DISSEMINATION OF *ESCHERICHIA COLI* O157:H7 TO THE SPINACH, *SPINACEA OLERACEAE* PHYLLOPLANE BY HOUSE FLIES, *MUSCA DOMESTICA* L.

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DISSEMINATION OF *ESCHERICHIA COLI* O157:H7

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CHAPTER I

INTRODUCTION

Since 1980, the number of food borne disease outbreaks related to the consumption of raw vegetables and ready-to-eat food products have increased. Many of these outbreaks are primarily due to the contamination of fresh fruits, vegetables and food products with enteric microbial pathogens. Bacterial pathogens include *Escherichia coli* O157:H7, *Campylobacter spp., Salmonella enterica,* and *Listeria monocytogens*. Two of these pathogens, *Salmonella* and *E. coli* O157:H7 have been found to survive in the plant phyllosphere. How they came in contact with plants has been to subject of much debate. One possible route of contamination is via insects which may move pathogens from bacteria-laden manure, compost or water to plants (Brandl, 2006). Insects have long been associated with bacterial contamination of food sources. Some of the filth flies have been documented to act as mechanical vectors of human pathogens and have caused food-borne diseases in humans (Moriya et al., 1999; Iwasa et al., 1999).

In 2006, a multistate outbreak of *E. coli* O157:H7 was reported and linked with the consumption of contaminated bagged spinach from a commercial brand sold in supermarkets. As a result of this 2006 outbreak, a funding effort by Fresh Express

Inc. simulated research to study the possible pathways of fresh produce contamination. In one of these studies it was observed that large numbers of filth flies (e.g. blow flies and house flies) were associated with lettuce in the field. Some collected fly samples were PCR positive for *E. coli* O157:H7 (Talley et al., 2009). In addition, large numbers of fly specks (regurgitation and defecation spots) were observed on the field lettuce. It was speculated that the fly specks or body parts that come in contact with the leaf surface may serve as sources of bacterial contamination (Talley et al., 2009).

In the Salinas Valley where the majority of spinach contamination in California by *E. coli* O157:H7 has occurred, vegetable production areas are surrounded by the coastal range mountains. These foothill areas are good for fly development because of the presence of range cattle. Although flies are well known vectors of bacteria to prepared food, the potential of filth flies to contaminate fresh produce with *E. coli* O157:H7 is not well understood. The relationship between *E. coli* O157:H7, flies, and spinach was the focus of this research. The filth fly selected to be the subject of this study is the house fly, *Musca domestica* L., because its life cycle can occur on cattle manure, a potential source of *E. coli* O157:H7 acquisition by flies as documented in Talley et al. (2009). This study is a continuation of that work and focuses on whether these bacteria can colonize and survive in the plant phylloplane after fly regurgitation and on the fly external body surfaces. The objectives of this study are to:

1. Determine if *E. coli* O157:H7 colonizes the spinach phylloplane following regurgitation by house flies on the leaf surface after acquisition of *E. coli* O157:H7 from various contaminated sources.

2. Determine if *E. coli* O157:H7 colonizes the external body surfaces (labellae and tarsi) of house flies after contact with various *E. coli* O157:H7 contaminated sources.

CHAPTER II

REVIEW OF LITERATURE

Food borne diseases

Most food borne diseases of humans are related to the contamination of food by enteric microbial pathogens. *Salmonella enteritidis, Campylobacter* spp., *Listeria monocytogens, Escherichia coli* O157:H7 and *Shigella* spp. are some of the bacterial pathogens that are associated with food borne disease outbreaks. Most of the reported food borne disease outbreaks are related to the consumption of undercooked meat, raw vegetables, salads, fresh fruits, and fruit juices. According to the data from food borne outbreak surveillance from 1973 to 1997, 190 produce-associated outbreaks have been reported and the most common food items implicated were salads, lettuce, fresh juices, berries, melons and sprouts. Of the outbreaks in which the pathogen was identified, 60% were caused by bacteria and of these 48% were caused by *Salmonella* spp. *E. coli* serotype O157:H7 was recognized as a pathogen during this time period (Sivapalasingham et al., 2004). According to CDC surveillance summaries of food borne diseases, during the period from 1998 to 2002, the number of reported outbreaks ranged from 1243 to 1417 per year (Lynch et al., 2006). Thirty-three percent of the outbreaks had a known etiological agent and 55% of those were caused by bacterial pathogens.

There are several reasons for the increased number of food borne disease outbreaks, including the increased popularity of minimally processed food, increased importation of fresh produce, increased consumption of fresh fruits, vegetables and salads and emergence of pathogens having low infectious doses (Tauxe et al., 1997). Contamination of produce prior to harvesting and during processing (Brandl, 2006) is also thought to enhance the occurrence of outbreaks. Brandl, (2006) illustrated multiple pathways by which produce can be contaminated in the field. Even though manure is a good source of fertilizer in organic farming practices, the application of improperly composted manure could serve as a potential source of enteric pathogens in the field. Insects often associated with these manure and compost piles could act as vectors in the movement of these pathogens to produce in the field. If pathogens internalize in the plant they could contaminate the seeds and increase their potential of dispersion. Application of contaminated water for irrigation as well as application of pesticides with contaminated water could serve as another method of contamination of produce in the field (Brandl, 2006).

E. coli O157:H7 disease outbreaks

E. coli O157:H7 was the causal agent of several of the largest outbreaks of enteric bacterial diseases in recent times (Brandl, 2006). Infection with *E. coli* O157:H7 was first recognized in 1982, associated with the consumption of contaminated hamburgers. In this case 47 people in Michigan and Oregon developed bloody diarrhea (Su and Brandt, 1995). The largest outbreak reported for this pathogen was in 1996 in Japan, where over 6300 school children were affected and two deaths were reported due to consumption of contaminated radish sprouts (WHO 2002; Brandl, 2006). In the United States, a

multistate outbreak of *E coli* O157:H7 infections associated with consumption of mesclun during May 28 to June 27, 1996 (Hilborne et al., 1999) was the first reported outbreak of *E. coli* O157:H7 infection associated with consumption of lettuce. This outbreak resulted in illness of 61 people, with 21 hospitalizations and three cases of hemolytic-uremic syndrome (Hilborne et al., 1999). In the period 1982 to 2002, 350 *E. coli* O157 outbreaks were reported from 49 states in United States (Rangel et al., 2005), of which 21% were produce-associated. Of this 21%, 34% were from lettuce, 18% from apple cider or apple juice, 16% from salad, 11% from coleslaw, 11% from melons, 8% from sprouts, and 3% from grapes (Rangel et al., 2005). During the period 1990 - 2004 *E. coli* O157:H7 was shown to be the causal agent of 48% of the food borne disease outbreaks associated with leafy vegetables, which consisted mostly of lettuce (Brandl, 2006).

In a 2006 *E. coli* O157:H7 outbreak, associated with consumption of contaminated bagged baby spinach, 205 illnesses and three deaths were reported. The same strain of *E. coli* O157:H7 that affected people was found in river water, cattle feces and wild pig feces. It was suggested that wild pig and cattle feces might have contaminated the waterways, resulting in the contamination. However the exact source of spinach contamination was not determined (http://www.marlerclark.com/ 2006 _Spinach_Report_Final_01.pdf).

Characteristics and evolution of *E. coli* O157:H7

Shiga toxin producing *E. coli* (STEC) is one of the pathotypes of *E. coli* that cause enteric diseases in humans. STECs that are responsible for causing hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans are known as enterohemorrhagic *E. coli* (EHEC) (Gyles, 2006). *E. coli* O157:H7 belongs to the EHEC class and produces the shiga-like toxins *stx*1 or *stx*2, also known as vero toxins VT1 and VT2 (Buchanan & Doyle, 1997). These toxins have a close resemblance to a cytotoxin produced by *Shigella dysenteriae* type I (Feng, 1995), which is cytotoxic to vero cells (Konowalchuck et al., 1977), derived from kidney epithelial cells of the African green monkey (Su and Brandt, 1995).

Shiga toxin has five B subunits and a single A subunit. The A subunit cleaves ribosomal RNA and disrupts protein synthesis (Gyles, 2006). The bacterium attaches to the host's colon epithelial cells and induces an effacing lesion (A/E lesion). The attachment is enhanced by a protein known as intimin, encoded by the *eae* gene (Tarr and Bilge, 1998). Following attachment, the bacterium produces shiga toxin in the epithelial cells of the colon. The toxin travels in the blood stream toward the kidney and damages renal endothelial cells, resulting in renal inflammation followed by hemolytic anaemia or acute renal failure, which ultimately leads to HUS and HC (Kaper et al., 2004).

E. coli O157:H7 is differentiated from other pathogenic *E. coli* in that is sorbitol negative, while 93% of all *E. coli* ferment sorbitol (Buchanan & Doyle, 1997). It does not hydrolyse MUG (4-methylumbelliferyl-β-D-glucuronide), does not grow at or above 44°C in culture and survives in lower temperatures (8°-10° C) (Buchanan and Doyle, 1997). Because of its high tolerance for acidic conditions and due to this *E. coli* O157:H7

disease cases are reported in association with fermented sausages, mayonnaise, apple juice and apple cider. Bacterial survival increases at low pH and low temperature conditions (Buchanan & Doyle, 1997). *E. coli* O157:H7 belongs to seropathotype A, the most virulent seropathotype of the STEC (Gyles, 2006). It is hypothesized that *E. coli* obtained the shiga toxin gene from *Shigella* via a bacteriophage during a pandemic that occurred in Central America in 1970s (Peacock et al., 2001).

The evolution of *E. coli* O157:H7, as proposed by Whittam, 1998, begins with an EPEC (enteropathogenic *E. coli*) like ancestor with the ability to express β-glucuronidase (GUD+) and ferment sorbitol (SOR+). This ancestral form gave rise to the O55:H7 from which had the pathogenicity island LEE (locus of enterocyte effacement). This pathogenecity island encodes for proteins, including intimin, that mediate bacterial attachment and subsequent production of attaching and effacing lesions (A/E). The transition of O55:H7 with GUD+, SOR+ form acquired the *stx*2 gene via a toxin-converting bacteriophage with *stx*2 and gave rise to O55:H7 with GUD+, SOR+ and *stx* 2 + form. The divergence from O55:H7 to O157:H7 was mainly due to the change in the somatic antigen change from O55 to O157 which was assumed to have occurred as a result of a lateral transfer and recombination of genes. From O157:H7 GUD+, SOR+ *stx* 2 + form, two distinct lines evolved. One line lost mobility but retained the *stx*2, GUD+ and SOR+ type. The other lineage lost the ability to ferment sorbitol but retained GUD+ and acquired stx1 via phage conversion. Subsequently this form lost the GUD activity, giving rise to a SOR-, GUD-, *stx*1+, and *stx*2+ strain, which is considered to be the immediate ancestor of the current O157:H7 form (Whittam, 1998).

Reservoirs of *E. coli* O157:H7

Ruminants, especially cattle, are major reservoirs of STEC and cattle-associated products harvested for human consumption are prone to be contaminated with these bacteria (Gyles, 2006). *E. coli* O157:H7 inhabits 6-9% of the range cattle and about 8.3% of the dairy cattle in the northern United States (Islam et al., 2004). *E. coli* O157:H7 can survive, replicate and move within the soil, and the presence of manure in the soil enhances its survival (Islam et al., 2004). After application of compost artificially contaminated with *E. coli* O157:H7 to the soil, bacteria persisted for 154-217 days. When lettuce and parsley was grown in that soil bacteria were detected on plant surfaces for up to 77-177 days (Islam et al., 2004). In cattle feces the concentration of *E. coli* O157:H7 can be as high as $4\text{-}10^7$ cfu/g, but generally between 10-100 cfu/g (Gyles, 2006). The bacteria are localized in the gastrointestinal tract, within the fore-stomach as well as distal sites. Deer and sheep also are carriers of *E. coli* O157:H7 (Buchanan & Doyle, 1997). Water also serves as a source for *E. coli* O157:H7 infections. In December 1989, a large *E. coli* O157:H7 outbreak in Missouri was associated with contamination of municipal water (Swerdlow et al., 1992).

***E. coli* O157:H7 infection in humans**

Symptoms of human infection with *E. coli* O157:H7 range from asymptomatic to severe and can include a variety of complications ranging from non bloody diarrhea to fatality. Bacteria that enter the human body with the consumption of contaminated food or water colonize in the intestine. The EHEC bacteria are highly acid tolerant and establish in the intestine (Gyles, 2006). *E. coli* O157:H7 has a very low infectious dose of between 50-100 cells (USDA-APHIS, 1997). Often reported symptoms are severe

abdominal cramps, mild or no fever, and watery to bloody diarrhea. There is a 3-4 day incubation period for this disease and diarrhea becomes bloody within two days (Su and Brandt, 1995). Other complications include the HUS, thrombotic thrombocytopenic purpura (TTP) and death. Hemorrhagic colitis was first reported in 1971, and after the 1982 outbreaks in Oregon and Michigan, *E. coli* O157:H7 became recognized for its potential to cause HC (Su and Brandt, 1995).

HUS, which is characterized by microangiopathic hemolytic anemia, acute renal failure and thrombocytopenia (Wachsmuth et al., 1991), can occur after seven days of gastrointestinal symptoms. Characteristic symptoms are pallor, intravascular destruction of red blood cells (microangiopathic hemolytic anemia), depressed platelet counts (thrombocytopenia), lack of urine formation (oligo-anuria), and acute renal failure (Buchnan and Doyle, 1997). Among survivors of diarrhea-associated HUS there is a significant increase in incidence of diabetes mellitus due to complete insulin deficiency (Suri et al., 2009). TTP symptoms are similar to those of HUS, but also includes fever and neurological abnormalities, but the distinction between HUS and TTP is not clear (Su and Brandt, 1995). Although kidneys are the main targets, many other organs (lungs, pancreas and heart) can also be affected. Children under five years of age and elderly people are more prone to severe complications with these diseases (Kaper, 2004).

Diagnostic methods

Early diagnosis is important in preventing the development of HUS and reducing associated mortality. For diagnosis, a patient's stool specimens are cultured on sorbitol MacConkey agar (SMAC) (Peacock et al., 2001) within 4-7 days after onset of illness

and prior to antibiotic exposure. *E. coli* O157:H7 ferments sorbitol slowly and develops colorless colonies within 24 hours (Peacock et al., 2001). These colonies can be further tested with commercially available O157 antisera or latex agglutination kits. SMAC has been modified with the antibiotics cefixime and tellurite for further specificity in identification (Mead et al., 1998). Another method for diagnosis of *E. coli* O157:H7 is the detection of shiga-like toxins, done with tissue culture assays using HeLa or vero cells (Su and Brandt, 1995). Toxins can be done also by using genetic probes and immune-specific assays. The immuno-specific assays, include ELISA (enzyme linked immunosorbent assays) with the use of monoclonal or polyconal antibodies against *stx* 1 or 2 (Su and Brandt, 1995). PCR amplification of the toxin genes is a reliable technique that allows the detection of low numbers of bacterial cells. Serological tests are also used to detect antibodies specific to the shiga-like toxins or O157 lipopolysaccharides (Su and Brandt, 1995).

No specific treatment is currently available for *E. coli* O157:H7 infections. Supportive therapy, including hydration and management of anemia and renal failure, is important (Centers for Disease Control and Prevention, 2008). Use of antibiotics can actually increase the risk of HUS development due to the elimination of natural bowel flora and release of the shiga toxins with the lysis of the *E. coli* O157:H7 cells due to antibiotic therapy (Su and Brandt, 1995). Treatment of diarrhea with colon antimotility drugs may increase the risk by allowing more time for the absorption of the toxin (Su and Brandt, 1995). Some *E. coli* O157:H7 isolates are resistant to erythromycin, metronidazole, vancomycin and tetracycline (Su and Brandt, 1995). CDC recommends thorough hand washing after using the bathroom, changing diapers or contact with

animals, especially cattle, all of which minimizes the chance of bacterial entry via the oral route. Meats, especially beef and related products, should be cooked thoroughly before consumption. Swallowing of water in the swimming pools lakes and other recreation areas should be avoided as a precaution.

E. coli O157:H7 on fresh produce

When considering the epidemiology of food borne illness related to the consumption of fresh fruits and vegetables, the phyllosphere has become an "intermediate niche" (Brandl, 2006) for enteric pathogens that ultimately may reach the gut. Human pathogens are often shed in the feces of animals and carried with fertilizer, irrigation water, or run-off from live stock pastures, or transported by insects, to reach the plants (Tyler and Triplett, 2008). In humans enteric pathogens usually reside in the mammalian gut, and are acclimated to warm body temperatures. In contrast to the animal gut where there is an adequate supply of nutrients and moisture in an anaerobic environment, the plant phylloplane has limited amounts of nutrients and moisture and bacteria living there are exposed to high doses of UV radiation and variable temperatures (Brandl, 2006). They must either overcome these hurdles on the plant surface or find a way to enter into the plant tissues. To become established on the phylloplane, the bacteria must attach to the surface (Brandl, 2006) and different human pathogens use various adhesion methods to attach to the plant surface.

E. coli O157:H7 expresses type III secretion system (T3SS) genes for adherence to spinach and lettuce leaf surfaces (Shaw et al., 2008). EspA filaments encoded by these genes also play a role in bacterial attachment to the mammalian host in early stages of

infection (Shaw et al., 2008). In a study focused on biofilm formation of *E. coli* strains, Prigent-Combaret et al. (2000) showed that the *E. coli* K-12 strains formed thin coiled fimbriae called curli, on cover slips, enabling them to attach to the surface as well as to each other. These curli were confirmed as such by anti-curlin immunogold labelleing methods (Prigent-Combaret et al., 2000). Xicohtencatl-Cortes et al. (2009) documented that mutations of the genes for adhesin intimin (*eae*) and the flagella major subunit (*fli*C) reduced the colonization and leaf invasion capability of EHEC strains on spinach and lettuce.

E. coli O157:H7 cells were found 20-100 µm beneath the cut surface of lettuce, attaching to the cut surface rather than the leaf surface (Seo and Frank, 1999; Takeuchi et al., 2000). Solomon and Matthews (2002) suggested that *E. coli* O157:H7 could enter the lettuce plants through the root system after being applied with contaminated manure or irrigation water and migrate to the lettuce leaves. The leaf surface is covered by a water repellent cuticle, so bacteria that attach to the unwounded surface are able to form hydrophobic attachments with the plant surface. Because *E. coli* O157:H7 has a high anionic surface charge but low surface hydrophobic properties, bacterial attachment to wounded or cut surfaces on plant leaves is higher than that on intact surfaces (Matthews et al., 2002). Therefore good hygienic practices are required in handling and processing of leafy greens as plant lesions or tissue damage which can occur during handling can promote the multiplication of *E. coli* O157:H7. Hassan & Frank (2002) found that surfactants with low hydrophilic/lipophilic balance (HLB) could disrupt the hydrophobic interactions between *E. coli* O157:H7 and the lettuce leaf surface. Also this pathogen can

survive well under modified atmospheric conditions such as temperatures between 10-15°C (Brandl, 2008).

Investigating the effect of plant maturity on the surface contamination and internalization of *E. coli* O157:H7, Shuaihua et al. (2009) reported that bacterial internalization occurred rarely under green house conditions. Surface contamination occurred in plants of around three weeks old but not in five week old plants. On a plant leaf surface the micro environment is heterogeneous, with an uneven distribution of sugar, moisture, and other components. Therefore, on the same leaf surface there can be different microsites which with varying suitability as habitats for human pathogens (Brandl and Amundson, 2008). *E. coli* O157:H7 and *Salmonella* both tend to aggregate at cell junctions between epidermal cells, rather than associating with biofilm structures (Warriner and Namvar, 2010). Leaf age and nitrogen content also affects the growth of *E. coli* O157:H7 on pre-harvest and post harvest lettuce. *E. coli* O157:H7 and *Salmonella enterica* increase in number on younger lettuce leaves than on the older leaves over time after inoculation (Brandl and Amundson, 2008). The survival of *E. coli* O157:H7 is lower on the leaf surface than in the rhizosphere (Warriner and Namvar, 2010).

Human pathogens can enter a viable but non-culturable state (VBNC) in which they fail to grow on culture media but remain alive in the environment (Oliver, 2009). EHEC strains, *Salmonella* and *Shigella* show very low levels of metabolic activity while in this state. Lack of nutrition, temperature and osmotic fluctuations, oxygen concentrations, heavy metals and exposure to white light are factors that induce the

VBNC state (Oliver, 2009; Oliver, 2005). It is possible that human pathogens enter this state when introduced to the plant surface.

Association of house flies with dissemination of human pathogens

House flies have been reported as mechanical vectors of many enteric bacterial pathogens, such as *E. coli* O157:H7, *Shigella* spp, *Vibrio cholera*, *Salmonella* (De Jesus et al., 2003) and *Campylobacter* (Kapperud and Rosef, 1982). They carry *Campylobacter fetus* subsp. *jejuni* from poultry and pig farms to humans in Norway (Kapperud and Rosef, 1982). *Salmonella* and *Shigella* species were isolated from feral house flies in Uturu, Nigeria (Ugbogu et al., 2006). These insects are also mechanical vectors of some protozoans that affect human health; *Cryptosporidium parvum* oocytes were carried on house fly adult and larval stages that developed on contaminated bovine feces. Adult fly defecation spots also carried numerous *C. parvum* oocytes after contact with contaminated bovine feces (Graczyk et al., 1999).

The role of house flies as potential vectors of *Campylobacter* and *E. coli* O157:H7 in United States has been investigated by PCR screening (Szalanski et al., 2004). Flies collected from several cattle, poultry and pig farms in Japan were positive for *E. coli* O157:H7, and the isolated *E. coli* O157:H7 colonies were positive for *stx*1and *stx*2 genes in their virulence plasmid (Iwasa et al., 1999). In an outbreak of *E. coli* O157:H7 related bloody diarrhea in a nursery school in Japan in 1996, the pathogen was mechanically transmitted by house flies that had contacted cattle in the village (Moriya et al., 1999). House flies carry *E. coli* O157:H7 between animals and to the neighboring environment. In a northeastern Kansas cattle feedlot, house flies collected from feed bunks, feed

storage sheds were positive for the *stx*1, *stx*2, *eaeA* and *fliC* genes (Alam and Zurek, 2004). The transmission of *E. coli* O157:H7 to cattle was tested experimentally by Ahmad et al. (2007), who caged house flies fed with an antibiotic resistant *E. coli* O157:H7 strain with cattle and later isolated the same strain from the feces up to 11 days after exposure. The highest number of bacteria was observed in the recto-anal mucosa of the cattle (Alam and Zurek, 2007).

In the life cycle of the house fly, larvae consume bacteria which are necessary for their development. When larvae were fed artificially with *E. coli*, the average rate of bacterial survival in the larvae was 62% within 48 hrs after ingestion (Rochon et al., 2004). The pupae and emerging adult flies also were infected with *E. coli* (Rochon et al., 2005). Kobayashi et al. (1999) showed that after were house flies exposed to *E. coli* O157:H7 lawns, the bacteria could multiply within the pseudotracheae of the house fly labellum. *E. coli* O157:H7 survived in the insects' intestines and were excreted for three days after ingestion. The authors, hypothesizing that the relationship between the fly and bacteria was more than simple contamination, providing a hospitable environment of the bacteria to multiply on the body surface, coined a new term: "bioenhanced transmission" (Kobayashi et al., 1999).

