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Evaluation of post-contamination survival and persistence of applied attenuated *E. coli* O157:H7 and naturally-contaminating *E. coli* O157:H7 on spinach under field conditions and following postharvest handling

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ABSTRACT

This study determined the variability in population uniformity of an applied mixture of attenuated *E. coli* O157:H7 (*attEcO157)* on spinach leaves as impacted by sampling mass and detection technique over spatial and temporal conditions. Opportunistically, the survival and distribution of naturally contaminating pathogenic *E. coli* O157:H7 (*Ec*O157), in a single packaged lot following commercial postharvest handling and washing, was also evaluated. From the main study outcomes, differences in the applied inoculum dose of 100-fold, resulted in indistinguishable population densities of approximately Log 1.1 CFU g^{-1} by 14 days post-inoculation (DPI). Composite leaf samples of 150 g and the inclusion of the spinach petiole resulted in the greatest numerical sensitivity of detection of *attEcO157* when compared to 25 and 150 g samples without petioles ($P < 0.05$). Differences in population density and protected-site survival and potential leaf internalization were observed between growing seasons and locations in California ($P < 0.05$). A Double Weibull model best described and identified two distinct populations with different inactivation rates of the inoculated *attEcO157*. Linear die-off rates varied between 0.14 and 0.29 Log/Day irrespective of location. Detection of *Ec*O157- *stx*1-negative and *stx2*-positive, resulting from a natural contamination event, was observed in 11 of 26 quarantined commercial units of washed spinach by applying the 150 g sample mass protocol. The capacity to detect *Ec*O157 varied between commercial test kits and non-commercial qPCR. Our findings suggest the need for modifications to routine pathogen sampling protocols employed for lot acceptance of spinach and other leafy greens.

1. Introduction

The spatial distribution of culturable epiphytic microbiota is widely recognized to be extremely non-uniform, typically log-normally distributed across a plant population and among individual leaves, and even between opposing leaf pairs, within a single plant (Andrews et al., 1980; Andrews and Harris, 2000; Lindow and Brandl, 2003; Leveau, 2006). Differential physical, environmental, and competitive dynamics occurring within the phyllosphere have been documented to result in this lognormal distribution (Crosse, 1959; Andrews et al., 1980; Hirano et al., 1982; Kinkel, 1997; McGrath and Andrews, 2007). Consequently, within studies of resident and transient phyllosphere colonizers, the experimental design and sampling strategy significantly influence the outcomes and, subsequently, interpretation of the data analysis (Kinkel et al., 1995; Meyer and Leveau, 2012; Wiken Dees et al., 2015).

In open environments, deterministic temporal factors affect the population flux and degree of heterogeneity following deposition of both bacterial plant epiphytes and bacterial pathogens of food safety concern on horticultural food crops (Leveau, 2006; Meyer and Leveau, 2012; Rastogi et al., 2012). The greatest concern for post-contamination survival, understandably, is associated with perishable horticultural foods typically consumed in a raw state. These temporally variable and interacting factors include the source of contamination (aqueous planktonic cells or aggregates imbedded in suspended sediments or an organic matrix), regional climate and field-scale microclimate, as well as plant developmental and anatomical/physiological traits (Rastogi et al., 2012). Bacterial populations on leaf surfaces are normally estimated by using standardized sample units, including leaf segments,

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individual leaves, or the entire plant (Tyler and Triplett, 2008; Morris and Lucotte, 1993). Scaling within a sample unit; in which size or weight have a direct influence on estimating bacterial numbers and spatial distribution among samples from within a plant population, has been a major focus of descriptive and quantitative phyllosphere microbiology (Williams and Marco, 2015; Widder et al., 2016), since sample size and plant organ may impact probability of detection and recovery efficiency.

Variability in bacterial phyllosphere populations has been correlated with leaf position, age and nutrient availability as well as the accessibility or exposure of leaves to the deposition of airborne microbiota, microclimates, growing season, leaf orientation, plant structure and the physicochemical condition of leaves (Mew and Kennedy, 1982; Plummer et al., 1992; Jacques et al., 1995; Ong et al., 1999; Redford and Fierer 2009; Lindow and Brandl, 2003; Aruscavage et al., 2006; Monier and Lindow, 2005; Redford et al., 2010; Jackson and Denney, 2011; Kroupitski et al., 2011; Burch et al., 2016).

Rastogi et al. (2012) evaluated the extent and presumptive sources of variability on the phyllosphere bacterial community composition on Romaine lettuce as a function of time, spatial separation within and between commercial fields, and seasonal environmental and geospatial differences. It was determined that the distance between fields or the timing of a natural environmental phenomenon and not Romaine cultivar or irrigation method explained differences in bacterial community composition between different commercial fields; suggesting that the mechanisms of bacterial community composition within plants and their subsequent variability are a function of environmental factors as suggested by Wiken Dees et al. (2015), Franz (2008), and Leff and Fierer (2013).

Leafy greens have consistently been implicated in foodborne disease outbreaks over the past 44 years (Herman et al., 2015). One response to this fact has been the implementation of routine audit standards compliance and lot acceptance monitoring of fresh produce for the presence of fecal indicators and bacterial human pathogens. These testing programs have increased dramatically following three unrelated STEC outbreaks on leafy greens in 2006 (Gil et al., 2014) As sampling strategies were not standardized, a keen interest emerged to better characterize population variability within a production lot and to improve the statistical validity and predictive capacity of any sampling strategy as suggested by Callahan et al. (2016).

Past and some current rapid test kits including Reveal (Neogen, Lansing, MI), SDI RapidChek (Strategic Diagnostics, Newark, DE), BAX O157 and BAX O157:H7 MP (DuPont Qualicon, Wilmington, DE) FDA BAM method (1998) and Assurance GDS O157:H7 (BioControl Systems, Inc., Seattle, WA) specify a 1:10 (w:w) ratio with a wash buffer for enumeration or enrichment detection protocols (APHA, 2001; AOAC, 2007). Test kit accompanying technical instructions refer to validated or performance-tested protocols with 10–25 g sample mass for an array of foods over a defined pre-detection enrichment period. This approach had raised concerns that this sample mass unit and paired detection systems may not have been fully characterized or validated for pathogen detection in fresh produce (UFPA, 2010 and D'Lima and Suslow, 2009). To that end, recent efforts by Lopez-Velasco et al. (2015) and Fang and Patel (2017) highlight the importance of using composite or pooled sample unit sizes greater than 200 g to improve the probability of pathogen detection in controlled and open-field environments of tender greens cropping systems with high seedling densities like baby spinach.

This current study was initiated to investigate the variability in populations of applied *attEcO157* on the surface of spinach leaves and associated plant tissue under field conditions. Of special interest was to determine the post-inoculation variability of leaf surface populations from the same plant and among groups of plants with and without a 1% silver nitrate surface-disinfection treatment known to effectively inactivate human pathogens in laboratory studies. During the course of these controlled field studies and a single naturally-occurring contamination even with *E. coli* O157:H7 (*EcO157*) was detected by industry collaborators. With the information from the controlled studies, we sought to asses in this natural contamination event; 1- the variability among independent 'contamination' incidents in distribution of surviving, culturable *EcO157* and; 2- the extent of variation in extraction and detection efficiencies differing among selected methods and between known and uncharacterized *Ec*O157 serotypes. Such comparisons were viewed to provide insights into qualitative assessments of the prevalence of *Ec*O157 based on sample mass distribution in commercially washed and packaged spinach and among individual leaves from known positive pre-market retained samples.

