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Pre-harvest internalization and surface survival of *Salmonella* and *Escherichia coli* O157:H7 sprayed onto different lettuce cultivars under field and growth chamber conditions

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ABSTRACT

Plant genotype has been advocated to have an important role in the fate of enteric pathogens residing in lettuce foliage. This study was therefore undertaken under the premise that different pathogen responses could occur in lettuce cultivars with cultivar selection being one of several hurdles in an overall strategy for controlling foodborne pathogens on field-grown produce. Up to eight lettuce cultivars (‘Gabriella’, ‘Green Star’, ‘Muir’, ‘New Red Fire’, ‘Coastal Star’, ‘Starfighter’, ‘Tropicana’, and ‘Two Star’) were examined in these experiments in which the plants were subjected to spray contamination of their foliage with pathogens. In an experiment that addressed internalization of *Salmonella*, cultivar was determined to be a significant variable ($P < 0.05$) with ‘Gabriella’ and ‘Muir’ being the least and most likely to exhibit internalization of this pathogen, respectively. Furthermore, antimicrobials (total phenols and antioxidant capacity chemicals) could be part of the plant's defenses to resist internalization as there was an inverse relationship between the prevalence of internalization at 1 h and the levels of these antimicrobials ($r = -0.75$ to -0.80, $P = 0.0312$ to 0.0165). Internalized cells appeared to be transient residents in that across all cultivars, plants sampled 1 h after being sprayed were 3.5 times more likely to be positive for *Salmonella* than plants analyzed 24 h after spraying (95% CI from 1.5 to 8.2, $P = 0.0035$). The fate of surface-resident *Salmonella* and *Escherichia coli* O157:H7 was addressed in subsequent growth chamber and field experiments. In the growth chamber study, no effect of cultivar was manifested on the fate of either pathogen when plants were sampled up to 12 days after spray contamination of their foliage. However, in the field study, five days after spraying the plants, *Salmonella* contamination was significantly affected by cultivar ($P < 0.05$) and the following order of prevalence of contamination was observed: ‘Muir’ < ‘Gabriella’ < ‘Green Star’ = ‘New Red Fire’ < ‘Coastal Star’. Nine days after spray contamination of plants in the field, no effect of cultivar was exhibited due primarily to the low prevalence of contamination observed for *Salmonella* (8 of 300 plant samples positive by enrichment culture) and *E. coli* O157 (4 of 300 plant samples positive by enrichment culture). Given the narrow window of time during which cultivar differences were documented, it is unlikely that cultivar selection could serve as a viable option for reducing the microbiological risk associated with lettuce.

1. Introduction

Consumption of fresh produce is advocated as an important component of a healthy and balanced diet. Given that advice and the increased diversity and year-long supply of popular fresh produce items, it is not surprising that within the U.S., consumption of raw or minimally processed vegetables increased by 52.7% from 1976 to 2009 (Cook, 2011). However, accompanying this trend has been an increased number of foodborne disease outbreaks or cases that have been attributed to fresh produce in several developed countries (Callejón et al., 2015). To offer a perspective as to the contribution of this commodity to the overall burden of foodborne outbreaks in different localities, 23% and 21.8% of total outbreaks were attributed to produce in the U.S. and Canada, respectively, (Butler et al., 2016; Jung et al., 2014), whereas only 10% and 4% of outbreaks in Europe and Australia, respectively, were associated with produce (EFSA, 2017; Lynch et al., 2009). A table

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detailing the relevant statistics of these produce-associated outbreaks (specific food vehicle, contaminant pathogen, number of cases and hospitalizations) has been provided in the review of Alegbeleye et al. (2018) along with the reference citation where additional information about the outbreak is published. Evident within that table is the realization that several produce items account for the majority of outbreaks. In particular, it was revealed in the review of Erickson and Doyle (2012) that during the period of 1998–2008, leafy greens, tomatoes, and melons accounted for 56%, 36%, and 17% of fresh-cut produce outbreaks that occurred within the U.S., respectively. Dissecting the data even further, attribution risk rankings of fresh produce-associated outbreaks in the U.S. identified enterohemorrhagic *Escherichia coli* in leafy greens as the leading pathogen-produce vehicle combination, followed by *Salmonella* spp. in tomatoes, and *Salmonella* spp. in leafy greens (Anderson et al., 2011).

Contamination of produce may occur at any point along the farm to fork continuum; however, commercial and retail interventions that may be applied after introduction of the pathogen are limited to reducing the contamination and not eliminating it. The logical solution to this dilemma would therefore be to prevent contamination at any point in the continuum. Sources that have been suggested as vehicles in the preharvest setting include: contaminated irrigation water or soil amendments, fecal wastes from encroaching wildlife, or humans who transfer fecal pathogens from their unwashed hands to produce during cultivation and harvesting operations (Alegbeleye et al., 2018; Doyle and Erickson, 2008). Based on those potential sources, governmental regulations have therefore advocated the implementation of practices that would minimize introduction of pathogens by those pathways (US FDA 2015). Despite these controls, a low incidence of contamination of produce has continued (Silva et al., 2017; Zhang et al., 2018) and implies that many of these pathogens are likely residents in fields, albeit heterogeneously distributed and at very low levels.

Under the premise that foodborne pathogens contaminate produce in the field despite appropriate safeguards, understanding the factors that contribute to the persistence of those pathogens is critical. Chief among those factors are the harsh conditions in natural environments to which pathogens are exposed. Such conditions, including solar radiation/ultraviolet light (Allende et al., 2017; Wood et al., 2010) and desiccation (Brandl and Mandrell, 2002; Liu and Micallef, 2017; López-Gálvez et al., 2018), are likely responsible for the rapid rate of decline seen when plants are initially exposed to high pathogen populations in a low nutrient medium (Bezanson et al., 2012; McKellar et al., 2014). The fate of surviving pathogens after that initial decline, on the other hand, has been much more difficult to predict as their disposition appears to be dependent on a number of interacting physical (e.g., structural morphology within and between plant cultivars (Brandl et al., 2013; Van der Linden et al., 2016)), biological (epiphytic natural microflora on produce surfaces (Klerks et al., 2007)), and chemical factors (constituent antimicrobials (Ruppel et al., 2008) and induced plant defense responses (Jang and Matthews, 2018; Markland et al., 2017; Roy et al., 2013)). Although such host plant-pathogen interactions are complex, a long-term solution may be to breed plants for resistance to colonization by enteric pathogens (Brandl et al., 2013). Nevertheless, in the short term, there already exists a large selection of cultivars that may inherently be less hospitable to contaminant enteric pathogens during cultivation. Supporting this approach, Lopez-Velasco et al. (2015) reported that survival of *Salmonella* and *E. coli* O157:H7 was affected by lettuce cultivar. The study reported in this paper is a continuation of this line of inquiry using cultivars not examined in the Lopez-Velasco et al. (2015) study. More specifically, within a growth chamber system, we examined eight lettuce cultivars for their degree of phyllosphere internalization of *Salmonella*. In addition, in a separate growth chamber trial, the fate of both *Salmonella* and *E. coli* O157:H7 residing either on or within the plant was examined to assist in screening which cultivars would be good candidates to utilize in a subsequent field study that was conducted.

