UCDAVIS CENTER FOR PRODUCE SAFETY

California Leafy Greens Research Program

CPS – California Leafy Greens Research Program

Project time period: April 1, 2009 – March 31, 2010
Final Report, April 30, 2009

Title of Research Proposal
Contribution of phyllosphere microbiota to the persistence of *Escherichia coli* O157:H7 ATCC 700728 on field-grown lettuce

Name of PI and name of Affiliated Institution of PI
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Project Objectives
Objective 1. Quantify differences in the culturable and total phyllosphere microbiota on drip and overhead irrigated field-grown Romaine lettuce plants for time points before and after the application of attenuated (non-pathogenic) *E. coli* O157:H7 ATCC 700728.

Objective 2. Identify strains of bacteria isolated from the phyllosphere of overhead and drip irrigated plants which inhibit the growth of *E. coli* O157:H7 ATCC 700728.

Background
This project was developed in complement to a concurrent CPS project led by Dr. Linda Harris *et al* investigating the persistence of *E. coli* O157:H7 (EcO157:H7) ATCC 700728 on field-grown Romaine lettuce in the Salinas valley.

Main conclusions
- Total bacterial phyllosphere population sizes on Romaine lettuce differed over time during the 4 week field-trials and season of planting (Spring and Fall 2009). During the Spring 2009, bacterial population amounts also differed significantly depending on method of irrigation and exposure to EcO157:H7. These results are preliminary and additional field studies evaluating the effects of season, time, and irrigation on the bacteria associated with Romaine lettuce are required to establish the dominant microbial patterns on the plants after an EcO157:H7 contamination event.
- Population sizes of the phyllosphere bacteria on plants in 2009 were negatively (Spring) and positively (Fall) correlated with the detection of viable EcO157:H7 on the plants. These results

are preliminary and additional field studies evaluating these relationships are required to determine the precise effects of indigenous population sizes on EcO157:H7 persistence.

- Thus far, a limited culture-independent analysis of the phyllosphere microbiota indicates that some plants contain only a few bacterial species while others harbor highly diverse microbial communities with members which are not easily cultivated on standard laboratory culture media. On-going analyses are aimed at identifying potential correlations between microbial diversity patterns or specific organisms which are associated with *E. coli* persistence on Romaine lettuce in the field.
- Field-grown Romaine harbors indigenous bacteria which are antagonistic towards the growth of virulent EcO157:H7. A total of 28 EcO157:H7 inhibitory isolates were identified in this project. The roles and applications of these stains to control EcO157:H7 amounts remains to be established.

Outcomes and Accomplishments

Objective 1. This objective achieved the isolation and enumeration of total culturable bacteria associated with field-grown romaine lettuce plants in the Salinas valley inoculated with EcO157:H7ATCC 700728. These analyses were performed in parallel with assessments of EcO157:H7 ATCC 700728 amounts by Dr L. J. Harris *et al* on plants from the same field in a concurrently funded CPS project. The proposal was focused on analysis of the phyllosphere microorganisms (microbiota) for the 2009 Spring field trial designed by Dr. Harris *et al*. However, as described below, we were able to extend the project to include analyses of the plant microbiota in the Fall 2009 field trial. We provide the outcomes for both trials below.

In the Spring 2009, total culturable bacteria associated with Romaine lettuce were quantified on field-grown plants in the Salinas valley which were either overhead (sprinkler) or drip (ground) irrigated and inoculated with EcO157:H7 ATCC 700728 or were not additionally modified (total of 4 treatment groups). Bacteria were enumerated on three different standard laboratory culture media (TSA, KB and MRS) at multiple time points over one month (t = 0, 2 h, 2 d, 7 d, 14 d, 21 d, and 28 d). These media enrich for different species of bacteria and resulted in the selective growth of multiple colony types (morphologies) at each time point. Estimates of bacterial population sizes on the lettuce during the Spring trial according to amounts of bacteria detected on TSA are provided in Figure 1. Bacterial population sizes on the lettuce according to KB and MRS are provided in the Appendix, Figures 1 and 2. In this study, TSA (Figure 1) and KB (Appendix Figure 1) yielded similar estimates of bacterial cell amounts; whereas different trends were observed for MRS, a cultivation medium designed to detect acid resistant lactic acid bacteria able to grow under anaerobic conditions (Appendix Figure 2). TSA and KB are among the most commonly applied media to quantify bacterial cell amounts in the phyllosphere, and therefore, we believe the results found on these media constituted dominant patterns for cultivatable phyllosphere-associated bacteria.

