CPS CENTER for PRODUCE SAFETY

## CPS – 2009 RFP

## FINAL PROJECT REPORT

### Project Title
Survival of *E. coli* on soil amendments and irrigation water in leafy green field environments

### Project Period
October 1, 2009 – March 31, 2011


### Principal Investigator
Name: Steven Koike
Department: UC Cooperative Extension – Monterey County
University/Organization: University of California
Email: stkoike@ucdavis.edu
Phone: 831.759.7356

### Co-Principal Investigator
Name: Mike Cahn
Department: UC Cooperative Extension – Monterey County
University/Organization: University of California
Email: mdcahn@ucdavis.edu
Phone: 831.759.7377


### Objectives

Objective 1: To further document survival of *E. coli* strains when introduced into a spinach production system

Objective 2: To monitor survival of *E. coli* strains when introduced into a lettuce field in the form of composts, organic amendments, and extracts such as teas

**FINAL REPORT**

**Abstract**
This project seeks to confirm how both generic and attenuated, non-toxigenic O157:H7 strains of *E. coli* survive when introduced to soil, water, and spinach plants in a commercial production setting. In a replicated field trial soil was inoculated with both *E. coli* strains, a spinach crop was grown in this field, and *E. coli* survival monitored by sampling and testing soil, water runoff, and spinach plants. In another experiment, we inoculated mature spinach plants, incorporated such plants into the field by disking, and tested soil for *E. coli* survival. Overall results are consistent with previous experiments conducted under commercial Salinas Valley agricultural environments. Both generic *E. coli* and attenuated *E. coli* O157:H7, when applied to soil, survived for relatively short periods of time. In addition, both inoculum types failed to move significantly into irrigation water runoff or move in the soil. Bacterial inoculum was not recovered from spinach plants that were grown in inoculated plots. However, when mature spinach plants were inoculated (with generic *E. coli* or attenuated *E. coli* O157:H7) and disked back into the soil, both types of bacteria were recovered from soil for an extended period of time. Additional studies would be appropriate to examine factors that could enhance decline of such bacterial populations on crop residue and in soil.

We also examined how generic and non-toxigenic O157:H7 strains of *E. coli* survive when introduced into field settings via production inputs. We inoculated compost and liquid supplements as standard amendment material, then introduced the contaminated materials into the soil and tested soil for survival of the inoculated strains. Plants from the romaine lettuce crop grown in these plots were evaluated for any indications of contamination. Contaminated compost, liquid supplements, and solid supplements did not result in persistent survival in soil and did not result in contaminated romaine grown in the plots. We also collected additional evidence that when *E. coli* is introduced to the roots of spinach plants, the plants do not absorb these bacteria and that “internalization” does not occur under field conditions.

**Background**
Outbreaks of foodborne pathogens on leafy vegetables occur at sporadic intervals and are not new developments. However, extensive and widely publicized foodborne pathogen outbreaks resulting in national-scale food recalls, such as the spinach case in 2006 and more recent lettuce recalls, have highlighted the need and increased the urgency for practical information on the dynamics of such organisms in agricultural systems. While the biology, ecology, and epidemiology of *E. coli* O157:H7 have been extensively researched and studied in animal and human contexts, such information is not well developed for *E. coli* as it occurs in leafy green production environments. The activity and dynamics of these pathogens on leafy vegetable plants have been mostly studied under laboratory and growth chamber environments. Until further information is available, such lab studies may have limited predictive value for food safety policies and practices as applied to commercial field vegetable production.

In addition to concerns about *E. coli* O157:H7 and other human pathogens, the leafy green vegetable industry and the produce industry as a whole must contend with the presence of

non-pathogenic, generic *E. coli* as well. Generic *E. coli* can be readily detected in many farm environments, yet the ecology, biology, and fate of these organisms are not well documented in this setting. Current food safety concerns, buyer contracts and conditions, and food safety metrics all list generic *E. coli* as an organism of concern because this bacterium is assumed to be a validated indicator of fecal contamination.

Because of the need for additional field oriented studies involving *E. coli*, there is need for further validating the usefulness of surrogate bacterial strains. Such surrogate strains may be used more readily, while pathogenic forms would not be allowed in field evaluations of *E. coli* survival, dispersal, detection (sampling methods and sensitivity), responses to environmental conditions, and role within plant/environment interactions.

Due to the extensive leafy green vegetable acreage in California, it is imperative to obtain more information on the biology, ecology, and epidemiology of both generic and pathogenic *E. coli* under coastal California agricultural field conditions. Applied field-oriented research increases our practical understanding of how *E. coli* operates in the field and assists industry and regulators in making informed decisions on growing practices, metrics, and regulatory food safety policies for the field. Because of the need for such information, we worked on two objectives: (1) To further document survival of *E. coli* strains when introduced into a spinach production system; (2) To monitor survival of *E. coli* strains when introduced into a lettuce field via contaminated composts, organic amendments, or liquid supplements.

**Research Methods and Results**

**Objective 1:** To further document survival of *E. coli* strains when introduced into a spinach production system.

**Part 1-a.** Pre-emergence soil inoculation

Spinach (cultivar: Avenger) was planted per commercial practice (42 lines on 80 inch wide beds, standard seed density at 3 million live seed/acre, and sprinkler irrigation) on May 7, 2010 in loamy-sand soil at the experimental site in the Salinas Valley. Plots consisted of two beds, each 100 feet long, with 4 randomized replicates of 5 treatments. For each plot, only the inner halves of the two beds were inoculated, allowing the unused half beds to function as untreated borders that would prevent cross-contamination.

The treatments (applied on May 10, 2010) were the following: generic *E. coli* (three rifampicin-resistant strains) as liquid inoculum, attenuated *E. coli* O157:H7 (two rifampicin-resistant strains) as liquid inoculum, generic *E. coli* (same three strains) on bagged sand medium, attenuated *E. coli* O157:H7 (same two strains) on bagged sand medium. The fifth treatment was an untreated control.