House flies fed *E. coli* O157:H7 lawns and then allowed to contact different foods disseminated bacteria onto the food surfaces by excretion for 24 hours (Sasaki et al., 2000). When foods with the excreta spots were incubated at 29° C, the bacteria proliferated 10^2-10^5 fold, suggesting that even a small number of bacteria in house fly excreta can serve as a potential source of inoculum that could lead to disease in humans

(Kobayashi et al., 2002). House fly regurgitation could play a role in dissemination of enteric bacterial pathogens to fresh produce in a field. Talley et al. (2009) documented that house fly regurgitation is a potential mechanism of *E. coli* O157:H7 dissemination onto the spinach phylloplane under laboratory conditions. Sukontason et al. (2006) studied the ultrastructure of the pulvilli from different fly families by scanning and transmission electron microscopy. The "electron-lucent area" of the setae (the distal end) of house flies plays an important role not only in their attachment to surfaces but also could serve as an adhesive surface for attachment of microorganisms (Sukontason et al., 2006). T

Most of the research on house fly movement of human pathogens is related to mechanical transport. House fly association with contamination of fresh produce in a field setting has not been documented but few studies have been carried out to show the house fly association with the contamination of food. Macovei et al. (2008) showed that house flies collected from cattle feedlot, when exposed to several ready-to-eat food sources contaminated the foods with different *Enterococci* species. Sensory hairs on the fly body are efficient in trapping bacteria (Sukontason et al., 2006). Because the relationships between flies and bacterial colonization of plants are not well understood, this research was designed to explore some of the gaps in our understanding under experimental conditions.

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CHAPTER III

MATERIALS AND METHODS

Common Procedures:

House fly colony maintenance: House flies, *Musca domestica*, were reared in an isolated room within the Veterinary-Medical Entomology building, Oklahoma State University. Wild caught house flies from the OSU Dairy, Stillwater, were reared up to 30 plus generations. Eggs were collected as follows: five g of Calf-Manna® (Manna Pro Products, Chesterfield, MO) was positioned on a black cotton cloth (15 cm x 15 cm) and tied with a rubber band to form a cone shape. The cones were moistened with warm water and placed in 8 oz styrofoam cups with 200 ml of water in each inside 12x12x12”collapsible aluminum cages (Bioquip, Rancho Dominguez, CA) containing adult house flies.

Egg masses deposited on the outer surface of the cone were collected and transferred to a four L plastic tub containing two L of wheat bran and 400 g of Calf-Manna® mixed with one L of water. The eggs were mixed into the medium and incubated at 21°C within a 14x14x24”collapsible rearing cage (Bioquip, Rancho Dominguez, CA) throughout larval development and pupal stages until the adult flies emerged. Newly emerged flies were maintained in the same facility with a temperature of 70° F, 12:12

light:dark photo period. When necessary the adult flies of two to five days old were collected and transferred to another collapsible cage that contained sugar cubes, powdered and water. Flies were transported to an arthropod containment biosafety level (BSL) 2 laboratory for all experiments.

Growth and maintenance of spinach plants: Spinach plants (*Spinacea oleracea*) variety Space F1 (Johnny's Selected Seeds, Winslow, Maine) were grown in a greenhouse. Seeds were sown (3-4 seeds/pot) in four inch geranium pots filled with Metromix 300® growing medium (Sun Gro Horticulture, Bellevue, WA) and kept in a green house at 27°C with a 12:12 light:dark photoperiod. After seedlings emergence, a complete fertilizer (N:P:K 20:10:2, Miracle-Gro®, Scotts Company, Marysville, Ohio) was supplied at 100 ppm N for five days per week. Tap water was applied to the plants two days a week. Four to five week old plants were used in all experiments.

***E. coli* O157:H7 cultures:** Green fluorescent protein (GFP) tagged *E. coli* O157:H7 strain ATCC 43888 (an attenuated strain lacking *stx*1 and *stx*2) was used for all experiments. The original culture was provided by Dr. Li Ma, University of Georgia, Griffin, GA and the cultures were stored as glycerol stocks in a -80° C freezer until used. For use in experiments, a loop of bacterial culture from the frozen stock was inoculated into 5 ml of Luria Bertani (LB) broth supplemented with ampicillin (100 µg/ml) and incubated at 37°C overnight with shaking at 100 rpm. A quantity of five ml of the culture broth was centrifuged at 10,000 rpm and the pellet was resuspended in sterile water. The bacterial concentration was estimated by using dark field optics and an Olympus BX2 microscope at 400x magnification. A 10 µl drop of bacterial suspension was placed on a glass slide covered with a 22x22 mm cover slip. Bacterial numbers from 10 random

fields were averaged and the concentration was adjusted to 10^7 cells/ml sterile water for use in all experiments.

As explained in Chapter 1 there was two objectives for this study. To study the first objective (determination of colonization of *E. coli* O157:H7 on the regurgitation spots of house flies after exposure to different acquisition sources) four experiments were designed:

1. Experiment 1: Detection and relative quantification of *E. coli* O157:H7 in the regurgitation spots left by house flies on spinach using relative quantitative PCR
2. Experiment 2: Quantification of bacteria-like organisms (BLOs) from regurgitation spots of spinach leaves using scanning electron microscopy
3. Experiment 3: Detection and relative quantification of *E. coli* O157:H7 in manually spotted *E. coli* O157:H7 droplets on spinach leaves using relative qPCR
4. Experiment 4: Quantification of bacteria-like organisms (BLOs) on spotted *E. coli* O157:H7 spinach leaf surfaces using scanning electron microscopy

To study the second objective (determination of colonization of *E. coli* O157:H7 on the external body surfaces of house flies after contact with different acquisition sources), two studies were carried out.

1. Experiment 5: Enumeration and quantification of *E. coli* O157:H7 on the external surfaces of exposed house flies (feet and head including the labellum) by microbiological methods

2. Observation of house fly external body parts (tarsi and labellae) exposed to different acquisition sources using scanning electron microscopy

Experiment 1: Detection and relative quantification of *E. coli* O157:H7 in regurgitation spots left by house flies on spinach using relative quantitative PCR

This experiment was conducted to determine if the *E. coli* O157:H7 DNA level in a house fly regurgitation spot on the spinach leaf surface changes over time. House flies were exposed to four different bacterial acquisition sources in Petri plates (diameter 100 mm, height 25 mm). These sources were: five g autoclaved cow manure mixed with five ml GFP-tagged *E. coli* O157:H7 to a final concentration of 10^7 cells/ml in sterile distilled water (EM); five g of autoclaved cow manure, mixed with five ml sterile water (SM); half of GFP tagged *E. coli* O157:H7 lawn on a LB ampicillin agar plate (half of the agar removed) (EP); and half of a LB ampicillin agar plate (LB); House flies were anesthetized with CO_2 gas to immobilize them and then transferred to the medium-free area of the plate. For each treatment only half of the agar plate contained the treatment, the other half providing a clear surface so as to minimize excessive mechanical contamination of flies with the acquisition source, which could occur during transfer of anesthetized flies by the researcher. Forty to 50 house flies were placed in each acquisition source plate, in five replicates. After two hours of exposure, flies were anesthetized by pumping carbon dioxide for ten seconds into the exposure chamber via a port cut into the lid of the Petri plate. Anesthetized flies were immediately moved onto spinach plants that were each enclosed inside a cylindrical plastic cage with one end

embedded into the growing medium and the top other covered with a net secured by a rubber band.

All caged plants with flies were placed inside a larger cage and left overnight inside the laboratory. After plant exposure flies were anesthetized and removed. Leaves were examined the next day and regurgitation spots in all the spinach plants were circled with a marker pen. Plants were kept in the laboratory for two weeks. During this period, plants were watered via the growing medium in the pot without splashing the leaves. Water was also sprayed with a spray bottle, about 1 foot above the cage to increase humidity inside the cage. Thirty regurgitation spots (leaf pieces including a single regurgitation spot) per day were collected using a 3 mm straw punch on 0 (day post overnight exposure with flies), 4, and 8 days after plant exposure to the flies. Regurgitation spots were used in relative quantification of the *E. coli* O157:H7 DNA using quantitative PCR (qPCR) (Schmittgen & Livak, 2008).

DNA isolation from plant tissues: Ten regurgitation leaf spots were pooled in a 2.0 ml centrifuge tube and total DNA was isolated using a QIAamp® DNA Mini-kit (Qiagen, Chatworth, CA). There were three replications per treatment. Prior to DNA extraction, 10 µl of 2.5 pg/µl pCR® 2.1 (3.9 kb) plasmid (TA Colning® Kit, Invitrogen Corporation, Carlsbad, CA) carrying the 200 bp target sequence, was added (internal control) to 200 µl of ATL buffer (tissue lysis buffer) (Qiagen, Chatworth, CA) per sample for the exogenous normalization in the relative qPCR method. DNA isolation was carried out by modifying the manufacturer's protocol.

Two metal beads were added and the sample was homogenized using a Mini-Beadbeater™-8 (BioSpec Products Inc., Bartlesville, OK) for 30 s. Fifty µl of Qiagen® Protease (proteinase K) (Qiagen, Chatworth, CA) was added and the homogenized mixture was incubated at 56°C for one hour. Then 200 µl of AL buffer (lysis buffer) (Qiagen, Chatworth, CA) was added to each sample and mixed by pulse vortex. The mixture was incubated at 70°C for 15 min and centrifuged at 10,000 rpm for 30 s and resuspended in 200 µl of 100% ethanol. The mixture was vortexed and briefly centrifuged as before. Both the supernatant and the pellet together were loaded on to a spin column (avoiding the beads) and spun at 8,000 rpm for one min. The flow through was discarded.

AW-1 buffer (wash buffer) (Qiagen, Chatworth, CA) was added (250 µl), the mixture was spun (8000 rpm for 1 min) and the flow through was discarded. A quantity of 250 µl of AW-2 buffer (wash buffer) (Qiagen, Chatworth, CA) was added. The column was centrifuged at 14,000 rpm for 3 min and the flow through was discarded. The column was inserted into a 1.5 ml tube, 25 µl of sterile distilled water was added, and the column and incubated at room temperature for 1 min and then centrifuged at 8000 rpm for 1 min to elute the DNA. The concentration of the DNA was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and samples were diluted to 10 ng/µl for use in relative qPCR.

A 7.5 µl quantity of FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany), 4.6 µl of sterile water, 0.45 µl of forward primer, 0.45 µl of reverse primer and 3 µl of DNA were mixed for a single reaction in a 15 µl final volume. Samples were mixed in 96 well plates and the reactions were performed in a Bio-Rad My

iQ™ Optical module real-time PCR machine (Bio-Rad Laboratories, Hercules, CA). Amplification conditions were 95 °C for 10 min, 50 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 20 s. Determination of *E. coli* O157:H7 DNA concentration was based on the *eae* gene amplification. The *eae* gene was selected as the target gene for the detection of *E. coli* O157:H7 because the strain we used was as attenuated strain which lacks *stx* genes and the only detectable gene was the *eae* gene which was a single copy gene.

In detecting the *E. coli* O157:H7 *eae* gene was selected as the target gene for amplification. The primer sequences were; *eae* forward primer 5' ATTAACCA CACCCCACCG 3', *eae* reverse primer 5' GTCATGGAAACCGTTGTCAC 3'. In the qPCR procedure for 200 bp fragment amplification (internal control), the annealing temperature was changed to 60 °C. The primer sequence for the amplification of the 200 bp target sequence was forward primer 5' GTCTACCAGGCATTCGCTTCAT'3, reverse primer 5' TGTGAATGCTGCGACTA CGAT 3'.

Statistical analysis of data: The delta Ct ($\Delta Ct = Ct_{eae} - Ct_{internal\ control}$) (Schmittgen & Livak, 2008) values from all treatments (EM, EP, SM, LB) were used to analyze significant differences between treated (EM & EP) and control groups (SM & LB) as well as time dependent variable within treatments (day 0-8). The ΔCt values were used to perform statistical analysis to determine a relative concentration and/or time-dependent and treatment dependent effects of EM, EP, SM and LB. Using the Statistical Analysis Software, (SAS, Version 9.1, 2009, SAS Institute, Cary, NC, USA), ΔCt values were tested using Analysis of Variance (ANOVA) tables generated through PROC-GLM (General linear model) allowing the utilization of a complete randomized design. P-values were analyzed through least square means (LSMEANS) for differences of ΔCt

values from each treatment group. This model was used to identify any significant differences between pre-planned comparisons of substrates inoculated with *E. coli* O157:H7 (EM & EP) vs. control substrates (SM & LB). Statistical differences were determined at $P \leq 0.05$. Results are presented as least square means ±S.E.M (standard error of the mean). The data were represented as fold changes compared to day 0 of each treatment. Fold changes were calculated using the ΔΔCt ($\Delta Ct_{\text{treatment}} - \Delta Ct_{\text{experiment control}}$) values, where fold change $= 2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008).

Experiment 2: Quantification of bacteria-like organisms (BLOs) from regurgitation spots of spinach leaves using scanning electron microscopy

This experiment was conducted to obtain another quantification of the number of bacteria in the regurgitation spots by counting the total observed BLOs on a scanning electron micrograph. Procedures for experiment 2 were the same as for experiment 1, except where otherwise noted. Regurgitation spots on spinach leaves from flies exposed to the four treatments (autoclaved manure mixed with *E. coli* O157:H7 suspended in sterile distilled water (EM), autoclaved cow manure mixed with sterile water (SM), *E. coli* O157:H7 lawn on LB ampicillin agar plate (EP) and LB ampicillin agar plate without bacteria (LB)) were excised and prepared for scanning electron microscopy. A total of 5-8 spots per treatment were processed and five of them were used in the analysis.

Plant tissue pieces were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 2 hours followed by two 10 minute 0.1 M phosphate buffer (pH=7.0) washes. The samples were incubated in 1% osmium tetroxide for incubated 1 hour, and washed twice

for 10 minutes with phosphate buffer. Fixed tissues were dehydrated through an increasing ethanol series of 30%, 50%, 70% 90%, 95% and 100% ethanol (2x), with 15 min incubation for each step. Samples were transported in 100% ethanol to the OSU Microscopy Laboratory for critical point drying and sputter coating with Au/Pd for 1min. Prepared samples were examined using the FEI Quanta™ 600 scanning electron microscope (SEM) (FEI Company, Hillsboro, Oregon) in high vacuum mode, operated at 15kV. Five spots were observed at 12,000x magnification, 10 images were taken from selected areas of hypothetical grid that covered the total area of the regurgitation spot. The images of 94 μm^2.area were printed and the rod-shaped BLOs (with a length of 1-2.5 µm and width of 0.25- 0.75 µm) on the leaf surface counted by marking each, were recorded for each treatment for day 0, 4 and 8 samples. A total of 50 images were analyzed for each treatment. These size dimensions for the bacteria were determined by measuring BLOs on several images obtained from the SEM images of the regurgitation spots of flies exposed to previously mentioned treatments.

Statistical analysis of data: The average number of rod-shaped BLOs of appropriate size from 10 images/spot (5 spots per treatment) for all the treatments were used to analyze significant differences between treatment groups (EM, EP, SM, & LB). Using SAS the average numbers of BLOs were tested using ANOVA tables generated through PROC-GLM. P-values were generated using the LSMEANS mean separation test for differences between means of average BLO numbers from each treatment group. This model was used for pre-planned comparisons between substrates inoculated with *E. coli* (EM & EP) vs. control substrates (SM & LB), and statistical differences were determined at $P \leq 0.05$. Results are presented as least square means ±S.E.M.

Experiment 3: Detection and relative quantification of *E. coli* O157:H7 in manually spotted *E. coli* O157:H7 droplets on spinach leaves using relative qPCR

This experiment served as a control to determine the response of *E. coli* O157:H7 on the spinach leaf surface without the influence of house flies or house fly regurgitant. Six well grown leaves per plant were selected and ten 1 cm diameter circles were drawn on the adaxial surface of each with a marker pen. *E. coli* O157:H7 10^7 cells/ml in sterile water and 1 µl drops were placed in the middle of the marked circles. Ten drops were used per leaf on a total of 6 leaves per plant for a total of five plants. For the negative control, sterile water drops were placed on a different set of spinach plant leaf surfaces in a similar manner. Treated plants were maintained in the laboratory (25°C and 12 hour white light:dark period) for two weeks. At 0, 2, 4, 6, 10, and 12 days post inoculation, 9-10 spots/leaf in three replicate samples (total of 30 spots) using 3-4 leaves were collected using a 0.6 mm diameter straw punch from a randomly selected plant from both treatments for qPCR analysis to determine the change of *E. coli* O157:H7 DNA concentration over time. The qPCR procedure was similar to that of experiment 1, with a reduction in the DNA volume to 2 µl for the *eae* amplification and 1µl for the 200 bp internal standard amplification.

Statistical analysis of data: The ΔCt values from both treatments (*E. coli* O157:H7 spotted leaves and sterile water spotted leaves) were used to analyze significant differences between treated (*E. coli* O157:H7 spotted leaves) and control groups (sterile water spotted leaves) as well as time dependent variable within treatments (day 0–12). Using SAS (Version 9.1, 2009, SAS Institute, Cary, NC, USA), ΔCt results were tested using ANOVA tables generated through PROC-GLM allowing the utilization of a

complete randomized design. P-values were analyzed through LSMEANS for differences of ΔCt values from each treatment group. This model was used to determine significant differences between the two treatments, and statistical differences were determined at $P \leq 0.05$. Results are presented as least square means ±S.E.M. The data were represented as fold changes compared to day 0 of each treatment and fold changes were calculated using the ΔΔCt values where fold change equals to $2^{-\Delta\Delta CT}$ (Schmittgen & Livak, 2008).

Experiment 4: Quantification of bacteria-like organisms (BLOs) on spotted *E. coli* O157:H7 spinach leaf surfaces using scanning electron microscopy

This experiment was conducted to obtain another quantification of the number of bacteria in the manually spotted *E. coli* O157:H7 on the spinach leaves by counting the total observed BLOs on a scanning electron micrograph. In a separate experiment carried out similarly to experiment 3, ten leaf spots were collected at 0, 2, 6 and 12 days post inoculation were collected using a 0.6 mm straw punch and processed for scanning electron microscopy. Five of the 10 spots were observed using SEM. The numbers of rod-shaped BLOs in the size range of 1.5-2 µm in five selected areas of a hypothetical grid which covered the total area within the spot were counted at 12000x magnification for each date and for each treatment.

Statistical analysis of data: The average number of BLOs from five images/spot (5 spots/day) from the *E. coli* O157:H7 spotted spinach leaf discs were used to analyze significant differences between day 0, 2, 6 and 12. Using SAS (Version 9.1, 2009, SAS Institute, Cary, NC, USA), the average number of bacteria-like organisms were tested

using ANOVA tables generated through PROC-GLM. P-values were analyzed using LSMEANS for differences in mean number of BLOs for each day. This model was used to test for significant differences between days at the $P \leq 0.05$ level. Results are presented as least square means ±S.E.M.

Experiment 5: Enumeration and quantification of *E. coli* O157:H7 on the external surfaces of exposed house flies (feet and head including the labellum) by microbiological methods

House flies carry several enteric bacterial pathogens but the persistence and colonization of these pathogens on the external body surfaces of the insect has not been documented. This study was conducted to evaluate the persistence and colonization of *E. coli* O157:H7 on the external body surfaces of house flies after exposure to different *E. coli* O157:H7 acquisition sources. House flies were exposed to the four acquisition sources as explained in experiment 1. Flies anesthetized with CO_2 gas as described previously transferred to the medium-free area of the plate. Forty to 50 house flies were placed onto each acquisition source in five replicates. After two hours of exposure, flies from each treatment were pooled together into 12x12x12" aluminum collapsible cages (Bioquip, Rancho Dominguez, CA), where they were fed sugar cubes, powdered eggs and water throughout the experiment.

Samples of 20 flies were removed from each cage on day 0, 2, 4, 6, 8, 10 and 13 post exposure and used for bacterial enumeration and quantification. Individual fly heads and legs were excised and placed in 100 µl of sterile water, vortexed at maximum speed

for 20s and the liquid was spread on LB ampicillin agar plates and incubated at 37° C overnight in the incubator. Five plates were randomly selected and ten isolated colonies from each plate were randomly picked and tested for the presence of *E. coli* O157 using a commercially available agglutination kit (Remel Wellcolex *E. coli* O157, Remel Europe LTD, Dartford, UK) by following the instructions provided by the manufacturer. The O157 positive colonies were further tested using end point PCR performed in a PTC-100 Thermal cycler (MJ Research Inc., Waltham, MA) using primers specific for the *eae* gene of *E. coli* O157:H7 (primer sequence was mentioned in experiment 1). Amplification conditions were 95 °C for 2 min, 35 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 40 s followed by 72 °C for 2 min extension.

Statistical analysis: The percent positives for *E. coli* O157 obtained from the agglutination test kit and the percent *E. coli* O157:H7 obtained from the PCR analysis of the agglutination positive colonies were used to analyze significant differences between treatment groups (EM, EP, SM, & LB). All data were converted to percent positive values before analysis. Using SAS (Version 9.1, 2009, SAS Institute, Cary, NC, USA), percent O157 positive results were tested using ANOVA tables generated through PROC-GLM and means were compared by a LSD mean separation test. The percent PCR positives were analyzed in the same manner previously described.

Some of the fly tarsi and fly heads from each treatment were prepared for scanning electron microscopy using the protocol outlined in experiment 2. These were examined at the OSU Electron Microscopy Laboratory using the FEI Quanta 600 field emission gun ESEM, high vacuum mode operated at 15 kV. Images were examined for the presence of BLOs and attachment-like structures.

CHAPTER IV

RESULTS & DISCUSSION

Experiment 1: Detection and relative quantification of *E. coli* O157:H7 in regurgitation spots left by house flies on spinach using qPCR (real-time PCR)

The data obtained in this study were examined in two ways; first fold change values were compared within a treatment to see how bacteria acquired from a particular source responded in a regurgitation spot over time. Second, pre-planned comparisons between bacterial substrates and its control (EM vs SM and EP vs LB) were made to determine the relative numbers of bacteria in the test treatments. Overall there were detectable differences in the relative amounts of the *E. coli* O157:H7 *eae* amplicon between the treatments ($df=11, 48$, $F=46.06$, $p<0.0001$). Figure 1 shows the fold change values changed within each treatment over time compared to day 0. Regurgitation spots deposited by flies exposed to autoclaved manure mixed with *E. coli* O157:H7 (EM) showed a significant increase in the amount of the *E. coli* O157:H7 *eae* amplicon by 18 folds from day 0 to day 4 ($p<0.0001$), which suggests that the *E. coli* O157:H7 bacterial number increased from day 0 to day 4. At day 8 the *eae* amplicon level dropped to eight folds which was significantly higher compared to day 0 ($p=0.001$) but not significantly different from day 4 ($p=0.1907$) (Figure 1.A).

For the regurgitation spots obtained from flies exposed to the EP treatment, the *eae* amplicon levels remained fairly level over time showing no significant difference in the fold change from day 0 to day 4 ($p=0.1188$), from day 0 to day 8 ($p=0.0539$) and from day 4 to day 8 ($p=0.6956$) suggesting no significant change in bacterial number over time (Figure 1.B). These ΔCt values (see appendix) were the lowest from the data set which means the *eae* amplicon levels were higher, suggesting a higher initial *E. coli* O157:H7 cell number compared to all other treatments. This difference in initial bacterial population was presumably due to the fact that the number of *E. coli* O157:H7 cells available to the flies were much higher on the bacterial lawn than in the manure-*E. coli* O157:H7 mixture. Within the regurgitation spot, competition for resources might have limited cell division or resulted in cell death. Also the bacterial cells may have been less adapted to survive on the leaf surface and in a regurgitation spot than a rich nutrient source such as LB agar.