2. Materials and methods

2.1. Field cultivation

Spinach seeds (*Spinacia oleracea* L. cv Barbados, Emilia and Blackhawk) were sown according to standard commercial practices during the months of May through November of 2008–2010 in Salinas (2008, 2010) and Soledad California (2009–2010), approximately 48 km separation north to south, respectively. Each cultivar was managed under standard fertilizer and pest management practices by cooperative growers at all locations. A total of 3 field trial experiments were performed at Soledad while 2 were performed at Salinas in parallel or separately. Growing conditions in Salinas: Plants were cultivated in a Chualar Loam soil composed of 14% Clay, 45% sand and 41% silt (Soilweb, 2017). Organic matter within the top 15 cm of the soil was determined to be 2.3% with an average pH of 7.2 and electrical conductivity (Ec) of 1.8 (dS/m). Nitrogen fertilization consisted of the following fertilizer program (6-00-20, 22-00-13 (liquid) and UN32 (urea and ammonium nitrate)) to achieve a final total applied N:NO3 of 225 kg/ha. Growing conditions in Soledad: Plants were cultivated in a Pico Fine Sandy Loam soil composed of 11% Clay, 72% sand and 17% silt (Soilweb, 2017). Organic matter within the top 15 cm of the soil was 1.2% with an average pH of 7.9 and Ec of 1.1 (dS/m). Nitrogen fertilization consisted of the following fertilizer program (ammonium nitrate and urea (UN32)) to achieve a final total applied N:NO3 of 180 kg/ha. At both growing locations plants were managed and harvested at dawn under standard commercial practices (Koike et al., 2011). Weather data at both growing locations was accessed through CIMIS (California Irrigation Management Information System) stations within 500 m from the position of each experimental setup and the monthly averages are provided in Fig. 1. Within each cultivated field plot dimension were 160 m long by 30 m wide. Each raised seed bed (n = 4), oriented west to east, was divided into 4 blocks (each 40 m long) across the length of the bed, each further divided into 2 additional subsections (each 10 m long). Inoculation and subsequent characterization of applied isolate persistence was conducted within this experimental design configuration (total 8 locations per bed). The remainder of the beds (total of 8) within the area selected were similarly managed to provide a sufficient cropping footprint, including buffer rows (Gutiérrez-Rodríguez et al., 2011). Inoculated leaves were combined from each block-bed zone for a total of 16 field replicates that were later combined into 8 replicates for laboratory analysis. Total average weight per replicate was 1.5 kg.

2.2. Inoculation studies

Plants at the 4–6 true leaf stage were spray-inoculated with an equal mixture of two *attEcO157* O157:H7 (PTVS 154 and PTVS 155 – *stx*1 and *stx*2 negative; collectively referred to in the remainder of this paper as *attEcO157*) in the afternoon just before dusk. This timing was selected based on previous studies under field conditions in Davis, CA where greater survival of the inoculated strains had been observed relative to applications prior to and spanning peak periods of solar UV flux (from CIMIS reference database; data not shown). Spray application

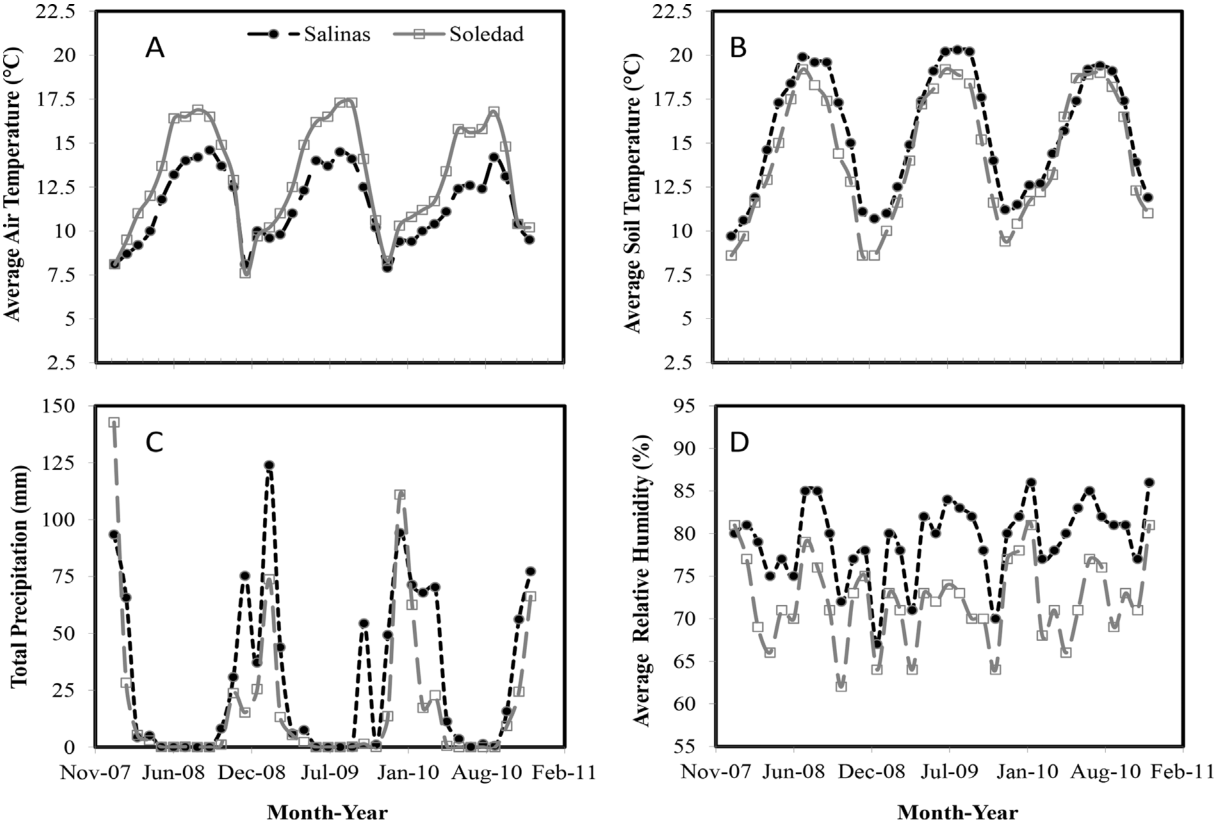

Fig. 1. Average air (A), soil temperature (B), precipitation (C) and relative humidity (D) in Salinas and Soledad California during November 2007 through February 2011 as reported by California Irrigation Management Information System (CIMIS) weather Station no. 114 and 116.

conditions and procedures, including inoculum confirmation and bacterial recovery, followed the methods described by Gutiérrez-Rodríguez et al. (2011). In brief, from single colonies following retrieval from −80 °C culture collection storage, inoculum growth was conducted in tryptic soy broth (TSB) amended with rifampicin (RIF) (80 mg L^{-1}) at 37 °C for 18 h and a confluent lawn was created by spread inoculation of the bacteria onto tryptic soy agar amended with 80 mg L^{-1} RIF (TSAR). Following 24–32 h growth at 37 °C the lawn was gently removed with a small, sterile rubber spatula. The cell mass was re-suspended and decanted from the TSAR plates with Butterfield's Phosphate Buffer (BPB) (Whatman Inc., Piscataway, NJ, USA) into a beaker containing 300 ml of BPB. The OD of the solution was adjusted to 0.725 at 600 nm (approximately Log 9 CFU ml^{-1}) using a spectrophotometer (Biomate, Fisher Scientific) and used to achieve a nominal inoculum concentration of log 2.0 and 4.0 CFU ml^{-1}. At time-zero, the prepared inoculum was then spray inoculated to each 10 m long section across the planted area. Each 10 m long section received 2 L of inoculum. Single time-point spray applications were made with a CO_2 pressurized sprayer fitted with a Tee-Jet 80 fan nozzle (TeeJet Technologies, Wheaton, IL) and pressurized headspace designed for large droplet size to maximize drift containment between plots and prevent drift beyond the designated trials.

2.3. Bacterial recovery

Plants were harvested by hand at dawn to minimize damage and excessive loss of turgor. Practical adherence to sterile technique (clean gloves after collecting each sample and in teams of two individuals) were used to prevent cross contamination between samples, regardless that all samples were inoculated with the same strain and inoculum dose. Plants were handled, stored and transported in pre-chilled coolers, with gel-ice, to the University of California Davis (Mann Lab) as described by Gutiérrez-Rodríguez et al. (2011). Sampling frequency was < 1, 7, 12–14 and 18–21 days post inoculation (DPI) to span anticipated reductions in viable populations, based on prior field studies in the region conducted by this lab group. A portion of the collected samples (individual leaves or 25 or 150 g samples) was treated with 1% $AgNO_3$ (silver nitrate) for stringent phylloplane disinfection as described by Matsumura et al. (2003), Franz et al., 2007 and Erickson et al., 2010. Survival of *attEcO157* was modeled by fitting the experimental data to the double Weibull survival model proposed by Coroller et al., (2006) and equally adopted by Ibekwe et al. (2011), Zhang et al., 2013 and many others to describe pathogen die-off irrespective of microbial growth patterns because of its ability to represent and describe most shapes of deactivation curves. We used the GInaFiT version 1.7 developed by Geeraerd et al., (2005). The die-off rates (DR) for *attEcO157* were calculated and expressed as Log CFU g/day.