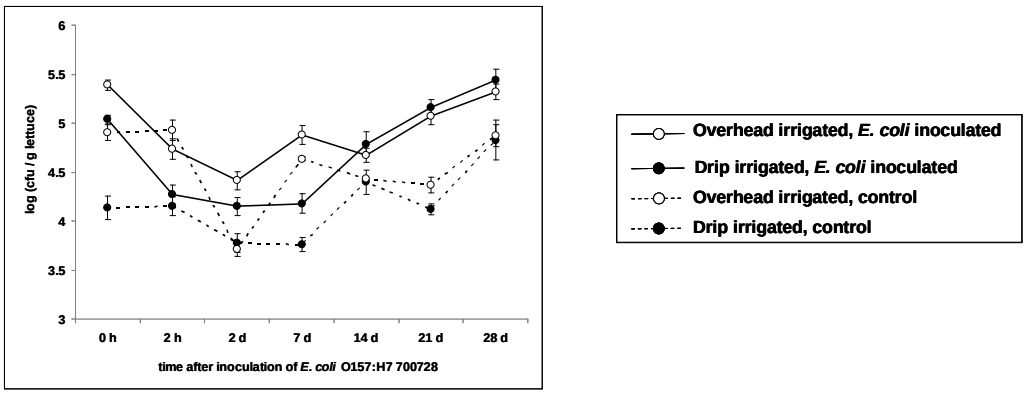


Figure 1. Total cultural bacteria on Romaine lettuce plants in Spring 2009 measured on tryptic-soy agar (TSA). Cell amounts were determined by plating dilutions of Romaine lettuce plant washes onto TSA and incubating at room temperature (21 °C) for 2 to 3 days. The average ± std error of 12 individual plants are shown for each treatment group and time point.

Bacterial population sizes were significantly different between the overhead and drip irrigated plants for time points t = 0 to 7 d (Figure 1). The overhead irrigated plants typically contained 2 to 5-fold more total bacteria. Similar effects of irrigation were found for the control plants and plants which were inoculated with the attenuated E. coli O157:H7 strain. Overall, these data are interesting with regard to the data of Dr. Harris *et al* which show that EcO157:H7 was more frequently detected on overhead irrigated compared to drip irrigated lettuce plants until 21 days after inoculation of the organism. Interestingly, the inoculated plants harbored significantly higher amounts of culturable bacteria ($p < 0.05$, student's t test) at most time points during the 28 day study. These differences were not due to the EcO157:H7 inoculum, because cultivatable amounts of this organism rapidly declined within the first 2 days after application onto the plants and were subsequently found in very low levels measurable only by enrichment (Moyne, Harris *et al personnal communication,* see also CPS report for Harris *et al*).

To identify relationships between the culturable populations of phyllosphere bacteria and *E. coli* persistence on the plants, enrichments for EcO157:H7 ATCC700728 were performed on the plants starting on day 3 of the field experiment. Comparisons between total bacterial amounts and the presence of culturable EcO157:H7 ATCC700728 showed a clear trend towards an inverse relationship between indigenous bacterial population sizes and the presence of EcO157:H7 in the phyllosphere ($p = 000014$, student's t-test) (Figure 2). This trend is opposite from the results of the Fall 2009 field trial (see below).

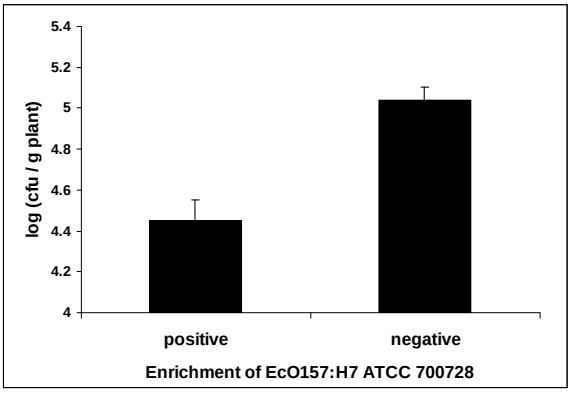


Figure 2. Average population sizes of bacteria associated with Romaine lettuce in Spring 2009 on plants which were positive or negative for the EcO157:H7 inoculant. Estimates of bacterial amounts were made using TSA. The average ± std error for all plants from the time points t = 3, 14, 21, and 28 d after inoculation (n = 28 positive and n = 64 negative). Enrichment results were not obtained for plants collected on day 7.