Similarly, the control treatment SM (sterile water mixed with autoclaved manure) showed no significant increase or decrease in the fold change from day 0 to day 4 ($p=0.9237$), from day 0 to day 8 ($p=0.1954$) and from day 4 to day 8 ($p=0.2309$) (Figure 1.C). The *eae* amplicon levels remained unchanged, showing no significant change in the fold differences from day 0 to day 4 ($p=0.2954$) from day 0 to day 8 ($p=0.0598$) and from day 4 to day 8 ($p=0.2945$) for the spots originating from the LB ampicillin agar (LB) exposed flies (Figure 1.D).

In a comparison of EM to SM, there were no significant differences in the fold change values observed on day 0 for both treatments. But on day 4 the *eae* amplicon levels increased 38 fold for EM, where as SM on day 4 had no significant change in the

eae amplicon levels ($p<0.0001$) (Figure 2). On day 8 a drop in the EM fold value was not statistically significant from that of EM on day 4, but still was significantly higher than that for SM on day 8. This finding suggests that the *eae* amplicon levels did not change in the SM treatment over time, but did so dramatically in the EM treatment, further supporting the conclusion that the *eae* amplicon level increase was due to the increase in *E. coli* O157:H7 bacterial number over time in the regurgitation spot. The material in the regurgitation spot, which contains material from both the manure and the fly gut, might have served as a nutrition source for the bacteria allowing them to multiply on the leaf surface. The *eae* amplicon levels of the positive control (EP), were 7000 fold higher on day 0 spots compared to that in the LB treatment on day 0 (Figure 3). Even though there were no significant differences in the *eae* amplicon levels over time in both treatments, the *eae* amplicon levels in EP was always significantly higher at all the time points compared to LB. ($p<0.0001$).

There was some amplification of *eae* in both of the negative controls. It is plausible that trace amounts of the *eae* gene were present in the digestive tract of colony flies. The Ct values from these treatments were the highest for the data set, suggesting very low *eae* amplicon levels in the sample. This hypothesis could be tested by including an additional control of fly regurgitation spots from obtained from house flies lacking exposure to any treatment was included.

There were some limitations in this experiment. It was difficult to obtain an adequate supply of regurgitation spots in a single experiment, in part because the visualization of the regurgitation spot on the leaf surface after the overnight period was difficult. Also, fly behavior inside the lab might have affected their regurgitation

frequency. Due to these constraints experiment 1 and 2 were done separately which might have affected contradiction of the results explained in next section.

This study is the first demonstration that *E. coli* O157:H7 persists and multiplies in fly regurgitation spots deposited on plant tissue. Whether the same relationships would be found under field conditions remains to be determined. Under the conditions of this experiment the regurgitation spots were protected from UV radiation and rain splash, which are normally encountered in field conditions. Further experiments should include an irrigation method similar to one used in field conditions. Furthermore we used an attenuated strain of *E. coli* O157:H7 lacking the *stx* genes, because of our use of highly mobile insects. The *stx* genes play an important role in pathogenicity of the bacterium (Gyles, 2006) and might play a role in plant colonization. Therefore future studies should be conducted using a pathogenic strain within a greenhouse, enabling researchers to determine the ability of a virulent *E. coli* O157:H7 strain to colonize on the leaf surface as well as the effects of environmental factors such as UV radiation and temperature fluctuations on the colonization of this pathogen on the spinach phylloplane.

Experiment 2: Quantification of bacteria-like organisms (BLOs) from regurgitation spots of spinach leaves using scanning electron microscopy

Scanning electron micrographs of housefly regurgitation spots revealed that the spots were approximately 200-500 µm in diameter. Some regurgitation spots were circular and some were irregular in outline (Figure 4). In the SEM images of the regurgitation spots of EM treatment exposed flies, much of the material observed was

probably material that the fly consumed from the autoclaved manure. For the ease of description, the regurgitated material is referred to as a matrix. Some images of the EM-exposed fly regurgitation spots showed cell division of the bacteria-like organisms (BLOs) embedded in the matrix material (Figure 5). Some coccus-like chains were also observed in these regurgitation spots (Figure 6). The regurgitation spots of EP treatment flies also contained many BLOs, but their size was smaller and the matrix was appeared different from those of the EM treatment spots. Bacteria were more clearly visible and numerous in the EP matrix surface (Figure 7). Some images showed cell division of BLOs (Figure 8) and some bacteria were observed on the surface of the guard cells of stomata (Figure 9). Flies exposed to the SM treatment produced regurgitation spots similar to those in the EM treatment and but few BLOs were observed in the matrix and those seen varied in size and morphology (Figure 10). Regurgitation spots of LB exposed flies lacked the dense matrix of the other treatments, but some thin filmy material containing a few BLOs of size and shape that differed from those in the EP treatment (positive control) was observed (Figure 11).

In counting the BLOs the size range (length 1-2.5 µm and width 0.25- 0.75 µm) was determined by taking average measurements of rod-shaped BLOs on randomly selected images for each treatment (Table 1). In general, many more BLOs were counted in spots from flies exposed to the *E. coli* O157:H7 plate (EP) than in spots from flies exposed to *E. coli* manure (EM) (Table 2) immediately after regurgitation. This difference was expected because many more bacteria were available for the flies to pick up from plates than were available from the *E. coli*-manure mixture. Over the course of the 8 day experiment, bacterial counts significantly declined or remained static,

depending upon the treatment (df=11, 599, F=22.98, p<0.0001). As shown in Figure 12, the SEM images of regurgitation spots of flies exposed to the EM treatment contained an average of 7.3 BLOs. The mean number declined to 3.16 and 4.06 BLOs on day 4 (p=0.2796) and day 8 (p=0.3993) respectively, these differences were not significant. In contrast, the mean number of BLOs observed for the EP regurgitation spots was significantly higher, but decreased from 45 on day 0 to 16 on day 4 (p<0.0001) and even further to 14 on day 8 (p<0.0001). Regurgitation spot BLO numbers from the SM treatment did not change significantly from day 0 to day 4 (p=0.924) or day 8 (p=0.6108). Similarly, regurgitation spots from LB-exposed flies showed no increase in BLO numbers from day 0 to day 4 (p=0.7504) or day 8 (p=0.6792). Overall, when comparing BLO numbers over time, surprisingly, there were no differences between the two manure treatments (EM and SM) as neither was shown to change in number over time using this method (Table 2). In contrast, differences between the EP and LB treatments were highly significant at all time intervals (Table 2).

The results obtained from this experiment do not correlate well with the results of qPCR data obtained in experiment 1 which indicated an increase in bacterial numbers (*eae* amplicon levels) from regurgitation spots from flies exposed to the EM treatment and no significant change in bacterial numbers for spots of flies exposed to EP treatment. One explanation for the lack of consistency between the two experiments could be that the bacteria responded differently in each of these two experiments. Experiment 1 and 2 were done separately, at two different time points because the number of regurgitation spots needed for both experiments could not be obtained at one time. Also, a new batch of house flies was used for each experiment. Individual fly behavior, regurgitation

frequency and regurgitation volume may have differed from one experiment to the other. Third, the mean number of BLOs counted may have been influenced by sampling error due to high level of magnification used (12,000x). This magnification was determined by observing several samples with 10,000x magnification and the ease of recognizing a rod shaped BLO in the regurgitation spots.

To improve results in future experiments, the number of images taken per spot should be increased, the use of lower magnification would allow coverage of a larger area and could result in a bacterial count more reflective of the actual number of bacteria present in a spot. Finally, a scanning electron microscopic image shows only the surface details. Since regurgitation spots have depth, the bacterial counts taken from an image are likely an underestimated number. During tissue fixation some of the matrix material in the regurgitation spot could have fallen off the leaf surface, leading to underestimation of the bacterial counts on the images. Finally the BLOs in these images cannot be definitively identified as *E. coli* O157:H7 because no immunological labeling of the target was carried out. However, it is likely that most of the BLOs observed were *E. coli* O157:H7 because the controls showed a lower number of BLOs compared to its treatment i.e. EM compared to SM and EP compared to LB.

This is the first report of observations of house fly regurgitation spots on spinach leaves by scanning electron microscopy. Also this study is the first to capture images of bacteria from a fly regurgitation spot and provide evidence of the presence of bacteria on the leaf surface, demonstrating the potential food safety risk when flies are present within a leafy greens field.

Experiment 3: qPCR detection and relative quantification of *E. coli* O157:H7 spotted on to spinach leaves

This experiment was conducted to study the responses of *E. coli* O157:H7 on the spinach surfaces without the influence of fly regurgitant. The *eae* gene was detected by qPCR in the leaf spot samples at all time points, but the ΔCt values showed no statistically significant changes over time (df=5, 31, F=2.1, p=0.0969), suggesting that the levels of the *E. coli* O157:H7 *eae* amplicon, and thus the number of bacteria, did not change over time (Figure 13). An explanation for the lack of change may be that the spotted *E. coli* O157:H7 did not survive on the leaf surface and died between day 2 and day 4. Even though the cells may have died, the DNA remained intact and was detected by qPCR.

Mitra et al. (2009) reported that spotting of *E. coli* O157:H7 on the abaxial surface of spinach resulted in bacterial survival on the phylloplane for 14 days post inoculation. They also documented that the bacterial titer increased over time and the leaf surface area increased of colonized. In contrast to these results, the *E. coli* O157:H7 DNA concentration of the regurgitation spots of flies exposed to manure mixed with *E. coli* O157:H7 (EM) treatment had an 18 fold increase on day 4 samples (experiment 1), suggesting that the fly regurgitation spots served as a nutrition source for the bacteria, allowing them to survive on the spinach phylloplane. The composition of house fly regurgitant and whether it can serve as a nutrient source for these bacteria remain to be determined.

Experiment 4: Quantification of bacteria-like organisms (BLOs) on spotted *E. coli* O157:H7 spotted spinach leaf surfaces using scanning electron microscopy

Scanning electron micrographs of the sterile water spots on spinach leaf surfaces showed no target BLOs, but a few slender, rod-shaped BLOs that were morphologically different from *E. coli* O157:H7 were observed (Figure 14). These non-target BLOs were probably residential or epiphytic bacteria, but they were not identified in this study. They were not counted in either the treatment or the control images. On day 0, SEM images of the *E. coli* O157:H7 spotted leaf surfaces showed many BLOs (Figure 15). Structures that can be suggested as attachment-like structures were observed between plant tissues and bacteria and among bacteria (Figure 16) were observed. The bacteria were primarily observed in the interclinal junctions between the epidermal cells and observed in large aggregates (Figure 17).

In a study of biofilm formation by *E. coli* strains, Prigent-Combaret et al. (2000), showed that the *E. coli* K-12 strains formed curli, or fimbriae, on cover slips which enabled them to attach to the glass surface and to each other. These fimbriae were confirmed as curli by anti-curlin immunogold labelleing (Prigent-Combaret et al., 2000). Shaw et al. (2008) showed that *E. coli* O157:H7 expresses type III secretion system (T3SS) genes for adherence to the spinach and lettuce leaf surfaces and EspA filaments, which were encoded by these genes and produced by the bacteria, allowed them to attach to the surface. Similarly Xicohtencatl-Cortes et al. (2009) documented that mutations of the adhesin intimin (*eae*) and flagella major subunit (*fli*C) reduced the colonization and leaf invasion capability of EHEC strains to spinach and lettuce. The attachment structures

observed in this study could be curli or flagella, but a definitive interpretation would require in depth analysis of these structures.

When the number and condition of spotted *E. coli* O157:H7 on spinach were considered over a period of time, images showed morphological changes in the BLOs on 2, 6 and 12 day post inoculation compared to day 0. On day 0 the BLOs were undamaged, rod-shaped cells with definite outlines (Figures 15, 16 and 17), but on days 2-12, the appearance of the cells changed sometimes dramatically. A large proportion of the cells appeared imperfect and/or damaged (Figure 18). The total number of BLOs (undamaged + damaged) and the number of BLOs that were undamaged (total- damaged) were counted and analyzed, but both analyses yielded the same trend. Bacterial numbers in the spots decreased until day 6, but then increased dramatically after 12 days. The total number and the number of undamaged BLOs decreased significantly from day 0 to day 6 ($p<0.0001$, df=3, 99, and $F=9.01$) (Figure 19).Compared to day 2, day 12 had a higher number of BLOs ($p=0.0329$) and it was higher compared to day 6 ($p<0.0001$).

Both the total number of BLOs and the number of undamaged BLOs showed the same pattern of number change over time (Figure 19). However, the drop in the number of BLOs on the leaf surface did not correlate well with the qPCR analysis of the total DNA concentration from spotted *E. coli* O157:H7 over time (experiment 3). Analysis of SEM images suggest that bacterial numbers fell immediately after *E. coli* deposition on the leaf surface whereas qPCR analysis suggested that bacterial number remained static after deposition. This discrepancy could have occurred because these two experiments were done at two different times and the bacterial titer of the inocula may also have varied. Second, the means were generated from only five images per spot. It is possible

that the number of images taken might not have been representative of the spot and analyzing more images might have resulted in a more accurate estimate of the number of BLOs in an individual spot.

On days 2, 6 and 12 post inoculation, the number of BLOs on the on the leaf surface declined, suggesting that the bacteria might have died and/or sloughed off of the leaf. This loss may have been due to any one of several reasons: lack of a nutrient source, dehydration or toxins secreted by the epiphytic /residential bacteria on the leaf surface. The shapes of the epiphytic bacteria were constant over time in both the *E. coli* O157:H7 treated and the control leaf samples (Figure 14), suggesting that the damage of the bacterial cell surface was not due to the chemicals used in tissue fixation for scanning electron microscopy.

Experiment 5: Enumeration and quantification of *E. coli* O157:H7 on the external surfaces of house flies (feet and head, including the labellum) by microbiological methods

E. coli O157:H7-exposed flies were tested at 2 day intervals for recovery of viable bacteria. There were significant differences in the numbers of the recovered *E. coli* O157 positives from flies exposed to different acquisition sources with the latex agglutination test (Remel Wellcolex *E. coli* O157, Remel Europe LTD, Dartford, UK) ($df=27, 138$, $F=11.20$, $p<0.0001$) (Figure 20). The number of positives were higher in the EP and EM exposed flies than in the other treatments, but many of the negative control flies (exposed to LB or SM) also were positive for the O157 antigen. When these positive samples were

re-tested by end point PCR for the presence of the *eae* gene, all of the bacteria recovered from the LB or SM exposed flies were negative, with significant differences between all treatments (df=27, 138 and F=27.80, p<0.0001) (Figures 21).

E. coli O157:H7 colonies were recovered from house flies exposed to *E. coli* O157:H7 lawn (EP) and autoclaved manure mixed with *E. coli* O157:H7 suspension (EM) up to 13 days post exposure. Initially, all recovered EP colonies were PCR positive, but the mean number of PCR positives dropped after day 6 (Figure. 21). A different pattern emerged from the EM exposed flies; 100% of the tested colonies of this group were positive for the *eae* gene on the day of exposure, but the number dropped to 0% positive on day 2. From day 2 to day 6 post exposure, the percentage of PCR positives for the EM-exposed flies increased from 0 to 46 and then dropped again to 0 at day 8.

The percent of recovered, PCR positive colonies showed a fluctuating pattern during the test period. None of the colonies recovered from the control treatment flies; LB agar plates (LB) and manure mixed with sterile water (SM), were PCR positive for *E. coli* O157:H7, but many were *E. coli* O157 positive by the serological agglutination test, indicating that the flies were likely already carrying a non-H7 antigen serotype. This result has implications for disease risk due to fly transmission of other pathogenic *E. coli*, but this factor is outside the scope of this study. What can be inferred from this work is that bacterial numbers increased after an initial decline, suggesting bacterial growth (replication) on the cuticular surface of flies. More importantly, *E. coli* O157:H7 persisted on fly body surfaces for up to13 days after the initial exposure. These results support the hypothesis of “bio-enhanced transmission” by Kobayashi et al. (1999), which

states that the relationship between flies and bacteria is more specific than just simple contamination of fly surfaces.

Scanning electron microscopy observations of fly tarsus (Figure 22) exposed to *E. coli* lawns (EP) showed rod-shaped BLOs in the sizes between 1 µm -2 µm on the sticky hairs of the pulvilli of the feet (Figure 23) and on the tarsus (Figure 24). BLOs appeared to be in higher numbers compared to those on flies exposed to the EM treatment, although numerical analysis was not done. EM treatment exposed flies had only a few BLOs on the sticky hairs of the pulvillus (Figure 25) and on the tarsus (Figure 26). No BLOs were observed on the LB and SM exposed fly tarsi (Figure 27). The labellae (Figure 28) exposed to EP treatment had aggregates of BLOs on the pseudotracheae (Figure 29) and some division of bacteria was observed between the grooves of pseudotracheae (Figure 30). No other treatment-exposed labellae or pseudotracheae showed any BLOs on their surfaces (Figure 31).

SEM observations of fly body parts revealed that the pseudotracheae of the labellae, body hairs, and the sticky setae of the fly tarsi may be a hospitable environment for *E. coli* O157:H7 multiplication on the house fly. Sukontason et al. (2006) studied the ultrastructure of the pulvilli from different fly families by scanning and transmission electron microscopy. The “electron-lucent area” of house fly setae (the distal end) plays an important role in their attachment to surfaces and could serve as an adhesive surface for attachment of microorganisms (Sukontason et al, 2006). The results from this research and the Sukontason study suggest that bacteria associated with fly feet could contaminate plant surfaces on which the insect land.

CHAPTER V

SUMMARY & CONCLUSIONS

The interaction of human pathogens with plants is a developing area in plant pathology and food protection oriented research. Insect involvement in the dissemination of human pathogens is not considered to be a major pathway; however some insects, such as filth flies, could be a source of fresh produce contamination. Brandl, (2006) noted that insects could be potential carriers of human pathogens in the field. Many other researches demonstrated that house flies are potential carriers of many human pathogens, such as *E. coli* O157:H7, *Shigella, Salmonella* and protozoans (De Jesus et al., 2003; Rosef and Kapperud, 1982; Kobayashi et al., 1999; Graczyk et al., 1999; Sasaki et al., 2000). The report by Talley et al. (2009) that house flies can disseminate *E. coli* O157:H7 to the spinach phylloplane under laboratory conditions was the first documentation of insect dissemination of *E. coli* O157:H7 to plants.

My research specifically examined the potential for subsequent colonization and persistence of the bacteria on the spinach phylloplane and on the insect external body surfaces under laboratory conditions. The results from experiment 1 suggest that bacteria

deposited by flies in regurgitation spots survived and multiplied, that fly regurgitant was a source of contamination of the spinach leaf surface, and that it served as a nutrition source for the bacteria. Scanning electron microscopy of the regurgitation spots of flies exposed to EM showed division of bacteria-like organisms in the spots, which suggests the bacteria were active. This finding is consistent with those of several studies in the literature. While not specifically addressing fly regurgitation spots, Macovei et al. (2008) showed that house flies collected from a cattle feedlot, when exposed to several ready-to-eat food sources, contaminated the foods with different *Enterococcus* species. Moriya et al. (1999) reporting on the *E. coli* O157:H7 disease outbreak in Japan in which the food and utensils of school children were contaminated with the pathogen noted that house flies were possible carriers of the pathogen, which moved to the nursery school from a nearby cattle feedlot. Sasaki et al. (2000) reported that when house flies were fed with *E. coli* O157:H7 and allowed to contact different foods they were able to disseminate bacteria onto the food surfaces by excretion within a 24 hour time.

Quantitative PCR analysis of the artificial spotting of *E. coli* O157:H7 on the adaxial surface of spinach leaves is consistent with an interpretation that the bacteria either died or sloughed off the leaf surface. The adaxial surface might not be a supportive micro-environment for bacterial survival. This inoculation method simulated contamination by rain splash or overhead irrigation. These data are inconsistent with results obtained by Mitra et al. (2009), who showed that *E. coli* O157:H7 survived for 14 days on the abaxial surface of the spinach leaves following leaf drop inoculation and the area of colonization and titer increased over the duration of the experiment. There were some differences between the two studies that might explain the discrepancy. In this

study, a single strain of *E. coli* O157:H7 was used (the attenuated ATCC strain # 43888) whereas a cocktail of five *E. coli* O157:H7 strains was used in the Mitra et al. (2009) study. Four of the five strains were pathogenic and may have had a greater ability to survive on the plant surface than the 43888 strain.

Interestingly, in the scanning electron microscopy study of leaf spotted bacteria, several of the BLOs appeared damaged on days 2-12 post inoculation. In contrast, the shapes of the epiphytic bacteria appeared undamaged over time in both the *E. coli* O157:H7 treated and the control leaf samples, suggesting that the damage of the bacterial cell surface was not caused by the chemicals used in tissue fixation. The most plausible explanation is that the spotted bacteria died on the phylloplane, leaving DNA that was detectable by qPCR. The inability to survive on the phylloplane may be due primarily to a lack of nutrition. Again, this also supports the conclusion that regurgitation spots may serve as a potential nutrition source allowing the *E. coli* O157:H7 to survive on the spinach phylloplane. It would be interesting to study the composition of the fly regurgitant as a nutrient reservoir for bacterial survival.

E. coli O157:H7 persisted on the fly body surfaces for 13 days post exposure to acquisition sources. The data suggest that the fly external body surface can support *E. coli* O157:H7 growth, at least for a few days. These results are consistent with those reported by Kobayashi et al. (1999), who documented that after exposing house flies to *E. coli* O157:H7 lawns, the bacteria remained in the house fly intestine and were excreted for three days after ingestion. The bacteria multiplied within the pseudotracheae of the house fly labellum. The authors hypothesized that the relationship between the fly and bacteria was more than simple contamination, and coined a new term: “bioenhanced

transmission" (Kobayashi et al, 1999). My study was carried out by exposing the house flies to a more natural source where the flies were exposed to cattle manure containing 10^7 cells/g of *E. coli* O157:H7 which is a concentration found naturally in cattle manure. The data of my study strongly supports the Kobayashi hypothesis because *E. coli* O157:H7 survived for 13 days post exposure to acquisition sources and bacteria multiplied on fly external body parts. Also my study was done using a higher sample numbers than the Kobayashi et al. (1999) study.

Overall, this study revealed that house flies are a mode of human pathogen dissemination to plants under laboratory conditions. Regurgitant may be a source of nutrition for *E. coli* O157:H7, allowing them to multiply on the leaf surface. Body hairs, sticky glandular hairs on the pulvillus and pseudotrachea on the labellum are additional potential niches in which the bacteria can survive. To more fully understand the colonization and persistence of *E. coli* O157:H7 on plant phylloplane future research should be extended to include green house and field studies that use pathogenic strains.

In the field there can be several factors that could attract the filth flies to the leafy green production areas. One can be the presence of honey dew which is the excretory substances on the leaf surfaces of plants infested with aphids which was observed by Drs. J. Talley and A. Wayadande (Personal Communication) in their visit to the leafy green production areas in the Salinas Valley, CA. Another factor is the application of composed or non-composted animal manure as fertilizers, which are excellent breeding sites for filth flies. The contact of filth flies with the leafy greens can introduce pathogens to the phylloplane as shown in this study which is an important mode of pathogen introduction but essentially ignored by growers. Therefore growers should be educated in the control

of filth flies in the production areas and to minimize the occurrence of the factors that could attract the flies to leafy green production areas in order to minimize the possible risks of introducing human pathogens to fresh produce.

Table 1. Average dimensions (length and width) of rod-shaped bacteria-like organism (BLOs) taken from regurgitation spots of flies exposed to different acquisition sources. (EM- autoclaved manure mixed with *E. coli* O157:H7 suspension, SM –autoclaved manure mixed with sterile water, EP - *E. coli* O157:H7 lawns on LB ampicillin agar, LB - LB ampicillin agar plates.)

