CENTER for PRODUCE SAFETY

CPS 2010 RFP
FINAL PROJECT REPORT

Project Title
Investigation of *E. coli* survival on contaminated crop residue

Project Period
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Objectives
The overall objective is to further document survival of *E. coli* and *Salmonella* strains in soil when a contaminated leafy-green crop (lettuce) is incorporated into soil and the site is prepared for subsequent plantings. Within this objective, the cultivation practices are varied to evaluate several different production scenarios.

Objective 1: Evaluate impact of different pre-incorporation practices, including mowing and ring-rolling of a contaminated crop, on bacterial survival.

Objective 2: Evaluate impact of additional irrigations on bacterial survival in contaminated soil.

Objective 3: Evaluate whether environmentally-adapted *E. coli* and *Salmonella* bacteria will transfer to a leafy-green crop re-planted in a previously contaminated soil.

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Abstract
Fresh market leafy green vegetables are periodically subject to contamination by foodborne human pathogens such as *E. coli*. Field aspects of such contamination events are not well understood and more information is needed on where and how *E. coli* comes in contact with leafy greens in the field, how *E. coli* survives there, and how production factors influence pathogen survival. We continue to develop information on the survival and ecology of *E. coli* under actual production environments for coastal California leafy greens. Our field studies in the Salinas Valley indicate that if lettuce or spinach crops were inoculated with *E. coli* (generic or attenuated O157:H7 strains), either prior to emergence from soil or early in their growth cycle, the plants at harvest were rarely contaminated and inoculated strains were rarely recovered from soil or runoff water. However, if a mature crop was contaminated with *E. coli*, disked into the field, and subsequently left undisturbed, we could recover *E. coli* from soil for long periods of time (over 85 days).

The present research expands our experience from these earlier findings regarding survival of *E. coli* and *Salmonella* when introduced to crop residues that are incorporated into the field soil. For this past research season, we simulated the contamination of lettuce by inoculating mature crops with generic *E. coli* and *Salmonella*. Lettuce plots were then differentially treated by mowing, ring-rolling, or leaving the contaminated crop undisturbed. After a holding period, the contaminated crops were disked into the soil. One set of plots, including all the soil the treatments, was subject to an irrigation treatment one week after disking. *E. coli* and *Salmonella* survival was monitored by sampling soil plus crop residue beginning with the day of incorporation and then at 1, 6, 11, 20, and 48 days post incorporation. After 21 days, a second lettuce crop was planted into the original plots and plants were sampled at thinning stage (27 days after emergence) to evaluate for transference of the inoculated strains to this replanted crop.

Following incorporation, target bacteria were quantifiable only in a few plots from the first sampling date, but were recovered by sample enrichment from most plots at all sampling dates up to 48 days after inoculation. No second crop lettuce plants tested positive for *E.* coli in any plot or in any of the two sampling sets. Second crop lettuce plants that tested positive for *Salmonella* were culturally and genetically confirmed as the inoculated isolate in six plots.

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 (Jay et al., 2007) and the lettuce recall in May 2010 (Fulton, 2010), have highlighted the need and increased the need for practical information on the dynamics of such organisms in agricultural systems. Recent studies (Moyne et al., 2011; Erickson et al., 2010) have contributed field generated information on such elements.

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 does react to the presence of non-pathogenic, generic *E. coli* as indicators of food safety risks. Generic *E. coli* can be readily detected in many farm environments, and the ecology, biology, and fate of these organisms

needs further documentation in this setting. Current food safety concerns, buyer contracts and conditions, and food safety metrics all list generic *E. coli* as a validated indicator of fecal contamination and product acceptance specifications are based on this assumption.