2. Materials and methods

2.1. Pathogen strains and their culture

Pathogenic strains used in growth chamber trials included *Salmonella enterica* (serovar Enteritidis ME 18, unknown origin and serovar Newport 11590K, beef isolate) and *Escherichia coli* O157:H7 (USDA 5, human isolate). Using a calcium chloride heat shock transformation method described by Ma et al. (2011), the *Salmonella* and *E. coli* O157:H7 strains had been labeled with the Clontech Gfpuv green- and the Clontech dsRed red-fluorescent protein (GFP and RFP) plasmids (Mountain View, CA), respectively. Each of these plasmids also contained an ampicillin (Amp)-resistant marker that aided in the detection of the strains.

Virulence attenuated pathogen strains used in the field trial included *Salmonella* Typhimurium χ3985 Δcrp-11, Δcya-12 (courtesy of Roy Curtiss III, University of Florida, Gainesville, FL) and *E. coli* O157:H7 (MD56 and MD58). The *E. coli* O157:H7 strains had been derived from the F4546 (1997 alfalfa sprout outbreak) and the K4492 (2006 lettuce outbreak) isolates, respectively, by knocking out the Shiga toxin and intimin genes (Webb et al., 2014). In addition, the attenuated *Salmonella* and *E. coli* O157:H7 strains had been labeled with the GFP- and RFP-plasmids, respectively.

Frozen cultures of each pathogen strain were thawed and streaked individually onto plates containing tryptic soy agar with 100 μg/ml ampicillin (TSA-Amp). The plates were incubated for 24 h at 37 °C after which individual fluorescent colonies were selected and subjected to two additional passages on TSA-Amp plates. At this point, several bright fluorescent colonies were removed from the final plate and subjected to either solid agar or liquid broth culture. In the case of conditions used for solid agar culture, the selected colonies were used to streak three TSA-Amp plates that were subsequently incubated at 37 °C for 24 h to generate a solid lawn of the isolate. Cells from the three plates were recovered by adding 4 ml of sterile 0.1% peptone water (PW) to each plate and dislodging the cells from the agar with the aid of a sterile loop. The rinse from each plate was poured into a sterile 50 ml centrifuge tube. An additional 4 ml of sterile 0.1% PW was added to each plate to remove the remaining cells and this rinse added to the first. The tube was centrifuged at 4050 ×*g* for 15 min at 4 °C and the cell pellet suspended in sterile 0.1% PW. This suspension was again centrifuged and the pellet re-suspended in 0.1% PW two additional times to wash the cells. The final suspension was adjusted to give an OD_{630} of 0.5 that was equivalent to ca. 10^9 CFU/ml. In the case of conditions used for liquid broth culture, one or more fluorescent colonies were added to tryptic soy broth with 100 μg/ml ampicillin (TSB-Amp). After incubating the mixture for 24 h at 37 °C to give a culture in stationary phase, the cells were recovered by centrifugation (4050 ×*g*, 25 min, 4 °C). At this point, the liquid-broth cultured pellet was washed and adjusted to a final concentration using conditions similar to those described above for the solid agar-cultured strains.

Stock inoculum cocktails were prepared from different combinations of the individual strain suspensions. For growth chamber trials, two cocktails were prepared. The stock cocktail used for an internalization trial contained the two non-attenuated GFP-labeled *Salmonella* strains. The non-attenuated RFP-labeled *E. coli* O157:H7 strain was not included in that cocktail due to the reported stronger induction of plant defenses by *E. coli* O157:H7 than *Salmonella* (Roy et al., 2013). The second cocktail prepared for the trial involving pathogen survival on the plants' surfaces included both non-attenuated GFP-labeled *Salmonella* and RFP-labeled *E. coli* O157:H7. For the field study, the stock cocktail contained all three attenuated strains (the one GFP-labeled *Salmonella* strain and the two RFP-labeled *E. coli* O157:H7 strains). Preparation of the stock inoculum mixtures involved combining equal volumes from each of the pathogen strain suspensions. These stocks were then diluted with sterile deionized water to give the working inoculum mixtures (ca. 5.3–5.8 log CFU/ml). Working mixtures for growth chamber studies were used within 3 h of preparation as the chambers were located near the biosafety laboratory facilities. In contrast for the field study, the field was located 240 km from the laboratory facilities. Hence, to minimize losses during transport, the stock was transported to the field and working mixtures prepared with the sterile deionized water on site.

2.2. Lettuce cultivars, source, and initial cultivation conditions

Seeds for six cultivars of green leafy lettuce (‘Green Star’, ‘Muir’, ‘New Red Fire’, ‘Starfighter’, ‘Tropicana’, and ‘Two Star’) and one cultivar of Romaine lettuce (‘Coastal Star’) were purchased from Johnny's Selected Seeds (Fairfield, ME). In addition, green leafy lettuce seeds for the ‘Gabriella’ cultivar were purchased from New England Seed Company (East Hartford, CT).

All cultivars were used in growth chamber studies with all activities (seed germination, cultivation, and challenge studies) being conducted in chambers having dimensions of 2.8 × 2.8 × 2.0 m. During these activities, the chambers were programmed to deliver a 10-h light cycle at 20 °C and 70% relative humidity and a 14-h dark cycle at 15 °C and 80% relative humidity. Germination involved sowing of seeds into trays containing Miracle-Gro Starting Potting Mix (The Scotts Company, LLC, Marysville, OH) and keeping the material moist. After one to two weeks, the seedlings were transferred to pots (7.6 cm diameter) containing Miracle-Gro Moisture Control Potting mix. Pots were then placed onto the capillary mat surface of 61 × 122 cm waterbed plant irrigation trays (Greenhouse Megastore, Danville, IL). Addition of water to the trays occurred as needed to keep the irrigation tray mat wet and in a state capable of delivering water to the soil within the pots through capillary action. The plants were grown under these conditions for approximately two to three weeks at which time they were used in pathogen challenge studies.