Treatment	Average. sizes of BLOs (µm)			
	Length range	Avg. Length	Width range	Avg. Width
EM (n=5)	1-1.6	1.28	0.44-0.78	0.6
EP (n=5)	1-1.4	1.18	0.56-0.67	0.62
SM (n=5)	1.3-1.8	1.62	0.44-0.56	0.55
LB (n=5)	1.1-1.6	1.26	0.33-0.56	0.44

Table 2. Mean number of rod-shaped bacteria-like organism taken from 10 images from five different regurgitation spots on spinach leaf surfaces (±SE). (EM- autoclaved manure mixed with *E. coli* O157:H7 suspension, SM - autoclaved manure mixed with sterile water, EP - *E. coli* O157:H7 lawns on LB ampicillin agar, LB - LB ampicillin agar plates)

	Days after exposure		
Acquisition Source	**0**	**4**	**8**
EM (treatment)	7.24 (0.95)	3.16 (0.53)	4.06 (1.65)
SM (control)	2.20 (0.42)	1.84 (0.80)	4.12 (1.65)
	P=0.1818	P=0.7264	P=0.9873
	Days after exposure		
Acquisition Source	**0***	**4***	**8***
EP‡ (treatment)	45.34a (7.39)	17.02b (2.99)	14.36b (4.02)
LB (control)	1.72 (0.43)	0.52 (0.19)	0.16 (0.07)
	P<0.0001	P<0.0001	P=0.0002

*Means significantly differences between treated and control acquisition sources within a sampling period.

‡Means followed by the same letter within a row are not significantly different

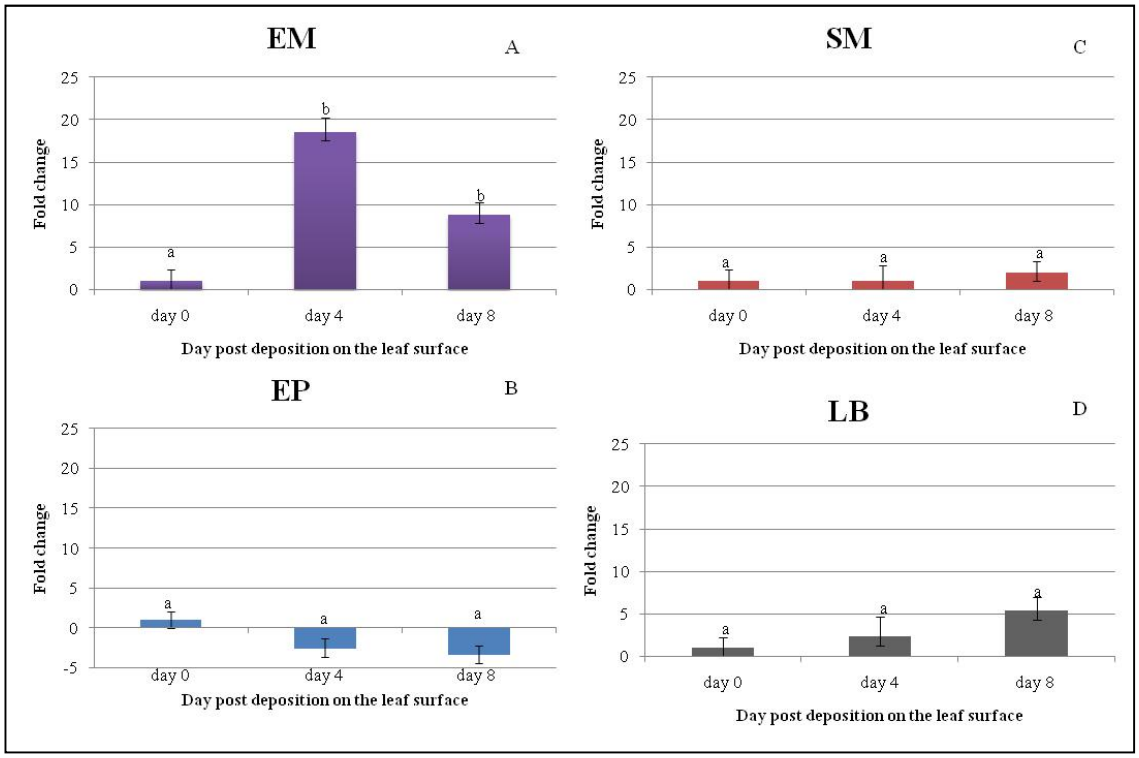

Figure 1. Relative quantitative PCR analysis of *E. coli* O157:H7 *eae* amplicon levels of the house fly regurgitation spots on the spinach leaf surfaces expressed as fold change compared to day 0 within different treatments. Bars represent standard error. Means with different letters are significantly different ($p<0.05$). A. EM = regurgitation spots originating from flies exposed to autoclaved manure mixed with *E. coli* O157:H7. B. EP = regurgitation spots originating from flies exposed to *E. coli* O157:H7 lawns on LB ampicillin agar. C. SM = regurgitation spots originating from flies exposed to autoclaved manure mixed with sterile water. D. LB = regurgitation spots originating from flies exposed to LB ampicillin agar plates.

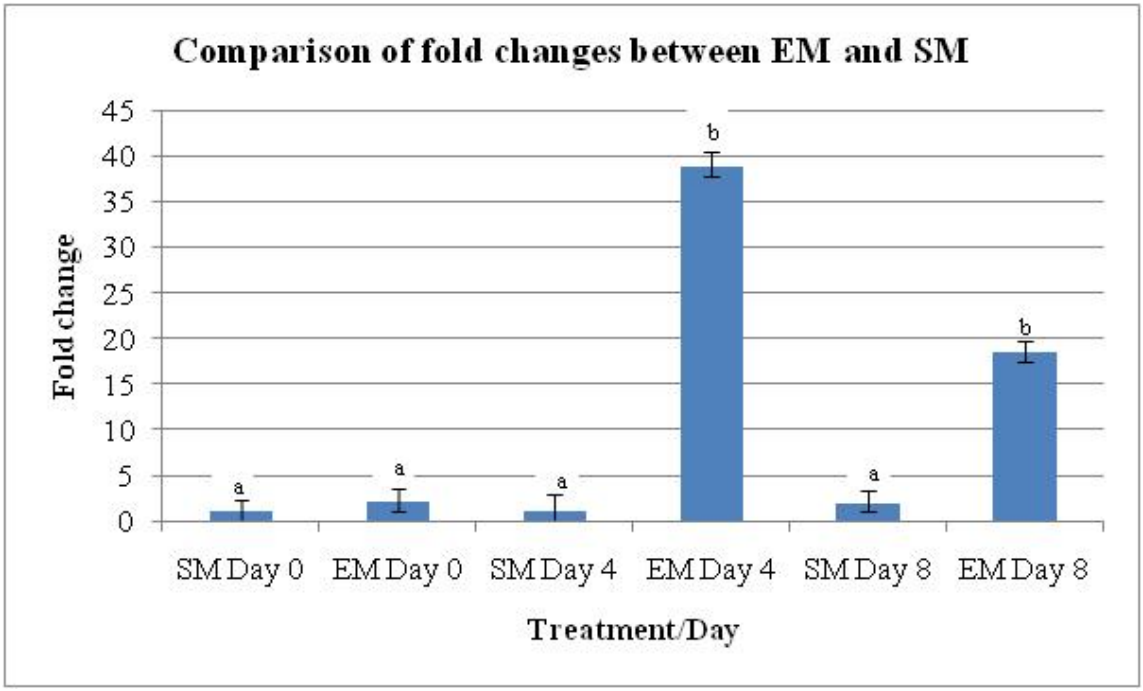

Figure 2. Relative quantitative PCR analysis of *E. coli* O157:H7 *eae* amplicon levels of the house fly regurgitation spots on the spinach leaf surface expressed as fold change between flies exposed to manure mixed with *E. coli* O157:H7 (EM) and flies exposed to manure mixed with sterile water (SM). The *eae* amplicon levels are represented as fold change. Bars represent standard error. Means with different letters are significantly different ($p<0.05$).

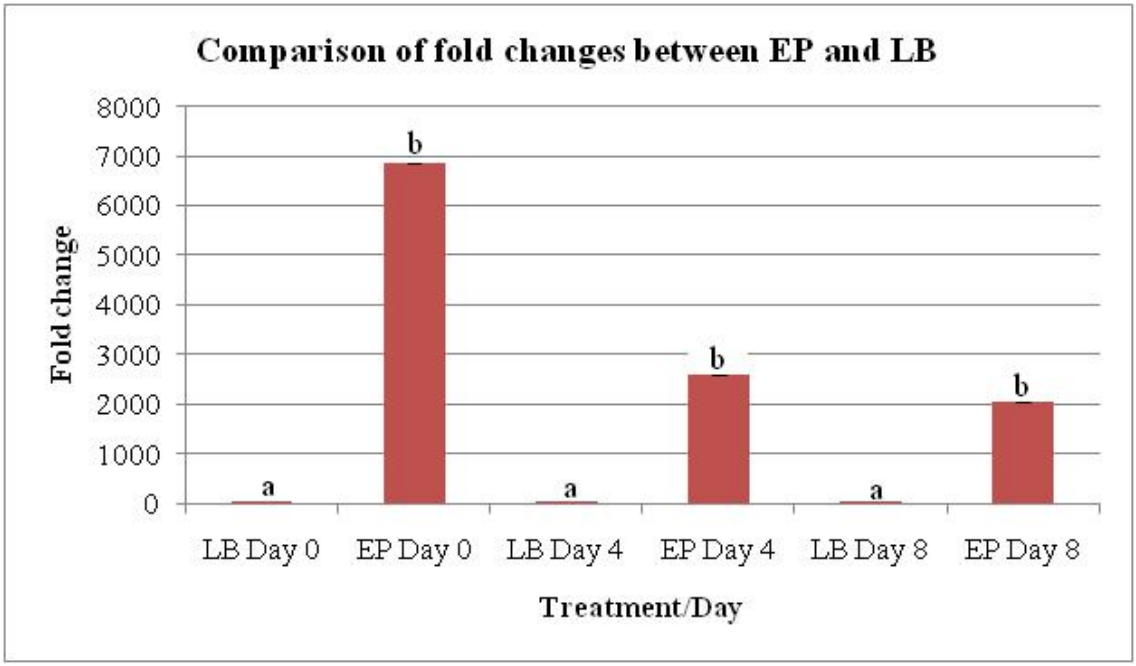

Figure 3. Relative quantitative PCR analysis of *E. coli* O157:H7 *eae* amplicon levels of the house fly regurgitation spots on the spinach leaf surface expressed as fold change between flies exposed to *E. coli* O157:H7 lawn on LB ampicillin agar (EP) and flies exposed to LB ampicillin agar plate (LB). Bars represent standard error. Means with different letters are significantly different ($p<0.05$).

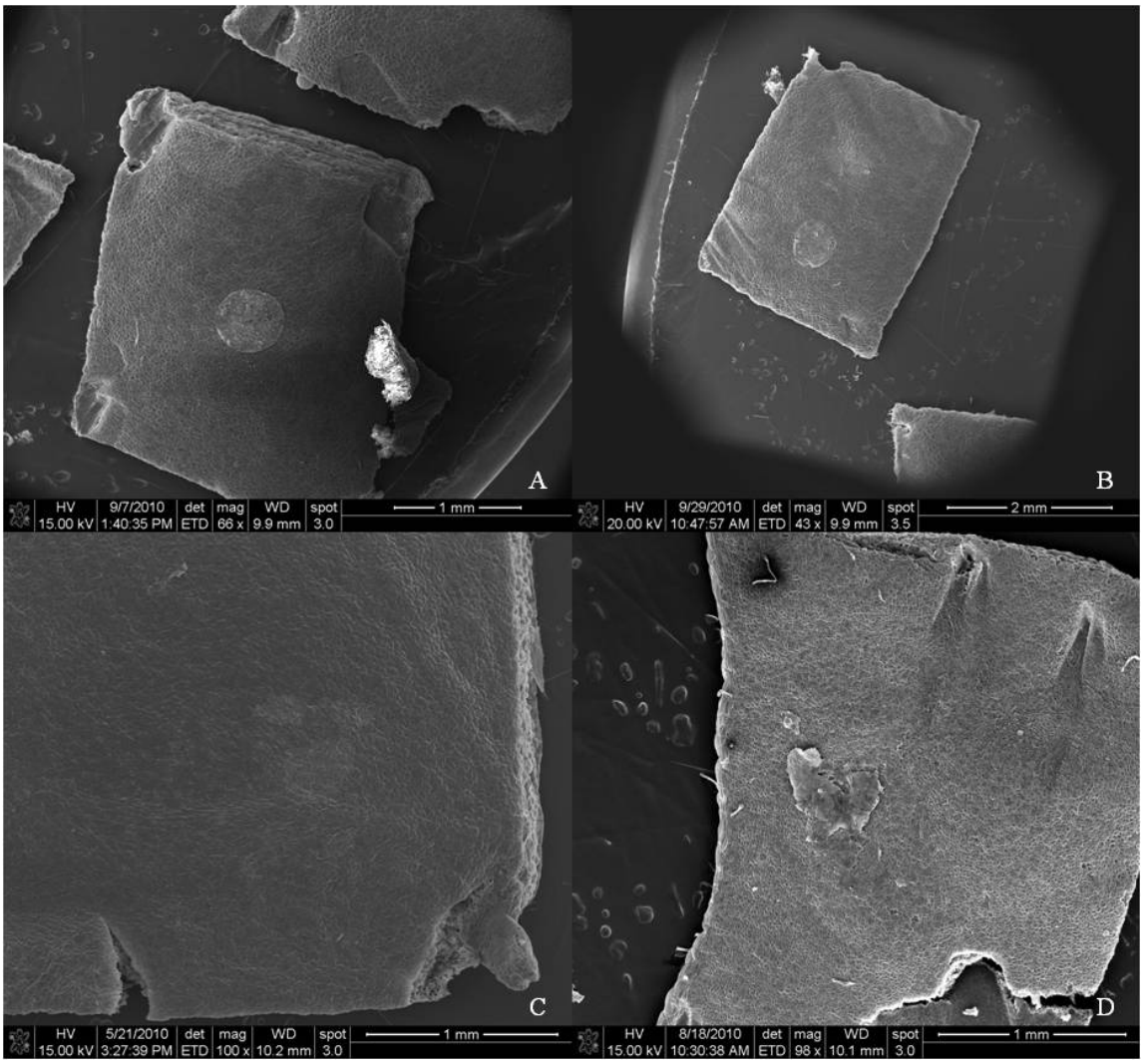

Figure 4. Regurgitation spots observed on the spinach leaf surface from flies exposed to different acquisition sources. A. Flies exposed to autoclaved manure mixed with *E. coli* O157:H7 (EM). B. Flies exposed to *E. coli* O157:H7 lawn (EP). C. Flies exposed to LB ampicillin agar (LB) D. Flies exposed to autoclaved manure mixed with sterile water (SM).

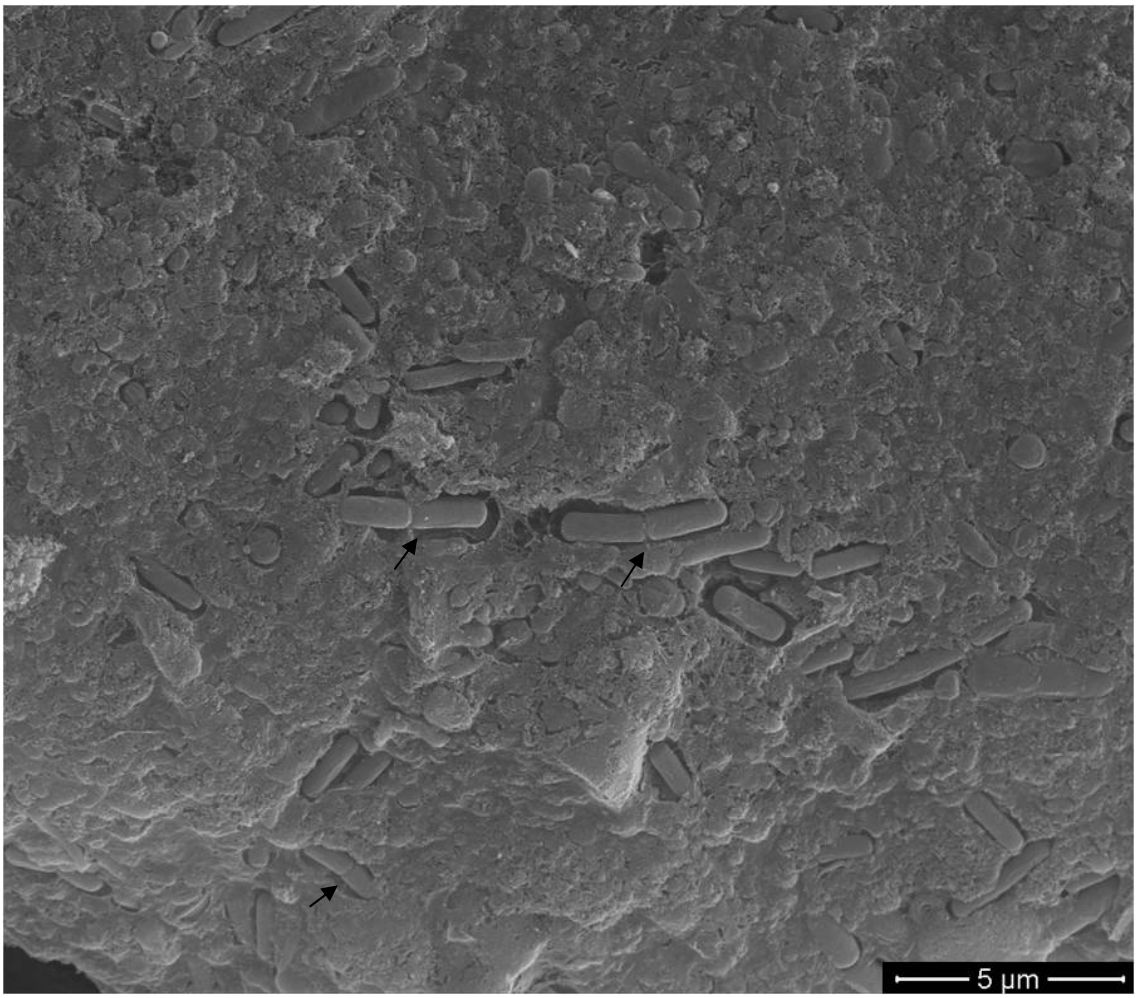

Figure 5: Rod-shaped bacteria like organisms embedded in the matrix of a regurgitation spot deposited on spinach leaf surface by a house fly exposed to EM treatment (autoclaved manure mixed with *E. coli* O157:H7 suspension). Magnification= 12000x Note the dividing cells are marked by arrows.

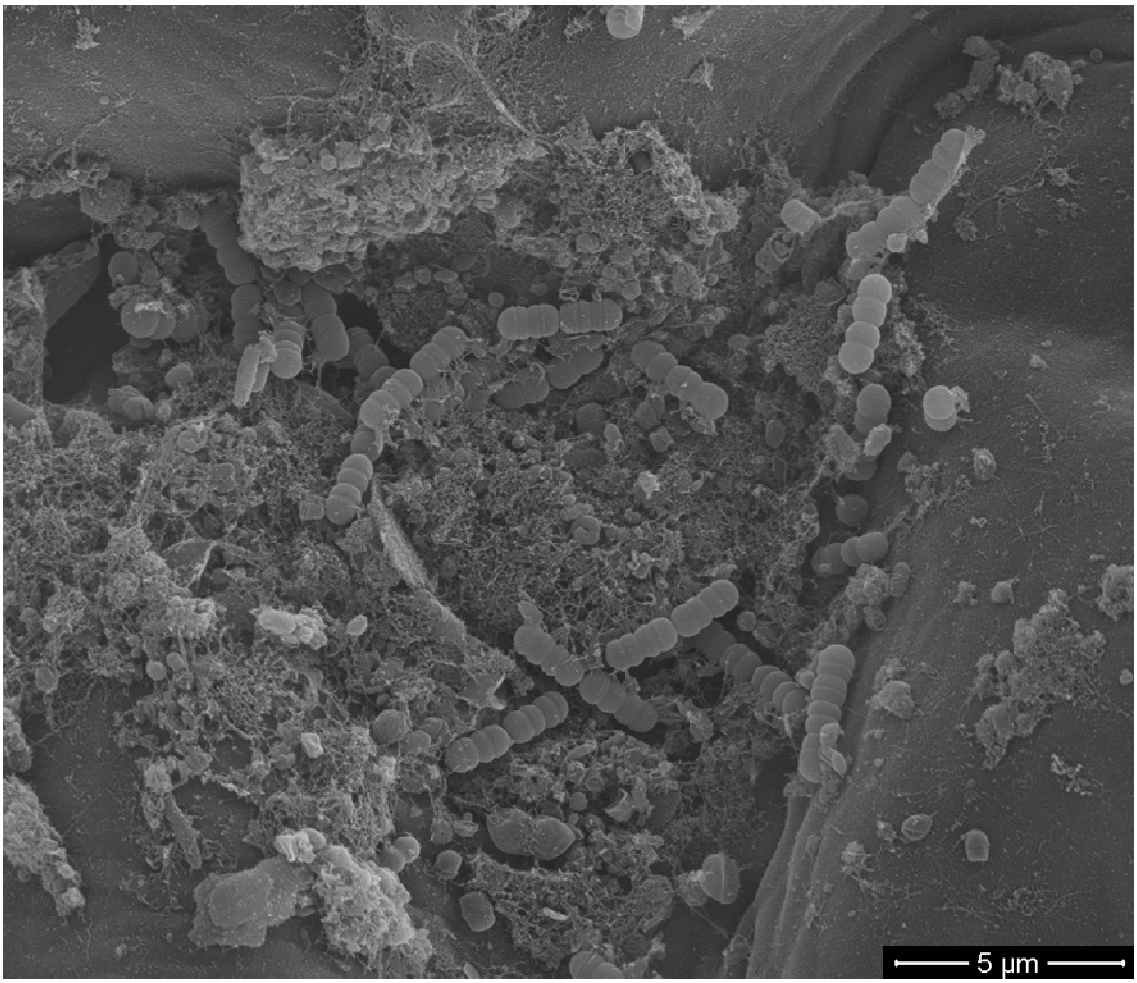

Figure 6. Chains of cocci bacteria-like organisms observed in a regurgitation spot from a house fly exposed to autoclaved manure mixed with *E. coli* O157:H7 (EM). Magnification = 12,000x.