Because results of the Spring 2009 field trial yielded new information regarding potential correlations between the indigenous phyllosphere microbiota and the persistence of EcO157:H7 ATCC700728 (see above), sampling of the phyllosphere microbiota was continued in the second field trial performed by Harris *et al* in 2009. This field trial was originally planned to be conducted in the Summer (July – August), 2009. However, difficulty with lettuce germination at the field site in Salinas resulted in the completion of the experiment during October, 2009. Hence, this field trial was termed the Fall 2009 field trial.

Enumeration of culturable phyllosphere microbiota was performed in the Fall trial using TSA (Figure 3) and MRS (Appendix Figure 3) according to methods described for the Spring field trial (see above). The

population dynamics of bacteria on these plants differed significantly compared to the Spring field trial (compare to Figure 1). Among the 4 plant treatment groups, differences between bacterial population sizes were limited to the day that *E. coli* was inoculated onto the plants (Figure 3). A major factor in the Fall field trial might have been the large rain storm in Salinas on day 2 of the trial (October 13, 2009). The population sizes of bacteria on all plants increased to similarly high levels (> 3 x 10^6 cells per gram lettuce) by day 3 and followed similar patterns of decline and survival during the remaining 25 days of the experiment.

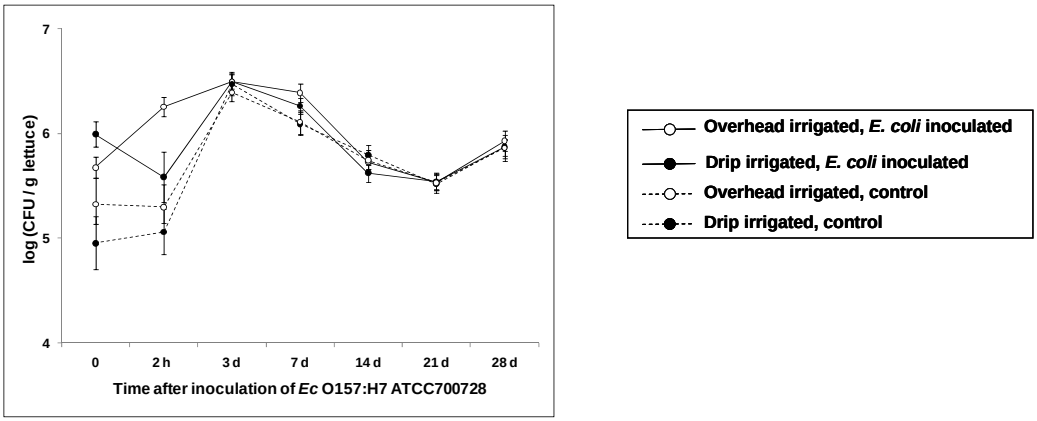


Figure 3. Total cultural bacteria on Romaine lettuce plants in Fall 2009 measured on tryptic-soy agar (TSA). Cell amounts were determined by plating dilutions of Romaine lettuce plant washes onto TSA and incubating at room temperature (21 °C) for 2 to 3 days. The average ± std error of 12 individual plants are shown for each treatment group and time point.

Comparisons between bacterial amounts recovered from the plants and EcO157:H7 ATCC700728 enrichments, showed a clear trend towards a direct relationship between indigenous bacterial population sizes and the presence of EcO157 in the phyllosphere (p = 000058, student's t-test) (Figure 4). This trend is opposite from what was observed during the Spring 2009 field trial (see above).

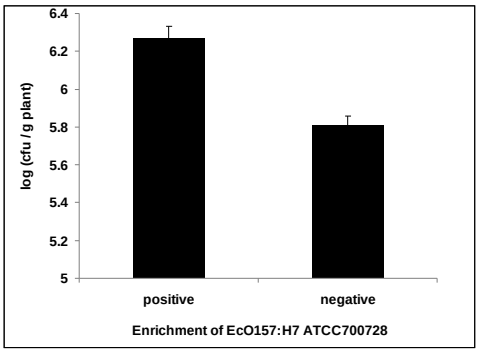


Figure 4. Average population sizes of bacteria associated with Romaine lettuce in Fall 2009 on plants which were positive or negative for the EcO157:H7 inoculant. Estimates of bacterial amounts were made using TSA. The average ± std error for all plants from the time points t = 3, 14, 21, and 28 d after inoculation (n = 48 positive and n = 72 negative).