The liquid inoculum treatment consisted of 4 liters (2 liters for each half-bed) of bacterial suspension containing either log 8.1 cfu/ml (generic *E. coli*) or log 8.0 cfu/ml (attenuated *E. coli* O157:H7) in 0.1 M potassium phosphate buffer. Inoculum was applied as a broadcast spray over the half-beds by using $CO_2$ powered backpack sprayers and two nozzle booms. The point

source mesh bags contained 50 grams of a sand mixture inoculated with log 7.4 cfu per generic teabag and 6.4 cfu per attenuated teabag in potassium phosphate buffer with 5% powdered milk. Generic and attenuated mesh bag treatment plots were inoculated by placing the mesh teabags on top of the center of each half bed at 20 inch intervals for a total of 10 bags per plot.

Soil sampling began approximately one hour after the spray application, hence defined as 0 days post inoculation (dpi), and was continued at 1, 3, 7, 14, 22, and 28 dpi. Soil samples were gathered with sterile disposable scoops at 5 evenly spaced locations per half-bed in a 10 x 10 sq. cm area and removed to an approximate depth of 2 cm. Soil samples from the mesh bag plots were taken in triplicate at designated distances (0 cm (=adjacent to the bag), 25 cm, and 50 cm) from the bags. Soil samples were processed in 18 oz stand up whirl-pak bags by mixing 100 grams of soil with 200 ml of sodium phosphate buffer. Bags were allowed to sit undisturbed for 20 minutes to allow the soil to settle. Then 250 µl of the supernatant were plated in duplicate on amended Tryptic Soy Agar (TSA-rif) that consisted of Tryptic Soy Agar, 100 µg/L rifampicin, 100 µg/L 4-methylumbelliferyl-beta-D-galactopyranoside, 100 µg/L PCNB, and 1 g/L pyruvic acid. Plates were incubated for 24 hours at 37° C to select for the target strains. If colonies were not detected by direct plating, 2 ml of the sample supernatant were centrifuged and the resulting pellets resuspended in 100 µl of buffer, plated and incubated for 24 hours at 37° C. Additionally, 10 ml of sample supernatant was added to 90 ml of amended Tryptic Soy Broth [100µg/L rifampicin (TSB-rif)] for an enrichment step. The mixture was incubated for 24 hours at 42° C. 250 µl of the enrichment mixture was subsequently plated to determine presence/ absence of target bacteria.

Ditches were cut across the field in front of each 100 foot plot to allow water to run off the plots without contaminating other plots. Sprinkler irrigation run-off samples were taken from each replication of each treatment at 23 and 30 dpi. Samples were collected in autoclaved 1 liter plastic Nalgene bottles after 3-4 hours of irrigation when water began to run down all furrows. Samples were transported in an iced cooler and processed within 24 hours of collection. Water samples were tested by centrifuging 15 ml of the sample and plating the pellets on TSA-rif for generic plots and Chromagar 0157-rif for attenuated plots, as well as using the Idexx colilert tray system.

Plant samples were taken at 14, 22, and 28 dpi. Plant material was gathered from 5 evenly spaced areas within each half bed to achieve a composite 150 g sample (except at 14 dpi when plants were too small to collect more than 75 g). Plant samples from the mesh bag plots were taken in triplicate at designated distances as mentioned above. Plant samples were processed by adding 300 ml (a 1:2 mass-to-volume ratio) of TSB-rif to the plant material in 55 oz whirl-pak filter bags and then massaging the bag thoroughly before plating 100 µl of the broth onto TSA-rif plates in duplicate. Following this step, an additional 100 ml of TSB-rif was added to the filter bags, which were then incubated for 24 hours at 42° C as an enrichment. After incubation, the broth was plated as described above to determine bacterial presence/absence.

**Part 1-b.** *E. coli* survival on crop residue

When spinach was planted for the soil inoculation experiment on May 7 (1-b above), an additional planting was put in place on the same day and grown for this crop residue study. This second planting was germinated and grown according to standard commercial practice. When the crop was mature, plots were set up in preparation for inoculation with generic and attenuated *E. coli* strains. Plots were comprised of two adjacent 80 inch beds that were each 50 feet in length and were replicated four times. Treatments used the same bacteria as described in 1-b and were the following: generic *E. coli* (three rifampicin-resistant strains) as liquid inoculum, attenuated *E. coli* O157:H7 (two rifampicin-resistant strains) as liquid inoculum. On June 14 each plot was sprayed with a total volume of 4 liters of inoculum at concentrations of log 8.2 cfu/ml (generic strains) or log 7.9 cfu/ml (attenuated strains). Immediately following the foliar sprays, the crop in each plot was disked into the soil. No other field production steps were taken until the field was irrigated on September 16 at 96 dpi and disked a second time on September 24 at 104 dpi.

Soil samples were taken at 0, 1, 3, 7, 14, 22, 28, 35, 63, 96 and 104 dpi. Samples were taken with hand trowels sterilized with ethyl alcohol and then rinsed with sterile distilled water. Each plot was sampled in 8 areas from which 4 scoops of soil, down to a 6 inch depth, were taken and mixed in a 5 gallon bucket also sterilized with alcohol and rinsed. The soil sample was mixed thoroughly with the hand trowel and a sub-sample was bagged and taken back on ice to the lab. Soil samples were processed in 18 oz stand up whirl-pak bags by mixing 100 grams of soil with 200 ml of sodium phosphate buffer. Bags were allowed to sit undisturbed for 20 minutes to allow the soil to settle. Then, 250 µl of the supernatant were plated in duplicate on TSA-rif and incubated for 24 hours at 37° C to select for the applied strains.

**Objective 2:** To monitor survival of *E. coli* strains when introduced into a lettuce field via contaminated composts, organic amendments, and liquid supplements.