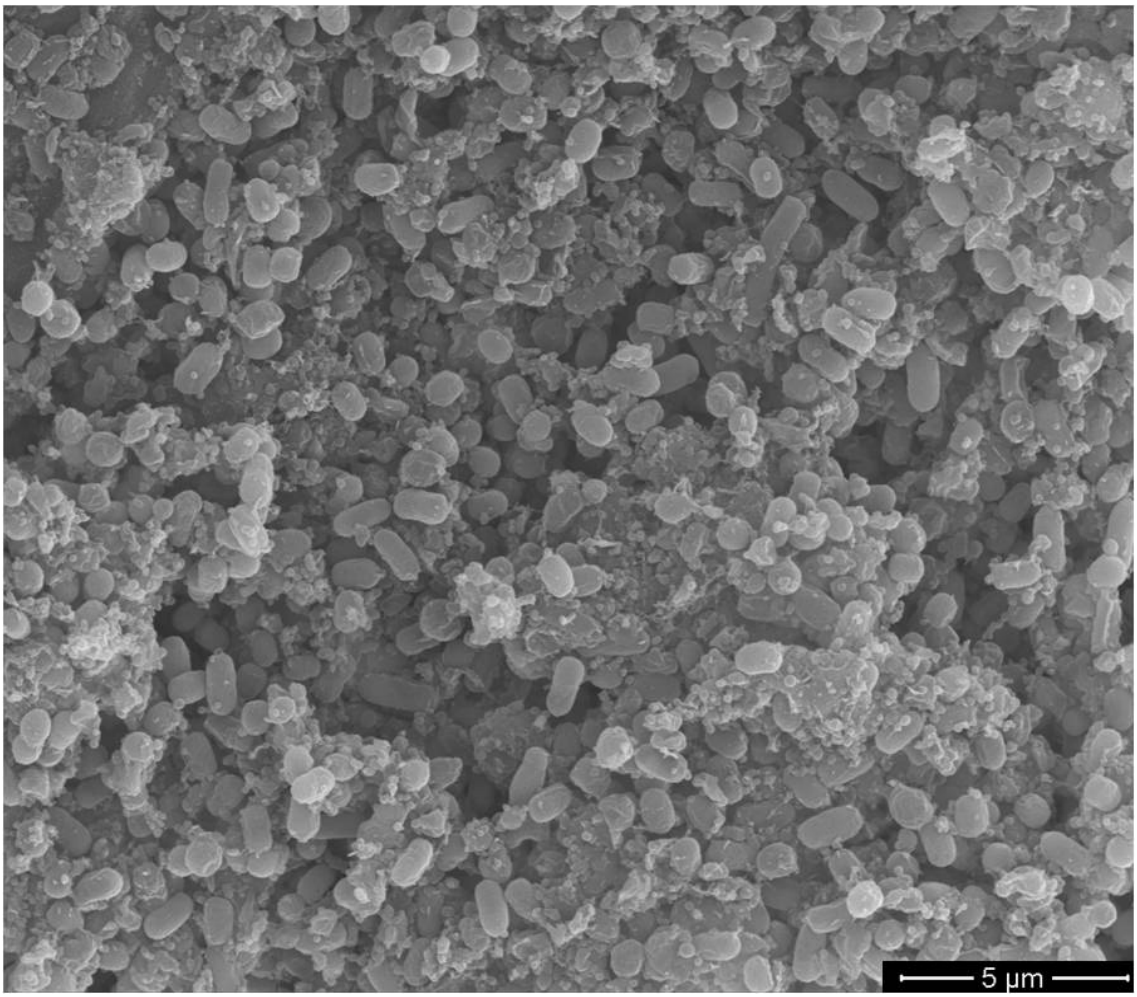

Figure 7. Numerous rod-shaped bacteria-like organisms observed in the regurgitation spots on the spinach leaf surface deposited by a house fly exposed to an *E. coli* O157:H7 lawns on LB ampicillin agar plates (EP). Magnification 12,000x.

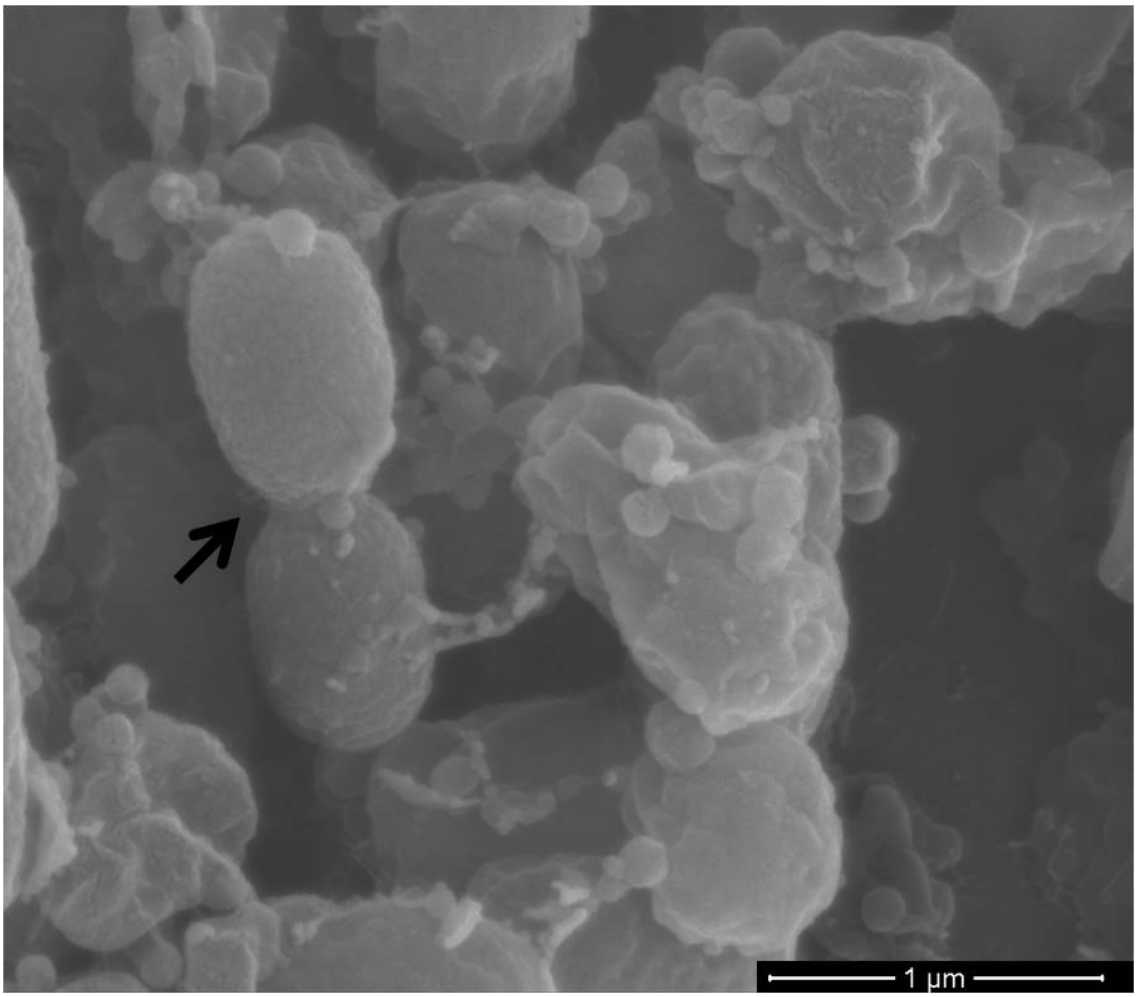

Figure 8. Division of bacterial cells (arrow) on the matrix of the regurgitation spot deposited by a house fly exposed to an *E. coli* O157:H7 lawn on LB ampicillin agar plates (EP). Magnification= 88,000x

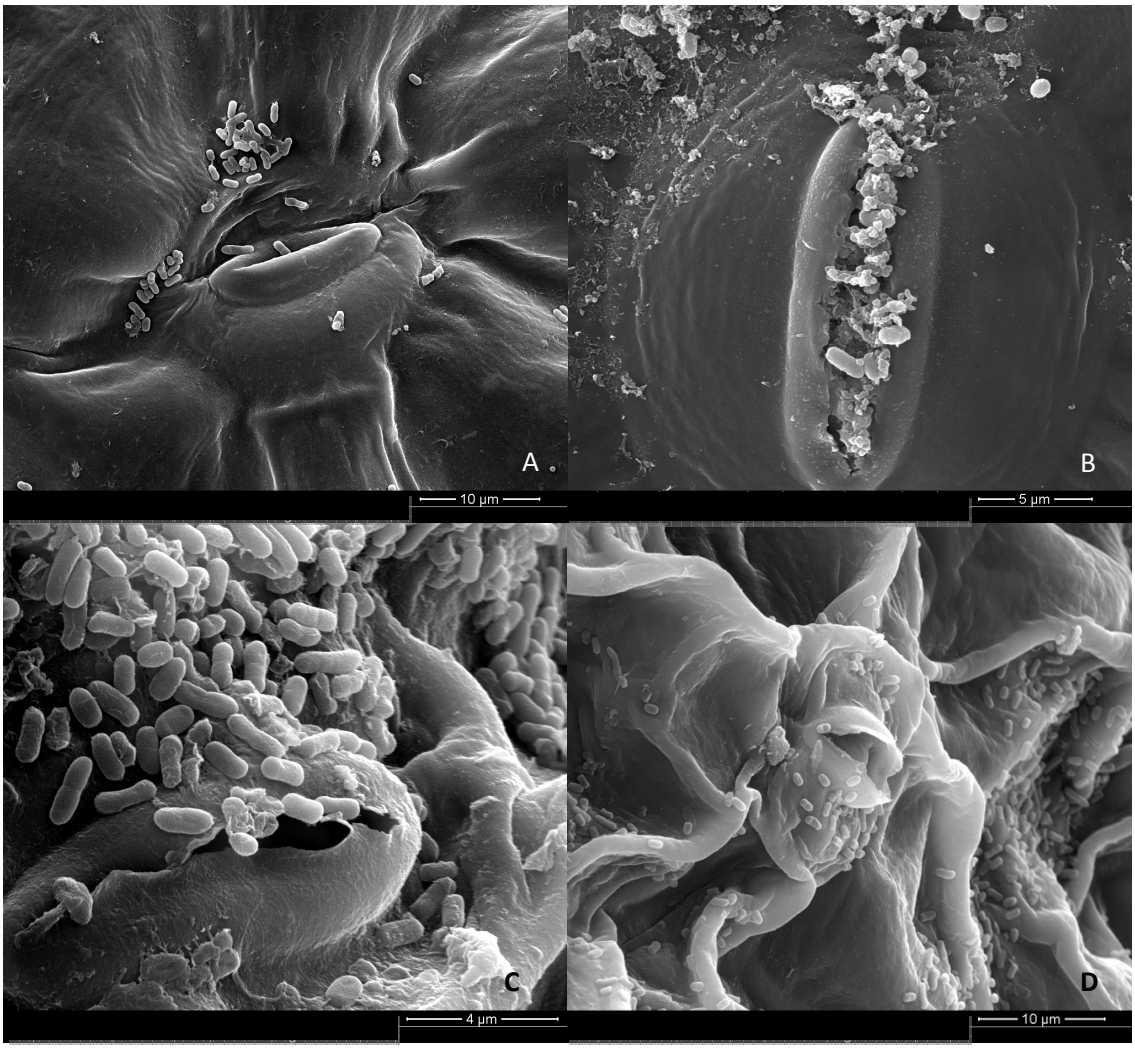

Figure 9. Four different SEM images of bacteria-like organisms observed on the guard cells of the spinach leaf stomata within regurgitation spots of house flies exposed to an *E. coli* O157:H7 lawn (EP). Images were taken under different magnifications; A. 6317x, B. 12,000x, C. 19,890x, D. 6660x

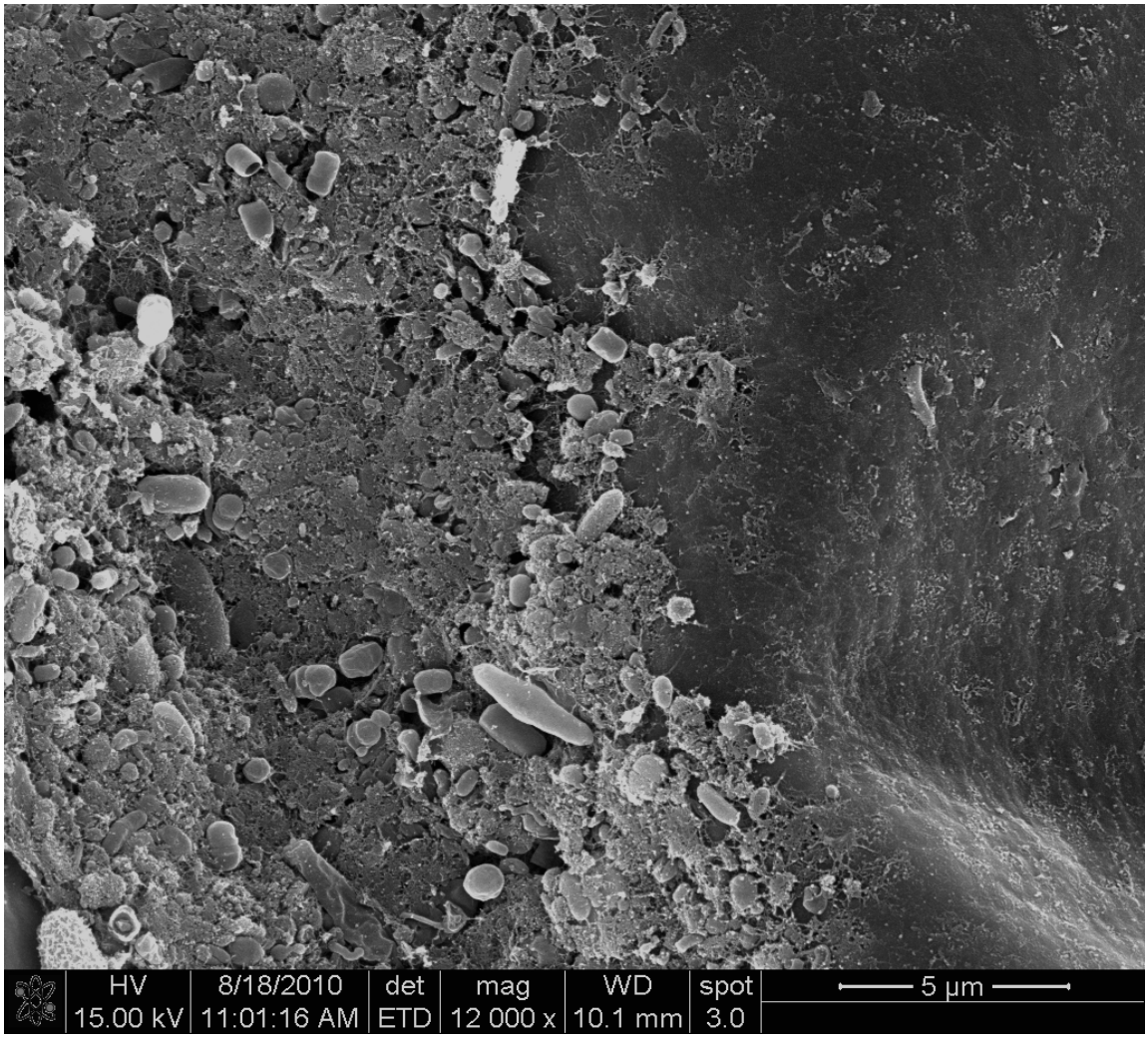

Figure 10. Image of a regurgitation spot deposited by a house fly exposed to autoclaved manure mixed with sterile water (SM). Bacteria-like organisms are embedded in the matrix. Magnification = 12,000x.

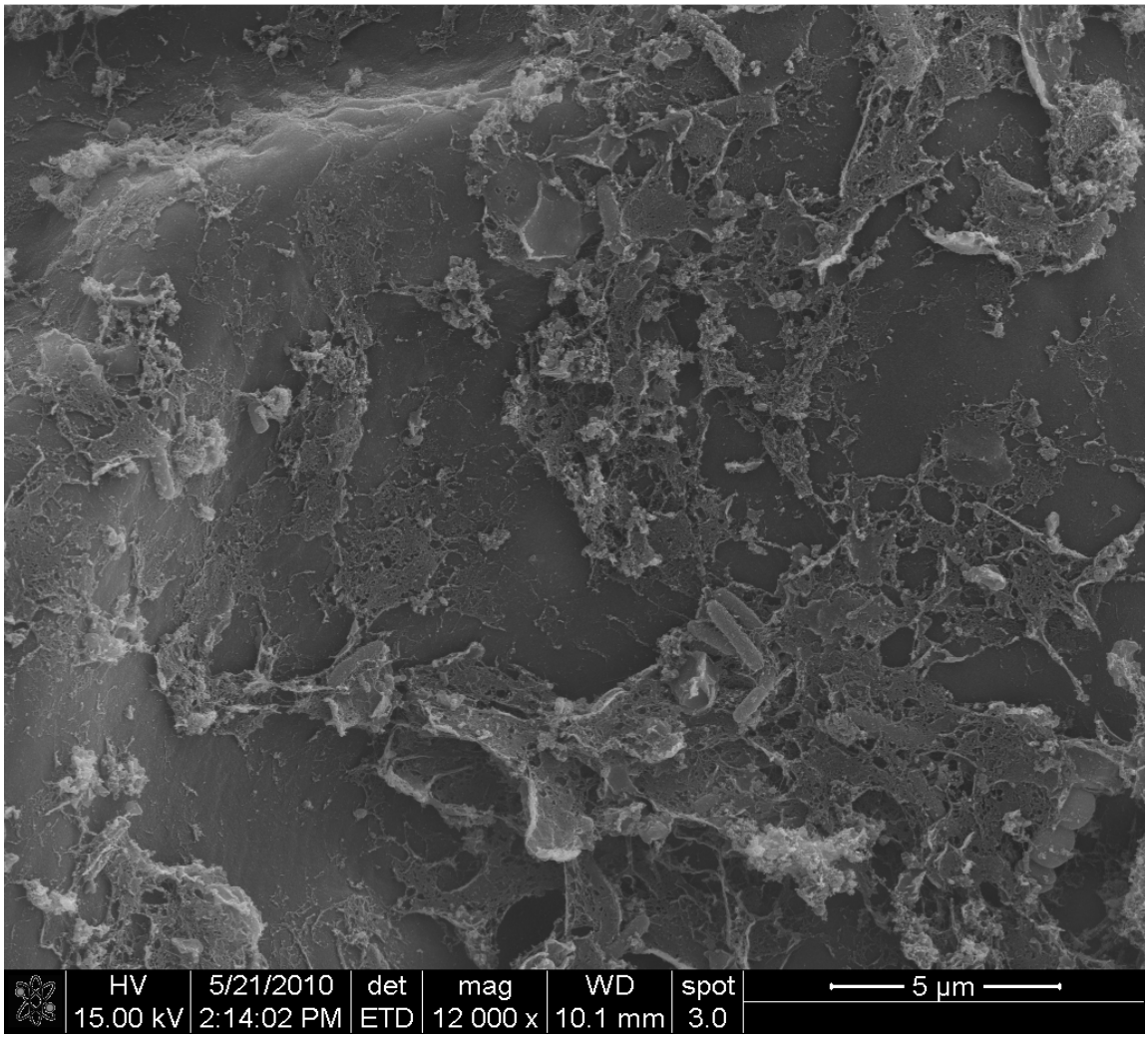

Figure 11. Regurgitation spot on the spinach leaf surface deposited by a house fly exposed to a LB ampicillin agar plate (LB). Magnification = 12,000x. Note the lack of a thick matrix.

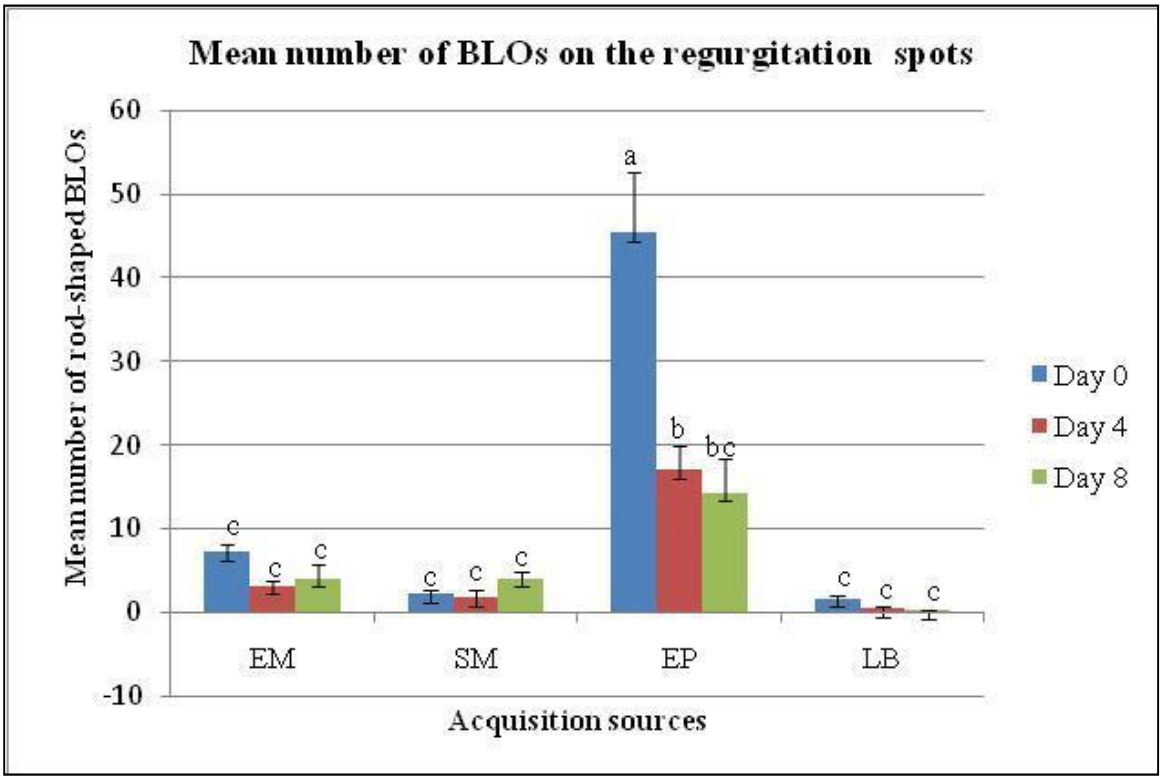

Figure 12. Mean number of rod-shaped bacteria-like organisms observed on the scanning electron microscopy images of the regurgitation spots on spinach leaves of flies exposed to different acquisition sources. Bars represent standard error. Means with different letters are significantly different ($p<0.05$). (EM- Flies exposed to autoclaved manure mixed with *E. coli* O157:H7 suspension in sterile water, EP- Flies exposed to *E. coli* O157:H7 lawn on LB ampicillin agar, SM- autoclaved manure mixed with sterile water, LB- Flies exposed to LB ampicillin agar plate)

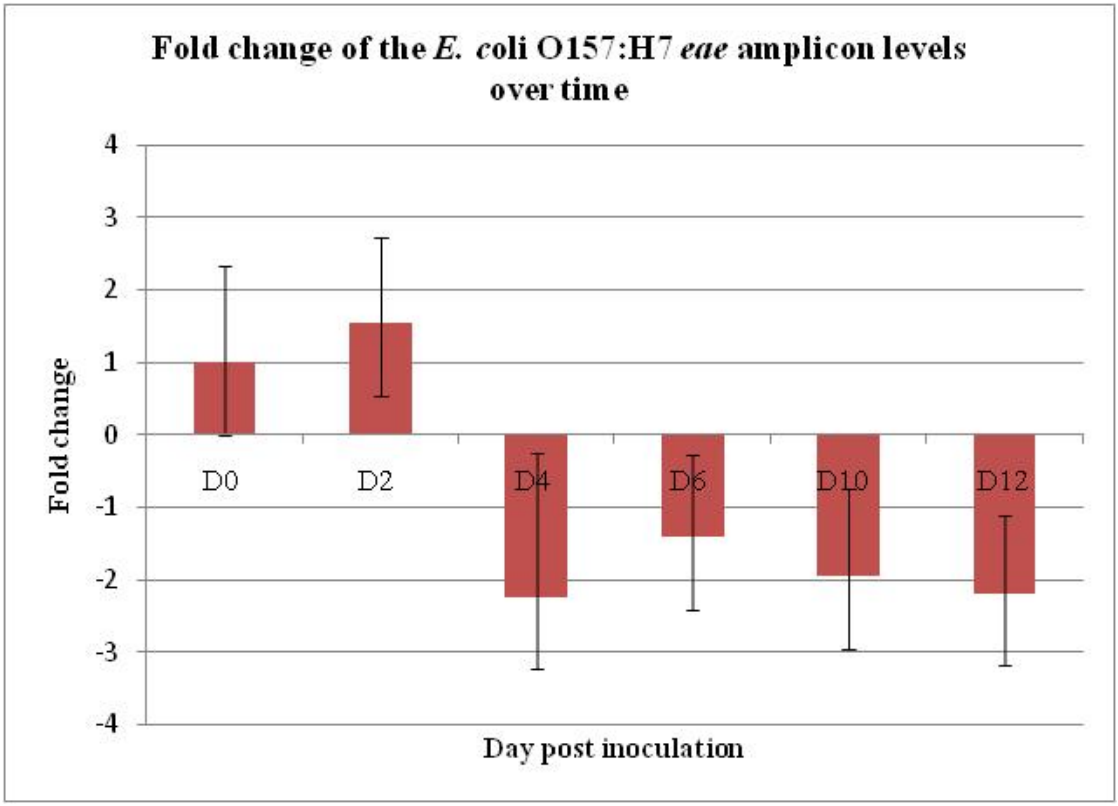

Figure 13. Relative quantitative PCR analysis of *E. coli* O157:H7 *eae* amplicon levels of the manually spotted *E. coli* O157:H7 on the spinach leaf surface over time. Bars represent standard error.

