CPS 2011 RFP
FINAL PROJECT REPORT

Project Title

Comparative assessment of field survival of *Salmonella enterica* and *Escherichia coli* O157:H7 on cilantro *(Coriandrum sativum)* in relation to sequential cutting and re-growth

Project Period

January 1, 2012 – December 31, 2012

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Objectives

1. To determine the quantitative and qualitative survival of *Salmonella enterica* and *Escherichia coli* O157:H7 on cilantro leaves after foliar applied contamination during pre-harvest production.

2. To determine the degree of persistence and dispersal of *Salmonella enterica* and *Escherichia coli* O157:H7 in sequential harvest and re-growth intervals.

3. Determine the impact of delays to cooling, specific temperature thresholds, and postharvest washing on growth potential and cross-contamination of applied pathogen-surrogates, including simulated industrial pilot-plant processing and simulated retail distribution.

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FINAL REPORT

Abstract

Since 2004, the FDA has confirmed the presence of *Salmonella* species and shiga-toxin producing *E. coli* in multiple fresh cilantro samples that was in, or entering into, commerce from both domestic and non-US origin. Cilantro is a culinary herb commonly consumed in its raw state without minimal processing. The purpose of this study was to assess the comparative post-contamination consequences on cilantro, in model commercial settings, with isolates of *S. enterica* and *E. coli* O157:H7 during open-field production, sequential harvest and re-growth, and in pilot plant postharvest operations.Two cultivars of cilantro, Santo and Leisure, were grown under open field conditions and spray-inoculated with two inoculum doses (log 4 and log 6 CFU/mL) an attenuated strain of *E. coli* O157:H7 and, inadvertently, a low-virulence isolate of *S. enterica* sv. Enteritidis rather than the intended attenuated *Salmonella* Typhimurium, due to a strain transfer error to the Suslow lab that originated in 2008. This mislabeling error from the source was not determined until after the completion of this project and only made possible by recently developed genetic sub-typing tools within the Suslow Lab specifically for the purpose or improved tracking of surrogate Salmonella in field and future pilot plant studies.

The survival of the applied research isolates statistically favors a greater outcome for net retained viability with the Salmonella than for the attenuated E. coli O157:H7 allowed for field applications by the UC Davis Biosafety Committee. As in prior CPS funded research in our lab, this indicates the importance of genus and strain selection and validation as it refers to environmental fitness and predictive fidelity of surrogates in modeling the behavior of pathogens. Populations as low as log 1.7 CFU/g were able to persist in the open environment for up to 12 days and survive postharvest disinfection hurdles. The persistence of *E. coli* O157:H7 and Salmonella on cilantro leaves was observed during the duration of cold-storage at 5C after 14 days post-washing or 26 days post-inoculation.

Cilantro was harvested at commercial maturity stage, processed in a model washing system treated with NaClO (50 mg/L, pH 6.5) and stored for 14 days at 5ºC or 12.5 ºC. Quantitative and qualitative survival of the pathogens was monitored after 12 h, 6 and 12 days post-inoculation (dpi), following disinfection, and 7 and 14 days post-washing. The effect of delays of cooling after harvest and the persistence of the pathogens in sequential cuttings and re-growth intervals was also evaluated.

No significant variability of attached *E. coli* O157:H7 and *Salmonella* was observed depending on the cilantro cultivar. *Salmonella* and *E. coli* O157:H7 populations declined after inoculation below the limit of quantitative detection, but still detected after 12 dpi by selective enrichment. The minimal processing with 50 mg/L of NaClO was not sufficient to disinfect the inoculated cilantro (log 6) prior to refrigerated storage. Viable populations of both pathogens were confirmed throughout storage, including the final time point. In relation to the potneial for re-growth on field cultivated cilantro, no culturable bacteria were detected 22 days after the first cut.

In addition, a wash water process control project was conducted in collaboration with SmartWash Solutions at their pilot plant facilities in Salinas, CA. A substantial in-kind funding, through technical and operational staff time and experimental materials, was generously contributed to the execution of this objective and was essential to obtaining the

data and results reported. This part of the project aimed to evaluate the removal and extent of cross-contamination control efficacy between cilantro and parsley inoculated with PTVS177 under pilot plant wash process operations. Four trials with pallet-load scale plant material, were conducted with varying conditions of challenge Salmonella dose on cilantro and sodium hypochlorite dose, with either citric acid or SmartWash (T-128) as the additional process water treatment aide. In brief summary of results, increase of chlorine dose to 15 ppm did not have an effect in further log reduction of Salmonella on contaminated cilantro compared to 4 or 10 ppm or in eliminating cross-contamination to non-inoculated and co-process washed parsley. The use of T-128 was observed to completely mitigate the presence of detectable levels of Salmonella in process wash water when contamination on cilantro was log 4 CFU/g or lower, but cross contamination due to plant to plant and plant to equipment contact needs to be considered for setting minimal dose thresholds.

This study provides a science-based approach to supply chain management that will be useful for the development and adoption of Best Practices in food safety management among cilantro growers and processors.

Background

Cilantro, parsley, basil, and other herbaceous culinary herbs have been implicated in several notable outbreaks and surveillance-initiated recalls since 1998. In the specific case of cilantro, since 2004, FDA has confirmed the presence of Salmonella spp. in 28 samples of cilantro in the market, from both U. S. and non-U. S. origin.

Cilantro is a widely enjoyed culinary herb commonly consumed in its raw state without a terminal kill step. Cilantro has become a popular fresh produce item in the United States for its usage in diverse ethnic cooking including Chinese, Southeast Asian, Mexican, Middle Eastern, Indian, and California Fusion styles. From November 2010 to February 2011, more than 8 brands and over 20 SKU's containing cilantro were recalled by major retailers in the US and Canada. In addition to the direct cost of implementing a recall, growers and shippers are typically required to remunerate their customers for a recall which may be 1000's of dollars in administrative fees, and hundreds of dollars in restocking fees per distribution center, SKU, and outlet store. Further indirect costs in lost reputation and continued erosion of consumer confidence are also of great concern to individual operations, the category, and the produce industry.

In March 2011, the FDA expressed concerns about positive sample findings for human pathogens on fresh cilantro in a guidance letter to companies that grow, harvest, sort, pack, or ship fresh cilantro. In this letter, the FDA recommends that the produce industry segment take action at three levels: 1) Review the current cilantro operations in the context of GAPs Guide, as well as other available information regarding reducing pathogens in fresh produce; 2) Assess hazards that are unique of the cilantro production and; 3) Develop commodity-specific preventive control strategies that would identify potential hazards that may be specific to fresh cilantro production and distribution, as it was previously done with tomatoes and leafy greens.

As one component of the response to the industry and public health concern, the Commodity Specific Food Safety Guidelines for the Production, Harvest, Post-Harvest, and Processing Unit Operations of Fresh Culinary Herbs (CSG Herbs 2013) was recently released. Our preliminary plant:pathogen post-contamination survival kinetic studies and

the in-progress outcomes of our CPS-funded research contributed to the development and content of this CSG document towards enhanced food safety management among cilantro growers and processors. The knowledge obtained during the development of this project is also expected to be largely transferrable to other leafy culinary herbs including parsley and basil and it is estimated that the quantitative and qualitative measurement of microbiological risks involved in the contamination of cilantro can set basis for the further development of commodity-specific guidelines and standards from production to processing and food service handling.

Research Methods and Results

OBJECTIVE A

To determine the quantitative and qualitative survival of* Salmonella enterica *and* Escherichia coli O157:H7 *on cilantro leaves after foliar applied contamination during pre-harvest production.

Materials and methods

<u>*Bacterial strains and inoculum preparation*</u>

Two different primary sources of inoculum, *E. coli* O157:H7 (*a*PTVS 155) and *Salmonella enterica* sv. Enteritidis (PTVS 177), were used in this study. The *E. coli* O157:H7 strain used is a non-toxigenic (*stx*1 and *stx*2 deficient) isolate (ATCC #700728) classified as suitable for Biosafety Level-1 (BSL-1) handling. At the initiation of this project the Salmonella strain used (originally cited as *a*PTVS 177) was believed to be an accurate derivative of *Salmonella enterica* sv. Typhimurium χ3985 an avirulent strain lacking adenylate cyclase and cyclic AMP receptor protein rendering it avirulent yet still immunogenic; this means it can be detected by immunoassays and PCR (Curtiss and Kelly, 1987; Hassan and Curtiss, 1990). This isolate has been used by the Suslow Lab since 2008, following modification of the isolate received by Material Transfer Agreement from Dr. Curtiss, University of Arizona. It was very recently discovered, during the course of development of a genetic sub-typing platform for isolate tracking in field trials and verification sero-typing, that the true attenuated isolate was not transferred due to a clerical error in culture collection document transfer at U of AZ years before our 2008 request. We have worked with Dr. Curtiss to confirm the error at the original source and the identity of the isolate we used for this and prior studies as *S. enterica* sv. Enteritidis 3895, a low virulence pathogen. There were no incidents of illness attributable to the handling of this isolate which, as a matter of standard policy, was still handled as a BSL2 strain, apart from the fact that it was used in field trials and pilot plant studies that would be likely to generate aerosols and allow by design for periods of environmental survival and exposure. Hereafter in this report we will refer to this isolate as *Salmonella* Enteritidis PTVS 177. An expanded explanation of this error is provided in the Appendix.

The use of these *E. coli* O157:H7 and *Salmonella* (as originally defined taxonomically) strains in both greenhouse and field studies was approved by the Office of Environmental Health and Safety (EH&S) of University of California, Davis. An antibiotic-resistant derivative strain for tolerance to rifampicin (80 mg/L) was isolated via spontaneous mutation and used to minimize interference with other bacteria and to facilitate the detection and recovery for each strain.

All strains were grown at 37ºC for 18 hours on tryptic soy agar (TSA) supplemented with 80 mg/L of rifampicin (TSA-rif). Several characteristic colonies were transferred to 5 ml of Butterfield's Phosphate Buffered Saline (PBS) to create a suspension of approximately log 9 CFU/ml and 100 µL were spread onto selective agar and incubated for 18 hours to create a uniform lawn. Cells were harvested by gently scraping the agar surface with a sterile rubber spatula, then washed in PBS, pelleted, re-suspended in PBS, and then serially diluted in PBS to the desired target dose. Two inoculum doses (log 4 and 6 CFU/mL) of each pathogen were prepared.