After each harvest interval, plant material from each field replicate was analyzed as groups of individual leaves and as composites of 25 and 150 g from the same population per field location. A total of 30 individual leaves and 8 to 12 replicates of composite samples with and without 1% silver nitrate disinfection treatment were analyzed per time point-field replicate. From the original 1.5 kg sample, individual leaves or composite samples were placed inside small, medium or large stomacher bags (NASCO Whirl-Pak®) as required to accommodate the sampling mass and a 1:1 (W:W) diluent addition was made with BPB. Non-disinfected individual leaves were pulsified (Pulsifier; Microgen Bio Products, Camberley, Surrey, UK) for 10 s, while 25 g and 150 g composites were pulsified for 20 and 40 s respectively. For bacterial enumeration, duplicate plates each with 200 µl of supernatant were plated on TSA + PPMR [Tryptic soy agar (TSA; BD Diagnostics, Sparks, MD, USA)] amended with MUG (4-methylumbelliferyl-b-D-glucuronide; Fisher Scientific Inc., Waltham, MA) (0.1 mg ml^{-1}), pentachloronitrobenzene (PCNB; Amvac Chemical Corp., Newport Beach, CA) (5 mg ml^{-1}) and rifampicin (RIF; Fisher Scientific, 100 mg l^{-1}) and incubated at 37 °C for 24–48 h. PCNB is added as an essential media amendment to inhibit the overgrowth of soil-borne fungi on plates during 48 h incubation periods that will allow any delayed colony development on the selective agar media, as described by Lopez-Velasco et al. (2012).

2.4. Leaf disinfection

Individual leaves and composite samples were disinfected with 1% silver nitrate following the method described by Erickson et al. (2010) and with minor modifications. In brief, individual leaf and composite (25 and 150 g) samples were immersed in the 1% silver nitrate for 30, 40 and 60s respectively, followed by two rinses of 2X volumes in sterile water. Disinfected samples were then placed inside small, medium or large stomacher bags based on sample mass and mEHEC™ (Biocontrol, Bellevue, WA, USA) amended with RIF (Fisher Scientific, 50 mg l^{-1}) was added at a 2:1 ratio (W:V). Samples were pulsified as described previously and incubated at 42 °C for 18–24 h, as specified for test kit protocols. The presence or absence of *attEcO157* was confirmed first by plating the enrichment on CHROMagarO157 (Becton Dickinson BBL, Franklin Lakes, NJ) amended with RIF (50 mg l^{-1}) (CHROM-O157-Rif), for characteristic colony appearance, and confirmation with TaqMan probe-based PCR targeting *stx*1, *stx*2 and *rfb*E genes as described by Tomas-Callejas et al. (2011).

2.5. Natural contamination event

During the course of this project with the applied att*EcO157*, an opportunistic study of a natural contamination event of spinach with *EcO157* became available in the region close to the Soledad trials. For systematic representation of information, we will denote this location as SaliSole. An environmental assessment of the specific associated farm production location was conducted in response to an unusual high deposition regional rainstorm under warm conditions and periods of dense fog. No clear point-source or crop input risk factors were evident and the experimental effort was limited to the quarantined palletized lot of washed and packaged baby spinach provided by a collaborating processor. Detection of *Ec*O157 within single-lot packages and leaf-to-leaf variability within individual STEC positive packages was assessed to determine the variability within packages and recovery procedures. The presumptive contaminated lot was identified in the course of routine lot-acceptance testing by a third-party laboratory using a single 25 g sample and detected using the BAX O157 platform. Three days after the initial positive result, our collaborator allowed sampling of the quarantined product, held in cold storage at approximately 3.5 °C, prior to a supervised disposal in a local landfill. Our collaborator also commissioned a positive culture confirm from the single, original the positive sample enrichment they tested coincident with our involvement. A total of 26 bags (900 g each) were randomly selected from 13 different pallets. Intact, labeled packages were transported back to the Mann Lab inside pre-cooled ice chests containing partially thawed gel-ice with a double layer of common, brown craft paper to prevent freeze-injury to the tender spinach. The packaged spinach was held for 18 h at 2.5 °C before processing. Samples were not direct plated on any selective or differential media because of the pre-tested inherently low population densities of this pathogen in the implicated bags. Instead, from each 900 g bag, 150 g samples, tempered to room temperature (approx. 22 °C) were placed inside large stomacher bags and mEHEC pre-conditioned at 42 °C was added at a 2:1 ratio (w:w) and directly enriched overnight (15 h) at 42 °C. The presence or absence of *Ec*O157 was determined by molecular screening with Bio Control GDS O157 (BioControl Systems, Inc., Seattle, WA), Qualicon BAX O157 MP (DuPont Qualicon, Wilmington, DE) or probe-based PCR as described elsewhere by Campbell et al. (2001) and Tomas-Callejas et al. (2011). The purpose of this test was to determine if sampling size within a known natural contamination event could accurately predict the presence of *Ec*O157 and if results matched those from commercial labs where 25 g samples were analyzed.

A subset of six 150 g samples coming from bags #5, 7 and 24 (total 18 samples) (identified as positive for *Ec*O157 by the techniques described previously) were analyzed separately by first rinsing the leaves with BPB +0.05% Tween 20 at a 2:1 ratio (w:w). Each bag was pulsified as described above, which does not result in macerated leaves, and the supernatant was separated and placed inside a 500 ml graduated cylinder. The 300 ml volume extracted was then divided into six 50 ml fractions by sequentially removing this amount from top to bottom of the cylinder with efforts to minimize bulk mixing between the 50 ml fractions. Each 50 ml fraction from each replicate was then mixed with the corresponding fraction from the additional 150 g samples into a final volume of 300 ml and combined at a 1:1 (w:w) ratio with (2X) mEHEC. This test was done to compare the variability within an extraction buffer coming from a known naturally contaminated sample and to determine if differences in detection efficiencies could be attributed to fractions within single extraction volumes. In some of the testing methods listed above and used by the industry, different portions or quantities of the extraction supernatant are used for pathogen testing. Using a portion instead of the totality of the extraction supernatant could lead to false negative results that could jeopardize public safety by identifying a lot free from pathogen contamination. The washed and pulsified leaves were then disinfected with silver nitrate as described previously followed by enrichment with mEHEC at a 2:1 (w:w) ratio. Both leaf and BPB supernatant were enriched overnight (15 h) at 42 °C. The presence or absence of *Ec*O157 was determined using GDS O157 (BioControl Systems, Inc., Seattle, WA), as described elsewhere by D'Lima and Suslow (2009).

From *Ec*O157 positive commercial bag # 7, a subset of 190 individual leaves (Roughly 170 to 220 leaves correspond to a 150 g composite sample) was used to further assess the distribution of *Ec*O157 naturally contaminating spinach using the molecular techniques GDS O157 and BAX O157 MP. Each individual leaf was place inside a small stomacher bag, mEHEC was added at a 2:1 (w:w) ratio, each leaf was pulsifed for 20s and each bag was incubated overnight at 42 °C. A subset of positive samples was later cultured confirmed and further confirmed by PCR amplification of diagnostic and cardinal virulence-associated markers (*eae*, *stx* 1, *stx* 2, *rfb*E, and *hyl*A) to verify results (data not shown) but not retained by request of our commercial partner and in view of prior culture confirmation. No direct plating was performed before enrichment from each bag because of the inherently low population densities of this pathogen in the implicated leaves.

