

## Synopsis 1

### **Subsurface contamination and internalization of *Escherichia coli* O157:H7 in pre-harvest lettuce**

Principal Investigator: Dr. Michael P. Doyle, Center for Food Safety, University of Georgia

Lettuce is one of the most commonly consumed leafy greens, with a farm value of over \$1.5 billion in 2005 in the United States. Contamination of vegetables by human pathogens can occur throughout the farm-to-fork continuum. Several outbreaks of Salmonella and *Escherichia coli* O157:H7 infections have been associated with consumption of fresh-cut leafy greens. Questions remain regarding the ability of these pathogens to become internalized within lettuce and spinach. Understanding whether or not internalization of foodborne pathogens occurs through plant roots and leaves will be helpful in conducting risk assessments and developing effective interventions to reduce pathogen contamination in produce.

The objectives of this project were (1) to label *E. coli* O157:H7 isolates with Green Fluorescent Protein (gfp) genes for lettuce studies; (2) to establish a root and shoot surface-disinfection method; (3) to determine survival and possible internalization of *E. coli* O157:H7 in lettuce as a function of site of contamination (abaxial versus adaxial leaf surfaces) and presence of soluble organic matter; (4) to determine strain differences among *E. coli* O157:H7 isolates to internalize, colonize, survive and grow in and on lettuce plants; (5) to determine the degree of internalization of *E. coli* O157:H7 and its subsequent survival and growth in different types of lettuce and at different phases of the plants growth cycle; (6) to evaluate influence of heat stresses (in combination with drought) on internalization of *E. coli* O157:H7 via the root system; (7) identify level of cross-contamination of *E. coli* O157:H7 from contaminated field coring tools to iceberg lettuce; (8) to determine the effect of insect damage on internalization of *E. coli* 157:H7 through contaminated lettuce leaves.

A plasmid-containing gfp gene was introduced into five strains of *E. coli* O157:H7 (ATC43888, EO122, K3995, K4992, F4546) through a CaCl<sub>2</sub>-heat shock method. These isolates are being used in subsequent studies outlined in our project as well as in studies at Oklahoma State University and UGA-Athens for projects funded by Fresh Express. Thirteen surface-disinfection methods were compared for their efficacy in killing *E. coli* O157:H7 on lettuce leaf and root surfaces using a 5-strain mixture of these gfp-labeled *E. coli* O157:H7. Dipping lettuce leaves or roots in 80% ethanol for 10 sec followed by immersion in 0.1% HgCl<sub>2</sub> for 10 min was determined to be the most effective surface-disinfection method for inactivating *E. coli* O157:H7 on lettuce leaves and roots, and was also validated for inactivating Salmonella and *L. monocytogenes*. This selected surface-disinfection method was used for the following internalization study. Romaine, iceberg, and leaf lettuces were grown in sandy soil in an envirotron to study internalization of *E. coli* O157:H7. Five strains of gfp-labeled *E.*

*E. coli* O157:H7 were used individually for soil inoculation study and as a mixture for leaf inoculation and heat-stress studies. Leaves were inoculated with 103 or 106 *E. coli* O157:H7 CFU/ml water or manure extract. In the first leaf inoculation trial, plants were inoculated on abaxial and adaxial sides of leaf surfaces at 3, 30, 60 days after transplantation and sampled at 19 and 54 days, 3, 7 and 25 days, and 3 and 7 days after inoculation, respectively. In the second leaf inoculation trial, plants were inoculated on abaxial and adaxial sides of leaf surfaces 7 days after transplantation and sampled at 15, 26, 35 and 44 days after inoculation. Inoculated *E. coli* O157:H7 survived longer on abaxial side of the leaves than on adaxial side. *E. coli* O157:H7 could survive in soil for at least 9 weeks. *E. coli* O157:H7 can survive on leaves for at least 25 days when exposed to pathogen 30-days post-transplantation. No *E. coli* O157:H7 were detected on leaves 54 days after inoculation when exposed to pathogen 3-days post-transplantation. Soil was inoculated at 103 and 106 *E. coli* O157:H7 CFU/g soil when lettuce plants were transplanted. In the first soil inoculation trial, lettuce plants and pot soil were sampled at 17, 45, and 60 days after transplantation. In the second soil inoculation trial, lettuce plants and pot soil were sampled at 26 and 60 days after transplantation. To evaluate the effect of heat stresses on internalization of *E. coli* O157:H7 via the root system, Romaine and iceberg lettuce were inoculated via pot soil at 103 and 106 *E. coli* O157:H7 CFU/g soil 35 days after transplantation. They were exposed to 32°C (90°F) during the day and 15.5°C (60°F) at night for 3 days after inoculation; or 36°C (97°F) during the day and 15.5°C (60°F) at night for 2 days after inoculation. Two water levels (wet and dry) were compared. *E. coli* O157:H7 assay was conducted for soil, leaf surface, ground leaves, rhizosphere, and ground roots. Internalization of *E. coli* O157:H7 via leaf or soil inoculation did not occur in lettuce regardless of plant age. Heat and water stresses did not enhance internalization of *E. coli* O157:H7 in lettuce.

Results revealed that there was little difference among bacterial isolates or lettuce types in *E. coli* O157:H7 internalization.

The field-core (cut-and-core) harvesting technique used for iceberg lettuce was evaluated as a potential means of cross-contamination with *E. coli* O157:H7. Chlorinated water was evaluated for its efficacy in removing or inactivating the pathogen on the blade portion of the field-coring device and on cored lettuce. The blade was immersed in soil containing *E. coli* O157:H7 at 3.74 or 6.57 log CFU/g; this resulted in 3.13 and 4.97 log CFU/blade, respectively. Treatment of inoculated blades by immersing in chlorinated water (200 µg/ml, total chlorine) for 10 s resulted in a reduction of 1.56 log CFU/blade, which was 1.42 log CFU/blade greater than achieved using water, but insufficient to eliminate the pathogen. Blades inoculated by contacting soil containing *E. coli* O157:H7 at 2.72 and 1.67 log CFU/g, then repeatedly used to cut and core ten lettuce heads, transferred the pathogen to ten and five consecutively processed heads, respectively. Lettuce cores remained positive for the pathogen after spraying with 100 µg/ml free chlorine for 120 s at 2.81 kg/cm<sup>2</sup> (40 psi), regardless of the inoculum level. The number of *E. coli* O157:H7 recovered from inoculated lettuce cores sprayed for 10 s with 200 ml of chlorine (100 µg/ml) was significantly ( $P < 0.05$ ) less than the number recovered from tissues treated with water. Dipping contaminated blades in chlorinated water may not be effective in killing the pathogen and controlling cross-contamination from head to head. Spraying

contaminated lettuce with chlorinated or untreated water reduces but does not eliminate *E. coli* O157:H7.

Forty-eight hours following mist application of *E. coli* O157:H7 to the top (adaxial) side of leaves, surface populations were 6.5 to 7.5 log CFU/leaf; however, recovery of internalized cells by enrichment also occurred in 3 of 10 and 4 of 10 lettuce and spinach leaves, respectively. Higher incidences of internalized *E. coli* O157:H7 occurred when the pathogen was applied as a mist to the under (abaxial) side of leaves. In these cases, the pathogen could be detected through enrichment in 7 of 10, 9 of 10, and 9 of 10 leafy green lettuce, Bibb lettuce, and spinach leaves, respectively. Increased internalization of *E. coli* O157:H7 was observed when the abaxial side of leaves was contaminated by spreading small droplets of inoculum gently over the leaf surface with a glass rod. It is hypothesized that this procedure either distributes the pathogen to more stomata or the gentle rubbing action causes abrasion of the surface and provides access of the pathogen to the underlying tissue. At 48 h after rubbing contaminated spinach and lettuce leaves, internalized populations were 2.7 log and 3.2 log CFU/leaf, respectively. Exposure of surface-contaminated leaves to insects (cabbage loopers, aphids, whiteflies, and thrips) led to fewer leaves having internalized *E. coli* O157:H7. This trend was particularly evident for cabbage loopers and thrips whose feeding activities, i.e., chomping and rasping, respectively, damage the tissue to a greater extent.