E. coli is not the only foodborne pathogen of concern for leafy green commodities. As a consequence of increased surveillance testing, in conjunction with a lesser pattern of involvement in outbreaks on leafy greens, *Salmonella* spp. has also become a serious concern for the recent frequency of costly recalls associated with detection. Diverse sub-types of *Salmonella enterica* have been recovered from lettuce and other leafy greens originating from all major production districts. As a soil or soil amendment and crop residue contaminant, *Salmonella* spp. has been recovered for long periods in agricultural environments. *Salmonella* is well-characterized as a zoonotic pathogen with a more robust environmental fitness, in general, than *E. coli* but specific survival, persistence, and details of soil transference potential to crops under field conditions is limited. Food safety management, preventive control standards, and audit criteria rely on such data to establish meaningful metrics.

In field oriented studies involving *E. coli*, we have validated 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. Because generic strains of *E. coli* have proven to survive as well or better than the available attenuated strains of O157:H7 *E. coli* in field environments over several years of studies, generic strains were selected for these field studies in 2011 and 2012.

Spinach and lettuce are high value leafy vegetable crops that are grown extensively in California. The coastal spinach and lettuce producing area is the most important and productive region for these commodities. Therefore, it is critical to develop practical information on how *E. coli* may behave in cropping systems under coastal California conditions and with the diverse biological setting found in the field (Anderson et al., 2006; Barker et al., 1999; Chandler and Craven, 1980; Gagliardi and Karns, 2002; Mubiru et al., 2000; Tate, 1978). Field-generated research conducted in the Salinas Valley can contribute significantly to our understanding of *E. coli* ecology and can assist industry in further refining management practices and metrics for dealing with this foodborne pathogen (Koike et al., 2008; Koike et al., 2009).

During the trials we conducted in 2011, we determined that the intensity of tillage treatments did not seem to have a significant impact on the survival and recovery of bacteria from a contaminated crop that had been incorporated into the soil. Also, different levels of biomass residue did not seem to impact the survival and recovery of bacteria from these plots. We did notice, however, that applications of water seemed to have a significant impact. Thus, for the 2012 research season, in addition to evaluating common methods for treatment of a contaminated crop before disk incorporation (mowing, ring-rolling), we aimed to develop further information on the impact of additional irrigations to fields where a contaminated crop had been incorporated. We also collected information on the contamination of a newly planted crop in a previously contaminated field.

Research Methods and Results

Project Design: Romaine lettuce (cultivar: Rome 59) was direct-seeded into six rows on 80 inch beds on June 4, 2012 in a loamy-sand soil at this experimental site in the Salinas Valley. The lettuce subplots, each 120 feet long and 6 beds wide, were thinned on July 3 and inoculated on

August 7 and 14. There were nine treatments, each with four replications (A to D), for a total of 36 plots.

Treatments 1-6: inoculate, wait 48 hours, apply soil treatment, wait one week, disk.

1. Untreated — No extra irrigation
2. Untreated — Extra irrigation after one week
3. Mowed — No extra irrigation
4. Mowed — Extra irrigation after one week
5. Ring-roll — No extra irrigation
6. Ring-roll — Extra irrigation after one week

Treatments 7-8: inoculate, wait 48 hours, then apply soil treatment and disk on same day.

7. Untreated — No extra irrigation
8. Mowed — No extra irrigation
9. Ring-roll — No extra irrigation

Inoculation: At crop maturity, plants were inoculated with liquid bacterial suspensions containing approximately log 6.0 cfu/ml of both generic *E. coli* (a cocktail of three rifampicin-resistant strains) and attenuated *Salmonella* sv. Typhimurium (one rifampicin-resistant strain). Preliminary lab studies determined that no significant growth interference or antagonism during co-culture of the four isolates in nutrient broth was observed. The inoculum was applied as a broadcast spray by using CO_2 powered backpack sprayers and hand-held, four nozzle booms. Two liters were applied to the center two beds of each plot (at a rate of one liter per 100 ft length of one 80 inch wide bed). All plots in treatments 1-6 were inoculated on August 7 and all plots in treatments 7-9 were inoculated on August 14.

Crop incorporation: Following inoculation (Aug. 7), the contaminated crops for all plots in treatments 1-6 were left standing for 48 hours. Subsequently, lettuce plants were mowed in treatments 3-4, were ring-rolled with a Schmeiser cultipacker attachment in treatments 5-6, and were left undisturbed in treatments 1-2. One week after incorporation, all plots in treatment 1-6 were incorporated into the soil with two passes of a standard disk.

