frontiers | Research Topics

Breeding crops for enhanced food safety

Edited by
Maeli Melotto, Wei Zhang and Max Teplitski

Published in
Frontiers in Microbiology
Frontiers in Plant Science

ISSN 1664-8714
ISBN 978-2-83252-013-0
DOI 10.3389/978-2-83252-013-0

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Breeding crops for enhanced food safety

Topic editors

Maeli Melotto — University of California, Davis, United States
Wei Zhang — Illinois Institute of Technology, United States
Max Teplitski — University of Florida, United States

Citation

Melotto, M., Zhang, W., Teplitski, M., eds. (2023). *Breeding crops for enhanced food safety*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83252-013-0

Table of contents

frontiers in Microbiology

EDITORIAL
published: 18 March 2022
doi: 10.3389/fmicb.2022.871247

Editorial: Breeding Crops for Enhanced Food Safety

Maeli Melotto[1]*, *Wei Zhang*[2] and *Max Teplitski*[3]

[1] Department of Plant Sciences, University of California, Davis, Davis, CA, United States, [2] Department of Food Science and Nutrition, Illinois Institute of Technology, Chicago, IL, United States, [3] International Fresh Produce Association, Washington, DC, United States

Keywords: breeding crops, food safety, microbial hazard, toxin, human health

Editorial on the Research Topic

Breeding Crops for Enhanced Food Safety

The demand for nutritious and safe food will likely increase as the human population is expected to reach 9.1 billion by 2050 along with increasing urbanization. Furthermore, healthy eating, including the consumption of fresh or minimally processed fruits and vegetables, has become increasingly popular as part of an integrated strategy to decrease the risk of serious diseases. Lamentably however, consumption of fresh produce does not reach the minimum daily recommendations. To this end, decision makers around the world now focus on investing into programs that will ensure nutritional security of populations (a term encompassing access to nutrient-dense rather than high-caloric foods). Achieving such nutritional security will require that all nutrient-dense foods are free of contaminants, including human pathogens.

Unfortunately, over the past decades, fresh fruits and vegetables have been associated with a number of high-profile outbreaks of human foodborne illness. Good agricultural practices, good handling practices, good manufacturing practices, and hazard analysis of critical control points remain the cornerstone of food safety management along the production chain (from farm to fork) as part of "multiple hurdle" approaches to limit produce contamination. Fresh or ready-to-eat produce, such as leafy vegetables and fruits, do not undergo thermal processes to inactivate human pathogens. Instead, they are often treated with aqueous solutions containing sanitizing chemicals to reduce potential cross-contamination and promote quality. The lack of an efficient kill step is one of the greatest challenges facing the fresh produce industry. Thus, novel and comprehensive approaches are needed to ensure the safety and quality of freshly consumed produce. One overlooked yet highly promising approach for reducing susceptibility of crops to colonization with human pathogens and toxin-producing organisms is plant breeding.

Thus far, most of the studies on plant interactions with human pathogens have focused on enterobacteria and toxin-producing fungi as they can grow on/in plants given the right conditions. Multiple examples of differential colonization of enterobacteria in several crop species have been reported and comprehensively reviewed by Henriquez et al. Importantly, these studies revealed that commercial cultivars that are more likely to be contaminated with human pathogens (Jacob and Melotto) represent a higher risk for disease outbreaks. When the crop has a narrow genetic basis, incorporating exotic germplasm compatible with commercial cultivars is an excellent alternative to improve crop safety, as in the case of almond (Gradziel).

Internalization and movement of bacterial cells to edible organs is a safety concern. There are several routes for bacterial internalization into plant organs; different bacterial species are likely to be specialized to preferentially use specific routes. Some human pathogens (such as *Salmonella enterica*) can produce mimics of plant hormones, thus creating openings for colonization of plant tissues. Although human pathogen internalization through lateral root junctions and leaf stomata

Edited and reviewed by:
Aldo Corsetti,
University of Teramo, Italy

***Correspondence:**
Maeli Melotto
melotto@ucdavis.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *07 February 2022*
Accepted: *11 February 2022*
Published: *18 March 2022*

Citation:
Melotto M, Zhang W and Teplitski M (2022) Editorial: Breeding Crops for Enhanced Food Safety. Front. Microbiol. 13:871247. doi: 10.3389/fmicb.2022.871247

has been confirmed previously, other points of entry are also possible. In cucumbers, for instance, *S. enterica* can more effectively penetrate blossoms and reach the fruit at high percentage than when entering through the roots (Burris et al.).

The foundation for plant breeding is the existence of genetic variability in the system under investigation, resulting in phenotypic variability (Melotto et al.). Variability in the colonization phenotype depends upon genetic factors of both the plant host and the enterobacteria, as well as environmental conditions. On the leaf surface, for instance, venation density (Doan et al.) and stomatal pore size (Jacob and Melotto) are associated with colonization traits of *E. coli* O157:H7 (i.e., leaf surface attachment and internalization). When plant tissues are able to mount strong and robust immune responses, such as rapid generation of reactive oxygen species and nitric oxide, they are less likely to be colonized by human pathogens (Ferelli et al.). However, at least *S. enterica* is able to evade plant immune responses, such as stomatal closure, and invade the plant tissue (Johnson et al.). A mutant screen revealed regions in the *S. enterica* genome that are required to subvert stomatal immunity (Montano et al.); however, their molecular functions are yet to be fully characterized.