2.6. Statistical analysis

A split-plot design was used in our field studies. During postharvest analysis of the collected samples, the unique spatial identity of field replicates was maintained throughout all evaluations. For commercial spinach naturally contaminated with pathogenic *Ec*O157, all provided bags were used in our evaluations and one bag was selected for further analysis of individual sub-sample prevalence of contamination based on the positive results observed with molecular markers used to detect *Ec*O157. One way ANOVA / 'Tukey test' with $\alpha = 0.05$ was used to determine whether there were significant differences between treatments and die-off rates. The GLM procedure, homogeneity of variance and analysis of variance (ANOVA)/'Tukey test' with $\alpha = 0.05$ was used to determine whether there were significant differences in the survival of the inoculated strains and after the data was transformed (percent recovery) to determine if there were difference in the recovery of *attEcO157* between treatments. When necessary, the Chi-Square analysis was performed to test independence between categorical variables. The double Weibull survival model described by Coroller et al., (2006) was calculated from experimental data from Salinas and Soledad samples and using the GInaFiT version 1.7 developed by Geeraerd et al., (2005).

3. Results

3.1. Meteorological profile

There are 48 km in north to south distance between Salinas and Soledad in the Salinas Valley, CA. Average differential in Air Temperature between these locations during the summer months of 2008 through 2010 was of 5.1 °C and the average difference in Relative Humidity was 7.5% (Fig. 1). The North Salinas Valley region, during this seasonal period, is characterized in early morning and late afternoon by low fog-layer conditions from off-shore ocean influences, while in the more southern Soledad region strong afternoon winds and warmer weather delay or prevent the intrusion of fog during most summer days.

3.2. Inoculation studies

Practical levels of routine preharvest pathogen testing may not provide the necessary information to determine the degree of risk contamination within field assessments prior to harvest. Consequently, harvest decision and potential lot acceptance depend on sampling depth, design and detection capacity of any rapid molecular-based testing platform. This project sought to address the preharvest and postharvest quantitative and spatial variation in pathogen contamination within controlled inoculation events of att*EcO157* under commercial spinach growing conditions.

3.2.1. Salinas, CA

Our success in recovery of *attEcO157* from a known applied quantity of marked-bacteria varied with inoculum dose, sampling date and analytical sample mass (Tables 1 and 2). This effect was different from results experienced from Soledad, C.A., despite both locations receiving equal amounts and concentrations of the inoculum within a given planted area. Both of these locations within the same growing season have significant temperature and relative humidity differences as described in Fig. 1. Sampling mass played a significant role in our ability to detect the presence of *attEcO157* after disinfection with 1% silver nitrate (Table 2). For simplicity, in the absence of specific plant-tissue spatial recovery, this evidence of viable recovery will be referred to as protected-site survival (PSS) for individual leaves and composites samples since liquid sanitizers may not be able to reach all plant sites due to differences in polarity and water tension. *attEcO157* was recovered from 25 to 150 g composite samples from Salinas at similar population densities for the low inoculum dose after 14 DPI (Table 1). However, this was not the case for the high inoculum dose between the 25 and 150 g composite samples (Table 1). At 14 DPI, population densities of *attEcO157* detected on 150 g composite samples from the low inoculum dose were less in Salinas than in Soledad irrespective of growing season (Salinas: 0.65 ± 0 vs Soledad May–June 1.6 ± 0.5 and Soledad June–July 0.95 ± 0, respectively, all values as Log CFU

Table 2

Percent recovery of positive composite spinach samples for att*EcO157* after disinfection with 1% silver nitrate according to growing season (Salinas, CA).

Field Trial	Inoculum dose Log CFU ml^{-1}	Sample Size (g)	Percent (%) of positive samples[a]	
			7DPI	14DPI
T1 June–July	2.0	25	0d	0d
		150	11c	0d
	4.0	25	11d	0d
		150	33b	11c
T2 August–September	2.0	25	0d	0d
		150	33b	0d
	4.0	25	22c	0d
		150	44a	22c

[a] Values represent the percent of positive composites for att*EcO157*after enrichment with mEHEC at 42 °C for 18 h and detection by GDSO157 or by characteristic colony development on CHROM-O157-Rif. Total number of 9 repetitions per sample size and time point. Different letters indicate significant differences between treatments, lsmeans "Tukey test" α = 0.05. Data are normally distributed. Samples were not analyzed at 21 days post inoculation as most results in early studies were negative by enrichment.

Table 3

Percent recovery of positive composite spinach leaves and petioles for *attEcO157* up to 14 days of inoculation with two inoculum doses without disinfection (Salinas, CA).

Inoculum dose	Sample type	Sample size (g)	Percent (%) of positive samples[a]		
			1DPI	7DPI	14DPI
Log 2.0 CFU ml^{-1}	Leaves	25	56	22	0
		150	78	33	11
	Petioles	25	44	44	33
		150	67	56	44
Log 4.0 CFU ml^{-1}	Leaves	25	78	44	11
		150	100	78	33
	Petioles	25	56	56	33
		150	78	89	56

[a] Values summarize results from two independent field trials grown during June–July and represent the percent of positives groups (18 repetitions per group) after enrichment with mEHEC for 18 h by GDS or CHROM-O157-Rif. Leaves were inoculated at 4–6 true leaf stage. Data was analyzed using the Chi-square test of independence in SAS to examine the relation between DPI, Inoculum, sample type and sample size. The only significant relation found between variables was DPI and sample type with X^2 (2, N = 216) = 11.96, $p < 0.01$ with petioles overtime significantly improving the probability to recover and detect *attEcO157* from the inoculated leaves when compared to leaves collected without petioles. Samples were not analyzed at 21 days post inoculation as most results in early studies were negative by enrichment.

Table 1

Die-off of *attEcO157* from composite spinach samples up to 14 days post-inoculation with two inoculum doses without leaf disinfection (Salinas, CA).

Inoculum dose (Log CFU ml^{-1})	Sample Size (g)	Recovery of att*EcO157* (Log CFU g^{-1})[a]			DR[c] (Log/Day^{-1})
		1DPI	7DPI	14DPI	
2.0	25	1.01 ± 0.39 d	0.65 ± 0 e[b]	0.65 ± 0 e[b]	0.29
	150	1.20 ± 0.36 d	0.73 ± 0.60 e	0.65 ± 0 e[b]	0.14
4.0	25	2.77 ± 0.59 b	1.26 ± 0.07 d	0.67 ± 0.07 e	0.14
	150	3.21 ± 0.53 a	1.52 ± 0.56 c	1.26 ± 0.07 d	0.29

Samples were not analyzed at 21 days post inoculation as most results in early studies were negative by enrichment.

[a] Values summarize results from two independent field trials (June–July and August–September) and represent averages of 11 groups of 25 or 150 g samples per inoculum dose and time point ± standard deviation. Leaves were inoculated at 4–6 true leaf stage.

[b] Limit of detection. Different letters indicate significant differences between treatments, Tukey test α = 0.05.

[c] DR = Die-off rates.

Table 4

Percent recovery of positive individual spinach leaves for *attEcO157* with and without disinfection with 1% silver nitrate up to 21 days post-inoculation with two inoculum doses (Salinas, CA).

Field Trial	Inoculum dose (CFU ml^{-1})	Postharvest Treatment[a,b]	Percent Recovery of *attEcO157*[c]		
			7DPI	14DPI	21DPI
T1 June–July	2.0	NDis	17b	3.3c	0c
		Dis	3.3c	0c	0c
	4.0	NDis	37a	20b	0c
		Dis	17b	7a	0c
T2 August–September	2.0	NDis	17b	0a	0c
		Dis	0c	0a	0c
	4.0	NDis	33a	10a	0c
		Dis	10c	3.3a	0c

[a] NDis = Non-disinfected leaves.

[b] Dis = Disinfected leaves.

[c] At 1 DPI all leaves from both inoculation doses were positive for *attEcO157* without disinfection. Values represent number of positives (30 individual leaves per treatment) by direct plating for non-disinfected leaves and for disinfected leaves after enrichment and detection by GDSO157 or by characteristic colony development on CHROM-O157-Rif. All leaves had petioles included when analyzed. Different letters indicate significant differences between treatments, lsmeans "Tukey test" $\alpha = 0.05$. Data were transformed to meet normality. Leaves for non-disinfected leaves that were negative by direct plating were enriched and tested with GDS to eliminate any potential bias associated with being below the limit of detection.

Table 5

Die-off of *attEcO157* from 150 g spinach samples after 14 days post-inoculation with two inoculum doses without leaf disinfection (Soledad, CA, 2010).