Four green leafy lettuce cultivars (‘Green Star’, ‘Gabriella’, ‘Muir’, and ‘New Red Fire’) and the one Romaine lettuce cultivar (‘Coastal Star’) were used in a field trial. Seeds of these cultivars were initially germinated in Fafard Super Germinating Mix (SunGro Horticulture, Agawam, MA) in greenhouses (22 to 25 °C). The seedlings were then held in these greenhouses for an additional four weeks after which seedlings were transplanted into the field.

The field site was located on the Horticultural Farm on the Tifton campus of The University of Georgia. The field (0.2-ha) was relatively flat (0% to 8% slope) and had been used in previous attenuated pathogen challenge studies (Erickson, 2010a, b, 2013, 2014). To serve as a deterrent for any run-off from the inoculated field, a 10-m wide strip of a pigeon pea (*Cajanus cajan*) crop surrounded the field.

In the field, lettuce seedlings were transplanted as plugs into 30 plots (6 plots/cultivar and 50 seedlings/plot) on raised beds (1.0 m width with 1.5-m alleys between beds). Individual plots (2.0 m length × 0.3 m width) within beds were separated by ca. 0.4 m. Cultivars were assigned to plots such that any one cultivar was found in only one plot in each row down the field and only one plot in each row across the field. Drip tube irrigation was used as the primary means of hydrating the plants.

2.3. Growth chamber challenge trials

2.3.1. Internalization trial

Three weeks after transplanting seedlings into pots, five plants from each cultivar were sampled to determine initial levels of total phenols and antioxidant capacity. In addition, fourteen plants from each of the cultivars were removed and placed in a separate growth chamber for application of the pathogen inoculum. To apply the inoculum to each plant, the potted plant was first placed within a spraying chamber box (open in front and partially at the top) to minimize dispersal of the pathogen within the chamber. The inoculum (3.5 ml) containing the two strains of liquid-cultured GFP-labeled *Salmonella* (Enteritidis ME18 and Newport 11590) at 5.8 log CFU/ml was applied to the plant's foliage as a fine mist (0.10–0.15 ml/squirt) from a distance ca. 5 to 8 cm using a 60-ml plastic Equate Fingertip Sprayer (Wal-Mart Stores, Bentonville, AR). The applied volume was designed to have all exposed surfaces sprayed to the point that the liquid was running off the plant. Immediately after spraying the plants, two plants from each cultivar were sampled for enumeration of total *Salmonella* in the sample. The remaining 96 potted plants were each placed into drip saucers, and thick paper collars placed around the base of each plant to prevent pathogen transfer to the plant's surface from contaminated soil. These plants were then held for up to 24 h on tables within the growth chamber. One and 24 h after spraying the plants with *Salmonella*, 6 plants from each cultivar were sampled for internalized pathogen.

2.3.2. Pathogen survival on plant's surface trial

Seedlings were cultivated for two weeks after transplantation. Five plants from each cultivar were again sampled from this batch to determine initial levels of total phenols and antioxidant capacity. In addition, 32 plants from each of the cultivars were removed and placed in a separate growth chamber for application of the pathogen inoculum. Spray contamination of each plant followed a similar protocol as described above for the internalization trial with the exception that the inoculum at 5.4–5.8 log CFU/ml contained solid-cultured strains of *E. coli* O157:H7 as well as *Salmonella* and the volume of inoculum sprayed on each plant was only 2.0 ml since these plants were smaller. Eight plants from each cultivar were removed from the growth chamber on days 0, 1, 4, and 8 to quantify *Salmonella* and *E. coli* O157:H7.

2.4. Field system challenge trial

Three batches of an attenuated cocktail of liquid broth-cultured *Salmonella* and *E. coli* O157:H7 (5.3–5.4 log CFU/ml) were initially prepared with application of each batch being made to one replicate plot of each cultivar. Two days later, another three batches of the cocktail were prepared at the same concentration with application of each batch being made to another replicate plot of each cultivar. On each inoculation day, two plants were first removed from each plot for chemical analysis. After this sampling operation, each plot received 300 ml of the inoculum as a fine mist with spraying being accomplished using a commercial garden 3.8-L sprayer (Flo Master Garden Sprayer, Lowell, MI). Uniform application was targeted by moving the sprayer head over the plants throughout the plot such that each plant received three to four applications and runoff from the foliage could be visibly seen. One hour after applying the inoculum, three plants were removed from each plot to determine the initial contamination levels of the pathogens. Then five and nine days after plots were contaminated, ten plants were removed from each plot for pathogen analysis. Average minimum and maximum daily temperatures, percent relative humidity, radiation, and evapotranspiration rate during the trial were 10.8 °C, 22.3 °C, 77%, 11.2 mJ/m^2, and 1.3 mm/day, respectively.

2.5. Sampling of plants for pathogen and chemical analyses

In both growth chamber and field studies, the entire phyllosphere tissue of the plant was used as one sample. To obtain this sample, the aerial portion of the plant was first grabbed with a sterile piece of paper towel and then the plant cut at its base, 1 to 2 cm above the soil's surface, using a sterile scissors. The sample, along with the paper towel, was placed in a Whirl-Pak bag (Nasco, Fort Atkinson, WI). The samples were held on ice during transport to the laboratory where they were either immediately analyzed for pathogens or frozen for subsequent chemical analysis.

2.6. Total phenols and antioxidant capacity analyses

Samples destined for chemical analysis were frozen with liquid nitrogen. Once frozen, the tissue was ground to a fine powder using a mortar and pestle and mixed to form as homogeneous a sample as possible. A portion of this sample was then placed in a 50-ml conical centrifuge tube and stored at −20 °C until aliquots (duplicate 0.5 g samples) were removed and extracted with cold 80% methanol. Total phenolic content of these extracts was then measured using the Folin-Ciocalteau assay and expressed as gallic acid equivalents/g tissue (Singleton et al., 1999). Antioxidant capacity of these extracts was also undertaken by measuring its radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and expressing the results as µmol Trolox equivalents/g tissue (Brand-Williams et al., 1995).

