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Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions

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ABSTRACT

Escherichia coli O157:H7 has been associated in multiple outbreaks linked to the consumption of whole produce and fresh-cut leafy vegetables. However, plant-based foods had not been traditionally recognized as a host for enteric pathogens until the elevated incidence of produce-related outbreaks became apparent. The survival dynamics of two cocktails of generic *E. coli* (environmental water, plant and soil isolates) and *E. coli* O157:H7 within the phyllosphere of Mizuna, Red Chard and Tatsoi during their production, harvest, minimal processing, packaging and storage over two greenhouse production cycles were studied. Genotyping of applied generic *E. coli* strains to evaluate their comparative survival and relative abundance in the phyllosphere by REP-PCR is also reported. The Mizuna, Red Chard and Tatsoi shoots were grown under standard greenhouse conditions and fertility management. Both *E. coli* cocktails were spray-inoculated separately and determined to result in an initial mean population density of log 4.2 CFU/cm^2. Leaves were harvested as mini-greens approximating commercial maturity, minimally processed in a model washing system treated with 3 mg/L of ClO_2 and stored for 7 days at 5 °C. Rapid decline of generic *E. coli* and *E. coli* O157:H7 populations was observed for all plant types regardless of the leaf age at the time of inoculation and the irrigation type across both seasonal growth cycle trials. The decline rate of the surviving populations for the fall season was slower than for the summer season. The minimal processing with 3 mg/L of ClO_2 was not sufficient to fully disinfect the inoculated leaves prior to packaging and refrigerated storage. Viable populations of *E. coli* and *E. coli* O157:H7 were confirmed throughout storage, including the final time point at the end of acceptable visual leaf quality. In this study, the ability of low populations of *E. coli* to survive during production and postharvest operations in selected mini-greens has been demonstrated. However, further field-based trials are needed to expand understanding of the post-contamination fate of enteric bacterial pathogens on leafy vegetables. In summary, this research work provides baseline data upon which to develop food safety preventive control guidance during the production and minimal processing of these crops.

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1. Introduction

Enteric diseases linked to consumption of fresh produce have dramatically increased in the last several decades (Scallan et al., 2011; Morris, 2011). *Escherichia coli* O157:H7 has been associated in multiple outbreaks linked to the consumption of whole produce and fresh-cut leafy vegetables (CDPH, 2004, 2005; CDC, 2006). Diverse opportunities for primary contamination and cross-contamination during preharvest phases and postharvest handling are recognized, including fecal contamination by animals or transmission by insects, use of untreated manure, application of contaminated irrigation or foliar contact water, flood water carrying human waste, and direct human hand contact (Beuchat, 1996; Suslow et al., 2003; Brandl, 2006). Prevention and sanitation become the most important tools for keeping microbial quality and safety of fresh-cut commodities. In response to repeated foodborne outbreaks associated with the consumption of fresh vegetables, the U.S. Food and Drug Administration (FDA) released its “Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables,” (Food and Drug Administration, FDA, 2008) which identified concerns and provided recommendations for food safety practices that are intended to minimize the microbiological hazards associated with fresh and fresh-cut plant products. Early in 2011, the FDA Food Safety Modernization Act was signed into law and has prompted the produce industry to anticipate minimum federal standards for production of fruits and vegetables based on known safety risks (FDA, 2011).

Consumption of leafy vegetables contaminated with *E. coli* O157:H7 poses an important risk for humans, as epidemiological studies have

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shown that the infectious dose may be as low as 10 cells (Jinneman et al., 1995). Infection with *E. coli* O157:H7 can cause many forms and severity of illness or morbidity, ranging from rapidly resolving diarrhea to severe hemorrhagic colitis and life-threatening hemolytic uremic syndrome, particularly in infants and elderly people (Delaquis et al., 2007). Plant-based foods had not been traditionally recognized as a host for life-threatening enteric pathogens until multiple factors converged to elevate both the incidence of produce-related outbreaks and the recognition of their role in overall foodborne illness (Warriner and Namvar, 2010).

Surface contamination of the edible tissues of leafy vegetables is often due to pathogen transference from soil or water (Delaquis et al., 2007). Survival of *E. coli* O157:H7 on plant surfaces is variable and controlled by numerous factors including: nutrient availability, competition with indigenous microflora, UV radiation, and relative humidity (Brandl, 2006). Unlike many true epiphytes, *E. coli* O157:H7 does not appear to produce enzymes that degrade plant cell walls and the fate of this enteric pathogen upon arrival in the phyllosphere is not fully understood (Teplitski et al., 2009). A study conducted by Islam et al. (2004) demonstrated that *E. coli* O157:H7 persisted for 154 to 217 days in soils amended with contaminated composts and was detected on lettuce and parsley for up to 77 and 177 days, respectively, after seedlings were planted. In a greenhouse experiment, when *E. coli* O157:H7 was sprayed inoculated (10^4 CFU/mL) on butterhead lettuce, it was detected after selective enrichment up to 30 days post-inoculation. However, a reduction of the dose level (10^2 CFU/mL) resulted in the absence of detection within 17 days post-inoculation (Salomon et al., 2003). Recently, Moyne et al. (2011) reported that *E. coli* O157:H7 spray-inoculated in a lettuce field was detected up to 28–35 days post-inoculation. Scientific based data modeling the postharvest survival of this enteric pathogen in the phyllosphere of leafy vegetables after processing and retail distribution is abundant but often contradictory (Sapers et al., 2006).

