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Natural variation and drought-induced differences in metabolite profiles of red oak-leaf and Romaine lettuce play a role in modulating the interaction with *Salmonella enterica*

Xingchen Liu [a], Yue Li [b], Shirley A. Micallef [a,c,*]

[a] Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, USA
[b] Department of Chemistry and Biochemistry, University of Maryland, College Park, MD, USA
[c] Center for Food Safety and Security Systems, University of Maryland, College Park, MD, USA

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ABSTRACT

Nutrients on produce surfaces are vital for successful enteric pathogen colonisation. In this study, we investigated natural variation in metabolite profiles of Romaine 'Parris Island Cos' and red oak-leaf lettuce 'Mascara' under regular and restricted watering conditions. We also investigated the impact of plant drought stress on the *Salmonella* – lettuce association. *Salmonella* Newport and Typhimurium were able to persist at higher levels on regularly watered Romaine than red oak-leaf lettuce. Drought treatment to lettuce impaired epiphytic *Salmonella* association, with *S.* Newport and Typhimurium being differentially affected. A higher log reduction of both serotypes was measured on drought-subjected red oak-leaf lettuce plants than controls, but *S.* Typhimurium was unaffected on water deficit-treated Romaine lettuce ($p < 0.05$). To assess *Salmonella* interaction with leaf surface metabolites, leaf washes collected from both cultivars were inoculated and found to be able to support *S.* Newport growth, with higher levels of *Salmonella* retrieved from Romaine washes ($p < 0.05$). The lag phase of *S.* Newport in washes from water restricted red oak-leaf lettuce was prolonged in relation to regularly-watered controls ($p < 0.05$). Untargeted plant metabolite profiling using electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) revealed natural variation between Romaine and red oak-leaf lettuce profiles for leaf tissue and leaf washes. Metabolite profile shifts were detected in both lettuce types in response to drought stress, but more unique peaks were detected in red oak-leaf than Romaine lettuce after drought treatment. Variation between the two cultivars was in part attributed to naturally higher levels of flavonoids and anthocyanins in red oak-leaf lettuce compared to Romaine. Moreover, red oak-leaf, but not Romaine lettuce, responded to drought by inducing the accumulation of proline, phenolics, flavonoids and anthocyanins. Drought stress, therefore, enhanced the functional food properties of red oak-leaf lettuce. *Salmonella* growth dynamics in lettuce leaf washes suggested that natural variation and drought-induced changes in metabolite profiles in lettuce could partly explain the differential susceptibility of various lettuce types to *Salmonella*, although the primary or secondary metabolites mediating this effect remain unknown. Regulated mild water stress should be investigated as an approach to lower *Salmonella* contamination risk in suitable lettuce cultivars, while simultaneously boosting the health beneficial quality of lettuce.

1. Introduction

As one of the leading enteric bacterial pathogens, *Salmonella enterica* subsp. *enterica* imposes the highest monetary cost of foodborne illness among 15 monitored foodborne pathogens in the US, exceeding $4 billion in 2018 estimates (Hoffmann and Ahn, 2021). Fruits and vegetables are highly implicated foods in multistate foodborne illness outbreaks in the US and accounted for 46 % of all reported foodborne illness in the US from 1998 to 2008 (Nguyen et al., 2015; Painter et al., 2013). An 8 % increase in outbreaks caused by raw produce was reported during 2010–2013 compared to 1998–2001 (Bennett et al., 2018). The *Salmonella*-produce commodity pair ranked 8th for impact to

* Corresponding author at: University of Maryland, Department of Plant Science and Landscape Architecture, Plant Sciences Building, Rm 2102, 4291 Fieldhouse Drive, College Park, MD 20742, USA.
E-mail address: smicall@umd.edu (S.A. Micallef).

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cost of illness and loss of quality adjusted life years (Morris et al., 2011).

Lettuce (*Lactuca sativa*) is the third most popular vegetable in the US (ERS, 2019a). Average per capita consumption of lettuce was estimated at approximately 5.8 kg year^{-1} using data from 2001 to 2008 (Lin et al., 2016). Consumer preferences have shifted over the past two decades with Romaine lettuce production growing over five-fold (ERS, 2021) while head lettuce dropping by almost 54% between 1997 and 2019 (ERS, 2019b). Simultaneously, leafy greens are the most commonly implicated vegetable associated with foodborne illness outbreaks in the US (Bennett et al., 2018; Interagency Food Safety Analytics Collaboration IFSAC, 2021). Among foodborne pathogens, *Salmonella* Newport was identified as the leading serovar in illnesses associated with salads, lettuce and tomatoes in the European Union (Callejón et al., 2015). Despite the huge economic loss and public health concerns caused by *Salmonella* on fresh produce, the interaction between *Salmonella* and plants remains unclear.

The contamination of produce with human pathogens can happen at any stage between preharvest to postharvest steps (Bennett et al., 2015; Harris et al., 2003). The principal way by which contamination is controlled during cultivation of fresh crops is by avoiding introduction of pathogens into fields and controlled environments, for instance via the implementation of water quality and soil amendment criteria, and animal exclusion. At least 20 % of contamination takes place during the preharvest stage, and once established in the plant niche, enteric pathogens are capable of persisting on harvested product, potentially leading to foodborne illness and outbreaks (Brandl and Mandrell, 2002; Yaron, 2014). Reducing or eliminating human pathogens from the plant microbiome is very challenging (Goodburn and Wallace, 2013; Parish et al., 2003) and no effective strategies that act at the production level exist. *Salmonella enterica* serovars exhibit a differential ability to colonise and grow on fruit and leaf surfaces, which can be partially explained by diversity in plant and bacterial traits that impact attachment and biofilm formation (Berger et al., 2009; Beuchat, 2002; Patel and Sharma, 2010). Therefore, exploring plant characteristics that favour or hinder *Salmonella* association with leaf vegetables could help devise novel solutions to control this human pathogen at the pre-harvest level.

Phytonutrients are crucial for successful bacterial colonisation of plants. Epiphytes depend on nutrients released to the plant surface (Hirano and Upper, 1990; Hirano and Upper, 2000; Mercier and Lindow, 2000). Under favourable environments, bacteria could reach up to 10^7 CFU g^{-1} of leaf (Hirano and Upper, 1990; O'brien and Lindow, 1989). Specifically, *Salmonella* can utilise sugars and amino acids as carbon and nitrogen resources to persist on plant surfaces (de Moraes et al., 2017; Kröger and Fuchs, 2009; Kwan et al., 2015; Megías-Pérez et al., 2020). Plant age, plant anatomical structure and cultivar impact the composition and levels of phytonutrients present and have been shown to mediate *Salmonella* association on produce surfaces (Brandl and Amundson, 2008; Han and Micallef, 2016; Quilliam et al., 2012). Investigating cultivar difference in metabolite profiles could help explain cultivar susceptibility to foodborne pathogens.