Field Trial	Inoculum dose (Log CFU m^{-2})	Recovery of *attEcO157* (Log CFU g^{-1})[a]			DR[c] (Log/Day^{-1})
		< 1DPI	7DPI	14DPI	
T1	2.0	1.64 ± 0.40 b	1.60 ± 0.85 b	1.51 ± 0.50 b	0.14
May–June	4.0	3.30 ± 0.17 a	1.70 ± 0.42 b	1.39 ± 0.34 b	0.28
T2	2.0	1.55 ± 0.34 b	1.30 ± 0.59 b	0.95 ± 0 c[b]	0.14
June–July	4.0	3.12 ± 0.23 a	1.42 ± 0.45 b	1.01 ± 0.14 c	0.28

Samples were not analyzed at 21 days post inoculation as most results in early studies were negative by enrichment.

[a] Values represent averages of 8 repetitions per trial ± standard deviation. Leaves were inoculated at 4–6 true leaf stage. Similar results were overserved for 2009 evaluations, data not shown.

[b] Limit of detection. Different letters indicate significant differences between treatments, Tukey test $\alpha = 0.05$.

[c] DR = Die-off rates.

g^{-1}) (Tables 1 and 5). This was not the case for the high inoculum dose where populations were not different between regions and field trials throughout the 14 DPI interval (Tables 1 and 5).

When comparing recovery of *attEcO157* from composite leaf samples collected in Salinas, CA, and disinfected with 1% silver nitrate, 150 g samples, as compared to 25 g masses, resulted in a greater number of positive samples after disinfection (Table 2). This observation was also determined for composite 150 g samples collected in Soledad (Table 7). Leaf PSS cannot be determined for the 150 g composite samples because of the practical difficulty of achieving adequate

Table 6

Percent recovery of positive composite spinach samples for *attEcO157* up to 21 days post-inoculation with two inoculum doses (Soledad, CA (2010)).

Inoculum dose Log (CFU m^{-2})	Sample Size (g)	Percent (%) of positive samples[a]			
		< 1DPI	7DPI	14DPI	21DPI
2.0	25	67b	0d	0d	0d
	150	92a	42b	25c	8d
4.0	25	92a	17c	4d	0d
	150	100a	79b	42b	21c

[a] Values summarize results from two independent field trials (May–June and June–July 2010) and represent the percent of positive groups (24 repetitions per group) after 18 h enrichment with mEHEC and detection by GDSO157 or by characteristic colony development on CHROM-O157-Rif. Leaves were inoculated at 4–6 true leaf stage. Similar results were overserved for 2009 evaluations, data not shown. Different letters indicate significant differences between treatments, lsmeans "Tukey test" $\alpha = 0.05$. Data were transformed to meet normality.

Table 7

Percent recovery of positive composite spinach samples for att*EcO157* after disinfection with 1% silver nitrate according to growing season (Soledad, CA (2010)).

Field Trial	Inoculum dose (CFU m^{-2})	Sample Size (g)	Percent Recovery (%) of *attEcO157*[a]		
			7DPI	14DPI	21DPI
T1 May–June	2.0	25	0d	0d	0d
		150	33b	17c	0d
	4.0	25	17c	0d	0d
		150	33b	33b	33b
T2 June–July	2.0	25	0d	0d	0d
		150	12.5c	12.5c	0d
	4.0	25	12.5c	0d	0d
		150	37.5b	25b	12.5c
T3 August–September	2.0	25	0d	0d	0d
		150	37.5b	25b	0d
	4.0	25	25b	0d	0d
		150	63a	37.5b	25b

b This time point was only performed for individual leaves and used for comparison purposes with Salinas data. Different letters indicate significant differences between treatments, lsmeans "Tukey test" $\alpha = 0.05$. Data were normally distributed.

[a] At 1 DPI all samples were positive for each inoculum dose. Values represent percent of positive composites (6–8 replications per treatment) after enrichment with mEHEC at 42 °C for 18 h and detection by GDSO157 or by characteristic colony development on CHROM-O157-Rif. Similar results were overserved for 2009 evaluations, data not shown.

immersion disinfection of this group of leaves while assuring prevention of penetration and translocation of silver nitrate into the leaf petiole opening.

At both locations cooler weather (August–September) was experienced due to an atypical weather pattern. However, the Soledad region was on average 2.5 °C warmer than Salinas (Fig. 1). Irrespective of location, viable *attEcO157* persisted in the field up to 21 DPI (Tables 2, 6 and 7). The average number of positive 150 g samples after disinfection (Tables 2 and 7) for Salinas and Soledad was 19.3% and 30.5%, respectively, while for 25 g samples, 4.1 and 4.5% for these respective locations was observed. Lesser samples masses for either location and at identical time points (Tables 2 and 7) were not different between locations.

The prevalence and persistence of *attEcO157* was also evaluated on composite leaves and petioles to determine if these parameters, separate or combined, had a significant influence on recovery of inoculated strains from composite 25 g and 150 g samples. Each spinach leaf had an average weight of 0.67 g (data not shown, range 0.35–1.27 g) which indicated that each 25 or 150 g composite sample had between 17 and 101 leaves, respectively. This is important because one acre of spinach could potentially have up to 15 million leaves (plating density of 3 million seed per acre and harvested at 5 true leaves) and this sampling size could represent 0.000011 and 0.000067% respectively of the totality in one acre. As expected, our success in recovery of *attEcO157* decreased with initial inoculum dose and time (Table 3); however, this effect was less pronounced in excised petioles than in leaves, irrespective of sampling mass and without leaf disinfection (Table 3). Following leaf surface disinfection with 1% silver nitrate, a greater initial inoculum dose resulted in a significantly greater frequency of PSS *attEcO157* detection from leaf surfaces, up to 14 DPI. No *attEcO157* was recovered from individual leaves with or without disinfection after 21 DPI (Table 4). This result was also observed on individual leaf samples from Soledad (Table 9). The detection prevalence was greatest at 14 DPI when the petioles were included in the plant tissue rather than sampling by excision of leaves at the transition point from the petiole in the field. This outcome was independent of sampling mass (Tables 4 and 9).

Table 8

Percent recovery of positive individual spinach leaves for *attEcO157* with and without disinfection with 1% silver nitrate after 18-days post-inoculation with two inoculum doses (Soledad, CA (2010)).

Field Trial	Inoculum dose (CFU ml^{-1})	Postharvest Treatment[a,b]	Recovery of *attEcO157* (% Positive)	
			7DPI	14-18DPI
T1 May–June	2.0	NDis	46 b	17d
		Dis	4	0
	4.0	NDis	67a	33c
		Dis	13d	0
T2 June–July	2.0	NDis	53 b	13d
		Dis	7	0
	4.0	NDis	77a	21d
		Dis	17d	7

[c]At 1 DPI all 24 or 30 leaves from both inoculation doses without disinfection were positive for *attEcO157*. Values represent number of positives by direct plating for non-disinfected leaves and for disinfected leaves after enrichment by GDSO157 or by characteristic colony development on CHROM-O157-Rif. Similar results were overserved for 2009 evaluations, data not shown. All leaves had petioles when analyzed. Different letters indicate significant differences between treatments, lsmeans "Tukey test" α = 0.05. Data were transformed to meet normality. Samples were not analyzed at 21 days post inoculation as most results in early studies were negative by enrichment. Leaves for non-disinfected leaves that were negative by direct plating were enriched and tested with GDS to eliminate any potential bias associated with being below the limit of detection.

[a] NDis = Non-disinfected leaves.

[b] Dis = Disinfected leaves.

Table 9

Double Weibull survival model for att*EcO157* inoculated at Log 4 CFU ml^{-1} from composite 25 and 150 g spinach samples collected from Salinas and Soledad, CA.