Asian baby leaf vegetables have recently grown in popularity as a base ingredient for fresh-cut mixed salads. Among those Tatsoi (*Brassica rapa* cv. rosularis), Mizuna (*B. rapa* cv. japonica) and baby Red Chard (*Beta vulgaris* cv. cicla) are the most commonly consumed. The specific crop cycles for these vegetables vary between 25 and 75 days depending on the environmental conditions and desired stage of maturity. The specific operations involved in fresh-cut preparation include combined light-handling methods such as washing, trimming or cutting, disinfecting, and packaging at chilled temperatures. Unitization is generally under polymeric films able to develop optimum modified atmosphere packaging (MAP) conditions (Artés and Allende, 2005). Baby leafy vegetables are frequently consumed raw, thus washing and disinfection are a key steps that contribute to effectively reduce microbial load across the supply chain (Suslow, 1997; Allende et al., 2004). Specific postharvest processing operations such as cutting or shredding can damage the plant tissues, rendering them more prone produce to support the survival and growth of pathogenic bacteria responsible for human illness. Postharvest handling practices can influence the fate of *E. coli*, as indicators of enteric contamination, and *E. coli* O157:H7 during processing and could increase the likelihood of cross-contamination (Delaquis et al., 2007; Luo et al., 2011). It has been demonstrated that plant lesions can promote the rapid multiplication of *E. coli* O157:H7 over a short period of time on lettuce during postharvest phases (Brandl, 2008). The application of chemical sanitizers during the washing step constitutes the available and a practical means for achieving some gains in decontamination of leafy vegetables and is one of the primary elements of a properly managed postharvest sanitation program. However, this step does not guarantee the total inactivation of human pathogens (Artés et al., 2009).

The purpose of the current study was to assess the fate of *E. coli* and *E. coli* O157:H7 in diverse baby leafy vegetables from preharvest to postharvest minimal processing, packaging and chilled storage conditions in a model system. Genotyping of applied generic *E. coli* strains to evaluate their comparative survival in the phyllosphere from production throughout processing is also reported.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Two different cocktails of generic *E. coli* (TVS 353, TVS 354 and TVS 355) and *E. coli* O157:H7 (PTVS 154 and PTVS 155) were used (Table 1). Generic *E. coli* strains were isolated from surface irrigation water (TVS 353), Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) (TVS 354) and sandy-loam soil (TVS 355) samples from the Central Coast near Salinas, California. Each isolate has a distinct DNA-fingerprint by repetitive extragenic palindromic PCR (Fig. 1). *E. coli* O157:H7 strains used in this study are non-toxigenic isolates (lacking *stx*1 and *stx*2) ATCC 700728 and ATCC 43888, classified as Biosafety Level-1 (BSL-1) and BSL-2, respectively. The use of both *E. coli* O157:H7 strains in greenhouse is currently approved by the Office of Environmental Health and Safety (EH&S) of University of California, Davis. An antibiotic-resistant derivative strain for growth in the presence of rifampicin (80 mg/L) was isolated via selection of spontaneous mutants and used to minimize interference with other bacteria and to facilitate the detection and recovery for each strain (Beuchat et al., 2001). Independent colonies growing on rifampicin amended media were purified and tested for retention of colony morphology and growth rate relative to the wild-type isolate under non-selective conditions. Both generic *E. coli* and *E. coli* O157:H7 strains were separately grown in 9 mL of tryptic soy broth (TSB) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin at 37 °C for 18 h. After incubation, cultures were centrifuged at 1500×g for 10 min. The pellet was re-suspended and washed twice with Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA). The final cell pellet was suspended in Butterfield's phosphate buffer to achieve a target initial cell density (OD_{600}) of approximately 0.7, which corresponds to a concentration of 10^8 CFU/mL and subsequently diluted to achieve a final concentration of 10^6 CFU/mL. The final concentration was confirmed by plating on Tryptic Soy Agar (TSA) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin (TSA-rif).

2.2. Baby leafy greens cultivation

Seeds of Mizuna (*B. rapa* var. japonica), Tatsoi (*B. rapa* var. rosularis) and Red Chard (*B. vulgaris* var. cycla) were supplied by Synergene Seed & Technology (Salinas, CA, USA). Each leafy vegetable was individually grown in aeration container pots of 18 cm in diameter and 10 cm in depth (Smart Pot, High Caliper Growing–Root Control, Inc., Oklahoma City, OK, USA) containing UC mix (33% peat, 25% sand, 42% fir bark). Common commercial plant densities of 900 seeds/m^2 for Red Chard and 1000 seeds/m^2 for Mizuna and Tatsoi were established. Pots were

Table 1
Characteristics of the *Escherichia coli* strains used in the study.