Cilantro cultivation and other culinary herbs

Two cultivars of cilantro, Santo and Leisure, were grown under open field conditions in the UC Davis Research Farm during fall 2011 and spring/summer 2012. Common commercial plant densities of 900 seeds/m^2 were established. In addition to cilantro, parsley cultivars Forest Green, Moss Curled, and Alizira as well as basil (Genoveses) were also seeded in the same field. Herb cultivation for field inoculation was planned and expected to take place at the UC Cooperative Extension field research site in the Salinas Valley, used in previous CPS funded research with S. Koike, but due to unanticipated limitations in availability of physical space to accommodate our trials, cultivation was done in Davis, CA to avoid delay of experiments.

The field setup consisted of 11 beds, each 200 ft long and 60 in wide. Each bed was divided in two subplots of 100 ft and three replicates or subplots of each cultivar were established planted (Fig. 1). Fields were previously fertilized and registered preplant herbicides applied to reduce the growth of weeds. The field was irrigated through sprinklers following regular agricultural practices. When necessary, weeds were manually removed.

Figure 1. Distribution of culinary herb cultivars and buffer zones in the field trial at UC Davis. Each cultivar was grown in triplicate in different subplots (n=3).

Field inoculation procedure

Each subplot was divided into 4 equal zones of 25 ft. each which corresponded to the two inoculum levels; log 4 and 6 CFU/mL as well as the two rifampicin-marked isolates; *Salmonella* PTVS 177 and *E. coli* O157:H7 (Fig. 2)

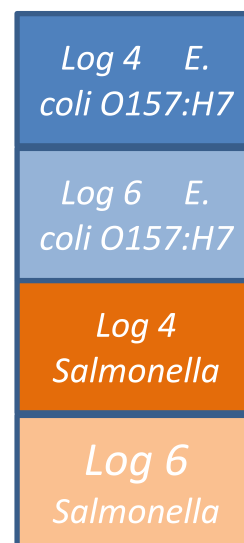

Figure 2. Subplot division and inoculation pattern.

Cilantro cultivars were inoculated 12 days before reaching a commercial maturity stage. Inoculum was applied in the pre-twilight hours with a CO_2 powered, handheld, backpack sprayer using a two nozzle spray boom and Teejet 8005 spray tips (selected to reduce spray drift); the sprayer was set at approx. 25-27 psi and it was sprayed 2 L/100ft. Each pathogen and dose was inoculated separately and during inoculation was momentarily sealed off with cardboards to prevent spraying to adjacent sections.

Harvest and Postharvest operations

Harvesting was done manually by directly cutting a desired amount of plant material and depositing directly into a sterile bag that was taken immediately to the laboratory for processing. Gloves were changed after collection of material from each subplot and disinfection of knives or scissors was also done within each block using 90% ethanol, sterile water, and wiping with a KimWipe.

Quantitative and qualitative survival of the pathogens was monitored after 12 hours, 6 and 12 days post-inoculation (DPI), following wash-processing (AW), and 7 and 14 days post-washing (DAW).

After harvest, approximately 600 g of each cultivar were processed separately at room temperature. The raw material was processed in a model wash systems amended with 50 mg/L of NaClO (pH 6.5 adjusted with a 1N citric acid solution) for 2 min and followed by a 30 s of rinsing with tap water. Then, the leaves were dewatered using a food service grade manual salad spin dryer (0.2 m^3 volume) to eliminate excess adhering water. An amount of 100 g of leaves were placed in commercial bags and stored in a controlled temperature room without light at 5 and 12.5 ^oC for up to 7 and 14 days, respectively. The 5 ^oC temperature was selected as the maximum limit recommended for storage and distribution, while 12.5 ^oC was selected as an abusive temperature during storage, distribution and retail sale. Three replicates for each cultivar, inoculum type/dose and storage temperature were prepared.

Recovery and detection

From each one of the treatments, a 100 g of plant samples were transferred into sterile plastic bags containing sterile modified EHEC media (mEHEC) or buffer peptone water (BPW) both supplemented with 80 mg/L of rifampicin in a 1:2 w/v (weight/volume) ratio for *E. coli* O157:H7 or *Salmonella*, respectively. Samples were then vigorously massaged by hand for 1 min and 100 µL of bacterial suspension was plated on TSA-rif and incubated at 37 ºC for 24 h to determine the surviving population by direct plating. Additionally, 1 and 5 mL of the wash buffer was filtered onto a 0.45 µm hydrophobic grid using the Neogen ISO-GRID system to increase the sensitivity in enumeration of viable and detectable applied isolates. TSA-rif plates were also supplemented with 1 g/L of sodium pyruvate {$C_3H_3NaO_3$;(TSARP)} during preparation to facilitate resuscitation of sub-lethally injured cells. Results of plate counts were reported as log CFU/g. A total of 5 samples per cultivar-subplot for field survival studies (n=15) were made. All samples for postharvest washing and storage studies were made in triplicate (n=3). After plating, the bags containing the homogenized plant samples and mEHEC or BPW supplemented with 80 mg/L of rifampicin were further supplemented with additional mEHEC or BPW supplemented with rifampicin to reach a 1:4 w/v ratio. Samples were incubated at 37ºC for 18h to evaluate bacterial populations below the limit of quantitative detection (LOD) by direct enumeration. For colony confirmation of the applied attenuated strains, the selective enrichments were plated onto ChromO157 (selective and differential for *E. coli* O157) and XLT-4 (selective and differential for Salmonella) supplemented with 80 mg/L of rifampicin and incubated at 37ºC for *E. coli* O157 and Salmonella. Typical mauve and black colonies were considered a positive result for *E. coli* O157:H7 and *Salmonella,* respectively.

Statistical analysis

If quantitative data was obtained, comparison using analysis of variance was performed with SAS statistical software using the GLM procedure and mean separation was done after Tukey analysis, however when only qualitative data was obtained, comparison was done using a generalized linear mixed model for categorical data using the GLIMIX procedure and Tukey for treatment separation to determine whether significant difference among treatments exist. Statistical significant difference was established when p-value <0.05.

Results

A controlled (fenced, bare soil buffered, and posted for restricted access) open field trial was conducted during fall 2011 at the UC Davis Plant Sciences Research Farm, as specified in the materials and methods section. Two cultivars of cilantro cv. Santo and cv. Leisure; three cultivars of Parsley cv. Moss Curled, cv. Alizira and cv. Green Forest; and a cultivar of basil cv. Genovese were seeded. Due to technical complications, weather conditions and a severe weed problem, parsley and basil seeds did not develop a useable stand for research purposes. Thus, this field trial was carried out only with cilantro cultivars.

Population dynamics of *E. coli* O157:H7 and *Salmonella* in the phyllosphere of cilantro during field production, postharvest washing and storage are presented in Table 1. The initial *E. coli* O157:H7 population was in the range of 3.80 – 3.81 and 1.84 – 1.97 log CFU/g after 12 hours of the inoculation event for the high (log 6) and the low (log 4) inoculum dose, respectively. No significant difference of attached *E. coli* O157:H7 in cilantro foliage samples was observed as a function of the cultivar. *E. coli* O157:H7 populations for all cultivars declined rapidly below the limit of quantitative detection (LOD) and no culturable bacteria were recovered by direct plating after 6 and 12 days post-inoculation. The viability of the bacterium was demonstrated 12 days post-inoculation, but significant differences among initial inoculum doses were observed. We determined that 100% of the samples (15/15) which were inoculated with the high dose were positive after selective enrichment for *E. coli* O157:H7, while only one sample out of 15 (6.7%) was positive for those samples inoculated with the low dose. For both inoculum doses, no differences in *E. coli* O157:H7 viability among cilantro cultivars were found. Statistical analysis of main factors showed that there was a significant effect of the dose (p=0.0031), as it refers to the day of harvest after 12 hours of inoculation, however no significant difference in survival was detected between days 6 or 12 post-inoculation (p=0.4047). After harvesting, cilantro samples were processed in a model wash system amended with 50 mg/L of NaClO and subsequently stored for up to 7 and 14 days at 12.5 and 5 ^{o}C. Overall, washing cilantro samples (log 6) with 50 mg/L of NaClO was insufficient to fully inactivate even these low populations of environmental stress-adapted *E. coli* O157:H7. The persistence of *E. coli* O157:H7 on cilantro leaves was observed during the duration of cold-storage at 5C after 14 days post-washing or 26 days post-inoculation.

A similar trend of persistence and population dynamics for *Salmonella* in the cilantro phyllosphere was observed (Table 1). *Salmonella* populations after application of inoculum ranged from 2.67 – 2.61 and 4.37 – 4.39 log CFU/g for the low and the high inoculum dose, respectively. In general, the recovery of viable cells from the delicate leafy culinary herb surface was greater for the applied *Salmonella* than for the applied *E. coli* O157:H7 for both inoculum sizes. Regardless, after 6 days post-inoculation all samples were below the LOD. In general, the percentage of positives after selective enrichment ranged from 70 – 93% and 100% for the low and the high inoculum dose, respectively, which showed a greater overall persistence in comparison with *E. coli* O157:H7 samples. Statistical analysis of main factors showed that there was a significant effect of the inoculum dose

(p=0.0105), as it refers to the day of harvest after 12 hours of inoculation statistical significant was established (p=0.0144) between the 6 and 12 days post-inoculum. The washing-disinfection step was unable to inactivate the low level of residual populations of *Salmonella* (<0.43 log CFU/g) attached to the high inoculum cilantro leaves, as similarly observed with *E. coli* O157:H7. Overall, all cilantro samples (log 6) were positive for *Salmonella* after selective enrichment during the storage regardless of the storage temperature and time. As stated above for *E. coli* O157:H7, no significant differences in *Salmonella* attachment and survival were observed as a function of the cultivar. In general, *Salmonella* persistence was greater than *E. coli* O157:H7 during the field production and postharvest washing and storage.

Populations of mesophilic bacteria were also monitored during post-harvest minimal process and storage (Fig. 3). An approximate 2-log reduction was determined after washing with sodium hypochlorite, however after seven days of storage the population size is re-established when stored at 2.5 ^{o}C and up to 3-log when exposed to temperature abuse even moderately above the recommend optimal or typical distribution cold-chain profile.

