0	40	38
12	36	31	33.7	31.1	32	0	95	85	90	NA	29.61	30.27	N	6.5	20.1	1.67	43.4	38.8	39	38
13	35	31	32.6	29.9	32	0	96	84	89	NA	29.60	30.26	NA	3.9	11.5	2.86	44.0	38.6	41	37
14	41	31	35.8	34.0	29	0	98	82	93	NA	29.52	30.17	ENE	5.8	15.0	2.34	44.1	39.1	41	37
15	38	25	32.1	29.9	33	0	99	80	92	0.07	29.36	30.01	NW	9.0	26.3	1.60	44.4	39.1	41	36
16	44	20	30.1	22.8	33	0	96	40	77	0.02	29.60	30.26	NA	4.1	15.5	11.69	42.8	36.2	38	35
17	55	23	38.1	24.3	26	0	99	27	64	0.00	29.54	30.19	NA	7.6	23.8	11.70	42.4	37.3	42	35
18	56	29	43.3	31.6	22	0	99	39	66	0.00	29.39	30.04	NA	6.6	21.6	11.23	43.6	40.2	47	36
19	63	24	42.0	34.3	22	0	100	37	78	0.00	29.37	30.01	NA	5.9	21.8	11.18	43.6	40.5	46	36
20	60	33	48.1	39.7	19	0	97	37	76	0.00	29.17	29.82	NA	3.9	13.4	11.06	45.4	45.5	51	42
21	65	28	46.8	35.9	18	0	100	30	72	0.00	29.11	29.76	NA	8.0	27.7	9.11	45.1	42.7	47	38
22	49	24	37.0	34.5	28	0	97	73	91	0.08	29.21	29.86	NW	10.5	35.6	0.69	45.9	42.3	45	38
23	48	17	31.1	21.2	32	0	92	33	69	0.01	29.56	30.21	NA	4.8	13.4	11.41	43.7	37.4	40	36
24	53	25	36.8	27.3	26	0	99	30	74	0.00	29.56	30.21	NA	2.6	11.4	11.38	43.5	39.0	45	36
25	57	25	40.4	24.5	24	0	96	23	59	0.00	29.38	30.03	NA	8.7	29.5	11.57	43.1	39.7	43	36
26	42	28	35.3	30.1	30	0	94	64	82	0.01	29.29	29.94	NA	5.2	16.2	3.70	43.1	37.9	40	36
27	38	33	35.3	32.6	30	0	98	82	90	0.00	29.34	29.99	NA	5.2	15.6	1.69	43.3	38.4	39	37
28	39	28	34.0	29.3	32	0	98	58	80	0.10	29.41	30.06	WNW	7.2	21.3	9.01	43.6	39.8	43	38
29	45	20	32.4	25.0	32	0	99	42	77	0.00	29.44	30.10	NA	4.2	16.2	10.69	42.6	37.8	41	36
30	54	24	35.4	22.9	26	0	97	21	69	0.00	29.30	29.95	NA	4.5	18.6	11.70	42.3	37.8	42	35
31	50	25	37.4	22.7	28	0	91	32	59	0.00	29.42	30.07	NA	8.4	28.7	9.69	41.8	37.0	40	35
	49	28	38.1	30.6	<- Monthly Averages ->						29.43	30.08	NA	6.8*	35.6*	6.81	45.6	40.8	44	38

Temperature - Highest: 70
Lowest: 17

Degree Days - Total HDD: 829
Total CDD: 0

Number of Days With:
Tmax ≥ 90: 0
Tmax ≤ 32: 1
Tmin ≤ 32: 25
Tmin ≤ 0: 0
Rainfall ≥ 0.01 inch: 9*
Rainfall ≥ 0.10 inch: 1*
Avg Wind Speed ≥ 10 mph: 4*
Max Wind Speed ≥ 30 mph: 4*

Rainfall: Monthly Total: 0.38* in.
Greatest 24 Hr: 0.10* in.