Following inoculation (Aug. 14), the contaminated crops for all plots in treatments 7-9 were left standing for 48 hours. Subsequently, lettuce plants were mowed in treatment 8, were ring-rolled with the Schmeiser in treatment 9, and were left undisturbed in treatment 7. Immediately following these treatments, all plots in treatments 7-9 were incorporated into the soil with two passes of a standard disk.

Following disking, ditches were cut at the boundaries of plots both length- and cross-wise to prevent irrigation runoff from crossing between plots. Overhead sprinkler irrigation was applied for 4 hours (for a total of 1.0 to 1.2 inches of water) to plots in treatments 2, 4 and 6 on August 23. Equipment in the field was operated parallel to bed lines and within the main plot boundaries to reduce the movement of soil that could result in cross contamination of plots.

Sampling and testing of soil: Following crop incorporation, soil and accompanying plant residues were sampled from all plots on the day of incorporation and then on 1, 6, 11, 20, and 48 days post incorporation. Samples were taken with hand trowels sterilized with ethyl alcohol and dried with a clean paper towel. From each of two beds in the central part of each plot, 10 scoops of soil were collected to a maximum depth of 10 cm, placed in a sterilized bucket, and thoroughly mixed. A sub-sample of approximately 500 grams was then transferred to a sterile Whirl-Pak bag and transported on ice to the laboratory. Soil samples were processed in 18 oz stand up

Whirl-Pak bags; 100 grams of soil were mixed with 200 ml of sodium phosphate buffer amended with 0.05% Tween 20 and shaken for 30 seconds. Bags were allowed to sit undisturbed for 15 minutes to allow the soil to settle. 100 µl of the supernatant were then plated in duplicate onto amended Chromagar-ECC as well as XLT4 medium, both of which contained 100 µg/liter rifampicin (ECC-rif and XLT4-rif). The soil suspension was held at 4º C until the results of the quantitative plating were known. Plates were incubated for 24 hours at 37º C to select for the target strains.

Blue colonies on ECC-rif were considered presumptive positives for generic *E. coli* and were enumerated. Black colonies on XLT4-rif were considered presumptive positives for *Salmonella* and were enumerated. If colonies were not detected by this direct plating method, a concentration step was added by taking 2 ml of the sample supernatant, centrifuging it at 3900 rpm for 10 minutes, and re-suspending the resulting pellets in 200 µl of buffer. This concentrated suspension was plated and incubated as above, then enumerated. Representative strains of presumptive *E. coli* and *Salmonella* were saved and later tested by PCR analysis to confirm that the recovered bacteria were the experimentally inoculated strains.

When bacterial colonies were not detected by this concentration technique, enrichment was done to further examine for the presence of the target bacteria. 25 ml of sample supernatant were added to 75 ml of amended tryptic soy broth that contained 100 µg/liter rifampicin (TSB-rif). This mixture was incubated for 24 hours at 37º C; 100 µl of the enrichment mixture were subsequently plated onto ECC-rif and XLT4-rif and incubated for 24 hours at 37º C. Due to very low bacterial recovery, beginning with the samples collected on Aug. 27 (11 dpi), a new enrichment procedure was used: 100g of soil were added to 200ml of concentrated buffered peptone water (BPW) amended with 100 µg/liter rifampicin (2x BPW-rif) within an 18 oz stand-up Whirl-Pak bag; the sample was shaken and massaged for 30 seconds, then incubated and plated as with the former procedure.

Replanting lettuce: To determine whether lettuce planted into the experimental plots would become contaminated from the previous crop residue, a second crop was planted into the same plots on September 6, 21 days after incorporation of the originally contaminated crop. Soils in the plots were prepared for planting according to commercial practices. Lettuce was then direct-seeded into the previously inoculated area of each plot and grown as per standard procedures.