**Part 2-a.** Compost amendments

The experiment was conducted on loamy sand soil in a field in Monterey County representative of the coastal vegetable production environment. Large strip plots, measuring 400 feet long by 30 feet wide each, were set up in a randomized complete block design with three replications. On August 3, two types of commercially available, solid compost (composted 100% yard waste and composted 60% cow manure/40% yard waste blend) were applied separately to designated plots at a rate of 5 tons/acre using a commercial compost-spreader truck. Treated areas were separated by 20 foot wide un-amended buffer strips. Within each large plot, two 30 x 30 sq ft sections were watered by a commercial water-truck (at a rate of 0.1 gallons per square foot). For plots receiving inoculum, these 30 x 30 sq ft sections were sprayed with bacteria immediately after the watering. The inoculum consisted of 3 generic strains of rifampicin-resistant *E. coli* at log 5.9 cfu/ml; 3 liters of solution per each 900 sq ft section were applied with $CO_2$ backpack sprayers through four-nozzle booms. Unamended buffer strips functioned as control plots that did not receive compost but were inoculated with bacteria.

The applied composts were chiseled into the field immediately following inoculation, and 24 hours later the field was prepared for planting by listing the field (incorporating the composts into the soil to approximately a 10 inch depth) and applying a pre-irrigation. Romaine lettuce (cv. Green Towers) was direct seeded into prepared 80 inch wide beds on August 11. The crop was subsequently grown according to commercial practices.

Soils were first sampled after the pre-irrigation on August 9 (at 6 dpi). Soils were collected to a depth of 12 inches using a 1 inch diameter soil probe, with each plot sample consisting of 10 probes. Soils were later sampled at 11, 19 and 43 dpi. Samples were processed in 18 oz stand up whirl-pak bags by mixing 100 grams of soil with 200 ml of sodium phosphate buffer. Bags were allowed to sit undisturbed for 20 minutes to allow the soil to settle. Then, 250 µl of the supernatant were plated in duplicate on TSA-rif and incubated for 24 hours at 37° C to select for the applied strains.

Plants were sampled at 48 dpi only, near harvest maturity, by cutting the lettuce plants at crown level just above the soil surface. Each plot sample consisted of 5 plants. In the laboratory, the outer leaves and upper and lower quarters of the lettuce head were removed. The remaining section was chopped into large pieces, mixed thoroughly, and a 150 g sub-sample placed into a 55 oz whirl-pak filter bag. Plant samples were processed by adding 300 ml (a 1:2 mass-to-volume ratio) of TSB-rif to the whirl-pak filter bags and then massaging the bag thoroughly before plating 100 µl of the liquid onto duplicate TSA-rif plates. An additional 100 ml of TSB-rif then was added to the filter bags which were incubated for 24 hours at 42° C as an enrichment step. The liquid was plated again as above to determine bacterial presence/absence. Water run-off was not collected for this experiment.

**Part 2-b.** Liquid and solid organic supplements

In another experiment, we examined survival of generic *E. coli* and *Salmonella* when introduced into a field as a contaminant in two commonly used nutrient supplements in organic lettuce production: fish emulsion (liquid) and chicken pellets (solid). Supplements were inoculated prior to application to the field with a mixture of 3 strains of generic *E. coli* plus 1 strain of attenuated *Salmonella* (all resistant to rifampicin). Prior to inoculating the supplements, a preliminary experiment was completed to determine whether the selected supplements would directly result in death or decline of the *E. coli* and *Salmonella* strains. Samples of the liquid and solid supplements were inoculated in the laboratory and then assayed using standard methods for viable *E. coli* and *Salmonella*. Neither fish emulsion nor chicken pellets showed significant inhibitory or mortality effects on the inoculated strains.

For the field experiment, conducted on a sandy-loam soil in the Salinas Valley, 40 inch wide beds were prepared according to commercial practice and then seeded with two rows of romaine (cv. Green Towers) per bed on August 27. Surface drip irrigation lines were then placed on top of the beds (one line per bed). Experimental plots measured two 40 inch beds wide by 160 feet long and were replicated four times. After planting, the field was sprinkle irrigated to germinate the crop. After germination the field was irrigated via surface drip lines. The crop was grown, thinned, and produced according to commercial practices. Field inoculation was

completed on September 29, when the crop had grown to the thinning stage. Treatments were the following:

- Inoculated liquid fish emulsion: 3.7 liters of concentrated fish emulsion were diluted with 36.3 liters of water and then inoculated with 400 ml of log 9 *E. coli* + *Salmonella*. This final mixture was injected into the appropriate drip lines over a period of 60 minutes. The final rate of fish emulsion applied to the field was 10 gallons/acre. To document the delivered concentration of the bacteria, samples were collected from the end of the drip line for each of the four replications 30 minutes after drip injection began. The actual concentration of inoculum delivered by the drip line was log 5.7 cfu/ml.

- Inoculated chicken pellets: Using a backpack sprayer, 1.6 liters of log 8 *E. coli* + *Salmonella* were applied to 50 lbs of chicken pellets that were spread out on plastic trays. After allowing pellets to dry for 15 minutes, approximately 12.5 lbs of contaminated pellets were applied to each bed for a final rate of 1000 lbs pellets/acre. Pellets were distributed onto the center 6 inch band for each inoculated bed. Pellets were assayed in the lab and the actual concentration of inoculum on the pellets was log 5.8 cfu/g of pellet.

- Uninoculated solid chicken pellets: Approximately 12.5 lbs of uninoculated chicken pellets were applied to each of the control plots.

Soils were sampled on 1, 7, 21, and 36 dpi. In chicken pellet plots, soil was gathered with sterile disposable scoops at 5 evenly spaced locations on one-half of the bed in a 10 x 10 sq cm area. Soil was taken to a depth of approximately 2 cm. For these samples, pieces of the pellet, which did not completely disintegrate over the course of the trial, were not separated from the sample. In fish emulsion plots, soils were sampled similarly but were taken slightly deeper, beneath the drip line, approximately 2-5 cm deep. Soil samples were processed in 18 oz stand up whirl-pak bags by mixing 100 gram of soil with 200 ml of sodium phosphate buffer. Bags were allowed to sit undisturbed for 20 minutes to allow the soil to settle. Then 250 µl of the supernatant were plated in duplicate on Chromagar ECC-rif and incubated for 24 hours at 37° C to select for the applied strains. Blue colored colonies on Chromagar-ECC are presumptive positives for generic *E. coli*. White colonies on Chromagar-ECC were re-streaked onto XLD-rif media, which selects for *Salmonella*. If bacterial growth turned black on XLD media, these colonies are presumptive positives for *Salmonella*. As with all other trials, samples of presumptive positives were tested by PCR analysis (Suslow lab) to confirm bacteria are the experimentally inoculated strains.

