Food Control 54 (2015) 165–175

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Factors affecting cell population density during enrichment and subsequent molecular detection of *Salmonella enterica* and *Escherichia coli* O157:H7 on lettuce contaminated during field production

Gabriela Lopez-Velasco*, Alejandro Tomas-Callejas, Adrian O. Sbodio, Xuan Pham, Polly Wei, Dawit Diribsa, Trevor V. Suslow

Department of Plant Sciences, University of California, One Shields Avenue, 124 Mann Laboratory, Mail Stop 3, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 31 December 2014
Received in revised form
27 January 2015
Accepted 29 January 2015
Available online 7 February 2015

Keywords:
Enrichment
Sample mass
Environmental exposure
Colony confirmation
Molecular detection
False negative
Salmonella
E. coli O157:H7

ABSTRACT

The aim of this study was to evaluate the field survival and subsequent detection of *Escherichia coli* O157:H7 and *Salmonella* on leafy greens to understand factors that influence their detection by molecular methods. Both applied microorganisms experienced a drop in population after 4 days of inoculation. and after 10 days, recovery was achieved only through enrichment. Field survival of microorganisms was affected by lettuce cultivar and better survival of *Salmonella* in comparison to *E. coli* O157:H7 was determined. Detection through real-time PCR was affected by duration of sample enrichment; shorter times (4–12 h) resulted in more false negatives, however after 18 h, detection was achieved in all samples. Red-pigmented lettuce cultivars caused inhibition of most PCR reactions. Preparation of tissue mass composites of 375 or 125 g in comparison to 25 g samples increased the rate of false negatives, the effect was pronounced when comparison was done between field-inoculated material compared to plant inoculated in the laboratory. In contrast, liquid compositing resulted a reliable strategy to reduce the number of molecular tests. Results highlighted the importance of specific commodity validation of molecular detection methods that consider sample matrix composition, natural microbiota and the cell stress of the pathogens.

1. Introduction

Substantial effort from government, industry and academic sectors has been invested to increasingly ensure that the produce supply chain provides safe food. Implementation of food safety systems, good agricultural and manufacturing practices, consistent and continuous training, and multidisciplinary research, have been applied to preventive systems towards controlling the major established and many of the less apparent causes of produce contamination. Preventive strategies in the form of various general and commodity-specific guidance documents and standards have been established over the past decade. However, microbial food-borne illnesses known or linked to produce consumption remain a considerable individual health, societal, and economic burden (Batz, Hoffmann, & Morris, 2012; Lynch, Tauxe, & Hedberg, 2009; Scallan, Griffin, Angulo, Tauxe, & Hoekstra, 2011). Pathogenic microorganisms may enter the food chain at many points that are not always obvious or controllable, in practical terms. In addition, their great versatility and genetic plasticity to adapt to the host and non-host environment (Winfield & Groisman, 2003) with inherent, or more recently acquired, genetic abilities allow broad survival and the potential for postharvest growth on most fruits and vegetable products.

As a contributor to food safety system management, monitoring for evidence of contamination in the produce supply chain has become an increasingly common approach as one of the food safety system verification tools, which has led to an increase in preharvest and final product testing for both indicators and pathogens (Gil et al., 2015; Havelaar et al., 2010; Hoorfar, 2011). Microbial testing has been traditionally done in the food industry to establish the, or established acceptable defect limit, of a pathogen and their toxins, as well as to meet general microbiological limits for quality and an anticipated correlation to shelf life stability and safety. Additionally, surveillance and monitoring at primary production levels require implementation of screening methods to determine the presence and even quantification of pathogens in food samples (Hoorfar,

* Corresponding author. Tel.: +1 530 754 8313.
E-mail address: gabylopezve@gmail.com (G. Lopez-Velasco).

http://dx.doi.org/10.1016/j.foodcont.2015.01.041
0956-7135/

2011). Currently, and largely as a result of *Escherichia coli* O157:H7, non-O157 shigatoxin producing *E. coli*, and *Salmonella* outbreaks associated with lettuce, baby spinach, other tender greens, and leafy culinary herbs, many growers and fresh processors have adopted preharvest testing and/or a finished goods 'test to release' approach before accepting a field lot for harvest or transporting product to processing operations or commercial distribution channels following a negative finding of target pathogens (D'Lima and Suslow, 2009; Suslow, 2013).

In contrast with other stable food products, leafy greens have a high respiration rate and are, therefore, highly perishable (Kader & Ben-Yehoshua, 2000). Thus a decision based upon detection of foodborne pathogens in fresh and minimally processed leafy greens in an acceptable timeframe becomes highly challenging. Traditional methods for pathogen detection, are not only labor intensive but, more importantly, time consuming; it may take 3–4 days to indicate a negative result and often up to 7 days, or longer, if colony confirmation is needed for confirmation of a positive outcome. In practice this is not a realistic timeframe for lot-acceptance decisions for most commodities, especially if testing is conducted on the final packed product where one or two days lost shelf-quality life quality may be economically damaging. Sufficient cold space for daily pre-shipping product load storage is also not always available during high volume periods and investment for that capacity may not be viable. Rapid methods were developed over the past decades to overcome the fundamental time-to-decision constraint challenges for perishable commodities (Bailey, 1998; de Boer, & Beumer, 1999; Hines, 2000). Current rapid methods offer results within a 12–24 h period for key bacterial pathogens and, in theory, allow risk managers to make yes/no decisions on a particular lot with a reasonable degree of confidence for microbiological risks.

Rapid methods for use in routine surveillance testing or product lot monitoring by industry are developed to require moderate operator skills, based on validated kit format and unequivocal visual or algorithm-based presence/absence detection outcomes. Equally they must meet cost-effectiveness, reproducibility, sensitivity and specificity expectations to adequately detect a target microorganism in the appropriate matrices (Havelaar et al., 2010). One of the major concerns regarding the implementation of rapid methods is the general lack of adequate validation on diverse produce commodities that consider comprehensive seasonal and crop management factors that might affect the performance of most rapid detection platforms (Havelaar et al., 2010; Hitchins, 2011; Hoorfar, 2011; Malorny et al., 2003; McKillip & Drake, 2004).

Methods based on the polymerase chain reaction (PCR) have been the basis for many commercial detection kits. PCR is highly sensitive and specific and often, small concentrations of target DNA can be detected (Harris & Griffiths, 1992). Quantitative real time PCR (qRTPCR), provides great target specificity, particularly among those that utilize target-specific probes. However independent studies have demonstrated that the accuracy of PCR based methods, in general, might be challenged when testing naturally contaminated samples as their sensitivity can be compromised by intrinsic or extrinsic factors (D'Aoust et al., 2007; Hoorfar, 2011). These challenges involve the inability of differentiating viable from dead cells, without significant additional sample processing, or those cells that can be in a viable but not culturable physiological condition (VBNC) (Moyne, Harris, & Marco, 2013). Furthermore, there may be substantial interference, difficult to predict in a non-validated matrix, of plant or food components that can cause inhibition of the PCR reaction (Harris & Griffiths, 1992; Jacobson, Gill, Irvin, Wang, & Hammack, 2012; Kim et al., 2012; Taskila, Toumola, & Ojamo, 2012). Commonly, low numbers of cells are present on contaminated samples that, alone, make detection difficult, but cells can also be sub-lethally injured or stressed which may delay recovery and reaching the critical detection threshold during an enrichment step (Havelaar et al., 2010; Kisluk, Hoover, Kneil, & Yaron, 2012; Stevens & Jaykus, 2004; Taskila et al., 2012). Although not currently a factor in lot acceptance decisions in fresh produce, enrichment greatly limits the opportunity to accurately quantify or even estimate the presence of a target pathogen. This also limits the conclusions derived from molecular detection based on an abbreviated enrichment phase followed by PCR and any one or combination of interferences could contribute to a false negative outcome that may expose consumers to avoidable and severe risk (D'Lima & Suslow, 2009). Therefore, more extensive validation of these methods becomes essential to support enhanced standardization of performance testing and certification on specific commodities and their diverse seasonal and regional production practices.

Pre-arrival or when contamination on a plant surface occurs during field production, pathogens are often exposed to harsh and stressful conditions that can affect their physiological status and thus their ability to grow during enrichment (Havelaar et al., 2010, Taskila et al., 2012). Most commercial kits based on PCR reactions are coupled to cultural enrichment, a physical or immuno-capture concentration step or both. Most methods require the enrichment end-point cell population density of target pathogens to reach about log 3 to log 4 CFU/mL for detection, thus in the majority of cases, pre-enrichment prior to application of even sensitive detection platforms is needed (Bennett, Greenwood, Tennant, Banks, & Betts, 1998; Franco, Hsu, & Simonne, 2010; Kisluk et al., 2012). The variation of culture enrichment among commercial and published methods is enormous, and it can involve non-selective enrichment, followed by a selective enrichment step, it can also be as short as 6 h or a minimum of 24 h depending on the target microorganism (D'Lima & Suslow, 2009; Taskila et al., 2012; Weber, Stephan, Druggan, Joosten, & Iversen, 2009). When non-selective enrichment media are utilized, primarily to minimize stress-cell inhibition but also to keep commercial test costs low, the risk of overgrowth from native microbiota due to nutrient competition increases (Kisluk et al., 2012; Weber et al., 2009). The general effect is to reduce the propensity for target pathogens to sufficiently increase their population to a cell density necessary to ensure transfer in the analytical volume required for the test kit, often 2–5 μl, thus resulting in a false negative outcome. When selective enrichment media are used, the physiological status of the microorganisms associated with environmental stress or potential cell injury, the result of applied sub-lethal doses of sanitizer processing aides and other antimicrobials, might also limit the ability of bacteria to grow in these media containing inhibitory, surfactant, and osmotic agents. Therefore it is necessary to determine the adequate sample mass and optimize the enrichment media and mass:media ratio utilized. In addition, methods often involve some type of sample preparation (homogenization) to detach or expose target bacteria prior to any analysis. This step, in the case of leafy greens, can potentially increase exposure to applied crop production materials (such as copper or fungicides with weak antibacterial activity) or release inhibitory plant components that can extend lag-phase or further impair bacterial growth during enrichment or inhibit the PCR reaction (Kim et al., 2012).