An important aspect of plant breeding is the design of reproducible, robust, fast, and relatively easy protocols for screening the desirable phenotype. Ideally, quantification of the phenotype should be validated with multiple methodologies prior to adoption into breeding programs. These considerations are particularly relevant to advance new fields such as the mechanistic understanding of human pathogen colonization of plants. One of the biggest challenges is to quantify bacterial internalization, persistence, and survival rate in leaves, especially when bacterial populations are small. Some efforts toward this direction have been made for *S. enterica* in leaves. Chahar et al. reported that bacterial internalization can be assessed by several methods and should be adapted to specific systems as steps in the procedure (namely surface sterilization) can interfere with the results. Additionally, a modified protocol for bacterial enumeration and recovery for downstream applications is available and can be adapted to different plant tissues (Oblessuc and Melotto).

Edible crops have been bred for millennia, resulting in crops that are essentially unrecognizable compared to their wild progenitors, and are superior in yield, taste, and a number of agronomic traits. Modern plant breeding tools offer an opportunity to explore the feasibility of breeding crops for their reduced susceptibility to human pathogens and toxin-producing organisms. "Breeding for food safety" does not need to be limited by the canons of the gene-for-gene hypothesis. Articles in this eBook identify crop phenotypes that, for a variety of reasons, are less conducive to human pathogens or toxin-producing microbes. They contributed to a significant advancement in the field of breeding crops for enhanced food safety, highlighting the most up-to-date research progresses, and identifying current knowledge gaps and future directions.

AUTHOR CONTRIBUTIONS

All authors served as co-editors to the Research Topic and also contributed to, critically read, discussed, and approved this Editorial.

Conflict of 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 24 January 2020
doi: 10.3389/fmicb.2020.00006

Salmonella enterica Serovar Typhimurium 14028s Genomic Regions Required for Colonization of Lettuce Leaves

*Jeanine Montano[1,2], Gabrielle Rossidivito[1,3], Joseph Torreano[1], Steffen Porwollik[4], Shlomo Sela Saldinger[5], Michael McClelland[4] and Maeli Melotto[1]**

[1] Department of Plant Sciences, University of California, Davis, Davis, CA, United States, [2] Plant Pathology Graduate Group, University of California, Davis, Davis, CA, United States, [3] Plant Biology Graduate Group, University of California, Davis, Davis, CA, United States, [4] Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA, United States, [5] Microbial Food Safety Research Unit, Department of Food Science, Agricultural Research Organization, Volcani Center, Rishon LeTsiyon, Israel

OPEN ACCESS

Edited by:
Min Suk Rhee, Korea University, South Korea

Reviewed by:
Bradley L. Bearson, United States Department of Agriculture (USDA), United States
Susan M. Bueno, Pontifical Catholic University of Chile, Chile
Carlos J. Blondel, Andres Bello University, Chile

***Correspondence:**
Maeli Melotto
melotto@ucdavis.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *25 July 2019*
Accepted: *03 January 2020*
Published: *24 January 2020*

Citation:
Montano J, Rossidivito G, Torreano J, Porwollik S, Sela Saldinger S, McClelland M and Melotto M (2020) Salmonella enterica Serovar Typhimurium 14028s Genomic Regions Required for Colonization of Lettuce Leaves. Front. Microbiol. 11:6. doi: 10.3389/fmicb.2020.00006

Contamination of edible produce leaves with human bacterial pathogens has been associated with serious disease outbreaks and has become a major public health concern affecting all aspects of the market, from farmers to consumers. While pathogen populations residing on the surface of ready-to-eat produce can be potentially removed through thorough washing, there is no disinfection technology available that effectively eliminates internal bacterial populations. By screening 303 multi-gene deletion (MGD) mutants of *Salmonella enterica* serovar Typhimurium (STm) 14028s, we were able to identify ten genomic regions that play a role in opening the stomatal pore of lettuce leaves. The major metabolic functions of the deleted regions are associated with sensing the environment, bacterium movement, transport through the bacterial membrane, and biosynthesis of surface appendages. Interestingly, at 21 days post inoculation, seven of these mutants showed increased population titers inside the leaf, two mutants showed similar titers as the wild type bacterium, whereas one mutant with a large deletion that includes the *Salmonella* pathogenicity island 2 (SPI-2) showed significantly impaired persistence in the leaf apoplast. These findings suggest that not all the genomic regions required for initiation of leaf colonization (i.e., epiphytic behavior and tissue penetration) are essential for continuing bacterial survival as an endophyte. We also observed that mutants lacking either SPI-1 (Mut3) or SPI-2 (Mut9) induce callose deposition levels comparable to those of the wild type STm 14028s; therefore, these islands do not seem to affect this lettuce defense mechanism. However, the growth of Mut9, but not Mut3, was significantly impaired in the leaf apoplastic wash fluid (AWF) suggesting that the STm persistence in the apoplast may be linked to nutrient acquisition capabilities or overall bacterial fitness in this niche, which are dependent on the gene(s) deleted in the Mut9 strain. The genetic basis of STm colonization of leaves investigated in this study provides a foundation from which to develop mitigation tactics to enhance food safety.

Keywords: food safety, leafy vegetable, *Salmonella* mutant screen, bacterial persistence, lettuce stress response

INTRODUCTION

Human pathogen contamination of produce was the leading cause of foodborne illnesses and outbreaks associated with a single-ingredient commodity between 2004 and 2013 (Fischer et al., 2015). Lack of visual evidence that indicates the presence of contamination on produce contributes to the estimated 9.4 million cases of foodborne illness in the United States annually (Dewey-Mattia et al., 2016). Various pathogen groups and toxins can be causal agents of foodborne illness associated with produce; however, non-typhoidal *Salmonella* ranks as the second leading cause of all illnesses associated with consumption of produce (DeWaal et al., 2008; Fischer et al., 2015; Dewey-Mattia et al., 2016).