Simultaneously, plants deal with adverse conditions by accumulating primary and secondary metabolites which provide defense against pathogens and herbivores and protect against oxidative damage generated by abiotic stresses (Kaur and Ganjewala, 2019; Zaynab et al., 2018). Controlled elicitation of primary and secondary metabolites is a new approach being explored for enhanced flavour, nutrition and for medicinal and beneficial value of crops (Thakur et al., 2019). This approach has potential to also enhance food safety (George and Brandl, 2021) but research is still needed in this area. Drought is one of the most severe stresses plants encounter. Lettuce responds to this stress by accumulating the amino acid proline and secondary metabolites including several polyphenols (Rajabbeigi et al., 2013). How the drought stress response in lettuce affects the lettuce-*Salmonella* association has not been explored. Thus, in this study we set out to investigate how natural variation and drought influenced the lettuce leaf and surface metabolome and levels of specific phytochemicals in two common lettuce types, Romaine and red oak-leaf lettuce. Moreover, we assessed how the lettuce response to drought impacted the epiphytic association of two *Salmonella enterica* serovars.

2. Material and methods

2.1. Lettuce growth and drought treatment condition

Lactuca sativa L. var. *longifolia* cv. 'Parris Island Cos' (Romaine) and *L. sativa* var. *crispa* cv. 'Mascara' (loose-leaf, red oak-leaf type) were used in this study. Lettuce seeds (Territorial Seed Co., OR, USA) were germinated in the potting mix soil (Sunshine LC1, Sungro Horticulture, Canada) at the Research Greenhouse Complex, University of Maryland, and grown under controlled light and temperature conditions (16 h light: 8 h dark photoperiod at 23 °C day and 18 °C night temperature). Irrigation was applied to the holding tray daily, ensuring no water came in contact with leaves. Four-week-old lettuce plants were subjected to water stress by restricting irrigation for 6 days or were continued to be watered regularly (controls) prior to experimentation. At the end of the watering treatment, lettuce plants were all 34 days old for subsequent analyses.

2.2. Lettuce leaf tissue extract and surface wash sample preparation

Whole lettuce leaves from both cultivars were flash frozen using liquid nitrogen and then ground into powders using a pestle and mortar and weighed immediately. Ground tissue was ready for proline and anthocyanin measurements. For phytochemical analyses, 200 mg of ground tissue was accurately weighed and extracted with 1.5 ml 70 % methanol (Sigma-Aldrich, MO, USA) with 0.5 % formic acid (VWR, PA, USA) by vortexing at maximum speed for 20 min at room temperature, then centrifuged at 500 ×*g* for 10 min to collect the supernatant. The supernatants were frozen at −20 °C until further analysis. Whole plant washes were collected to capture leaf surface compounds by placing whole lettuce plants in Whirl-pak bags (Nasco, Wi, USA) immersed in 30 ml 5 % methanol/water solution while leaving the root ball out of the bag and shaken at 150 rpm for 24 h at room temperature. Solutions containing leaf surface compound were filter-sterilised through 0.2 μm cellulose acetate syringe filters (VWR) for microbial growth evaluation and metabolite profiling.

2.3. Salmonella enterica inoculum preparation

Salmonella enterica serovar Newport, an environmental isolate collected from an irrigation pond that matched a recurring tomato outbreak strain (Greene et al., 2008) and *Salmonella* serovar Typhimurium, an isolate recovered from river water in Maryland (Callahan et al., 2019), were used in this study. Both *S.* Newport and *S.* Typhimurium had been adapted to rifampicin (rif) (MilliporeSigma, MA, USA) and were maintained at −80 °C in Brucella broth (BD, MD, USA) containing 15 % glycerol (VWR) and 80 μg ml^{-1} rifampicin and revived by streaking onto Trypticase Soy Agar (TSA, BD) plates amended with 50 μg ml^{-1} rifampicin (TSA-rif) overnight at 35 °C. Inocula of *S.* Newport or *S.* Typhimurium were made by suspending fresh bacterial colonies in 0.1 % peptone water (PW, BD) to an $OD_{600} = 0.5$, to obtain a cell density of ~10^8 CFU ml^{-1}. The growth of *Salmonella* in plant washes was only evaluated using *Salmonella* Newport. Actual inoculum level for *S.* Newport or *S.* Typhimurium was determined by plating serial dilutions onto TSA-rif plates for direct quantification prior to plant or wash inoculation.

2.4. Lettuce surface and wash inoculation with S. enterica

The third or fourth true leaf of either Romaine or red oak-leaf lettuce under both watering conditions was gently marked on the stalk with a black marker. One hundred μl aliquots of *S.* Newport or Typhimurium

suspension were applied to the leaf, avoiding the midrib, by pipetting 10 drops on the abaxial side of each marked leaf. Plants were kept still to allow the droplets to air dry without running off the leaf. An estimated total inoculum dose of 10^6 CFU was inoculated per leaf (Table 1). Inoculated lettuce leaves were kept at room temperature (20 °C) and harvested 24 h post-inoculation (hpi). Each leaf was clipped off the stem aseptically with scissors and placed in a sterile Whirl-pak bag. Inoculated leaves were then immersed in 30 ml 0.1 % PW, hand massaged for 30 s and sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonics Corporation, CT, USA) for 1 min at maximum speed to dislodge *Salmonella* cells from the lettuce leaf surfaces, then shaken at 150 rpm for 10 min. Serial dilutions from each leaf rinsate were plated on TSA-rif plates for S. Newport or Typhimurium enumeration after 24 h of incubation at 35 °C.

For growth assessment in leaf washes, 20 µl of *S.* Newport inoculum were added to 2 ml sterile lettuce leaf wash to achieve an initial concentration of *Salmonella* Newport at ~10^4 CFU ml^{-1} and then incubated at 35 °C at 150 rpm in a shaking incubator. *S.* Newport cell counts were recorded at 0, 2, 4, 6 and 24 hpi. At each time point, serial dilutions were plated on TSA-rif plates for enumeration. Sterile 5 % methanol (2 ml) inoculated with 20 µl S. Newport served as the negative control.

2.5. Metabolite profiling of lettuce leaf extracts and washes using electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS)

Metabolite profiling was conducted on lettuce leaf tissue and wash samples. One ml aliquots of lettuce leaf extracts and 30 ml filter-sterilised leaf wash solutions were lyophilised into powder and resuspended in 100 µl 70 % methanol and 0.5 % formic acid. To ensure all compounds were fully dissolved in the extraction liquid, all tubes were sonicated in a water bath for 2 h at maximum intensity and then transferred into vial inserts for ESI-MS analysis.