## Synopsis 2

### **A novel approach to investigate internalization of *Escherichia coli* O157:H7 in lettuce and spinach**

Principal Investigator: Manan Sharma, Agricultural Research Service, USDA

Co-investigator: Michael Donnenberg, University of Maryland–Baltimore

A molecular method based on chromosomal integration of the green fluorescent protein (*gfp*) gene into *E. coli* O157:H7 was used in this project to provide an alternative and potentially more accurate approach to the measurement of *E. coli* O157:H7 internalization in fresh produce. Use of the *gfp* marker in previous studies typically involved integration of the *gfp* gene into plasmid DNA along with an antibiotic resistance gene marker, which may lead to detection of fewer *E. coli* O157:H7 cells than actually present under conditions of physiological, nutritional, or antimicrobial stress. The chromosomal integration method used in this study determine whether and to what extent internalization of *E. coli* O157:H7 occurs in fresh produce.

The project applied this novel approach to evaluate the following hypotheses: (1) that *E. coli* O157:H7 strains isolated from produce associated outbreaks are better able to be internalized into plants than are strains isolated from beef-associated outbreaks or non-pathogenic

*E. coli* strains; (2) that *E. coli* O157:H7 requires an intact stress response to survive in plants; and (3) that established *E. coli* O157:H7 virulence factors are heightened, or up-regulated, during growth on leafy greens compared with growth in ground beef.

These specific objectives have been achieved in this project:

- 1) The chromosomal integration of the green fluorescent protein (*gfp*) gene was successfully accomplished into four nalidixic acid resistant *E. coli* strains: two O157:H7 strains from produce outbreaks, 4407 and 5279, one O157:H7 strain from a beef-associated outbreak, 86-24h11, and a non-pathogenic commensal isolate (HS). The *gfp* gene was amplified with a consensus sigma-70 promoter and cloned into the pGRG36 *attTn7* vector to create pXLW45. The construct pXLW45 was introduced by conjugation into the four *E. coli* strains. An *rpoS*-deficient strain of *E. coli* O157:H7 was not able to be transformed with *gfp* because *rpoS*-functionality is thought to be required for this transformation. The fluorescence of these strains was confirmed using epi fluorescent microscopy.
- 2) No *E. coli* populations were detected by spiral plating from homogenized internal tissues of baby spinach plants grown in pasteurized soils. However, in some cases, *E. coli* cells were visualized in root tissue by fluorescent microscopy. No cells were visualized in shoot tissues. Three separate *gfp*-labeled inocula, *E. coli* O157:H7 strains 4407 and 5279 (Inoculum 1), *E. coli* O157:H7 strain 86-24h11 (Inoculum 2), and *E. coli* HS (Inoculum 3), were grown in a sterile fecal slurry and were applied to sterile soils at two inoculum levels (ca 7 log CFU/g or ca. 3 log CFU/g) in which a baby spinach variety was planted. In the 28-day study, no *E. coli* strains were recoverable by spiral

plating from surface-sanitized internal tissues of spinach plants. Populations of all three inocula in the rhizosphere declined over the 28 d study; however, Inoculum 1 survived at significantly higher populations in the rhizosphere than Inoculum 3 after 21 and 28 d, indicating that produce outbreak strains of *E. coli* O157:H7 may be more viable in soils than non-pathogenic *E. coli* isolates.

- 3) Cells of *E. coli* O157:H7 86-24h11 (Inoculum 2) applied to hydroponic medium at a ca 7 log CFU/ml were recovered by spiral plating from the shoot tissues of spinach plants in hydroponic media after 14 and 21 days. Internalized cells were also recovered from shoot tissues 7 d after transplantation to pasteurized soils. Cells of Inoculum 2 were visualized in shoot tissue by fluorescent microscopy. In contrast to inocula applied to pasteurized soils, *E. coli* populations either grew in hydroponic media from ca 3 to 7 log CFU/ml or maintained their levels at higher populations (ca 7 log CFU/ml). The higher levels of *E. coli* maintained in the hydroponic environment immediately surrounding the root tissue may partially account for the recovery of internalized cells.
- 4) The virulence factors *rfbE* (O157-antigen) and *ehx* (hemolysin) were expressed at significantly higher levels when *E. coli* O157:H7 was grown in ground beef compared to on cut lettuce at 37°C. However, no differences in the expression of *eae* (outer membrane adhesin intimin), *espA* (type III secretion filament), *ihaA* (adherence factor), and *stxII* (shiga toxin 2) were observed when *E. coli* O157:H7 cells were grown on ground beef, lettuce, or in laboratory growth medium. These data indicate that iceberg lettuce supported statistically similar levels of expression of four of six virulence factors of *E. coli* O157:H7 as ground beef.

Overall, root uptake of *E. coli* O157:H7 cells containing a chromosomal *gfp* insert was not observed in spinach plants when grown in pasteurized soils for up to 28 days. However, when exposed to a high inoculum, *E. coli* O157:H7 was recovered and microscopically observed from shoot tissues of spinach plants grown in hydroponic media inoculated with *E. coli* O157:H7. Lettuce was shown to support expression of virulence factors of *E. coli* O157:H7 similar to those of ground beef in most cases.

### Synopsis 3

#### **Interaction of *Escherichia coli* O157:H7 with fresh leafy green produce**

Principal Investigator: Jorge A. Girón, University of Arizona

Contributors: Juan Xicohténcatl, Ethel Sánchez, and John M. Leong, University of Arizona

Enterohemorrhagic *E. coli* (EHEC) O157:H7 uses a repertoire of surface and secreted factors to interact with epithelial cells and to ultimately colonize and survive within their natural (bovine) or accidental (human) hosts. Outbreaks of human enteric disease due to the consumption of EHEC-tainted spinach and other leafy produce indicate that the bacteria are capable of also interacting and surviving industrial decontamination processes on the surface of leafy greens. In this study, we aimed to: (i) determine the environmental, plant, and bacterial factors that favor colonization of leafy green produce by EHEC O157:H7; (ii) determine the mechanism by which EHEC O157:H7 colonizes leafy green produce and evades and survives decontamination; and (iii) identify safe technology for eradication of EHEC in leafy green produce.

To accomplish these aims, we have employed genetic, molecular and cell biology, and ultrastructural approaches to compare the adherence capabilities of wild type and isogenic mutants defective in production of surface adhesins, flagella, the type 3 secretion system (T3SS), and effector molecules (e.g. Tir, EspF, EspFU), which are responsible for reorganizing cytoskeletal components in mammalian eukaryotic cells. The adhesins tested include intimin, curli, cellulose, and the newly described *E. coli* common pilus (ECP), hemorrhagic coli F17 (HCF17A), and the type 4 hemorrhagic coli pilus (HCP). Additionally, we tested a collection of 16 mutants of *E. coli* O157:H7, which are affected in genes encoding novel effectors and other proteins of unknown function and that are present in several O-islands of EDL933.

#### **Spinach and lettuce colonization by EHEC EDL933**

By studying the dynamics and the growth environmental factors that influence adherence and colonization of lettuce and spinach by *E. coli* O157:H7, we found that the bacteria colonize spinach or lettuce leaves at similar rates independently of the infection temperature (e.g. 25°C, 37°C or 42°C). Infections in DMEM tissue culture medium or tap water showed that the bacteria are able to colonize in either condition, although higher bacterial counts were obtained when the experiments were performed with DMEM than in tap water. These data would mean that the bacteria are able to replicate and survive on the leaves in the presence of municipal water.

#### **Role of adhesins in spinach and lettuce colonization**

We provided ultrastructural and functional compelling data showing that EHEC colonizes spinach and lettuce leaves through flagella, curli, and the T3SS. Among the several strains tested, only the flagella, T3SS, and curli mutants showed the most dramatic effect in reduction in colonization. Mutants in intimin, Tir, cellulose, and the new pili: ECP, HCP and HCF17A did not show any defect in colonization, suggesting that these factors, which are important for bovine or human gut colonization, are not apparently required for spinach or lettuce colonization.

### **Role of the LEE in spinach and lettuce colonization**

When the virulence-associated LEE region of EHEC was introduced into non-adherent *E. coli* K-12 DH5, we observed comparable adherence rates as those seen in the wild type EHEC EDL933; suggesting that the LEE contains genes required for colonization of spinach and lettuce. This is consistent with our observations that the T3SS, which is encoded on the LEE, is required for plant colonization.