In a pre-harvest setting, enteric pathogenic bacteria are introduced to fresh produce through many routes, including low-quality irrigation water, use of contaminated organic fertilizers, close proximity to livestock operations, wildlife intrusions, improper worker hygiene, or contaminated equipment (Critzer and Doyle, 2010; Barak and Schroeder, 2012). Once on the leaf surface, bacteria are faced with harsh conditions, such as UV irradiation, low nutrient and water availability, and unfavorable weather (Hirano and Upper, 1983; Lindow and Brandl, 2003). Bacteria may escape these conditions by attaching to the leaf surface and forming biofilms (Kroupitski et al., 2009) or by transitioning to an endophytic lifestyle through internalization into the leaf extracellular space (i.e., apoplast) via natural pores or wounds (Kroupitski et al., 2009; Critzer and Doyle, 2010; Roy et al., 2013). While leaf internalization is likely to confer some protection to the bacteria, it is not without some disadvantages. Plants can detect endophytic bacteria in the apoplast through pattern recognition receptors (PRRs) localized at the cell membrane that perceive conserved microbial molecules known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Nicaise et al., 2009; Newman et al., 2013). PRR-PAMP binding leads to initiation of PAMP-triggered immunity (PTI) (Jones and Dangl, 2006), which functions to prevent further internalization of bacteria (Melotto et al., 2006; Kroupitski et al., 2009; Roy et al., 2013) and to eradicate those that have already entered the apoplast (Jones and Dangl, 2006; Nicaise et al., 2009). This suggests that internalization trades one challenge for another (i.e., those of the phylloplane for those of the apoplast), and only bacteria that can cope with these challenges will be able to colonize leaves successfully.

Previous studies have shown that *Salmonella* spp. interact with plants in a sophisticated manner, although the exact mechanisms are not fully understood (Melotto et al., 2014). For instance, similar to some plant pathogens, *Salmonella enterica* serovar Typhimurium (STm) SL1344 can modulate stomatal movement in Arabidopsis leaves, where it induces a transient stomatal closure and re-opening at 4 h post incubation (hpi) (Roy et al., 2013). Stomatal closure can diminish bacterial internalization and subsequent contamination of internal leaf tissues. Bacterium-induced re-opening of stomata can lead to higher pathogen load in the leaf apoplast (reviewed by Garcia and Hirt, 2014; Melotto et al., 2014).

The mechanism for stomatal re-opening by the phytopathogen *Pseudomonas syringae* pv. *tomato* (Pst) is through the action of coronatine (Melotto et al., 2006), a polyketide phytotoxin (Bender et al., 1999). However, the genomes of STm strains LT2 and 14028s do not encode genes for coronatine synthesis (McClelland et al., 2001; Jarvik et al., 2010). Furthermore, stomatal re-opening is not a ubiquitous response to human pathogens. For instance, *Escherichia coli* O157:H7 induces a lasting stomatal closure in lettuce and Arabidopsis for at least 4 and 8 h, respectively (Melotto et al., 2006; Roy et al., 2013).

Beyond the ability to modulate stomatal movement, STm SL1344 can survive at a higher titer within the apoplast of Arabidopsis leaves than O157:H7 after surface-inoculation (Roy et al., 2013) and *S. enterica* serovar Thompson strain RM1987 can survive at high titers on the surface of romaine lettuce leaves (Brandl and Amudson, 2008). Therefore, *S. enterica* may either induce a weak plant immune response or can counteract plant immunity and consequently persist on and in leaves (Garcia and Hirt, 2014; Melotto et al., 2014). Internalization and persistence within the apoplast are arguably the most important targets for managing contamination of produce by *Salmonella*, as endophytic populations cannot be removed through typical washing tactics (Heaton and Jones, 2008; Gil et al., 2009; Goodburn and Wallace, 2013).

Here, we provide details of multiple genomic regions required for internalization and persistence of STm 14028s into lettuce (*Lactuca sativa* cv. Salinas) leaves. These genomic regions were identified with a genetic screen of multi-gene deletion (MGD) mutants of STm 14028s (Porwollik et al., 2014) to pinpoint proteins and metabolic pathways responsible for stomatal re-opening and apoplastic persistence. Selected MGD mutants were further characterized regarding their ability to survive in the apoplast, induce hallmark plant defenses, and replicate in apoplastic wash fluid (AWF). While all mutants induced a prolonged stomatal closure when applied to the leaf surface, the mutants were found to vary in other aspects of phyllosphere survival.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Salmonella enterica STm 14028s and 303 mutants derived through lambda red-mediated gene recombination (**Supplementary Table S1**) were obtained from the McClelland laboratory at the University of California, Irvine (Porwollik et al., 2014). The mutants have a MGD that removes 2–70 genes from each strain and the mutant collection covers 3476 genes (65.25%) from the 5327 genes in the STm 14028s genome, not including the 1124 genes (21.10%) that code for essential genes (e.g., tRNA, rRNA) (Porwollik et al., 2014). All bacterial strains, including the isolate STm SL1344 MB282 (Hoiseth and Stocker, 1981) were maintained in glycerol stock at −80°C and streaked on Low-Salt Luria-Broth (LSLB) agar plates at the time of use. Cultures grown on solid medium were kept stationary and incubated at 28°C until colonies were formed (∼24 h). Cultures grown in liquid

LSLB medium were incubated at 28°C with rotational shaking (225 rpm). LSLB broth was prepared using 10 g Tryptone (VWR, J859-500G), 5 g Yeast Extract (BD, 212750), and 5 g NaCl (VWR, X190-1KG) per liter of water. LSLB Agar was prepared similarly with the addition of 15 g/L of Bacteriological Agar (VWR, J637-1KG). Medium was supplemented with either 60 μg/mL kanamycin or 20 μg/mL chloramphenicol to grow the STm mutant strains.

Bacterial cells of *P. syringae* pv. *tomato* (Pst) DC3000 or its non-polar mutant *hrc*C$^-$ (Hauck et al., 2003), were grown in liquid LSLB medium at 28°C for all experiments. Cells were streaked on solid medium from frozen glycerol stocks for inoculum preparation. Medium was supplemented with rifampin (100 μg/mL) to grow Pst strains.