A Time-of-Flight mass spectrometer (AccuTOF, JEOL, USA, Inc., Peabody, MA, USA) equipped with an ESI ion source was used for mass spectrometric analysis of the lettuce tissue extracts. Mass spectra were acquired in positive and negative modes at a rate of one spectrum s^{-1} (m/z range of 50–800 Da), averaging mass spectra over ~1 min. The AccuTOF MS settings were as follows: needle voltage = 2100 V, orifice 1 temperature = 100 °C, orifice 1 = 30 V, orifice 2 = 5 V, ring = 10 V. The desolvating chamber temperature was set at 250 °C, and the flow rates of the nebulizing and desolvating gases were 0.6 and 3.0 l min^{-1}, respectively. The sample injection volume was 10 µl with a flow rate of 0.25 ml min^{-1}. Ten µl methanol were injected prior to and following the analysis of each sample and measurement taken to monitor possible contaminants or carryover. Calibration for exact mass measurements was accomplished using 5 mM caesium iodide as the internal standard. For all samples, measurements were repeated at least twice to ensure reproducibility and data from the second injection were used.

Table 1
Counts of *Salmonella enterica* on Romaine and red oak-leaf lettuce leaf surfaces grown under regular watering (CON) and irrigation withholding (DR) conditions, retrieved after 24 h of bacterial inoculation. Statistically significant differences in *Salmonella* counts are denoted by letters; small letters indicate differences by treatment within the same cultivar (Student's *t*-test, $p < 0.05$), capital letters denote differences by cultivar within the same watering treatment (Student's *t*-test, $p < 0.05$). Values are means of replicates ± standard error (SE), with units of log CFU/ plant.

	S. Newport (log CFU/plant)		*S.* Typhimurium (log CFU/ plant)	
Watering condition	Inoculum	Decline†	Inoculum	Decline†
Romaine lettuce 'Parris Island Cos'				
CON	5.7–5.9	0.7 ± 0.3 a, A	6.0–6.5	1.4 ± 0.5 a, A
DR		1.4 ±0.4 a[y], A		1.9 ± 0.5 a, A
Red oak-leaf lettuce 'Mascara'				
CON	5.7–6.0	2.0 ± 0.2 a, B	6.0–6.5	2.5 ± 0.4 a, A[z]
DR		2.7 ± 0.2 b, B, ‡		3.4 ± 0.2 b, B, ‡

† Data represent the average log CFU/plant reduction between initial inoculum and 24 h retrieval [log (initial inoculum count/retrieval count)] in at least four independent experiments with at least four replicates. [y] $p < 0.1$ for the comparison CON vs. DR for Romaine; [z] $p < 0.1$ for the comparison CON vs. CON between two cultivars. The log decline on red oak-leaf plants under water restriction was higher for *S.* Typhimurium than *S.* Newport ($p < 0.05$, denoted by ‡).

2.6. Proline measurement of lettuce leaves

Lettuce ground tissue (200 mg) was mixed with 1.5 ml of 3 % aqueous sulfosalicylic acid (Avantor, PA, USA) in a 2 ml microcentrifuge reaction tube. Samples were fully vortexed and centrifuged at 6800 ×*g* for 30 min at 4 °C. Extract supernatant (300 µl) was mixed with 300 µl ninhydrin (Avantor) and 300 µl glacial acetic acid (VWR Chemicals, BDH) in 2 ml reaction tubes, vortexed thoroughly, incubated at 90 °C on a digital heat block (VWR, PA, USA) for 1 h then placed on ice for 5 min. Toluene (VWR Chemicals, BDH) (900 µl) was added to the reaction tube and thoroughly mixed before going through centrifugation at 5600 ×*g* for 5 min and 200 µl of supernatant transferred to a microplate reader (Synergy HTX; Biotek, VT, USA) for absorption measurements at 520 nm. Pure proline (Avantor) was used to make a standard curve and results were expressed as mg proline/g of leaf ground tissue according to the standard curve (Bates et al., 1973).

2.7. Antioxidant capacity and phytochemical analyses of lettuce leaf extracts

2.7.1. Antioxidant capacity

Modifications were made to the method previously described by Stratil et al. (2006). ABTS solution was prepared by dissolving 31.7 mg 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (TCI, OR, USA) and 8.6 mg $K_2S_2O_8$ (potassium persulfate; (Aldon Corp SE, NY, USA)) in 10 ml water and allowed to stand at room temperature in the dark for 16 h to form a stable radical. The solution was diluted to an absorption of ~1.3 at 734 nm and 100 µl aliquots prepared, to which 20 µl sample extracts were added and allowed to stand for 15 min. Absorption was measured at 734 nm using a microplate reader. A standard curve from the blank-corrected absorption at 734 nm of Trolox (TCI, OR, USA) was plotted and antioxidant capacity of lettuce leaves was calculated as Trolox equivalent.

2.7.2. Total flavonoids

The method was adapted from Zhishen et al. (1999). Aliquots of 100 µl leaf extracts were added to 20 µl 5 % $NaNO_2$ (Avantor, PA, USA). After 5 min, 20 µl 10 % $AlCl_3$ (Alfa Aesar, MA, USA) were added, followed by 100 µl 1 M NaOH (VWR, PA, USA) 1 min later. Absorption of the mixture was measured at 510 nm on the microplate reader. A standard curve from the blank-corrected absorption at 510 nm of the catechin standard (Enzo Life Sciences, NY, USA) was plotted and total flavonoid content was calculated as catechin equivalent (Ketnawa et al., 2020) using the standard curve.

2.7.3. Total phenolic content

Using a method adapted from previous publications (Kao et al., 2014; Singleton and Rossi, 1965), 80 µl lettuce leaf extracts were mixed in a 96-well plate with 90 µl 10 % Folin Ciocalteu reagent (MP Biomedicals, CA, USA), then 40 µl 700 mM Na_2CO_3 (VWR Chemical, PA, USA) added. Samples were vortexed in the microplate reader and allowed to stand for 1 h prior to absorption measurements at 765 nm. A standard curve from the blank-corrected absorption values at 765 nm of the gallic acid (Acros, Organic Fair Lawn, NJ, USA) standard was plotted and total phenolics were calculated as gallic acid equivalents.

2.7.4. Anthocyanin measurement

This method was modified from Neff and Chory (1998). Lettuce tissue powder (200 mg) was mixed with 300 μl methanol (VWR Chemicals) with 1 % (v/v) HCl (VWR Chemicals) and incubated overnight at 4 °C in the dark. To each lettuce sample tube, 200 μl of water and 500 μl of chloroform (VWR Chemicals) were added and tubes centrifuged at 6800 ×*g* for 5 min at room temperature. The supernatant was transferred into a new tube and 60 % methanol containing 1 % (v/v) HCl was used to adjust the volume to 800 μl. Aliquots were transferred to 96-well plates for absorption measurements at 530 nm and 657 nm using 60 % methanol-1 % HCl as blank. Anthocyanins were calculated based on the following equation:

$$\text{Anthocyanins} = (Ab_{530} - Ab_{657})*1000*\text{powder weight (mg)}^{-1}$$

2.8. Statistical analysis

Independent experiments were repeated with new sets of plants and freshly prepared bacterial inocula. Treatments were plants subjected to regular watering or water restriction, as previously described. Each experiment was repeated at least three times with 3–10 plants in each treatment, depending on assay and date of experiment. Data from repeated experiments were pooled for statistical analysis. Bacterial counts were taken in duplicate and averaged. The retrieval of *S. enterica* Newport and Typhimurium count data on lettuce leaf surface in CFU/plant and *S. enterica* Newport data in lettuce washes in CFU ml^{-1} were log transformed prior to Student's *t*-test. Proline, antioxidant capacity, phytochemical measurements and anthocyanins were analyzed by one-way analysis of variance (ANOVA). One-way ANOVA and Tukey's HSD and Student's *t*-test were conducted using JMP Pro ver. 15.2.0 (SAS Institute Inc., Cary, NC).