### **EHEC opens stomata after 4 hours of infection and this event is mediated by the T3SS**

High-resolution electron microscopy studies performed on infected spinach leaves showed bacteria internalized within plant stomata, with visible flagella-like structures emanating from them. These observations led us to further investigate the possibility that the bacteria were inducing stomal opening promoting their own penetration. Kinetics of spinach infection to monitor stomal opening by light microscopy, immunofluorescence, and scanning electron microscopy showed that most stomata are closed before 4 h of infection. However, beyond this time point the number of stomata opened increases as the bacteria replicate in situ. The presence of flagella on the colonizing bacteria was demonstrated by immunofluorescence using anti-flagella antibodies. Thus, EHEC subverts the mechanisms of stomata opening resulting in the internalization of the bacteria. This means that the bacteria must inject a yet unidentified effector that acts at the level of the signal pathway responsible for stomal opening and closure.

### **Identification of EspFU as an effector responsible for stomal opening**

Very importantly, we have identified a T3SS effector that we believe is responsible for opening spinach stomata. Analysis of a collection of mutants in genes located within several O-islands led to determine that an EspFU mutant was unable to open the leaf stoma and significantly deficient in colonization and stomata opening. This is, we believe, a mechanism employed by *E. coli* O157:H7 to manipulate the leaf stoma for its benefit and to colonize and survive within the safe niche of the stoma.

### **Treatment of tainted spinach with ozone-treated water eliminates colonizing bacteria**

Based on our findings, we hypothesized that bacteria within the stomata are protected from bactericidal compounds or decontamination solutions. It is important that we find ways to kill the bacteria within the stoma without affecting the organoleptic properties of the plant. We subjected infected leaves to decontamination with several concentrations of chlorine, Tsunami and ozone-treated water. Our data show that the bacteria within stomata are resistant to washing treatment with 1% and 0.1% chlorine, as viable bacteria were recovered after treatment. When Tsunami and Ozone were tested as potential ways of cleaning EHEC-contaminated spinach leaves, we found that treatment of with ozonized water kills 99.9998% of the bacteria, while Tsunami treatment does not eradicate all the internalized bacteria.

### **Concluding remarks**

It is clear that the interplay between EHEC and its mammalian or plant hosts is multifactorial. We have found that curli, flagella, and the T3SS, are important bacterial surface components for colonization of spinach and lettuce. Plant pathogens, such as *Pseudomonas syringae*, utilize T3SS and effector molecules to manipulating stomal

opening in order to gain access to internal tissues and cause disease in plants. We found that EHEC mimics this pathogenesis strategy by using the T3SS and associated effectors, in particular EspFU, to open and hide out within the nutrient-rich environment of the stoma protected from exterior foes in order to survive in the environment before animal or human infection. This may explain why EHEC evades industrial decontamination processes. When injected into human cells, EspFU helps in the nucleation of actin and other cellular components that participate in the formation of attaching and effacing lesions. It remains to be determined how EspFU triggers plant stomal opening.

### Synopsis 3

#### **Sanitization of leafy vegetables by integrating gaseous ozone treatment into produce processes**

Principal Investigator: Ahmed Yousef, Ohio State University

Co-investigator: Sudhir Sastry, Ohio State University

Ozone is a potent antimicrobial agent that is well-suited for use with fresh produce. The aim of this study is to take advantage of the sanitizing potency of ozone gas to overcome fresh produce contamination with *Escherichia coli* O157:H7. Consistent with this aim, the following project objectives were established:

1. Adapting existing vacuum cooling operations for leafy green produce to include a sanitization step by repressurizing with gaseous ozone. The goal is to sanitize fresh produce with gaseous ozone during application of vacuum cooling, a process referred to as San-Vac.
2. Determine San-Vac processing parameters that best meet the requirements of vacuum cooling, and show promise for product decontamination/sanitization.
3. Optimize San-Vac processing parameters for maximum product decontamination while minimizing product damage by the potent gaseous ozone and changes in pressure.
4. Explore the feasibility of an additional decontamination step that involves a long-term treatment with ozone to simulate fresh produce sanitization during transportation or storage.

The following is our approach to meet these objectives:

#### **Develop a process for simultaneous vacuum cooling and sanitizing (San-Vac)**

A process has been developed where fresh produce (baby spinach) is treated with vacuum to induce cooling; subsequently, the vessel is repressurized with an ozone-containing mixture of gases. Cooling was accomplished at rates comparable to those used in the fresh produce industry. Additionally, the controlled release of the sanitizing gas mixture allowed measurement of the dose of sanitizer, product exposure time, temperature, and pressure. To assure treatment uniformity, ozone concentration was measured at various locations in the treatment vessel. The equipment setup and processing trials were carried out in a biosafety level-2 facility.

#### **Determine San-Vac optimum processing parameters**

Several San-Vac processing variables were tested. These include vacuum level, vessel pressure after ozone introduction, ozone concentration, treatment time, average temperature, and product loading. Combinations of select parameters were varied systemically and the corresponding reduction in the population of *E. coli* O157:H7 was determined. Results are arranged in surface-response figures; these results show several promising combinations of ozone concentration, pressure and treatment time. These combinations resulted in a maximum decontamination of 2.4 log, but optimum processing combinations produced 1.8-1.9 log reduction in *E. coli* O157:H7. Viability of *E. coli* O157:H7 during refrigerated storage of the treated product is being monitored.

### **Long-term ozone treatment to simulate decontamination during transportation and storage**

The fresh produce (baby spinach) was held at refrigeration temperature and continuously flushed with low concentrations of ozone, for up to 3 days. Natural microbiota and inoculated *E. coli* O157:H7 were monitored during the treatment. Flushing stored spinach with 10 ppm ozone for one day decreased *E. coli* population by one log and product quality was comparable with the control (untreated) sample. The investigation will include combining the short-term and long-term treatment to maximize process efficacy.

In conclusion, we successfully adapted existing vacuum-cooling technology and included a sanitization step involving repressurization of produce-containing treatment chamber with gaseous ozone. Our approach involved sequentially application of vacuum, gaseous sanitizer, and pressurized gases. The new setup was tested at different ozone concentrations, holding times, and gaseous pressures, and the lethality of *E. coli* O157:H7 on fresh produce was monitored. Additional atmospheric modifications in the treatment chamber included using carbon dioxide gas. The study also included a long-term treatment of spinach with ozone-containing atmosphere to mimic product sanitization during transportation and storage. Results are encouraging and are likely transferable to future fresh produce processing equipment. Therefore, sanitizing fresh produce during the vacuum cooling seems feasible.

## Synopsis 5

### **Quantifying the Risk of Transfer and Internalization of *Escherichia coli* O157:H7 during Processing of Leafy Greens**

Principal Investigator: Elliot Ryser, Michigan State University

Co-Investigators: Bradley Marks and Ewen Todd, Michigan State University

This project evaluated the role of 5 key processing steps—shredding, conveying, fluming, shaker table drying and centrifugal drying—in the potential transfer of bacterial pathogens during pilot-plant scale processing of iceberg lettuce and baby spinach. Based on the quantitative bacterial transfer data, several mathematical models have been developed to predict the extent and likelihood of bacterial transfer during commercial processing of leafy greens. The long-term goal of this work is to develop a scientific basis for minimizing the risk of contaminating fresh-cut leafy greens during commercial processing and provide the industry with a series of recommendations on cleanability and sanitary design of commercial processing equipment for leafy greens.

The project's specific objectives were to:

- Determine the numbers of *E. coli* O157:H7 transferred from dip-inoculated iceberg lettuce and baby spinach to equipment during shredding, conveying, fluming, shaker table drying and/or centrifugal drying.
- Determine the extent of *E. coli* O157:H7 internalization during vacuum-cooling of iceberg lettuce and baby spinach.
- Develop a mathematical model to predict the quantitative transfer of *E. coli* O157:H7 during processing of iceberg lettuce and baby spinach, which can then be used to identify candidate risk mitigation strategies.