Much of the lettuce and leafy greens industry has incorporated pathogen testing into their food safety programs; therefore it is important to provide adequate methods that are validated for each specific commodity group in order to offer cost-effective strategies with high sensitivity and specificity. Leafy greens can become a complex matrix during pathogen testing due to their composition and other variations associated with cultivar (D'Lima & Suslow, 2009), including a diverse population of native associated

bacteria, many of them within the same phylogenetic group as most common enteric bacterial pathogens, which adds difficulty to pathogen detection methods. In addition, there is an interest from the produce industry to improve sensitivity of detecting random contamination in a lot by increasing the sample mass tested while, at the same time, reducing cost by keeping the number of molecular test kits utilized per lot constant. However, interference due to nutrient competition during enrichment and increased inhibitory compounds from the plant mass could also increase the chance of false negatives.

This study was developed to better understand the factors that influence the detection of *E. coli* O157:H7 and *Salmonella enterica* on leafy greens, particularly lettuce. Environmental stress, pathogen population size, duration of incubation and type of enrichment as well as sample mass were considered to assess the capabilities of available molecular methods to detect these two most common vegetable associated enteric pathogens.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

A non-toxigenic isolate of *E. coli* O157:H7 was used in this study (ATCC700728). This isolate lacks *stx1* and *stx2* genes and it is classified as Biosafety Level-1 (ATCC; Rockville, MD). Its use is currently approved for greenhouse and field trials by the Office of Environmental Health and Safety (EH&S) of University of California, Davis. The *Salmonella* isolate used in this study was received in 2009 (*S. enterica* sv. Typhimurium χ3895) from R. Curtiss (Professor of Life Sciences, School of Life Sciences and Director, Center for Infectious Diseases and Vaccinology; The Biodesign Institute, Arizona State University). The requested strain was classified as an attenuated *S. enterica* sv. Typhimurium (Curtiss and Kelly, 1987) for which EH&S permission was obtained to be used during greenhouse and field trials. A rifampicin-resistant derivative, PTVS177, was selected with all appropriate precautions for culture integrity during transfers and presumptively considered a faithful version of the attenuated strain that lacked adenylate cyclase and cyclic AMP receptor protein rendering it avirulent yet still immunogenic and previously utilized as surrogate in other model systems prior to this study. In preparation for a large field trial in 2013, after the completion of this study, during implementation of an enhanced quality control test performed to laboratory strains it became apparent that the strain PTVS177 was in fact derived from *S. enterica* var. Enteriditis 3985 that was inadvertently transferred from the Curtiss laboratory in 2009. The strain has been removed from the surrogate culture collection and the mistaken strain identity and its use in greenhouse and controlled-access field research was immediately reported to EH&S. The strain presumptively categorized as attenuated *Salmonella* (PTVS177) was utilized in this and other studies without knowledge of this issue at the time. Following a thorough review of all data, and confirmation by Professor Curtiss of the clerical error by staff, not further actions were required by the Institutional Biosafety Committee (IBC).

Both *E. coli* O157:H7 and *S. enterica* sv. Enteriditis (PTVS155 and PTVS177 respectively) correspond to derivative rifampicin-resistant isolates selected via spontaneous mutation for tolerance to 80 mg/L of rifampicin (Rif^{80}), which facilitates detection and recovery and minimizes interference from background bacteria during field studies. Both derivative strains were verified to have an *in vitro* growth rate in several media indistinguishable from the parent strain and a plating efficiency exceeding 85% following 20 generations without rifampicin amendment (data not shown).

PTVS155 and PTVS177 were cultured at 37 °C for 18 h on tryptic soy agar (TSA, BD Diagnostics, Sparks MD, USA) supplemented with 80 mg/L of rifampicin (Rif^{80}; Fisher Scientific; TSA/Rif^{80}). Approximately five colonies were re-suspended in 5 mL of Butterfield's phosphate buffered saline (BPBS; Whatman Inc. Piscataway, NJ, USA). A total of 100 μL were spread onto TSA/Rif^{80} and incubated for 18 h to allow the formation of a confluent lawn of cells in early stationary phase (Suslow & Schroth, 1982; Theofel & Harris, 2009; Wilson & Lindow, 1993). Cells were harvested and suspended in BPBS. The bacterial suspension was washed three times by centrifuging at 1500 × *g* for 10 min. The pellet was re-suspended in BPBS and the optical density at 600 nm was adjusted to an absorbance of approximately 0.750, corresponding to log 9 CFU/mL. The inoculum was diluted to the desired concentration for field and laboratory trials (see below). Final inoculum was serially diluted and plated on TSA/Rif^{80} to determine the nominal estimated concentration of applied cells.

2.2. Description of the field trial

Lettuce seeds (*Lactuca sativa*) of Green Romaine, Tango, Green Oak, Green Leaf, Red Oak and Lolla Rosa (Snow Seed Co. Salinas CA, USA) were planted during late spring and fall of 2010 and 2011 at the University of California Davis Research Farm facility. Plant watering was performed through standard overhead sprinkler emitters, and pre-plant fertilization and herbicide treatments typical of commercial lettuce production were done. Seeds were planted on beds of approximately 30 m of length (150 cm width), each bed was seeded with four rows of seeds (Yolo silt clay loam; class 1 soil). For each lettuce variety a total of 3 beds were planted, having a total of 15 beds that were randomly distributed. Each bed was further divided into four blocks that were inoculated, at the point close to commercial harvest maturity for tender greens, with log 3 and log 5 CFU/mL of PTVS177 or PTVS 155. With this sub-plot configuration, each quarter of a bed corresponded to one block and thus each inoculum concentration and applied microorganism was repeated in triplicate blocks within the experimental plot.

Leafy greens were inoculated 7–10 days, depending on season, before reaching a commercial baby leaf harvest stage, typically developing 5 to 8 true leaves. Inoculum was applied in the pre-twilight hours with a CO_2 powered, handheld, backpack sprayer using a two nozzle spray boom and Teejet 8005 spray tips (selected to reduce spray drift); the sprayer was set at approx. 25–27 psi and applied by pre-calibrated walking speed to deliver spray at 2 L/30 m. Each pathogen and dose was inoculated separately but in each treatment the lower doses were applied first.

2.3. Behavior of E. coli O157:H7 and S. enterica on leafy greens during field production

Recovery and detection of both *E. coli* O157:H7 and *S. enterica* was conducted after 12 h, 4 and 10 days post inoculation. Plants were manually harvested by cutting approximately 2 cm above the soil with sterile scissors. Plant samples were collected from each subplot and placed in a sterile polymer bag. Plants were transferred to the laboratory, stored at 2.5 °C and processed within 8–10 h after collection. In the laboratory, 25, 75 or 150 g of plant material were tested for recovery of applied strains. Plant samples were transferred to sterile plastic bags (Whirl −Pak; Nasco, Modesto CA, USA) containing sterile mEHEC (Biocontrol, Bellevue WA, USA) or buffered peptone water (BPW; BD Diagnostics, Sparks MD, USA) in a 1:2 ratio (plant to liquid media), both supplemented with Rif^{80} for *E. coli* O157:H7 or *Salmonella*, respectively. Samples were massaged by hand for 1 min and 0.1 and 1 mL of the bacterial suspension were plated on TSA/Rif^{80} supplemented with 1 g/L of sodium pyruvate facilitate resuscitation of sub-lethally injured cells. Plates were incubated for 24 h at 37 °C and further incubated for up to 32 h if no

colony development was observed. Colony forming units (CFU) were quantified and results were transformed to log CFU/g of leaf material.

In parallel with immediate plating procedures, additional liquid media (mEHEC/Rif^{80} and BPW/Rif^{80}) was added to the remaining cell suspension to achieve a 1:4 ratio (plant to liquid media). Bags were incubated at 37 °C for up to 18 h of enrichment of *E. coli* O157:H7 and *Salmonella* to evaluate their presence in samples below the limit of detection by direct agar plate enumeration. For colony confirmation, 40 μL of enrichment were plated onto CHROMagarO157 (ChromO157; Becton Dickinson BBL, Franklin Lakes NJ, USA) or selective agar Xylose Lactose Tergitol 4 (XLT-4; BD Diagnostics, Sparks MD, USA) both supplemented with Rif^{80} for confirmation of *E. coli* O157:H7 or *Salmonella*, respectively. Incubation of ChromO157/Rif^{80} and XLT-4/Rif^{80} plates was done at 42 and 37 °C, respectively. Analyses were repeated in triplicate within each block except for those samples containing 150 g in which only 1 sample per block was analyzed.