To explore relationships among lettuce samples, ordination of the metabolite profiles acquired from ESI-MS was performed using multi-dimensional scaling (MDS). Briefly, ESI-MS data consisting of the *m/z* ratio and relative signal intensity of each compound were imported into RStudio (version 1.3.1093). Distance matrices were generated using the Bray-Curtis dissimilarity coefficient using relative peak with the vegan package (version 2.5-7) and MDS analysis was performed in JMP Pro ver. 15.2.0. For significance testing of sample data, analysis of similarity (ANOSIM) was conducted in PRIMER 6 (Plymouth Routines in Multivariate Ecological Research version 6.1.15; PRIMER-*E*-Ltd., Plymouth, United Kingdom) using Bray-Curtis or Jaccard (based on presence/absence of peaks) distance matrices. The *R* statistic in ANOSIM ranges from 0 to 1, with a value closer to 0 when samples from different treatments are similar and a value closer to 1 when replicates within a treatment are closer to each other than to samples from other treatments.

3. Results

3.1. Epiphytic persistence of Salmonella on leaf surfaces of regularly watered lettuce was dependent on cultivar

Decline of *S.* Newport and *S.* Typhimurium populations after 24 h compared to inoculum was measured for both cultivars under a regular watering regime. A larger *S.* Newport log reduction of 2.0 log CFU/plant was detected on red oak-leaf 'Mascara' compared to 0.7 log CFU/plant reduction on Romaine 'Parris Island Cos' ($p < 0.01$). The discrepancy was also apparent for *S.* Typhimurium, which decreased by 2.5 log CFU/plant on red oak-leaf compared to 1.4 log CFU/plant on Romaine lettuce ($p = 0.07$) (Table 1). On both cultivars under regular watering conditions, *S.* Newport declined less than *S.* Typhimurium, however, the differences were not statistically significant. The results indicate that the Romaine leaf surface was a more supportive niche for *Salmonella* than the red oak-leaf surface, and the effect could be serotype specific.

3.2. Salmonella persistence on lettuce leaf surfaces was influenced by drought

The population of *S.* Newport declined more on drought-subjected Romaine lettuce leaf surfaces than on regularly watered Romaine, with a 0.7 log CFU/plant difference in log reduction ($p = 0.06$) (Table 1). The decline of *S.* Newport was also higher on drought-exposed red oak-leaf lettuce than on control plants and a 0.7 log CFU/plant difference in log reduction was measured between the two watering conditions ($p < 0.01$). No statistical difference was found between the log reduction of *S.* Typhimurium on Romaine lettuce grown under different watering conditions (Table 1). By contrast, drought-exposed red oak-leaf lettuce supported lower counts of *S.* Typhimurium than regularly watered plants with a discrepancy in log reduction of 0.9 log CFU/plant ($p < 0.05$) (Table 1).

Cultivar comparison for plants grown under water restriction showed the same trends as for regularly watered plants. After 24 h, the population of both serotypes showed less decline on drought-treated Romaine than red oak-leaf lettuce, with a 1.3 and 1.5 log CFU/plant discrepancy in log reduction between the two cultivars for *S.* Newport ($p < 0.01$) and Typhimurium ($p < 0.05$), respectively (Table 1). Comparing serotype behaviour on the same cultivar under drought treatment, no statistical difference in population decline between the two serotypes was noted on Romaine lettuce. By contrast, a discrepancy of 0.7 log CFU/plant reduction was measured between *S.* Typhimurium and *S.* Newport on red oak-leaf lettuce under drought, with greater log decline observed for *S.* Typhimurium ($p < 0.05$) (Table 1). Taken together, the results suggest that plants under drought provide a less favourable niche for *Salmonella* and the effect is cultivar-dependent, being more prominent on red oak-leaf than Romaine lettuce and varies by serotype.

3.3. Growth of Salmonella Newport in leaf surface washes was affected by cultivar and drought over a 24 h period

Growth data of *Salmonella* Newport in plant surface washes at 2, 4, 6 and 24 h were collected and growth of *Salmonella* was calculated based on the population level change in relation to the corresponding 0 h samples. Wash samples collected from both red oak-leaf and Romaine lettuce were able to support the growth of *Salmonella* Newport over 24 h under both watering conditions, with lower levels of *Salmonella* reported in red oak-leaf lettuce washes (both $p < 0.05$; Table 2). A cultivar-dependent effect was detected in *Salmonella* Newport growth curve progression. In washes from control plants, *Salmonella* growth was noted after 2 h in red oak-leaf washes, but after 4 h in Romaine washes. At the 24 h time point, population level change was higher in Romaine than red oak-leaf by 1.4 log CFU ($p < 0.05$; Table 2).

Subjecting plants to drought had an impact on *Salmonella* growth in red oak-leaf leaf washes, but less so on Romaine lettuce. Little growth was detected in washes from drought-subjected red oak-leaf before the 24 h timepoint and *Salmonella* population levels differed from controls at all timepoints ($p < 0.05$ for 2, 4 and 6 h and $p = 0.09$ at 24 h). This apparent longer lag phase was also apparent in Romaine washes, with a weak difference at 2 h ($p = 0.08$), but the lag did not result in a difference in population levels compared to controls at 24 h (Table 2).

3.4. Metabolite profiles of lettuce leaf tissue and surface wash were distinct and differed by cultivar

The differential *Salmonella* response to Romaine and red oak-leaf lettuce under different watering regimes prompted us to conduct untargeted metabolite profiling of leaf tissue and wash samples to explore leaf tissue and leaf surface metabolome shifts that could explain the *Salmonella*-leaf interaction. Altogether, 1008 peaks were detected in positive mode and 469 peaks were detected in negative mode (Table 3). The two cultivars yielded an approximate number of peaks. In Romaine lettuce, more peaks were found to be in common between tissue and

Table 2
Counts of *Salmonella enterica* Newport in Romaine and red oak-leaf lettuce leaf surface washes grown under regular (CON) and restricted (DR) watering conditions taken 2-, 4-, 6- and 24-h post-inoculation and reported as change from 0 h. Small letters indicate differences by treatment within same cultivar and timepoint and capital letters denote differences by cultivar within same watering treatment ($p < 0.05$). Values are means of replicates ± standard error (SE), with units of log CFU ml^{-1} of leaf wash.