Fresh-cut, pre-washed heads of California iceberg lettuce and baby spinach were initially dipped in Glo-Germ™ (a fluorescent compound) and then processed using a commercial shredder followed by a conveyor, flume tank, shaker table and/or centrifugal dryer to identify a total of 50 product contact areas on the equipment for subsequent sampling. Thereafter, 22.7 kg of cored iceberg lettuce heads and baby spinach were immersed in a 4-strain cocktail of *E. coli* O157:H7 containing the green fluorescent protein (gfp) so as to contain approximately  $10^6$ ,  $10^4$  or  $10^2$  log CFU/g and then drained for 1 hour. Numbers of *E. coli* O157:H7 transferred to various equipment surfaces on the shredder, conveyor, flume tank, shaker table and/or centrifugal dryer and to the processing water were determined by direct plating in combination with membrane filtration. In general, approximately 90% of the inoculum transferred from the lettuce and spinach to the water during fluming with about 30% of the *E. coli* O157:H7 population present on the lettuce or spinach after shaker table dewatering shed with the water during centrifugal drying. Greatest attachment was seen to the shredder and conveyor during lettuce processing followed by the centrifuge and shaker table with the sides of the flume tank yielding the lowest numbers of *E. coli* O157:H7. When 22.7 kg of uninoculated lettuce or spinach was processed followed by 22.7 kg of inoculated product ( $\sim 10^6$ ,  $10^4$  or  $10^2$  log CFU/g) and then 90.8 kg of uninoculated product, *E. coli* O157:H7 populations steadily decreased in the 90.8 kg of previously uninoculated product. However, the pathogen typically remained

quantifiable in the last 5 of 45 leafy green samples exiting the processing line, indicating that consumption of such product still poses a risk for consumers.

Transfer of bacteria during the processing of leafy greens is being modeled as a complex, multi-modal process that involves the following six transfer scenarios: product-to-water, product-to-equipment, water-to-equipment, and all three reverse transfers. A dimensional analysis model has been formulated for each of these transfer events, which includes the relevant system variables (e.g., flume dimensions, product mass flow, water velocity, product contact surface area on the equipment) that affect the rate of transfer. A simplified version of the model is being used to quantify the aggregate rates of transfer for a specific complete process. For example, initial results from the processing of inoculated lettuce have shown that a total of ~92% of the *E. coli* O157:H7 inoculum on the product is transferred to various components of the processing line. The percentages transferred to the surfaces of shredder, conveyor, flume, shaker table, and centrifugal dryer were approximately 0.20, 0.67, 0.01, 0.01, and 0.37%, respectively, with the rest (~91%) transferring to the process water. For baby spinach (not a shredded product), a smaller fraction was transferred from the inoculated product to the equipment surfaces. The next phase of the modeling effort will demonstrate the sensitivity of the transfer rates to the key system variables and incorporate the transfer model into a numerical solution for unsteady-state transfer in a continuous processing system.

A probabilistic model has been constructed in Excel to further evaluate the *E. coli* O157:H7 transfer data collected during processing. This probabilistic model accounts for *E. coli* cross contamination when contaminated lettuce or spinach enters a processing line for fresh cut produce. Simulations of the model are being performed using @Risk Palisade<sup>®</sup> Software with the end result providing an estimate of *E. coli* populations in commercially bagged product. In addition, the impact of different sanitation regimes and disinfection processes at different steps in the processing line (e.g., shredder, shaker table, and conveyor) on the prevalence and concentration of *E. coli* O157:H7 in the bags of product can also be evaluated using this model.

In separate work, the extent of *E. coli* O157:H7 internalization into iceberg lettuce and baby spinach during experimental vacuum-cooling is being determined prior to processing. After vacuum-cooling the dip-inoculated (~10<sup>6</sup> CFU/g) iceberg lettuce and baby spinach, the samples are either being homogenized by stomaching or surface-sterilized by immersion in 1% silver nitrate, rinsed and ground, and then plated to quantify the numbers of external and internalized cells of gfp-labeled *E. coli* O157:H7, respectively. In addition, selected samples are being examined for internalized cells using Confocal Scanning Laser Microscopy with these results forthcoming.

In summary, mechanical shredding, conveyance on a conveyor belt or through a flume tank, and dewatering by shaking or centrifugation have proven to be important multidirectional transfer points for *E. coli* O157:H7 during processing of iceberg lettuce and baby spinach with the flume water, shredder and conveyor belt generally yielding the highest transfer rates. The predictive model being developed is the first reported attempt at modeling bacterial transfer during processing of leafy greens and will be further refined as more data are collected. These findings, along with the probabilistic risk assessment model being developed, reinforce the importance for proper cleaning,

sanitizing and maintenance of the equipment to minimize the transfer and spread of bacterial pathogens during commercial processing of leafy greens.

## Synopsis 6

### **Movement of *Escherichia coli* O157:H7 in spinach and dissemination to leafy greens by insects**

Principal Investigator: Jacqueline Fletcher, Oklahoma State University

Co-investigators: Astri Wayadande, Justin Talley, and Stanley Gilliland, Oklahoma State University

We evaluated *E. coli* O157:H7 colonization patterns and titers in spinach plants, based on the route of exposure and/or internalization and the type of spinach cultivar. We also examined the potential role of key insect species in transmitting or disseminating *E. coli* to leafy greens. The likelihood of insect transmission of *E. coli* O157:H7 from animal production areas to leafy green produce is not known, although flies have been previously implicated in the transmission of *E. coli* O157:H7 to cattle, feed, and water. The results will inform the development and prioritization (based on bacterial internalization sites, titer and translocation propensity) of strategies used in commercial spinach production operations to prevent or interrupt bacterial dissemination and/or invasion of plant tissues, including choice of cultivar, pest management practices, and bacterial removal or inactivation treatments.

The study was designed to explore the following hypotheses:

1. *E. coli* O157:H7 colonizes different locations (surfaces and tissues) of the spinach plant, and achieves different titers, depending on the site and method of introduction.
2. *E. coli* O157:H7 enters plants through natural openings.
3. *E. coli* O157:H7 translocates within spinach plants.
4. Insects common to both cattle production areas and spinach production operations are capable of transmitting *E. coli* O157:H7 to spinach plants.

#### General Experimental Methods

##### **Bacteria**

Five strains of *E. coli* O157:H7, each tagged with the green fluorescent protein (GFP), were obtained from Michael Doyle at the University of Georgia. Each was cultured separately in broth, and concentrations were adjusted to the titer of the least concentrated strain. All 5 were mixed in equal numbers immediately before inoculation.

##### **Inoculation and pathogen detection**

Commercial cultivars Tyee (highly lobed savoy), Space (semi-savoy), and Bordeaux (smooth, arrow-shaped leaves) were used, although all were not used in every objective. Including all three experimental replications, a total of 25 plants were inoculated with each treatment. Inoculation methods were designed to mimic different natural or artificial conditions:

1. **Leaf drop:** mimics rainsplash.
2. **Stab wounding** with a multi-pronged fork, mimics physical wounding by insects or wind-blown dust.
3. **Root drenching:** mimics soil infiltration of irrigation water or incorporation of contaminated manure into planting soil.

4. **Pressure inoculation:** positive control, assessment of translocation.

**Microscopy**

Plant tissues were examined by confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and/or fluorescence microscopy (FM) at intervals post-inoculation, and GFP bacteria-inoculated plants were compared with those of buffer-inoculated controls.

**Bacterial cultivation**

Whole-plant samples were surface-sterilized, ground in buffer and dilution-plated on VRBA agar. Some were also enriched in GN Hajna broth + antibiotics and inoculated onto Sorbitol McConkey agar and Chrom agar O157.

**Polymerase chain reaction**

*E. coli* O157:H7 in enriched samples was assessed by PCR using the Bax PCR system.

**Experiments and Results**

**Objective 1: Assess whether a green fluorescent protein-tagged *E. coli* O157:H7, introduced into different plant tissues, colonizes and moves through intercellular spaces, the vascular system, or both.**

*E. coli* O157:H7 was introduced to 4-leaf stage, cv. Tyee, spinach plants via the four methods shown above. No fluorescence was detected in any of the PBS controls. Bacteria were consistently observed by CLSM on spinach inoculated via leaf drop and pressure inoculation, but inconsistently or not observed in stab-inoculated tissues or drenched roots. Bacterial numbers increased on the surface of leaf-drop inoculated spinach over a 2-week period; bacteria were observed occasionally in intracellular spaces of the plant mesophyll. In pressure-inoculated plants, bacteria were observed near the inoculation site but there was no evidence of translocation or lateral spread. Plate enumeration and BAX PCR of surface sterilized plants revealed *E. coli* O157:H7 in spinach plants at multiple time points over the 2-week test period, regardless of the inoculation method. Bacterial numbers, assessed by plate enumeration and BAX PCR, were highest following pressure inoculation, followed by leaf drop, root drench, and stab inoculation, in order. Titers dropped over time, particularly for the root drenched plants.