Sampling and testing of plants from the second crop: Lettuce plants from the second crop were collected at thinning stage (27 days after planting) to evaluate for presence of the inoculated strains of bacteria. Two sample sets were collected per plot. Due to the lateness of the season, the crop growth was slow and not uniform across the field, so plant sample sizes varied between plots. We intended to collect at least 75 g of leaves per plot, but in some sections this was not possible. There were no plant samples collected for plots 2A, 5A, 5D, and 9B. Tools and gloves were sterilized with ethanol between plots.

Plants were cut just above the soil surface and placed into a 55 oz Whirl-Pak filter bag, then transported on ice to the lab. Samples were processed by adding a 1:1 mass-to-volume ratio of BPW amended with 100 µg/liter rifampicin (BPW-rif). The mixture was then massaged thoroughly before reserving 1ml of the broth for enumeration by direct plating. Then another 2x volume of BPW-rif was added to the bag (for a total 1:3 mass to volume ratio), which was massaged again before being incubated at 37º C for 24 hours. After incubation, 100 µl of the enriched broth was plated onto duplicate ECC-rif and XLT4-rif plates and incubated for 24 hours at 37º C to determine presence or absence of the target bacteria.

On October 11, additional plant samples were taken from the plots where the original samples were positive for the inoculated bacteria (six plots total). For the second sampling, inner and outer plant leaves were separated at the time of collection and processed as distinct samples using the methods described above.

Outcomes and Accomplishments

The limit of detection for bacterial recovery in this study is defined as log 1.43 cfu/g for direct plating of 100 µl and log 0.13 cfu/g for plating a 2 ml concentration. Only a few samples could be enumerated as such, and the majority of the data reflects only the presence or absence of the target bacteria as determined by the enrichment procedure.

Soil:

In the first two days after crop incorporation, target bacteria could only be enumerated by concentrating a 2 ml sample; about 15% of the plots had visible colonies and these were present at very low numbers. Therefore, processing after August 17 (11 dpi) was done exclusively by enrichment and no numerical trend data are available for bacterial recovery.

Generally, the number of soil samples testing positive for S*almonella* after enrichment increased with time from the day of crop incorporation (Figure 1). After irrigation (August 23) in plots 2, 4, and 6, there does not appear to be a significant change in number of plots testing positive for the inoculated strains.

Figure 1. Total (out of four replications) number of samples per treatment with positive results for *Salmonella* after enrichment of soil sample.

	Sampling Date					
Treatment	**16-Aug**	**17-Aug**	**22-Aug**	**27-Aug**	**5-Sep**	**3-Oct**
1	0	1	1	2	4	4
2	1	2	2	1	4	3
3	1	1	0	3	2	3
4	2	3	4	4	4	4
5	0	1	0	2	4	2
6	2	1	3	4	3	3
7	0	0	0	2	3	2
8	3	3	2	3	3	2
9	0	0	0	1	4	2

Very few samples tested positive for *E. coli* throughout the trial, and there does not appear to be a trend in *E. coli* recovery over time (Figure 2). There were no positive samples for *E. coli* for treatments 1 and 9 for all sampling points.

Figure 2. Total (out of four replications) number of samples per treatment with positive results for *E. coli* after enrichment of soil sample.

	Sampling Date					
Treatment	**16-Aug**	**17-Aug**	**22-Aug**	**27-Aug**	**5-Sep**	**3-Oct**
1	0	0	0	0	0	0
2	0	0	0	2	0	1
3	1	0	0	0	1	0
4	0	1	0	0	0	1
5	0	0	0	0	0	1
6	0	0	0	0	1	1
7	0	0	0	0	0	1
8	1	1	1	0	1	1
9	0	0	0	0	0	0

Plants:

No second crop lettuce plants tested positive for *E. coli* in any plot or in any of the two sampling sets. Second crop lettuce plants that tested positive for *Salmonella* were confirmed in six plots. When these six plots were re-tested eight days later, there were no positive results for *Salmonella.*