2.7. Pathogen analyses

Samples destined for pathogen enumeration were first weighed and then sterile 0.1% PW (4:1 vol/wt) was added to the Whirl-Pak bag. The tissue was pummeled for 1 min at 260 rpm using a Stomacher 400C (Seward Laboratory Systems, Inc., Port Saint Lucie, FL). Portions (0.1 or 0.25 ml) of the extract were removed from the bag directly or from 10-fold dilutions of the extract and applied to TSA-Amp plates for incubation (37 °C, 18–24 h) and subsequent counting of red (*E. coli* O157:H7) or green (*Salmonella*) colonies under UV light. After aliquots had been removed, an equivalent volume of 2× TSB-Amp was added to the Whirl-Pak bag. This broth mixture was incubated at 37 °C for 24 h after which an aliquot (0.1 ml) was spread over a fresh TSA-Amp plate using a sterile plastic loop. This plate was also incubated at 37 °C for 24 h. After this period, the plate was exposed to UV light and the presence or absence of any red- or green-fluorescent colonies indicated a positive and negative result, respectively.

Samples collected at 1 and 24 h after inoculation in the internalization growth chamber trial were immediately processed by first disinfecting the surface with $AgNO_3$ (Erickson et al., 2014). After macerating the tissue in 0.1% PW using a mortar and pestle and rinsing the vessel with additional 0.1% PW, an equivalent volume of 2× TSB-Amp was added to the macerated mixture. At this point, the enrichment culture followed the same protocol as was applied to extracted samples that were enriched.

In anticipation that field samples collected on any day but the day of inoculation would be below the limit of detection for enumeration of pathogens, all the samples were directly enriched (i.e., no sample extraction step). However, before the enrichment medium was added to any one sample, all the samples were first weighed. Then a fixed volume of 1× TSB-Amp, that was at least four times the average weight of those samples, was added to each sample. This broth mixture was then subjected to the same series of steps as described above for enrichment culture and confirmation of the presence or absence of fluorescent colonies on streaked plates.

2.8. Statistical analyses

The StatGraphics Centurion XVI software package (StatPoint, Inc., Herndon, Virginia) was used for all statistical analyses of the data. When differentiating pathogen populations in samples, enumeration data were first converted to log CFU/plant. At that point, the transformed data along with the chemical data was subjected to analysis of variance (ANOVA). When statistical differences were observed by ANOVA ($P < 0.05$), the sample means were differentiated using the least significant difference test. In the growth chamber survival study, differentiation of the data into two groups that were collected for Day 0's plant weights, and *Salmonella* and *E. coli* O157:H7 populations were accomplished through Cluster Analysis and confirmation by ANOVA. When differentiating sample groups as to the prevalence of pathogen contamination, the binary data (presence = 1; absence = 0) was subjected to logistic regression analysis. Variables were considered significant in those analyses when the P value was < 0.05. Simple regression was used to establish any potential relationships ($P < 0.05$) between the average chemical and biological values obtained for the individual lettuce cultivars used in either the growth chamber or field trials. In addition, simple regression was used to determine the relationship between individual plant weights and their initial pathogen populations in the growth chamber survival trial.

3. Results and discussion

3.1. Pre-harvest internalization of Salmonella within growth chambers

Three weeks after transplanting eight lettuce cultivars, the plants in a growth chamber were sprayed with water containing *Salmonella* at 5.8 log CFU/ml. No significant differences between cultivars were observed in the amount of *Salmonella* retained on the plants, the average across all cultivars being 4.4 ± 0.5 log CFU/plant (Table 1). One and 24 h after spraying, six plants of each cultivar were analyzed for internalized *Salmonella*. Across all cultivars, plants sampled 1 h after being sprayed were 3.5 times more likely to be positive for *Salmonella* than plants analyzed 24 h after spraying (95% CI from 1.5 to 8.2, $P = 0.0035$). However, due to the low number of samples analyzed at each of the time periods, no significant differences could be discerned between cultivars at either of these time periods ($P > 0.05$). However by merging the prevalences for *Salmonella* internalization for the two time periods, cultivar was determined to be a significant variable

Table 1
Pre-harvest internalization of *Salmonella* into different lettuce cultivars cultivated in growth chambers.

			0 h PI[a]	1 h PI	24 h PI	
Cultivar	Antioxidant capacity (µg Trolox/g, Mean ± S.D.)[b]	Total Phenols (µg gallic acid/g, Mean ± S.D.)[b]	Total (log CFU/plant[c], Mean ± S.D.)	Internalized (# positive by enrichment culture/# plants sampled)		Odds ratio[d] 1&24 h PI $P = 0.0053$ (95% CI)
Gabriella	130 ± 20d	323 ± 54e	4.3a	1/6	0/6	–
Green Star	60 ± 4bc	277 ± 24cd	4.9 ± 0.3a	4/6	2/6	11 (1 to 118)
Romaine	71 ± 18c	191 ± 19b	4.7 ± 0.4a	5/6	1/6	11 (1 to 118)
New Red Fire	73 ± 7c	241 ± 14c	4.6 ± 0.6a	5/6	3/6	22 (2 to 243)
Starfighter	49 ± 9ab	289 ± 55de	4.0 ± 0.3a	4/6	4/6	22 (2 to 243)
Tropicana	47 ± 8ab	243 ± 37c	3.9 ± 0.5a	4/6	4/6	22 (2 to 243)
Two Star	51 ± 5ab	256 ± 17cd	4.0 ± 0.4a	6/6	3/6	33 (3 to 386)
Muir	43 ± 4a	147 ± 11a	4.7 ± 0.3a	6/6	4/6	55 (4 to 727)

[a] PI = post-inoculation.
[b] Values within a column followed by a different letter are significantly different from each other ($P < 0.05$).
[c] There were no statistical differences in plant weights in response to cultivar and average weight across all cultivars was 5.8 ± 4.8 g/plant.
[d] Odds ratio is relative to the 'Gabriella' cultivar and includes the *Salmonella* prevalence from both 1 and 24 PI.

($P = 0.0053$) with 'Gabriella' being the least likely to exhibit internalization of this pathogen and 'Muir' having the greatest likelihood (Table 1). These results were similar to observations made by Golberg et al. (2011) in which the degree of *Salmonella* internalization varied between different lettuce types that had been immersed in a pathogen inoculum. In that study, the prevalence for internalization of *Salmonella* into iceberg lettuce had been nearly four times that observed in romaine or red leafy lettuce ('Ruby Red' cultivar).