**Objective 1 Summary.** As expected, the presence/absence and titer of *E. coli* O157:H7 on or within spinach plants varied depending on the mode and location of introduction and the inoculum titer. Bacterial titers in the samples, determined by plate counts, were low in most of the treatments except for pressure infiltration, which forced bacteria into the plant interior. Results of some treatments in the 3 replications were not completely consistent. The inability to see bacteria in plant tissues at some time points at which bacteria were recovered into culture is likely not due to quenching of the fluorescent signal, as it remains strong, but could indicate loss of the GFP-plasmid. Overall, Objective 1 results suggest that under some of our treatment conditions *E. coli* O157:H7 can and do colonize spinach leaf surfaces. When forced into plant interiors, the pathogen survived for several days but there was no evidence of titer increase or within-plant translocation. Whether such colonization can, or is likely to, occur in nature remains unknown.

**Objective 2: Test susceptibility to *E. coli* 0157:H7 for a variety of spinach cultivars.**

The number of bacteria observed by CSLM and SEM, and the area in which bacteria were observed, increased over time for savoy (Tyee), semi-savoy (Space), and smooth (Bordeaux) types. On Tyee, most bacteria were located in the crevices and furrows between adjacent epidermal cells. Cv. Space had fewer bacteria, covering a smaller leaf surface area, and most were seen near stomata. Very few bacteria were present on Bordeaux. While direct-plating of leaf extracts yielded no colonies at any time point (tested at 1, 7 and 14 dpi), enrichment culturing revealed *E. coli* 0157:H7 in a very small proportion of samples (2/60 Tyee and 3/60 Bordeaux).

The fact that *E. coli* 0157:H7 persisted in large numbers on leaves of Tyee, despite harsh washes and fixation, suggests that mechanisms for surface adherence were operating. Findings by others that this species can form biofilms on surfaces would be consistent with this finding and is an important topic for future research. The detection of *E. coli* 0157:H7 in a few samples of surface sterilized leaves is consistent with an interpretation of very occasional internalization; however, we cannot completely rule out the possibility that a few bacteria could have escaped the surface sterilization process, thereby remaining on the leaf surfaces. Confirmation of these findings will be an important goal of future research.

**Objective 2 Summary.** Bacterial numbers and colonization patterns on spinach leaf surfaces varied on the three spinach cultivars. Higher numbers of bacteria, and localization to leaf surface depressions, characterized *E. coli* 0157:H7 colonization of cv. Tyee. The leaf texture of Space, on which fewer bacteria were seen, is less bumpy, and Bordeaux, in which we found very little colonization, is smooth-surfaced and more delicate. It is tempting to speculate that the different leaf morphologies of the three spinach cultivars impacts the propensity for, and patterns of, bacterial colonization; however, the number of cultivars tested was too small to state this conclusively. Factors other than leaf morphology may have had a role in the differences observed. Future work should assess additional spinach cultivars having diverse leaf surface morphology. If the relationships are consistent among many spinach cultivars, then certain cultivars might be more desirable with respect to susceptibility to colonization by human pathogenic strains of *E. coli*. Similar experiments involving root drench inoculation are in progress at this time.

**Objective 3: Survey cattle pastures and nearby lettuce/spinach growing areas for insects.**

**Insect collection**

In May 2007 A. Wayadande and J. Talley visited the Salinas Valley of California in leafy greens production areas and in adjacent or nearby rangeland that currently or previously housed cattle. Collection sites included a conventional farm and three sites on organic farms. Sampling was by (1) sweeping foliage with nets (2) deploying yellow sticky traps for 2 days. Trap catches were sorted to insect order or family level for direct comparison of species composition in leafy greens production areas vs rangeland. A second trip to Salinas was made in May 2008. A single farm was revisited and flies were collected from four environmental sites: mature lettuce, mature cauliflower, fresh cowpats from the cow pasture adjacent to the farm, and the

commercial compost piles located in the center of the farm. Flies were processed for recovery of bacteria and plated on ChromAgar O157.

## Results

**2007 Survey:** Very few leafhopper and whiteflies were found, and insect diversity in the two target sampling habitats was low. Of significance were the several filth fly species associated with mature lettuce. Of 18 Calliphorid blowflies collected from mature lettuce fields, 11 tested positive for *E. coli* O157:H7. Although we have no evidence that the captured flies originated in the pasture area adjacent to the lettuce, the finding does suggest a possible route of contamination from pasture to leafy greens production areas.

The presence of filth flies in mature lettuce at one site and two muscid flies in mature romaine at another was unexpected because these flies develop in manure, compost, rotting vegetation, decomposing carcasses, or similar environments. To our knowledge, they have not previously been associated with leafy greens. The numbers of flies in direct contact with the lettuce and the evidence of fly fecal and regurgitation spots on leaf surfaces may indicate a possible route for contamination of leafy greens by *E. coli*-bearing filth flies.

**2008 Survey:** Fly populations at this site were significantly lower than the previous year. Significantly fewer flies were captured from plants than from compost or manure. Fly homogenate plated on ChromAgar O157 resulted in a color positive for several flies collected from manure and compost, but not from lettuce or cauliflower. However, we were unable to confirm the identity of these bacteria using *E. coli* O157:H7 specific primers and serological tests.

**Objective 3 Summary:** The high number of filth flies and the species composition on mature lettuce in some Salinas valley vegetable farms in 2007 were unexpected. Several flies were PCR positive for *E. coli* O157:H7 and may have been capable of transmission to plants. Our inability to culture *E. coli* O157 from live flies collected in the same area in 2008 may indicate an ephemeral presence of this pathogen in certain settings. More intriguing questions were generated than answered, prompting us to continue examination of the filth fly-*E. coli* ecological relationship in commercial greens.

### **Objective 4a: Confirm that houseflies acquire *E. coli*.**

Flies were poorly attracted to *E. coli* contaminated water, bacterial plates or contaminated manure. GFP-*E. coli* was easily detected by fluorescent microscopy from manure-exposed or bacterial plate exposed insects, confirming previous research by others that documented housefly acquisition of *E. coli*.

### **Objective 4b: Test phytopathogen vectors for their capacity to acquire pathogens from the above sources and from artificial feeding sachets.**

*E. coli*-fed leafhoppers (*Circulifer tenellus*) or aphids (*Acyrtosiphon pisum*) had not deposited bacteria into artificial feeding sachets after 3, 10, or 14 dpi. Mortality of leafhoppers, but not aphids, fed on *E. coli* was higher than that of controls.

**Objective 4 Summary:** This objective was designed to determine if these insect models were capable of transmission. As expected, flies easily acquired *E. coli* and were later shown to transfer it to plants or to agar plates. Leafhoppers and aphids, however, did not transmit the bacterium via typical routes (salivation, stylet probing), suggesting that these two insect models are unlikely to be involved in casual *E. coli* transmission to plants.

**Objective 5a: Determine dispersal flight distances of flies and cabbage looper butterflies.**

This objective, scheduled to be completed in the summer of 2007, was dropped due to excessive rain in Oklahoma and lower than anticipated fly populations necessary for completion of the study.

**Objective 5b: Assess propensity of contaminated flies, butterflies to alight on and contaminate greens.**

Spinach exposed to houseflies that had acquired bacteria from contaminated manure had very low numbers of bacterial cells on the plant surface after 1, 3, 5, or 7 days (determined by fluorescence microscopy). Spinach exposed to flies that acquired bacteria from plates had small aggregates of a few bacteria scattered on the leaf surface after 1, 3, 5, and 7 days.

**Objective 5c: Evaluate *E. coli* vector competence of leafhoppers and aphids; *C. tenellus*, *A. pisum*, *M. persicae* and *Bemesia tabaci*.**

Leafhoppers fed GFP-tagged *E. coli* in feeding sachets were caged with spinach plants. After 7 days, large pools of leafhopper honeydew fluoresced on the leaf surface of the plants exposed to GFP-fed leafhopper. In contrast, plants exposed to control leafhoppers (feeding solution only, no bacteria) had few fluorescing areas, most of these were autofluorescing salivary sheaths left behind by the feeding insects. It is likely that the ingested GFP-tagged *E. coli* was digested within the leafhopper gut and that the excreta contained still-fluorescent GFP proteins.