Strain	Source of information
TVS 353	*E. coli* W778, selected rifampicin resistant. Isolated from irrigation water (Salinas Region, CA, USA).
TVS 354	*E. coli* P149, selected rifampicin resistant. Isolated from lettuce leaves (Salinas Region, CA, USA).
TVS 355	*E. coli* S19, selected rifampicin resistant. Isolated from sandy loam soil (Salinas Region, CA, USA).
PTVS 154	*E. coli* O157:H7. ATCC # 43888, does not possess *stx1* and *stx2* genes (BSL-2). Selected rifampicin resistant.
PTVS 155	*E. coli* O157:H7. ATCC # 700728, non toxigenic (BSL-1). Selected rifampicin resistant.

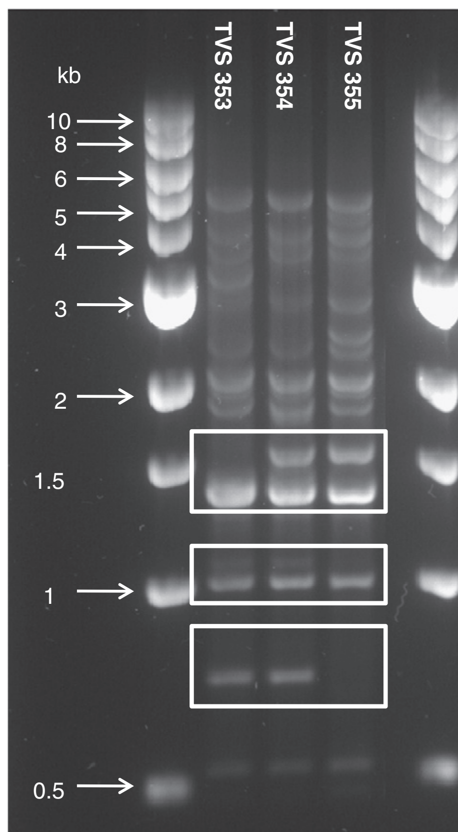

Fig. 1. A PCR fingerprint patterns of *E. coli* strains TVS 353, TVS 354 and TVS 355 strains by REP-PCR.

randomly distributed across a greenhouse bench. Greenhouse conditions ranged from 18 to 21 °C and from 65 to 75% of relative humidity (RH). Plants were watered daily and fertilized as needed with 50% Hoagland's Solution following standard practices in a research greenhouse of the University of California, Davis. Two development stages of the baby leafy greens were defined for experimental sampling as 3 and 4 week old post-planting plants.

2.3. Greenhouse trials and inoculation procedure

Two trials were conducted during summer (July–August) and fall (October–November) 2010. A factorial combination of the following conditions was evaluated: irrigation type (overhead and drip irrigation), leaf age at inoculation time (3 and 4 weeks post-planting), and inoculum type (generic *E. coli* and *E. coli* O157:H7). Each grouping treatment consisted of 3 pots of each leafy vegetable ($n = 9$), resulting in 54 pots per trial. Each pot was considered as a replication within the same grouping treatment. For the fall trial, only drip irrigation was used.

Hand-spray bottles were used to inoculate the 3 and 4 week old plants. The bottles were previously calibrated to release 4 mL of the 10^6 CFU/mL inoculum solution in two single manual pumps of the spray-trigger per pot. The inoculation was always performed between 8 and 8:30 am to prevent acute heat stress. The final bacterial load achieved in each pot was determined to be approximately log 4.2 CFU/cm^2. The final concentration was confirmed by plating on TSA-rif.

Inoculated 3 and 4 week old plants were harvested at commercial baby leaf stage after reaching 31 and 38 days post-emergence, respectively, and were subjected to further postharvest operations.

2.4. Postharvest operations

After harvest, each grouping treatment was processed separately at room temperature. Leaves with defects such as yellowing, decay, cuts and bruising were carefully discarded. The raw material was washed for 90 s with tap water containing 3 mg/L ClO_2, adjusted from a liquid concentrate, as the disinfectant agent and followed by a 1 min of rinsing with tap water. Then, the leaves were centrifuged using a foodservice grade manual salad spin dryer (0.2 m^3 volume) to eliminate excess water. An amount of 20 g of leaves were placed in 23×15 cm commercial salad bags and stored in a controlled temperature room without light at 5 °C for up to 7 days. The 5 °C temperature was selected as the maximum limit recommended for short-term storage and distribution and the most commonly used for fresh-cut plant commodities throughout its commercial retail sale. Three replicates for each grouping treatment were prepared. All the postharvest experimentation was conducted in the BioSafety Level 2 containment laboratory of the Louis Mann Postharvest Laboratory Facility (T. Suslow, Director) at University of California, Davis.

2.5. Plant sampling, bacterial recovery and detection

Recovery and detection of generic *E. coli* and attenuated *E. coli* O157:H7 were conducted after 2, 7 and 10 days post-inoculation, following disinfection (AW), and at 7 days of chilling storage (AS). To collect samples, baby leaves were removed by cutting immediately above the soil level with sterile scissors. Plant samples were transferred into sterile plastic bags (Whirl-Pak, Nasco, Modesto, California, US) containing sterile TSB supplemented with 80 mg/L of rifampicin in a 1:2 w/v ratio. Samples were then massaged by hand for 1 min and 100 μL of bacterial suspension was plated on TSA-rif and incubated at 37 °C for 24 h. 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 (Knudsen et al., 2001). Due to the different availability of plant material during the greenhouse development, and in order to homogenize the sample size, an amount of 5 to 15 g of sample was taken for the 2, 7 and 10 days post-inoculation time points and 20 g following disinfection and storage. Results from plate counts were reported as log CFU/g. All analyses were made in triplicates.