In conjunction with the internalization experiment conducted in this study, plants of each of the eight cultivars were also analyzed for two chemical groups (antioxidant capacity chemicals and total phenols) that in the past have been associated with antimicrobial activity (Friend, 1979). In this study, significant differences in the levels of these chemical groups were found between cultivars (Table 1, $P < 0.05$). Moreover, the levels of antioxidant capacity and total phenols were found to have a significant but inverse relationship to the degree of internalization observed at 1 h by each of the cultivars ($r = -0.80$, $P = 0.0165$ and $r = -0.75$, $P = 0.0312$, respectively) supporting the idea that the chemicals may be a component in the plant's defenses against pathogen invasion.

3.2. Survival of Salmonella and E. coli O157:H7 on lettuce cultivars within growth chambers

In a separate study designed to address the overall survival of both internalized and surface-resident *Salmonella* and *E. coli* O157:H7, plants from each of the eight cultivars were sprayed with water containing 5.8 and 5.4 log CFU/ml, respectively. Despite the plants being of similar ages (three weeks post-transplantation), three of the cultivars ('Muir', 'Romaine', and 'Two Star') were noticeably smaller (average weight of 3.3 ± 4.7 g/plant) than the other five cultivars ('Gabriella', 'Green Star', 'New Red Fire', 'Starfighter', and 'Tropicana', average weight of 17.6 ± 10.2 g/plant). Given that such size disparities between cultivars had not manifested for plants used in the internalization study, it was discovered that the two groups of plants had been transplanted into potting soils from different bags. This unintended consequence highlights that investigators should be cautious in using commercial potting soil due to the possibility that constituents within the matrix affecting growth may vary from batch to batch. Notwithstanding these plant weight differences, the challenge study was still initiated with all cultivars; however, as expected the populations of both *Salmonella* and *E. coli* O157:H7 for each cultivar on Day 0 (Table 2) were significantly correlated to that cultivar's plant size ($r = 0.76$, $P = 0.03$ and $r = 0.74$, $P = 0.03$, respectively). Furthermore, cluster analysis as well as ANOVA of the initial population levels for both pathogens revealed that there were two sub-groups within the large plant cultivar group ($P < 0.05$), one sub-group including the 'Gabriella', 'New Red Fire', and 'Green Star' cultivars and the other sub-group including the 'Starfighter' and 'Tropicana' cultivars. Different plant topologies or adherence characteristics of cultivars within these two sub-groups could account for their differential ability to retain pathogens on their surfaces as has been observed in the study by Hunter et al. (2015). Nevertheless, the pathogen levels in these two sub-groups decreased over the next four days to levels that were indistinguishable on day 4 ($P > 0.05$) and this trend aligns with the previous observation that rates of population decay increased as inoculum level increased (McKellar et al., 2014). However, these results along with observations by Bezanson et al. (2012) (i.e., similar initial decay rates by *E. coli* O157:H7 on lettuce grown at two different sites with different climate and soil conditions) would suggest that the inherent hardiness of the bulk of the pathogen's population is the major determinant in rates of inactivation immediately after inoculation. Furthermore, it is conjectured that these initial decay rates are indicative of the inability for the majority of cells grown in nutrient-rich media to adapt to the harsh environmental and nutrient-poor conditions encountered on plant surfaces (Allende et al., 2017). Hence, the rates are meaningless as they are an experimental artifact that is dictated by the culture conditions and holding conditions to which the inoculum is subjected prior to application to the plants. Instead, the leveling in populations of a specific pathogen which occurred across cultivars suggests that this group is a sub-population that has adapted to each plant's environment. Therefore, to ultimately understand and potentially minimize prevalence of contamination, it is important to focus on the fate of this sub-population. With that focus in mind, plant samples from all cultivars were collected at day 8 when the pathogens in the large plant group were still capable of being detected by enumeration (Table 2) and on day 12, when the pathogens could only be detected through enrichment culture (data not shown). Comparison of population declines by the two pathogens between days 4 and 8 demonstrates that inherent differences to tolerate environmental stresses were minimal with *Salmonella* being slightly more resistant to inactivation than *E. coli* O157:H7 for three of the five cultivars (Table 2). In any event, lettuce cultivar had no significant effect on the populations of either *Salmonella* or *E. coli* O157:H7 on day 8 when the pathogen could still be detected by enumeration (Table 2) nor were there any differences in prevalence of contamination among the cultivars twelve days after initial contamination (85%–100% positive by enrichment culture).

3.3. Survival of Salmonella and E. coli O157:H7 on lettuce cultivars in field systems

Although experimental studies conducted in growth chambers permitted the use of pathogen strains suspected to be virulent, the chamber's size limited the number of plants and cultivars that could be

Table 2
Pre-harvest survival of *Salmonella* and *E. coli* O157:H7 on the phyllosphere tissue of different lettuce cultivars held in a growth chamber[a].

Plant size group	Cultivar	Antioxidant capacity (μg/Trolox/g)	Total phenols (μg gallic acid/g)	*Salmonella* log CFU/plant				*E. coli* O157:H7 log CFU/plant			
				Day 0	Day 1	Day 4	Day 8	Day 0	Day 1	Day 4	Day 8
Large	Gabriella	235 ± 65c	302 ± 73b	5.1 ± 0.2b	4.7 ± 0.3c	3.7 ± 0.3a	3.2 ± 0.3a	4.6 ± 0.2b	4.3 ± 0.3c	3.0 ± 0.5a	1.0 ± 1.4a
	New Red Fire	112 ± 44b	232 ± 43ab	5.0 ± 0.2b	4.2 ± 0.4a	3.7 ± 0.9a	3.7 ± 0.6a	4.6 ± 0.1b	3.7 ± 0.6ab	2.9 ± 1.4a	3.2 ± 1.4a
	Green Star	86 ± 39ab	217 ± 54a	5.0 ± 0.5b	4.4 ± 0.2b	3.7 ± 0.2a	3.4 ± 0.5a	4.5 ± 0.6 b	4.0 ± 0.4bc	2.6 ± 1.2a	1.4 ± 1.9a
	Starfighter	48 ± 22a	197 ± 62a	4.5 ± 0.8a	4.0 ± 0.2a	3.5 ± 0.3a	2.9 ± 0.5a	4.0 ± 0.8a	3.5 ± 0.2a	2.8 ± 0.5a	2.0 ± 1.7a
	Tropicana	49 ± 15a	177 ± 28a	4.1 ± 0.2a	3.9 ± 0.2a	3.9 ± 0.6a	3.5 ± 0.9a	3.6 ± 0.3a	3.5 ± 0.3a	3.0 ± 1.4a	1.9 ± 1.6a
Small	Muir	29 ± 11a	308 ± 211a	3.3 ± 0.6a	2.7 ± 0.8a	2.9 ± 0.6a	nd[b]	2.8 ± 0.6a	1.2 ± 1.4a	1.9 ± 1.2a	nd
	Two Star	36 ± 9a	269 ± 29a	2.9 ± 1.2a	2.4 ± 0.8a	1.5 ± 1.4a	nd	2.4 ± 1.1a	2.0 ± 1.6a	1.0 ± 1.5a	nd
	Romaine	44 ± 6a	174 ± 15a	2.8 ± 0.7a	1.9 ± 0.5a	1.4 ± 1.3a	nd	2.3 ± 0.6a	0.5 ± 0.7a	0.3 ± 0.9a	nd

[a] Mean ± S.D. Values within a column for each plant size group followed by a different letter are significantly different from each other ($P < 0.05$).
[b] nd = not detectable by plate count enumeration.