Plants were sampled on 7, 21, and 36 dpi. Whole lettuce plants were cut at the crown just above the soil surface. Each sample consisted of 5 plants per plot. In the laboratory, the outer leaves and upper and lower quarters of the lettuce head were removed. The remaining section was chopped into large pieces, mixed thoroughly, and 150 g of the lettuce measured into a 55

oz whirl-pak bag. Plant samples were processed by adding 300 ml (a 1:2 mass-to-volume ratio) of TSB-rif to the whirl-pak filter bags and then massaging the bag thoroughly before plating 100 µl of the liquid onto duplicate Chromagar ECC-rif plates. An additional 100 ml of TSB-rif was then added to the filter bags, which were then incubated for 24 hours at 42° C as an enrichment step. After incubation, the liquid was plated as described above to determine bacterial presence/absence.

**Objective 3:** Determine whether field-grown spinach will absorb and transport *E. coli* from roots to foliage (internalization).

At the same location as the fish emulsion/chicken pellet supplement experiment (described above), additional 40 inch beds were prepared separately for this spinach experiment. Spinach (cv. Avenger) was seeded in six lines per bed using a Planet Junior hand planter on August 27. A surface drip irrigation line was placed in the center of each bed. Each plot was one bed wide by the length of the field (160 feet). The two treatments were a cocktail of the previously mentioned generic and attenuated O157:H7 *E. coli* strains and an untreated control. There were two replications per treatment. On September 24 inoculum was applied by injecting the bacteria into the drip lines. The concentrated inoculum (2 liters of log 9 cfu/ml) was added to 5 gallon buckets of water and injected with diaphragm injectors (Blue-White Industries model C-600) over a 30-minute period with a 6.25 liter per minute flow rate to achieve an actual dose within the drip line of log 7.4 cfu/ml. The final drip line concentration was determined by collecting water from drip line emitters at 15 and 25 minutes after starting the injection. The plots were first irrigated in the morning before inoculum injection began to pre-wet the soil. After all inoculum was injected into the line, irrigation continued for an additional 15 minutes to flush remaining inoculum out of the line. At the time of inoculation, spinach plants were at the four to six leaf stage.

Soil samples were collected at 0, 6 and 20 dpi. Soil samples were gathered with sterile disposable scoops at 5 evenly spaced intervals per half-bed in a 10 x 10 cm area from 2-5 cm deep, beneath the drip line in the rooting zone. Soil samples were processed in 18 oz stand up whirl-pak bags by mixing 100 grams of soil with 200 ml of sodium phosphate buffer. Bags were allowed to sit undisturbed for 20 minutes to allow the soil to settle. Then 250 µl of the supernatant were plated in duplicate on TSA-rif, Chromagar 0157-rif, and Chromagar ECC-rif and incubated for 24 hours at 37° C to select for the applied strains.

At least 100 individual plants were harvested at both 6 dpi and 20 dpi. The plants from 6 dpi were processed as follows: whole plants were washed in Nanopure water (NPW) and surface sterilized by submersion for 1 min in 1 liter of 1% $AgNO_3$ (1 liter for 60 plants). Individual leaves were cut at stem level and washed in two NPW rinses for 1 min each (leaves from individual plants were kept together throughout processing). Sterilized leaves were then macerated in a whirl-pak bag with a handheld homogenizer. Each bag then received 50 ml mEHEC+high Rif and was incubated for 18 h at 42° C. After enrichment 2 replications of each sample were streaked on TSA-rif and incubated for 18 h at 42° C.

The plants from 20 dpi were processed as follows: whole plants were washed in NPW and surface sterilized by submersion for 30 seconds in 1 liter of 0.1% AgNO3 (1 liter for 30 plants). After sterilization, each leaf belonging to the same plant was cut at the base of the stem, followed by another cut dividing the leaf between the blade (lamina) and petiole. Small leaves (≤ 2cm long) were processed as one unit. Leaf blade (lamina), petiole, and small leaves were rinsed in 2 NPW wash baths for 1 min each and then macerated in a whirl-pak bag with a handheld homogenizer. Each bag then received 20 ml TSB+high rif and was incubated for 18 h at 42° C. After enrichment, samples were spot plated on TSA-rif and incubated for 18 h at 42º C. (The minor differences in the sterilization treatments were evaluated in the laboratory to have no impact on the results.)

**Outcomes and Accomplishments**
**Objective 1:**

**Part 1a.** Pre-emergence soil inoculation

1. Spray inoculation and recovery from soil: Both generic and attenuated O157 strains of *E. coli* survived similarly in the field after spray inoculation and were not recoverable from soil in direct plating assays at the lower limit of detection within 14 days after inoculation (Fig. 1). Control plots did not test positive for any generic or attenuated strains on any of the testing dates. (For these *E. coli* experiments, the upper limit of detection (direct plating) is log 1.43 cfu/g and the lower limit (after concentration procedure) is log 0.13 cfu/g.)