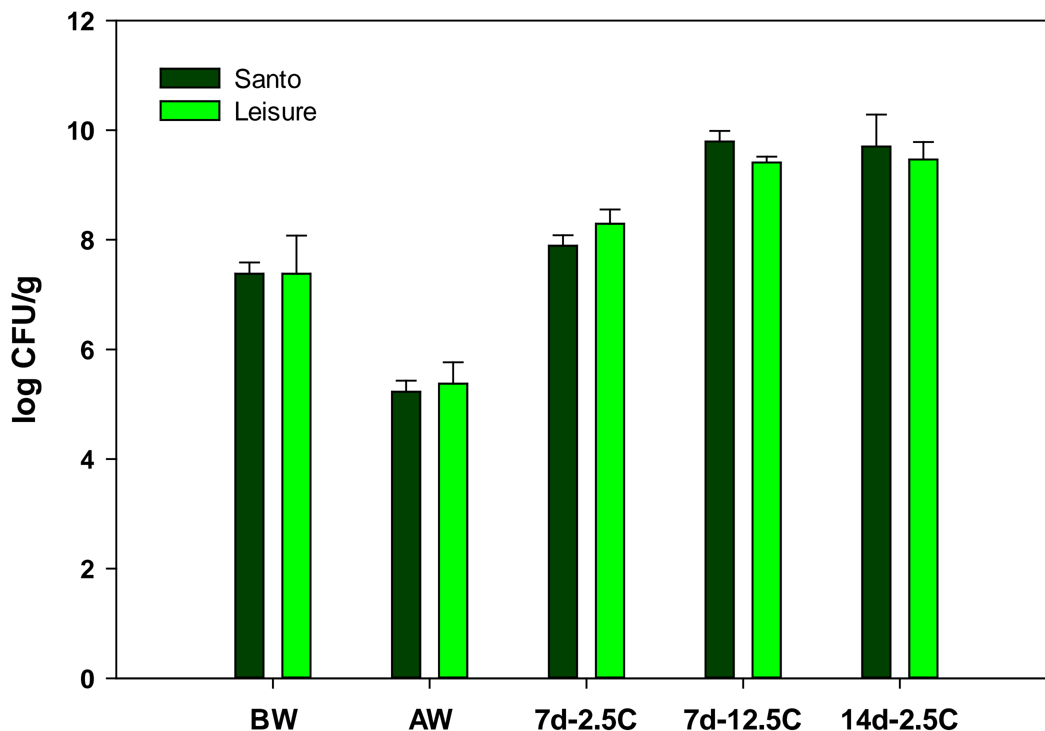

Figure 3. Population of total mesophiles on two cultivars of cilantro leaves (Santo and Leisure) after postharvest minimal processing and during storage. BW: before washing, AW: after washing

Table 1. Qualitative and quantitative survival of *E. coli* O157:H7 and Salmonella on cilantro leaves from preharvest through postharvest conditions. Fall 2011

***Escherichia coli* O157:H7**		**PREHARVEST**			**POSTHARVEST**				
		Days after inoculation				**7DAW & 19DPI**		**14DAW & 26DPI**	
Variety	**Inoculum level**	**0.5**	**6**	**12**	**AW**	**2.5°C**	**12.5°C**	**2.5°C**	**12.5°C**
Santo	**Log 4**	1.84[A]	(1/15)[B]	(1/15)	(0/3)	(0/3)	(0/3)	(0/3)	nd
	Log 6	3.80	(15/15)	(12/15)	(1/3)	(1/3)	(2/3)	(2/3)	nd
Leisure	**Log 4**	1.97	(4/15)	(1/15)	(0/3)	(0/3)	(0/3)	(0/3)	nd
	Log 6	3.81	(15/15)	(15/15)	(1/3)	(3/3)	(2/3)	(2/3)	nd

Salmonella		**PREHARVEST**			**POSTHARVEST**				
		Days after inoculation				**7DAW & 19DPI**		**14DAW & 26DPI**	
Variety	**Inoculum level**	**0.5**	**6**	**12**	**AW**	**2.5°C**	**12.5°C**	**2.5°C**	**12.5°C**
Santo	**Log 4**	2.67[A]	(14/15)[B]	(1/15)	(0/3)	(0/3)	(0/3)	(0/3)	nd
	Log 6	4.37	(15/15)	(14/15)	(3/3)	(3/3)	(3/3)	(3/3)	nd
Leisure	**Log 4**	2.61	(9/13)	(1/15)	(0/3)	(1/3)	(0/3)	(0/3)	nd
	Log 6	4.39	(15/15)	(14/15)	(3/3)	(3/3)	(3/3)	(3/3)	nd

(A) Numerical results denote the mean of n=15 samples in log CFU/g of cilantro (nd) not determined; DAW – Days after postharvest washing; DPI – days post-inoculation

(B) Ratio denotes the number of replicates testing positive for the target pathogen by selective enrichment. LOD = 0.43 log CFU/g

NOTE:

In addition to the first trial carried out during fall 2011, a second open field trial was conducted during late spring/summer 2012 at the UC Davis Plant Sciences Research Farm. For this experiment, two cultivars of cilantro cv. Santo and cv. Leisure; two cultivars of parsley cv. Moss Curled and cv. Green Forest; and one cultivar of basil, cv. Genovese, were used in this study. Due to weather conditions, parsley cultivars were not able to establish a stand suitable for research, thus they were not included in this trial.

The experimental design, sampling and recovery procedures were carried out as specified in the original proposal and the first field trial (fall 2011). Two inoculum levels of of *Salmonella* Enteritidis (PTVS 177) and *E. coli* O157:H7 (*a*PTVS 154) were prepared and adjusted to log 4 and log 6 CFU/mL. The foliar application of the inoculum was performed using standard procedures as previously described at approximately 80% seedbed cover.

Cilantro and basil samples were collected 12 hours after inoculation and at 6 and 12 days post-inoculation (dpi). Due to weather conditions, cilantro plants bolted (prematurely developed a seed stalk) during the course of the experiment, thus cilantro samples were not collected at 12 dpi as the impact of this physiological and anatomical change on downstream experimental outcomes are unknown and could not be predicted. The sampling regime consisted of 5 samples of 100 g for each subplot (3 subplots per cultivar). Direct plating and enrichment-based recovery, as previously described, were used to determine viable and culturable populations of PTVS 177 and *a*PTVS 155 and their temporal survival in the phyllosphere under simulated commercial conditions in an open field environment.

Overall, similar results to the first trial where found in this second experiment. No significant variability of attached *E. coli* O157:H7 and *Salmonella* in cilantro and basil foliage samples were observed as a function of the cultivar after inoculation. Both *Salmonella* and *E. coli* O157:H7 population declined rapidly below the limit of quantitative detection (LOD) and no culturable bacteria were recovered by direct plating after 6 dpi for cilantro and basil samples and 6 and 12 dpi for basil samples. The viability of the PTVS177 and aPTVS 155 was demonstrated 12 dpi for basil samples, but significant differences among initial inoculum doses were observed as was described above during first cilantro trial. It is important to point out that viability of Salmonella was greater than *E. coli* O157:H7 on basil phyllosphere (Data not shown).

Basil plants were harvested after 12 days of the contamination event and placed in a 2.5 ^{o}C cold room for further minimal processing. The plant material was washed for 2 min with tap water containing 50 mg/L NaClO, pH 6.5 adjusted with citric acid, as the disinfectant agent and followed by a 1 min of rinsing with tap water. Washed leaves were dewatered by centrifugal action using a foodservice grade manual salad spin dryer (0.2 m3 volume). Basil plants were placed in commercial polybags and stored in a controlled temperature room without light at 5 °C for up to 7 days.

Overall, washing with 50 mg/L of NaClO was not sufficient to disinfect the inoculated basil (log 6) prior to refrigerated storage for *Salmonella* samples. Viable populations of Salmonella were

confirmed throughout the storage interval but no populations of *E. coli* O157:H7 were recovered after enrichment for washed basil samples held for up to 7 days at 5 ^{o}C.

KEY CONCLUSIONS DERIVED FROM OBJECTIVE A

- *The survival of applied research isolates statistically favors a greater outcome for net retained viability with Salmonella than for the available attenuated E. coli O157:H7. As in prior CPS funded research in our lab, this indicates the importance of genus and strain selection and validation as it refers to environmental fitness and predictive fidelity of surrogates in modeling the behavior of pathogens. Populations as low as log 1.7 CFU/g were able to persist in the open environment for up to 12 days and survive postharvest disinfection hurdles.*

- *The effect of the inoculation dose and time were statistical significant in the survival of both applied surrogates.*

- *Postharvest treatments with hypochlorite, after field-establishment of both pathogens on the cilantro and basil phyllospheres, were insufficient to mitigate their presence, further supporting the critical role of preventive measures to minimize the chance of contamination as the primary food safety principle.*

- *During storage an increase in the population of mesophilic bacteria was determined, particularly when exposed to temperature abuse conditions (12.5 ^{o}C). Other studies have demonstrated the influence of background bacteria on the behavior of human pathogens; therefore it would be of importance to investigate whether the fate of E. coli* and *Salmonella during storage is associated with the growth of other bacterial groups.*

OBJECTIVE B

To determine the degree of persistence and dispersal of* Salmonella enterica *and E. coli O157:H7 in sequential harvest and re-growth intervals.

Materials and methods

After the first harvest cut, cilantro plants were allowed to re-grow for a second cut which took 22 days during the seasonal timeframe. Harvested crop areas were divided in plots and inoculated with a single dose of log 4 CFU/mL of PTVS 177 or *a*PTVS 155 with the following pattern

- Subplot 1: No inoculation after the first cut
- Subplot 2: Inoculation of the crop and residue 4 days after the first cut
- Subplot 3: Inoculation of the crop and residue 14 days after the first cut
- Subplot 4: Inoculation of the 20 days after the first cut

Cilantro plants from each plot (3 plots per cultivar; 3 samples per plot; n=9) were collected 2 days after each inoculation event and after the 22 days re-growth period during the fall 2011 trial. Samples were processed for quantification and recover from cilantro plants as previously detailed in Objective A. A third cut after and re-growth cycle was not performed due to weather restrictions.

This research objective was done after the finalization of the survival trial described in objective B during the fall 2011 trial.

Results

The persistence of *E. coli* O157:H7 and *Salmonella* in sequential harvest and re-grow intervals of cilantro is shown in Tables 2 and 3, respectively. Overall, no culturable bacteria were recovered by direct plating for all cilantro samples 2 days after the inoculation event with a single dose of log 4 CFU/mL of *a*PTVS155 or PTVS177.

For *E. coli* O157:H7 (Table 2), only one out of 9 samples (11%) was positive 2 days post-inoculation when plants were not re-inoculated after the first cut, this was observed for both cilantro cultivars. In general, for cilantro samples that were not inoculated after the 1st cut or 4 days after the 1st cut, no applied bacteria were recovered at the 22 day re-growing period. For the rest of the treatment conditions (inoculation event 14 and 20 days after the 1st cut), the presence of *E. coli* O157:H7 was confirmed in the 11 and 22% of the samples for both cultivars.