Plant Material and Growth Conditions

Commercial iceberg lettuce (*L. sativa* L. var *crispa* cv. Salinas) seeds were a gift from the Michelmore lab at the University of California, Davis. Seeds were germinated for 2 days on damp paper towels at 20°C with a 12-h daylight cycle at 100 μE photosynthetic active radiation and 65 ± 5% air relative humidity (RH). Seedlings were transplanted to dampened Peat Pellets (Jiffy, 70000116) and incubated for 3 weeks with a 12-h daylight cycle at 200 μE photosynthetic active radiation. Conditions for temperature and air RH were 19 ± 1°C and 75 ± 4% RH during the day and 18 ± 1°C and 92 ± 2% RH at night. 3-week old plants were either used in some assays or transplanted into 8 cm^2 pots (Kord Products, Toronto, SQA3500) for later use. Pots contained SunGro Professional Growing Mix supplemented with 20 mL of fertilizer solution (Peters® Excel pH Low 19-11-21 at a concentration of 14.8 g/L) and treated with Gnatrol WDG (Outlaw Hydroponics, AJ-WYC7-RK9U) (1 g/L). Transplanted plants were used at 5 weeks of age and kept in a controlled environment chamber for the duration of the experiments.

Seeds of *Arabidopsis thaliana* (L. Heyhn.) wild type ecotype Columbia (Col-0, ABRC stock CS60000) were sown in a 1:1:1 (v:v:v) mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite. Plants were grown in controlled environmental chambers at 22 ± 2°C, 60 ± 10% RH, and a 12-h photoperiod under light intensity of 100 μE photosynthetic active radiation. For all experiments, 4–5-week old plants were used.

Stomatal Circadian Movement Evaluation

Stomatal aperture widths were measured as described by Montano and Melotto (2017). Briefly, sections from four leaves from 3-week-old lettuce plants were excised and the abaxial surface was imaged with a Nikon Eclipse Ni-U upright microscope (Nikon Corporations, Shinagawaku, Tokyo, Japan). Images were recorded every 2 h during the daylight time (0–12 h) using the Nikon NIS Elements Imaging Software Version 4.13.04 and stomatal aperture was determined as mean (n = 80 ± standard error) of eight biological replicates (i.e., four different leaves and the experiment performed twice). Ten stomata were imaged per leaf. Significant differences among the means were tested by ANOVA with *post hoc* Tukey HSD test ($p < 0.05$).

MGD Mutant Library Screen

Multi-gene deletion mutants were screened for their ability to stimulate stomatal re-opening at 4 h post-inoculation (hpi) as compared to the parental strain STm 14028s. Bacterial liquid cultures were allowed to grow to an OD_{600} of 0.8–1.0. Cells were harvested by centrifugation (1360 × *g*, 20°C) and resuspended in water to generate an inoculum concentration of 1×10^8 CFU/mL (0.2 OD_{600}). Each bacterial inoculum (3 mL) was dispensed in one well of a 12-well plate and two sections of 3-week old lettuce leaves were floated abaxial side down on the inoculum as reported by Chitrakar and Melotto (2010). Images of abaxial surface stomata were captured at 4 hpi as previously described (Montano and Melotto, 2017). Mean stomatal aperture width (n = 20) and standard error (SE) were calculated from two independent leaves and compared by Student's t-test.

Owing that a large number of mutants were not able to re-open the stomatal pore in this first screening (177 MGD strains), we functionally annotated the missing genes in these strains based on the description of the mutants described in **Supplementary Table S2** from Porwollik et al. (2014) as another criterion for selecting mutants for further investigations. Functional annotation of genes and operons were conducted by BLAST searches using NCBI[1] and KEGG (Kanehisa et al., 2017) databases. We then selected 51 mutants to be re-tested as described above, but this time using three biological replicates. Further selection of ten MGD mutants was based on their consistent inability to open the stomatal pore and predicted function of mutated genes. A workflow of the functional genetic screen is depicted in **Supplementary Figure S1**.

Genotyping of Mutant Strains

Genome mutation in the ten selected MGD strains was confirmed by genome sequencing and PCR analyses. Genomic DNA (gDNA) was extracted from MGD strains and the wild type STm 14028s using DNeasy UltraClean Microbial Kit (Qiagen, Germantown, MD, United States) according to the manufacturer's instructions. High quality gDNA was submitted for library preparation and shotgun sequencing by the UC Davis DNA Technology Core using a MiSeq platform (PE300; Illumina, San Diego, CA, United States). Reads were assembled into scaffolds using A5-miseq for KBase[2] and aligned to published *Salmonella* genomes, STm 14028s (Jarvik et al., 2010) (NCBI accession number NC_016856.1) and STm LT2 (McClelland et al., 2001) (NCBI accession number NC_003197.2), using the NCBI megablast tool (Tatusova and Madden, 1999) with default parameters. All MGD strain sequences and our STm 14028s isolate aligned to the published STm 14028s with >99% identity (E-values = 0.0). The deleted region of each MGD mutant strain was identified and functionally annotated. Missing functional units were inferred through analysis of the functional annotation of the STm 14028s and STm LT2 regions available

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi

[2] http://kbase.us

at NCBI. The STm 14028s annotation is current as of February 2017 and was created using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). The LT2 annotation is current as of September 2017 and was created using the programs GLIMMER (Delcher et al., 1999) and GeneMark (Borodovsky and McIninch, 1993).