	Salmonella Newport log change†			
Timepoint/ h	2	4	6	24
Romaine lettuce 'Parris Island Cos'				
Control	−0.01 ± 0.12 a, A	0.20 ± 0.10 a, A	0.55 ± 0.21 a, A	**3.71 ± 0.30** a, A
Drought	**−0.25 ± 0.11** a[y], A	**0.09 ± 0.11** a, A	**0.30 ± 0.20** a, A	**3.64 ± 0.27** a, A
Red oak-leaf lettuce 'Mascara'				
Control	**0.11 ±0.03** a, A	0.31 ± 0.07 a, A	0.61 ± 0.18 a, A	**2.30 ± 0.22** a, B
Drought	**0.02 ± 0.04** b, B	**0.14 ± 0.04** b, A	**0.02 ± 0.10** b, A	**1.80 ± 0.29** a[z], B

† Data represents the average log CFU ml^{-1} difference from count at 0 h in at least three independent experiments with at least four replicates. The mean count at 0 h was 3.20 ± 0.06 log CFU ml^{-1}. Bold type denotes counts that are significantly different from 0 h count for that treatment. [y] $p < 0.1$ for the comparison CON vs. DR for Romaine. [z] $p < 0.1$ for the comparison CON vs. DR for red oak-leaf.

Table 3
Number of metabolite peaks acquired from leaf tissue and wash samples from Romaine and red oak-leaf lettuce plants grown under regular (CON) or restricted (DR) watering regimes based on phytochemical profiling with ESI-MS under positive and negative modes.

ID	Sample type	Total peaks detected	Shared peaks		Treatment/ unique peaks	
			Leaf-wash	CON-DR	CON	DR
Peaks detected in positive ionization mode						
Romaine	Leaf	125	15	71	35	19
Romaine	Wash	353		287	55	13
Red oak-leaf	Leaf	120	46	56	28	36
Red oak-leaf	Wash	410		220	122	68
Peaks detected in negative ionization mode						
Romaine	Leaf	129	66	122	7	0
Romaine	Wash	147		118	20	9
Red oak-leaf	Leaf	110	7	55	35	20
Red oak-leaf	Wash	83		44	24	15

wash samples than for red oak-leaf lettuce (81 versus 53). Within each cultivar, several peaks were shared between plants grown under different watering regimes. However, unique peaks by watering treatment (detected only in control or water restricted plants) were also abundant (Table 3).

The most distinct divergence was detected between tissue and surface metabolite profiles, regardless of cultivar (Fig. 1). The global R statistics acquired from ANOSIM for sample type (leaf tissues versus leaf surface) were 0.26 for positive and 0.35 for negative mode (both $p < 0.001$), respectively (Table 4).

Comparing the two cultivars, leaf tissue profiles of Romaine were distinct from red oak-leaf lettuce profiles for plants under both regular and restricted watering (both $R = 1$, $p < 0.01$ in positive and negative mode; Table 4, Fig. 1). Differences in leaf surface metabolite profiles obtained from Romaine and red oak-leaf leaf washes were also detected in positive and negative modes (both $R = 1$, $p < 0.01$; Table 4; Fig. 1). Twenty peaks were shared between the cultivars in positive mode and 27 in negative mode.

3.5. Drought induced shifts in leaf tissue and leaf surface metabolomes

ANOSIM analysis supported metabolite profile shifts in Romaine and red oak-leaf lettuce tissue in response to water deficit under both positive and negative modes (Fig. 1A). Leaf tissues of Romaine lettuce under positive mode yielded 125 peaks and while 71 of these were detected in samples under both regular watering and drought-subjected plants, 19 were detected only in plants under restricted watering and 7 were unique to controls (Table 3). Nonetheless, ANOSIM based on the presence or absence of peaks was not significant for Romaine leaf tissue under negative mode and the difference between the treatments was attributed mostly to variations in levels of shared compounds (Table 4). Similarly, under positive mode detection, the response of red oak-leaf tissue profile to drought was also likely driven by changes in levels of compounds, since no significant difference to control plants was detected using the Jaccard similarity coefficient. By contrast, in negative mode, changes in the presence or absence of peaks between treatments contributed to leaf tissue profile divergence ($R = 0.31$, $p < 0.05$) (Table 4).

Surface metabolite profiles of Romaine lettuce plants under different watering conditions diverged mostly prominently under positive ($R = 0.38$, $p < 0.05$; Fig. 1A) compared to negative mode ($R = 0.24$, $p = 0.07$; Tables 3 and 4, Fig. 1B). Metabolite profiles of wash samples from drought-exposed red oak-leaf lettuce were markedly different to profiles from regularly watered lettuce under both positive ($R = 0.59$, $p < 0.05$) and negative ($R = 0.21$, $p < 0.01$; Table 4) modes, and in both cases, both relative amounts and presence/absence of peaks contributed to the profile shifts detected.

Taken together, results suggest that metabolite profile differences between the two lettuce cultivars were greater than the variations induced by drought within the same cultivar. ESI-MS in positive mode was more sensitive in identifying changes in metabolite profiles in lettuce. Importantly, metabolite profile shifts were identified in both leaf tissue and on leaf surfaces. The surface metabolome (in leaf wash) of red oak-leaf was more strongly influenced by drought than Romaine lettuce.

3.6. Proline, phytochemical compounds and antioxidant capacity varied by lettuce cultivar and were induced by drought treatment in red oak-leaf lettuce

Proline and various phytochemicals were measured in leaf tissue extracts to better explain the metabolome shifts revealed in Romaine 'Parris Island Cos' and red oak-leaf lettuce 'Mascara'. Proline and phytochemical levels varied by cultivar. Proline content was significantly higher in red oak-leaf lettuce than in Romaine lettuce grown under both regular and restricted watering ($p < 0.05$) (Fig. 2A). Red oak-leaf lettuce under regular watering condition also showed significantly higher antioxidant capacity (Fig. 2B; $p < 0.05$) and accumulated more flavonoids (Fig. 2C; $p < 0.05$) than Romaine lettuce. Red oak-leaf lettuce proline (Fig. 2A), flavonoids (Fig. 2C), phenolics (Fig. 2D) and anthocyanins (Fig. 2E) were induced by drought (all $p < 0.05$ compared to control), and all phytochemicals and antioxidant capacity were higher than Romaine lettuce measurements under drought treatment (Fig. 2A–D, all $p < 0.05$). Water restriction only had an impact on the phytochemical content and proline accumulation of red oak-leaf lettuce. Total flavonoid and phenolic levels of Romaine lettuce were unaffected by drought treatment, resulting in an enhanced difference in phytochemical levels between Romaine and red oak-leaf lettuce than when grown under restricted watering.