After plating, the bags containing the homogenized plant samples and TSB supplemented with 80 mg/L of rifampicin were incubated at 37 °C for 18 h to evaluate bacterial populations below the limit of detection by direct enumeration. For confirmation of generic *E. coli* enrichment was plated onto Chrom-ECC agar (Chrom Agar, Paris, France) supplemented with 80 mg/L of rifampicin and incubated at 37 °C. Typical blue colonies were considered a positive result. For detection of *E. coli* O157:H7, amplification of *rfbE* was done using probe based real-time PCR. Amplicons were generated using forward primer *rfbE*-F (5′-GATGCCAATGTACTCG-GAAAAAT-3′), reverse primer *rfbE*-R (5′-CCACGCCAACCAAGATCCT-3′) and *rfbE* probe (NEDCAAAAGCACCCTATAGCTMGBNFQ). Each 20 μL reaction contained 10 μL of a 2× Taqman® Gene expression master mix (Applied Biosystems Inc., Foster City, CA, USA), 0.5 μM of forward and reverse primers, 2.5 pmol of probe targeting *rfbE* (Applied Biosystems Inc., Foster City, CA, USA) and 2 μL of enrichment that was previously boiled for 95 °C for 10 min. Amplification was carried in a thermocycler (7300 Real Time PCR System, Applied Biosystems Inc., Foster City, CA, USA) with a protocol consisting in one cycle of 50 °C for 5 min, one cycle of denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and annealing at 60 °C for 1 min. Amplification greater or equal to the Ct value of a standard containing 1 copy of *rfbE* was classified as positive.

2.6. Repetitive extragenic palindromic PCR (REP-PCR)

Genotyping of individual generic *E. coli* contained in the cocktail was by amplification of repetitive extragenic palindromic (REP) elements to evaluate the distribution of the strains in the phyllosphere of each leafy

vegetable from preharvest to postharvest conditions. Ten percent of the total *E. coli* colonies recovered throughout the entire experimental time-frame were isolated and used for this assay. Isolated colonies were purified by streaking them on TSA and incubating at 37 °C for 24 h, repeated twice. Purified colonies were re-suspended on Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA) to yield an OD_{600} of 0.4–0.5. The cell suspension was subsequently centrifuged at 13,000 g for 1 min and resuspended in 200 μL 1× TE buffer. Template DNA was prepared by heating at 95 °C for 10 min. Amplification was done with primer REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I: (5′-ICGICTTATCIGGCC-TAC-3′) (Versalovic et al., 1991). Each 25 μL reaction contained 5 μL 5× colorless GoTaq® buffer (Promega Corp. Madison, WI, USA) supplemented with 62.5 mmol of MgCl (Invitrogen Corp. Carlsbad, CA, USA), 0.2 mM of each dNTP (Applied Biosystems Inc., Foster City, CA, USA), 0.5 μM of REP1R-I and REP2-I primers 1% of DSMO and 1.25 U of GoTaq® (Promega Corp. Madison, WI, USA), 7.75 μL H_2O and 1.5 μL of template DNA. Amplicons were generated in thermocycler (GeneAmp®, PCR System 2700, Applied Biosystems Inc., Foster City, CA, USA) with an initial denaturation (4 min, 94 °C) followed by 30 cycles of denaturation (94 °C, 1 min), annealing (40 °C, 1.5 min) and extension (65 °C, 8 min), followed by a single final extension (65 °C, 16 min). Amplicons were separated on 1:2 (w/v) agarose for electrophoresis during 2.5 h at 120 mV. Isolates showing identical band patterns were grouped manually.

2.7. Statistical analysis

Statistical analysis was carried with Statistical Analysis Software V. 9.2 (SAS Institute Cary NC) using a factorial design. To assess significant difference among treatments during the time, the MIXED procedure was utilized and Tukey pair wise comparison was applied for mean separation. A p-value lower than 0.05 was utilized to establish significant difference among treatments. Data was previously analyzed for normality and homogeneity of variance using the UNIVARIATE procedure, function of SAS.

3. Results

3.1. Environmental conditions during vegetable production

Weekly average temperature, RH and hours of daylight at the greenhouse for both summer and fall trials are shown in Table 2. During the summer trial, the average temperature ranged from 20 to 22 °C and the RH around 69–74% during the 5 weeks of experimental baby leaf production. Similar conditions of temperature and RH were recorded during the fall trial; temperature varied from 20 to 21 °C and RH from 65 to 72%. Although temperature and RH were similar during both consecutive growing seasons, differences in daylight hours were recorded and light spectrum and quality were correspondingly lower. Average daylight during summer trial ranged from 14.1 to 13.1 h while for the fall trail daylight ranged from 11.7 to 10.6 h, which represents a difference of 2.4 to 2.5 h of daylight exposure. Supplemental light was not used.

Table 2
Climatic data summary for each trial. Values are the average weekly temperature, relative humidity and daylight hours.