To confirm the *in silico* prediction of the deletion site, gDNA from the MGD strains was amplified with primers flanking each predicted deleted region, while the corresponding wild type STm 14028s genomic regions were amplified using a forward primer flanking the deletion start site and a reverse primer located within the deleted region, except for Mut9, for which both forward and reverse primers were located within the deleted region. PCR reactions were carried out using 200 ng gDNA, 100 nM of each primer (**Supplementary Table S4**), and GoTaq Green Master Mix (Promega, Madison, WI, United States) diluted with nuclease free water to a final volume of 50 μl. Thermocycler (Bio-Rad, Hercules, CA, United States) conditions included one initial 2 min period of 95°C and 30 cycles of 95°C for 30 s, 53°C for 30 s, and 73°C for 2 min, followed by a final 5 min period of 72°C. PCR products and Bio-Rad EZ Load^{TM} 1 kb Molecular Ruler #1708355 (Bio-Rad) were visualized using SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, United States) in 1% agarose gel and purified with Promega Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, United States). Purified DNA fragments were submitted for Sanger sequencing at the UC Davis Facility[3] to confirm the exact start and end sites of the deletion.

Stomatal Movement in Response to Selected Mutants in Mature Lettuce Plants

Five-mL of LSLB bacterial cultures were grown in 14.0 mL culture tubes on a rotary shaker (150 rpm) to an OD_{600} of 0.8–1.0. Cells were harvested by centrifugation (1360 × *g*, 20°C) and resuspended in water to an OD_{600} of 0.002 (1×10^6 CFU/mL). Inoculum was infiltrated with a needleless syringe into leaves of 5-week-old lettuce plants that were kept under the same environmental conditions used for plant growth. Stomatal bioassay was conducted as previously described (Montano and Melotto, 2017) at 2 and 4 hpi. Mean stomatal aperture widths (n = 120; 40/biological replicate) from three independent leaves and SE were calculated. The difference between the means (mutant versus wild type) was compared by Student's *t*-test to determine statistical significance.

Bacterial Motility Assay

Swimming motility was assessed by analyzing movement within low-percentage agar (VWR, United States) as previously described (O'May and Tufenkji, 2011). Briefly, bacterial cultures were grown in LSLB liquid medium at 160 rpm to an OD_{600} of 0.8–1.0. Cells were harvested via centrifugation (1360 × *g*, 20°C) for 20 min and resuspended in sterile water to an OD_{600} of 0.2 (1×10^8 CFU/mL). One microliter of this bacterial culture was inoculated within the swim agar medium (LSLB with 0.3% agar) followed by incubation for 24 h at 30°C. Turbidity resulting from bacterial migration from the inoculation point through the low-percentage agar was assessed. Qualitative results were recorded by imaging the culture plates. The assay was repeated three times with three replicates each time.

To assess bacterial swarming motility based on the protocol described by O'May and Tufenkji (2011), freshly streaked LSLB agar plates grown overnight was used to obtain single colonies of a similar size and age. One single colony was used to inoculate on the surface of a swarm agar medium (LSLB with 0.6% agar). Medium plates were incubated at 95 ± 5% humidity at 30°C for 24 h. Motility was assessed by measuring the distance of the swarm from the point of inoculation. Mean swarm distance (n = 8 ± SE) was calculated from eight independent plates assessed in two separate assays and statistical significance between the means (mutant versus wild type) was assessed using Student's *t*-test.

Bacterial Persistence in the Leaf Apoplast

Bacterial cultures were grown in LSLB liquid medium at 160 rpm to an OD_{600} of 0.8–1.0. Cells were harvested by centrifugation (1360 × *g*, 20°C) and resuspended in water to an OD_{600} of 0.002 (1×10^6 CFU/mL). This inoculum was infiltrated into 5-week-old lettuce leaves with a needleless syringe as previously described (Katagiri et al., 2002). Apoplastic bacterial titer was evaluated by serial dilution plating technique as previously described (Jacob et al., 2017) at 0, 3, 7, 14, and 21 days post-inoculation (dpi). Briefly, the inoculated leaf was detached from the plant, surface-sterilized (1 min 2% sodium hypochlorite solution, 1 min 70% ethanol, 1 min sterile deionized water), followed by mechanical maceration of leaf disks with known area and plating. Data represents the mean of four technical replicates and three biological replicates (i.e., three leaves) per strain per time point, repeated twice at different days. Mean (n = 24 ± SE) bacterial population size was calculated and statistical significance was evaluated using the Student's *t*-test comparing each mutant to STm 14028s at each time point.

Callose Deposition Assay

To assess the strength of apoplastic defenses of lettuce cultivar Salinas against *Salmonella* strains and compare to that of the Arabidopsis/*Pseudomonas* system, a callose deposition assay was performed as previously described (Hauck et al., 2003; Nguyen et al., 2010). Briefly, attached whole leaves of 5-week old plants were infiltrated with either water (mock treatment) or 1×10^8 CFU/mL of bacterium inoculum using a needless syringe as described by Katagiri et al. (2002). After 24 h, leaves were harvested and chlorophyll was cleared by immersing the leaves into 95% ethanol and kept at 37°C until chlorophyll was removed completely. Ethanol was replaced whenever necessary. Cleared leaves were rinsed consecutively in 70% ethanol and water followed by a 1-h incubation with 150 mM K_2HPO_4 containing 0.01% aniline blue. For microscopy, leaves were mounted on slides using 50% glycerol and imaged under a Nikon Eclipse Ni-U upright microscope equipped with DAPI filter. Damaged areas,

[3]http://dnaseq.ucdavis.edu/

mid vein, and leaf edges were avoided for imaging to prevent false positive results. Light intensity settings were set to 1350–2047 and LUTs were set to 850–2047. Analysis of the images was performed using the NIS Elements Imaging Software Version 4.13.04 (Nikon). Six images from each of four biological replicates (i.e., four leaves) per treatment were recorded and analyzed and the assay was repeated three times. Mean ($n = 12 \pm$ SE) callose deposits/mm^2 was calculated and statistical significance was determined with a one-way ANOVA with *post hoc* Tukey HSD ($p < 0.05$) using XLSTAT version 19.4.