In contrast, a greater viability and survival of *Salmonella* (Table 3) in comparison with *E. coli* O157:H7 under similar conditions was observed. Overall, no applied Salmonella was recovered after the 22 day re-growing period for cilantro samples that were not re-inoculated after the 1st cut. For the remainder of the treatments, the presence of Salmonella was confirmed in 11, 43 and 100% of cilantro samples that were inoculated 4, 14 and 20 days after the 1st cut respectively.

No significant variability of attached *Salmonella* and *E. coli* O157:H7 was observed as a function of the cultivar.

Table 2. Persistence of *E. coli* O15:H7 in sequential harvest and re-growth intervals

Cultivar	Inoculation after 1st cut	Recovery	
		2 DPI	22 days after 1st cut
Santo	None	*(1/9)	(0/9)
	4 days	(9/9)	(0/9)
	14 days	(9/9)	(2/9)
	20 days	(9/9)	(2/9)
Leisure	None	(1/9)	(0/9)
	4 days	(9/9)	(0/9)
	14 days	(9/9)	(1/9)
	20 days	(9/9)	(1/9)

(*)Ratio denotes the number of replicates testing positive for the target pathogen by selective enrichment. LOD = 0.43 log CFU/g

Table 3. Persistence of *Salmonella* in sequential harvest and re-growth intervals

Cultivar	Inoculation after 1st cut	Recovery	
		2 DPI	22 days after 1st cut
Santo	None	*(1/9)	**(0/9)**
	4 days	(9/9)	(1/9)
	14 days	(9/9)	(4/9)
	20 days	(9/9)	(9/9)
Leisure	None	(1/9)	**(0/9)**
	4 days	(9/9)	(1/9)
	14 days	(9/9)	(3/9)
	20 days	(9/9)	(9/9)

(*)Ratio denotes the number of replicates testing positive for the target pathogen by selective enrichment. LOD = 0.43 log CFU/g

KEY CONCLUSIONS DERIVED FROM OBJECTIVE B

- *After the primary inoculation event performed before the first cut (see objective A), none of the applied bacteria was detected after 22 days of the re-growth period in the absence of field re-inoculation post-cutting. This observation may indicate that the basis for commercial field contamination events may be more likely from irrigation or foliar application events close to harvest or other inputs (such as non-synthetic fertilizer applications between growth cycles) and environmental sources between each harvest interval. A timeframe of approximately 20 days may be sufficient to mitigate a microbial contamination event following overhead irrigation. Caution should be taken, at this time, in extending these preliminary results to commercial standards as seasonal and regional differences that may significantly alter persistence outcomes are not known.*

- *Contamination events from irrigation, foliar applications, fertilizer treatments, or environmental sources close to each sequential cut and, especially, harvest would appear to present a more substantial risk to the subsequent crop due to higher probability of survivors. Knowledge of water sources through pathogen-specific testing and quantitative characterization could be considered an important parameter to*

determine the risk of possible contamination within the growth and harvest cycle time frames and climatic conditions.

OBJECTIVE C

Determine the impact of delays to cooling, specific temperature thresholds, and postharvest washing on growth potential and cross-contamination of applied pathogen-surrogates, including simulated industrial pilot-plant processing and simulated retail distribution

a. Delays of cooling and post-harvest washing

Materials and methods

Cilantro plants were harvested in bunches of approximately 50-70 g and unitized with a rubber band after 12 days of the first inoculation event (see materials and methods in objective A). The distribution and sampling size description specified in the proposal grant was carefully followed. Cilantro bunches were subjected to three regimes of delays to cooling right after harvesting:

a) Placement on ice right after harvesting
b) Placement on ice 60 min after harvesting
c) Placement on ice 120 min after harvesting

Samples were subsequently stored at 2.5 or 12.5 ^{o}C for up to 14 and 7 days, respectively. Cilantro samples were analyzed for pathogen detection and quantification, as described in the materials and methods for objective A, after 7 and 14 days of postharvest storage, which corresponds with 19 and 26 days after the on-farm inoculation event.

A second set of bulk cilantro plants was harvested after 12 days of the contamination event and placed in a 2.5 ^{o}C cold room for further minimal processing. Pathogen inactivation and growth potential during postharvest storage. The results corresponding to pathogen inactivation and growth potential were included in Objective A (Table 1).

Results

The impacts of delays to cooling on growth potential of *E. coli* O157:H7 and *Salmonella* on cilantro bunches are shown in table 4 and 5, respectively.

In general, *E. coli* O157:H7 was not detected during postharvest storage for those samples that were inoculated with log 4 CFU/mL, regardless of the delayed cooling exposures, storage temperature and cilantro cultivar (Table 4). However, viable cells of *a*PTVS155 were recovered from those samples that were inoculated with the high dose (log 6 CFU/mL) and the statistical analysis showed a significant difference attributable to inoculation dose (p=0.0003). These short delays to cooling did not significantly affect the qualitative/quantitative survival of the surrogates during the cold storage timeframe (p=0.8786).

Table 4. Impact of the delay of cooling on the growth potential of *E. coli* O157:H7

			7DAH & 19DPI		**14DAH & 26DPI**	
Variety	**Inoculum level**	**Delay of cooling (min)**	**2.5C**	**12.5C**	**2.5C**	**12.5C**
Santo	log 4	0	(0/6*)	(0/6)	(0/6)	nd
		60	(0/6)	(1/6)	(0/6)	nd
		120	(1/6)	(0/6)	(0/6)	nd
	log 6	0	(2/6)	(4/6)	(3/6)	nd
		60	(2/6)	(5/6)	(5/6)	nd
		120	(5/6)	(6/6)	(6/6)	nd
Leisure	log 4	0	(1/6)	(0/6)	(0/6)	nd
		60	(1/6)	(0/6)	(0/6)	nd
		120	(0/6)	(1/6)	(1/6)	nd
	log 6	0	(4/6)	(5/6)	(6/6)	nd
		60	(6/6)	(6/6)	(5/6)	nd
		120	(5/6)	(6/6)	(4/6)	nd

(*)Ratio denotes the number of replicates testing positive for the target pathogen by selective enrichment. LOD = 0.43 log CFU/g. DAH – days after harvest; DPI – days post-inoculation

Significant difference was determined between the inoculation doses (p=0.0030), however delays of cooling, the collection days or the cultivar were not significant (p=0.8786, 0.2537 and 0.5782, respectively)

Similar results were found for *Salmonella*, with a significant difference between the two inoculation doses (p=0.0009), but no significant effect was determined for delays of cooling (p=0.4389) (Table 5). However, the survival of PTVS177 was greater than *a*PTVS155, especially for those samples that were inoculated in the open research environment with the low dose (log 4 CFU/g).

Table 5. Impact of the delay of cooling on the growth potential of *Salmonella*

			7DAH & 19DPI		**14DAH & 26DPI**	
Variety	**Inoculum level**	**Delay of cooling (min)**	**2.5C**	**12.5C**	**2.5C**	**12.5C**
Santo	log 4	0	(1/6*)	(2/6)	(2/6)	nd
		60	(0/6)	(3/6)	(1/6)	nd
		120	(1/6)	(3/6)	(0/6)	nd
	log 6	0	(6/6)	(6/6)	(6/6)	nd
		60	(6/6)	(5/6)	(6/6)	nd
		120	(5/6)	(5/6)	(6/6)	nd
Leisure	log 4	0	(1/4)	(1/4)	(1/6)	nd
		60	(1/4)	(2/4)	(1/6)	nd
		120	(0/4)	(3/4)	(0/6)	nd
	log 6	0	(6/6)	(6/6)	(6/6)	nd
		60	(5/6)	(5/6)	(6/6)	nd
		120	(5/6)	(6/6)	(6/6)	nd

(*)Ratio denotes the number of replicates testing positive for the target pathogen by selective enrichment. LOD = 0.43 log CFU/g. DAH – days after harvest; DPI – days post-inoculation

Significant difference was determine between the inoculation doses (p=0.0009), however delays of cooling, the collection days or the cultivar were not significant (p=0.4389, 0.2650 and 0.5345 respectively)

KEY CONCLUSION OBJECTIVE C.a

- *Overall for both surrogates, the role of limited delays to cooling during postharvest handling does not seem to promote their growth or survival. This study only evaluated a maximum time of 120 min of cooling delay, based on industry input for initiating studies considering commercial conditions, within the field environment, however other factors including humidity, free moisture during harvest, temperatures at harvest and transport, and extended periods of cooling delay temperature abuse should also be explored in future studies.*

b. To determine the effectiveness of a novel chlorine stabilizer T-128 during postharvest washing under semi-industrial conditions to prevent Salmonella cross contamination

This subpart of the project was conducted in collaboration with New Leaf Foods (now dba - SmartWash Solutions) – pilot plant facilities in Salinas, CA. A substantial in-kind funding, through technical and operational staff time and experimental materials, was generously contributed to the execution of this objective and was essential to obtaining the data and results reported. This part of the project aimed to evaluate the removal and extent of cross-contamination control efficacy between cilantro and parsley inoculated with PTVS177 under pilot plant wash process operations. The specific objectives of this project were

a) To evaluate removal of inoculated *Salmonella* from cilantro surfaces under pilot plant conditions.

b) To assess the potential of cross-contamination from inoculated material to non-inoculated material via processing water.

General description of materials and methods (specific conditions are described within each trial)

Inoculation of plant material was done with attenuated *Salmonella* PTVS177. Approximately **<u>8-10 pallets</u>** of cilantro were inoculated with log 6 CFU/mL of the bacterium using spray application; cilantro was placed on a belt and while homogenous spraying occurred. Inoculated plant material was stored in the SmartWash Solutions research cold room for approximately 18 h prior to processing in the washing line to allow acclimation and attachment of the inoculum to the leaves. The initial proposal to CPS described the inoculation of cilantro in the field prior to processing, unfortunately we were unable obtain sufficient space at the UCCE site in an adequate time frame, thus Taylor Farms generously agreed to source and provide the harvested plants necessary to perform the inoculation in the SmartWash pilot plant facility.

The SmartWash pilot plant is located in Salinas, CA. The wash line is a NON FT Jacuzzi style wash line, with two stages (primary and secondary). The primary stage or first flume contains 900 gallons of water, circulated from a bottom holding tank into the processing tank, and back through coarse filtration screens to remove product that has overflowed the main wash body. The second stage or second flume contains approximately 800 gallons of water and the water recirculation regime is similar to the primary system.