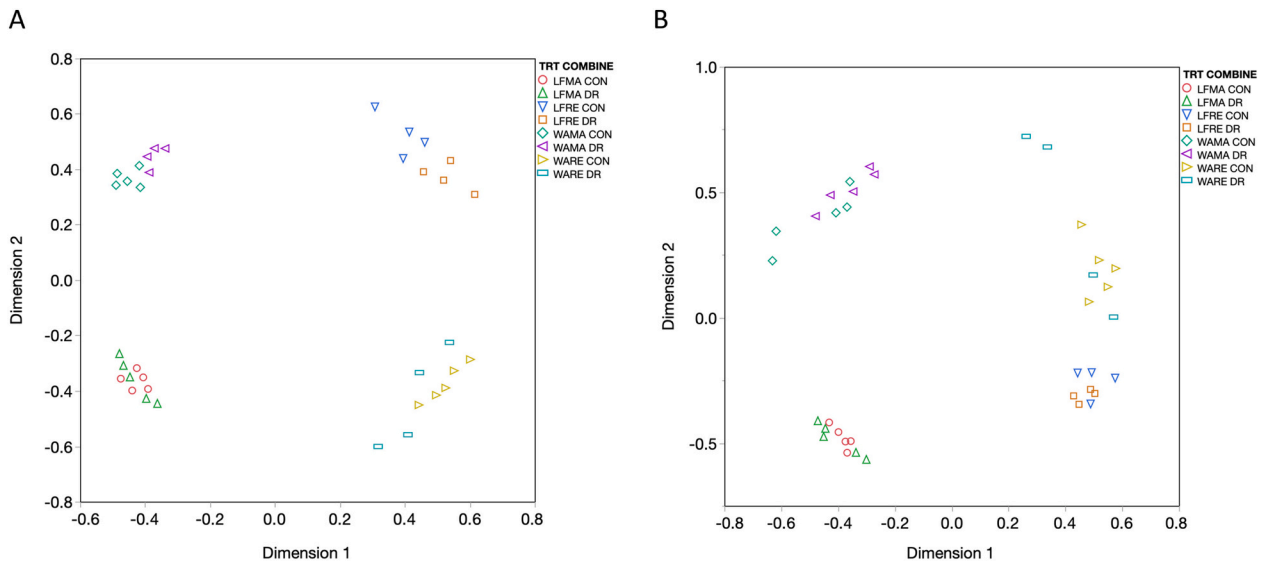

Fig. 1. Multidimensional scaling (MDS) plots of phytochemical profiles of lettuce leaf tissue (LF) and leaf surface washes (WA) for both Romaine 'Parris Island Cos' (RE) and red oak-leaf 'Mascara' (MA) lettuce based on ESI-TOF-MS under both (A) positive and (B) negative modes. Stress was 0.19 in both plots.

Table 4
ANOSIM pairwise comparison of Romaine and red oak-leaf lettuce leaf extracts and leaf surfaces washes based on phytochemical profiling with ESI-MS under positive and negative modes. Plants were 34 days old at time of experimentation and subjected to regular (CON) or restricted (DR) watering. The *R* statistic spans 0–1, with 0 indicating no relationship and 1 indicating highest relatedness between sample profiles. Two distance measures were used to construct the distance matrix for ANOMSIM analysis, Bray-Curtis dissimilarity coefficient which uses relative peak height data and Jaccard Index which considers only the presence or absence of peak.

Lettuce type	Watering condition	Positive mode		Negative mode	
		R (*p*-value)			
		Bray-Curtis	Jaccard	Bray-Curtis	Jaccard
Leaf tissue extracts					
Romaine	CON vs. DR	0.26 (<0.05)	0.443 (<0.05)	0.417 (<0.05)	0.125 (NS)
Red oak-leaf	CON vs. DR	0.272 (<0.05)	0.04 (NS)	0.2 (NS)	0.312 (<0.05)
Romaine vs. Red oak-leaf	CON	1 (<0.01)	1 (<0.01)	1 (<0.01)	1 (<0.01)
Romaine vs. Red oak-leaf	DR	1 (<0.01)	1 (<0.01)	1 (<0.01)	1 (<0.01)
Leaf surface washes					
Romaine	CON vs. DR	0.375 (<0.05)	0.538 (<0.01)	0.244 (<0.1)	0.238 (<0.1)
Red oak-leaf	CON vs. DR	0.581 (<0.05)	0.413 (<0.05)	0.208 (<0.05)	0.256 (<0.05)
Romaine vs. Red oak-leaf	CON	1 (<0.01)	1 (<0.01)	1 (<0.01)	1 (<0.01)
Romaine vs. Red oak-leaf	DR	1 (<0.05)	1 (<0.05)	1 (<0.01)	1 (<0.01)

4. Discussion

In this study we assessed the natural variability in the leaf and leaf surface metabolome, and in targeted phytochemical accumulations between two lettuce types (Romaine and red oak-leaf), and the effects elicited by drought. In addition, we investigated how the drought stress response in lettuce modulated the *Salmonella*-lettuce association in these two cultivars. Our results showed that regularly watered Romaine lettuce accumulated a lower level of secondary metabolites and had an overall inferior antioxidant capacity than red oak-leaf lettuce. Moreover, we detected metabolite profile differences in lettuce leaf tissue and leaf washes between the two cultivars, and in response to drought stress. The data raise the possibility that the enhanced decline of *Salmonella* populations on red oak-leaf lettuce compared to Romaine leaf surfaces, and the growth lag in leaf washes, could be partly explained by the differential accumulation of metabolites between these two lettuce types. More evidence to support this hypothesis was provided by the further impairment of *Salmonella* on water-restricted red oak-leaf lettuce, in which phytochemical induction was concomitantly measured, effects not observed in Romaine.

Differences in primary and secondary metabolites in leaf tissues explain the divergent metabolite profiles between the two cultivars grown under regular watering condition. In this study we revealed an inherently higher level of flavonoids and anthocyanins in red oak-leaf lettuce tissue, with anthocyanins not being detected in Romaine. Red lettuce cultivars have been shown to have higher total phenolics and flavonoids, antioxidant capacity and anthocyanins levels than green lettuce (Ozgen and Sekerci, 2011; Tsormpatsidis et al., 2010). Moreover, the red oak-leaf lettuce in this study responded more strongly to abiotic elicitation via drought, with an induction of proline, which can serve as an osmoprotectant in lettuce plants (Rajabbeigi et al., 2013; Shin et al., 2021). This elicitation also induced accumulation of flavonoids, phenolics and anthocyanins in red oak-leaf lettuce, a response that was not detected in Romaine. Nevertheless, metabolite changes were detected in Romaine following drought treatment, as revealed by untargeted metabolite profiling of both leaf tissue and leaf washes, suggesting that the response to water deficit in Romaine elicited changes we were not measuring directly. The accumulation of bioactive compounds with antioxidant and anti-inflammatory properties could be a strategy to enhance the functional properties of lettuce crops. Regulated water stress could be optimized to boost these properties in controlled environment agriculture, including hydroponic systems.