Bacterial Growth in Apoplastic Wash Fluid

Apoplastic wash fluid (AWF) was extracted from 5-week-old lettuce leaves, omitting the cotyledons, using an infiltration-centrifugation method as previously described (O'Leary et al., 2014). To ensure that plant cellular contamination did not occur during extraction, AWF was evaluated for cellular contaminants using the Sigma-Aldrich® Glucose-6-Phosphate Dehydrogenase Assay Kit (Sigma-Aldrich, MAK015-1KT). None of the AWF used exhibited detectable levels of G6PDH (data not shown). AWF was saved in aliquots to limit freeze-thaw cycles and stored at −20°C and filter sterilized at the time of use.

Bacterial cultures were grown in LSLB liquid medium on an orbital shaker to an OD_{600} of 0.8–0.1. Cells were harvested by centrifugation (1360 × *g*, 20°C) and resuspended in water to an OD_{600} of 0.2 (1×10^8 CFU/mL). An aliquot of this inoculum was added to each medium (water, LSLB, or AWF) to achieve an initial bacterial culture concentration of 5×10^6 CFU/mL in a 96-well plate format. Growth curves were obtained by growing cultures stationary, except for a 30-s rotation prior to each OD_{600} reading using a BioTek EPOCH 2 Microplate Spectrophotometer (BioTek, Winooski, VT, United States). OD_{600} readings were obtained every 30 min throughout a 24-h period and blanks (sterile media) were included as a control. This experiment was performed three times with three technical replicates each time. Mean OD_{600} ($n = 9 \pm$ SE) for each time point of the growth curve was calculated after subtracting the mean blank value and subsequently converted to bacterial cell number per mL of culture.

Growth rates (generations/hour) in the log-phase of growth were determined using the formula $N_0 \, x \, 2^n = N_f$ where N_0 is the number of bacteria at the first time point of interest, N_f is the number of bacteria at the final time point of interest, and n is the number of generations. The formula was rearranged to $log_2 \frac{N_f}{N_0} = n$ to calculate n and the number of generations n was divided by the time to obtain the number of generations per hour as previously described (Todar, 2012).

RESULTS

Salmonella Mutant Screening for the Inability to Re-open Lettuce Stomata

We utilized a collection of MGD bacterial mutants derived from STm strain 14028s (Porwollik et al., 2014). We first confirmed that this strain induces a similar stomatal response to that of STm strain SL1344 (Roy et al., 2013). We evaluated changes in the stomatal aperture width in leaves of young lettuce plants by floating leaf pieces onto bacterial inoculum as previously reported (Chitrakar and Melotto, 2010). Both STm strains induced an initial stomatal closure at 2 h post inoculation (hpi) followed by re-opening at 4 hpi (**Figure 1A**), suggesting that the MGD library could be useful to identify STm genomic regions required for successful stomatal re-opening at 4 hpi. Second, to ensure that lack of re-opening was due to deletion of genes required for stomatal re-opening by STm 14028s rather than temporal factors, the circadian movement of lettuce stomata was determined. This analysis indicated that the stomatal aperture was widest at 6 h after first light (hafl) (**Figure 1B**). We therefore, chose to start the stomatal bioassay at 2 hafl to ensure that the 4 hpi time point corresponded to a time with maximum expected stomatal aperture width.

A primary screen of 303 MGD strains with a single biological replicate indicated that 177 mutants were unable to re-open stomata, suggesting a high rate of false-positives. Thus, we functionally annotated the predicted deleted genes in these 177 mutants (Porwollik et al., 2014). Considering the current knowledge of STm epiphytic behavior (Kroupitski et al., 2009), we reasoned that genes involved in secretion, perception of environmental signals, signaling, and regulatory functions could be involved in opening of the stomatal pore. Thus, we selected 51 MGD mutants based on their functional annotation for re-testing with at least three biological replicates. The primary functional units missing in these 51 mutants are described in **Supplementary Table S2**. From this confirmation screen, only eight mutants (named Mut1/2/4/5/6/7/8/10) were unable to re-open lettuce stomata consistently (**Supplementary Table S2**) and they were selected for further characterization. Furthermore, previous results indicated that mutants for the *Salmonella* Pathogenicity Island 1 and 2 (SPI-1 and SPI-2) were unable to open lettuce stomatal pores (S. Sela, unpublished data). Thus, we also analyzed two MGD strains from our collection (Mut3 and Mut9) that have a predicted deletion of these regions in addition to a few adjacent genes (**Figure 1C** and **Table 1**).

To confirm that the lack of stomatal re-opening using leaf pieces floating on bacterial inoculum (i.e., surface inoculation of detached leaves) was a reproducible response that can also be observed in leaves still attached to the plant, we designed a stomatal bioassay that included infiltration of mature lettuce leaves with STm 14028s, Mut3, or Mut9 bacterium suspensions. In this assay, bacteria are placed in the leaf apoplast, including the sub-stomatal chamber, where they can be in contact with the guard cells. All three strains induced a strong stomatal closure at 2 hpi (**Figure 2A**), similar to observations made using surface inoculation of mature, whole plants (Roy et al., 2013; Montano and Melotto, 2017). Furthermore, the wild type strain STm 14028s, but not the mutant strains, induced stomatal re-opening at 4 hpi (**Figure 2A**), suggesting that this response is robust. To rule out the possibility that the infiltration procedure induced an unpredictable stomatal movement, we assessed the circadian stomatal movement in untreated lettuce leaves as well as leaves infiltrated with water (mock control), STm 14028s,