2.4. Molecular detection of E. coli O157:H7 and S. enterica on leafy greens in relation to time and enrichment media, environmental exposure, sample mass and sample compositing

2.4.1. Effect of duration of incubation and type of media during enrichment

Three cultivars; Green Oak, Green Romaine and Red Oak were used in this study. For analysis, 25 g of the plant material were collected after 3 and 10 days of inoculation. A total of 6 replicates of 25 g per block were collected (n = 18 per cultivar and inoculation dose). Plant material was enriched with 225 mL of BPW or brain heart infusion (BHI; BD Diagnostics, Sparks MD, USA) for enrichment of *Salmonella* at 37 °C and with 225 mL of tryptic soy broth (TSB; BD Diagnostics, Sparks MD, USA) or MP media (Dupont; Wilmington DE, USA) for enrichment of *E. coli* O157:H7 incubated at 42 °C. Before incubation, samples were vigorously massaged by hand to ensure total exposure of plant material and the entire leaf sample was pressed below the surface of the media in the bag before static incubation. In this case, addition of Rif^{80} to the enrichment broth was not done, to allow full competitive interactions, and all media were pre-conditioned to the final incubation temperature before addition to the bags. Aliquots of the enrichment were collected after 4, 8, 12 and 18 h of incubation. Aliquots were subjected to standard pathogen testing using Taqman® probe based real time PCR targeting *invA* (Sbodio, Maeda, Lopez-Velasco, & Suslow, 2013) and *rfbE* (Tomas-Callejas et al., 2011) genes for detection of *Salmonella* and *E. coli* O157:H7, respectively. Amplification lower or equal to the Ct value of a standard containing 5 copies of the target gene was classified as positive. Additionally, enrichments were also analyzed using the BAX® system for *Salmonella* and the BAX® system real-time PCR assay for *E. coli* O157:H7 (Dupont; Wilmington DE, USA). For some portions of the study, as a whole, variations in sample mass and enrichment conditions deviated slightly from the specific validated protocols detailed in the BAX® technical instructions to allow for comparative analysis. However, post-enrichment sample preparation for the PCR reaction was done following manufacturer instructions, with the exception that enrichments were previously washed twice with BPBS due to inhibition of the PCR reaction for both sample and internal amplification controls, due to intrinsic properties of the plant material, particularly with red-pigmented lettuces. A result was considered as a presumptive negative only if the internal amplification control (IAC) provided the correct positive result.

After enrichment, all samples were subjected to colony confirmation by conducting a brief secondary enrichment with 200 μL of the enriched sample that was placed in 1.8 mL of BHI supplemented with Rif^{80}; secondary enrichments were incubated at 37 °C for 6 h prior to plating for confirmation on ChromO157/Rif^{80}or XLT-4/Rif^{80} as described above. A sample was considered a definite positive only when colony confirmation of the characteristic rifampicin resistant applied strains on differential media was achieved.

2.4.2. Sample mass (plant compositing) and effect of environmental exposure

Leaf tissue mass of cultivars Green Oak, Green leaf, Red Oak, and Lolla Rosa inoculated with log 3 CFU/g and collected after 3 days post-inoculation in the field trial, were utilized for this part of the study. Comparative tissue mass samples of 375, 125 or 25 g of plant material were individually placed in sterile bags. Due to the amount of leaf mass in larger samples and to reflect trends being implemented by the produce industry, bags containing 375 or 125 g were enriched with liquid media in a 1:4 ratio (plant:liquid media) and they were compared with an industry standard sample of 25 g containing liquid enrichment media in a 1:10 ratio (plant:liquid media). Enrichment broths utilized were MP media (Dupont; Wilmington DE, USA) coupled with detection through the BAX® system for both *Salmonella* and *E. coli* O157:H7 and BHI (BD Diagnostics, Sparks MD, USA) for *Salmonella* and mEHEC (Biocontrol, Bellevue WA, USA) for *E. coli* O157:H7 coupled to the Assurance GDS® system (Biocontrol, Bellevue WA, USA) for both *Salmonella* and *E. coli* O157:H7. Sample preparation for GDS system samples was done following manufacturer instructions with some modifications to enhance detection; prior to immuno-magnetic bead separation (IMS) prescribed within this kit platform enrichments were washed twice as described above to prevent inhibition of PCR reaction. All media were pre-conditioned to 42 °C before incubation.

All samples were also analyzed using Taqman® probes targeting *invA* and *rfbE* gene as previously described. Additionally all samples were also subjected to colony confirmation after secondary enrichment with Rif^{80} as a selective enrichment. Positive outcomes by molecular detection were compared with matched-sample positives obtained through culture-based colony detection and confirmation. All enrichments were analyzed after 8 and 18 h of incubation.

In order to assess the effect of field inoculation and environmental stress exposure on recovery of the target pathogens, a set of non-inoculated Green Oak and Lolla Rosa plots from the same field planting was collected and subjected to the same enrichment protocols and molecular detection as previously described. However, in this case tissue samples were inoculated in the laboratory with 10 CFU/sample of either PTVS177 or PTVS155 that were directly applied to the plant material contained in sterile bags. Inoculated material was stored at 5 °C for 16–18 h before enrichment in order to pre-condition the cells to a lower metabolic activity (Jacobson et al., 2012; Kase et al., 2012).

2.4.3. Effect of liquid compositing

As an alternative to plant compositing, enrichment of 25 g samples followed by compositing of post-enrichment media into a single analytical sample was assessed. For this purpose, a triplicate of 10, 5 or 3 samples containing 25 g of inoculated leaf tissue from the field material (log 5 CFU/mL; 10 days post-inoculation) were enriched with BPW and MP media for *Salmonella* and *E. coli* O157:H7 for up to 18 h at 37 and 42 °C, respectively. In each set only 1 out of the 10, 5 or 3 plant samples (Green Romaine) was a known positive (by culture-based detection, colony confirmation, and molecular detection). One milliliter of the positive sample was mixed with 1 mL of the other 9, 4 or 2 mL of sample enrichments that came from non-inoculated plant material so that we constructed final liquid composites of 10:1, 5:1 or 3:1 ratio (negative

enrichment: positive enrichment). Enrichments were mixed in a Falcon® tube (BD Biosciences; San Jose CA, USA) and vortexed thoroughly; 1 mL of the mixture was washed twice with BPBS to prevent PCR amplification inhibition. Samples were analyzed through molecular detection methods using Taqman® targeting *invA* and *rfbE* and BAX® system as previously described.

2.5. Statistical analysis

Results of quantification of bacterial populations through ten-fold serial dilution plate counts on rifampicin amended media were transformed to log CFU/g of plant material. Transformed data were subjected to analysis of variance and mean comparison using Tukey's multiple comparisons of means with Statistical Analysis System 9.2 (SAS Institute, Cary, NC). Statistical significance was established when p-values were lower than 0.05.

3. Results

3.1. Behavior of Salmonella and E. coli O157:H7 on lettuce leaves during field production

Inoculation of several varieties of lettuce leaves with *Salmonella* and *E. coli* O157:H7 was done during field production to a commercial harvest maturity, commonly referred to as baby-leaf or tender greens, and survival of both microorganisms was followed for up to 10 days. Population dynamics showed a net decrease in recoverable target populations within the first 12 h for *E. coli* O157:H7 with approximately 1-log reduction (Fig. 1). In contrast populations of *Salmonella* on lettuce inoculated with log 5 CFU/mL, maintained their population after this time, with exception of cultivar Tango (p-value < 0.05) (Fig. 1A). When inoculation was done with log 3 CFU/mL, a significant reduction (p-value < 0.05) of about 0.5-log and 1-log was determined for *Salmonella* and *E. coli* O157:H7, respectively (Fig. 1B and D). After 3 days post-inoculation a rapid decline in population was determined for most varieties inoculated with log 5 CFU/mL, however significantly greater populations (p-value < 0.05) were recovered from green cultivars (Green Romaine, Tango Green Oak and Green leaf) than on red-pigmented cultivars (Lolla Rosa and Red Oak) for both applied pathogens (Fig. 1A and C). In contrast, inoculation with log 3 CFU/mL resulted in detection of viable target populations only after sample enrichment (Fig. 1B and D). After 10 days of inoculation, no significant difference in bacterial survival was determined for all cultivars (p-value > 0.05), and for both initial cell density inoculation levels; viable detection was only achieved through sample enrichment (See Supplementary Tables 1 and 2 and Fig. 1). In addition to an effect on survival associated with lettuce cultivar and initial inoculation dose, comparison between strains indicates that lower populations of *E. coli* O157:H7 were recovered following the first 12 h after inoculation as compared to *Salmonella* populations (p-value < 0.05).