Table 3
The fate of *Salmonella* and *E. coli* O157:H7 sprayed on different lettuce cultivars grown under field conditions and relationship to potential antimicrobial constituents[a,b].

Lettuce cultivar	Plant wt (g) Day 0	Total phenols (μg gallic acid/g)	Antioxidant capacity (μg Trolox/g)	*Salmonella*			*E. coli* O157:H7		
				log CFU/plant	# positive by enrichment culture/# samples		log CFU/plant	# positive by enrichment culture/# samples	
				Day 0	Day 5	Day 9	Day 0	Day 5	Day 9
Gabriella	11.8 ± 1.8ab	355 ± 131ab	209 ± 67a	4.6 ± 0.2a	26/60	4/60	4.5 ± 0.2a	7/60	1/60
Green Star	14.1 ± 3.7b	455 ± 148b	132 ± 24a	4.7 ± 0.1a	34/60	0/60	4.5 ± 0.1a	1/60	0/60
Muir	12.7 ± 0.7ab	197 ± 105a	122 ± 52a	4.6 ± 0.1a	17/60	0/60	4.5 ± 0.1a	3/60	0/60
New Red Fire	8.5 ± 2.0a	976 ± 252c	1026 ± 228b	4.4 ± 0.2a	34/60	4/60	4.3 ± 0.2a	8/60	0/60
Romaine	21.4 ± 6.7c	360 ± 108ab	144 ± 29a	4.6 ± 0.2a	39/60	0/60	4.5 ± 0.1a	3/60	3/60

[a] An inoculum containing 5.4 ± 0.1 and 5.3 ± 0.1 log CFU/ml of *Salmonella* (GFP-labeled *Salmonella* Typhimurium X3985, attenuated) and *E. coli* O157:H7 (MD56 and MD 58 lacking stx_1, stx_2, and/or stx_{2c} genes), respectively, was sprayed (300 ml) onto 3 plots of each cultivar (ca. 50 plants/plot). Two days later, a freshly prepared inoculum containing 5.2 ± 0.1 and 5.3 ± 0.1 log CFU/ml of *Salmonella* and *E. coli* O157:H7, respectively, was sprayed onto another 3 plots of each cultivar.

[b] Mean ± S.D. Values within a column followed by different letters are significantly different from each other ($P < 0.05$).

compared simultaneously and thus could have prevented the discernment as to whether lettuce cultivar may have influenced the fate of enteric pathogens on those plants. Consequently, two green leafy (‘Green Star ‘and ‘Muir’), one romaine (‘Coastal Star’) and two red leafy (‘Gabriella’ and ‘New Red Fire’) lettuce cultivars were selected for cultivation in a field system. One of the most surprising differences that occurred was the vibrant red leaf color of the ‘New Red Fire’ when grown in the fields compared to just a hint of red color on its leaves when plants were grown in the growth chambers. At the same time, the appearance of the other red leafy cultivar, ‘Gabriella’, in the two systems did not display striking differences. Ultraviolet light, that is known to activate the production of the red-pigmented anthocyanins (Krizek et al., 1998; Tsormpatsidis et al., 2008), contributes very little of the spectrum emitted by growth chamber bulbs, but is a major component of sunlight. Under those contrasting conditions, it is therefore assumed that anthocyanins were the principal component responsible for the red pigmentation in the ‘New Red Fire’ cultivar. Confirmation of that hypothesis is based on the antioxidant activity assay to which anthocyanins contribute (Llorach et al., 2008). Antioxidant activity in field-grown ‘New Red Fire’ plants had 9.2 times the levels found in growth chamber-grown plants, whereas the levels of ‘Gabriella’ in field- and growth chamber systems were similar (Tables 2 and 3). Furthermore, the three remaining cultivars had levels that ranged from 1.5 to 4.2 times higher for plants grown in fields versus growth chambers (Tables 2 and 3).