Location	Sample Mass (g)	Survival Parameters				
		α	Delta 1	p	Delta 2	RMSE
Salinas	25	2.7 (0.37)	0.67 (0.23)	1.72 (0.98)	18.19 (5.60)	0.270
	150	2.60 (0.38)	0.70 (0.32)	1.33 (0.93)	22.36 (9.47)	0.306
Soledad	25	2.66 (0.71)	0.78 (0.40)	3.02 (4.63)	20.66 (10.82)	0.327
	150	2.21 (4.17)	1.10 (9.78)	6.00 (648.41)	30.90 (2612)	0.342

Models were constructed by combining data from 3 experiments performed at Soledad and 2 experiments performed at Salinas. Each model was constructed using data from 19 repetitions per location. Inactivation model identified was N=N0/(1 + 10^alfa)*(10^(-((t-1)/delta1)^p + alfa) + 10^(-((-1)/delta2)**p)), and α = $\mathrm{Log}_{(10)}$ (f/(1-f)). N is the number of survivors, N0 is the inoculum size; t is the time; p is the shape parameter, when $p > 1$ a convex curve is observed; when $p < 1$ a concave curve is observed, when $p = 1$ a linear curve is observed. The scale parameter, delta, represents the time needed for first decimal reduction. The f parameter varies from 0 to 1 and represents the fraction of subpopulation 1 in the population while α varies from negative infinity to positive infinity and is obtained by logit transformation of. Values in parenthesis represent the approximate standard errors for each corresponding parameter. RSME represents the root mean square error.

3.2.2. Soledad, CA

The ability to recover *attEcO157* from the phyllosphere of inoculated spinach leaves was influenced by initial inoculum dose and subsequent sampling mass (Tables 5 and 6). Detectable, viable populations of *attEcO157* after 1 DPI were different between inoculum doses but this difference was not observed at 7 and 14 days DPI, irrespective of growing season (Table 5). Greater number of positive samples for *attEcO157* were recovered for composite leaf samples receiving the high inoculum dose, irrespective of growing season (Table 7).

Recovered populations of *attEcO157* without surface disinfection from composite spinach samples of 150 g followed a similar trend as populations from individual leaves. However, less variability was observed in the recovered populations and a greater number of positive samples was determined for both composite samples when compared to individual leaves (Tables 7 and 8). Residual populations of *attEcO157* for trial 1 (May–June) were greater than those from trial 2 (June–July) after 14 days DPI (Table 5). Nonetheless, the number of positive composite samples analyzed without a disinfection step, and after enrichment with mEHEC, was greater for trial 2, irrespective of sampling mass (Table 6). A clear difference in the capacity to recover *attEcO157* from 25 to 150 g composite samples and individual leaf samples was observed between inoculum doses but not between regions (Salinas vs Soledad) (Tables 1 and 5 at 14 DPI (population densities), Tables 2 and 6 at 7 and 14DPI (percent recovery, Tables 4 and 8 at 7 and 14DPI (percent recovery NDIs and Dis)).

Individual leaf populations of *attEcO157* were Log 1.40 ± 0.59 and 2.88 ± 0.44 ($p \leq 0.05$) cfu/g after 1 day of inoculation for each inoculum those and Log 1.30 ± 0.92 and 1.68 ± 0.85 cfu g^{-1} after 7 days of inoculation (average data for experiments from May–June and June–July). Direct plate recovery after 14 days of inoculation was only observed in 24 individual leaves out of 216 analyzed with 70% represented by the high inoculum dose. (Data not shown). The low frequency of detection in both situations limits the significance of ascribing seasonal effects to this observation and as a consequence, in order to clarify and substantiate future guidance expanded population size studies seem warranted.

Leaf PSS viability was attempted for the 150 g composite samples but determined to have sufficient practical difficulty of achieving adequate immersion disinfection of this large group of leaves without the application of greater mechanical force to achieve full immersion of the blades. With a 25 g sample, gentle immersion of the leaf blades by holding the cut ends of the petioles, as a group, above the silver nitrate solution was simple and reproducible. As we were concerned about uniformity of the treatment across groups of 150 g samples and the much greater potential for penetration and translocation of silver nitrate into the leaf petiole opening, this assessment was not conducted". During all three trials less than 3% of samples were positive for *attEcO157* PSS among the 25 g composite samples (Table 7). For composite samples of 150 g, the trend was different in that a greater number of PSS samples were determined irrespective of inoculum dose (Table 7). For individual leaves, inoculum dose determined our ability to recover *attEcO1*57 by enrichment and after disinfection, which may include both surface aggregates in protected epidermal locations and cells internalized into leaves (Table 8). At 7 DPI both low and high inoculum doses were observed to have positive individual leaf samples after disinfection and at 14 day, only the June–July trials had 7% of samples positive after disinfection and enrichment for *attEcO157*.

Die-off kinetics of composite 25 and 150 g samples were determine for both Salinas and Soledad and the double Weibull survival model best described the characterized recovery of *attEcO157*. Data combined results from all experiments for each region and suggests that the shape parameter (p) for each region follows a convex approach consistent with biphasic bacterial survival (Table 9). At both locations the survival of *attEcO157* the RMSE was small and at or below 0.35. The model for composite 150 g samples from Soledad presented the greatest variability in the shape parameter and RMSE. However, at both locations the models generated from the experimental data were similar. Delta values between composite 25 and 150 g samples were similar between locations; however, delta 1 and delta 2 were significantly different indicating that the two sub-populations behave differently, but similarly between locations. In general the sub-population with greater delta values will decline at a reduced rate than the corresponding population with a smaller delta value. The linear die-off rates for composite 25 and 150 g samples were similar at both locations (Tables 1 and 5) and reflect the time it took to reach the limit of detection by enrichment.

3.3. Natural contamination event (SaliSole)

The distribution of pathogenic *Ec*O157 associated with naturally contaminated, commercially washed baby spinach was evaluated on 26 bags, each of 0.9 kg, following the same bacteria recovery procedures used in our controlled inoculation field studies in the Salinas Valley. The commercial real-time PCR kit GDS O157 gave a positive reaction for *Ec*O157 on 11 of 26 samples analyzed (Table 1). For the Taqman-based PCR probe system, *stx*1 was present in only 3 of 26 samples analyzed whereas *stx*2 was detected in 17 of 26 samples. The *eae*A gene target was present in 10 of the 26 samples analyzed. The presence of *stx*2 was only associated with the presence of *stx*1 in three samples (5, 12 and 24), while the pairing of *stx*2 with *eae*A co-occurred in 10 of 26 samples (Table 10). Only 6 of 26 samples were identical between both techniques (GDS and TaqMan) and identified sample as positive for the *stx*2 and *eae*A genes. As with GDS O157 and TaqMan qPCR, duplicate samples were not always positive for the same genes analyzed with each molecular technique.

The efficacy of silver nitrate to eliminate the potential presence of naturally contaminating *Ec*O157 was tested on a group of 150 g samples coming from molecular detection positive bag samples 5, 7 and 24. These samples tested positive for 8 of 10 combined molecular markers analyzed with GDS O157 and TaqMan qPCR.

From the pre-disinfection wash water (conducted after chlorinated commercial wash system treatment), 2 out of 17 samples tested positive for *Ec*O157 with GDS O157. In both cases, these samples corresponded to the penultimate and ultimate portion of the supernatant separated from the washed spinach. Observations during aliquot removal made it apparent that these sub-samples contained visible particulates and sediment that had settled after addition of the combined wash water factions to the graduated cylinder. After $AgNO_3$ disinfection, none of the composite leaf mass samples associated with the wash were positive for *Ec*O157 (Table 11). A total of 180 individual leaves were also taken from product sample bag number 7 for further testing of the presence of *Ec*O157 using BAX O157 and GDS O157. BAX O157 was included in this final analysis since this was the detection platform used by our industry partner to detect the presence of *Ec*O157. From the 24 eight-leaf composite samples analyzed, ten positives were observed but only in one occasion did both techniques identify the same sample as positive for *Ec*O157 (Table 12). BAX O157 determined 7 positive composite samples of 24 analyzed, which corresponds to 31% of total 180 leaves analyzed while GDS O157 detected 4 of 24 samples corresponding to 16% of the total groups (Table 12). If we average these two results (31 and 16%) we have a 23.5% chance of finding a positive 25 g sample from every 7 analyzed.

Table 10
Probe-based molecular detection of naturally occurring *Ec*O157 from enrichments of spinach samples each of 150 g collected from 0.9 kg retail bags.