This objective also aimed to identify the indigenous bacteria in the Romaine lettuce phyllosphere using culture-independent (DNA-based) methods. In particular, we focused our attention on Spring 2009 plant samples at time points 7 d, 14 d, and 21 d after application of the attenuated strain, as these time points illustrated unique differences between the plant treatment groups. Application of DNA microarrays

containing 16S rRNA genes for bacterial identification was described in the original aims of this project. However, this aim encountered several challenges. Firstly, we found that the stomacher approach used for washing the lettuce by Harris *et al* was quite damaging to the plants. Although this washing method yields consistent and accepted measurements of viable bacterial cells from lettuce, it also releases large amounts of plant DNA and likely other compounds (e.g. phenolics, carbohydrates, and humic acids) which inhibit downstream DNA analysis. Hence, we investigated multiple DNA extraction methods for the microorganisms associated with the plant samples collected by using the stomacher method. We successfully overcame this challenge well within the time-line of the project (late Summer 2009),and identified a suitable, although not a 100% effective method, which involves the use of CTAB, phenol, and Qiagen columns for DNA purification. The second challenge with the culture - independent analysis was with the facilities applying high-throughput methods for bacterial identification. DNA microarray Phylochip analysis were met with considerable delays (samples sent in October, 2009). Difficulties with the Phylochip processing facility were eventually resolved, and in March, 2010 (data not shown), however, this delay prompted us to explore pyrosequencing, a technology which is being applied in another CPS project (Coaker *et al*). Pyrosequencing provides similar microbial identification opportunities as the Phylochips. For this purpose, we initiated contact with the Core for Applied Genomics and Ecology (CAGE), The University of Nebraksa, Lincoln which specializes in pyrosequencing with the 454 Life Sciences, GS FLX Titanium platform. The results for two samples examined by pyrosequencing are summarized in Appendix Figure 4. Notably, both the Phylochips and pyrosequencing yielded similar conclusions: (1) a limited culture-independent analysis of the phyllosphere microbiota confirmed the presence of highly diverse microbial communities associated with Romaine lettuce which are not easily cultivated on standard laboratory media; (2) Substantial plant to plant variation exists in microbial diversity patterns on lettuce. Enterobacteriaceae**,** the bacterial Family to which EcO157:H7 belongs, were found in both plant samples. However, real time RT-PCR for detection of EcO157:H7 ATCC700728 indicated that this organism was not found in measurable quantities on either plant. This result was not in agreement with enrichment tests which showed that one of the plants (Appendix Figure 4 (A)) tested positive for EcO157:H7.

Due to the satisfactory results obtained for the first 2 samples, we redirected our attention to pyrosequencing to complete the sequencing on the remaining 16 samples budgeted in this project. The pyrosequencing technology has some advantages compared to the Phylochips including cost savings, availability of advanced data analysis tools, quantification options, and overall accessibility through service centers (e.g. CAGE) throughout the USA. These samples were provided to CAGE in March, 2010. However, this method was also encumbered with delays due to reagent reformulations by Roche, 454 Life Sciences, preventing completion of our sample analysis. This issue applies to all pyrosequencing users, and hence affects all researchers using this instrument (See letter attached to the back of the Appendix). We are currently working with CAGE and Roche to find a rapid resolution to the reagent reformulations, and we have the highest expectations to complete the intended analyses in advance of the CPS symposium in June, 2010.

Objective 2. This objective was designed to identify strains of bacteria isolated from the phyllosphere of the overhead and drip irrigated plants which inhibit the growth of *E. coli* O157:H7. Growth-inhibition studies were performed on attenuated and pathogenic EcO157:H7 strains using an agar overlay method. A total of 98 isolates collected from the Spring and Fall field trials (80 from the Spring and 18 from the Fall) were examined. Among these phyllosphere isolates, twenty-eight were able to inhibit the growth of one or more of EcO157:H7 strains including EcO157:H7 ATCC700728 and 5 isolates from human and bovine sources associated with apple-juice, spinach, or sprout outbreaks. Isolates collected during the

Spring 2009 conferred lower levels of inhibition compared to isolates from the Fall field trial (Appendix Table 1). Specifically, several isolates from the Fall yielded the highest levels of inhibition (≥3 mm). The identified species are targets for identification in the phyllosphere by pyrosequencing or real-time RT-PCR to determine if their amounts are inversely correlated with EcO157:H7 levels on the plants.