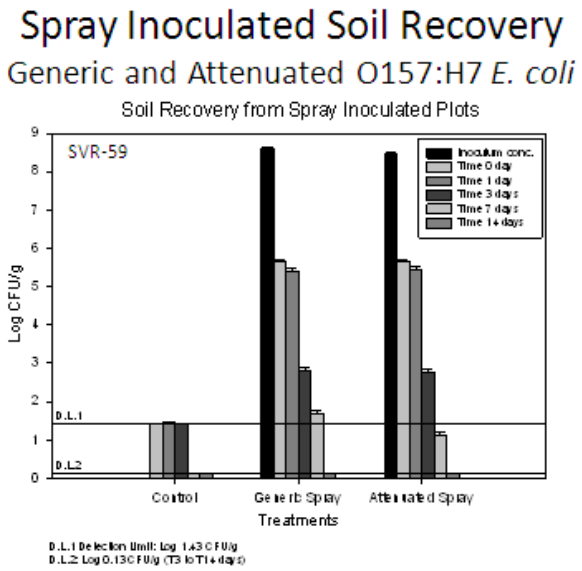
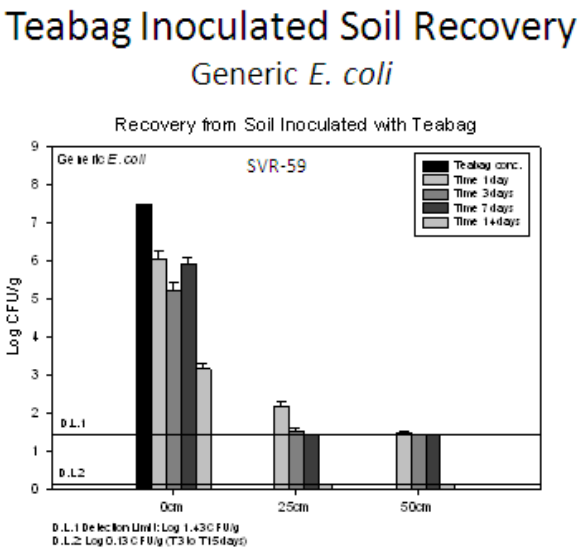
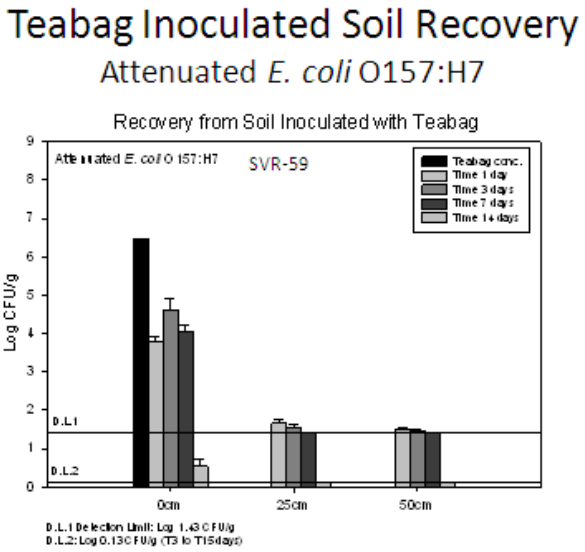



Figure 1. Soil recovery of generic and attenuated O157:H7 *E. coli* from spray inoculated plots.

2. Teabag inoculation and recovery from soil: Although the attenuated *E. coli* teabag concentration was one log less than that of the generic *E. coli* teabag (log 6.4 cfu/bag attenuated vs. log 7.4 cfu/bag generic), bacterial survival was almost identical throughout the experiment (Fig. 2A and B). At 0 cm from the teabag, direct plating recovery from soil remained above the lower limit of detection after 28 days. Direct plating recovery from soil at 25 cm away from the bags fell below the limit of detection around 3 days. At the 50 cm distance, recovery was at or below the limit of detection for all sampling days.




**A**




**B**

Figure 2. Soil recovery of generic (A) and attenuated O157:H7 (B) *E. coli* from teabag inoculated plots.

3. Irrigation water runoff: Generic and attenuated O157:H7 *E. coli* strains were not detected by iso-grid membrane filtration for all runoff samplings.

4. Spray Inoculation and recovery from spinach: All direct plate assays of spinach plants grown in the spray inoculated soil plots were negative for generic and attenuated *E. coli* strains, so enrichments were subsequently conducted. At 14 days after inoculation to soil, there were positive enrichments from plant samples collected from the spray-inoculated plots for both generic (3 positives out of 8 samples) and attenuated O157 (1 positive out of 8 samples) *E. coli* (Table 1). *E. coli* was not recovered from enrichments in later samplings (22 and 28 days) of these plots (Table 1).

Table 1. Enrichment recovery from spinach in spray-inoculated plots

| | **Generic** | **Attenuated O157:H7** |
|---|---|---|
| T14D | 3/8 | 1/8 |
| T22D | 0/8 | 0/8 |
| T28D | 0/8 | 0/8 |

All generic and attenuated 0157:H7 *E. coli* were confirmed by Rep-PCR.

5. Teabag Inoculation and recovery from spinach: All direct plate assays of spinach plants grown in the teabag inoculated soil plots, were negative for generic and attenuated *E. coli* strains, so enrichments were subsequently conducted. At 14 days, generic *E. coli* was recovered from enrichments from plant samples collected at the 0 cm location (next to teabags; 6 positives out of 8 samples). All other 14 day plant samples (generic at 25 and 50 cm; attenuated at 0, 25, and 50 cm) were negative for the inoculated strains (Table 2).

At 22 days, generic *E. coli* was recovered from enrichments from plant samples collected at the 0 cm (6/8) and 25 cm (1/8) locations. The attenuated strain was only recovered through the enrichment step at the 0 cm location (4/8) (Table 2).

At 28 days, generic *E. coli* was recovered from enrichments only from plants collected the 0 cm location (2/8). The attenuated strain was not recovered from spinach at any location (Table 2).

Table 2. Enrichment recovery from spinach in teabag-inoculated plots

| | **Generic** | | | **Attenuated O157:H7** | | |
|---|---|---|---|---|---|---|
| | **0cm** | **25cm** | **50cm** | **0cm** | **25cm** | **50cm** |
| T14D | 6/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |
| T22D | 6/8 | 1/8 | 0/8 | 4/8 | 0/8 | 0/8 |
| T28D | 2/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |

All generic and attenuated 0157:H7 *E. coli* were confirmed by Rep-PCR.