	Summer 2010			Fall 2010		
	T (°C)	RH (%)	Daylight hours	T (°C)	RH (%)	Daylight hours
Week 1	21.3	72.5	14.1	20.8	71.6	11.7
Week 2	21.0	73.4	13.8	19.8	69.4	11.4
Week 3	20.1	72.8	13.6	20.2	68.4	11.2
Week 4	21.8	69.4	13.3	19.2	71.8	10.9
Week 5	21.1	71.4	13.1	19.1	65.6	10.6

3.2. Survival of generic E. coli

A cocktail of three generic strains of *E. coli* was spray-inoculated onto 3 week old or 4 week old Mizuna, Tatsoi and Red Chard leaves. The initial inoculum level was about 4.2 log CFU/cm^2. In general, applied *E. coli* was detected at all the sample points from production to storage regardless of the season, leaf age at inoculation time and irrigation type for all three baby leafy greens evaluated.

For the summer trial, *E. coli* counts 2 days post-inoculation were 2.45 and 1.87 log CFU/g for sprinkle and drip irrigation, respectively, for the 3 week-old Mizuna leaves, while *E. coli* populations on 4 week-old Mizuna leaves, were below the quantitative limit of detection (1.43 log CFU/g) (Table 3). According to the analysis of variance, the leaf age had no effect on the bacterial population ($p>0.05$) during the crop growing for each irrigation type. After 7 and 10 days post inoculation, all the samples were below the limit of detection (LOD) but were detected after selective enrichment. The wash disinfection of Mizuna leaves with ClO_2 was unable to fully inactivate the inoculated bacteria as evidenced by enrichment-detection of *E. coli* survival after processing and storage up to 7 days at 5 °C. During the fall trial (Table 4A), *E. coli* populations on the 3 and 4 week-old Mizuna leaves after 2 days post-inoculation were 2.51 and 3.47 log CFU/g respectively. In this trial, the effect of the leaf age on the survival of *E. coli* was significant ($p<0.05$) only at 2 days post-inoculation. *E. coli* populations dropped by approximately 0.75 and 1.66 log CFU/g after 10 days for the 3 and 4 week-old Mizuna leaves, respectively. In contrast with the summer trial, *E. coli* was recovered by direct-plating on selective culture media and the colony counts were above the LOD up to 10 days post-inoculation. Washing harvested material with 3 mg/L of ClO_2 resulted in a reduction of *E. coli* population below the LOD. Similarly to the summer trial, the target bacteria were recovered from all samples after commercial chilling storage of 7 days at 5 °C.

In the summer trial for Tatsoi leaves (Table 3), the effect of the leaf age on the survival of *E. coli* was not significant. The *E. coli* populations after 2 days were 3.22 and 3.50 log CFU/g for the 3 week-old leaves subjected to sprinkle and drip irrigation, respectively, and 2.72 and 3.05 log CFU/g for the 4 week-old leaves. The populations remained unchanged during the growing period regardless of the irrigation type and dropped by 0.7–1 log CFU/g after the wash disinfection

Table 3
Persistence of *E. coli* inoculated on 3 week-old (A) and 4 week-old (B) Mizuna, Tatsoi and Red Chard baby leaves from production to processing and storage (summer 2010).

		Time after inoculation[a]				
Crop	Irrigation type	2	7	10	AW	AS
A						
Mizuna	Sprinkle	2.45_A	(3/3)[b]	1.59_A	1.79_A	1.52_A
	Drip	1.87_A	(3/3)	(3/3)	(2/3)	(2/3)
Tatsoi	Sprinkle	3.22_A	3.14_A	3.49^c_A	3.37_A	2.34_A
	Drip	3.50_A	(3/3)	(3/3)	(3/3)	(3/3)
Red Chard	Sprinkle	2.91^c_A	(3/3)	(3/3)	2.33^c_{AB}	2.10_{AB}
	Drip	2.62_B	(3/3)	2.92^c_A	1.94_B	(3/3)
B						
Mizuna	Sprinkle	(3/3)	(3/3)	(3/3)	(2/3)	(2/3)
	Drip	1.52_A	(3/3)	(2/3)	(3/3)	(3/3)
Tatsoi	Sprinkle	2.72_{AB}	(3/3)	2.97_A	2.28_{AB}	2.12_{AB}
	Drip	3.05_A	2.31_A	2.86_A	1.87_A	1.90_A
Red Chard	Sprinkle	3.11_A	2.95_A	3.05_A	3.21_A	2.04_A
	Drip	3.39_A	2.96_A	3.38_A	2.94_A	2.86_A

Different capital letters denote significant difference ($p<0.05$) within the same row.
[a] Levels of *E. coli* are expressed as log CFU/g (n = 3).
[b] Ratio denotes the number of replications tested positive for *E. coli* by selective enrichment in TSB + rif^{80} and confirmed by surface plating on CHROMECC + rif^{80} per total. Limit of detection is 1.43 log CFU/g.
[c] Denotes significant difference ($p<0.05$) between the irrigation type for the same crop and time point.

Table 4
Persistence of *E. coli* (A) and *E. coli* O157:H7 (B) inoculated on Mizuna, Tatsoi and Red Chard baby leaves from production to processing and storage (fall 2010).