Methods and resources used to gather data

Several routes of data collection were used in this project:

(1) Field-site, USDA, Salinas, CA (Harris *et al*)
(2) Standard microbiological laboratory culture media (TSA, MRS, and KB), protocols, and equipment were used for phyllosphere bacteria enumeration and bacterial isolate collection. Agar overlays were used to identify EcO157:H7 growth - inhibitory bacterial isolates.
(3) Standard molecular biology protocols were used as well as advanced molecular technologies for bacterial identification (DNA microarrays (Phylochips) and pyrosequencing (454 Life Sciences).

Unexpected events in planning/implementing the project

There were a couple unexpected outcomes during the implementation of the project. Firstly, results from the Spring 2009 indicated interesting trends between indigenous phyllosphere bacteria population sizes and the persistence of EcO157:H7 on the plants. Hence, the project was extended to include analysis of bacterial population sizes in the Summer 2009 field trial. As described above, the Summer field-trial was substantially delayed and therefore not performed until the Fall 2009.

Additionally, the project met challenges with the extraction of sufficiently pure DNA from the stomached lettuce plants. This small hurdle was overcome in the (late) Summer 2009. Culture-independent analysis of the phyllosphere microbiota was then substantially delayed due to errors in the processing of the Phylochips in a manner which was beyond our control and control of the Weimer lab. Finally, pyrosequencing of the 16S rRNA in the plant samples is currently delayed due to reagent changes in essential components of the pyrosequencing reactions (see attached letter). However, we are confident that the major hurdles in the technical set-up of both technologies are now resolved and we expect the results of pyrosequencing in advance of the CPS symposium in June 2010.

Collaborative efforts

Implementation of this project was dependent on the field trials planned in a concurrent CPS project under the direction of Dr. Linda Harris, UC Davis. This collaboration was successful as our labs were well integrated for the experimental work performed in the project. This was illustrated by the combined efforts in lettuce sampling and washing. Moreover, because we followed the same protocol for washing the lettuce (0.1% peptone water for 2 min at medium speed in a Seward Stomacher), the results of culturable bacteria associated with the plants are directly comparable to assessments of EcO157 determined by the Harris lab. In addition, enrichments performed on plants used for measurements of the phyllosphere microbiota were performed by Dr. Moyne (Harris *et al*), thereby enabling consistency in EcO157 detection methods between the two projects.

This project also described a collaboration with Professor Bart Weimer, UC Davis. The role of this lab was to provide assistance with DNA microarray, Phylochip, analysis of the leaf phyllosphere microbiota. Our first samples were provided to the Phylochip facility in October 2009, well within the time-line described by the project. Due to circumstances not in Dr. Weimer's control, we experienced considerable delays due to poor initial results with the Phylochips due to slow and incomplete information provided by the phylochips processing facility and technical difficulty due to the contamination of chloroplast and

mitochondrial DNA in the plant samples. Considerable effort was made to correct the errors made at that facility and to process representative plant samples by Prof. Weimer's lab and the Marco lab in February-March 2010. These efforts resulted in the successful examination of 2 plant samples on the Phylochips. We are working with CAGE and Prof. Weimer to resolve the current delay in the culture-independent analysis portion of this project.

Budget

This project was budgeted at $50,800. The budget costs were allocated toward personnel and materials/reagents. Personnel costs were also partially off-set by the teaching salary (3 months) provided to Thomas Williams, the PhD graduate student in the Marco lab working on the project. Due to this arrangement, an extension on the project budget has been approved until June 31, 2010.

- **Budget allocation:**

The budget was divided mainly between personnel (45%) and materials/consumables (55%).

- **Changes to original budget:**

A post-doctoral researcher was described in the project budget at a level of 0.5 FTE for 12 months. Due to the initial difficulty in finding a 50% post-doc and the strict time-line for completion of the project, a PhD student in the Marco lab, Thomas Williams performed the experimental work described in the project. Tom's efforts on the project have been outstanding and remarkable for a PhD student in the 2nd year of study.

Because the Phylochip analysis could not be performed within the time-line of the project, we redirected some resources to analyze our samples using Roche 454 Pyrosequencing. This technology can be performed at a cost savings, used independently without the cost burden of purchasing large quantities of chips, and is part of the *next generation* of DNA sequencing techniques which are being actively and intensively developed in many research laboratories. A portion of this budget is remains due to delays with the sequencing facility which were well beyond our control (see above). However, availability of funds in this project were extended until June 31st which will enable the completion of the project.