The equipment is controlled by an ASAP ® unit which is used to provide continuous monitoring and control of pH and free chlorine of the wash line. The unit is capable of maintaining pH within pH ± 0.5 of control set-point and free chlorine within ± 1 ppm of control set-point.

Quantification and cell recovery were performed on 50 g of plant material in triplicate for each time point collected (see below), quantification was done through direct plating on TSA-rif and if the anticipated population was below the LOD, enrichment was done with BPW supplemented with rifampicin, cell detachment, incubation and colony confirmation were done as described above (see materials and methods for objective A). Additionally water samples were collected during each trial at different time points (see below). Water was analyzed to monitor pH, oxidation-reduction potential (ORP), chlorine concentration and presence of applied *Salmonella,* which was determined after enrichment of 50 mL with double strength BPW supplemented with 80 mg/L of rifampicin, followed by colony confirmation on selective media XLT-4 supplemented with 80 mg/L of rifampicin.

Trial 1.

Two runs of 30 min period were performed:

a. Consisted of 4 mg/L of sodium hypochlorite with pH adjusted to 6.5 with citric acid at a constant rate for 15 min for both primary and secondary flumes. After 15 min, no chlorine injection was done for the remaining 15 minutes to conduct a depletion analysis.

b. Consisted of 4 mg/L of sodium hypochlorite supplemented with T-128 (SmartWash SW) at a constant rate for 15 min for both primary and secondary flumes. After 15 min, no chlorine injection was done for the remaining 15 minutes to conduct a depletion analysis.

The flow rate of plant material consisted on a 1:1 ratio of parsley:'inoculated cilantro' (cross-contamination donor crop) that were independently and intermittently fed into the wash line at a constant rate, curly parsley was chosen as a 'cross-contamination' receiving product to facilitate distinction between the two types of recipient:donor plant material. Samples of water and plant material were collected at 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 21-24, 27-30 min blocks.

Results trial 1

Significant differences before and after washing were determined for both treatments; citric acid and T-128 ($p<0.0001$), with approximately a 2-log reduction attributable to washing, however no significant difference was determined during the time of processing between treatments (Figs. 4 and 5).

Specific analysis of each time point showed that there is significant difference between citric acid and T-128 ($p=0.0007$) when comparing log-reduction of *Salmonella*, overall, larger reductions where associated with the presence of T-128 than when processing took place with citric acid, however after 21 min of processing (no injection of chlorine), there is no difference between the two disinfection treatments.

Evaluation of the cross contamination to parsley surfaces from contaminated cilantro, showed very variable results and no significant difference established between the two disinfection treatments when main effects were statistical analyzed ($p=0.6169$) (Fig. 6). However cross contamination cannot only be attributed to water contact but also to direct plant to plant surface contact and plant to equipment contact surface, given the levels of viable Salmonella in the process water (Fig 7). Evaluation of water, showed that *Salmonella* can be detected in all flumes for both treatments. When citric acid was used as the acidulant for the hypochlorite disinfectant agent, *Salmonella* was detected in 8 of the 12 time points while, in contrast, utilization of T-128 as the acidulant and stabilizer resulted in detection in only 4 of the samples collected in the both flumes over the time course and not after the initial 15 min (Table 6).

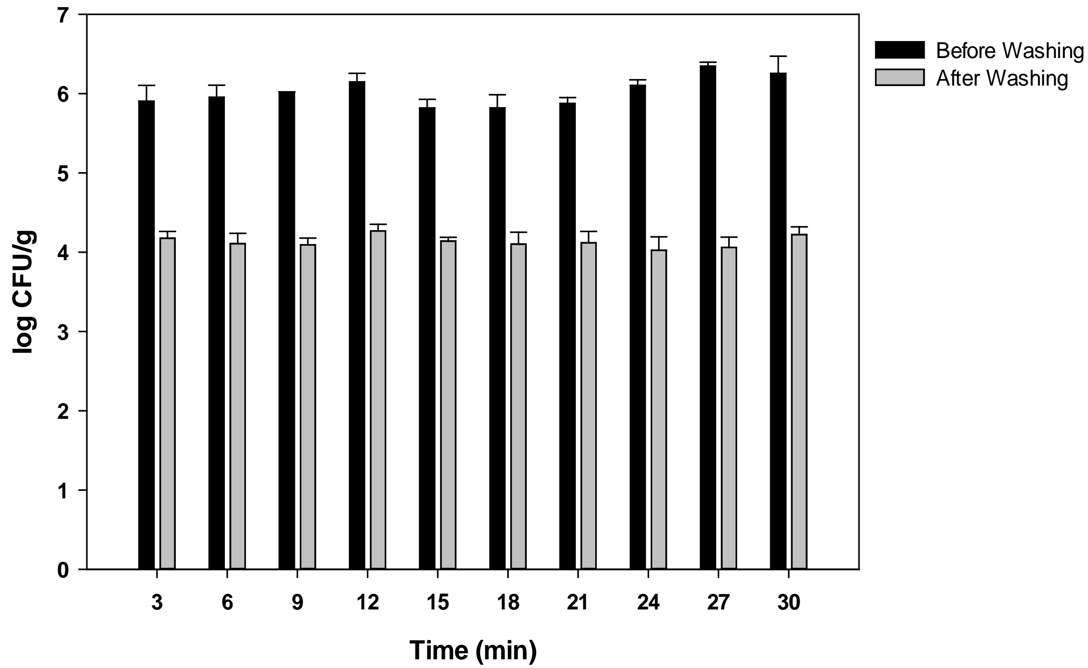

Figure 4. Population of applied *Salmonella* (PTVS177) before and after washing with 4 mg/L of chlorine adjusted to pH of 6.5 with citric acid (CA)

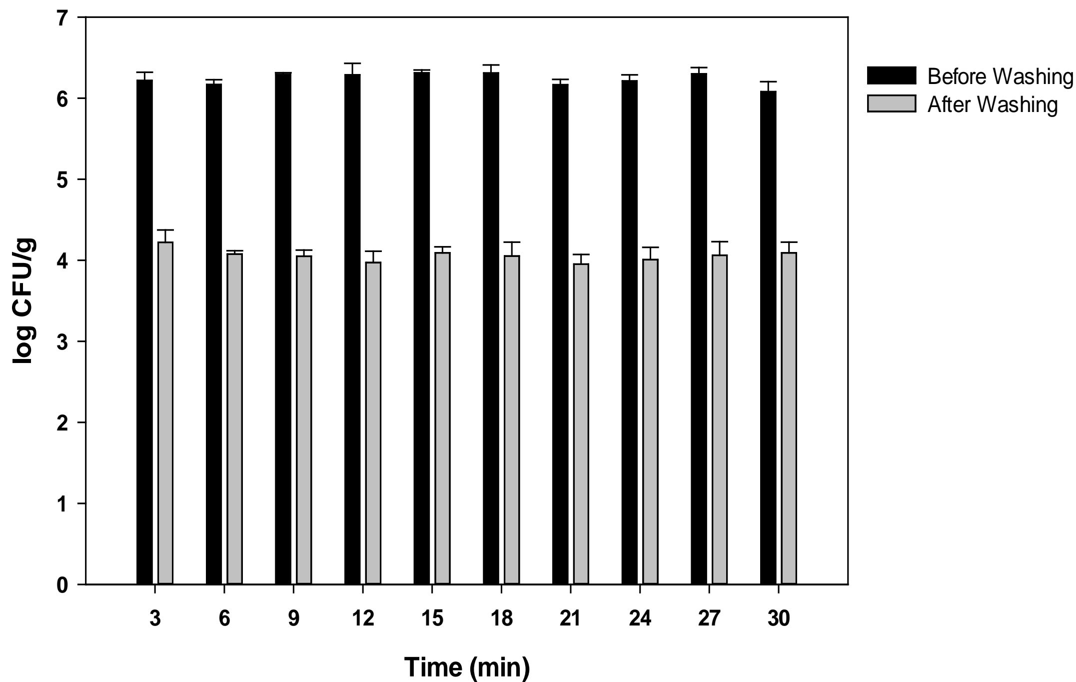

Figure 5. Population of applied *Salmonella* (PTVS177) before and after washing with 4 mg/L of chlorine and T-128 (SmartWash)

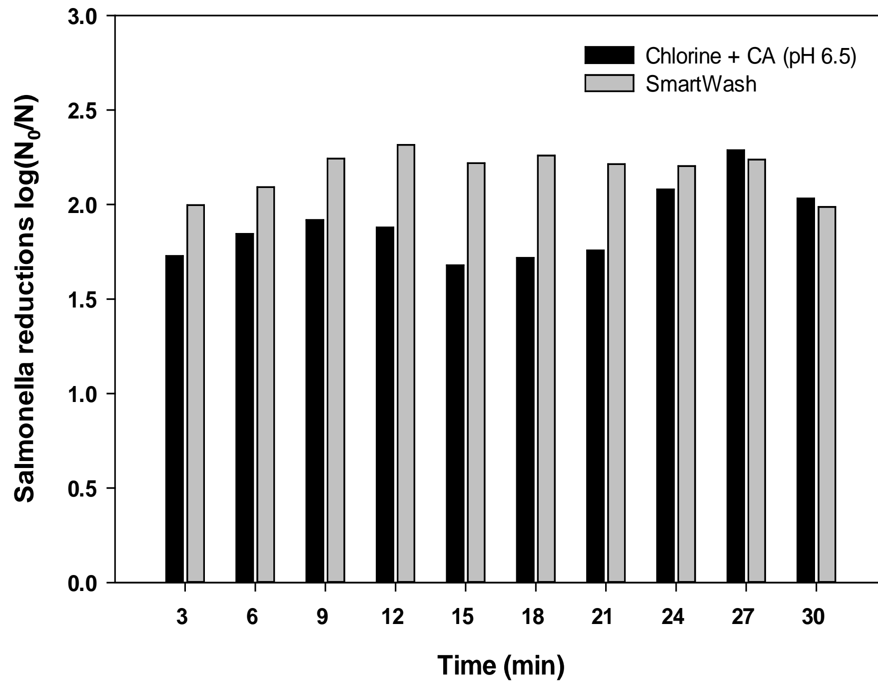

Figure 6. Comparison of the log reduction of *Salmonella* between chlorine adjusted with citric acid (CA) or with T-128 (Smart wash)