Humidity - Highest: 100
Lowest: 21

* Denotes incomplete record

monthly data generated on wednesday, april 01, 2009 at 10:14 am

Oklahoma Mesonet Climatological Data Summary for Bixby in January 2008

MESONET CLIMATOLOGICAL DATA SUMMARY — January 2008 — Time Zone: Midnight-Midnight CST
(BIXB) Bixby — Nearest City: 2.0 NE Bixby — County: Tulsa
Latitude: 35-57-46 — Longitude: 95-51-58 — Elevation: 604 feet

DAY	TEMPERATURE (F) MAX	MIN	AVG	DEWPT	DEG DAYS HDD	CDD	HUMIDITY (%) MAX	MIN	AVG	RAIN (in)	PRESSURE (in) STN	MSL	WIND DIR	SPEED (mph) AVG	MAX	SOLAR (MJ/m2)	4" SOIL TEMPERATURES SOD	BARE	MAX	MIN
1	38	23	31.1	12.7	34	0	88	25	49	0.00	30.00	30.66	NW	10.1	34.9	11.98	41.1	35.6	37	35
2	30	15	22.1	9.4	42	0	91	33	57	0.00	30.20	30.86	N	6.1	19.5	12.09	39.7	34.3	35	34
3	41	14	28.2	12.4	37	0	93	26	55	0.00	29.91	30.57	NA	12.4	30.2	10.12	38.8	33.4	34	33
4	56	30	44.1	32.8	22	0	78	51	65	0.00	29.49	30.15	S	17.0	38.2	8.18	39.8	33.8	38	33
5	72	53	61.8	48.8	2	0	84	41	64	0.00	29.17	29.82	S	14.7	33.8	10.34	43.5	45.1	50	38
6	74	61	66.4	53.3	0	3	76	46	64	0.00	29.14	29.78	SSW	15.9	32.4	10.29	47.0	51.6	55	49
7	74	58	67.0	57.2	0	1	88	51	71	0.15	29.13	29.77	S	13.4	31.0	4.01	49.6	54.7	57	53
8	67	29	45.7	40.7	17	0	99	57	93	0.76	29.32	29.97	NA	7.9	36.8	4.95	50.0	50.6	55	44
9	55	25	40.3	31.8	25	0	100	36	75	0.00	29.36	30.01	NA	7.6	24.7	12.17	46.8	42.8	47	39
10	49	36	43.0	31.4	23	0	85	42	64	0.00	29.13	29.78	WNW	10.1	31.9	4.22	46.3	42.0	44	39
11	57	22	38.6	26.3	25	0	97	27	66	0.00	29.25	29.89	NA	5.1	18.3	11.76	44.8	40.1	45	37
12	51	30	38.9	30.6	25	0	92	49	73	0.00	29.35	30.00	NA	4.5	17.4	5.02	44.3	39.5	43	37
13	45	25	33.5	25.5	30	0	92	45	74	0.00	29.58	30.24	NA	4.4	13.7	12.26	43.5	39.0	44	36
14	52	24	35.7	28.3	27	0	98	39	78	0.00	29.64	30.29	NA	3.0	11.6	12.45	42.6	39.1	45	36
15	62	23	41.0	27.0	23	0	98	21	65	0.00	29.42	30.07	NA	7.0	25.2	12.48	42.5	39.4	44	36
16	47	29	43.3	35.7	27	0	97	49	76	0.14	29.27	29.91	SE	10.0	25.9	2.14	43.9	41.2	43	40
17	34	20	26.1	17.0	38	0	90	41	69	0.00	29.48	30.14	NA	7.4	27.6	11.96	42.7	39.0	41	36
18	49	19	31.2	19.1	31	0	85	31	64	0.00	29.55	30.20	NA	8.3	29.0	11.78	40.8	35.9	38	35
19	28	15	20.9	7.8	43	0	78	34	58	0.00	29.81	30.47	N	8.0	24.1	11.99	39.8	34.5	36	34
20	43	15	29.6	12.8	36	0	79	26	53	0.00	29.73	30.39	SSE	12.0	27.9	13.15	38.8	33.5	34	33
21	37	29	33.1	24.0	32	0	98	49	71	0.06	29.60	30.25	SE	10.3	21.4	1.59	39.2	33.3	33	33
22	34	16	24.6	17.2	40	0	98	46	74	0.01	29.70	30.35	NA	6.9	22.7	11.79	39.2	33.3	33	33
23	42	13	27.3	14.4	38	0	95	29	63	0.00	29.57	30.22	NA	5.7	29.4	13.28	38.1	33.1	33	33
24	30	13	22.1	5.5	44	0	76	25	51	0.00	29.80	30.45	N	8.2	24.3	13.32	37.6	32.8	33	33
25	37	23	32.4	24.8	35	0	97	51	74	0.00	29.63	30.29	S	10.3	24.6	2.79	37.6	32.7	33	33
26	43	24	34.3	32.9	31	0	100	76	95	0.00	29.56	30.22	NA	4.9	16.4	3.85	39.0	32.8	33	33
27	62	24	40.2	29.7	22	0	100	25	73	0.00	29.53	30.18	NA	7.2	22.1	11.54	39.5	35.4	40	33
28	61	48	53.7	45.6	10	0	89	55	75	0.00	29.08	29.72	S	17.0	39.8	3.03	41.8	42.2	48	38
29	64	22	43.5	31.6	22	0	86	26	64	0.00	29.11	29.75	SSW	12.0	42.9	11.05	43.9	45.6	49	38
30	50	20	35.7	14.4	30	0	87	17	49	0.00	29.31	29.95	SSE	12.6	38.1	14.36	41.2	36.9	39	35
31	40	25	34.0	27.8	33	0	99	44	90	0.10	29.30	29.95	ESE	10.3	26.1	2.35	41.1	35.6	37	35
	49	27	37.7	26.7	<- Monthly Averages ->						29.49	30.14	NA	9.4	42.9	9.11	42.1	38.6	41	37