Publications and presentations

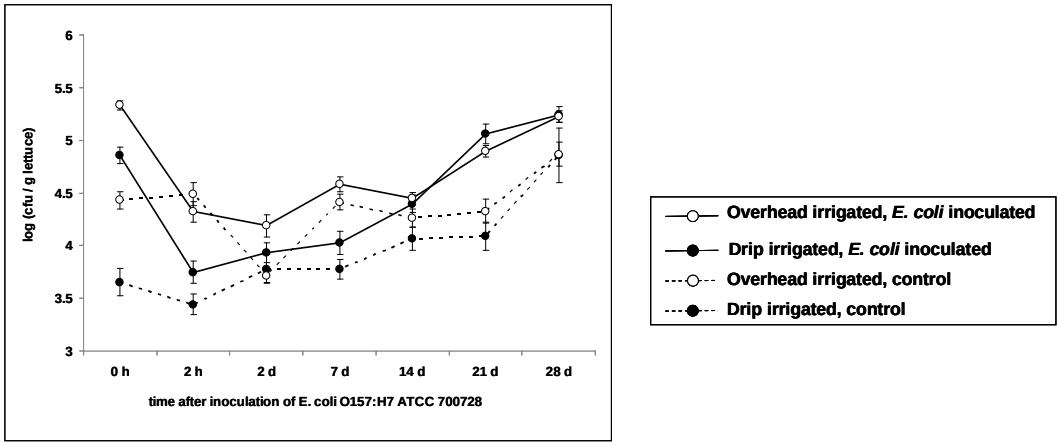
Because of the preliminary nature of this study, no manuscripts were prepared during the course of this year. Presentations were provided at the mid-term and annual CLGRB meetings.

Appendix

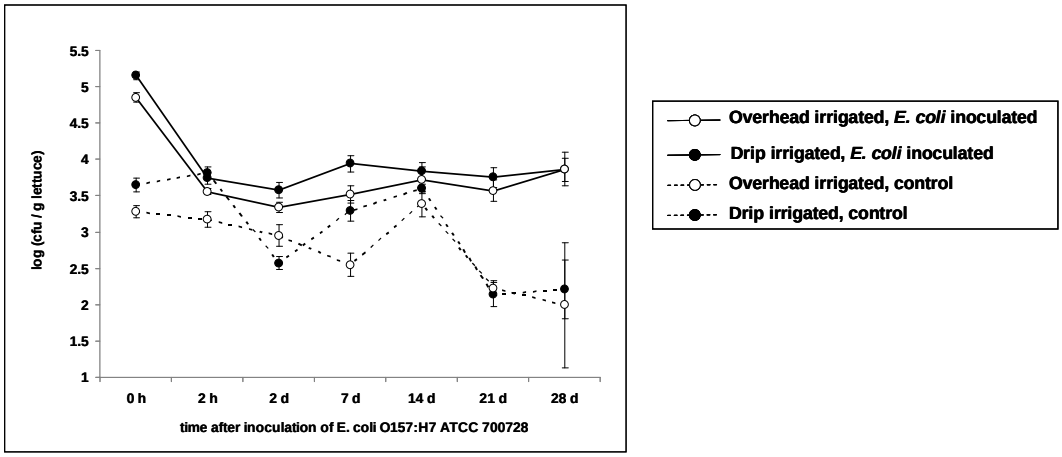
The appendix contains 4 Figures, 1 Table, and a Letter from the Core for Applied Genomics and Ecology.

Do you have any requests of the Center for Produce Safety or the California Leafy Greens Research Program regarding this project? None, thank you.