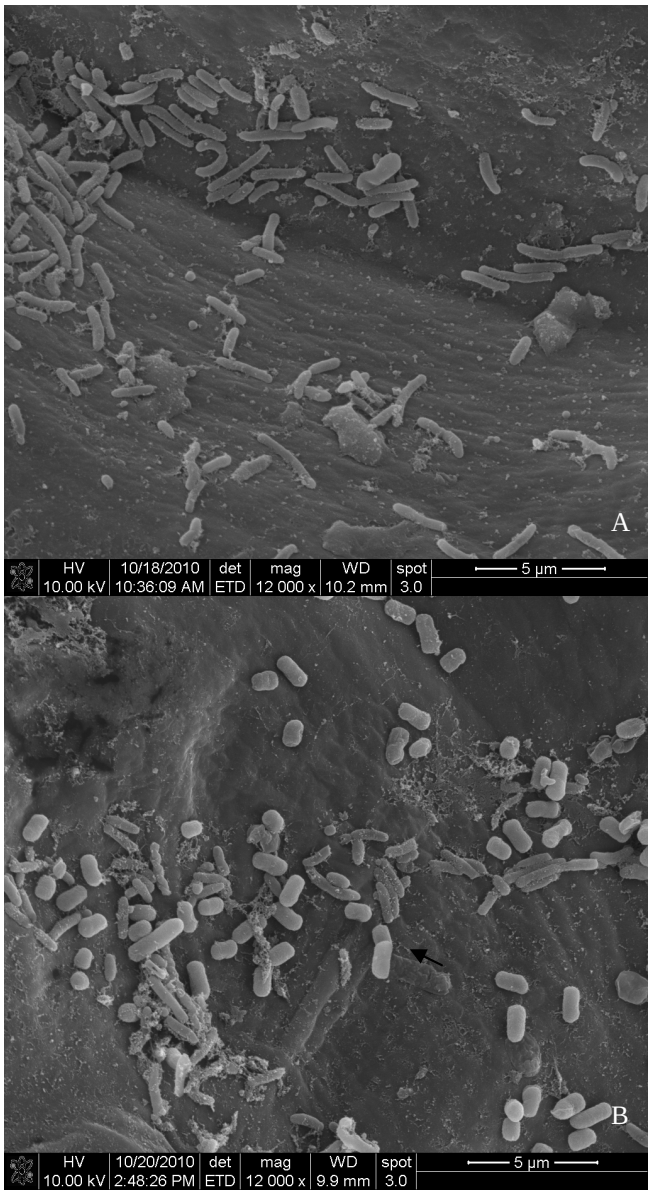

Figure 14. Slender, non-target, rod-shaped bacteria-like organisms observed on the spinach leaf samples. A. Non-target bacteria (arrows) observed on the control (spotting of sterile water on the leaf surface). B. Non-target bacteria (arrows) and the target (*E. coli* O157:H7) observed on the leaf surface of test samples. Magnification = 12,000x.

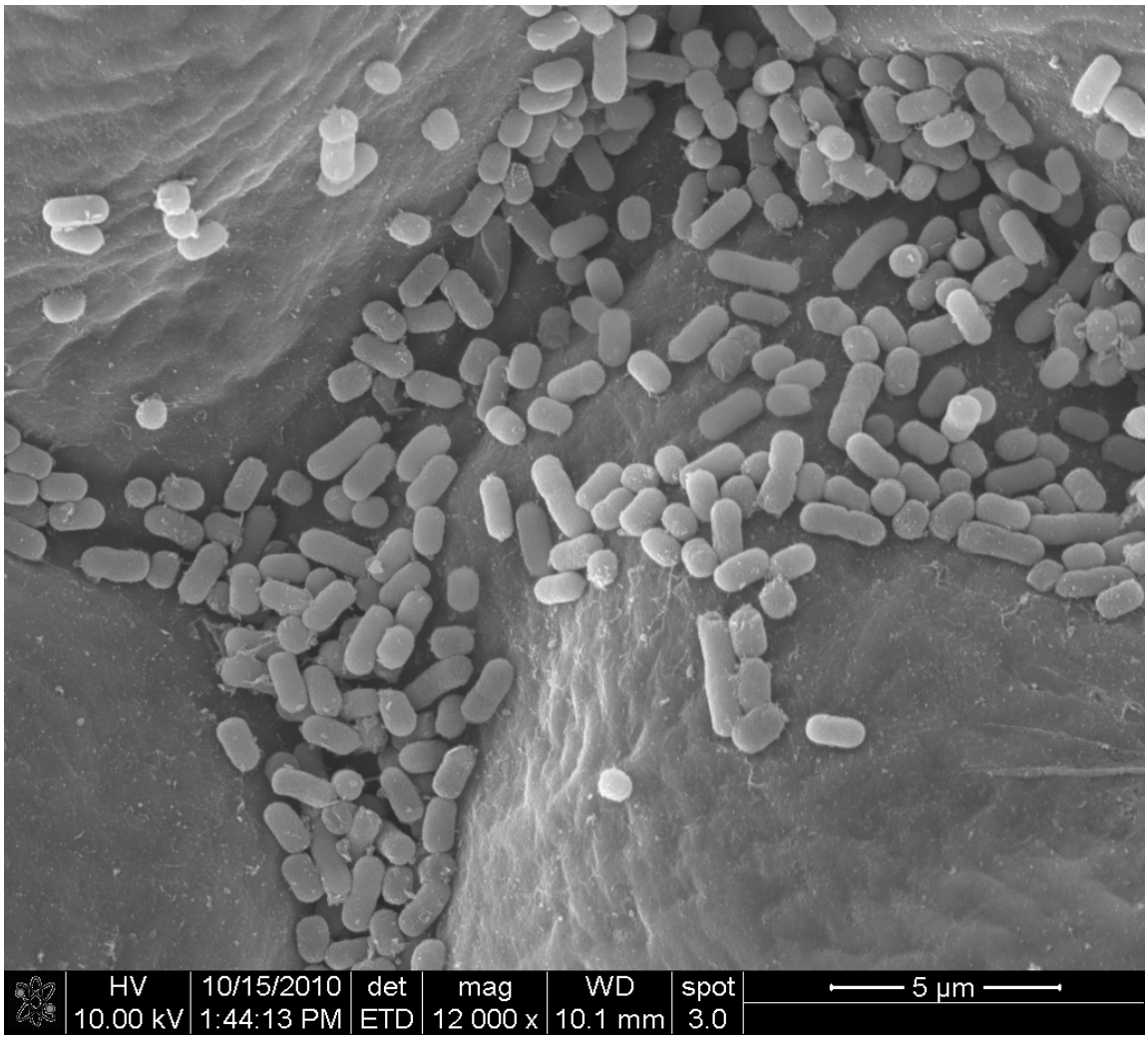

Figure 15. Bacteria-like organisms observed on the *E. coli* O157:H7-spotted spinach surface on Day 0. Note that most of the cell shapes and structures are intact. Magnification = 12,000x.

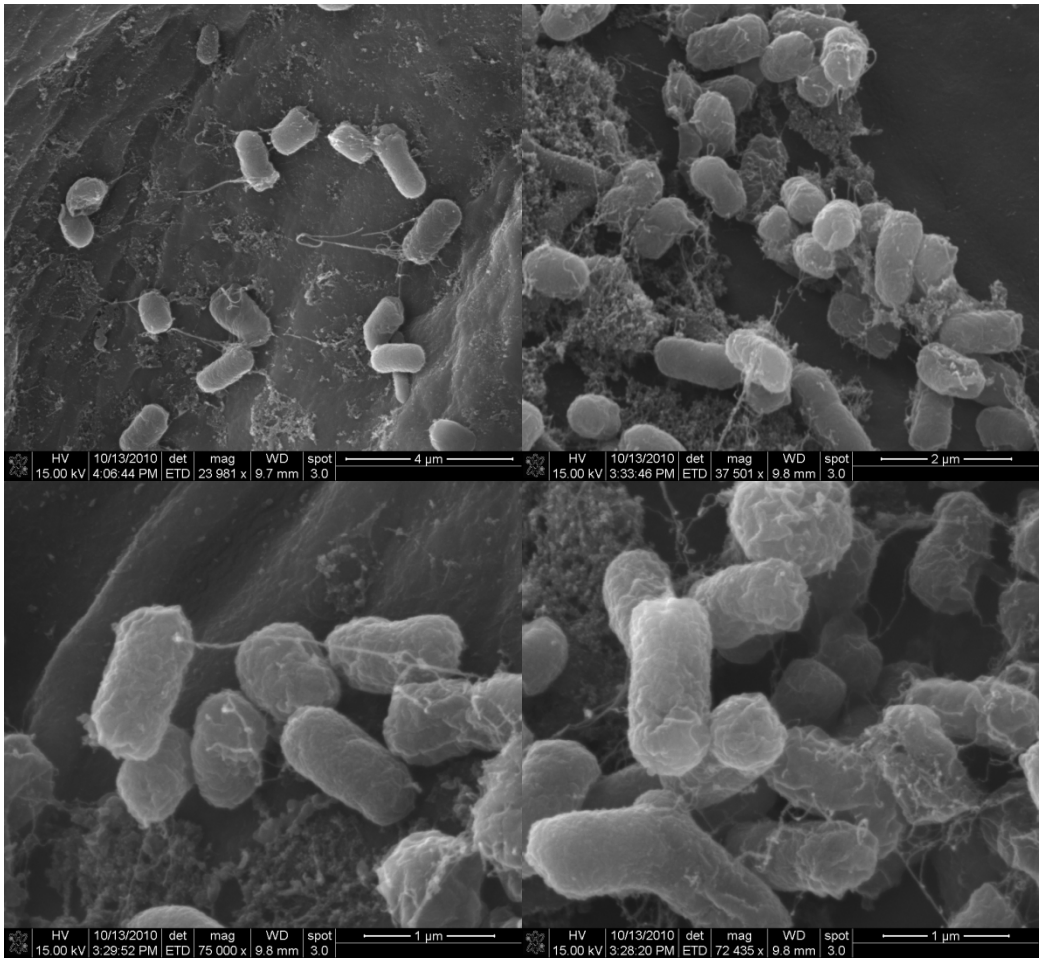

Figure 16. Attachment structures between bacteria and between the bacteria and the plant on *E. coli* O157:H7-spotted leaves on day 0.

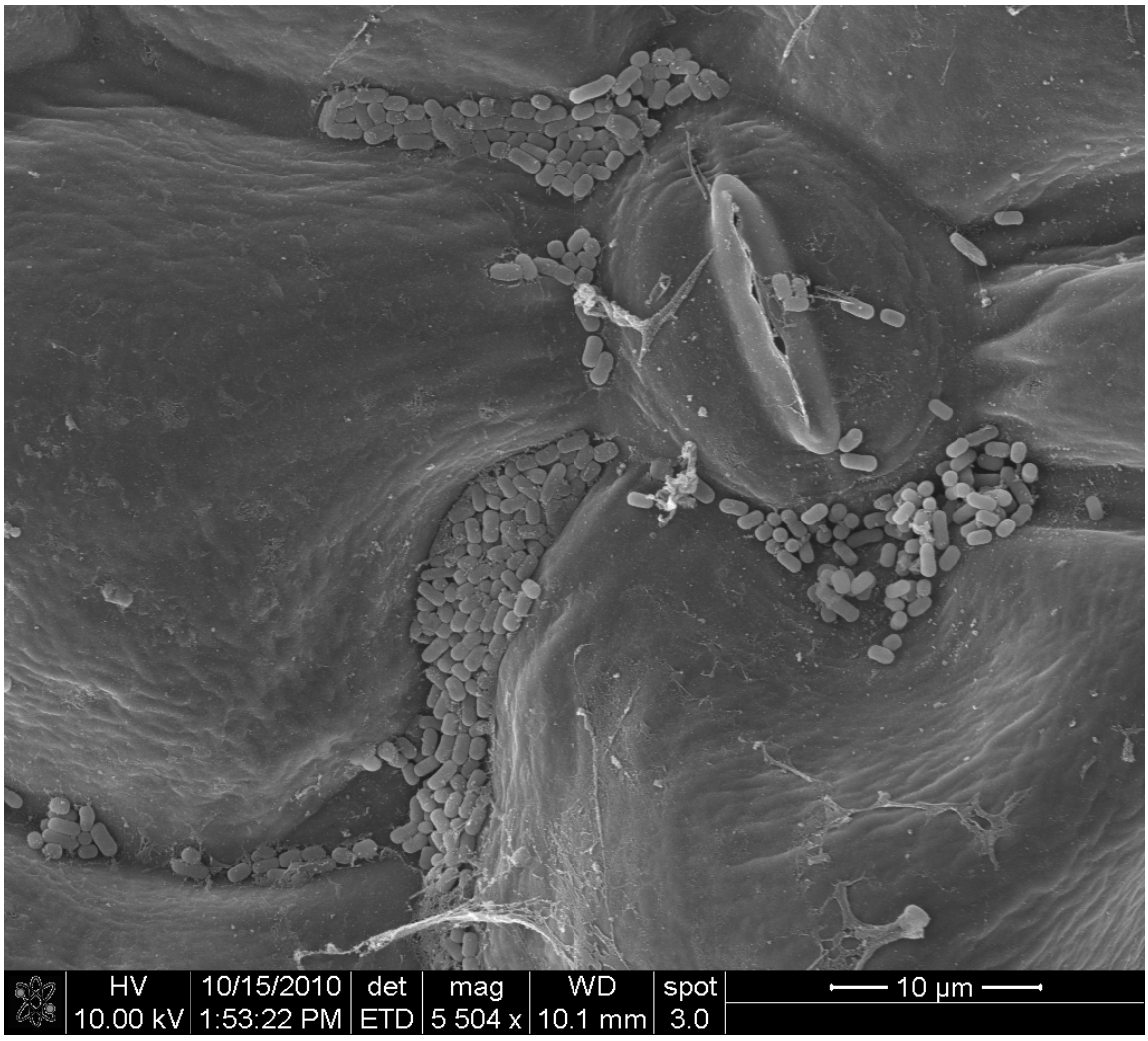

Figure 17. Distribution of bacteria-like organisms on the leaf surface immediately after *E. coli* O157:H7 deposition on day 0. Note that bacteria are observed in aggregates and in the interclinal groves between the epidermal cells. Magnification = 5,504x.

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Figure 18: Bacteria-like organisms observed on spinach 2 days after manual spotting of *E. coli* O157:H7 on the leaf surface. Note the presence of damaged cells (arrows) on the leaf surface. Magnification = 12,000x.

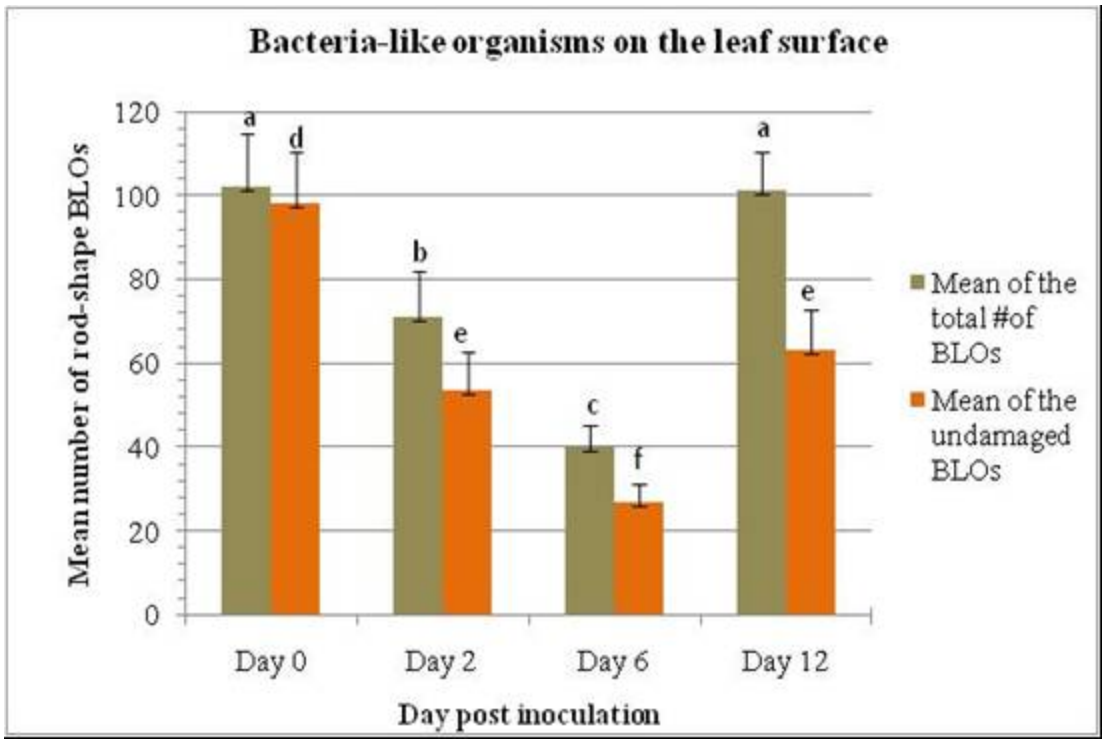

Figure 19. Mean number of rod-shaped bacteria-like organisms observed on the scanning electron microscopy images of spinach leaves spotted with *E. coli* O157:H7. Means with different letters or numbers of + are significantly different ($p<0.05$).

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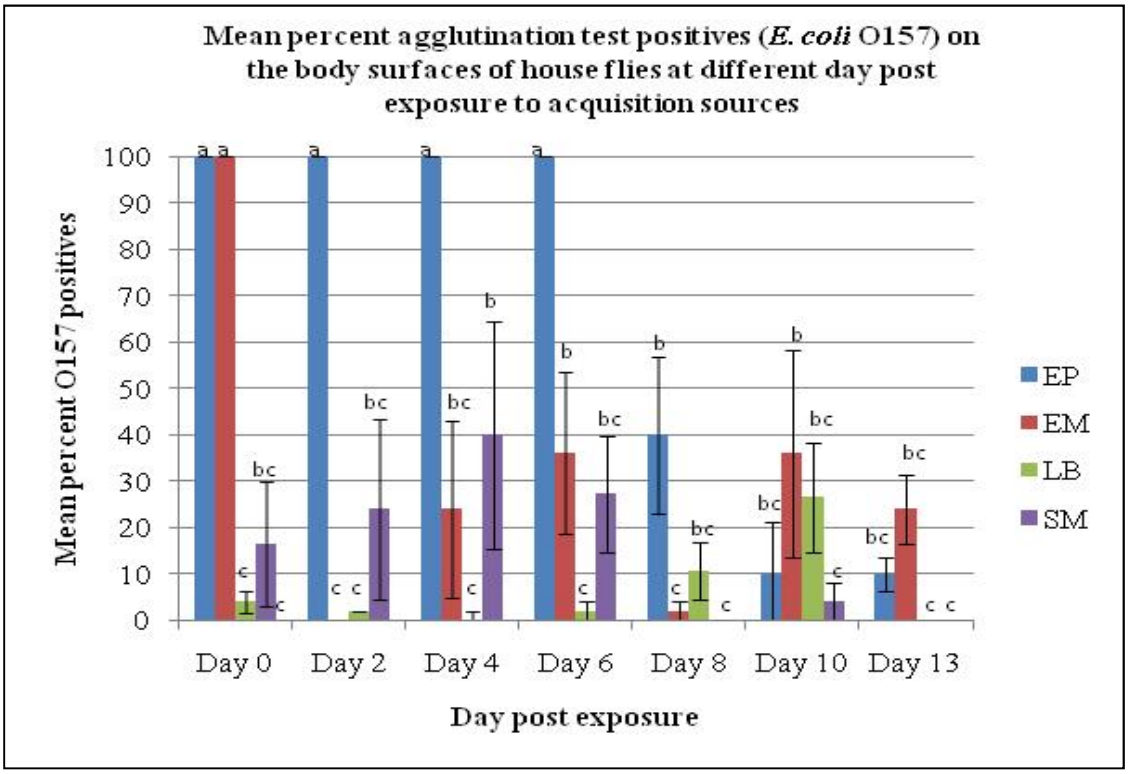

Figure 20. Comparison of mean percent *E. coli* O157 positive colonies obtained from agglutination test from house flies exposed to different acquisition sources. Bars represent standard errors. Means with different letters are significantly different ($p<0.05$). (EP- *E. coli* O157:H7 lawn on LB ampicillin agar, EM- Autoclaved cattle manure mixed with *E. coli* O157:H7 suspension in sterile water, LB- LB ampicillin agar plate, SM- Autoclaved cattle manure mixed with sterile water

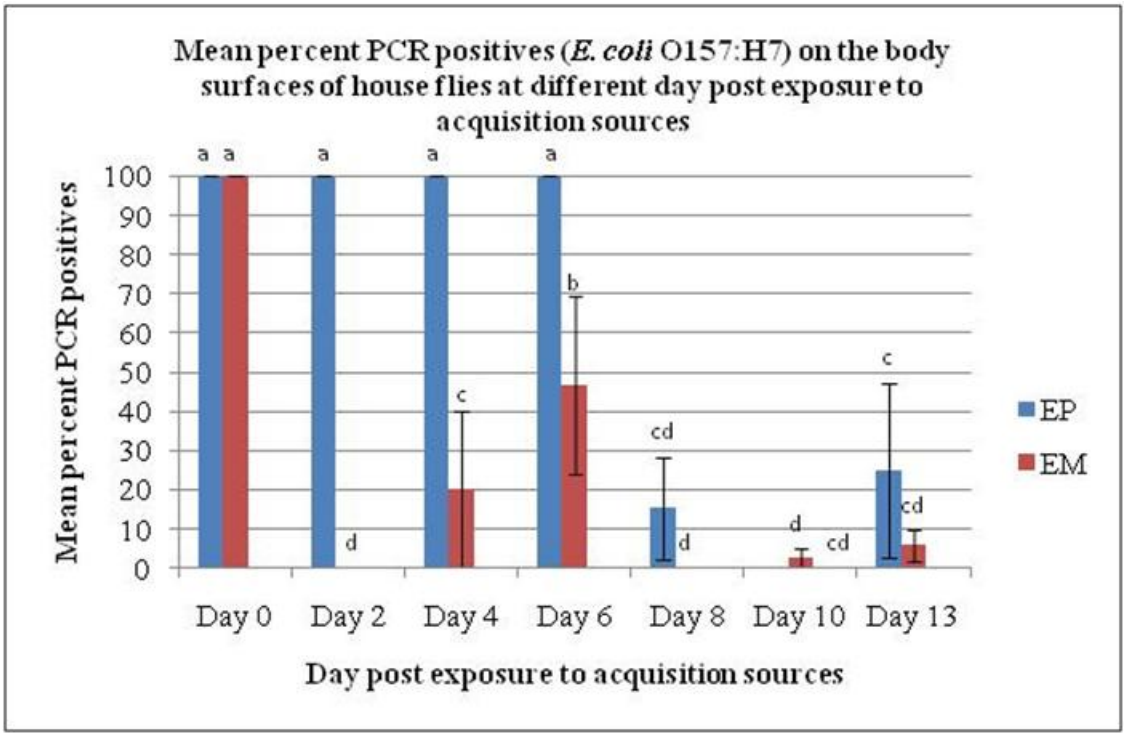

Figure 21: Comparison of mean percent PCR positive colonies (*E. coli* O157:H7) obtained from house flies exposed to different acquisition sources. Bars represent standard errors. Means with different letters are significantly different ($p<0.05$). (EP- *E. coli* O157:H7 lawn on LB ampicillin agar, EM- Autoclaved cattle manure mixed with *E. coli* O157:H7 suspension in sterile water)