Temperature - Highest: 74
Lowest: 13

Degree Days - Total HDD: 845
Total CDD: 4

Number of Days With:
Tmax ≥ 90: 0
Tmax ≤ 32: 3
Tmin ≤ 32: 26
Tmin ≤ 0: 0
Rainfall ≥ 0.01 inch: 6
Rainfall ≥ 0.10 inch: 4
Avg Wind Speed ≥ 10 mph: 15
Max Wind Speed ≥ 30 mph: 11

Rainfall: Monthly Total: 1.22 in.
Greatest 24 Hr: 0.76 in.

Humidity - Highest: 100
Lowest: 17

* Denotes incomplete record

monthly data generated on wednesday, april 01, 2009 at 14:14 utc

Oklahoma Mesonet Climatological Data Summary for Bixby in May 2008

MESONET CLIMATOLOGICAL DATA SUMMARY — May 2008 — Time Zone: Midnight-Midnight CST
(BIXB) Bixby — Nearest City: 2.0 NE Bixby — County: Tulsa
Latitude: 35-57-46 — Longitude: 95-51-58 — Elevation: 604 feet

DAY	TEMPERATURE (F) MAX	MIN	AVG	DEWPT	DEG DAYS HDD	CDD	HUMIDITY (%) MAX	MIN	AVG	RAIN (in)	PRESSURE (in) STN	MSL	WIND SPEED (mph) DIR	AVG	MAX	SOLAR (MJ/m2)	4" SOIL TEMPERATURES SOD	BARE	MAX	MIN
1	83	67	74.0	61.1	0	10	76	49	65	0.00	28.86	29.50	S	19.1	43.0	19.42	61.5	69.7	77	64
2	75	51	63.9	41.3	2	0	93	22	48	0.69	29.02	29.67	WNW	11.5	48.5	28.86	62.4	67.0	72	61
3	66	44	54.2	33.8	10	0	91	23	49	0.00	29.40	30.05	NA	7.4	27.3	29.28	60.9	60.9	68	55
4	74	38	57.1	40.5	9	0	98	23	62	0.00	29.45	30.10	NA	2.5	11.1	28.75	59.6	60.7	70	51
5	82	47	66.0	52.6	1	0	95	39	66	0.00	29.41	30.06	NA	5.3	18.3	27.60	60.6	64.2	73	55
6	76	62	66.9	62.0	0	4	94	65	85	0.09	29.24	29.89	SSE	9.7	25.7	11.68	62.2	65.9	70	63
7	68	59	64.3	62.3	2	0	96	86	93	2.38	28.96	29.60	ESE	8.4	42.2	1.75	62.7	64.6	66	63
8	77	61	67.6	59.0	0	4	96	51	76	0.01	29.02	29.66	NNW	7.4	24.7	24.67	63.6	67.4	75	62
9	79	59	68.7	58.0	0	4	96	41	71	0.00	29.11	29.75	N	5.7	18.8	25.31	65.0	69.0	76	63
10	86	55	68.4	57.1	0	6	95	35	70	0.00	29.04	29.69	NW	11.8	39.6	16.17	64.7	66.6	73	63
11	69	47	56.5	35.8	7	0	94	23	50	0.00	29.37	30.02	NA	8.4	36.2	30.09	63.5	62.5	70	56
12	77	42	63.2	43.5	5	0	97	26	55	0.00	29.21	29.85	NA	11.7	31.8	28.85	61.8	61.4	68	54
13	79	66	71.8	62.4	0	7	91	55	73	0.02	29.11	29.76	S	12.6	27.0	11.58	63.1	65.2	70	61