Sample Number	GDS-O157	Taqman		
		*stx*1	*stx*2	*eae*A
1	–	–	–	–
2	+	–	+	+
3	–	–	–	–
4	–	–	–	–
5[a]	+	+	+	–
6	–	–	+	–
7[a]	+	–	–	+
8	+	–	–	–
9	+	–	–	–
10	–	–	+	+
11	+	–	+	+
12	–	+	+	–
13	–	–	–	–
14	+	–	+	–
15	–	–	+	+
16	–	–	+	+
17	–	–	+	+
18	–	–	+	–
19	–	–	–	–
20	+	–	+	+
21	+	–	+	+
22	+	–	+	+
23	–	–	+	+
24*	+	+	+	–
25	–	–	–	–
26	–	–	+	–

[a] Indicates selected samples used for further analysis to determine the impact of sample size on disinfection efficacy and capacity to detect naturally contaminated plant material with *EcO157*.

Table 11
Percent of presumptive positive spinach samples of 150 g naturally contaminated with *Ec*O157 by GDS-O157 after washing and disinfection with 1% silver nitrate.

Sample Number	Wash Water[a]	Disinfected leaf mass[a]
5	0	0
7	17	0
24	17	0

[a] Values represent number of positive groups for *Ec*O157 after 18hr enrichment at 42 °C with mEHEC and detection by GDSO157 or by characteristic colony development on CHROM-O157-Rif. Total number of repetitions per group was 5 or 6.

Table 12
Probe-based molecular detection of naturally-occurring *Ec*O157 from enrichments of individual spinach leaves collected from retail bag 7 as determined by two commercial test kits.

Sample Number	Composite[a]	BAX O157[b]	GDS-O157[b]
1	1–8	–	–
2	9–16	+	–
3	17–24	–	–
4	25–32	–	–
5	33–40	+	–
6	41–48	–	–
7	49–56	–	–
8	57–64	+	–
9	65–72	–	–
10	73–80	–	+
11	81–88	+	–
12	89–96	–	–
13	97–104	–	+
14	105–112	–	+
15	113–120	+	–
16	121–128	–	–
17	129–136	+	–
18	137–144	–	–
19	145–152	–	–
20	153–160	+	+
21	161–168	–	–
22	169–176	–	–
23	177–184	–	–
24	185–190	–	–

[a] Total number of leaves analyzed was 190.

[b] Each sample represents a composite of eight leaves enriched individually and one aliquot of each enrichment composited for GDS-O157 and BAX O157 analysis.

4. Discussion

Test-and-hold approaches and field risk assessments adopted by major producers and suppliers of packaged salads rely on the ability to rapidly determine the presence or absence of a selected panel of human enteric pathogens before product is harvested, at raw material receiving, or prior to finished product release to distribution channels ultimately reaching consumers. At the core of these testing procedures, the scaling within a sample unit, lot or field, the physicochemical characteristics of the crop, the source of contamination and the molecular techniques used for pathogen detection influence the final outcome of the analysis (Gutiérrez-Rodríguez et al., 2011; D'Lima and Suslow, 2009; Ahmed et al., 2009; Brandl and Amundson, 2008; Jacques et al., 1995; Kinkel et al., 1995; UFPA, 2010). In this study we evaluated the temporal traits of bacterial pathogen heterogeneity development by comparing the standardized and validated sample size of 25 g for detection of enteric pathogens from leafy vegetable tissue (FDA BAM, 1998; APHA, 2001; AOAC, 2007) with sampling units of one to 180 leaves and composites of 150 g over different spatial and temporal field production conditions.

Recovered populations from inoculated spinach leaves with *attEcO157* were similar for composite 25 and 150 g samples across inoculum doses and field locations. However greater variability in the number of positive samples and culturally determined population density was observed on individual leaves. Kinkel (1997) indicated that variability of microbial populations in the phyllosphere is correlated with leaf position, age and plant species that have upright habitats. Individual leaves of spinach were analyzed based on leaf stage (data not shown), however no clear pattern was observed between leaves according to basipetal position hierarchy 1–2 (older) and 3–4 (younger) collected from both Salinas and Soledad. Within the spinach canopy, leaves 3–4 are close to the center of the plant at an upright position, while leaves 1–2 are normally at a more horizontal plane and partially covering the younger tissue because of the commercial plant density. Similar observations were determined under greenhouse conditions for artificially inoculated spinach leaves with the same mixture of at-t*EcO157* by Gutiérrez-Rodríguez et al. (2011). Pu et al. (2009) determined that spinach leaf surface contamination occurred occasionally and peak events clustered between 3 and 5 weeks of age, but not among leaves younger than 3 weeks of ages. Under our experimental conditions, leaves were inoculated at 4 weeks when plants had reached 4–5 true leaves. Following spray-inoculation, bacterial survival and the potential to reach protective tissue sites, which avoid a strong surface disinfectant treatment, was monitored over 14 or 21 days. The differential prevalence to reach or develop in protective sites, assessed as presence or absence following selective enrichment, varied by production location and season. The specific mechanism or spatial distribution of post-treatment survival, following a topical silver nitrate wash, was not determined. However, such survival has been attributed to internalization, microsites of leaf surface hydrophobicity, and association with established microbial aggregates, and other features of the phylloplane physical environment (Poza-Carrion et al., 2012). During this period of time, bacterial population clustering was observed between sampling masses but no differences in att*EcO157* populations were associated with leaf maturity from the single inundative contamination event. Burch et al., 2016, associated survival and persistence of *EcO157* on Romaine and baby spinach with protected growth conditions and the microbial community in the leaves, which supports our findings.

Growing conditions in Soledad and Salinas, CA are quite different with regard to air temperature and relative humidity (Fig. 1). These differences in local hourly and daily climatic conditions could not be correlated with differences in bacterial populations from individual leaves. Net-surviving populations of att*EcO157* among composite 25 and 150 g samples recovered from plants receiving an initial 100-fold variance in inoculum dose were quantitatively and qualitatively different between the geospatial locations. Kinkel et al. (1995) studied the influence of sample scale on the estimation of the mean epiphytic bacteria population size from potato leaf disks, entire leaflets and plants. It was determined that populations were highly variable among sampling units suggesting that there was no optimal scale at which sample variance was minimized. Under our experimental conditions variability within individual leaves was greater than determined with composite samples; nonetheless all three had similar population densities except for composite samples from Salinas. When individual leaves and composite samples were surface disinfected with silver nitrate the variability was more pronounced, but composite samples consistently had a greater number of positive leaves for att*EcO157* than individual leaves with 150 g samples presenting the highest number of positive samples. This outcome tends to support the widely held industry belief that accuracy and improved statistical validity of lot acceptance testing on leafy greens is enhanced if greater sample mass per unit area was included in pathogen detection plans. However, Lopez-Velasco et al. (2015) reported that the improvement in probability of detection is often offset by competitive interference from leaf associated microbiota if the tissue mass to buffer ratio is reduced to accommodate larger single samples. The conclusion supported a post-enrichment composite of 25–125 g sub-samples per lot rather than a single 375 g sample at the industry ratio of 1:4 or 1:3 tissue mass to broth in a single enrichment. This same concern is reflected in the revised FDA BAM (2017) protocols for detection and recovery of diarrheagenic *Escherichia coli.* Our findings supports the use of 150 g for sampling purposes to increase pathogen detection.

Gutiérrez-Rodríguez et al. (2011) found great variability in population densities of artificially inoculated att*EcO157* from spinach developmental leaf position 6 (older) and 12 (younger) but not from unexpanded leaves and the basal shoot tissues. In our experimental conditions, simulated contaminant deposition, using att*EcO157,* occurred when young and older spinach leaves were present in the high-density plant cropping system and greater detection of the pathogen was observed when petioles were included in the analysis. These outcomes suggest that interactions with the physicochemical condition or component maturity of the plant (Leveau, 2006; Lindow and Brandl, 2003; Monier and Lindow, 2005; Redford and Fierer, 2009) and accessibility to locations protective from solar UV exposure (Wood et al., 2010) and greater persistent free-moisture and nutrient content (Tecon and Leveau, 2012; Rastogi et al., 2012; Meyer and Leveau, 2012) can influence or determine att*EcO157* bacterial population survival. Likewise, differences in mean population densities and survival of att*EcO157* observed between Salinas and Soledad could be associated with different interactions with native microbiota present at each location as suggested by Lindow and Brandl (2003) and Rastogi et al. (2012). In contrast to our results in spinach, Brandl and Admunson (2008) associated greater population sizes of EcO157 on young romaine lettuce leaves, grown under warm temperature greenhouse conditions, than on middle leaves in the presence of persistent leaf wetness. In addition to protected growth conditions, the microbial community and growth behavior of EcO157 on Romaine and baby spinach develop differently (Burch et al., 2016 and Sharma et al., 2008).