Figure 22: Scanning electron micrograph of a house fly tarsus. a. tarsus, b. claw, c. pulvillus. Magnification=350x

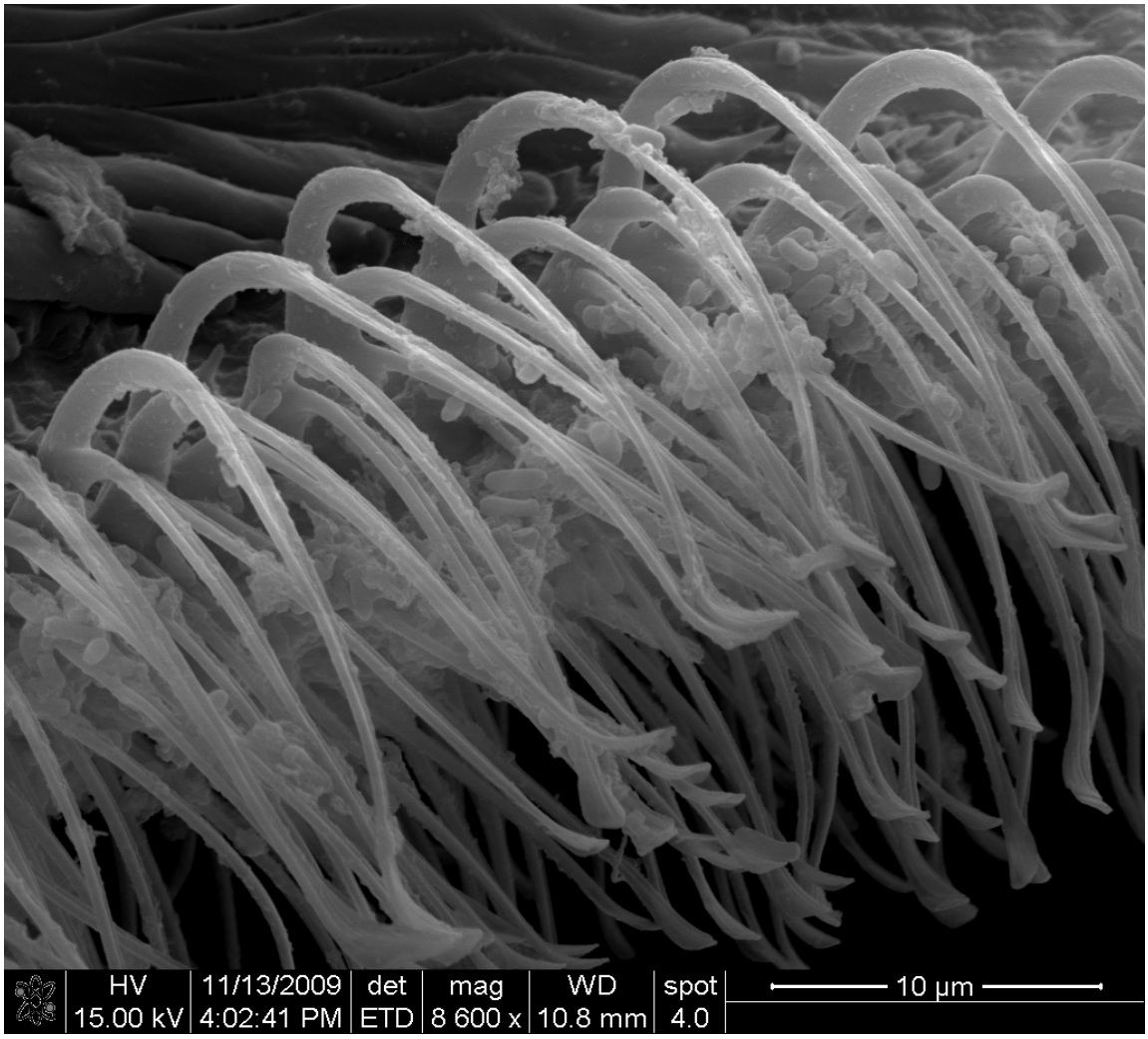

Figure 23. Presence of bacteria-like organisms on the sticky hairs of the pulvillus of a house fly exposed to an *E. coli* O157:H7 lawn. Magnification = 8,600x.

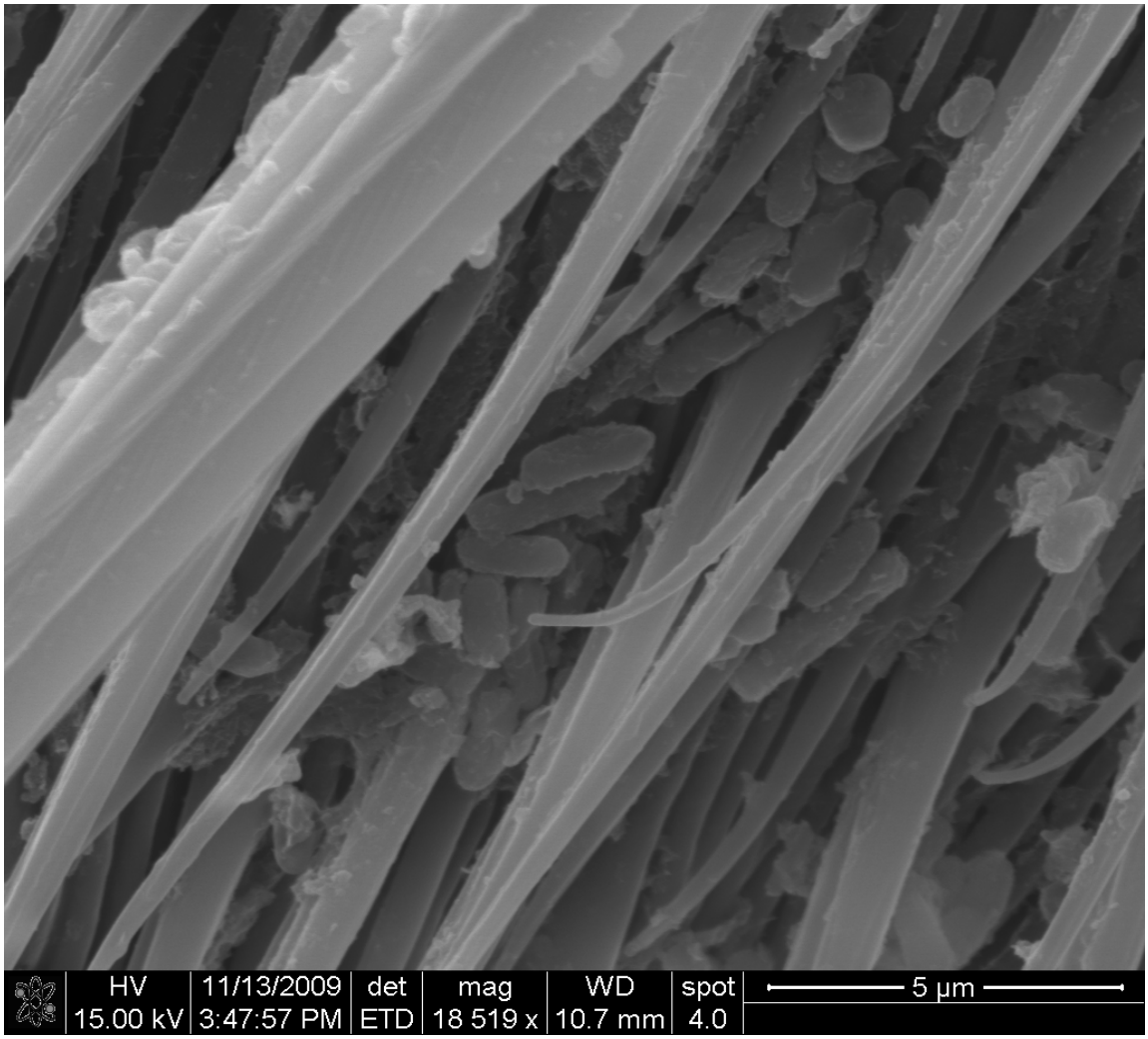

Figure 24. Presence of bacteria-like organisms on the setae (hairs) of the tarsus of a house fly exposed to an *E. coli* O157:H7 lawn. Magnification = 18,600x.

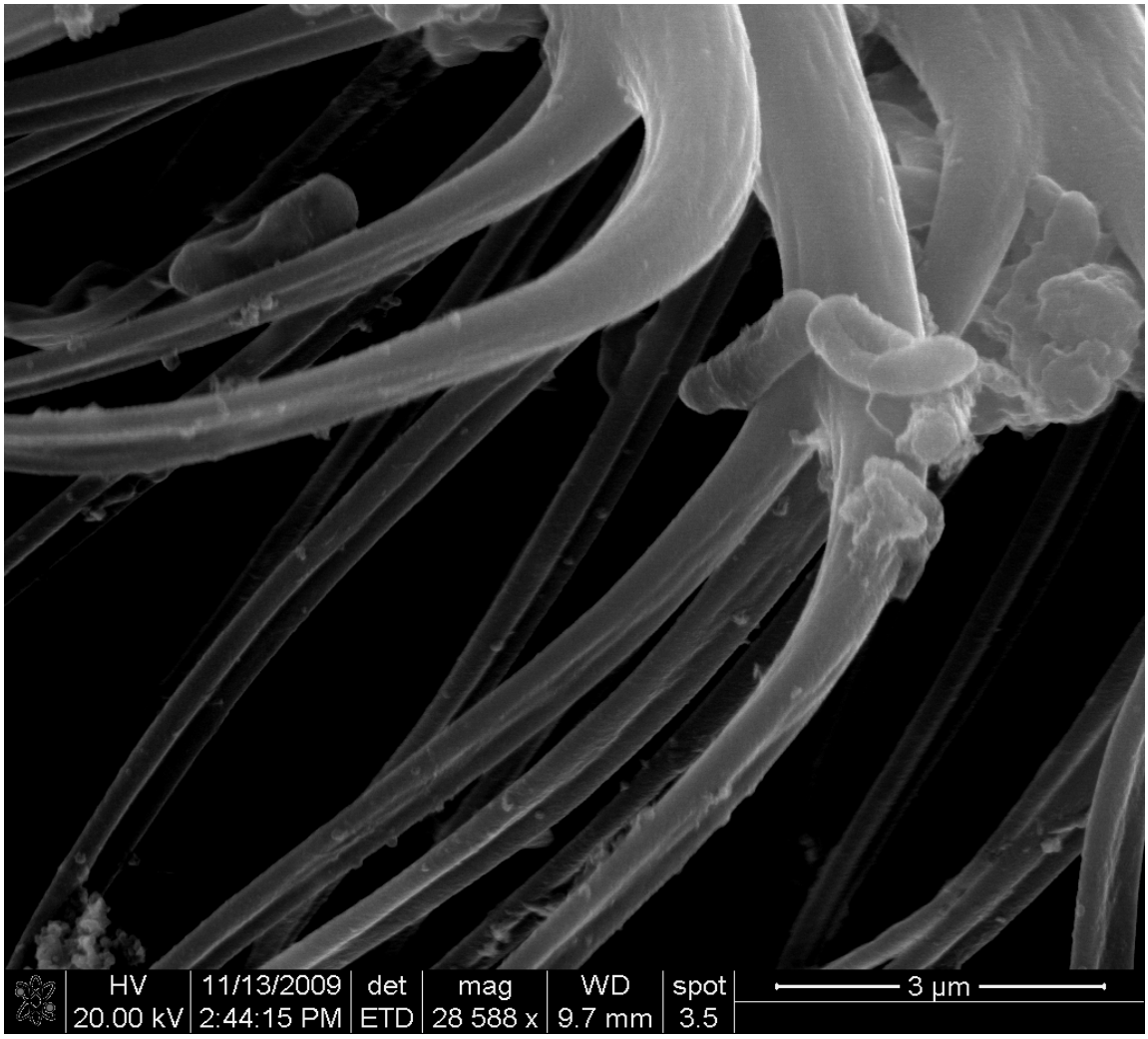

Figure 25. Presence of bacteria-like organisms on the sticky hairs of the pulvillus of a house fly exposed to *E. coli* O157:H7 mixed with manure. Magnification = 28,588x.

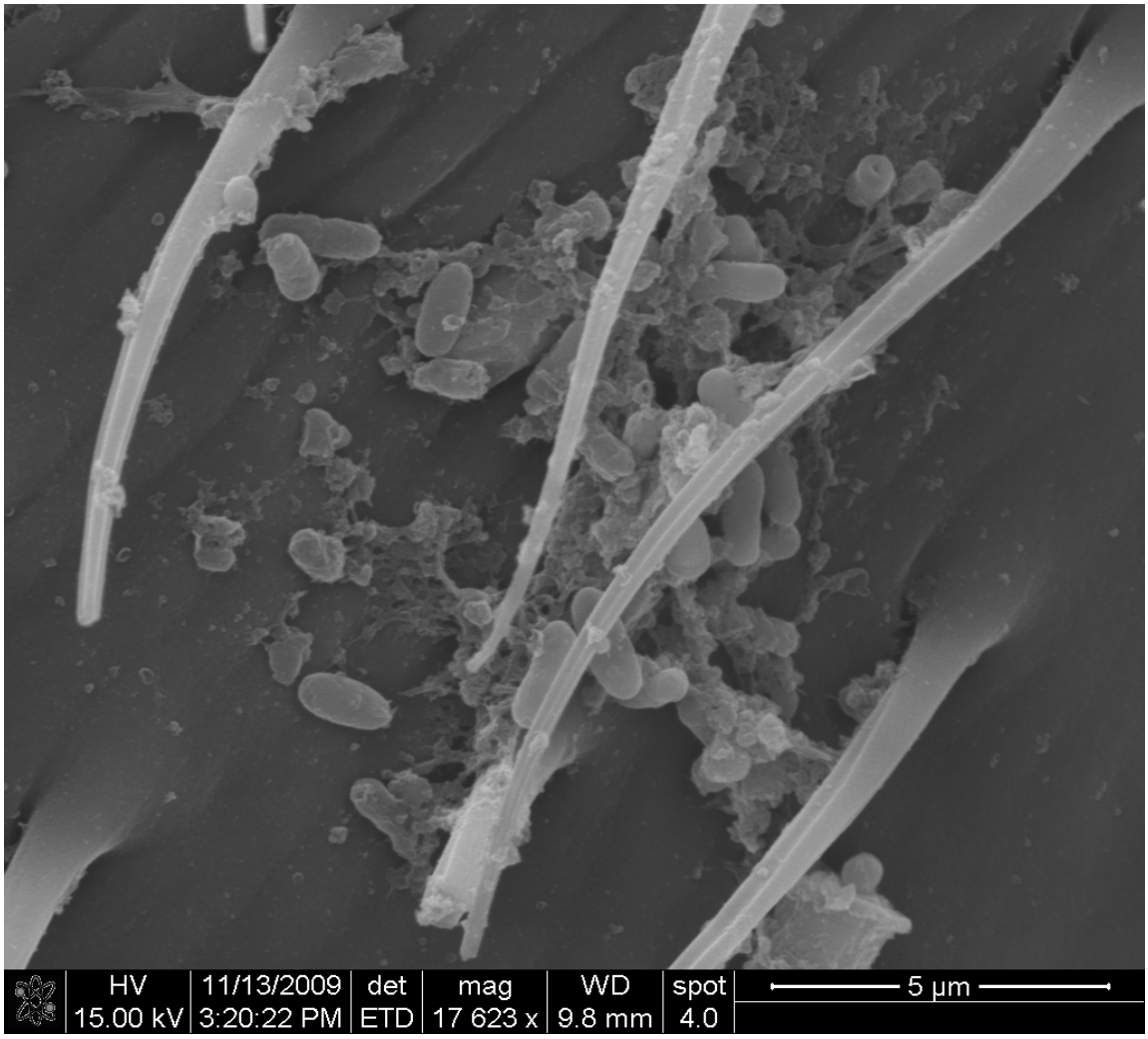

Figure 26. Presence of bacteria-like organisms on the tarsal hairs of the tarsus of a house fly exposed to *E. coli* O157:H7 mixed with manure. Magnification = 17,623x.

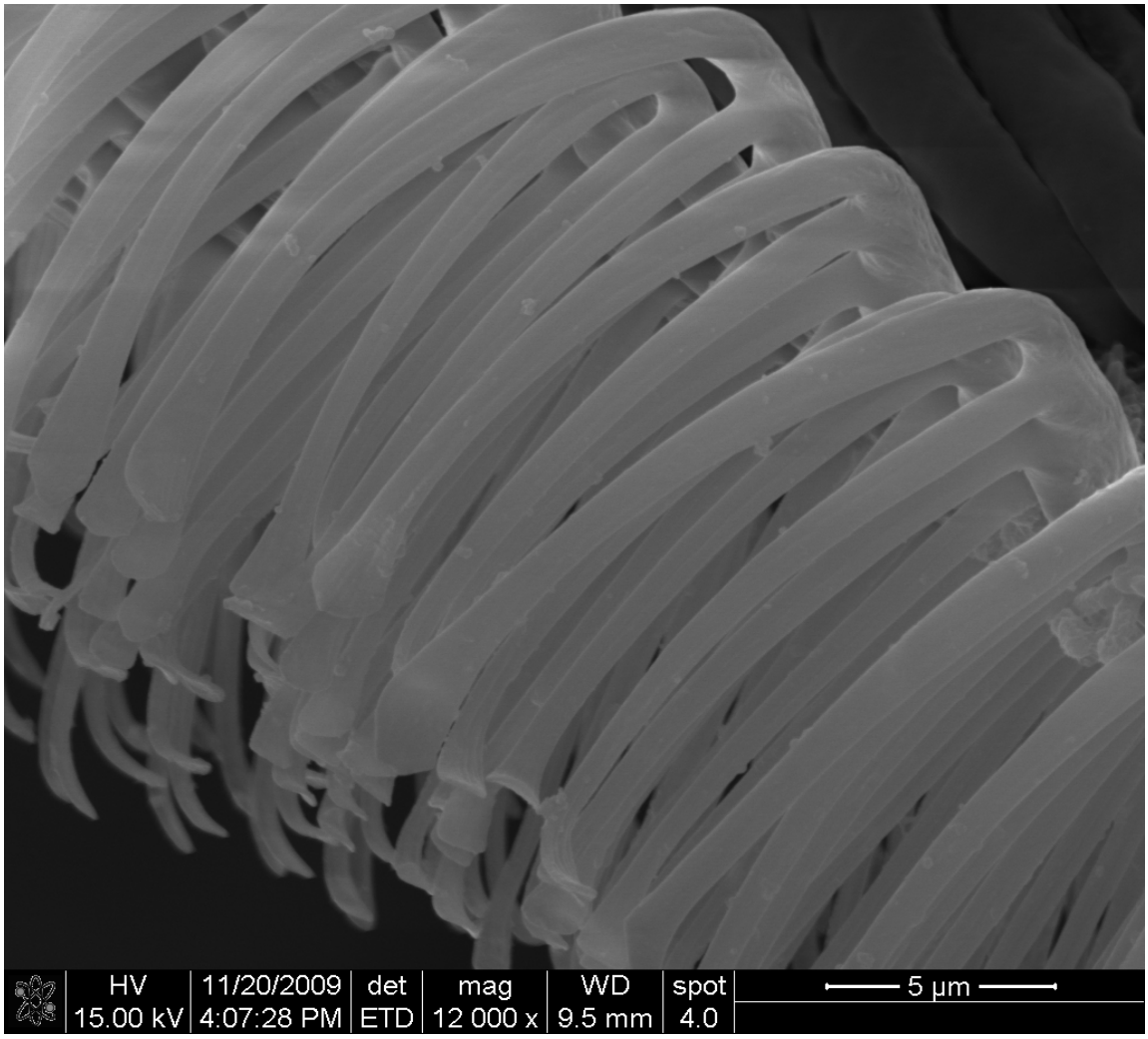

Figure 27. Pulvillus of a house fly exposed to LB ampicillin agar showing no bacteria-like organisms. Magnification = 12,000x.

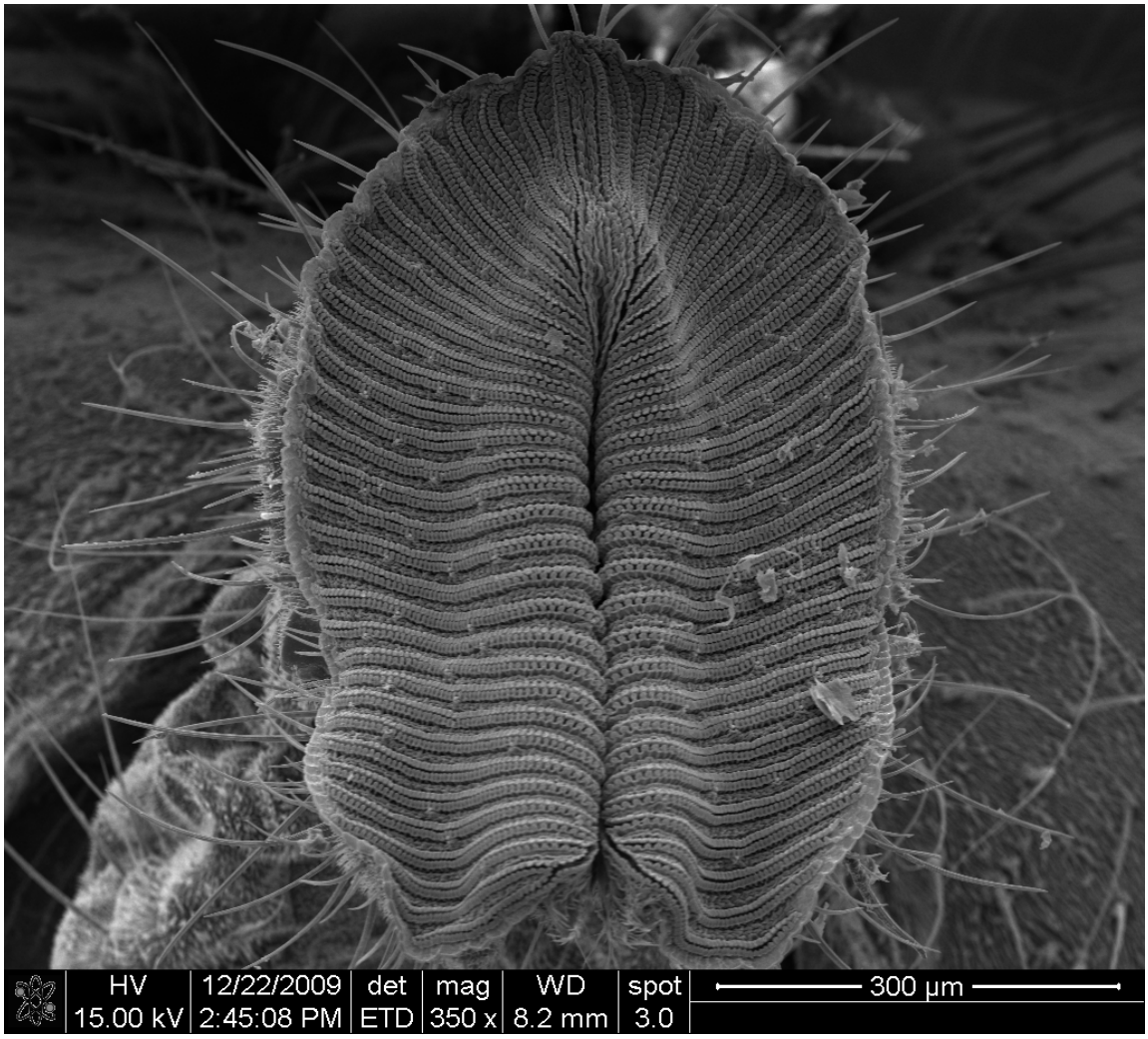

Figure 28. Labellum of a house fly showing numerous pseudotracheae. Magnification = 350x.