3.2. Influence of enrichment media and duration of incubation on the detection of Salmonella and E. coli O157:H7 from lettuce inoculated during field production

Samples of leafy greens (Green Oak, Green Romaine and Red Oak) collected after 3 and 10 days of inoculation with either log 3 or log 5 CFU/mL were enriched for up to 18 h in various media

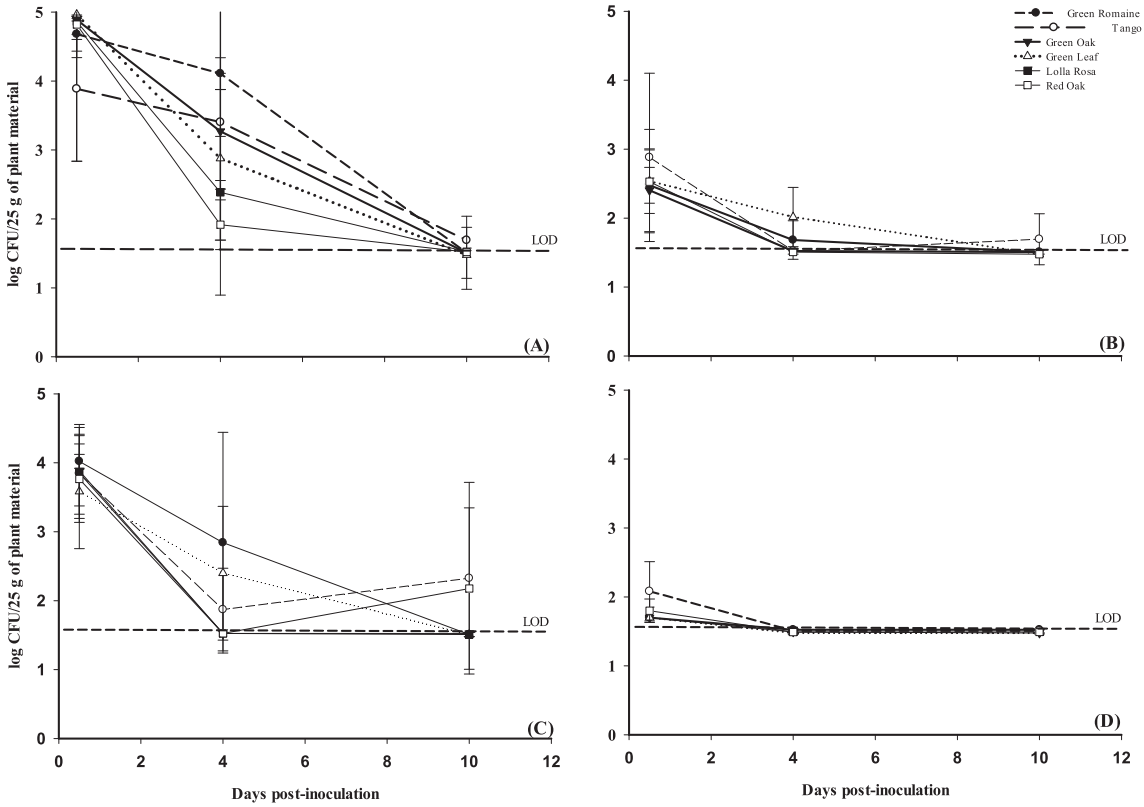

Fig. 1. Survival and behavior of *Salmonella enterica* and *Escherichia coli* O157:H7 on lettuce surfaces following a simulated field contamination incident. (A) *S. enterica* inoculated with log 5 CFU/mL, (B) *S. enterica* inoculated with log 3 CFU/mL, (C) *E. coli* O157:H7 inoculated with log 5 CFU/mL, (D) *E. coli* O157:H7 inoculated with log 3 CFU/mL. Each point represent the average of n = 3 replicates of 75 g of plant material and bars indicate the standard deviation of the mean obtained through direct plating on tryptic soy agar supplemented with 80 mg/L of rifampicin. Data was normalized to log CFU/25 g of sample, values below the limit of detection (LOD = log 1.52 CFU/25 g) represent non-parametric data that was obtained through sample enrichment and thus it only indicates presence or absence of the target pathogen.

recommended for further molecular detection (See Supplementary Table 3 and Section 2.4.1 for methodology description). Aliquots progressively taken for detection during the course of enrichment were initiated after 4 h to ensure fractional positive outcomes at leading time points up to and beyond the validated kit endpoint. After enrichment in media without supplementation of rifampicin for up to 18 h, all samples were subjected to colony confirmation using selective agar supplemented with Rif^{80}. For *Salmonella*, within 4 and 12 h in either BHI or BPW between 42 and 58% of the final total positive samples, at 18 h, were confirmed as positive after colony confirmation (Table 1). After 18 h, the number of fractional positives of *Salmonella* was greater than for *E. coli*. For lettuces inoculated with *E. coli* O157:H7, colony confirmation, of fractional positive samples from field inoculation, was achieved in up to 96% of the samples enriched in TSB after 6 h and in up to 88% of the samples when enriched in MP after 12 h. As occurred with *Salmonella*, from all known positive samples, 100% of the samples were positive by colony confirmation after 18 h of enrichment (Table 1). Extended lag phase recovery of environmentally stressed or sub-lethally injured cells from antimicrobial treated wash water may result in lower than necessary cell densities of the target pathogen for detection, depending on the specific test kit utilized.

Using the same enrichments, samples were subjected to molecular detection using Taqman® probes as well as the BAX® system. The analyzed enrichments were those collected after 10 days of inoculation with log 5 CFU/mL to ensure fewer negative outcomes. On average, between 8 and 35% of the samples were positive after 4 h of enrichment, between 32 and 63% after 8 h and between 38 and 58% after 12 h of enrichment and 100% after 18 h (Table 2). Reliance on the duration of incubation of the enrichment could be a key factor to increase the probability of detection (POD) when sample analysis is based solely on rapid test methods using molecular detection.

3.3. Effect of sample mass and environmental exposure in the molecular detection of Salmonella and E. coli O157:H7

During the spring seasonal trial, in addition to studying the behavior of the applied pathogens during field production, we also evaluated whether an increase of the sample mass (usually 25 g) resulted in an enhanced detection or reduction of false negatives obtained in a sampled product lot (Refer to Section 2.4.2 for methodology description). Increasing in sample mass also increases the amount of broth needed during enrichment and space for incubation to keep ratios for analysis constant. While desirable, this is not practical or feasible for the majority of laboratories. To assess maintaining a manageable sample size during laboratory handling, we evaluated the use of a 1:4 ratio in samples containing 375 and 125 g that, at the time of the study, this ratio had already been adopted by some commercial contract testing labs following undisclosed self-validation studies. This volume was sufficient to cover the entire amount of plant material. The increased sample mass was compared to replicate control samples containing 25 g and a 1:10 ratio (plant material: liquid broth).

Recovery of applied *Salmonella* and *E. coli* O157:H7 after enrichment with BPW and mEHEC, respectively, both supplemented with Rif^{80} did not show a trend that supports the premise that a larger sample size would necessarily enhance the detection of the pathogens, this considering that initial contamination event was uniform across a lot at least after the 4 days of inoculation (Supplementary Tables 1 and 2). However, by day 10 post-inoculation, when populations were predominantly below the limit of enumerative detection, greater numbers of positive samples were obtained from 150 g samples than from 75 or 25 g, presumably due to the increasing heterogeneity in persistence across the treated blocks (See supplementary Tables 1 and 2).

To further assess the POD in standard preharvest assays, a green and a red cultivar (Green Oak and Lolla Rosa, respectively) were collected in the field from blocks not inoculated with PTVS 155 or PTVS177. A total of 375, 125 or 25 g of plant material were weighed in sterile bags and 10 CFU per sample of both strains were applied directly to the plant material. Plants were stored for 16–18 h at 5 °C to allow inoculum conditioning before enrichment without the addition of Rif^{80}. The same enrichment was subjected to colony confirmation and molecular detection (Table 3). By colony confirmation, all samples were positive irrespective of sample mass, however using molecular detection as a principal screening tool resulted in more false negatives for the 375 and 125 g samples than either the 25 g controls or their counterparts screened by colony confirmation (Table 3). This outcome indicated that increasing sample mass in a single unit test, without the benefit of a strong selective agent or process, could be susceptible to a higher risk of false negatives in product testing.

Table 1
Influence of enrichment media and incubation time on the detection of *Salmonella enterica* and *E. coli* O157:H7 from field inoculated lettuce.

Time of enrichment (h)	*Salmonella*		*E. coli* O157:H7	
	Brain heart infusion	Buffered peptone water	TSB	MP
	% of positive detection by colony confirmation[a]			
4	42	38	67	82
6	nd[c]	Nd	96	nd
8	58	51	100	82
12	58	52	100	88
18	100	100	100	100
Ratio of positive samples/total samples analyzed[b]	69/108	61/108	28/108	34/108

[a] The percentages reported are based on the total number of positives that were culture confirmed after 18 h of enrichment in non-selective media followed by secondary enrichment in media supplemented with Rif^{80}.
[b] Positive samples correspond to samples where the attenuated pathogens were enriched in absence of rifampicin for 18 h in the respective media and then colony confirmation was done through secondary enrichment with rifampicin-selective media. Results for all inoculation levels and lettuce cultivars were pooled.
[c] (nd) not determined for this time-point.

Table 2
Detection of *Salmonella enterica* and *E. coli* O157:H7 through molecular detection after 10 days of inoculation in the field.

Time of enrichment (h)	*Salmonella*		*E. coli* O157:H7	
	Taqman®	BAX®	Taqman®	BAX®
	% of positive samples[a]			
4	35	20	33	8
8	63	32	50	33
12	55	38	58	42
18	100	100	100	100
Total number of positive samples/total samples analyzed[b]	43/54		12/54	

[a] The percentages reported are based on the total number of positives that were culture confirmed after 18 h of enrichment from samples collected after 10 days of inoculation with the high dose of inoculum (log 5 CFU/mL).
[b] Positive samples correspond to samples where the attenuated pathogens were enriched in absence of rifampicin for 18 h in the respective media and then colony confirmation was done through secondary enrichment with rifampicin-selective media. Results for all lettuce cultivars were pooled (See Supplementary Table 3 for specific information of each variety).

Table 3
Effect of sample mass in the molecular detection of (A) *E. coli* O157:H7 and (B) *Salmonella* in leafy greens lab-inoculated prior to enrichment.