Crop	Leaf age	Time after inoculation[a]				
		2	7	10	AW	AS
A						
Mizuna	3 weeks	2.51_A	1.82_B	1.76_B	(3/3)[b]	(3/3)
Tatsoi		3.25_A	1.52_B	1.74_B	1.50_B	(3/3)
Red Chard		2.16_A	2.22_A	2.34_A	2.08_A	1.86_A
Mizuna	4 weeks	3.47_A	(3/3)	1.75_B	(3/3)	(3/3)
Tatsoi		3.33_A	2.76^{c}_{B}	1.75_C	(3/3)	(3/3)
Red Chard		3.11^{c}_{A}	2.83_{AB}	2.56_{AB}	2.32_{AB}	2.07_B
B						
Mizuna	3 weeks	3.15_A	(3/3)[b]	1.74_B	(3/3)	(1/3)
Tatsoi		2.69_A	1.75_B	(3/3)	1.93_{AB}	(2/3)
Red Chard		3.91_A	2.46_B	2.31_B	2.28_B	2.07_B
Mizuna	4 weeks	2.68_A	2.02^{c}_{B}	1.91_B	1.69_B	(1/3)
Tatsoi		3.32_A	2.01_B	(3/3)	(2/3)	(2/3)
Red Chard		3.37_A	2.18_B	2.28_B	2.22_B	1.54_B

Different capital letters denote significant difference ($p<0.05$) within the same row.
[a] Levels of *E. coli* are expressed as log CFU/g (n = 3).
[b] Ratio denotes the number of replications tested positive by selective enrichment in TSB + rif and confirmed by surface plating on CHROMECC + rif for generic *E. coli* or, through amplification of *rfbE* by qRT-PCR for *E. coli* O157:H7 per total. Limit of detection is 1.43 log CFU/g.
[c] Denotes significant difference ($p<0.05$) between the leaf age for the same crop and time point.

Table 5
Persistence of *E. coli* O157:H7 inoculated on 3 (A) and 4 (B) week-old Mizuna, Tatsoi and Red Chard baby leaves from production to processing and storage (summer 2010).

Crop	Irrigation type	Time after inoculation[a]				
		2	7	10	AW	AS
A						
Mizuna	Sprinkle	1.87_A	1.88_A	(1/3)	(2/3)	(2/3)
	Drip	2.45_A	(3/3)[b]	(1/3)	(1/3)	(2/3)
Tatsoi	Sprinkle	1.80_A	(3/3)	(3/3)	(3/3)	(3/3)
	Drip	2.17_A	(3/3)	(2/3)	(1/3)	(2/3)
Red Chard	Sprinkle	2.16_A	(3/3)	1.57_A	(3/3)	(3/3)
	Drip	1.71_A	(3/3)	(2/3)	(2/3)	(2/3)
B						
Mizuna	Sprinkle	1.66_A	(3/3)[b]	1.73_A	1.65_A	1.77_A
	Drip	1.95_A	(3/3)	1.69_A	(3/3)	(3/3)
Tatsoi	Sprinkle	2.27_A	2.46_A	2.06_A	1.76_A	(3/3)
	Drip	3.37^{c}_{A}	2.82_A	3.39_A	2.72_A	2.30_A
Red Chard	Sprinkle	2.10_A	(3/3)	(3/3)	(3/3)	(3/3)
	Drip	2.97_A	(3/3)	1.85^{c}_{AB}	1.85_B	1.79_B

Different capital letters denote significant difference ($p<0.05$) within the same row.
[a] Levels of *E. coli* O157:H7 are expressed as log CFU/g (n = 3).
[b] Ratio denotes the number of replications tested positive for *E. coli* O157:H7 through amplification of *rfbE* by qRT-PCR per total.
[c] Denotes significant difference ($p<0.05$) between the irrigation type for the same crop and time point.

with ClO_2 for the 4 week-old leaves. In general, with the exception of the 3 week-old leaves under drip irrigation, *E. coli* populations from all the sample points were above the quantitative LOD (Table 3A). As expected, *E. coli* was still recovered after storage at 5 °C for 7 days. A similar trend for the trial performed in fall was observed. *E. coli* population declined in approximately 1.5 log CFU/g after 10 days compared to the population recovered from 2 days post-inoculation. In contrast with the summer trial, the reduction observed for each leaf age was significantly different. After the cold storage, survival of target bacteria was only confirmed by selective enrichment.

In contrast with the previous results, the effect leaf age was significant on the survival of *E. coli* on Red Chard leaves after 2 days post-inoculation for both summer and fall trials. No difference between irrigation types was observed. The number of culturable bacteria declined rapidly from the initial date of inoculation until harvesting and subsequent postharvest washing for the 3 week-old Red Chard leaves in the summer trial. However, the *E. coli* population remained constant from day 2 up to the period of chilling storage and no significant differences ($p>0.05$) were recorded. The same behavior was recorded for *E. coli* population during the fall trial.