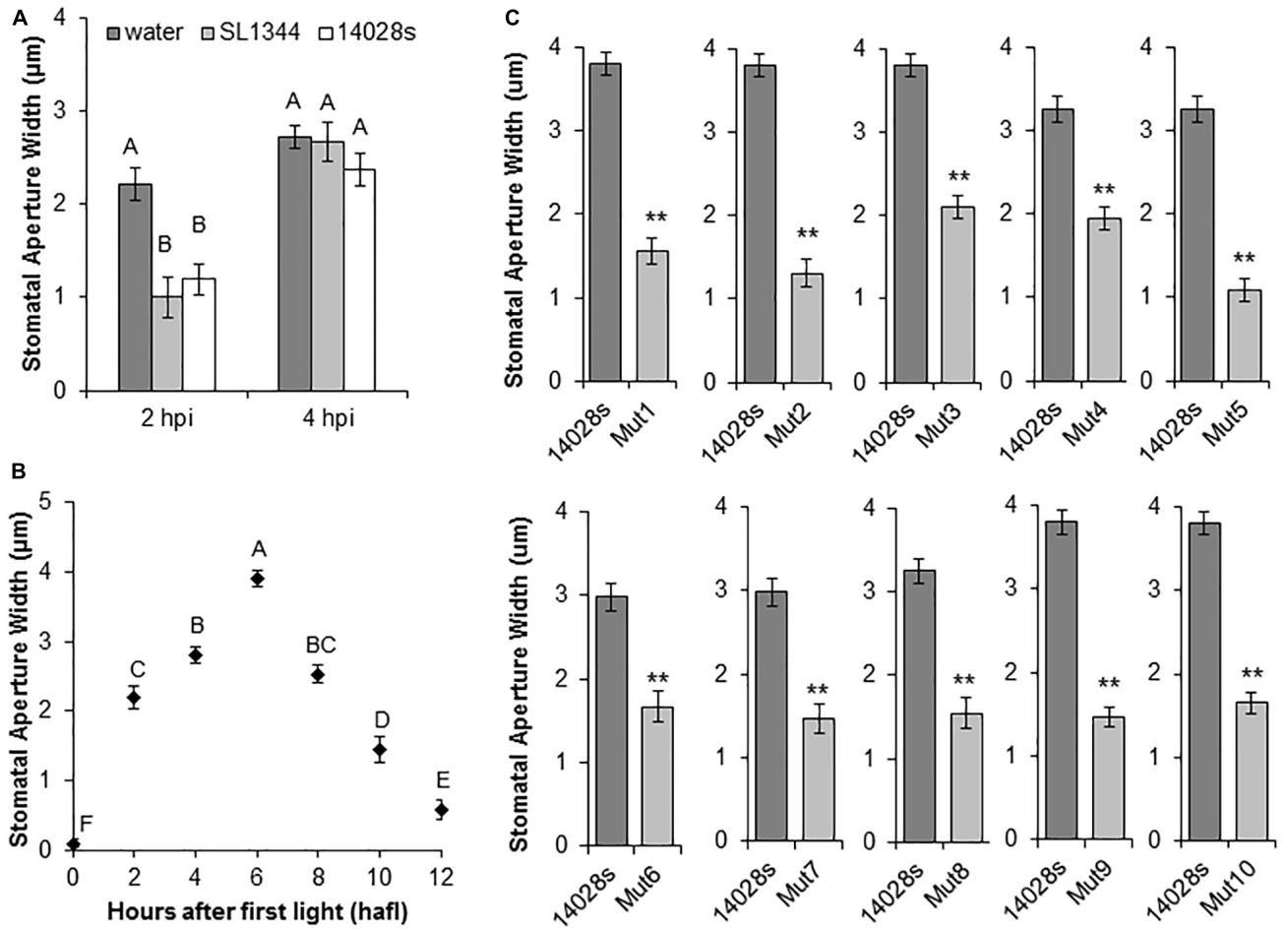


FIGURE 1 | *Salmonella enterica* serovar Typhimurium (STm) mutant screening analysis. **(A)** STm 14028s and STm SL1344 induce similar stomatal reactions in lettuce leaves at 2 and 4 h post-inoculation (hpi). Leaf sections were floated on bacterial suspensions (1×10^8 CFU/mL). Results are shown as the mean ($n = 20$) ± standard error (SE) calculated from two independent leaves. Different letters above the bars indicate statistically significant differences among the means (ANOVA with *post hoc* Tukey HSD test; $p < 0.05$). **(B)** Circadian rhythm of stomatal movement on non-inoculated lettuce leaves. Mean stomatal aperture widths ($n = 80$ ± SE) of eight biological replicates (i.e., four different leaves and the experiment performed twice). Ten stomata were imaged per leaf. Different letters above the bars indicate statistically significant differences among the means (ANOVA with *post hoc* Tukey HSD test; $p < 0.05$). **(C)** Stomatal response to selected bacterial mutants. Lettuce leaf sections were floated on bacterial inoculum (1×10^8 CFU/mL) and stomatal aperture widths were measured at 4 hpi. Results are shown as mean stomatal aperture width ($n = 120$; 40/biological replicate) from three independent leaves and SE. Statistical difference between the means (STm 14028s vs. mutant) was determined using Student's *t*-test (**$p < 0.01$).

TABLE 1 | Functional unit annotation for the ten MGD mutant strains selected for further characterization.