	(A)	Colony confirmation[a]			BAX®			Taqman® (*rfbE*)		
		Total number of positives/total number of samples[b]								
	Sample size (g)	375	125	25	375	125	25	375	125	25
Media	Cultivar									
MP	Green Oak	1/1	3/3	3/3	0/1	2/3	3/3	1/1	3/3	3/3
	Lolla Rosa	1/1	3/3	3/3	0/1	1/3	3/3	1/1	3/3	3/3
	Total	**2/2**	**6/6**	**6/6**	**0/2**	**3/6**	**6/6**	**2/2**	**6/6**	**6/6**
		Colony confirmation			GDS_{O157}			Taqman (*rfbE*)		
mEHEC	Green Oak	1/1	3/3	3/3	0/1	3/3	2/3	1/1	3/3	2/3
	Lolla Rosa	1/1	3/3	3/3	0/1	0/3	3/3	0/1	3/3	3/3
	Total	**2/2**	**6/6**	**6/6**	**0/2**	**3/6**	**5/6**	**1/2**	**6/6**	**5/6**
	(B)	Colony confirmation[a]			BAX			Taqman (*invA*)		
		Total number of positives/total number of samples[b]								
	Sample size (g)	375	125	25	375	125	25	375	125	25
Media	Cultivar									
MP	Green Oak	1/1	3/3	3/3	0/1	1/3	3/3	0/1	0/3	3/3
	Lolla Rosa	1/1	3/3	3/3	0/1	3/3	2/3	0/1	2/3	3/3
	Total	**2/2**	**6/6**	**6/6**	**0/2**	**4/6**	**5/6**	**0/2**	**2/6**	**6/6**
		Colony confirmation			$GDS_{Salmonella}$			Taqman (*invA*)		
BHI	Green Oak	1/1	3/3	3/3	0/1	3/3	3/3	1/1	3/3	3/3
	Lolla Rosa	1/1	3/3	3/3	1/1	3/3	3/3	1/1	3/3	3/3
	Total	**2/2**	**6/6**	**6/6**	**1/2**	**6/6**	**6/6**	**2/2**	**6/6**	**6/6**

[a] The same final-point enrichment was subjected to both culture confirmation and molecular detection with BAX, GDS or Taqman. Culture confirmation was done from the 18 h enrichment which was subjected to secondary enrichment with Rif[80] and plating on selective chromogenic media supplemented with Rif[80].
[b] Results were obtained after 18 h of enrichment. Sampling was done after 8 h, in which samples containing 25 g of leaf material were positives by all detection methods. The ratio of leaf material to enrichment broth was 1:4 for 375 and 125 g and 1:10 for 25 g samples.

Table 4
A)Effect of sample mass on the molecular detection of *E. coli* O157:H7 from lettuce inoculated during field production. B) Effect of sample mass on the molecular detection of *Salmonella* in field inoculated leafy greens.

	(A)	Colony confirmation[a]		BAX®		Taqman® (*rfbE*)	
		Total number of positives/total number of samples[b]					
	Sample size (g)	375	125	375	125	375	125
Media	**Lettuce cultivar**						
MP	Green Oak	3/3	6/6	0/3	0/6	0/3	1/6
	Green leaf	1/3	0/6	0/3	0/6	0/3	0/6
	Red Oak	0/2	2/6	0/2	0/6	0/2	0/6
	Lolla Rosa	3/3	1/6	0/3	0/6	2/3	0/6
	Total	**7/11**	**9/24**	**0/11**	**0/24**	**2/11**	**1/24**
	Sample size (g)	Colony confirmation		GDS_{O157}		Taqman (*rfbE*)	
		375	125	375	125	375	125
mEHEC	Green Oak	3/3	6/6	0/3	2/6	2/3	2/6
	Green leaf	3/3	4/4	0/2	2/4	0/3	1/4
	Red Oak	0/0	0/0	0/0	0/0	0/0	0/0
	Lolla Rosa	3/3	6/6	0/3	6/6	1/3	4/6
	Total	**9/9**	**16/16**	**0/8**	**10/16**	**3/9**	**7/16**
	(B)	Colony confirmation[a]		BAX®		Taqman® (*invA*)	
		Total number of positives/total number of samples[b]					
	Sample size (g)	375	125	375	125	375	125
Media	**Lettuce cultivar**						
MP	Green Oak	3/3	6/6	2/3	5/6	3/3	4/6
	Green leaf	2/3	2/6	0/3	0/6	0/3	1/6
	Red Oak	2/3	5/5	1/3	0/6	0/3	0/5
	Lolla Rosa	3/3	4/6	1/3	1/6	1/3	1/6
	Total	**10/12**	**17/24**	**4/12**	**6/24**	**4/12**	**6/24**
	Sample size (g)	Colony confirmation		$GDS_{Salmonella}$		Taqman (*invA*)	
		375	125	375	125	375	125
BHI	Green Oak	3/3	4/4	0/3	0/4	3/3	4/4
	Green leaf	3/3	6/6	0/3	1/6	3/3	4/4
	Red Oak	1/1	5/5	0/1	0/2	1/1	5/5
	Lolla Rosa	3/3	6/6	1/3	1/6	3/3	6/6
	Total	**10/10**	**21/21**	**1/10**	**2/18**	**10/10**	**19/19**

[a] The same final-point enrichment was subjected to both culture confirmation and molecular detection with BAX, GDS or Taqman on field inoculated material, recovered after 3 days of inoculation. Culture confirmation was done from the 18 h enrichment, which was subjected to secondary enrichment with Rif[80] and plating on selective chromogenic media supplemented with Rif[80].
[b] Results were obtained after 18 h of enrichment. Sampling was done after 8 h, in which samples containing 25 g of leaf material were positives by all detection methods. The ratio of leaf material to enrichment broth was 1:4 for 375 and 125 g and 1:10 for 25 g samples.

The above experiment was repeated with samples of four field-grown lettuce cultivars with samples collected after three days of inoculation with *E. coli* O157:H7 (Table 4A) or *Salmonella* (Table 4B) applied at with log 3 CFU/mL. This evaluation assessed the potential impacts associated with environmental exposure in addition to sample mass. Detection after an 8 h enrichment interval was negative for all samples by both culture confirmation with Rif[80] and molecular detection. For the case of *E. coli* O157:H7 inoculated samples, culture confirmation with Rif[80] was observed in 70–100% of the collected samples, however the use of molecular detection methods with 375 g resulted in 0–40% detection of positives and 0–40% for 125 g samples (Table 4A), which were both a lower POD as compared to leaf material from the same field source period inoculated with the same isolates at 10 CFU/sample in the laboratory (Table 3).

There is greater quantitative and temporal survival and prevalence of *Salmonella* than *E. coli* O157:H7 after inoculation as previously indicated (Supplementary Table 3). But similar to *E. coli* O157:H7 samples, those containing 375 g of plant material inoculated with *Salmonella* had a lower detection rate than with 125 g and in both cases there were fewer positive samples determined by molecular detection kits than by colony confirmation with Rif[80] (Table 4B) and than those in which inoculation of validation samples occurred in the lab, as is most common. Results indicate that increase in sample mass, as a single analytical unit, could contribute to an increased population of background bacteria competing in the enrichment for nutrient resources, at the reduced broth ratio. In combination with competition, exposure to environmental stress could impair the rate of recovery in reaching log-phase growth during the enrichment and failure to achieve cell densities adequate to the sensitivity of a specific molecular detection method. Therefore, increasing sample mass without consideration of the enrichment volume (although economically attractive and clearly functional in some instances) could potentially increase the risk of a false positive outcome and, therefore, decision to ship contaminated product. In these studies, detection of false positives was never encountered among all the detection methods utilized in control samples of non-inoculated lettuce varieties.

A statistically valid alternative strategy would be to perform the enrichment of multiple standard, validated sample size (25 g; 1: 10) and then composite the final enrichments into a single analytical sample for pathogen detection processing (Jarvis, 2007). In this study, we combined aliquots of a sample of a Green Romaine enrichment culture that was positive by both molecular detection and culture confirmation with Rif[80] with 2, 4 or 9 dual detection method negative samples followed by a repeat molecular detection assay (Table 5). All mixed ratio composites resulted in a positive detection by molecular kit methods. Therefore, preparation of a post-enrichment broth culture composite rather than a plant mass composite pre-enrichment could minimize molecular kit utilization, generally the most costly aspect of product testing, without compromising the final result of a lot.

Table 5
Effect of sample pooling in the detection of *E. coli* O157:H7 and *Salmonella* on Romaine lettuce using molecular detection methods.

Mixing ratios[a]	Taqman®		BAX®	
	E. coli O157:H7 (*rfbE*)	*Salmonella* (*invA*)	*E. coli* O157:H7	*Salmonella*
	Ratio of positive samples/total analyzed composites[b]			
10:1	3/3	3/3	3/3	3/3
5:1	3/3	3/3	3/3	3/3
3:1	3/3	3/3	3/3	3/3

[a] Total number of negative samples:total number of positive samples. The total volume used to create the composite was of 1 mL from each sample.
[b] Enrichment was performed in BPW and MP media for enrichment of *Salmonella* and *E. coli* O157:H7 respectively for 18 h with a 1:10 ratio of plant material and enrichment broth. Initial inoculation dose was of 10 CFU.

4. Discussion

Detection of foodborne pathogens through molecular techniques based on real time PCR has become a common approach to assess their presence in various food matrices. The performance of such methods and their sensitivity can vary depending on the food matrix and its preparation for molecular detection. These and other factors that influence the rate of bacterial pathogen growth or PCR inhibition underscore the importance of adequate validation, under appropriate challenge conditions of these methods on specific commodities to ensure an adequate and dependable performance. Validation assessments should consider bacterial pathogen physiologic status in field and process environment that mirror actual use conditions. As produce testing becomes more common across diverse commodities such standardized validation criteria and protocols will become essential to support the expanding application by research, private contract, and public health laboratories.