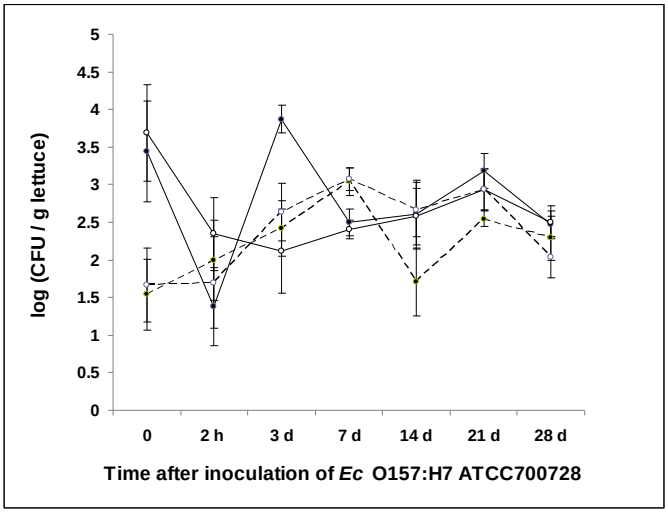
Appendix
Final report, Marco *et al*



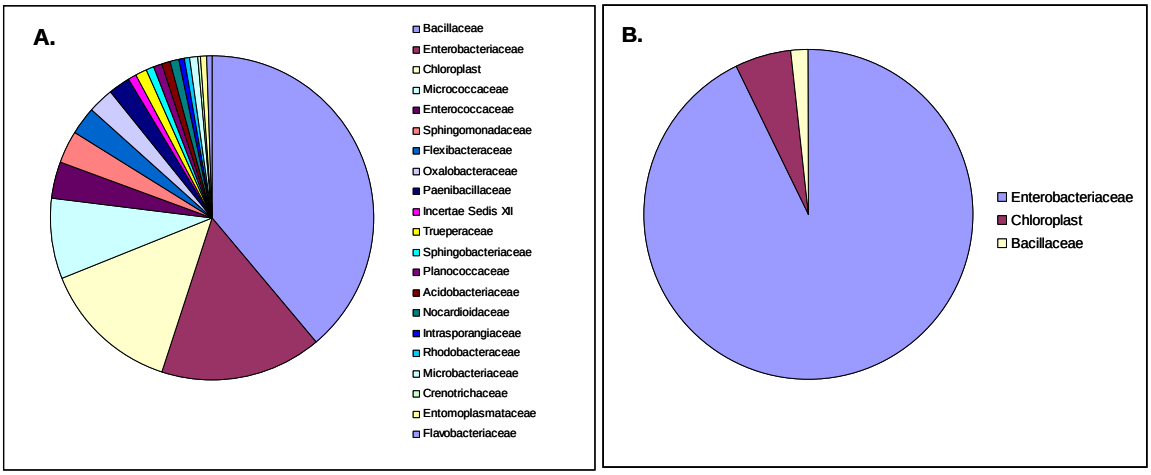
Appendix Figure 1. **Bacterial population sizes on Romaine lettuce during the Spring 2009 field trial according to <u>King's B agar (KB)</u>.** Cell amounts were determined by plating dilutions of Romaine lettuce plant washes onto KB and incubating room temperature (21 °C) for 2-3 days. The average ± std error of 12 individual plants are shown for each treatment group and time point.



Appendix Figure 2. Bacterial population sizes on Romaine lettuce during the Spring 2009 field trial according to <u>Mann-Rogosa-Sharpe agar (MRS)</u>. Cell amounts were determined by plating dilutions of Romaine lettuce plant washes onto MRS and incubating at 30 °C for 4 days. The average ± std error of 12 individual plants are shown for each treatment group and time point.



Appendix Figure 3. Bacterial population sizes on Romaine lettuce during the Fall 2009 field trial according to Mann-Rogosa-Sharpe agar (MRS). Cell amounts were determined by plating dilutions of Romaine lettuce plant washes onto MRS and incubating at 30 °C for 4 days. The average ± std error of 12 individual plants are shown for each treatment group and time point.



Appendix Figure 4. Bacterial families associated with Romaine lettuce according to 454 pyrosequencing. The plants were collected on day 14 of the field trial and consisted of **(A)** bacteria contained on a plant which as was overhead irrigated and inoculated with EcO157:H7 ATCC700728; and **(B)** bacteria contained a plant which was drip irrigated and not inoculated with the attenuated pathogen. Chlorophyll sequences represented approximately 15% of total sequences reads per plant. Plant represented by (A) harbored viable EcO157:H7 ATCC700728 according to enrichment, however, neither plant yielded a positive identification for EcO157:H7 ATCC700728 by real-time PCR.

Appendix Table 1.Bacterial isolates from the Romaine lettuce phyllosphere with growth inhibitory activity towards EcO157:H7.