Another noticeable difference in the field cultivars at the time when they were subjected to spray contamination with the pathogens was their size. This observation was confirmed by the plant weights for the samples taken on Day 0 with the smallest plants being the red leafy cultivars (‘New Red Fire’ and ‘Gabriella’) and the largest being ‘Coastal Star’ (Table 3). The smaller size of the red leafy cultivars was anticipated as it has been previously documented that growth is sacrificed when anthocyanin production is increased (Tsormpatsidis et al., 2008). In any event, the wide range in size of the plants did not significantly affect the load of either *Salmonella* or *E. coli* O157:H7 deposited on the plant initially (Table 3, $P > 0.05$). However, there was a significant inverse relationship between the antioxidant capacity and the initial population of *Salmonella* and *E. coli* O157:H7 ($r = -0.978$, $P = 0.0069$ and $r = -0.965$, $P = 0.0078$, respectively) suggesting that the compounds contributing to antioxidant activity may either directly or indirectly affect the fate of those pathogens. Given that an inverse relationship existed between antioxidant levels of a cultivar and its ability to internalize *Salmonella* (Table 1), it is conceivable that some of the initial, albeit small, differences in population levels between cultivars observed in this field study may be attributed to the disruption of internalization by antioxidant capacity chemicals. In contrast, such a mechanism does not appear to be playing a role in the pathogen's subsequent survival as neither the levels of antioxidant activity chemicals nor total phenols affected the prevalence of plants contaminated with *Salmonella* or *E. coli* O157:H7 five days after the plants were sprayed with the pathogen ($P > 0.05$, Table 3). Meanwhile, at the 5-day time point and across all cultivars, a plant was 12.6 times more likely to be positive for *Salmonella* than for *E. coli* O157:H7 (95% CI from 7.8 to 20.8, $P \leq 0.0001$). Pathogen prevalence in plants continued to decrease (Table 3) with the probability of finding a plant positive for either *Salmonella* or *E. coli* O157:H7 on day 9 than on day 5 being 36.5 and 5.9 times less likely, respectively (95% CI from 17.4 to 76.4, $P \leq 0.0001$ and 1.99 to 17.23, $P = 0.0002$, respectively). Indeed, the low pathogen prevalence observed nine days after plants were contaminated likely contributed to an inability to detect any differences between the cultivars for either pathogen ($P > 0.05$). However, five days after contamination, cultivar was a significant variable in the prevalence of plant contamination by *Salmonella* ($P < 0.05$) with ‘Muir’ exhibiting the least numbers of plants contaminated. Accordingly, the ‘Gabriella’, ‘Green Star’, ‘New Red Fire’, and ‘Coastal Star’ cultivars were 1.9, 3.3, 3.3., and 4.7 times more likely to have a positive plant than the ‘Muir’ cultivar on Day 5 (95% CI from 0.9 to 4.1, 1.5 to 7.1, 1.5 to 7.1, and 2.2 to 10.2, respectively, $P = 0.0004$). The ability for cultivar to influence the fate of *Salmonella* agrees with the field trial conducted by Lopez-Velasco et al. (2015). However, in their trial where plants were sprayed with 5 log CFU/ml, significantly greater populations were recovered from green cultivars (‘Green Romaine’, ‘Tango Green’, and ‘Green Leaf’) than on red-pigmented cultivars (‘Lolla Rosa’ and ‘Red Oak’) for both *Salmonella* and *E. coli* O157:H7 ($P < 0.05$). In this study, only one of the two red leafy cultivars (i.e., ‘Gabriella’) showed reduced survival compared to the green leafy cultivars, suggesting that cultivar color may not be used as a criterion to predict the microbiological risk associated with any one cultivar. Hence, additional studies will need to be conducted to decipher the contribution of physical, chemical, and biological elements associated with each cultivar as to their role in affecting the survival of *Salmonella* and *E. coli* O157:H7. However, given the narrow window of time during which cultivar differences may be documented, it is unlikely that cultivar selection would make an impact on minimizing the microbiological risk associated with lettuce.

3.4. Concluding remarks

Due to the diverse topological, biological, and chemical diversity associated with lettuce cultivars, it has been conjectured that cultivars could be bred with a reduced propensity for enteric pathogens to survive. Although that approach may still be warranted, an alternative approach was to select eight cultivars currently available commercially as subjects for cultivation in growth chambers and fields. After spraying the plants' phyllosphere tissue with water containing either *Salmonella* or *Salmonella* and *E. coli* O157:H7, the 'Gabriella' cultivar was shown to have reduced internalization compared to the other cultivars. An inverse relationship between the level of chemicals exhibiting antioxidant activity and the prevalence of internalized pathogens suggested that this may be one tool used by plants to resist invasion by enteric pathogens although other mechanisms (i.e., number of stomata, pathogenesis-related gene expression, etc.) not explored in this study may also play a role. In regards to overall survival in the phyllosphere tissue, cultivar was not a significant variable when plants were raised in growth chambers. However, lettuce cultivar did exhibit a significant effect on the prevalence of *Salmonella* contamination when the plants were grown in fields and sampled five days after being sprayed with the pathogen. Although the cultivar effect was not significant for *Salmonella* nine days after being sprayed with the pathogen or for *E. coli* O157:H7 on either sampling day, low pathogen prevalence generally occurred and therefore a larger sample pool would have been needed to distinguish differences in response by the pathogens to the different cultivars.

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References

Alegbeleye, O.O., Singleton, I., Sant'Ana, A.S., 2018. Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: a review. Food Microbiol. 73, 177–208.

Allende, A., Castro-Ibáñez, I., Lindqvist, R., Gil, M.I., Uyttendaele, M., Jacxsens, L., 2017. Quantitative contamination assessment of *Escherichia coli* in baby spinach primary production in Spain: effects of weather conditions and agricultural practices. Int. J. Food Microbiol. 257, 238–246.

Anderson, M., Jaykus, L.-A., Beaulieu, S., Dennis, S., 2011. Pathogen-produce pair attribution risk ranking tool to prioritize fresh produce commodity and pathogen combinations for further evaluation (P^3ARRT). Food Control 22, 1865–1872.

Bezanson, G., Delaquis, P., Bach, S., McKellar, R., Topp, E., Gill, A., Blais, B., Gilmour, M., 2012. Comparative examination of *Escherichia coli* O157:H7 survival on Romaine lettuce and in soil at two independent experimental sites. J. Food Prot. 75, 480–487.

Brandl, M.T., Mandrell, R.E., 2002. Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. Appl. Environ. Microbiol. 68, 3614–3621.

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

Brand-Williams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. LWT Food Sci. Technol. 28, 25–30.

Butler, A.J., Pintar, K.D.M., Thomas, M.K., 2016. Estimating the relative role of various subcategories of food, water, and animal contact transmission of 28 enteric diseases in Canada. Foodborne Pathog. Dis. 13, 57–64.

Callejón, R.M., Rodríguez-Naranjo, M.I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M.C., Troncoso, A.M., 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. Foodborne Pathog. Dis. 12, 32–38.

Cook, R., 2011. Tracking demographic and U.S. fruit and vegetable consumption patterns. Available at: http://ucanr.edu/datastoreFiles/234-2795.pdf, Accessed date: 26 June 2018.

Doyle, M.P., Erickson, M.C., 2008. Summer meeting 2007 – the problems with fresh produce: an overview. J. Appl. Microbiol. 105, 317–330.

Erickson, M.C., Doyle, M.P., 2012. Plant food safety issues: linking production agriculture with one health. In: Institute of Medicine (Ed.), Improving Food Safety through a One Health Approach. Workshop Summary. National Academies Press, Washington, DC, pp. 140–175.

Erickson, M.C., Webb, C.C., Díaz-Pérez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L.E., Payton, A.S., Liao, J., Ma, L., Doyle, M.P., 2010a. Infrequent internalization of *Escherichia coli* O157:H7 into field-grown leafy greens. J. Food Prot. 73, 500–506.

Erickson, M.C., Webb, C.C., Díaz-Pérez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L.E., Payton, A.S., Liao, J., Ma, L., Doyle, M.P., 2010b. Surface and internalized *Escherichia coli* O157:H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. J. Food Prot. 73, 1023–1029.