In this study we monitored the population dynamics of *E. coli* O157:H7 and *S. enterica* on the lettuce phyllosphere during field production, after harvest, the evaluation of real time PCR-based molecular methods for detection of applied bacteria was done. This assessment included the consideration of key factors that could potentially impact the POD and result in a false negative outcome of a single commercial lot acceptance test, which is relevant as it is the typical method supporting marketability decisions, after pathogens have been exposed to field conditions.

Population dynamics of both *Salmonella* and *E. coli* O157:H7 showed a rapid decline within the first 4 days after inoculation. Following 10 days post-inoculation, populations remained below the limit of standard quantitative detection and thus recovery was addressed by an enrichment scheme (Fig. 1 and Supplementary Table 3). This survival profile is not uncommon for both phyllosphere-competent isolates (Lindow & Brandl, 2003) and surrogates of enteric pathogens introduced to the phyllosphere by inoculation of lab-grown (Delaquis, Bach, & Dinu, 2007; Gutierrez-Rodriguez et al., 2012; Moyne et al., 2011; Tomas-Callejas et al., 2011; Wood, Bezanson, Gordon, & Jamieson, 2010). Moyne et al. (2011) using the same attenuated strain of *E. coli* O157:H7 as was used in this study, were able to recover the applied microorganism from lettuce surfaces by sample enrichment only after 2 days of inoculation with log 5 CFU/mL of a broth inoculum culture and up to 14 days post-inoculation. In contrast, studies reported by Gutierrez-Rodriguez et al. (2012), showed that inoculation with log 4 CFU/mL cocktail of two attenuated *E. coli* O157:H7, sprayed on spinach samples, resulted in quantitative recovery of culturable populations of up to log 1.2 CFU/g after 14 days of inoculation. Using the same *Salmonella* strain as in this study to inoculate melon surfaces during field production, a significant reduction of the population was observed during the first few days (Lopez-Velasco, Sbodio, et al., 2012). These and other studies, provide evidence that there is an initial loss of viability or recovery after enteric bacteria are inoculated on plant surfaces, however surviving bacteria may establish and persist at very low cell numbers that require concentration or enrichment for their detection (Moyne et al., 2013; Poza-Carrion, Suslow, & Lindow, 2013). How these data-based observations reflect the post-arrival fate of natural sources of contamination and the factors that influence this potential, other than atypical practices such as non-treated wastewater used for irrigation (Pachepsky, Shelton, Mclain, Patel, & Mandrell, 2011; Suslow, 2010), are largely unknown.

Additionally, we observed significantly greater populations of *Salmonella* than *E. coli* O157:H7 on lettuce surfaces including a larger number of survivors after populations dropped below the limit of detection. This suggests an effect during field survival associated with individual bacterial strains that could alter initial establishment of pathogen on the plant phyllosphere. This difference may also be attributed to individual strain differences associated with physiological and genetic traits or, has been reported, a general observation that *Salmonella* has superior environmental fitness relative to *E. coli* (Brandl, Clayton, & Teplitski, 2013; Harris et al. 2012; Harris et al., 2013; Winfield and Groisman, 2003). It is likely that the fact that the *Salmonella* isolate, inadvertently not being attenuated as explained above, was better fit to survive environmental hurdles that would more likely effect the attenuated *E. coli* O157:H7 used in this study, as previously reported (Gutierrez-Rodriguez, Gundersen, Sbodio, & Suslow, 2012).

Significant differences in survival associated with lettuce cultivar were also observed in this study (Fig. 1), specifically after 4 days of inoculation, with lower populations on red or red-purple–pigmented colored than on solely green lettuce cultivars. Differences in survival associated with plant cultivar have also been demonstrated in other species. Barak, Kramer, and Hao (2011) demonstrated that differences in tomato plant colonization by *Salmonella* were cultivar dependent, mostly the result of preferential colonization on plant structures including trichomes and stomata. Lopez-Velasco, Welbaum, Falkinham III, and Ponder, (2011) found a significant correlation with the number of total bacterial populations on the phyllosphere of various cultivars of spinach, which was associated with the number of colonization sites on the plants that varied among cultivars. Therefore, it would be important to study more deeply the inherent lettuce leaves characteristics or agricultural practices that can help to restrict or to enhance the establishment of bacterial pathogens.

Once population dynamics and survival of two of the most common pathogens reported in fresh produce was characterized, field-inoculated material was utilized to evaluate molecular detection methods based on real time PCR. Recovery of target pathogens and growth amplification during enrichment to a critical cell density threshold is one of the main challenges for most molecular detection methods, which varies by the specific platform sensitivity. To our knowledge, this is the first study that utilizes plant material inoculated during field production to evaluate various aspects that could affect the detection of pathogens using rapid methods.

Quantitative data regarding the levels of contamination of most bacterial pathogens on the phyllosphere is mostly unknown but very low levels of contamination are expected, based on investigative studies of naturally-contaminated fields, which makes sample enrichment necessary. Previous to molecular detection, enrichment of target pathogens through culture media is often used as a preparatory step in sample preparation, this not only increases the required analytical-threshold sensitivity but also favors the recover injured and stressed cells after exposure to hostile conditions in the field (Kisluk et al., 2012; Stevens & Jaykus, 2004).

There are numerous studies and commercial protocols available for this purpose. Molecular detection methods are commonly targeted as "rapid methods", minimizing the time of analysis is a practical challenge and thus early adoption in commercial applications with validated kits often used short culture enrichment times, outside the validated times specified by the kit developer, that ranged from 5 to 8 h (D'Lima & Suslow, 2009). Rapid test developers have also specified culture enrichment times of up to 24 h depending on the pathogen and food matrix. In this study, evaluation of the duration of enrichment of field inoculated lettuce was done (Tables 1 and 2). Enrichment in BHI, BPW, TSB or MP media showed similar results; short times of enrichment (<12 h), were not able to detect applied bacteria on field inoculated material and an increase of enrichment time for up to 18 h was needed. Additionally, when culture confirmation was achieved through the addition of rifampicin to favor the applied bacteria, a lower number of false negatives was obtained by direct molecular detection through qPCR. Although only two molecular methods were tested in this aspect of our study, other platforms and commercial test kits, including newer versions of the kits used during the timeframe of this study, might be efficacious within a reduced enrichment interval before molecular detection. However, as is true for all such methods, the cell density of the target might not be sufficient to produce an amplification signal that can be detected without some post-enrichment processing. This does not suggest that a culture confirmation approach would be a more practical or sensitive method, as in this case; it was applied with the use of rifampicin as a selective agent so we can ensure and facilitate the recovery of the applied strain if it was present in the samples against a more numerous background microbiota. These results do provide evidence that even in preconditioned media, if the target bacteria have been exposed to environmental stressors, an enrichment interval greater than the 10–12 h specified in many test kits might be necessary to avoid false negatives during the application of molecular detection platforms to routine produce testing. However our data indicates, that even in temperature pre-conditioned media, if the target bacteria has been exposed to environmental stress, a time of enrichment greater than 12 h might be necessary to avoid false negatives during the application of molecular detection platforms. Although media selected in this study for enrichment of *E. coli* O157:H7 seem to allow shorter enrichment times, an enrichment less than 18 h could result in higher risk of obtaining a false negative outcome in any single, randomly collected commercial product field sample or post-process sample. D'Lima and Suslow (2009), also reported that a short duration of culture enrichment resulted in larger numbers of false negatives, not only by PCR but also by other type of platforms including immunoassays, lateral flow devices, and immunomagnetic separation (IMS). Other studies have provided evidence that the effect of enrichment on the POD and rate of false negatives is also associated with low number of cells that develop during enrichment. Most molecular detection methods, need a cell density of the target pathogen at or above log 3–4 CFU/mL, particularly when limited aliquot transfers from the enrichment culture, typically 2–5 μl, to initiate detection reactions are used (Bennett et al., 1998; Franco et al., 2010; Kisluk et al., 2012). Samples with bacterial populations below the limit of detection (<log 1.5 CFU/25 g), as it is expected after days of exposure to the environment, should consider the length of enrichment or post-enrichment processing, such as IMS or other capture-concentration techniques, when selecting molecular test methods.

Some of the main concerns during the enrichment include competition by background bacteria. Native microbiota associated with vegetables can be initially at greater numbers at sampling and compete for nutrients during enrichment, reaching high cell densities. To limit these competitive interactions, studies have suggested the use of direct selective enrichment to favor the growth of a target pathogen, however this approach has been discouraged because of their low effectiveness in recovering injured cells particularly if they are present in low populations (D'Aoust, Sewell, & Warburton, 1992; Taskila et al., 2012; Wu, 2008). Some alternatives to culture enrichment include immobilization with bacteriophages, magnetic beads, buoyant density centrifugation among others (Reviewed in Hoorfar, 2011). Studies on leafy greens have shown that the population of coliforms and mesophiles can increase up to 6- and 2-log, respectively after 8 h of enrichment (D'Lima & Suslow, 2009) that could potentially affect sensitivity of molecular detection methods. *In vitro* studies have demonstrated that co-culture of human pathogens like *E. coli* O157:H7 and *Salmonella* can be out-competed by coliforms and several other *Enterobacteria* that are part of the phyllopshere (Bennett et al., 1998; Cooley, Chao, & Mandrell, 2006; Cooley, Miller, & Mandrell, 2003; Franco et al., 2010; Lopez-Velasco, Tyddings, et al., 2012), and thus interfere with accurate detection and increasing the chance of obtaining either a false positive or negative outcome. Several efforts using high throughput technologies have demonstrated the enormous diversity on the plant phyllosphere (Delmotte et al., 2009; Lopez-Velasco, Davis, Boyer, Williams, & Ponder, 2010; Rastogi et al., 2012) and therefore the interplay of these complex communities during enrichment with pathogenic bacteria during culture enrichment should be considered.