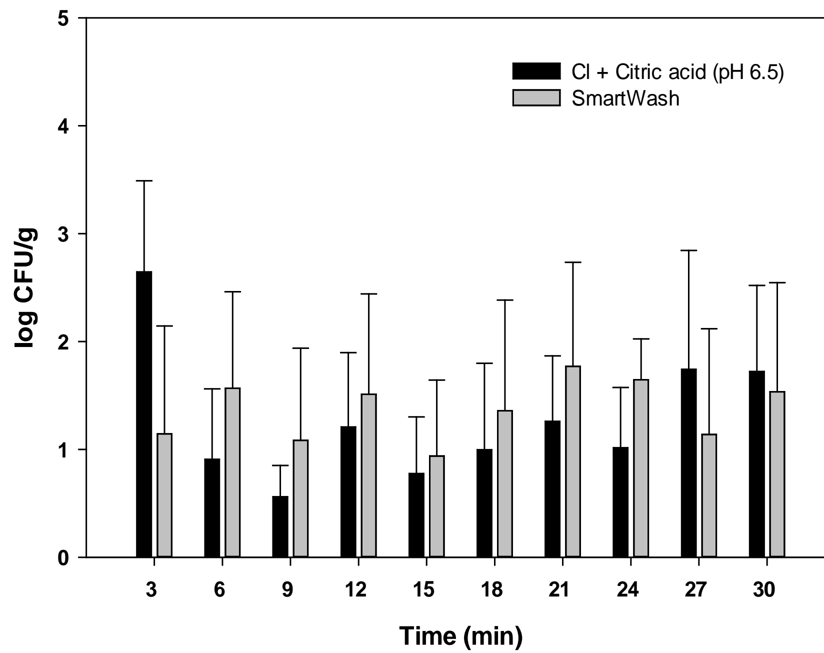

Figure 7. Population of *Salmonella* on parsley as a result of cross contamination from contaminated cilantro.

Table 6. Detection of Salmonella in system water during cilantro and parsley processing through the wash line.

	Chlorine + Citric Acid		**Chlorine + T-128 (SmartWash)**	
	Flume 1	Fume 2	Flume 1	Flume 2
0 - 3 min	**-**	**+**	**+**	**-**
3 - 6 min	**+**	**-**	**-**	**+**
6 - 9 min	**+**	**-**	**-**	**-**
9 - 12 min	**+**	**+**	**-**	**-**
12 - 15 min	**-**	**-**	**+**	**-**
15 - 18 min	**+**	**-**	**-**	**+**
18 - 21 min	**+**	**-**	**-**	**-**
21 - 24 min	**-**	**-**	**+**	**-**
24 - 27 min	**+**	**-**	**ND**	**ND**
27 - 30 min	**ND**	**ND**	**-**	**-**

(+) and (-) indicate presence or absence of *Salmonella* in processing water respectively.

(ND) not determined for that particular time point.

KEY OBSERVATIONS

- *Although the use of T-128 appears to be more effective in preventing the presence of the pathogen in water over run-time and reducing cross contamination, other sources of cross-contamination through direct plant to plant and plant to equipment surface contact still could play a major role and may have impaired a better comparison of the cross-contamination associated exclusively to processing water. The low level of free chlorine, 4 ppm, may also have been significant in allowing measureable levels of persistent Salmonella to survive in the recirculating water.*

Trial 2.

Two runs of 30 min period were performed:

a) Consisted of 4 mg/L of sodium hypochlorite with pH adjusted to 6.5 with citric acid at a constant rate for 15 min followed by a 10 min pause to adjust the concentration of chlorine to 10 mg/L which completed the test run for an additional 15 minutes.

b) Consisted of 4 mg/L of sodium hypochlorite supplemented with T-128 (SmartWash SW) at a constant rate for 15 min followed by a 10 min pause to adjust the concentration of chlorine to 10 mg/L which completed the test run for an additional 15 minutes.

Inoculation of the plant material as well as processing conditions and microbiological analysis were performed as described above. *Inoculation level was log 6 CFU/mL*

Results trial 2.

Variations in the log reduction of the *Salmonella* attributed to different disinfection treatments and dose were analyzed (Fig. 8). A statistical difference was determined after main effect analysis for different disinfection treatments (CA or SmartWash; $p<0.0001$) but no significant effect was attributed to the dose of chlorine utilized ($p=0.1203$). A greater reduction of *Salmonella* was determined in presence of citric acid during the first 18 min of the run, but after that time point no significant difference was found when the system was elevated to 10 mg/L.

Similar to results obtained in trial 1, cross-contamination from cilantro to non-inoculated parsley resulted in the presence of log 1 to log 2 CFU/g of assayed recipient parsley. Although results were variable across the time points, larger populations were found in presence of T-128 than when pH was adjusted with citric acid (Fig. 9). Analysis of main effect showed that there was not a significant difference associated to the dose of chlorine utilized ($p=0.8307$) however significant difference was associated with the disinfectant treatment (CA or SmartWash; $p=0.0066$). It is not possible to determine whether this was a function of normal random variability between runs or due to some other uncontrolled experimental parameter between treatment evaluations over time.

Salmonella was also present in processing water and no differences seem to be attributed to the dose of chlorine or the presence of chlorine stabilizer (Table 7).

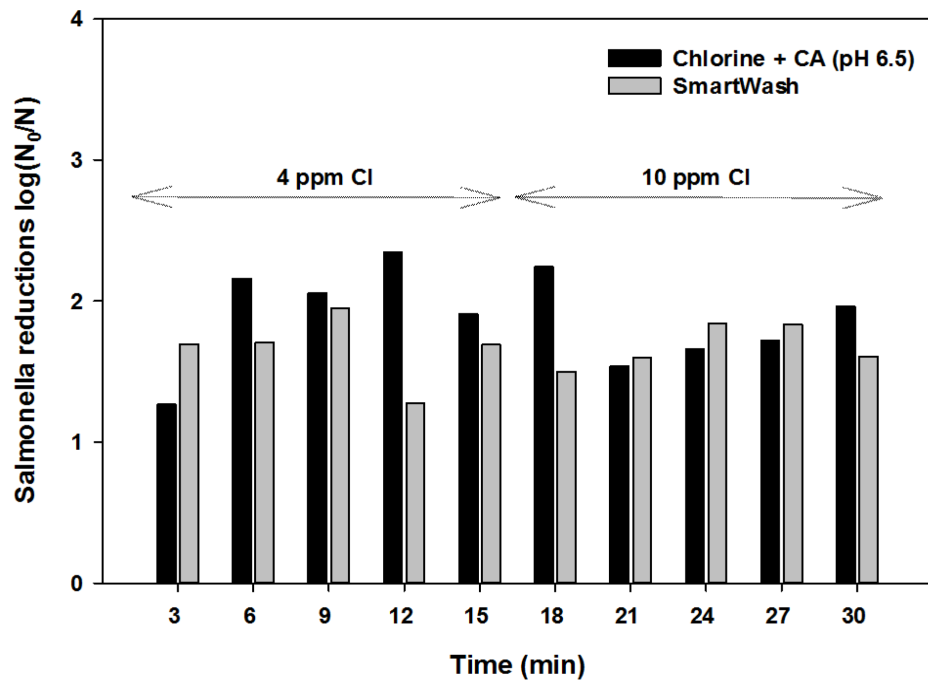

Figure 8. Effectiveness of different doses of sodium hypochlorite in presence of citric acid (CA) or T-128 (Smarwash) on the log reduction of *Salmonella*. Initial inoculation level was log 6 CFU/g

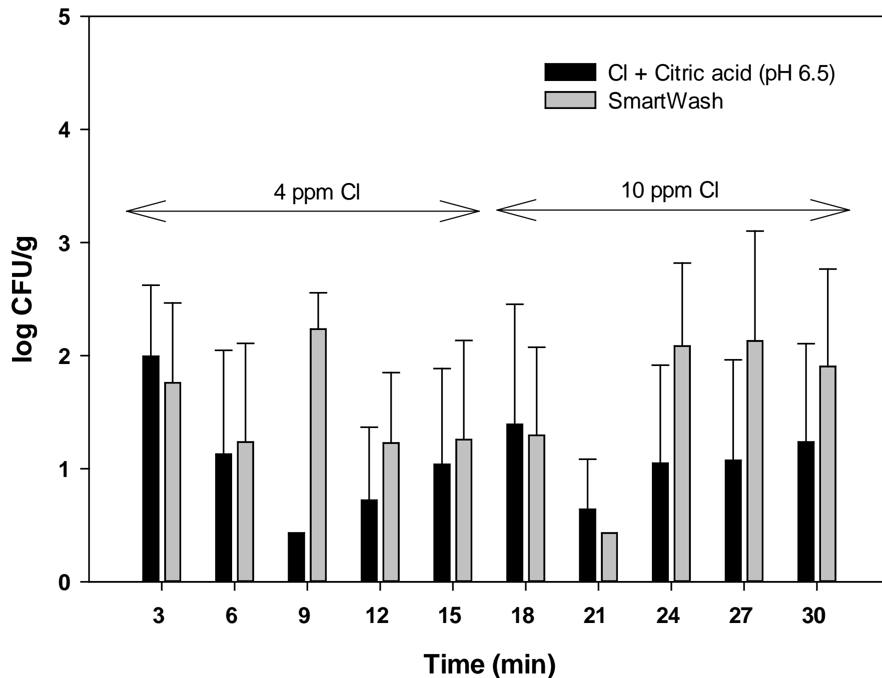

Figure 9. Population of *Salmonella* on parsley resulting from cross contamination associated with detachment from contaminated cilantro.

Table 7. Detection of Salmonella in system process water during cilantro and parsley processing through the wash line.

	Chlorine + Citric Acid		**SmartWash**	
	Flume 1	**Fume 2**	**Flume 1**	**Flume 2**
0 - 3 min	-	-	-	-
3 - 6 min	-	-	+	-
6 - 9 min	-	-	-	-
9 - 12 min	+	-	-	-
12 - 15 min	+	-	+	-
15 - 18 min*	-	-	-	-
18 - 21 min	-	+	-	+
21 - 24 min	-	-	-	-
24 - 27 min	-	-	-	-
27 - 30 min	+	+	+	-

(+) and (-) indicate presence or absence of *Salmonella* in processing water respectively.