**Part 1b.** *E. coli* survival on crop residue

After the mature spinach crop was inoculated and then disked into the soil, both inoculated strains were recovered from the soil+crop residue samples (Fig. 3). Bacterial populations generally increased within the first week after inoculation and then gradually declined. However, despite an additional application of water at 95 dpi and disking at 103 dpi, bacterial numbers did not reach the limit of detection even after 105 days post-inoculation. Recovery of attenuated O157:H7 was lower, overall, than recovery of generic *E. coli* (Fig. 3).

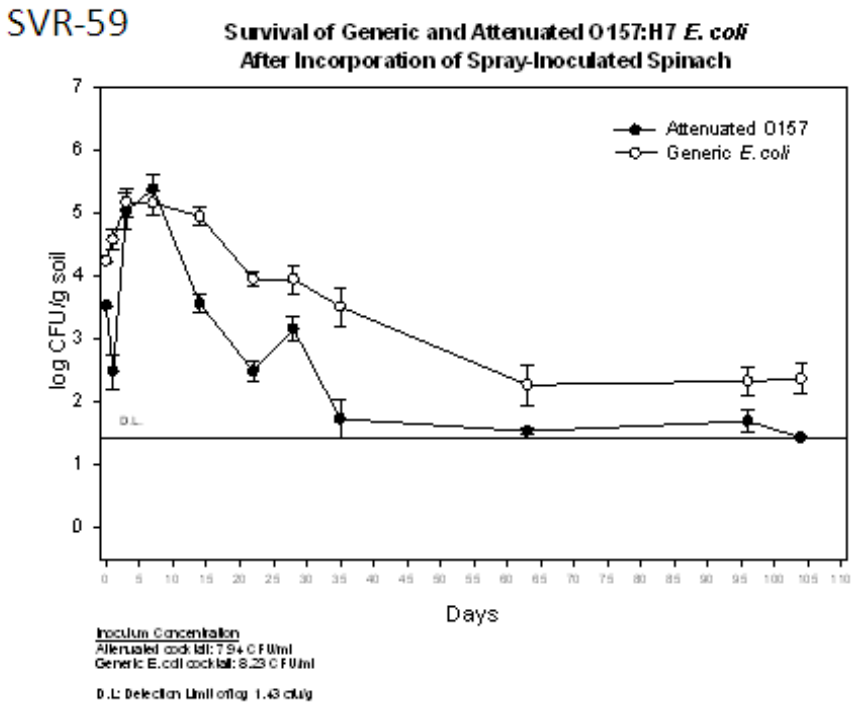



Figure 3. Survival of generic and attenuated *E. coli* strains on inoculated and soil-incorporated spinach crop residue.

**Objective 2: Contaminated composts, organic amendments, and liquid supplements**

**Part 2a.** Compost amendments

1. The first soil sample was collected 6 days post-inoculation (dpi). Few bacterial colonies were recovered from the soil for any of the treatments at 6, 11 and 19 dpi. Recovered colonies were analyzed by PCR (Suslow lab) and confirmed that they were the inoculated generic strains. By 43 dpi, no colonies were recovered at the limit of detection. There were no significant differences between yard waste compost (YW), yard waste+manure blend (M), or inoculated soil without compost (B). At no time were bacterial colonies recovered from the control soils (C).

Table 3. Recovery of bacteria from soil that received inoculated composts

| **Soil** | Values: *E. coli* LOG cfu/g soil | | | |
|---|---|---|---|---|
| **Time (dpi)** | **YW** | **B** | **M** | **C** |
| **6** | 1.12 | 1.54 | 1.35 | 1.03 |
| **11** | 1.03 | 1.08 | 1.04 | 1.03 |
| **19** | 1.04 | 1.04 | 1.03 | 1.03 |
| **43** | 1.03 | 1.03 | 1.03 | 1.03 |
| Limit of detection: LOG 1.03cfu/g | | | | |

YW: 100% composted yard waste
B: soil inoculated with no amendment added
M: composted 60% cow manure/40% yard waste blend
C: control – one non-inoculated control for each of the amended soils and the un-amended soil
All generic *E. coli* were confirmed by Rep-PCR.

2. Romaine plants grown in the plots were sampled at 48 dpi. Enrichment of the plant material showed that the inoculated generic *E. coli* strains were absent from all plant samples.

3. To determine whether the characteristics of the different compost sources have an impact on the survival of the inoculated generic *E. coli* strains, samples of the compost substrates used in this trial were tested in the laboratory to evaluate *E. coli* survival over time when applied as a liquid inoculum to the compost and subject to different moisture and temperature treatments. The overall conclusions from this subtrial are:

- The *E. coli*$^{rif}$ inoculum survived similarly on both types of compost material.

- Inoculum dose was very important in bacterial survival: colonies were recoverable at a very low rate and for a much shorter duration from compost inoculated with a concentration of $10^3$ colonies/ml relative to that inoculated with a concentration of $10^6$ colonies/ml.

- Moisture was important in supporting and prolonging bacterial survival. When substrates were moist, bacteria survived similarly well whether stored at room temperature or at 4° C, up to 23 dpi. Dried substrates did support minimal survival for up to 14 dpi when inoculated with a bacterial concentration of $10^6$ colonies/ml.

**Part 2b.** Liquid and solid organic supplements

1. Total bacterial recovery from soil (averaged from all subsamples of each treatment) is shown on Table 4 as log CFU/g. The presence of either generic *E. coli* or attenuated *Salmonella* is listed under Enrichment and is a ratio of the number of positive tests per either four or eight total subsamples. Bacteria were recovered from all soil samples for each of the sampling days (0, 1,

7, 13, 21, and 35 days post inoculation) in both contaminated solid and liquid supplement treatments (Table 4). Bacteria were not recovered from control plots at the limit of detection, log 1.43 cfu/g.