In this study, the effect of background bacteria competition with *E. coli* O157:H7 and *Salmonella* on molecular detection was demonstrated (Tables 3 and 4). Increasing plant sample mass, ostensibly to improve lot acceptance decision-making while minimizing cost per test, is being implemented (and in some commercial applications put into practice without adequate validation (Suslow, 2013)). One of the obstacles is that the ratio of plant material to enrichment broth needs to be reduced, for practical realities, so the sample can be feasibly handled but still sufficient to cover the plant material during enrichment culture. Initial experiments, in which plant mass was increased and enrichment was performed in presence of rifampicin, did not show a particular trend in the detection outcome and the amount of plant material for *Salmonella* however it did result in a greater rate of positive detection for *E. coli* O157:H7 associated with larger plant mass (Supplementary Tables 1 and 2). Conversely, plant material that was spiked in the laboratory with *Salmonella* or *E. coli* O157:H7 followed by a period of adaptation of up to 18 h at 5 °C and enrichment without any selective agent, resulted in more false negative outcomes by molecular detection in samples with larger sample mass (375 or 125 g) than the control samples (25 g of plant enriched with 225 mL of broth; Table 3). This information suggests that a substantial increment in the amount of plant material employed for a lot-qualifying or investigative detection system could be a factor that can contribute to the frequency of false negative outcomes. These observations may be the result of a reduced threshold number of the targeted pathogen cells after enrichment, likely resulting from competition with background bacteria. Therefore, following manufacturer instructions and validated or process-approved protocols for use, adhering to both the amount of sample and the corresponding ratio with the enrichment broth, is essential. Alternatively, or complimentary to increasing plant mass for improved detection in a lot, composite, liquid pooling or compositing post-enrichment appears an efficient strategy to reduce the number of molecular tests without compromising the sensitivity of the analysis (Table 5).

The rate of false negatives was more pronounced when plant material was inoculated during field production (Table 4A–B); recovery by culture confirmation was observed in 70–100% of the collected samples, however the use of molecular detection

methods with 375 g of samples resulted in 0–30% positive detection and 0–40% for 125 g samples. In each case the result was a lower rate of detection as compared to leaf material taken from the field and inoculated in the laboratory using protocols typical of validation studies (Table 3). These results emphasize the importance of the physiological status of the pathogen prior to the detection-recovery interval, which has not received much attention during the evaluation and validation of most published protocols and suggests that conditioning of the inoculum on the plant material at low temperatures for limited periods might not be sufficient for the expected test kit accuracy. Environmental stress with other technologies including ELISA and IMS, has shown an influence in the reaction success associated with temperature, salt concentrations, and oxidative stress resulting in a reduction of up to 48% of efficiency reaction for both *E. coli* and *Salmonella* (Hahm and Bhunia, 2005). Certainly field inoculation with enteric pathogens to test or validate detection methods is not an available approach for most research groups and it is definitely not suggested as a required strategy, however the physiological status, result of environmental exposure and environmental stress should be strongly considered during method validation. Currently there are a large number of bacterial genomes available that in conjunction with functional genomic tools can provide insight regarding stress response and plant-bacterial pathogen interactions (Abee, van Schaik, & Siezen, 2004; Muller & Ruppel, 2014). In addition, in enteric bacteria, changes in expression of genes associated with stress response or adaptation traits in enteric bacteria inoculated on leafy greens, particularly lettuce and spinach, have been previously reported (Brandl et al., 2013; Carey, Kostrzynska, & Thompson, 2009; Lopez-Velasco, Welbaum, Boyer, et al., 2011; Sharma et al., 2011). Similar studies could assist in induction of gene expression profiles that result from environmental exposure through other stress trigger factors such as heat shock or starvation could prime the inoculum used to validate target cell enrichment prior to molecular detection methods.

5. Conclusions

Molecular detection of pathogenic bacteria in produce can be impacted by multiple factors including the duration of incubation and composition of enrichment media, sample mass and composition, background microbiota and the physiological status of the target microbe. Although this study primarily utilized a Taqman-based system and commercially available BAX® pathogen detection platforms, the study was not intended to specifically compare these methods or to negatively evaluate their efficiency outside of the validated and approved test method specified in user directed technical-information as intended for current commercial application by qualified laboratories. The results of this study did fulfill the purpose of highlighting the importance of validation of molecular detection platforms that include environmental conditions specific and applicable for leafy greens. Similar studies should be performed for any commercial kits that are intended for pathogen detection across a diversity of fresh produce.

Disclosure

This study was not performed with the intent to favor, endorse or discourage the utilization of a particular media or commercial kit.

Acknowledgments

This research was supported by the California Leafy Green Research Program. Authors gratefully acknowledge the time and technical assistance contributed by Alex B. Camacho, Lee Ann Richmond, Eduardo Gutierrez-Rodriguez, Heather Abbott, Tam Vo, Jocelyn Lam, Laura Huang, and Ravi Nakrani for their assistance in field planting, sample collection and processing and to Jim Jackson and Fred Stewart for the field trial management. We deeply appreciate the support of DuPont® that provided the equipment, detection kits and media related to the BAX® system to be able to perform this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2015.01.041.

References

Abee, T., van Schaik, W., & Siezen, R. J. (2004). Impact of genomics on microbial food safety. *Trends in Biotechnology, 22*, 653–660.

Bailey, J. S. (1998). Detection of *Salmonella* cells within 24 to 26 hours in poultry samples with the polymerase chain reaction BAX system. *Journal of Food Protection, 61*, 792–795.

Barak, J. D., Kramer, L. C., & Hao, L. Y. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Applied and Environmental Microbiology, 77*, 498–504.

Batz, M. B., Hoffmann, S., & Morris, J. G., Jr. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *Journal of Food Protection, 75*, 1278–1291.

Bennett, A. R., Greenwood, D., Tennant, C., Banks, J. G., & Betts, R. P. (1998). Rapid and definitive detection of *Salmonella* in foods by PCR. *Letters of Applied Microbiology, 26*, 437–441.

de Boer, E., & Beumer, R. R. (1999). Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology, 50*, 119–130.

Brandl, M. T., Clayton, E. C., & Teplitski, M. (2013). *Salmonella* interactions with plants and their associated microbiota. *Phytopathology, 103*, 316–325.

Carey, C. M., Kostrzynska, M., & Thompson, S. (2009). *Escherichia coli* O157:H7 stress and virulence gene expression on Romaine lettuce using comparative real-time PCR. *Journal of Microbiological Methods, 77*, 235–242.

Cooley, M. B., Chao, D., & Mandrell, R. E. (2006). *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *Journal of Food Protection, 69*, 2329–2335.

Cooley, M. B., Miller, W. G., & Mandrell, R. E. (2003). Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. *Applied and Environmental Microbiology, 69*, 4915–4926.

Curtis, R., 3rd, & Kelly, S. M. (1987). *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor are avirulent and immunogenic. *Infection and Immunity, 55*, 3035–3043.

D'Aoust, J. Y., Pagotto, F., Akhtar, M., Bussey, J., Cooper, C., McDonald, C., et al. (2007). Evaluation of the BAX gel and fluorometric systems for the detection of foodborne *Salmonella*. *Journal of Food Protection, 70*, 835–840.

D'Aoust, J. Y., Sewell, A. M., & Warburton, D. W. (1992). A comparison of standard cultural methods for the detection of foodborne *Salmonella*. *International Journal of Food Microbiology, 16*, 41–50.

D'Lima, C. B., & Suslow, T. V. (2009). Comparative evaluation of practical functionality of rapid test format kits for detection of *Escherichia coli* O157:H7 on lettuce and leafy greens. *Journal of Food Protection, 72*, 2461–2470.

Delaquis, P., Bach, S., & Dinu, L. D. (2007). Behavior of *Escherichia coli* O157:H7 in leafy vegetables. *Journal of Food Protection, 70*, 1966–1974.

Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., et al. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proceedings of the National Academy of Sciences, 106*, 16428–16433.

Franco, W., Hsu, W. Y., & Simonne, A. H. (2010). Survival of *Salmonella* and *Staphylococcus aureus* in Mexican red salsa in a food service setting. *Journal of Food Protection, 73*, 1116–1120.

Gil, M. I., Selma, M. V., Suslow, T., Jacxsens, L., Uyttendaele, M., & Allende, A. (2015). Pre- and post-harvest preventive measures and intervention strategies to control microbial food safety hazards of fresh leafy vegetables. *Critical Reviews in Food Science, 55*, 453–468.

Gutierrez-Rodriguez, E., Gundersen, A., Sbodio, A. O., & Suslow, T. V. (2012). Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *Journal of Applied Microbiology, 112*, 109–118.

Hahm, B. K., & Bhunia, A. K. (2005). Effect of environmental stress on antibody-based detection of *Escherichia coli* O157:H7, *Salmonella enterica* serotype *Enteriditis* and *Listeria monocytogenes*. *Journal of Applied Microbiology, 100*, 1017–1027.