(*) Chlorine concentration was increased from 4 to 10 mg/L

KEY OBSERVATIONS

- *Increasing of the chlorine dose does result in a major increase of the effectiveness to mitigate the presence of Salmonella on plants or to minimize the cross contamination.*
- *Comparison of trials 1 and 2 indicate that the effect of CA or SmartWash in log reduction is not consistent and likely variation among trial runs, complicating data interpretation between independent tests, would be anticipated until the threshold for practical elimination of cross-contamination was achieved.*

Trial 3

Two runs of 30 min period were performed:

a) Consisted of 4 mg/L of sodium hypochlorite with pH adjusted to 6.5 with citric acid at a constant rate for 15 min followed by a 10 min pause to adjust the concentration of chlorine to 10 mg/L which completed the test run for an additional 15 minutes.
b) Consisted of 4 mg/L of sodium hypochlorite supplemented with T-128 (SmartWash SW) at a constant rate for 15 min followed by a 10 min pause to adjust the concentration of chlorine to 10 mg/L which completed the test run for an additional 15 minutes.

Inoculation of the plant material as well as processing conditions and microbiological analysis were performed as described above. *Inoculation level was log 4 CFU/mL*

Results trial 3.

Following the same conditions in trial 2, we evaluated the effect of CA and SmartWash when the material was inoculated with a lower dose of *Salmonella* (log 4 CFU/g). As occurred in previous trials, a significant difference was determined before and after washing for all treatments ($p<0.0001$). However the effect of the dose in the log reduction was only significant when citric acid was utilized as the acidulant ($p=0.0268$), but not for SmartWash. Additionally, no significant difference was found between the use of CA or SmartWash in the log reduction, under these experimental conditions, which was in a range of log 1 to log 1.5 (Fig. 10).

Compared to trials 1 and 2 that utilized log 6 CFU/g as initial inoculum concentration, lower populations of *Salmonella* were found on the recipient parsley surface as a result of cross-contamination (Fig. 11). Similar to previous observations of log reduction, the effect of the variable dose in the log reduction was only significant when citric acid was utilized as the acidulant ($p=0.0025$), but not with SmartWash addition to the water ($p=0.3952$), under test conditions.

In this trial, the presence of *Salmonella* was only detected in flume water when CA was utilized as the acidulant but not in the presence of T-128 as a chlorine stablizer.

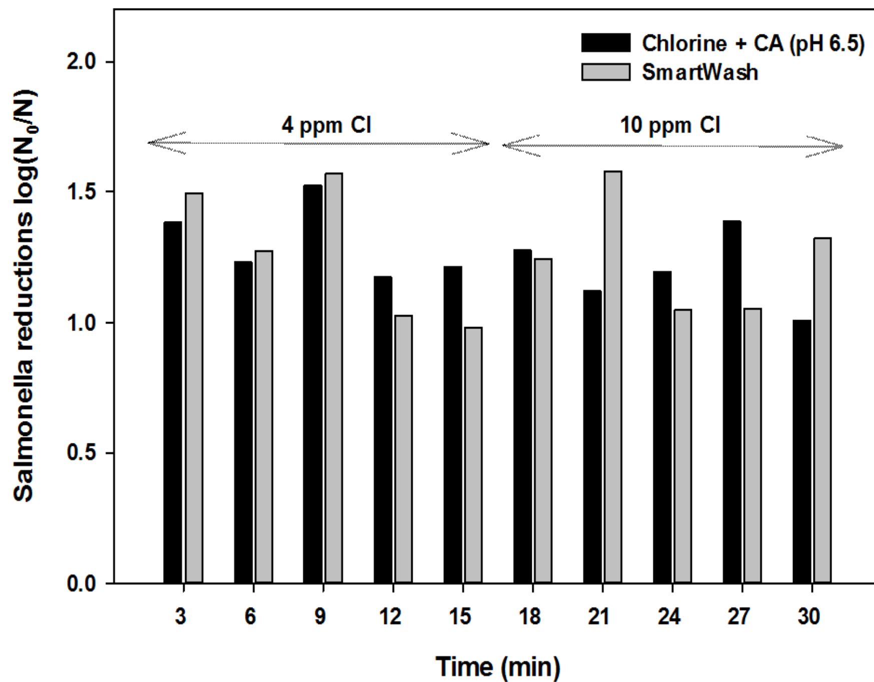

Figure 10. Effectiveness of different doses of sodium hypochlorite in the presence of citric acid (CA) or T-128 (SmartWash) on the log reduction of *Salmonella* on plant product. Initial inoculation level was log 4 CFU/g

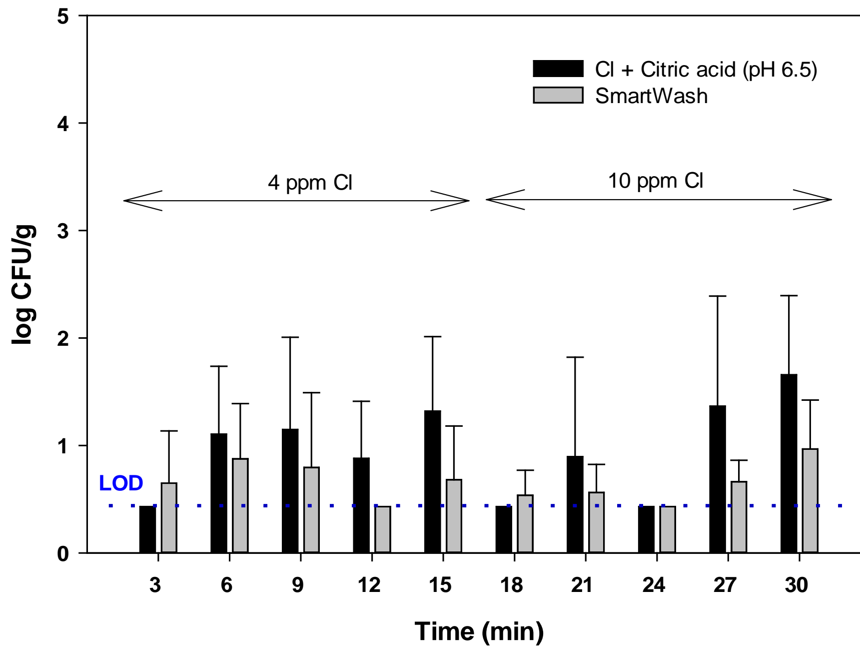

Figure 11. Population of *Salmonella* on parsley as a result of cross-contamination associated with detachment from, contaminated cilantro. (LOD) Limit of detection

Table 8. Detection of Salmonella in system process water during cilantro and parsley processing through the wash line.

	Chlorine + Citric Acid		**SmartWash**	
	Flume 1	**Fume 2**	**Flume 1**	**Flume 2**
0 - 3 min	-	-	-	-
3 - 6 min	-	-	-	-
6 - 9 min	+	-	-	-
9 - 12 min	-	-	-	-
12 - 15 min	-	-	-	-
15 - 18 min*	-	-	-	-
18 - 21 min	-	-	-	-
21 - 24 min	+	+	-	-
24 - 27 min	-	-	-	-
27 - 30 min	+	+	-	-

(+) and (-) indicate presence or absence of *Salmonella* in processing water respectively.

(*) Chlorine concentration was increased from 4 to 10 mg/L

KEY OBSERVATIONS

- *Effectiveness of CA or T-128 as chlorine acidulant or stabilizer to prevent practical cross-contamination of Salmonella is reasonably likely to be dependent on the initial contamination level and rate of detachment of the pathogen from crop surfaces or non-product sources, such as soil. Under these research conditions, our results suggest that at leaf concentrations of log 4 CFU/gm, T-128 would be more efficient in controlling the presence of Salmonella in processing water and thus minimize or prevent cross contamination within a lot.*

Trial 4.

Two runs of 30 min period were performed:

c) Consisted of 4 mg/L of sodium hypochlorite with pH adjusted to 6.5 with citric acid at a constant rate for 15 min followed by a 10 min pause to adjust the concentration of chlorine to 15 mg/L, which completed the test run for an additional 15 minutes.

a.

d) Consisted in 4 mg/L of sodium hypochlorite supplemented with T-128 (Smartwash SW) at a constant rate for 15 min followed by a 10 min pause to adjust the concentration of chlorine to 15 mg/L, which completed the test run for an additional 15 minutes.

Inoculation of the plant material as well as processing conditions and microbiological analysis were performed as described above. *Inoculation level was log 4 CFU/mL*

Results trial 4.

Increasing the dose of sodium hypochlorite from 4 to 15 mg/L was evaluated in this trial when plants were inoculated with log 4 CFU/mL. As occurred with all previous trials, a significant difference was determined before and after washing ($p<0.0001$), with a reduction of approximately 1 to 1.2 log. After washing, a significant difference was determined between the two process water aides ($p=0.0287$), however the increase in the hypochlorite dose had no effect in the log reduction of *Salmonella* achieved on cilantro leaves ($p=0.2627$). Overall, greater log-reductions were determined when SmartWash was used as a chlorine stabilizer, as compared to citric acid as an acidulant (Fig. 12).

With exception of the first 9 minutes following combined deliver to the wash system, cross-contamination was limited to populations below the limit of detection on parsley (Fig. 13), but no significant effect was determined for the chlorine dose ($p=0.2820$) or the chlorine process aide utilized ($p=0.2657$).

As determined in trial 3, detection of *Salmonella* in processing water was only observed in the second flume when citric acid was injected but not when T-128 was utilized to stabilize free chlorine availability (Table 9).