The incidence of PSS among individual spinach leaves with att*EcO157* was also different between growing locations; where greater numbers of leaves with detectable PSS populations were determined in Salinas field plots. Although a limited number of leaves with *attEcO157* PSS was evaluated, these differences could be associated with either practical limitations of sample size or the availability of growth-limiting nutrients on the surface of the leaves. Salinas plants received foliar applied fertilizer (standard grower-applications) while in Soledad all N fertilization amendments were incorporated into the soil. Additionally, in Salinas, spinach leaves frequently experienced extended periods of leaf wetness due to morning and late afternoon fog, potentially providing favorable conditions for the net survival of att*EcO157*. Extended leaf wetness may have allowed motility or dispersal and access to protective sites on or within sub-epidermal spaces of the leaves (Gomes et al., 2009; Hora et al., 2005; Mitra et al., 2009; Torres et al., 2005; Wood et al., 2010; Erickson et al., 2010; Xicohtencatl-Cortes et al., 2009; Wood et al., 2010, 2010).

The desire to establish and improve the statistically validity and predictive capacity of any sampling strategy within a produce safety management scheme has been of concern for industry leaders and public health officials and regulators. Since the conduct of this project, it has been recognized that the conventional standard tissue sampling mass of 25 g may not be adequate for the detection of human pathogens in natural contamination situations. In response, many leafy greens processors require a larger mass or a multiple 25 g post-enrichment composite in their lot acceptance testing schemes (D. McDonald, Taylor Farms; personal contact information).

Any generalized conclusions regarding persistence of att*EcO157* under the field conditions in Soledad and Salinas test sites necessitate consideration of the variation observed between season, growing location and sampling mass. However, greater consistency in pathogen detection, irrespective of field location, was determined for the 150 g composite samples when compared to individual leaves or composites of 25 g. Differences in our ability to detect att*EcO157* from spinach leaves with and without disinfection during different growing seasons could be attributed to differences in temperature at each location, sampling mass and sample type (leaves vs leaves and petioles) and to differences on leaf physicochemical characteristics as suggested by Mitra et al. (2009), Xicohtencatl-Cortes et al. (2009), Gutiérrez-Rodríguez et al. (2012) and Khalil and Frank (2010). Under our experimental conditions differences in the ability to recover att*EcO157* from non-disinfected and disinfected spinach leaves was different than those results reported by Pu et al. (2009), Mitra et al. (2009), Patel et al. (2009) and Erickson et al. (2010). These differences could be mainly attributed to greater sampling mass used in our experimental conditions, to differences in strain fitness between experimental systems, recovery procedures for inoculated bacteria and the different growing conditions experienced in each experiment.

Detection of human pathogens from a natural contamination event on produce depends on several factors including background microbiota, sample size, the microbiological technique used in the analysis, chemical inhibitors and type of matrix analyzed (Feldsine et al., 2005; Hall et al., 2001; Burnett and Beuchat, 2001; Sivapalasingam et al., 2004; Lopez-Velasco et al., 2015).

Inactivation profiles and intervention strategies that are able to reduce pathogen contamination from *Salmonella* spp*., L. monocytogenes* and *EcO157* are needed. Of special interest is the need to better understand the change in pathogenic bacterial populations in open field environments along the cropping cycle and what sort of inactivation and predictive microbiological tool could estimate human bacterial pathogen response to different environmental conditions and stressors. This is of special importance with the implementation of the FSMA Produce Safety Rule that provides growers a die-off rate of 0.5 Log CFU per day, based on indicator *E. coli*, over a period of 4 consecutive days between the last irrigation event and harvest. Such provision assumes a potential linear die-off of any pathogen that if followed could compromise the safety within the produce supply chain since several other studies, besides this one, have shown that die-off can follow an exponential or Weibull function. Double Weibull models are based on the assumption that there are two subpopulations, and that each population, differs in the level of resistance to environmental stress, or physiological state of the cells and to the biphasic deactivation nature of pathogens on bacterial populations over time (Coroller et al., 2006), leading to different survival rates. Our results were best described using this model for composite 25 and 150 g samples and suggest that the applied att*EcO157* behaved in a biphasic nature irrespective of location within the Salinas Valley. Further, it identifies two populations in which the lesser population of att*EcO157* is projected to last significantly longer when compared to the initial inoculum doses (significant differences in delta values (Delta 2 ≫ > Delta 1)). The initial sharp decrease in att*EcO157* at both locations might be largely attributed to the faster die-off of bacterial subpopulations with smaller deltas (delta 1). The bacterial subpopulation with greater (delta 2) lead to slower and steadier decline of cells and this could explain why DR were in the range of 0.14 and 0.29 Log CFU per day even at a linear scale. Similar observations for cabbage and different soil types were reported by Ongeng et al., (2011) and Ma et al., 2011.

Opportunistically, we also report in this study the influence of sample size on the probability of pathogen detection from a natural contamination event; using molecular detection and confirmation techniques commonly used by third party laboratories for lot acceptance programs for leafy greens consumed fresh. As expected, great variability was observed between molecular techniques targeting the shiga toxin genes *stx*1 and *stx*2 and the virulence factor *eae*A (Feng and Lampel, 1994; Barkocy-Gallagher et al., 2004; Bonardi et al., 2004; Feng et al., 2017). This variability was observed between sampling masses and between subsamples coming from a single lot of pre-washed, commercially packaged spinach. Observed differences could be attributed to the detection limit associated with each technique, where for example BAX O157 has a reported limit of less than 5 CFU/65 g while GDS O157 and TaqMan qPCR have reported limits of less than 2 CFU/65 g (D'Lima and Suslow, 2009). Since the completion of this research study, both commercial platforms have had substantial changes to improve the performance and specificity of the certified test methods.

Furthermore, differences of up to 200 fold in the template-sample reaction aliquot used on each technique, according to technical kit instructions, increase the chance of a false negative outcome between and within evaluated samples. Similarly, each technique may interact differently to chemicals coming from the plant material analyzed and from the terminal enrichment culture, as suggested by D'Lima and Suslow (2009). Despite the potential for increasing the sensitivity of detection of *Ec*O157, within a lot, by increasing the sampling mass from 25 g to 150 g we did not detect a homogeneous contamination among the subsamples, despite passing through a common single-line wash system, emanating from the same palletized lot or individual bag from this lot.

Our ability to detect *Ec*O157 from different sampling masses, the wash buffer and disinfected composite samples was further explored by selecting specific bags (5, 7 and 24) previously identified by our initial pathogen assessment as contaminated with *Ec*O157. From this analysis the enrichment of the wash buffer increased our probability to detect *Ec*O157 when compared to plant enrichments. The analysis of composite wash water from multiple 25 g subsamples may increase detection of *Ec*O157 in routine and investigative testing.

When individual leaves were analyzed, greater variability in our ability to detect *Ec*O157 was determined mainly due to the overall small mass (total leaf population) analyzed (~160 g). Differences in our ability to detect *Ec*O157 from the same samples were observed between molecular techniques. Only in one occasion did both the GDS O157 and BAX O157 pathogen detection kits used in this study coincided with the detection of a positive sample, despite the former using 1 ml of the enrichment when compared to 5 µl used by BAX O157. D'Lima and Suslow (2009) found GDS O157 to have a lower limit of detection for *Ec*O157 than BAX O157 but our experience is often that specific sample enrichments are negatively impacted by failure of the immuno-magnetic separation used in the GDS-specified protocol, during the research period, and the desired target is lost during the paramagnetic bead-washing procedures. Overall, more than 4 kg of product were analyzed under our experimental conditions and this corresponds to more than one hundred and sixty 25 g samples. It is clear that single 25 g samples, often the extent of Test and Hold sampling for a given lot, limit our ability to detect *Ec*O157, or other pathogens, and that the sampling scheme and the molecular platform used for pathogen detection strongly influence the final outcome. Our analysis established that the bulk field-harvested and commercially washed spinach was non-uniformly contaminated with a viable *Ec*O157 in the final packaged product.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.08.013.

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