14	66	56	60.8	52.3	4	0	92	54	74	0.36	29.30	29.95	NNE	8.3	22.8	9.90	63.2	63.4	66	61
15	70	52	60.4	52.6	4	0	95	50	77	0.37	29.30	29.95	NA	8.5	24.6	18.31	62.4	62.5	67	59
16	77	47	63.0	50.8	3	0	97	33	70	0.00	29.41	30.06	NA	2.7	11.8	29.44	62.6	65.0	75	56
17	84	51	69.5	52.1	0	3	95	29	59	0.00	29.22	29.87	NA	5.4	17.1	29.54	63.7	67.0	75	59
18	85	58	72.1	50.1	0	6	93	21	52	0.00	29.13	29.78	NA	4.8	17.4	29.71	64.9	68.8	76	62
19	94*	57*	76.6*	NA	0*	10*	NA	NA	NA	0.00*	29.00*	29.65*	NA	7.2*	NA	26.31*	65.8*	69.5*	77*	62*
20	81	58	72.4	NA	0	5	NA	NA	NA	0.00	29.16	29.81	NA	7.3	22.7	26.01	66.5	70.2	74	66
21	79	53	68.2	NA	0	1	NA	NA	NA	0.00	29.05	29.69	NA	8.6	26.0	23.35	65.4	67.5	73	62
22	85	68	76.4	NA	0	12	NA	NA	NA	0.00	28.90	29.54	SE	14.4	29.8	17.10	66.6	70.2	75	66
23	83	70	75.8	NA	0	12	NA	NA	NA	0.00	29.07	29.71	SE	14.0	29.1	14.82	68.0	71.9	76	69
24	86	68	77.4	NA	0	12	NA	NA	NA	0.00	29.22	29.87	SE	11.7	28.1	17.52	68.9	73.4	78	70
25	90	74	80.8	NA	0	17	NA	NA	NA	0.00	29.21	29.85	S	9.5	23.8	22.75	70.0	76.2	82	71
26	87	63	75.4	NA	0	10	NA	NA	NA	2.44	29.20	29.85	SSE	10.2	33.9	21.15	69.8	74.9	80	69
27	80	65	71.3	NA	0	7	NA	NA	NA	1.72	29.28	29.93	NA	7.5	37.0	16.40	70.2	73.3	78	70
28	84	68	74.3	NA	0	11	NA	NA	NA	0.00	29.45	30.11	NA	4.5	12.2	20.51	71.2	75.0	81	70
29	84*	67*	74.5*	NA	0*	11*	NA	NA	NA	0.00*	29.39*	30.04*	SSE*	10.3*	NA	22.20*	72.3*	75.1*	80*	71*
30	87	71	79.3	69.2	0	14	92	52	73	0.00	29.23	29.88	S	12.3	28.7	27.09	73.3	76.0	81	72
31	87	66	77.7	69.2	0	12	95	57	76	1.49	29.26	29.91	SSE	9.1	33.5	22.74	72.8	75.9	81	72
	80*	58*	69.3*	53.3*	<- Monthly Averages ->						29.19*	29.84*	NA	9.0*	48.5*	21.90*	65.5*	68.4*	74*	63*

Temperature - Highest: 94*
Lowest: 38*

Degree Days - Total HDD: 47*
Total CDD: 177*

Number of Days With:
Tmax ≥ 90: 1* — Rainfall ≥ 0.01 inch: 10*
Tmax ≤ 32: 0* — Rainfall ≥ 0.10 inch: 7*
Tmin ≤ 32: 0* — Avg Wind Speed ≥ 10 mph: 11*
Tmin ≤ 0: 0* — Max Wind Speed ≥ 30 mph: 9*

Rainfall: Monthly Total: 9.57* in.
Greatest 24 Hr: 2.44* in.