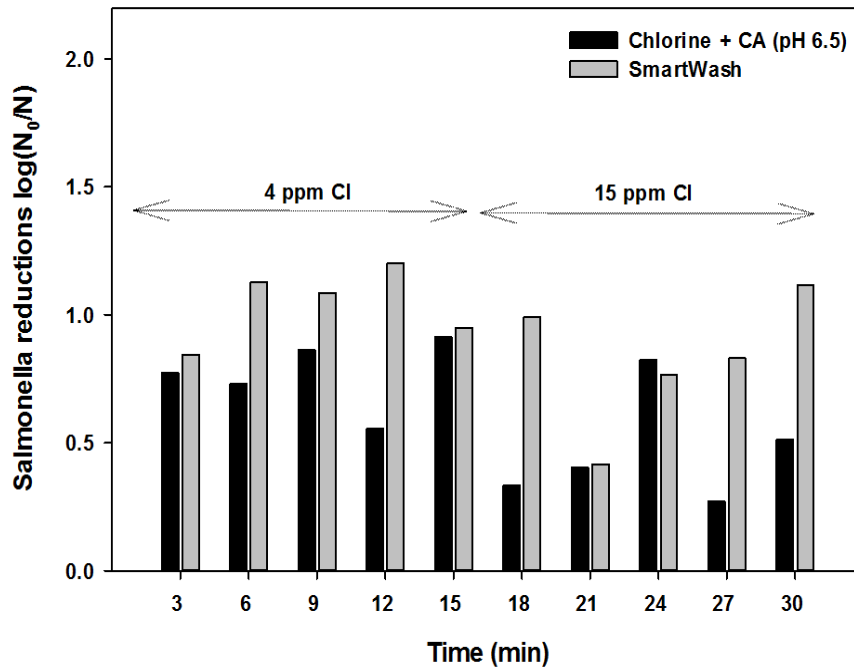

Figure 12. Effectiveness of different doses of sodium hypochlorite in the presence of citric acid (CA) or T-128 (SmartWash) on log reduction of *Salmonella* on inoculated cilantro. Initial inoculation level was log 4 CFU/g

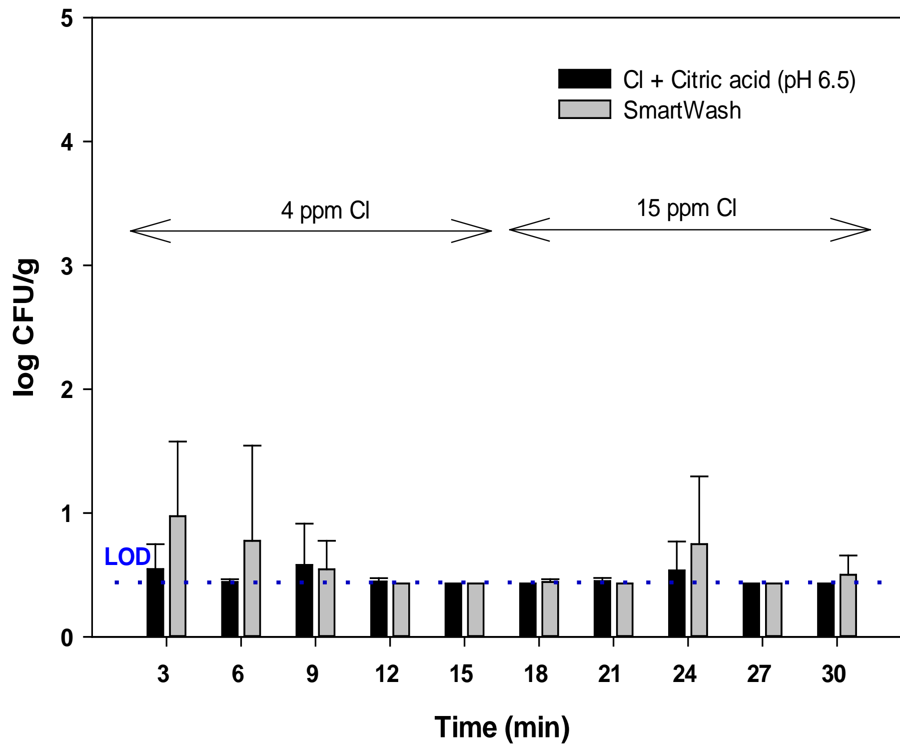

Figure 13. Population of *Salmonella* on parsley resulting from cross-contamination associated with detachment from contaminated cilantro.

Table 9. Detection of Salmonella in system process water during cilantro and parsley processing through the wash line.

	Chlorine + Citric Acid		**SmartWash**	
	Flume 1	**Flume 2**	**Flume 1**	**Flume 2**
0 - 3 min	-	-	-	-
3 - 6 min	-	-	-	-
6 - 9 min	-	-	-	-
9 - 12 min	-	+	-	-
12 - 15 min	-	+	-	-
15 - 18 min*	-	-	-	-
18 - 21 min	-	-	-	-
21 - 24 min	-	-	-	-
24 - 27 min	-	-	-	-
27 - 30 min	-	-	-	-

(+) and (-) indicate presence or absence of *Salmonella* in processing water respectively.

(*) Chlorine concentration was increased from 4 to 15 mg/L

KEY OBSERVATIONS

- *Increase of chlorine dose to 15 ppm did not have an effect in further log reduction of contaminated cilantro compared to 4 or 10 ppm or in eliminating cross-contamination to non-inoculated plant material.*
- *The use of T-128 seem to completely mitigate the presence of Salmonella in process wash water when Salmonella contamination of plant material is log 4 CFU/g or lower, but cross contamination due to plant to plant and plant to equipment contact needs to be considered for setting minimal dose thresholds.*

Summary of Findings and Recommendations

Culinary herbs could be at risk of persistent contamination with human bacterial pathogens that may follow from preharvest events to postharvest handling and distribution. Though not a novel observation, it is clear that once contamination occurs, establishment and firm attachment of the pathogens to the surface of cilantro is likely to occur and be persistent for at least 12 days, depending on many physical and biological factors (Poza-Carrion et al., 2013). Furthermore, persistent under field conditions is likely to be dependent on a combination of dose and physiological adaptation to acute stress, associated with the nature of the associated matrix (water vs. fecal matter) following deposition.

The persistence of enteric bacteria can extend beyond the phases of non-lethal postharvest processing including commercial washing with disinfectants and typical supply-chain conditions of commercial storage, particularly if temperature abuse occurs. The efficiency of different commercial washing and disinfection strategies is also dependent on the level of contamination and detachment of the pathogen, however there is always a risk of cross-contamination through the water or through the plant material contact-points

Supporting Citations

CSG Herbs. 2013. Commodity Specific Food Safety Guidelines for the Production, Harvest, Post-Harvest, and Processing Unit Operations of Fresh Culinary Herbs. Western Growers. http://www.wga.com/sites/default/files/herb-document.pdf accessed March 1, 2013.

Curtiss, R. III and Kelly, S.M. (1987) Salmonella Typhimurium deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect Immun 55, 3035–3043

Hassan, J.O. and Curtiss, R. III. (1990) Control of colonization by virulent Salmonella Typhimurium by oral immunization of chickens with avirulent DcyaDcrp S. Typhimurium. Res Microbiol 141, 839–850.

Poza-Carrion, Cesar, T. Suslow, and S. Lindow. 2013. Resident bacteria on leaves enhance survival of immigrant cells of *Salmonella enterica*. Phytopathology. 103:341-51

APPENDICES

Publications and Presentations (required)

Alejandro Tomás-Callejas, Gabriela López-Velasco, Adrian Sbodio and Trevor V. Suslow. 2012. Risk assessment of field survival of *Salmonella enterica* and *Escherichia coli* O157:H7 surrogates on cilantro in relation to sequential cutting, re-growth and postharvest washing and storage. Technical Presentation (Oral) by A. Tomás-Callejas at IAFP 2012 Providence, RI.

Budget Summary (required)

All allocated funds will have been expended by the termination of this project, including travel costs associated with presentation of the Final Report at the CPS Symposium 2013.

Tables and Figures (optional)

Integrated in Results Section above

Suggestions to CPS (optional)

The PI is disappointed with the overall performance of field aspects of this project in relation to providing full project deliverables across the proposed objectives, some of which may have been preventable and others significantly influenced by the whims of nature. I believe the research teams, including the contributions by SmartWash Solutions staff and management, have reported here a substantial body of new knowledge that may be applied to cilantro, other fresh culinary herbs, and wash water management in general. Our suggestion for CPS is merely to keep the original objectives in mind as priorities for future funding are considered.

Explanation of Incorrect Surrogate Isolate of Salmonella

The following is an abbreviated explanation of the unexpected and surprising after-the-fact discovery that the Salmonella isolate used in this project was incorrect and not the attenuated version originally requested from the provider.

In 2008, Dr. R. Curtiss, Arizona State University Center for Infectious Diseases and Vaccinology Biodesign Institute, generously provided to PI Suslow the requested research isolate of *S. enterica* serovar Typhimurium χ3985 for lab and controlled field research on fresh produce (See Citations). Upon receipt the strain properly identified as the *Salmonella* Typhimurium and was stored in dual vials in our permanent culture collection and as working culture stocks held at -80C. We took all precautions in transfer and colony purification prior to broth culture for storage of the original isolate and spontaneous rifampicin resistance selection to facilitate greenhouse and environmental field recovery. These have been very helpful in our research on produce food safety and we have published four journal articles citing the attenuated Salmonella from the Curtiss laboratory and as the parental attenuated Salmonella..

During late 2012 and into 2013, outcomes in our research program and field permitting application renewals caused me to further characterize both the original isolate and the rif-mutant in comparison to other Salmonella Typhimurium in our collection from Univ of GA, ATCC, FDA, and CDC. The results of our characterization tests placed the isolates as **S. sv. Enteritidis** and not Typhimurium.

The basic evidence so included;

1. Serotyping by California Animal Health & Food Safety Laboratory System places both original and rif-mutant isolates as Enteritidis
2. Two-enzyme PFGE show both as indistinguishable from each other but highly differentiated from several Typhimurium, including a few other avirulent mutants, in our collection which are essentially indistinguishable from each other
3. Neither original and rif-mutant isolates produce an amplicon when using Typhimurium specific primers but all other presumptive Typhimurium in our collection produce one of the expected size.
4. Indeterminate growth (±) on maltose rather than the expected negative utilization of the χ3985 double-mutant.

Upon this confirmed realization my primary concern has been for the safety of those handling these isolates. Although we treat them consistent with BSL 2 procedures, with regards to biohazardous waste, we have put several very large field trials out over the past four years, sometimes with very large cell densities in the inoculum. There have been no incidents of health issues reported.

Dr. Curtiss was instrumental in helping to identify the source of the initial error. An error appeared on the Material Transfer Agreement from his lab to the Suslow Lab when the responding technical collection curator transposed the request from χ3985 to χ3895. In the course of his review of all the history, Dr. Curtiss noted in his response that when someone copied the information from their old 3 X 5 card filing system (started many years before computers) to a File Maker Pro system, 'Enteritidis' was inadvertantly changed to 'Typhimurium' for the strain χ3895. The strain on receipt was labeled as S. Typhimurium cya crp mutant Chi3895. PI Suslow did not catch this possible strain-transfer error at the time and believed it to be a *bona fide* attenuated strain.

Upon sharing the true identity of the isolate we received, Dr. Curtiss wrote in response that his lab has tested many S. Enteritidis strains over the years in chickens (mostly white leghorn SPAFAS birds) and in BALB/c mice and have never found any that are as naturally virulent as the commonly used S. Typhimurium strains (generally 2 to 5 log higher LD50). While not great solace for the substantial effort to correct the errors in Biosafety permit applications and factual errors in our publications, it is fortunate that the specific isolate we did use was also a low virulence cya crp mutant and that the isolate error was not introduced upon receipt in the Suslow lab as a function of handling and improper controls and technique between our pathogenic strains and the attenuated forms in our own collection.