**Objective 5 Summary:** A GFP-based bacterial detection system gave more consistent results than did PCR for testing potential vector insects. Although the insect objectives are not yet complete, our data suggest that our homopteran models, aphids and leafhoppers, are unlikely to transmit *E. coli* O15:H7 to spinach. However, filth flies transferred GFP-tagged bacteria to plants after acquisition from manure or plates. Whether or not these few observed bacteria could form the basis for plant phyllosphere colonization is not known. Fly behavior, the role of contaminated regurgitant or excreta, and the role of mechanical damage by insects in the contamination of food plants by *E. coli* O157:H7 are all critical elements of future research projects planned by our group.

**Significance of research**

Although it remains premature to draw final conclusions from our work to date, the project is generating significant new information on plant colonization by *E. coli* O157:H7 and on the potential for insects to play a role in dissemination of the pathogen. Our data suggest that *E. coli* O157:H7 colonizes the leaf phyllosphere and may be internalized under the experimental conditions in our laboratory. It also appears that there are differences in colonization susceptibility among different spinach types. As a result of the California insect survey and PCR testing of collected insects, we have identified blowflies, a taxon not previously considered, as potential

vehicles of leafy greens contamination and have shown that houseflies move *E. coli* to spinach. We plan to pursue further studies on these interactions and submit grant proposals to funding agencies for future work. These findings will facilitate the development and prioritization of strategies, such as cultivar choice, pest management practices, and bacterial removal or inactivation treatments that can be used by Fresh Express, Inc., and other fresh vegetable producers for preventing or interrupting bacterial dissemination and/or invasion of plant tissues.

## Synopsis 7

### **Factors that influence the ability of *E. coli* O157:H7 to multiply on lettuce and leafy greens**

Principal Investigator: Linda Harris, University of California–Davis

Co-investigators: Mysore Sudarshana and Trevor Suslow, University of California–Davis

This project investigated pre- and post-harvest factors that may influence the behavior of *E. coli* O157:H7 on lettuce and leafy greens under three major objectives. We hypothesized that conditions of the plant (variety, age, growing conditions, season {day length}, method of harvest, moisture at harvest, soluble nutrients), of the bacteria (pre-inoculation stress and growth conditions) and of the post-inoculation environment will all influence how well *E. coli* O157:H7 is able to survive or grow on lettuce and leafy greens.

**Objective 1: *Pre-Harvest Factors.*** To characterize the growth of inoculated *E. coli* O157:H7 on spinach with an evaluation of the effects of variety, age of plant, growing conditions, fertility management, growth rate, and day length.

Spinach was grown under controlled greenhouse conditions using hydroponic culture and evaluated for the singular and interactive effects of nitrogen fertilization regimes, varying levels of photosynthetically active radiation (PAR), spinach variety, and developmental leaf age on leaf morphology and histology, leaf texture and toughness, total leaf sugars, preharvest colonization by *E. coli*, postharvest colonization by *E. coli* O157:H7, and potential for resistance to surface disinfection or internalization. Key outcomes from this series of studies collectively indicate that nitrogen and PAR have the dominant impact on spinach leaf morphology and texture, affecting characteristics that may have important roles in post-contamination events. Primary results may be summarized as follows; Total sugars per unit leaf mass are reduced with increasing nitrogen availability. Cell size is increased and cell number decreased in the leaf palisade layer and spongy mesophyll tissue is restructured during growth under conditions of excess nitrogen availability. Leaf development under this condition leads to a morphology with increased intercellular spaces. Excess nitrogen or low PAR reduces leaf toughness. None of these statistically significant differences in plant morphology and constituent composition significantly affected the colonization potential of *E. coli* O157:H7, within the methodology utilized. Neither variety nor developmental leaf age influenced the colonization of *E. coli* O157:H7, under the test conditions. Survival of *E. coli* was greatest on whole plants at the basal crown area and among the newly developing leaf whorls. Microscopy of colonized leaves was suggestive of a very low frequency of internalization under hydroponic culture. These observations suggest that more advanced techniques will need to be applied to discriminate the potential for the clear changes in leaf structure, nutrient composition, and texture to alter pathogen growth and spatial distribution on spinach in model systems and field environments.

**Objective 2: *Pre-inoculation Bacterial Growth Conditions.*** To characterize the behavior of inoculated *E. coli* O157:H7 on washed and bagged Romaine lettuce and

Romaine lettuce plants under different pre-inoculation bacterial growth conditions and stresses.

*E. coli* O157:H7 inoculum was prepared: on solid or liquid medium; in nutrient rich or poor medium; at minimal, moderate, and optimum temperatures; in different inoculum carriers (irrigation water, MilliQ water, and 0.1% peptone); to early or late stationary phase; and held for different times between preparation of inoculum and inoculation.

Very few pre-inoculation growth conditions had significant impact on the behavior of *E. coli* O157:H7 inoculated onto lettuce. Factors that had the greatest impact included growth of the culture at 25°C rather than 15 or 37°C, growth to exponential rather than stationary phase and growth in nutrient rich broth. Impacts were more pronounced on survival of the organism at 5°C rather than growth at 20°C. These data suggest that a range of pre-inoculation growth conditions used by different researchers may be directly compared and used collectively to develop risk assessment models. These data may also be used by researchers to as a guide to methods development and data evaluation in inoculation studies.

**Objective 3:** *Post-harvest Environmental Conditions.* To characterize the growth of inoculated *E. coli* O157:H7 on washed and bagged romaine lettuce and spinach at different static and fluctuating holding temperatures.

Sub-optimal temperature management during harvest, processing, distribution and pre-consumption handling of bagged lettuce and leafy greens allows significant growth of *E. coli* O157:H7 to occur prior to product reaching a point of clear consumer aversion.

Fresh-cut Romaine lettuce and chopped or grated iceberg lettuce were inoculated with different levels of *E. coli* O157:H7 and held at static temperatures or under conditions where the temperature is shifted for various periods of time from refrigerated to temperatures that permit growth of *E. coli* O157:H7.

When stored at 4°C levels of *E. coli* O157:H7 decreased slowly over time; a 1 log reduction was generally noted at the end of 5 days. At 10°C, *E. coli* O157:H7 survived well without any change in the net population over 5 days. In contrast at 20°C, *E. coli* O157:H7 populations increased by 2 to 3 log after 24 h. When short temperature shifts were made, populations of *E. coli* O157:H7 did not increase within 6 h of storage at 20°C; increases were not observed until 12 h or more of storage. This suggests that short fluctuations in storage temperature should not increase food safety risks. Populations of *E. coli* O157:H7 behaved similarly when inoculated at 300, 3,000, or 30,000 CFU per sample suggesting that moderate inoculum levels can be used to predict the behavior of *E. coli* O157:H7 when present significantly lower levels. Although overall increases were greater when initial populations were lower the final population was generally greater when higher levels were inoculated.

## Synopsis 8

### **Fate of *Escherichia coli* O157:H7 on fresh and fresh-cut iceberg lettuce and spinach in the presence of normal background microflora**

Principal Investigator: Mark Harrison, University of Georgia

Co-investigators: William Hurst and William Kerr, University of Georgia

The objective of this study was to determine the ability of *E. coli* O157:H7 to multiply in the presence of normal background microorganisms on iceberg lettuce and spinach under conditions that mimic actual practices between production and retail sale, including:

- Transportation from harvest field to cooler
- Refrigerated storage
- Transportation and distribution as (1) cored product in a nitrogen atmosphere (lettuce), (2) open returnable plastic totes (spinach), or (3) finished packaged chopped lettuce at both common and abusive temperatures.

By simulating conditions and practices of the fresh-cut industry, this study allowed for the evaluation of the fate of *E. coli* O157:H7 on produce during typical handling practices. The information gained provides insight into how *E. coli* O157:H7 on iceberg lettuce and spinach interacts with naturally-occurring bacteria, which may have competitive or antagonistic influences on the growth of *E. coli* O157:H7. This knowledge can be used to identify handling and packaging routines that reduce the potential for contamination of fresh produce by *E. coli* O157:H7.