Humidity - Highest: 98*
Lowest: 21*

* Denotes incomplete record

monthly data generated on wednesday, april 01, 2009 at 14:14 utc

Oklahoma Mesonet Climatological Data Summary for Bixby in June 2008

MESONET CLIMATOLOGICAL DATA SUMMARY — June 2008 — Time Zone: Midnight-Midnight CST
(BIXB) Bixby — Nearest City: 2.0 NE Bixby — County: Tulsa
Latitude: 35-57-46 — Longitude: 95-51-59 — Elevation: 604 feet

DAY	TEMPERATURE (F) MAX	MIN	AVG	DEWPT	DEG DAYS HDD	CDD	HUMIDITY (%) MAX	MIN	AVG	RAIN (in)	PRESSURE (in) STN	MSL	WIND SPEED (mph) DIR	AVG	MAX	SOLAR (MJ/m2)	4" SOIL TEMPERATURES SOD	BARE	MAX	MIN
1	82	65	74.3	68.5	0	9	95	62	83	0.83	29.27	29.92	ESE	8.1	55.5	14.61	72.7	74.3	78	71
2	88	69	79.8	69.7	0	14	93	52	70	0.00	29.17	29.81	S	13.8	32.9	28.53	73.7	75.8	81	71
3	89	77	83.0	69.7	0	18	76	51	63	0.00	28.96	29.60	SSW	15.0	33.2	28.33	74.8	76.7	81	73
4	88	77	82.3	69.7	0	18	74	53	64	0.00	28.87	29.52	S	16.7	37.3	25.90	75.1	76.5	81	73
5	89	69	82.7	69.1	0	13	95	53	64	0.52	28.91	29.55	S	22.3	62.0	20.13	74.9	76.0	79	73
6	86	69	78.7	72.0	0	13	94	67	80	0.18	29.14	29.78	SSW	9.6	35.5	18.42	75.0	76.5	81	72
7	88	78	82.1	71.3	0	18	82	55	71	0.00	29.22	29.87	S	15.2	32.1	21.34	75.7	77.4	81	75
8	88	67	81.2	70.3	0	13	93	55	70	0.02	29.18	29.83	S	14.3	33.5	19.10	75.8	77.0	80	74
9	70	59	65.2	63.0	1	0	98	78	93	2.37	29.27	29.92	NA	7.1	32.9	4.15	72.5	70.7	76	68
10	83	59	71.6	66.0	0	6	98	65	84	0.01	29.29	29.94	SSE	8.8	25.6	26.57	71.8	NA	NA	NA
11	88	71	79.4	69.9	0	14	92	58	74	0.00	29.14	29.79	SSE	16.0	33.8	27.87	74.0	NA	NA	NA
12	88	76	81.5	69.5	0	17	78	55	68	0.00	29.17	29.81	S	15.5	32.7	26.78	75.0	NA	NA	NA
13	78	69	72.9	67.5	0	8	95	75	84	0.00	29.31	29.96	NNE	7.3	20.0	8.36	74.5	NA	NA	NA
14	86	63	75.2	68.3	0	10	98	59	81	0.00	29.34	29.99	SSE	6.0	16.5	29.51	74.3	NA	NA	NA
15	91	72	80.8	72.5	0	16	95	60	77	0.00	29.23	29.88	SSE	9.1	26.5	25.40	75.5	NA	NA	NA
16	76	65	68.5	65.2	0	5	96	73	89	2.57	29.35	30.00	N	9.0	41.0	4.21	73.1	NA	NA	NA
17	80	63	70.6	66.0	0	7	98	59	86	0.13	29.41	30.06	NA	4.0	12.6	16.80	72.4	NA	NA	NA
18	85	66	73.6	67.3	0	10	97	52	83	0.91	29.31	29.96	NA	4.0	25.2	23.22	73.1	NA	NA	NA
19	87	68	77.5	68.9	0	13	97	48	77	0.00	29.28	29.93	NA	4.8	15.6	26.90	74.4	NA	NA	NA
20	85	69	76.8	68.8	0	12	96	51	78	0.00	29.34	29.98	NA	4.5	16.8	24.82	75.4	NA	NA	NA
21	89	65	77.7	64.7	0	12	98	37	69	0.00	29.44	30.09	NA	2.5	16.1	30.50	75.4	NA	NA	NA
22	92	64	79.1	66.8	0	13	98	39	70	0.00	29.35	30.00	NA	4.3	14.3	29.36	75.6	NA	NA	NA
23	87*	67*	76.9*	68.4*	0*	12*	93*	57*	76*	0.28*	29.34*	29.99*	S *	9.1*	NA	18.55*	75.5*	NA	NA	NA
24	90*	71*	79.7*	69.1*	0*	16*	92*	44*	69*	0.00*	29.40*	30.05*	S *	9.3*	NA	26.31*	75.8*	78.0*	84*	73*
25	90	71	80.6	71.1	0	15	92	58	74	0.00	29.37	30.02	S	9.2	20.8	27.22	76.2	79.1	85	74
26	89	71	81.2	70.8	0	15	88	58	72	0.00	29.28	29.93	S	10.6	22.7	27.95	76.9	80.3	86	75
27	87	71	79.0	71.0	0	14	94	66	77	0.18	29.22	29.87	S	10.2	28.1	16.93	76.7	79.1	82	76
28	83	71	76.8	70.7	0	12	96	71	82	0.05	29.29	29.94	NA	8.0	30.0	12.30	76.6	77.2	80	75
29	84	63	74.9	59.2	0	9	98	30	64	0.01	29.45	30.10	NA	5.0	20.2	30.75	75.8	76.9	83	72
30	88	59	74.4	57.7	0	8	98	27	63	0.00	29.46	30.11	NA	2.8	20.8	30.75	74.9	77.3	86	69
	86*	68*	77.3*	69.0*	<- Monthly Averages ->						29.26*	29.91*	S *	9.4*	62.0*	22.39*	74.8*	76.7*	81*	73*