Table 4. Recovery of bacteria from soil that received inoculated liquid and solid supplements

| | Control | | | | Chicken Pellet | | | | Fish Emulsion | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TOTAL COUNT | log CFU/g | Std error | Enrichment E. coli | Enrichment Sal | log CFU/g | Std error | Enrichment E. coli | Enrichment Sal | log CFU/g | Std error | Enrichment E. coli | Enrichment Sal |
| T0D* | nd | nd | nd | | 5.88 | 0.23 | nd | | 5.74 | 0.06 | nd | |
| T1D** | 1.43 | 0.00 | 0/4 | 0/4 | 6.05 | 0.23 | 4/4 | 4/4 | 6.20 | 0.06 | 4/4 | 4/4 |
| T7D | 1.43 | 0.00 | 3/4 | 3/4 | 4.31 | 0.24 | 4/4 | 4/4 | 3.82 | 0.56 | 4/4 | 4/4 |
| T13D | 1.43 | 0.00 | 0/4 | 0/4 | 4.36 | 0.26 | 4/4 | 4/4 | 4.28 | 0.00 | 1/4 | 1/4 |
| T21D | 1.43 | 0.00 | 1/8 | 0/8 | 3.37 | 0.27 | 8/8 | 8/8 | 2.87 | 0.42 | 8/8 | 8/8 |
| T35D | 1.43 | 0.00 | 1/8 | 0/8 | 1.97 | 0.17 | 6/8 | 5/8 | 2.24 | 0.21 | 7/8 | 6/8 |

*Fish Emulsion expressed in log CFU/ml and Chicken Pellet in log CFU/g of pellet, both for T0D only.
** Total Count from TSA+PPMR
nd = no data
Genetic identification by Rep-PCR and qRT-PCR invA analysis for all generic *E. coli* and *Salmonella* presumptive positives is not yet complete for these samples.

2. Romaine plants grown in the plots were sampled at 7, 13, 21, and 35 dpi. Inoculated bacteria (both generic *E. coli* and *Salmonella*) were recovered by direct plating from plant samples at 7, 13 and 21 dpi but not at 35 dpi. However, generic *E. coli* and *Salmonella* were recovered by enrichment of plant samples on all sampling dates for both solid and liquid supplement treatments (Table 5).

Table 5. Recovery of bacteria from romaine grown in plots that received inoculated liquid and solid supplements

| | Control | | | | Chicken Pellet | | | | Fish Emulsion | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lettuce | log CFU/g | Std error | Enrichment E. coli | Enrichment Sal | log CFU/g | Std error | Enrichment E. coli | Enrichment Sal | log CFU/g | Std error | Enrichment E. coli | Enrichment Sal |
| T7D | 1.26 | 0.00 | 1/4 | 1/4 | 1.36 | 0.07 | 4/4 | 4/4 | 1.44 | 0.02 | 4/4 | 4/4 |
| T13D | 1.26 | 0.00 | 0/4 | 0/4 | 1.67 | 0.00 | 2/4 | 1/4 | 1.26 | 0.00 | 1/4 | 1/4 |
| T21D | 1.26 | 0.00 | 1/8 | 0/8 | 1.28 | 0.02 | 8/8 | 7/8 | 1.30 | 0.04 | 8/8 | 5/8 |
| T35D | 1.26 | 0.00 | 0/8 | 0/8 | 1.26 | 0.00 | 2/8 | 2/8 | 1.26 | 0.00 | 1/8 | 1/8 |

All generic *E. coli* confirmed^ by Rep-PCR and all *Salmonella* confirmed^ by qRT-PCR invA.
(^Except for all samples from day T13, as bacterial colonies died before genetic identification could be completed.)

3. In a final test to determine the presence/absence of introduced bacterial strains on lettuce grown with the supplements, three replicates of 60 leaves each (collected when plants were at

harvestable size) were randomly taken from throughout the contaminated solid supplement plots. Samples were collected as composites of 25, 75, or 125 g per plot and were enriched in TSB-rif. All enrichment tests were negative. Composites of the lettuce enrichments spiked with log 3 cfu *Salmonella*/sample, as positive detection controls, were all positive. (In 18 h of enrichment time, typically 2 cfu/25 g would reach at least log 5.2/sample, accounting for expected lag phase recovery.)

4. A small subtrial was conducted to evaluate the bacterial recovery from chicken pellets relative to soil sampled from the inoculated chicken pellet plots. The purpose of the subtrial was to determine whether bacteria survived primarily upon the pellets, or if they were thriving within the soil. Bacterial recovery per gram of pellets was significantly higher than per gram of soil, suggesting that bacterial survival was enhanced by association with the pellet material.

**Objective 3: Internalization of field grown spinach**

1. Large numbers of *E. coli* colonies were recovered from soil samples taken immediately following drip-irrigation inoculation (0 dpi) in the treated plots (beds A and B) while no colonies were recovered from the untreated plots (controls 1 and 2), indicating successful inoculation and no cross contamination (Table 6). At 6 dpi, soil samples contained significantly fewer recoverable colonies than at 0 dpi but both generic and attenuated *E. coli* persisted. PCR analysis (Suslow lab) was done on 40 colonies isolated and purified from the soil sample at 6 dpi; colonies were confirmed to be the inoculated generic or attenuated *E. coli* strains. By 20 dpi, only the generic *E. coli* strains were recovered from the soil taken from the root zone.

Table 6. Recovery of bacteria from soil irrigated with drip-applied inoculum

| | Day 0 | | | | Day 6 | | | | Day 20 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Near injection 15 min | Near injection 25 min | End of drip 15 min | End of drip 25 min | Generic *E. coli* | | Attn O157:H7 | | Generic *E. coli* | | Attn O157:H7 | |
| | log CFU/ml | | | | log CFU/g | sterror | log CFU/g | sterror | log CFU/g | sterror | log CFU/g | sterror |
| Control 1 | 0.95 | 0.95 | 0.95 | 0.95 | 1.43 | 0.00 | 1.43 | 0.00 | 1.43 | 0.00 | 1.43 | 0.00 |
| Control 2 | | | | | 1.43 | 0.00 | 1.43 | 0.00 | 1.43 | 0.00 | 1.43 | 0.00 |
| Bed A | 6.97 | 8.16 | N/A | 7.06 | 2.98 | 0.06 | 2.30 | 0.13 | 2.50 | 0.02 | 1.43 | 0.00 |
| Bed B | | | | | 2.44 | 0.12 | 1.60 | 0.17 | 2.85 | 0.05 | 1.43 | 0.00 |
| Detection limit | 0.95 | | | | 1.43 | | | | 1.43 | | | |

All generic and attenuated 0157:H7 *E. coli* were confirmed by Rep-PCR.