3.3. Survival of E. coli O157:H7

Population dynamics of *E. coli* O157:H7 in the phyllosphere of Mizuna, Tatsoi and Red Chard baby leaves are shown in Tables 4B and 5 for summer and fall trials, respectively. In general, the effect of the irrigation type and leaf age on the survival of *E. coli* O157:H7 was not significant for all leafy green types and trials. For all 3 week-old mini-greens, average *E. coli* O157:H7 population was in the range of 1.8–2.4 log CFU/g after two days post-inoculation. However, bacteria were not recovered by direct plating after 2 days post-inoculation from any sampling point. The evidence of *E. coli* O157:H7 survival throughout the simulated processing chain and refrigerated storage conditions was confirmed exclusively by qRT-PCR. Decrease of *E. coli* O157:H7 populations was detected after 2 days post-inoculation for the 4 week-old baby leaves during the summer trial, however populations tended to remain constant from day 7 and during storage. Processing operations, including a washing disinfection step with 3 mg/L of ClO_2, were unable to efficiently remove and/or inactivate the attached *E. coli* O157:H7 from the plant surfaces. The bacteria populations before and after the washing-disinfection operation were not significantly different for all the leafy green.

Seasonal effect for both *E. coli* O157:H7 and generic *E. coli* was evaluated. Significant effect of the growing season was determined for the *E. coli* O157:H7 for the three leafy green varieties, in contrast to the effect of season on generic *E. coli* populations which was found to be only significant for Mizuna but not for Tatsoi or Red Chard.

3.4. Distribution of E. coli strains in the leafy greens phyllosphere

A cocktail of three different *E. coli* strains (TVS 353, TVS 354 and TVS 355) which were originally isolated from irrigation water, Romaine lettuce leaves, and sandy loam soil cultivated with Romaine lettuce in the Salinas Region (California, USA), was used in this study. REP-PCR, as a genotypic identification method, was utilized in the current study to differentiate the three different strains, which have a different DNA-fingerprint (Fig. 1), and evaluate their relative abundance and distribution in the phyllosphere of the leafy vegetables.

During the summer trial, TVS 353 (water isolate) represented more than 50% of the culturable bacteria for Mizuna and Red Chard while TVS 355 (soil isolate) population ranged from 20 to 33% for all the leafy green types. TVS 354 (plant) was the least abundant strain with a relative abundance of 9, 16, and 27% for Tatsoi, Mizuna and Red Chard respectively (Fig. 2A).

Similar results were observed for the fall trial where TVS 353 was the most persistent strain. On average, a relative abundance of 56, 46 and 42% of TVS 353 compared to the total populations of applied *E. coli* corresponding to Mizuna, Red Chard and Tatsoi. TVS 355 represented 33 and 35% of the total for Red Chard and Mizuna while it represented 40% for Tatsoi leaves (Fig. 2B).

In general, *E. coli* strains could be ranked according to their persistence in the phyllosphere of the assayed leafy greens as follows: TVS 354 (plant) < TVS 355 (soil) < TVS 353 (water), being the *E. coli* isolated from environmental plant samples the least persistent under the conditions of this study.

4. Discussion

This study provides an assessment of indicator/surrogate strains and pathogen behavior under model conditions for the production

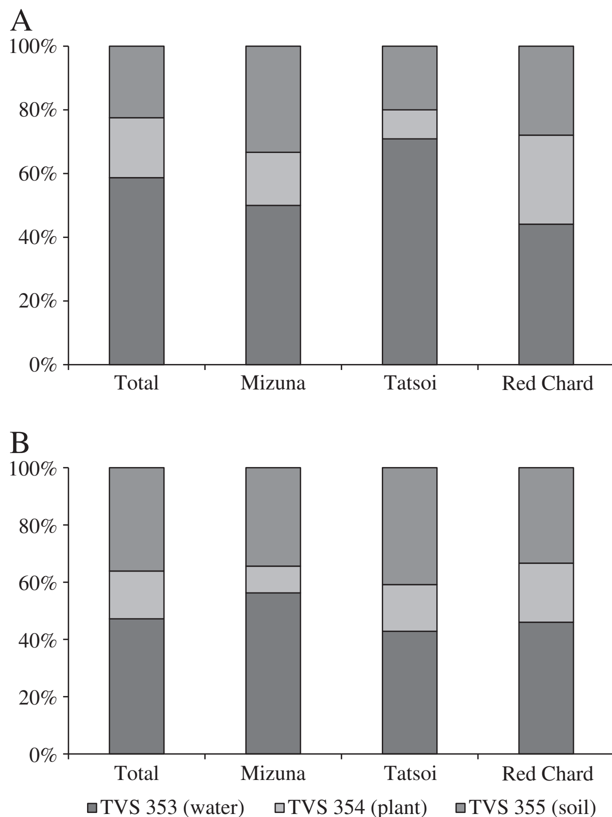

Fig. 2. Relative abundance of *E. coli* strains TVS 353, TVS 354 and TVS 355 in the phyllosphere of Mizuna, Tatsoi and Red Chard during the summer (A) and fall (B) trial.

of baby Mizuna, Tatsoi and Red Chard and including postharvest minimal processing and chilling storage, mimicking commercial conditions. A mixture of three strains of generic *E. coli* and a mixture of two avirulent strains of *E. coli* O157:H7, safe surrogates of the pathogen, were used in this study.