Naturally-occurring bacteria on the product under the different conditions were screened for their ability to inhibit the pathogen. Variations on the way iceberg lettuce and spinach are handled in commercial operations and packaged produce were reflected in the sampling plans.

Green fluorescent pigment-expressing (GFP) *E. coli* O157:H7 was used in the experiments to allow tracing of the organism on inoculated product during the handling and storage treatments. Fresh iceberg lettuce and spinach were harvested from fields in central California and shipped overnight to the UGA Department of Food Science and Technology in Athens, Georgia. Prior to inoculating the greens, they were removed from the cooler and equilibrated to 25°C (~75°F) or 32°C (~90°F). Using these temperatures allowed for a comparison of two harvest conditions (one that is desirable and one that is abusive). The outer leaves of the iceberg lettuce heads were removed and the heads cored by hand. Investigators wore gloves to minimize introduction of microorganisms at this point.

Samples were taken to enumerate the background microflora (aerobic mesophilic and psychrotrophic bacteria, coliforms, yeasts and molds, and lactic acid bacteria) on the lettuce and spinach.

The lettuce and spinach leaves were inoculated with GFP-expressing *E. coli* O157:H7 per cm<sup>2</sup>. After the inoculum dried, sections of the inoculated leaves were removed and the *E. coli* O157:H7 were enumerated to determine the initial contamination level. Heads of lettuce inoculated with the GFP-expressing *E. coli* O157:H7 were placed in plastic totes, held at 25°C (~75°F) and 32°C (~90°F), and sampled for GFP-expressing *E. coli* O157:H7 and background microflora for up to 10 hours. Temperatures and times represent reasonable conditions and abusive conditions for transporting greens from the field to the cooling facility. After sampling, the lettuce was vacuum cooled to approximately 40°F within 20-40 min. Totes were held at either 4°C (~40°F) or 12°C (~55°F) and the inoculated heads were sampled for GFP-expressing *E. coli* O157:H7 and background microflora at 0, 10, and 72 hours. Sampling after 10 hours represented transit to a fresh-cut operation in the general area where the lettuce was harvested, while 72 hours represented the typical transit time from central California to eastern areas like Atlanta under a reasonable transit temperature and an abusive refrigeration temperature.

Lettuce was chop-cut (1.5-2" pieces) and held in chilled water (3-4°C/37-39°F) with or without chlorine (pH adjusted) with agitation to simulate contact with water in fresh-cut operations. After dewatering and bagging, lettuce samples were held at desired refrigeration and abusive storage temperatures and sampled over several days. Times and temperatures included abusive conditions that could occur during distribution of the retail product.

Spinach was handled in a similar manner as the lettuce except the baby spinach was forced air cooled to approximately 4°C (~40°F) and bagged in macroperforated bags. In addition, the leaves were not chopped or shredded before packaging.

Randomly selected isolates from the plates used to enumerate the various types of bacteria, yeasts and molds present on the lettuce and spinach at the different stages of handling and processing were screened to determine if they possessed any notable competitive or antagonistic effect on *E. coli* O157:H7. An agar spot method was used to screen isolates for antimicrobial activity to *E. coli* O157:H7. All isolates with inhibitory activity were evaluated to see if they were able to multiply at both 4 and 10°C.

### **Lettuce Results:**

#### *E. coli* O157:H7 populations

- We examined the effects of field temperature, precool, postcool, transportation temperature and wash treatment on *E. coli* O157:H7 counts before the lettuce was stored in retail bags. Field temperature ( $p = 0.0013$ ), precool ( $p < 0.0001$ ), postcool ( $p = 0.0028$ ) and wash treatment ( $p < 0.0001$ ) are important factors that affect the number of *E. coli* O157:H7 present on the lettuce, while transportation temperature was not significant ( $p > 0.2$ ). *E. coli* O157:H7 numbers were lower when the field temperature was 25°C and the precool time was minimal. Washing is an important step, since washing with chlorine was found to be significantly better than washing without chlorine, and washing without chlorine was significantly better than no washing, in terms of reducing the numbers of *E. coli* O157:H7.

- On days 2 and 5 of the storage period, *E. coli* O157:H7 counts were mainly affected by precool ( $p < 0.0001$ ), wash treatment ( $p = 0.0392$ ), and storage temperature ( $p < 0.0001$ ) with the lowest numbers of *E. coli* O157:H7 encountered when the precool time was minimal, the lettuce was washed with chlorine and the storage temperature was 4°C. Field temperature and transportation temperature are not important once the storage period starts and the treatment of storage temperature becomes effective.
- To determine the ability of *E. coli* O157:H7 to multiply during storage, the data was analyzed by fixing storage temperature at 4°C and 25°C, respectively. When stored at 4°C, precool ( $p < 0.0001$ ), postcool ( $p = 0.0015$ ), washing ( $p < 0.0001$ ) and storage day ( $p < 0.0001$ ) have significant effects on *E. coli* O157:H7. At 4°C, the longer lettuce/spinach is stored, the less *E. coli* O157:H7 is found on samples (day 0 > day 2 > day 5 > day 10 > day 18). When stored at 25°C, precool ( $p < 0.0001$ ), washing ( $p < 0.0342$ ) and storage day ( $p < 0.0001$ ) are still important, whereas postcool becomes insignificant ( $p = 0.2309$ ). At 25°C, the level of *E. coli* O157:H7 increases over storage time (day 0 < day 2 < day 5).

#### Psychrotrophs populations

- Important factors that affected psychrotrophic populations were postcool ( $p < 0.0001$ ), transportation temperature ( $p = 0.0028$ ) and wash ( $p < 0.0001$ ). As expected, fewer psychrotrophs were present on samples subjected to shorter postcool times (0 hour < 10 hours < 72 hours) and lower transportation temperature (4°C < 12°C). The effect of washing on psychrotrophs is similar to the effect on *E. coli* O157:H7, with washing with chlorine corresponding to the lowest level of psychrotrophs and no washing corresponding to the highest level. Field temperature and precool are not significant factors for psychrotrophic numbers.
- When storage days at 2 and 5 were fixed to compare different storage temperatures, psychrotroph counts were affected by precool ( $p = 0.0006$ ), postcool ( $p < 0.0001$ ), transportation temperature ( $p = 0.037$ ), wash ( $p = 0.0016$ ) and storage temperature ( $p < 0.0001$ ). Field temperature was not important ( $p = 0.4806$ ).
- The number of psychrotrophs increased significantly over storage days at both 4 and 25°C.

#### Mesophilic bacteria, lactic acid bacteria, coliform (non-*E. coli*), and yeasts and mold populations

- Shorter precool time, shorter postcool time and lower transportation temperature led to lower populations of these microbial groups. Lower field temperature resulted in lower populations of all the groups except the yeasts and molds. Washing significantly reduced levels of each group, with washing with chlorine being more effective than washing without chlorine.
- Storage temperature ( $p < 0.0001$ ) had a significant effect on each microbial group with greater amounts of growth at the higher temperatures.
- There were significant increases in the population size for all the groups except the lactic acid bacteria when product was stored at 4°C. There was no significant increase in the numbers of lactic acid bacteria on samples stored for the first 10 days.

## Spinach Results

### *E. coli* O157:H7 populations

- Prior to storage, field temperature ( $p = 0.0003$ ), precool ( $p = 0.0019$ ) and wash ( $p < 0.0001$ ) significantly affected *E. coli* O157:H7 levels, while postcool ( $p$ -value=0.7878) was not important. Spinach held for a minimum time before cooling had lower levels of *E. coli* O157:H7. Washing with chlorine significantly reduced *E. coli* O157:H7 to a greater degree than washing without chlorine.
- To compare 4 and 25°C storage temperatures, days of storage was fixed at 2. When spinach was stored for 2 days, the effect of field temperature on *E. coli* O157:H7 counts was not significant ( $p = 0.9785$ ). Significant effects were precool ( $p = 0.0231$ ), wash ( $p < 0.0001$ ) and storage temperature ( $p < 0.0001$ ). The *E. coli* O157:H7 level was significantly lower at 4°C storage temperature, compared to storage at 25°C.
- Overall *E. coli* O157:H7 populations were reduced over time at 4°C storage temperature (day 0 > day 2 > day 5 = day 10), but increased over time at 25°C storage temperature.