Erickson, M.C., Webb, C.C., Díaz-Pérez, J.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Phatak, S.C., Doyle, M.P., 2013. Internalization of *Escherichia coli* O157:H7 following spraying of cut shoots when leafy greens are regrown for a second crop. J. Food Prot. 76, 2052–2056.

Erickson, M.C., Webb, C.C., Díaz-Pérez, J.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Phatak, S.C., Doyle, M.P., 2014. Absence of internalization of *Escherichia coli* O157:H7 into germinating tissue of field-grown leafy greens. J. Food Prot. 77, 189–196.

European Food Safety Authority (EFSA), 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. European Centre for Disease Prevention and Control. EFSA J. 15, 5077.

Friend, J., 1979. Phenolic substances and plant disease. In: Swain, T., Harbone, J.B., Van Sumere, C.F. (Eds.), Biochemisry of Plant Phenolics. Recent Advances in Phytochemistry. 12. Springer, Boston, MA, pp. 557–588.

Golberg, D., Kroupitski, Y., Belausov, E., Pinto, R., Sela, S., 2011. *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. Int. J. Food Microbiol. 145, 250–257.

Hunter, P.J., Shaw, R.K., Berger, C.N., Frankel, G., Pink, D., Hand, P., 2015. Older leaves of lettuce (*Lactuca* spp.) support higher levels of *Salmonella enterica* ser. Senftenberg attachment and show greater variation between plant accessions than do younger leaves. FEMS Microbiol. Lett. 362, fnv077.

Jang, H., Matthews, K.R., 2018. Influence of surface polysaccharides of *Escherichia coli* O157:H7 on plant defense response and survival of the human enteric pathogen on *Arabidopsis thaliana* and lettuce (*Lactuca sativa*). Food Microbiol. 70, 254–261.

Jung, Y., Jang, H., Matthews, K.R., 2014. Effect of the food production chain from farm practices to vegetable processing on outbreak incidence. Microb. Biotechnol. 7, 517–527.

Klerks, M. M., Franz, E., van Gent-Pelzer, M., Zijlstra, C., van Bruggen, A.H.C., 2007. Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. ISME J. 1, 620–631.

Krizek, D.T., Britz, S.J., Mirecki, R.M., 1998. Inhibitory effects of ambient levels of solar UV-A and UV-B radiation on growth of cv. New Red Fire lettuce. Physiol. Plant. 103, 1–7.

Liu, X., Micallef, S., 2017. Water stress limits the growth of Salmonella on the lettuce leaf surface. In: IAFP Annu. Mtg. Abstract P3-57, Tampa, Florida, July 9–12.

Llorach, R., Martínez-Sánchez, A., Tomás-Barberán, F.A., Gil, M.I., Ferreres, F., 2008. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. Food Chem. 108, 1028–1038.

López-Gálvez, F., Gil, M.I., Allende, A., 2018. Impact of relative humidity, inoculum carrier and size, and native microbiota on *Salmonella* ser. Typhimurium survival in baby lettuce. Food Microbiol. 70, 155–161.

Lopez-Velasco, G., Tomas-Callejas, A., Sbodio, A.O., Pham, X., Wei, P., Diribsa, D., Suslow, T.V., 2015. 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. Food Control 54, 165–175.

Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiol. Infect. 137, 307–315.

Ma, L., Zhang, G., Doyle, M.P., 2011. Green fluorescent protein labeling of *Listeria*, *Salmonella*, and *Escherichia coli* O157:H7 for safety-related studies. PLoS One 6, e18083.

Markland, S.M., Bais, H., Kniel, K.E., 2017. Human norovirus and its surrogates induce plant immune response in *Arabidopsis thaliana* and *Lactuca sativa*. Foodborne Pathog. Dis. 14, 432–439.

McKellar, R.C., Peréz-Rodríguez, R., Harris, L.J., Moyne, A.-l., Blais, B., Topp, E., Bezanson, G., Bach, S., Delaquis, P., 2014. Evaluation of different approaches for modeling *Escherichia coli* O157:H7 survival on field lettuce. Int. J. Food Microbiol. 184, 74–85.

Roy, D., Panchal, S., Rosa, B.A., Melotto, M., 2013. *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL 1344. Phytopathology 103, 326–332.

Ruppel, S., Krumbein, A., Schreiner, M., 2008. Composition of the phyllospheric microbial populations on vegetable plants with different glucosinolate and carotenoid compositions. Microb. Ecol. 56, 364–372.

Silva, B.N., Cadavez, V., Teixeira, J.A., Gonzales-Barron, U., 2017. Meta-analysis of the incidence of foodborne pathogens in vegetables and fruits from retail establishments in Europe. Curr. Opin. Food Sci. 18, 21–28.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 199, 152–178.

Tsormpatsidis, E., Henbest, R.G.C., Davis, F.J., Battey, N.H., Hadley, P., Wagstaffe, A., 2008. UV irradiance as a major influence on growth, development and secondary products of commercial importance in Lollo Rosso lettuce 'Revolution' grown under

polyethylene films. Environ. Exp. Bot. 63, 232–239.
U.S. Food and Drug Administration (FDA), 2015. Final rule: standards for the growing, harvesting, packing, and holding of produce for human consumption. Available at: https://www.gpo.gov/fdsys/pkg/FR-2015-11-27/pdf/2015-28159.pdf, Accessed date: 26 June 2018.
Van der Linden, I., Eriksson, M., Uyttendaele, M., Devlieghere, F., 2016. Is there a relation between the microscopic leaf morphology and the association of *Salmonella* and *Escherichia coli* O157:H7 with iceberg lettuce leaves? J. Food Prot. 79, 1784–1788.
Webb, C.C., Erickson, M.C., Davey, L.E., Payton, A.S., Doyle, M.P., 2014. Construction and characterization of outbreak *Escherichia coli* O157:H7 surrogate strains for use in field studies. Foodborne Pathog. Dis. 11, 893–899.
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. Int. J. Food Microbiol. 143, 198–204.
Zhang, G., Chen, Y., Hu, L., Melka, D., Wang, H., Laasri, A., Brown, E.W., Strain, E., Allard, M., Bunning, V.K., Parish, M., Musser, S.M., Hammack, T.S., 2018. Survey of foodborne pathogens, aerobic plate counts, total coliform counts, and *Escherichia coli* counts in leafy greens, sprouts, and melons marketed in the United States. J. Food Prot. 81, 400–411.