Temperature - Highest: 92*
Lowest: 59*

Degree Days - Total HDD: 1*
Total CDD: 358*

Number of Days With:
Tmax ≥ 90: 2*
Tmax ≤ 32: 0*
Tmin ≤ 32: 0*
Tmin ≤ 0: 0*
Rainfall ≥ 0.01 inch: 13*
Rainfall ≥ 0.10 inch: 9*
Avg Wind Speed ≥ 10 mph: 10*
Max Wind Speed ≥ 30 mph: 13*

Rainfall: Monthly Total: 8.06* in.
Greatest 24 Hr: 2.57* in.

Humidity - Highest: 98*
Lowest: 27*

* Denotes incomplete record

monthly data generated on wednesday, april 01, 2009 at 14:16 utc

VITA

Jessica Ong

Candidate for the Degree of

Master of Science

Thesis: ASSESSING THE RISK OF IN-FIELD MICROBIAL CONTAMINATION OF LEAFY GREENS USING INOCULATED SOIL

Major Field: Food Science

Biographical:

Personal Data:
Born in Jakarta, Indonesia on April 24th, 1984.

Education:
Graduated from Stillwater High School, Stillwater, Oklahoma in May 2001.
Earned Bachelor of Science in Nutrition at Oklahoma State University, Stillwater, Oklahoma in December 2006.
Completed the requirements for the Master of Science in Food Science at Oklahoma State University, Stillwater, Oklahoma in July, 2009

Experience:

Professional Memberships:
Institute of Food Technologists Association (2008-2009)
Phi Kappa Phi Honor Society (2008-2009)
Golden Key International Honor Society (2008-2009)
Gamma Sigma Delta, the Honor Society in Agriculture (2008-2009)

Name: Jessica Ong Date of Degree: July, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: ASSESSING THE RISK OF IN-FIELD MICROBIAL CONTAMINATION OF LEAFY GREENS USING INOCULATED SOIL

Pages in Study: 80 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope and Method of Study:

The objective of the study was to investigate the survival of non-pathogenic *Escherichia coli* in soils using different inoculation methods and to determine whether the microorganism would be transmitted to edible parts of the plants when planted in contaminated soils. Different field studies were conducted starting fall 2007 through early spring 2009 at Oklahoma State University Vegetable Research Station in Bixby, Oklahoma. Treatments included soils applied with previously inoculated spent mushroom compost, wood shavings, and direct inoculation using water-bacteria mixture sprayed to soils with tilling and without tilling. Two different variety of leafy greens were planted in treated soils include Top White Globe turnip greens and spinach variety Padre. Soil samples were collected in weekly and monthly basis for all experiments and analyzed for *E. coli* populations using direct plating method. Ground leaves of turnip greens and spinach were also analyzed for presence of *E. coli* with and without surface sterilization.

Findings and Conclusions:

Results from different experiment suggested that media used for inoculation, season variability and inoculation methods to land influence survival rate of *E. coli* in soils. *E. coli* survived better in winter season than in spring due to temperature differences and moisture in soils. *E. coli* was able to survive in soils for at least 161 days in soils amended with inoculated wood shavings, whereas spraying of water bacteria mixture to soils had the lowest survival of *E. coli*. Flooding over green turnip crops caused transmission of *E. coli* from contaminated soils to leaves although the site of attachment was not determined. *E. coli* was not recovered from any spinach leaf samples. However, the maximum amount of sample assayed was very small. Therefore, future studies should include assay of larger amount of spinach sample.

ADVISER'S APPROVAL: Dr. William McGlynn