Harris, L. J., Bender, J., Bihn, E. A., Blessington, T., Danyluk, M. D., Delaquis, P., et al. (2012). A framework for developing research protocols for evaluation of

microbial hazards and controls during production that pertain to the quality of agricultural water contacting fresh produce that may be consumed raw. *Journal of Food Protection, 75*, 2251–2273.
Harris, L. J., Berry, E. D., Blessington, T., Erickson, M., Jay-Russell, M., Jiang, X., et al. (2013). A framework for developing research protocols for evaluation of microbial hazards and controls during production that pertain to the application of untreated soil amendments of animal origin on land used to grow produce that may be consumed raw. *Journal of Food Protection, 76*, 1062–1084.
Harris, L. J., & Griffiths, M. W. (1992). The detection of foodborne pathognes by the polymerase chain reaction (PCR). *Food Research International, 25*, 457–469.
Havelaar, A. H., Brul, S., de Jong, A., de Jonge, R., Zwietering, M. H., & Ter Kuile, B. H. (2010). Future challenges to microbial food safety. *International Journal of Food Microbiology, 139*(Suppl. 1), S79–S94.
Hines, E. (2000). PCR-based testing: unraveling the mystery. *Food Quality, 7*, 22–28.
Hitchins, A. D. (2011). The determinacy of reproducibility assessments of qualitative microbial food borne pathogen methods detecting a few microbes per analytical portion. *Food Microbiology, 28*, 1140–1144.
Hoorfar, J. (2011). Rapid detection, characterization, and enumeration of foodborne pathogens. *APMIS, 119*, 1024.
Jacobson, A. P., Gill, V. S., Irvin, K. A., Wang, H., & Hammack, T. S. (2012). Evaluation of methods to prepare samples of leafy green vegetables for preenrichment with the Bacteriological Analytical Manual *Salmonella* culture method. *Journal of Food Protection, 75*, 400–404.
Jarvis, B. (2007). On the compositing of samples for qualitative microbiological testing. *Letters of Applied Microbiology, 45*, 592–598.
Kader, A. A., & Ben-Yehoshua, S. (2000). Effects of superatmospheric oxygen levels on postharvest physioloy and quality of fresh fruits and vegetables. *Postharvest Biology and Technology, 20*, 1–13.
Kase, J. A., Maounounen-Laasri, A., Son, I., Deer, D. M., Borenstein, S., Prezioso, S., et al. (2012). Comparison of different sample preparation procedures for the detection and isolation of *Escherichia coli* O157:H7 and Non-O157 STECs from leafy greens and cilantro. *Food Microbiology, 32*, 423–426.
Kim, S. R., Yoon, Y., Kim, W. I., Park, K. H., Yun, H. J., Chung, D. H., et al. (2012). Comparison of sample preparation methods for the recovery of foodborne pathogens from fresh produce. *Journal of Food Protection, 75*, 1213–1218.
Kisluk, G., Hoover, D. G., Kneil, K. E., & Yaron, S. (2012). Quantification of low and high levels of *Salmonella enterica* serovar Typhimurium on leaves. *LWT – Food Science and Technology, 45*, 36–42.
Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Applied and Environmental Microbiology, 69*, 1875–1883.
Lopez-Velasco, G., Davis, M., Boyer, R. R., Williams, R. C., & Ponder, M. A. (2010). Alterations of the phylloepiphytic bacterial community associated with interactions of *Escherichia coli* O157:H7 during storage of packaged spinach at refrigeration temperatures. *Food Microbiology, 27*, 476–486.
Lopez-Velasco, G., Sbodio, A., Tomas-Callejas, A., Wei, P., Tan, K. H., & Suslow, T. V. (2012). Assessment of root uptake and systemic vine-transport of *Salmonella enterica* sv. Typhimurium by melon (*Cucumis melo*) during field production. *International Journal of Food Microbiology, 158*, 65–72.
Lopez-Velasco, G., Tydings, H. A., Boyer, R. R., Falkinham, J. O., 3rd, & Ponder, M. A. (2012). Characterization of interactions between *Escherichia coli* O157:H7 with epiphytic bacteria in vitro and on spinach leaf surfaces. *International Journal of Food Microbiology, 153*, 351–357.
Lopez-Velasco, G., Welbaum, G. E., Boyer, R. R., Mane, S. P., & Ponder, M. A. (2011). Changes in spinach phylloepiphytic bacteria communities following minimal processing and refrigerated storage described using pyrosequencing of 16S rRNA amplicons. *Journal of Applied Microbiology, 110*, 1203–1214.
Lopez-Velasco, G., Welbaum, G. E., Falkinham, J. O., III, & Ponder, M. A. (2011). Phyllopshere bacterial community structure of spinach (*Spinacia oleracea*) as affected by cultivar and environmental conditions at time of harvest. *Diversity, 3*, 721–738.
Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology and Infection, 137*, 307–315.
Malorny, B., Tassios, P. T., Radstrom, P., Cook, N., Wagner, M., & Hoorfar, J. (2003). Standardization of diagnostic PCR for the detection of foodborne pathogens. *International Journal of Food Microbiology, 83*, 39–48.
McKillip, J. L., & Drake, M. (2004). Real-time nucleic acid-based detection methods for pathogenic bacteria in food. *Journal of Food Protection, 67*, 823–832.
Moyne, A. L., Harris, L. J., & Marco, M. L. (2013). Assessments of total and viable *Escherichia coli* O157:H7 on field and laboratory grown lettuce. *PloS One, 8*, e70643.
Moyne, A. L., Sudarshana, M. R., Blessington, T., Koike, S. T., Cahn, M. D., & Harris, L. J. (2011). Fate of *Escherichia coli* O157:H7 in field-inoculated lettuce. *Food Microbiology, 28*, 1417–1425.
Muller, T., & Ruppel, J. (2014). Progress in cultivation-independent phyllosphere microbiology. *FEMS Microbial Ecology, 87*, 2–17.
Pachepsky, Y. A., Shelton, D. R., Mclain, J. E., Patel, J. R., & Mandrell, R. E. (2011). Irrigation waters as a source of pathogenic microorganisms in produce: a review. *Advance Agronomy, 113*, 73–138.
Poza-Carrion, C., Suslow, T., & Lindow, S. (2013). Resident bacteria on leaves enhance survival of immigrant cells of *Salmonella enterica*. *Phytopathology, 103*, 341–351.
Rastogi, G., Sbodio, A., Tech, J. J., Suslow, T. V., Coaker, G. L., & Leveau, J. H. (2012). Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME Journal, 6*, 1812–1822.
Sbodio, A., Maeda, S., Lopez-Velasco, G., & Suslow, T. V. (2013). Modified Moore swab optimization and validation in capturing *E. coli* O157:H7 and *Salmonella Enterica* in large volume field samples of irrigation water. *Food Research International, 51*, 654–662.
Scallan, E., Griffin, P. M., Angulo, F. J., Tauxe, R. V., & Hoekstra, R. M. (2011). Foodborne illness acquired in the United States- unispecified agents. *Emerging Infectious Diseases, 17*, 16–22.
Sharma, M., Lakshman, S., Ferguson, S., Ingram, D. T., Luo, Y., & Patel, J. (2011). Effect of modified atmosphere packaging on the persistence and expression of virulence factors of *Escherichia coli* O157:H7 on shredded iceberg lettuce. *Journal of Food Protection, 74*, 718–726.
Stevens, K. A., & Jaykus, L. A. (2004). Bacterial separation and concentration from complex sample matrices: a review. *Critical Reviews in Microbiology, 30*, 7–24.
Suslow, T. (2010). *Analysis of the standards for irrigation and Foliar Contact water*. UC Cooperative Extension. Available at http://www.producesafetyproject.org/ Accessed date: May 2014.
Suslow, T. V. (2013). *Personal communication*.
Suslow, T. V., & Schroth, M. N. (1982). Rhizobacteria of sugar beets: effects of seed application and root colonization on yield. *Phytopathology, 72*, 199–206.
Taskila, S., Toumola, M., & Ojamo, H. (2012). Enrichment cultivation in detection of food-borne *Salmonella*. *Food Control, 26*, 369–377.
Theofel, C. G., & Harris, L. J. (2009). Impact of preinoculation culture conditions on the behavior of *Escherichia coli* O157:H7 inoculated onto romaine lettuce (*Lactuca sativa*) plants and cut leaf surfaces. *Journal of Food Protection, 72*, 1553–1559.
Tomas-Callejas, A., Lopez-Velasco, G., Camacho, A. B., Artes, F., Artes-Hernandez, F., & Suslow, T. V. (2011). Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. *International Journal of Food Microbiology, 151*, 216–222.
Weber, C., Stephan, R., Druggan, P., Joosten, H., & Iversen, C. (2009). Improving the enrichment procedure for *Enterobacteriaceae* detection. *Food Microbiology, 26*, 565–572.
Wilson, M., & Lindow, S. E. (1993). Effect of phenotypic plasticity on epiphytic survival and colonization by *Pseudomonas syringae*. *Applied and Environmental Microbiology, 59*, 410–416.
Winfield, M. D., & Groisman, E. A. (2003). Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology, 69*, 3687–3694.
Wood, J. D., Bezanson, G. S., Gordon, R. J., & Jamieson, R. (2010). Population dynamics of *Escherichia coli* inoculated by irrigation into the phyllosphere of spinach grown under commercial production conditions. *International Journal of Food Microbiology, 143*, 198–204.
Wu, V. C. (2008). A review of microbial injury and recovery methods in food. *Food Microbiology, 25*, 735–744.