2. At 7 dpi, 60 spinach plants each from bed A and B were collected and processed for presence of the inoculated strains. 10 control plants were also tested. No colonies were recovered from the control plants. A total of 5 plants (3 from bed A and 2 from bed B) returned presumptive positives. PCR analysis confirmed that 40 colonies isolated from these samples and purified for analysis were from the inoculated strains.

At 21 dpi, 39 plants from bed A and 32 plants from bed B were collected and processed for presence of the inoculated strains. 10 control plants were also tested. These larger plants were subdivided into mature leaf blades, petioles, and small leaves for a total of 983 subsamples. No target bacteria were recovered from any of these samples.

**Summary of Findings and Recommendations**

We investigated how generic and attenuated, non-toxigenic O157:H7 strains of *E. coli* survive when introduced to soil, water, and spinach plants in a commercial production setting. Our overall results are consistent with previous experiments conducted under commercial Salinas Valley agricultural environments. Both generic *E. coli* and attenuated *E. coli* O157:H7, when applied to soil, survived for relatively short periods of time. In addition, both inoculum types failed to move significantly into irrigation water runoff or move in the soil. Bacterial inoculum was not recovered from spinach plants that were grown in inoculated plots.

However, when mature spinach plants were inoculated with either *E. coli* strain and disked back into the soil, both types of bacteria were recovered from soil and crop residues for an extended period of time (over 100 days). This was an unexpected out come and additional studies would be appropriate to examine factors that could enhance decline of such inoculum.

We also examined how generic and non-toxigenic O157:H7 strains of *E. coli* survive when introduced into field settings via production inputs. We inoculated compost, a liquid supplement, and a pelleted supplement as standard amendment materials, then introduced the contaminated materials into the soil and tested soil for survival of the inoculated strains. Plants from the romaine crops grown in these plots were evaluated for any indications of contamination. Contaminated compost did not result in persistent survival in soil and did not result in contaminated romaine. For liquid and solid supplements, which were inoculated with generic *E. coli* and non-toxigenic *Salmonella*, the lettuce had low levels of recoverable bacteria until day 35, at which time recovery was negative. We also collected additional evidence that when *E. coli* is introduced to the roots of spinach plants, the plants do not absorb these bacteria and that “internalization” does not occur under field conditions.

Spinach and lettuce are both high-value leafy vegetable crops that are extensively grown in California. The coastal spinach and lettuce producing area is the most important and productive region for these commodities. Because spinach and lettuce have been subject to *E. coli* contamination, it is critical to develop practical information on how *E. coli* may behave in these cropping systems under coastal California conditions. Field-generated research information developed in commercial coastal California conditions contributes significantly to our understanding of *E. coli* ecology and assists the industry in further understanding the dynamics of this foodborne pathogen. Field studies conducted under coastal California conditions are not widely available, and this research reduces such information gaps.

**Research team**

Steven T. Koike, Mike Cahn, Trevor Suslow, Richard Smith

**Acknowledgments**
We acknowledge the support of the Center for Produce Safety (CPS), California Leafy Greens Research Board, and the leafy greens industry in California. We thank Bonnie Fernandez and Leslie Maulhardt (CPS) and June Rasmussen (UC Cooperative Extension) for help with project administration. This project would not be possible without our industry cooperators: David Costa and Gilbert Hernandez. Special thanks to Laura Murphy, Adrian Sbodio, and Grace McClellan for overseeing the project, and to Joe Sproul, Mike Hardoy, and Kevin Vaughn for assistance with field operations. We thank NewStar and Brian Lopez for planting the spinach. Thanks to the following for their help with this project: Patty Ayala, Jianlong Bi, Keith Day, Mike German, Kat Kammeijer, Ian Kile, Eric Lauritzen, Tim Sugishita, Kim Vu.

## APPENDICES

### 1. Publications and Presentations

#### Publications

Koike, S. T. 2010. Ground zero: food safety research and extension in California's Salinas Valley. Abstract for APS Special Session Presentation. Assuring the safety of fresh produce: Issues and strategies. Phytopathology 100:S155.

Koike, S. T. 2010. Examination of the survival and internalization of E. coli on spinach under field production environments. Abstract. Produce Research Symposium. June 23. Center for Produce Safety.

Koike, S. T., Cahn, M., Suslow, T., and Smith, R. 2010. Field survival of *E. coli* in a spinach production system. California Leafy Greens Research Board. Progress report. October 5.

Koike, S. T., Cahn, M., Suslow, T., and Smith, R. 2010. Survival of *E. coli* under a commercial spinach production environment. Abstract. Food Safety and Water Quality Co-management Forum. Watsonville. December 8.

#### Presentations

Koike, S. T. 2010. Examination of the survival and internalization of E. coli on spinach under field production environments. Produce Research Symposium. Center for Produce Safety. UC Davis. June 23.

Koike, S. T. 2010. Outreach to growers: Extension and food safety in California. Symposium: Human pathogens associated with edible plants. International Association for Food Protection annual meeting. Anaheim. August 2.

Koike, S. T. 2010. Ground zero: Food safety research and extension in California's Salinas Valley. Symposium: Assuring the safety of fresh produce—Issues and strategies. American Phytopathological Society annual meeting. Charlotte, North Carolina. August 10.

**2. Budget Summary**

Expenditure of funds was completed as planned. The majority of the funding covered personnel expenses for technicians and assistants with UC Cooperative Extension (Monterey County: Koike lab) and UC Davis (Suslow lab). Other expenses included the following: laboratory and field supplies for the experiments, inoculations, sampling, and testing; grower expenses for growing the crops; travel for UC Cooperative Extension personnel to the field site; travel for UC Davis personnel to Monterey County. The budgeted funding was sufficient to fully complete this project.