Untargeted metabolite profiling of leaf washes detected shifts in the composition of the metabolome due to cultivar and water deficit. Leaf washes also allowed for the direct assessment of leaf surface released metabolites on *Salmonella*, eliminating other possible plant-microbe interactions. Romaine leaf washes provided a more favourable medium for *Salmonella* than red oak-leaf lettuce washes. Moreover, growth

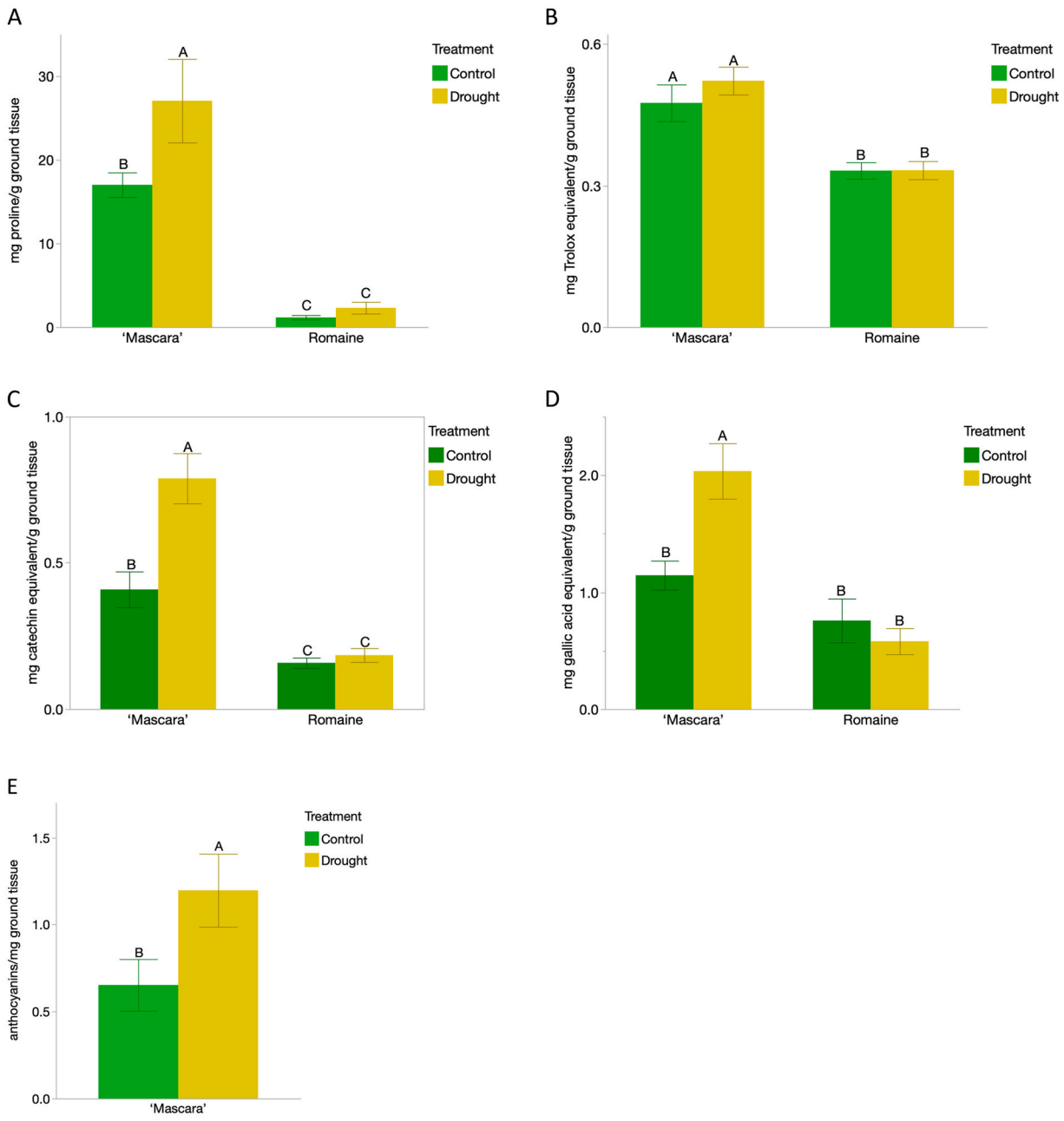

Fig. 2. Levels of (A) proline, (B) antioxidant capacity, (C) total flavonoids, (D) total phenolics, in Romaine 'Parris Island Cos' and red oak-leaf 'Mascara' lettuce leaf tissues, and (E) anthocyanin content in red oak-leaf lettuce, under regular and restricted watering regimes (there were no detectable anthocyanins in Romaine lettuce). Different capitalised letters represent significant differences due to watering conditions and cultivar by ANOVA and Tukey's HSD test ($p < 0.05$).

of *Salmonella* Newport in washes from red oak-leaf lettuce subjected to drought showed a prolonged lag phase compared to controls, resulting in a lower maximum population after 24 h. Growth in Romaine washes showed a slight lag initially, but the populations recovered in relation to controls in this cultivar. The constituents mediating these growth dynamics in the leaf washes affecting *Salmonella* growth remain to be identified, however they indicate that the differential epiphytic *Salmonella* associations observed between Romaine and oak-leaf lettuce could in part be modulated by metabolites on the plant surface.

Salmonella populations could have decline on regularly watered Romaine and red oak-leaf lettuce as a result of nutrient limitations (Brandl and Mandrell, 2002; Leveau and Lindow, 2001). The log reduction of *S.* Newport and *S.* Typhimurium on either regularly watered Romaine or red oak-leaf lettuce was not markedly different, suggesting no dramatic serotype effect. Similar results were reported using Romaine leaf pieces, with the retrieval of *S.* Newport and Typhimurium showing no differences 2 h post-incubation at 25 °C and 3 days at 30 °C (Kroupitski et al., 2009). However, in our study, *Salmonella* Newport and Typhimurium decline on Romaine lettuce was lower than on red oak-leaf lettuce, with highest *Salmonella* counts obtained from Romaine under regular watering. Taken together, these results suggested that Romaine lettuce provided a more favourable niche for *Salmonella*, such as in the form of nutrients which would allow the population to persist at higher levels.