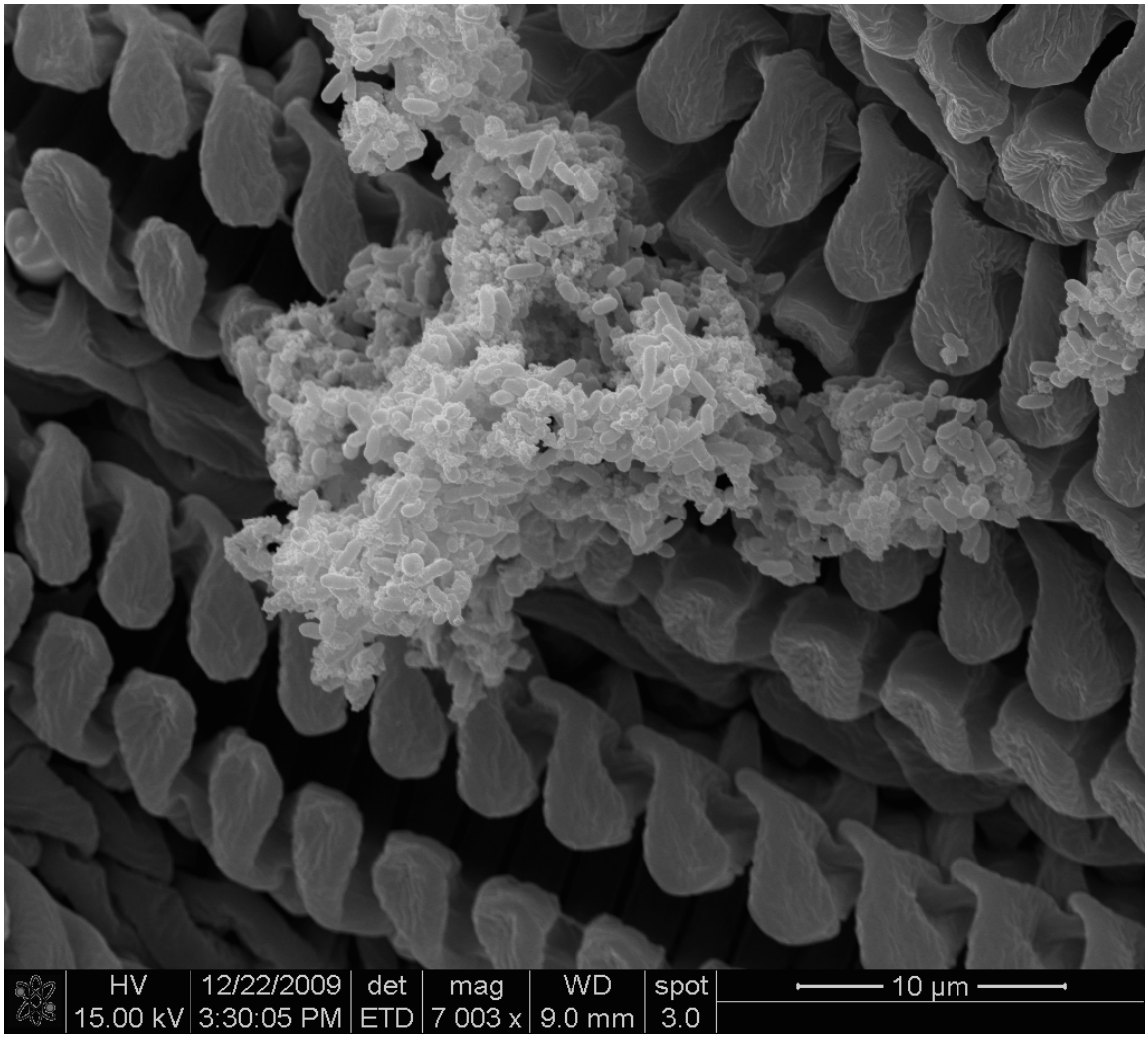

Figure 29. Aggregates of bacteria-like organisms observed on the pseudotracheae of the labellum of a house fly exposed to an *E. coli* O157:H7 lawn. Magnification = 7,003x.

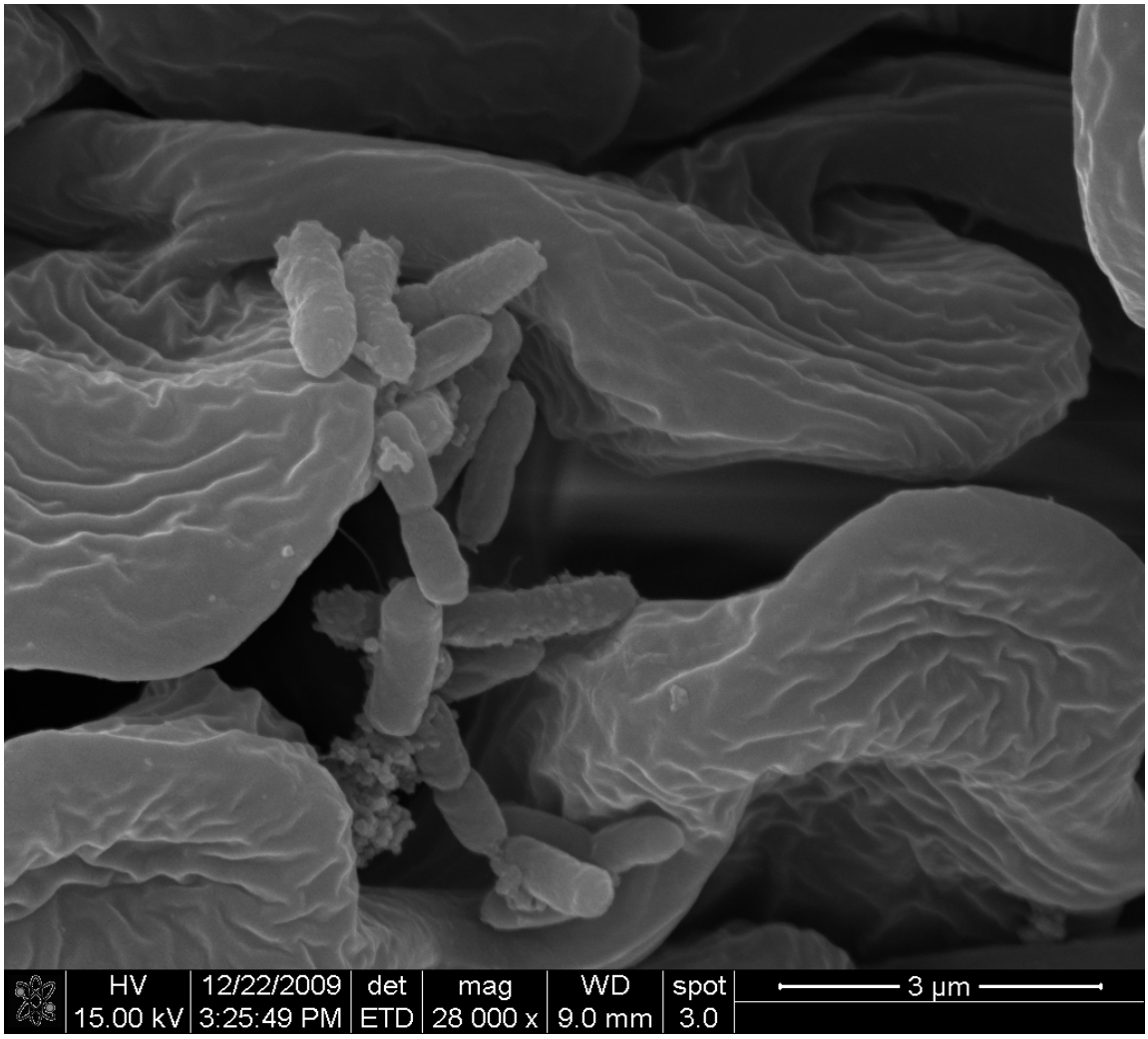

Figure 30: Division of bacteria observed within the grooves of the pseudotracheae of the labellum of a house fly exposed to an *E. coli* O157:H7 lawn. Magnification = 28,000x

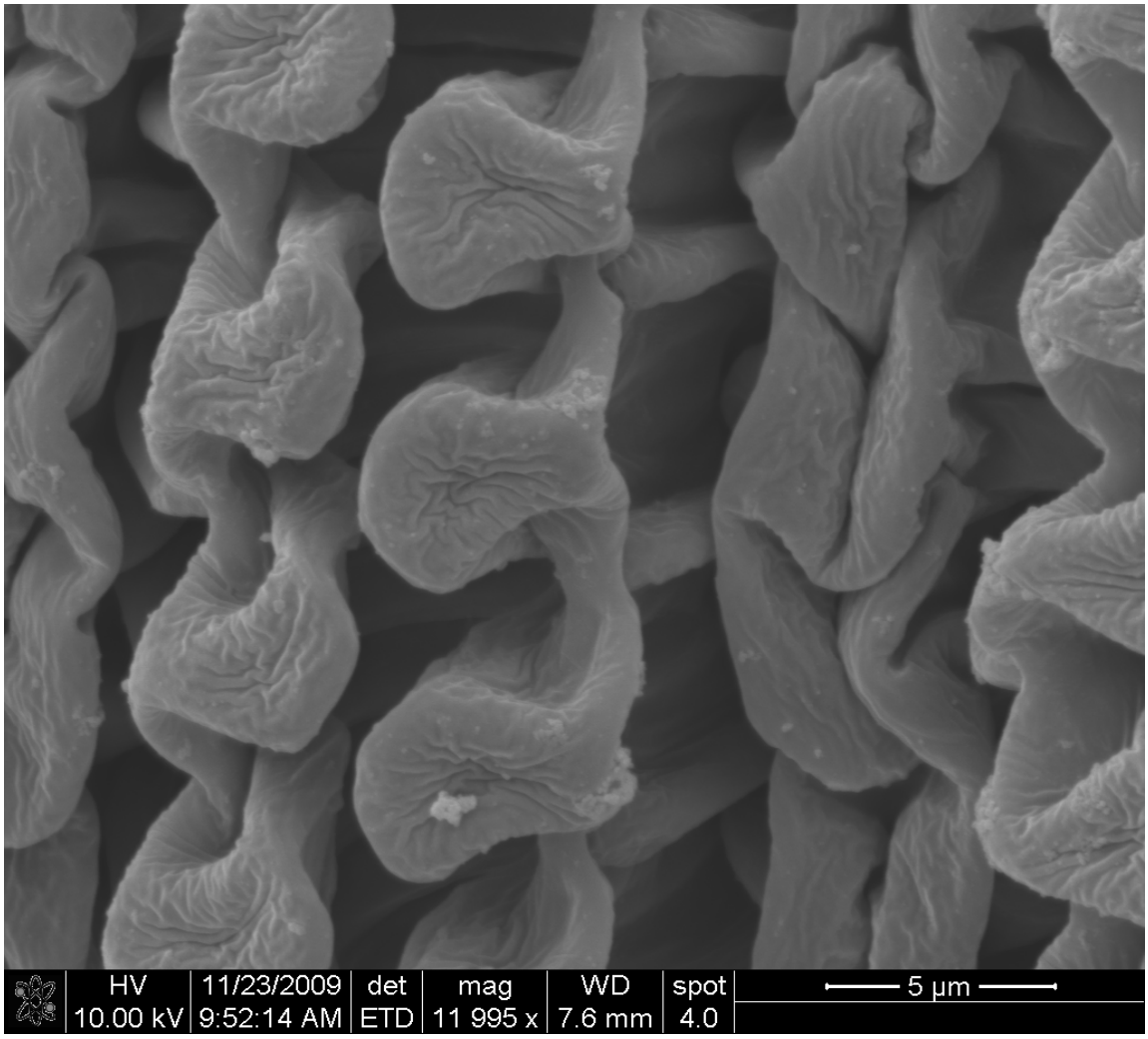

Figure 31: Pseudotrachea of a house fly labellum exposed to *E. coli* O157:H7 mixed with manure. Note the lack of bacteria-like organisms. Magnification = 11,995x.

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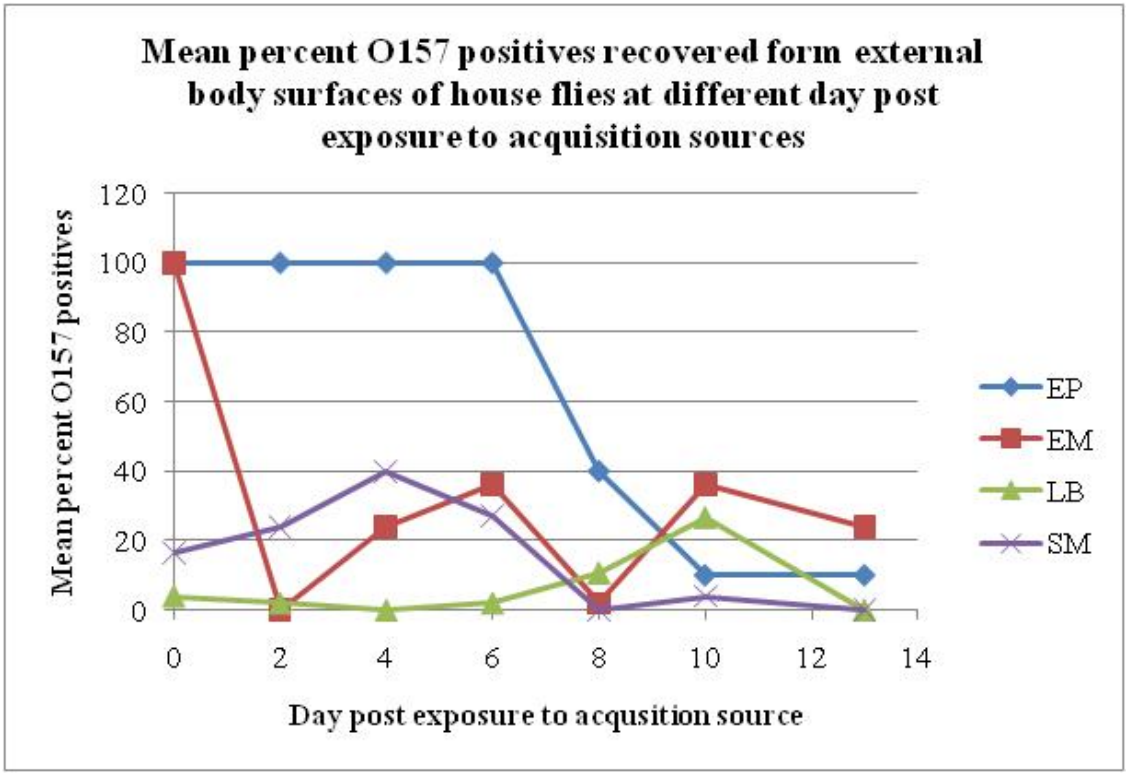
APPPENDIX

Mean dCt values and fold change values obtained from the qPCR analysis of the regurgitation spots of house flies exposed to different acquisition sources

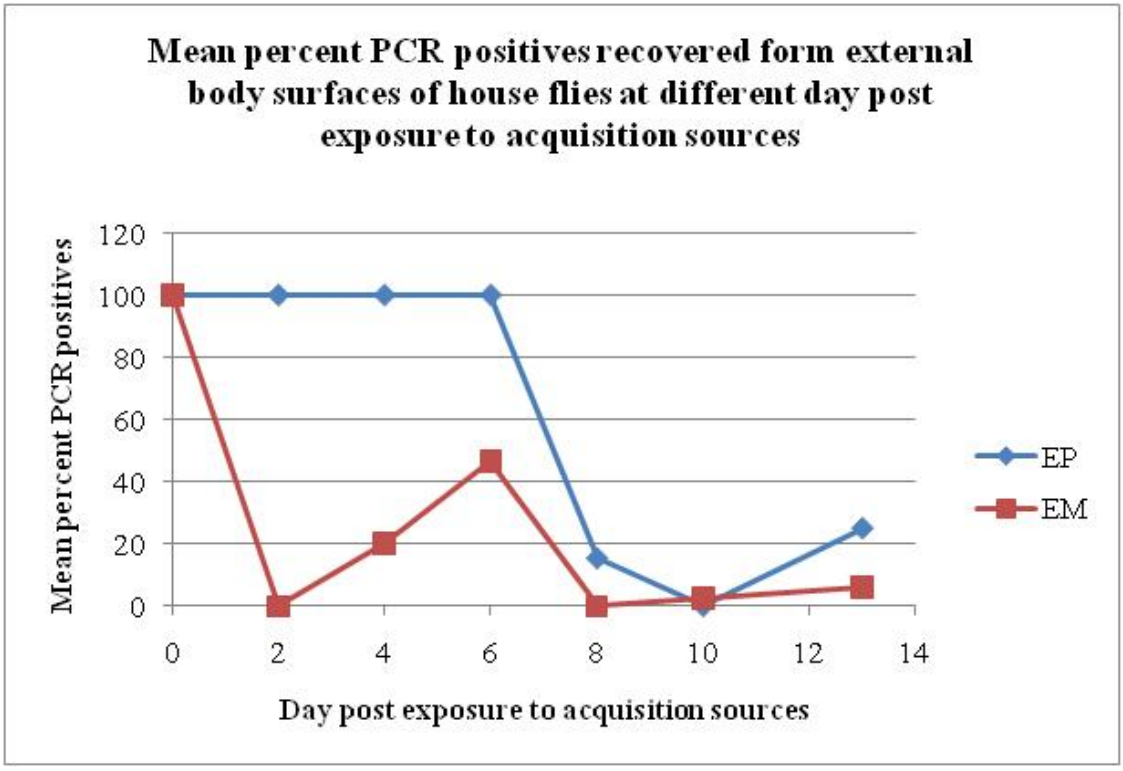
trt	ID	dCt	ddCt	fold change	correction of fold		
1	EM Day 0	12.52	0	1		1	1
2	EM Day 4	8.305	-4.215	18.5712626		18.57126	18.57126
3	EM Day 8	9.3775	-3.1425	8.83052982		8.83053	8.83053
4	EP Day 0	3.025	0	1		1	1
5	EP Day 4	4.4325	1.4075	0.37696435	-1.4075	2.652771	-2.65277
6	EP Day 8	4.78	1.755	0.29627319	-1.755	3.375263	-3.37526
7	SM Day 0	13.582	0	1		1	1
8	SM Day 4	13.506	-0.076	1.05409142		1.054091	1.054091
9	SM Day 8	12.58667	-0.99533	1.99354103		1.993541	1.993541
10	LB Day 0	15.77	0	1		1	1
11	LB Day 4	14.56	-1.21	2.31337637		2.313376	2.313376
12	LB Day 8	13.35	-2.42	5.35171022		5.35171	5.35171

Mean dCt values and fold change values obtained from the qPCR analysis of the manually spotted *E. coli* O157:H7 on spinach leaves at different time points

Day	dCt	ddCt	correction	fold change
D0 E	-0.08	0		1
D2 E	-0.70167	-0.62167		1.5386517
D4 E	1.08	1.16	-1.16	2.2345743
D6 E	0.416667	0.496667	-0.496667	1.4109501
D10 E	0.89	0.97	-0.97	1.9588406
D12 E	1.048333	1.128333	-1.128333	2.18606



Comparison of mean percent *E. coli* O157 positive colonies obtained from agglutination test from house flies exposed to different acquisition sources (df=27,138, F=11.2, $p<0.0001$). Bars represent standard errors. (EP- *E. coli* O157:H7 lawn on LB ampicillin agar, EM- Autoclaved cattle manure mixed with *E. coli* O157:H7 suspension in sterile water, LB- LB ampicillin agar plate, SM- Autoclaved cattle manure mixed with sterile water)



Comparison of mean percent PCR positive colonies (*E. coli* O157:H7) obtained from house flies exposed to different acquisition sources. Bars represent standard errors. (EP- *E. coli* O157:H7 lawn on LB ampicillin agar, EM- Autoclaved cattle manure mixed with *E. coli* O157:H7 suspension in sterile water)

VITA

Lakmini Priyangika Wasala

Candidate for the Degree of

Master of Science

Thesis: DISSEMINATION OF *ESCHERICHIA COLI* O157:H7 TO THE SPINACH, *SPINACEA OLERACEA* PHYLLOPLANE BY HOUSE FLIES, *MUSCA DOMESTICA* L.

Major Field: Plant Pathology

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International Association for Food Protection
American Association for the Advancement of Science
Golden Key International Honor Society

Name: Lakmini P. Wasala Date of Degree: December, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DISSEMINATION OF *ESCHERICHIA COLI* O157:H7 TO THE SPINACH, *SPINACEA OLERACEA* PHYLLOPLANE BY HOUSE FLIES, *MUSCA DOMESTICA* L.

Pages in Study: 94 Candidate for the Degree of Master of Science

Major Field: Plant Pathology

Scope and Method of Study: Insects may be involved in the dissemination of human pathogens to fresh produce. This study aimed to determine if *E. coli* O157:H7 could colonize the spinach phylloplane via regurgitation spots deposited by house flies after exposure to *E. coli* O157:H7 acquisition sources. Attachment and colonization of *E. coli* O157:H7 on the external body surfaces of the house flies was also studied. Flies were exposed to different acquisition sources with and without *E. coli* O157:H7. Exposed flies were transferred to spinach plants and the regurgitation spots on days 0, 4 and 8 were analyzed by relative quantitative PCR. Also bacteria were spotted onto spinach leaves subjected to relative qPCR to understand the fate of the *E. coli* O157:H7 without the insect involvement on the phylloplane from 0-12 days post inoculation. Exposed fly legs and heads were dissected and *E. coli* O157:H7 were enumerated by microbiological methods from 0-13 days post exposure. Mouthparts and legs of bacteria-exposed flies were examined by scanning electron microscopy.

Findings and Conclusions: The relative qPCR of the regurgitation spots showed that the *E. coli* O157:H7 DNA concentration increased on day 4 which suggested that the bacteria multiplied within the regurgitation spots when flies acquired the bacteria from inoculated manure. The relative qPCR of the artificial spotting did not show any significant change in the *E. coli* O157:H7 DNA levels on the phylloplane. *E. coli* O157:H7 persisted on the fly external body surfaces for 13 days and a colonization period was suggested from days 2-6. Overall the research data suggested that fly regurgitation is an important mode of human pathogen dissemination under laboratory conditions. Regurgitant may be a potential nutrition source for the bacteria. Body hairs and pseudotracheae may be potential niches for the bacteria to survive until they reach a more suitable environment. These data show the potential of house flies to contaminate spinach under laboratory conditions. Future studies will be aimed on to study the bacterial survival after regurgitation under field conditions.

ADVISER'S APPROVAL: Dr. Astri Wayadande