Plant enrichment results for detecting Salmonella

sampling date		3-Oct	9-Oct
1	A		
	B		
	C		
	D		
2	A	-ns-	
	B		
	C	(+)	(-)
	D	(+)	
3	A		
	B		
	C		
	D		
4	A		
	B		
	C	(+)	(-)
	D		
5	A	-ns-	
	B		
	C		
	D	-ns-	

sampling date		3-Oct	9-Oct
6	A	(+)	(-)
	B	(+)	(-)
	C		
	D		
7	A		
	B		
	C	(+)	(-)
	D		
8	A		
	B		
	C		
	D		
9	A		
	B	-ns-	
	C		
	D		

-ns- indicates that no sample could be collected from this plot

Summary of Findings and Recommendations

There were several differences between the inoculation procedures in this trial as compared with previous years. First, a lower concentration of inoculum was applied: log 6.0 cfu/ml this year as compared to log 8 cfu/ml in previous years. Also, for this year only half the volume of inoculum was applied per square foot as compared with previous years. Finally, the crop was left intact for a minimum of 48 hours after inoculation before being incorporated into the soil whereas in previous trials the crop had been immediately incorporated after inoculation. All of these factors likely contributed to the lower rate of recovery of the inoculated strains once sampling began.

The decision to decrease the volume was based primarily on practical considerations of the size of the experiment and the application methods available. The decision to decrease the concentration was intended, in part, to bring the experiment closer to a possible real-world contamination scenario. Despite the decreases in inoculum concentration and volume, a very large amount of bacteria was applied to the trial crops, an amount exponentially greater than what would be anticipated in a likely irrigation water contamination event. For practical and resource considerations, we did not test the difference between incorporating an inoculated crop immediately versus waiting 48 hours before incorporation. It is likely that the exposure to the elements during the 48 hour hold period in this experiment, as has been shown in our previous studies, had a great impact on the frequency of recovery of the inoculated strains.

Salmonella appears to have been more persistent in the environment than *E. coli*, with a greater number of samples testing positive for *Salmonella* than for *E. coli* across the experiment. *Salmonella* also appears to have thrived (or competitors that interfere with detection declined) as it adapted to the environment, as greater numbers of overall samples tested positive for *Salmonella* as time progressed in the sampling season.

Treatments 2, 4, 6, and 8 returned at least one positive replication of *Salmonella* for every sampling date. All other treatments had at least one sampling date with no positive replications. While treatments 2, 4, and 6 received an additional irrigation one week after incorporation, treatment 8 did not share this characteristic. Genetic confirmation is still pending for the presumptive positive results reported here.

This project builds upon data from previous years in examining the survival of generic strains of *E. coli* when inoculated into leafy-green production systems. The purpose was to provide documented information on the ecology and survival of *E. coli* and *Salmonella* under field conditions and to address practical questions facing growers. If a mature, presumably contaminated crop is not harvested and is disked down, what is the fate and survival of the bacteria on crop residue and in the soil? Such information, strengthened by multiple years of data, could be useful in addressing regulatory and metric concerns faced by growers in California.

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APPENDICES

Publications and Presentations

CA Leafy greens Research Board mid-year research meeting. Summary of studied field ecology of E. coli. Huron. March 20, 2012. S. T. Koike.

CA Leafy greens Research Board: Grower series on food safety. Field studies: E. coli/Salmonella survival. Salinas. September 6, 2012. S. T. Koike.

23rd annual fall desert crops workshop. Survival of E. coli and Salmonella in contaminated soil and current research towards remediation and on-farm and postharvest sources of Listeria contamination. El Centro. November 27, 2012. T. V. Suslow.

No publications were made during this period.

Budget Summary

The budget was divided between the Koike lab (UC Cooperative Extension, Monterey County) and the Suslow lab (UC Davis). Koike used the majority of the funds for hiring field personnel and laboratory technicians to assist in the research. Other funds were used to cover expenses such as field supplies, transportation costs, and crop growing costs. We did have sufficient funds to fully cover this project.

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