Carbon- and nitrogen-containing nutrients leached from plant leaves are critical resources for enteric pathogen colonisation, and total sugars on plant surfaces were positively related to epiphyte populations (Brandl and Amundson, 2008; Leveau and Lindow, 2001; Mercier and Lindow, 2000). Cuticular permeability may be one of the most important determinants of epiphytic bacterial colonisation, and stomata and

trichomes have been identified as sites where diffusion of polar compounds, such as ions, sugars and amino acids, can occur (Schreiber, 2005). Epiphytic bacteria may also release biosurfactants that enhance cuticular permeability, altering diffusion rates of nutrients and hence improving the habitability of the niche (Schreiber et al., 2005; van der Wal and Leveau, 2011). This suggests that the background microbiota, which could be influenced by cultivar (Hunter et al., 2010), could aid persistence of enteric pathogens such as *Salmonella.* The composition of the cuticle and other properties such as stomatal density, which vary by cultivar (Lu et al., 2015), could also affect permeability and nutrient release to the leaf surface. Indeed, Lu et al. (2015) measured higher amounts of alkanes, fatty acids and total wax content in 'Outredgeous' Romaine compared to two green loose-leaf lettuce varieties, which could affect permeability, providing one possible explanation for differential cultivar differences in *Salmonella* association between Romaine and loose-leaf lettuce. Moreover, *Arabidopsis* plants subjected to water deficit had a lower leaf water content, higher amounts of cuticular wax and thicker cuticles (Kosma et al., 2009). Thicker cuticles in response to drought could explain the reduced bacterial association with lettuce in water deficit. The dehydration rate of red oak-leaf lettuce after water deficit treatment was higher than that of Romaine, which seemed unaffected by the drought treatment. The reduced water content in leaves, therefore, could also have negatively impacted epiphytic *Salmonella* populations.

In addition to cuticular permeability, sugar levels also vary by cultivar. Fructose and glucose were the major soluble sugars detected in both oak-leaf and Romaine lettuce (Allende et al., 2006; López et al., 2014), however oak-leaf lettuce had a lower sugar content than Cos (Romaine) lettuce (Missio et al., 2018). Glucose is a carbon source that can be readily utilised by *Salmonella* (Gutnick et al., 1969; Nur et al., 2016). Malic and citric acid, the predominate organic acids found in oak-leaf were also found in Romaine lettuce (Allende et al., 2006; López et al., 2014). These organic acids are vital nutrients for commensal bacteria on leaves, probably including *Salmonella* (Wilson and Lindow, 1994). Moreover, amino acids in leaf tissue could serve as nutrients for epiphytes (Wilson and Lindow, 1994) and are highly involved in *Salmonella* colonisation on lettuce surface (Goudeau et al., 2013). A difference in nutrient availability on the leaf surface between red oak-leaf and Romaine lettuce should, therefore, be investigated as a mechanism for the differential *Salmonella* interaction on these two lettuce types. Finally, it should be noted that *Salmonella* attachment and internalization have been reported to vary by serotype and cultivar, and may be attributed to a variety of factors including leaf characteristics such as stomatal density and plant immune responses (Jacob and Melotto, 2020; Patel and Sharma, 2010; Van der Linden et al., 2016).

The drought effect on lettuce was measurable via induction of secondary metabolites (phenolics, flavonoids and anthocyanins). Although secondary metabolites in red oak-leaf lettuce were strongly induced by drought, phenolics and flavonoids are located in highest concentrations in the vacuole of cells. Phenolics are also a minor component of the cutin polymer, which makes up the cuticle of plant surfaces (Reynoud et al., 2021). However, to what degree bacteria in the phyllosphere interact with phenolics or other components of the cuticle is unclear (Aragón et al., 2017). We did not directly measure phytochemicals in washes, but Hunter et al. reported that leaf surface phenolic content increased with leaf age in iceberg ('Saladin') and batavian ('Iceberg') lettuce, as well as in wild lettuce *L. serriola* (Hunter et al., 2015). The retrieval of lower *Salmonella* levels from washes of drought-treated plants compared to controls is an intriguing finding and raises the question of which metabolites might be reaching the leaf surface and mediating bacterial interactions, potentially explaining differential cultivar susceptibilities to enteric pathogen colonisation.

Regulated, mild water stress has been suggested as an approach to enhance the health beneficial properties of lettuce by eliciting accumulation of antioxidant compounds such as phenolic compounds including chicoric, chlorogenic and caffeic acids and vitamins C and E (Oh et al., 2010; Paim et al., 2020; Toscano et al., 2019). Depending on timing of abiotic stress application, biomass, yield, and physicochemical and sensory quality of the harvest can be affected, and therefore suitable cultivars must be identified for this approach (Malejane et al., 2018; Paim et al., 2020). Organoleptic properties cannot be overlooked, since the accumulation of secondary metabolites and osmoprotectants can affect the ratio of bitterness to sweetness and colour, while cuticle thickening in response to drought can affect firmness, which all influence consumer preferences (Chadwick et al., 2016). Carefully adjusted timing and extent of water deficit induced in suitable cultivars, however, can optimize the balance for enhanced nutritional quality without compromising yield and preferred consumer traits (Paim et al., 2020). This approach has appeal for controlled environment agriculture (CEA), an industry that is growing and developing more sophisticated ways to integrate precisely controlled environmental conditions with biological control while meeting sustainability goals and the needs of local communities (Tan et al., 2021). Our study adds another objective to integrate into CEA that also considers food safety. A deeper understanding of the mechanisms that impede enteric pathogen establishment on plants can help develop effective management practices for leafy greens that aim to enhance food safety, possibly while simultaneously also boosting health benefits.

5. Conclusion

In this study, we investigated the metabolite profiles of Romaine and red oak-leaf lettuce and the lettuce-*Salmonella* association. As an epiphyte, successful *Salmonella* colonisation on leaf surfaces is highly dependent on availability of nutrients. Results from this study suggest that Romaine lettuce, the most popular leafy green in the US, is more favourable for *Salmonella* association compared to oak-leaf lettuce, possibly due to the composition of the surface metabolome which has the potential to support or restrict the *Salmonella*-lettuce association. Results from this research could help farmers in decision making when selecting cultivars in areas where *Salmonella* is prevalent in the environment or in irrigation water. Our results show that Romaine lettuce characterized by naturally reduced accumulation of antioxidant compounds, presented a more favourable niche to *Salmonella* compared to red oak-leaf lettuce. We demonstrate that mild drought stress further reduced the favourability of the red oak-leaf lettuce surface and elicited secondary metabolites with bioactive properties, providing evidence that certain cultivars may be suitable for deficit irrigation approaches that not only boost health beneficial compounds but also enhance food safety. In summary, it is feasible to manipulate *Salmonella* populations through environmental interventions and natural cultivar variation. Future work should assess the mechanisms of bacterial restriction and interactions with other pathogens such as *Escherichia coli* O157:H7, which has caused numerous foodborne illness outbreaks with Romaine lettuce (CDC, 2018, 2019). Future studies are also needed to further decipher metabolome differences in lettuce cultivars and their response to drought and to identify specific compounds that may possess *Salmonella* growth-limiting properties.

Author contributions

SAM and XL conceptualised the study and designed the experiments. XL conducted the experiments. YL and XL designed and conducted the ESI-TOF-MS experiments. XL and SAM analyzed and interpreted the data and wrote the manuscript. YL reviewed and edited the manuscript. SAM acquired funding and administered the project.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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