					Zone of inhibition conferred by plant isolates against *E. coli* O157:H7 isolates (mm)					
					E. coli - ATCC700728	E. coli - 0557	E. coli - 1187	E. coli - 538	E. coli - 1189	E. coli - 1184
Isolate	Season	Time of collection	Control '(C) or inoculated (I)	Srinkler (S) or Drip (D) irrigation	attenuated strain; field-trial	Apple cider associated outbreak; human isolate	2006 Spinach outbreak	Sprout outbreak; human isolate	2006 spinach outbreak; bovine isolate	2006 spinach outbreak
Lactobacillus plantarum [a]					6 [b]	6	6	4	6	7
Curtobacterium_flaccumfaciens	Spring	2 h	C	S		1.5	0.5			
Bacillus sp	Spring	2 h	C	S		1.5	0.5			
Bacillus_cereus	Spring	48 h	C	S	1.5					
Bacillus_thuringiensis	Spring	48 h	C	S	0.5	1.5	1.5	< 0.5	< 0.5	
Bacilllus_cereus	Spring	0 h	I	D		0.5	0.5	N/A	N/A	N/A
Enterobacteriaceae_sp.	Spring	0 h	I	S	1.5	0.5	1	N/A	N/A	N/A
Lysinibacillus sp.	Spring	2 h	C	S		0.5				
Bacillus_sp.	Spring	2 h	C	D		1	1			
Bacillus_megaterium	Spring	2 h	C	S	1.5	1				
Enterococcus faecalis	Fall	28 d	I	S	1	0.5	0.5	0.5	< 0.5	< 0.5
Enterococcus sp	Fall	28 d	I	D	< 0.5	< 0.5	< 0.5	0.5	1	< 0.5
Enterococcus mundtii	Fall	28 d	I	S	5	1	1	1	1.5	< 0.5
Carnobacterium species	Fall	7 d	I	S	1			0.5	< 0.5	< 0.5
Uncultured sp	Fall	21 d	I	D	3	1	4	0.5	0.5	< 0.5
Enterobacteriacea	Fall	21 d	C	D	4	< 0.5	3	< 0.5		
Lactococcus lactis	Fall	21 d	I	D	6	6	4	N/A	N/A	N/A
Enterococcus sp	Fall	7 d	I	S	4	3	2	1	2	1
Enterococcus sp	Fall	14 d	I	S	3	3	2	1	2	1
Enterococcus sp	Fall	14 d	I	D	6	6	6	0.5	1	0.5
Leuconostoc sp	Fall	14 d	C	S	6	6	5	1.5	2.5	2.5
Unculturerd bacterium	Fall	14 d	C	S	1.5	< 0.5	1	1	1	1
Leuconostoc sp	Fall	14 d	C	D	4	4	2	2	4	1.5
Enterococcus faecalis	Fall	14 d	C	S	< 0.5	< 0.5		< 0.5	1	1

[a] *L. plantarum* was used as a positive control.
[b] Zone of inhibition of *E. coli* growth around the plant isolate according to the agar overlay method.

CAGE
core for applied genomics and ecology

University of Nebraska Lincoln

April 28, 2010

Dr. Maria Marco
Assistant Professor
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Dear Dr. Marco:

I regret to inform you that due to technical problems we have incurred significant delays in sequencing your plant samples for microbiome analysis.

Your samples had been processed using our validated protocols for generating amplicons with 16S fusion primers and running the pooled amplicons with the Roche titanium shotgun kits. However, at the beginning of April, Roche implemented an upgrade to their Lib-L Titanium shotgun kits and their emPCR kits that we had been using. We were initially assured that our validated process would be compatible with these new kits.

Unfortunately, the changes Roche has made in their emPCR kits are incompatible with our established process. Using the new kits and titrating across a range from 1-20 copies per bead, we get no significant emPCR bead recovery at any concentration for amplicons, even with pooled samples that had previously been run successfully. We have eliminated all obvious sources of the problem with our amplicons and are obtaining successful results with genome libraries using these same kits. At this point, we have no choice but to redesign all of our fusion primers and change our amplicon protocols so that they are compatible with the newly released Roche amplicon emPCR kits.

The performance of the new primers will also have to be validated against our old data to ensure that the new fusion primers perform to our standard. This is an extraordinary amount of bad luck and circumstance, and unfortunately, I doubt that we will be able to give you the data from your samples until the beginning of May.

Here is our best-case scenario for generating your data:

April 19th:	New Fusion Primers designed and ordered
April 23rd-26th	Anticipated delivery of new Fusion Primers
April 26th-30th	Validation runs on new primers
April 30th	PCR prep and emPCR reaction on your samples

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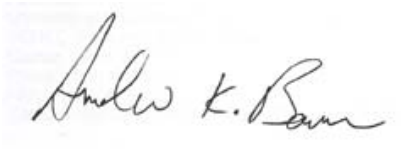
May 3rd	Bead recovery and run on your samples
May 4th-6th	Data QC and processing
May 7th	Data released

I am sincerely sorry about this situation. Unfortunately, it has been due to circumstances that are beyond our control. In the meantime, we can only thank you for your patience and assure you that we are working around the clock to provide the high-quality results our clients have to come to expect.

Sincerely,



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