### Psychrotrophic populations

- Prior to storage, field temperature ( $p < 0.0001$ ), precool ( $p < 0.0001$ ), postcool ( $p = 0.005$ ) and wash ( $p < 0.0001$ ) significantly affected psychrotrophs. Lower field temperature (25°C), shorter precool time (0 hour) and shorter postcool time (0 hour and 10 hours, as opposed to 72 hours), and washing with chlorine resulted in lower psychrotrophic counts.
- In the storage stage, field temperature ( $p < 0.0001$ ), postcool ( $p = 0.0019$ ) and storage temperature ( $p < 0.0001$ ) significantly affected psychrotrophs. In particular, at 4°C storage temperature, the psychrotrophic levels were significantly lower.
- Psychrotrophic levels increased over time at both 4 and 25°C storage temperatures.

### Mesophilic bacteria, lactic acid bacteria, and yeasts and mold populations

- Prior to the storage stage, field temperature ( $p < 0.0001$ ), precool ( $p < 0.0001$ ), postcool ( $p < 0.0001$ ) and wash (at least  $p < 0.0417$ ) significantly affected these microbial groups. Lower field temperature (25°C), shorter precool time (0 hour), shorter postcool time (0 hour and 10 hours, < 72 hours), and washing with chlorine resulted in lower levels of each group.
- In the storage stage, in addition to field temperature, precool, postcool and wash, storage temperature ( $p < 0.0001$ ) significantly affected mesophilic levels resulting in lower levels when spinach was stored at 4°C.
- Levels of mesophilic bacteria, lactic acid bacteria and yeasts and molds increased over time, at both 4 and 25°C storage temperatures.

## Competitive or Antagonistic Activity

- Over 36,900 isolates were selected from the lettuce samples and screened for their potential to be competitive or antagonistic towards *E. coli* O157:H7. Samples were representative of the various steps in the handling and storage of the lettuce. Of the isolates screened, 309 were determined to have some competitive or antagonistic activity.
- Over 17,500 isolates were selected from the spinach samples and screened for their activity with 470 isolates showing some degree of activity.
- Of the isolates that showed activity:

- Approximately 45% of them were Gram negative rods selected from the violet red bile agar plates. Only 5 isolates were selected from the media used to select for yeasts and molds.
- Approximately 70% were from samples initially held at 25°C.
- Approximately 60% were from samples that were processed immediately after cooling.
- Approximately equal numbers of isolates were selected from samples held at 0, 10 or 72 hours postcool.
- Approximately 45% were isolated from samples after the chlorine wash. *Burkholderia cepacia* and *Pseudomonas putida* were the most prevalent isolates exhibiting activity and were found on samples from each step in the processing and storage. Other commonly isolated genera included *Pantoea*, *Klebsiella*, *Enterobacter*, *Burkholderi*, and *Aeromonas*.

### **Overall Summary**

The objectives of this study to determine the ability of *E. coli* O157:H7 to multiply in the presence of normal background microflora on iceberg lettuce and spinach under conditions that mimic those from the field to finished, packaged product were met. On chopped iceberg lettuce and whole leaf spinach that was packaged and stored at 4°C, *E. coli* O157:H7 contamination can still be detected after typical handling practices, although the populations decreased from the initial levels in many cases by at least 1.5 logs. In some abusive cases, populations did not decrease as much or may have increased on leaves. In these cases the product quality quickly deteriorated. Evidence of a variety of naturally-occurring microorganisms on fresh lettuce and spinach with possible antagonistic activity toward *E. coli* O157:H7 was documented.

## Synopsis 9

### **Determining the environmental factors contributing to the extended survival or regrowth of foodborne pathogens in composting systems**

**Synopsis 1** Principal Investigator: Xiuping Jiang, Clemson University  
Co-investigators: Geoff Zehnder and Feng Luo, Clemson University

This study examines the effectiveness of composting to inactivate pathogens in manure, given that raw or inadequately composted animal waste applied to growing fields is a potential pre-harvest source of produce contamination. The primary mechanism for pathogen inactivation during outdoor composting is microorganism-related heat generation. In practice, the effectiveness of pathogen inactivation varies with environmental factors, including temperature, rainfall, nutrient sources, compost ingredients, and pathogens' induced heat resistance.

Key environmental factors related to the regrowth and survival of three foodborne pathogens in compost are evaluated in this study. Data from this project can be applied to the design of composting practices in California, the identification of environmental factors conducive to pathogen regrowth, and to the prediction of pathogen inactivation during on-farm composting.

#### **Specific objectives:**

- Identify the optimal conditions for *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* regrowth in composts under field application. The most desirable environmental conditions such as heavy rain/overhead sprinkling/flooding, nutrient sources (types of compost), and warm temperatures, were evaluated for pathogen regrowth in compost. A cocktail of three strains for each pathogen were adapted to low nutrient condition and then inoculated into the compost with low initial populations. The inoculated compost was maintained inside a greenhouse for up to 4 weeks and sampled periodically for pathogen analysis. Different varieties or ages of compost were used for studying pathogen regrowth.
- Determine the fate of heat-adapted avirulent pathogens during composting on the farm; Composting practices in California were followed and simulated, using on-farm composting of vegetables with dairy manure and ingredients used in California (which can also be found in South Carolina.) The green wastes from either summer or fall organic crops were collected from Clemson University's Calhoun Field Laboratory farm. Duplicate compost heaps were set up on site. The survival of compost-adapted and heat-adapted avirulent strains of *E. coli* O157:H7, *Salmonella* spp., and *L. innocua* was determined at 3 locations (surface, center, and bottom) of composting heaps. Field studies were conducted during summer months (25-35°C) and winter months (5-15°C) to determine the influence of outdoor temperatures and precipitation on the fate of pathogens.

- Study the impact of initial heat resistance of pathogens on their survival during composting. Three strains for each pathogen were heat-shocked at 47.5°C prior to the inoculation of compost mixture separately. Cultures without heat-shock treatment were used as controls. The inoculated compost held in a Tyvek® pouch was kept in a humidity-controlled incubator at a range of temperatures (50, 55 and 60°C) to simulate the conditions inside compost heaps during thermophilic composting. At certain intervals, samples were taken out and analyzed for surviving bacterial counts. Mathematic models based on these data were developed to predict the thermal inactivation of pathogens during composting.

**Outcomes:**

Objective 1: The regrowth of *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* were examined in both dairy compost and compost extract. Our study has demonstrated that a few pathogenic cells (less than 10 cfu) can regrow in diluted compost extract at the presence of ca. 5 log cfu background microflora/ml, and in dairy compost with moisture content maintained at least 20% and less than 3 log cfu/g of other microorganisms. All ages of compost can support the pathogen regrowth under certain conditions. Bacterial cultures adapted to low nutrient media have high regrowth potential in compost at room temperature. Strain variation on regrowth potential was observed in compost, and spinach-outbreak *E. coli* O157:H7 strain grew faster in both compost extract and compost.

Objective 2: Two trials of on-farm composting were conducted in this study. The results indicate that on-farm composting of dairy waste and vegetable residues can eliminate pathogens inside the compost heaps within 5 days provided the thermophilic condition was maintained. Heat-adapted cultures survived relatively longer either inside or on the surface of compost heap than the control cultures without prior heat-shock treatment. Both control and heat-adapted cultures declined very slowly in the similar rates throughout the mesophilic composting heaps under field condition due to the inadequate heating of the heaps. Results from both trials revealed that indicator and pathogenic bacteria may remain viable on the compost surface for weeks to months. In addition, the seasonality plays an important role for pathogen inactivation by on-farm composting.

Objective 3: The thermal tolerance of *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* in finished compost was tested at the selected composting temperatures inside a humidity chamber. As compared with control cultures, heat-adapted cultures survived longer ( $p < 0.05$ ) in compost when tested at 50, 55, and 60°C. However, non-linear thermal inactivation of three pathogens in compost was observed at above temperatures. The thermal inactivation data were modeled mathematically for predicting the survival of pathogens during composting.

The results so far have revealed that the physiological stages of bacterial inoculum, nutrient availability, indigenous microbial populations, moisture content, and temperature all have impact on the regrowth and persistence of pathogens during composting. On-farm thermophilic composting can inactivate pathogens inside the heaps rapidly, but previously heat-adapted cultures increase the thermal resistance at elevated composting temperatures. Furthermore, environmental conditions

conducive for mesophilic composting inside the heaps permit the extended survival of pathogens in compost.