In general, a rapid decline of generic *E. coli* and *E. coli* O157:H7 population for all the leafy vegetables regardless of the leaf age and the irrigation type for both seasonal trials was observed. Our results agree with previous findings reported by Wood et al. (2010) for population dynamics of *E. coli* inoculated in growing spinach. In that field study, *E. coli* population was reduced by 3–5 logs after 72 h and culturable bacteria were recovered only up to 6 days post inoculation. Moyne et al. (2011) evaluated the survival of *E. coli* O157:H7 (ATCC 700728) on lettuce plants using a relative low inoculum level (5 log CFU/mL) in a summer field setting experiment. After 2 days post inoculation no culturable *E. coli* O157:H7 were recovered but the target bacteria were detected by selective enrichment up to 14 days post inoculation. A recent study conducted by Erickson et al. (2010) reported that *E. coli* O157:H7 was not recovered after 7 days post-inoculation in an artificially inoculated spinach and lettuce field at 10^4 CFU/mL via irrigation water. In contrast with this behavior, *E. coli* O157:H7 was detected up to 77 and 177 days in lettuce and parsley respectively (Islam et al., 2004). Other studies have reported the influence of the irrigation method (Salomon et al., 2002) on the transmission and persistence of the pathogen as well as the leaf age (Brandl and Amundson, 2008) as a risk factor of contamination with *E. coli* O157:H7. In the current study, the leaf age at the time of inoculation as well as the irrigation method used did not have a significant effect on the survival of *E. coli* and *E. coli* O157:H7 during growing phases in a greenhouse.

Solar ultraviolet (UV) radiation and desiccation have been identified as important factors influencing pathogen survival in the phylloplane (Heaton and Jones, 2008). UV radiation contributes to inactivate phyllosphere bacteria and foodborne pathogens of produce (Jacobs and Sundin, 2001; Allende and Artés, 2003). Experiments under greenhouse conditions provide an effective formula to protect the crops from natural winds and dryness. However, our results did not differ from previous studies, performed under field conditions (Erickson et al., 2010; Wood et al., 2010; Moyne et al., 2011). The decline rate of the survival populations for the fall season was slower than for the summer season, likely result of the differences the duration of daylight exposure among seasons, potentially attributed to longer exposure to solar radiation and a consequence of desiccation at the leaf-air boundary, causing additional stress to bacterial cells on the plant surface.

In the current study we inoculated the leafy vegetables using a cocktail of *E. coli* or *E. coli* O157:H7 strains. This study presents the distribution of the three environmental isolates of generic *E. coli* strains in the phyllosphere of Mizuna, Tatsoi and Red Chard. A heterogeneous distribution of the isolates was observed (Fig. 2). In general, a different relative abundance for each strain was observed, with isolate TVS 353 (water isolate) the most tolerant or best adapted. This corroborates that the use of a mixture of different strains provides a better approach to a real world scenario compared to the use of a single strain.

The minimal processing of the Mizuna, Tatsoi and Red Chard baby leaves included a disinfection step with 3 mg/L of ClO_2 as a disinfection agent. In general, reductions of the population of generic and pathogenic *E. coli* less than 1 log unit for all treatments and leafy green types were achieved. In most cases, these reductions were not significant and ClO_2 was not able to fully disinfect the inoculated leaves. Similar findings have been reported for other minimally processing leafy vegetables such as spinach (Lee and Baek, 2008) and lettuce (Keskinen et al., 2009). Results of this study suggest that the sanitizer was not able to reach the bacteria during the washing step, probably due to their location on the leaves or association within aggregates (Lindow and Brandl, 2003). In fact, bacteria on plant surfaces tend to concentrate where there are more binding and protected sites (Parish et al., 2003), with preferential occurring at the base of trichomes, on the outer rim of stomata, and in cell grooves along veins (Monier and Lindow, 2004). A study of the bacterial distribution on lettuce surface treated with NaClO by scanning electron microscopy showed that the most of the bacteria were located either around or infiltrating stomata or as groups of clustered cells (López-Gálvez et al., 2010). These findings mentioned above suggest that the location of bacteria in sheltered sites could protect them from chemical sanitizers.

After the minimal processing of Mizuna, Tatsoi and Red Chard, they were stored at 5 °C which is one of the most commonly used temperatures for minimal processed vegetables throughout its commercial distribution and retail sale. By the end of the simulated shelf-life at 5 °C, evidence of survival of *E. coli* and *E. coli* O157:H7 was confirmed by both culture and enrichment or RT-PCR methods for all minimally processed products. In general, the population remained constant or a limited decrease during the cold storage was achieved. Similar results have been reported by other authors. Oliveira et al. (2010) studied the survival and growth of *E. coli* O157:H7 inoculated onto shredded lettuce packaged under MAP at commercial cold storage conditions. After 10 days at 5 °C populations of *E. coli* O157:H7 decreased approximately in 1 log unit. This minimal effect on survival of *E. coli* O157:H7 is similar to other studies with leafy vegetables where no changes in population or small decrease in iceberg lettuce (Delaquis et al., 2002) and spinach (López-Velasco et al., 2010) stored at 5 °C and 4 °C respectively were found.

The fate of *E. coli* and *E. coli* O157:H7 during production, harvest, processing and storage of Mizuna, Tatsoi and Red Chard baby leaves is reported in the current study. The ability to survive during production and after disinfection and storage of low levels of *E. coli* in the assayed mini greens has been demonstrated. However, field-based trials under

realistic conditions to understand the fate of the pathogen should be further studied. In summary, this research work provides useful data to develop an adequate science-base risk assessment during the production and minimal processing of these crops.

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