Mutant	Plate-well position	Deleted nucleotides	Deletion size (bp)	Deleted gene loci (ID)
Mut1	K_77/78_C08	4538998–4572514	33,516	STM14_RS22490 to STM14_RS22630
Mut2	K_77/78_F03	3618835–3626190	7,355	STM14_RS18330 to STM14_RS18370
Mut3	C_03_H10	2998648–3042149	43,501	STM14_RS15195 to STM14_RS15425
Mut4	C_01_H4	2451061–2455149	4,088	STM14_RS12670 to STM14_RS12690
Mut5	C_01_G2	2016000–2046442	30,442	STM14_RS10460 to STM14_RS10615
Mut6	C_01_F12	1948041–1981245	33,204	STM14_RS10090 to STM14_RS10285
Mut7	C_01_E9	1572754–1583690	10,936	STM14_RS08285 to STM14_RS08335
Mut8	C_01_E6	1534872–1548754	13,882	STM14_RS08105 to STM14_RS08175
Mut9	K_71/72_E4	1462743–1511389	48,646	STM14_RS07705 to STM14_RS07965
Mut10	C_02_G4	1051514–1057804	6,290	STM14_RS05455 to STM14_RS05480

The plate-well position corresponds to the original MGD collection reported by Porwollik et al. (2014). Full annotation of deleted genes is listed in ***Supplementary Table S3****.*

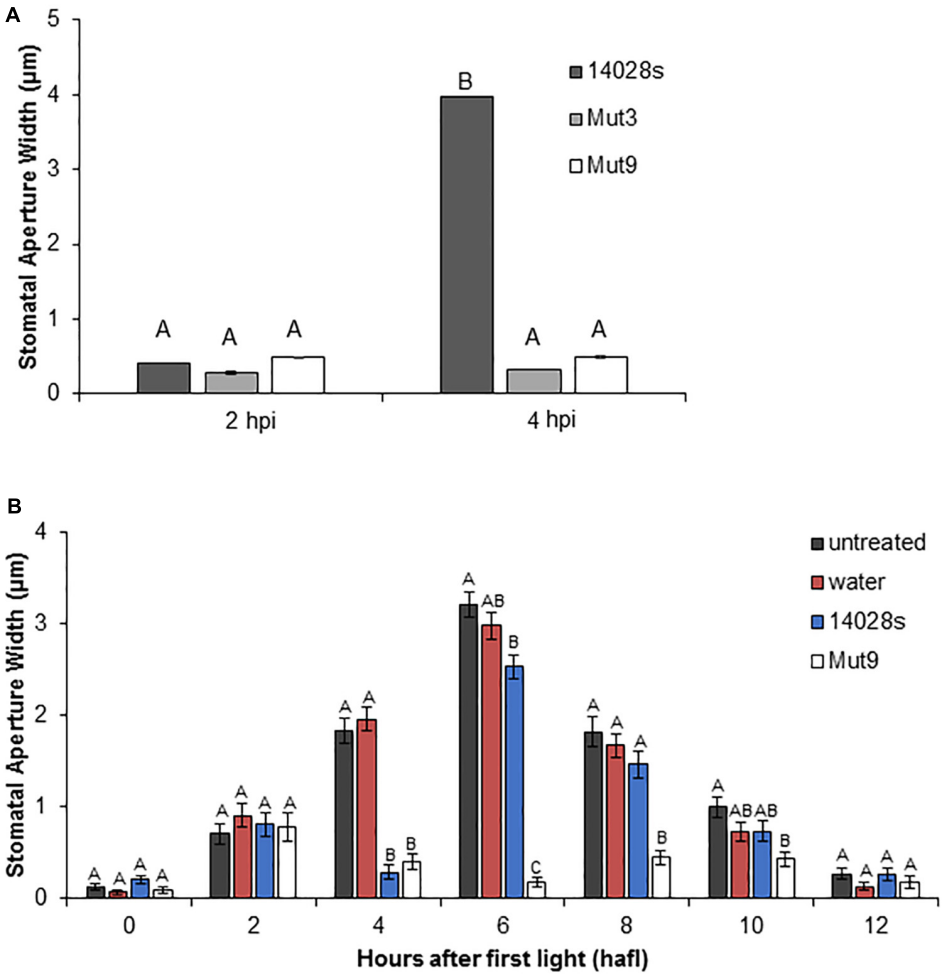


FIGURE 2 | Stomatal response to Mut3 and Mut9. **(A)** Bacterial inoculum (2×10^6 CFU/mL) of *Salmonella* mutants and 14028s was syringe-infiltrated into 5-week-old *L. sativa* cv. Salinas leaves. Inoculation of leaves was performed 2 h after first light (hafl). Results are shown as the mean ($n = 120 \pm$ SE) stomatal aperture width in three independent leaves measured at 2 and 4 hpi. Note that error bars are too small to appear in the graph. Different letters above adjacent bars indicate statistically significant differences among the means within each time point as determined by two-way ANOVA with *post hoc* Tukey HSD test ($p < 0.01$). **(B)** Long-term impact of inoculation with 14028s or Mut9 on the stomatal circadian rhythm on intact *L. sativa* cv. Salinas plants from 0 hafl until darkness (12 hafl). Leaves either remained untreated or were infiltrated with water or a bacterial inoculum (2×10^6 CFU/mL) after 2 hafl. Mean stomatal aperture width ($n = 80 \pm$ SE) of eight biological replicates (i.e., four different leaves and the experiment performed twice). Ten stomata were imaged per leaf. Statistical significance of samples within each time point was determined using a one-way ANOVA with *post hoc* Tukey HSD test ($p < 0.01$). Different letters above adjacent bars indicate statistically significant differences among the means.

or Mut9. Mock-treated and untreated leaves showed an almost identical movement pattern, stomata of Mut9-infiltrated leaves remained closed throughout the daylight period, and STm 14028s-infiltrated leaves showed a transient reduction in stomatal aperture width at 2 hpi that corresponded to 4 hafl (**Figure 2B**).

Genotypic and Phenotypic Characterization of Selected Mutants

Upon completion of this screening procedure, the genome position of the deleted region for each mutant was identified at the nucleotide level by whole genome sequencing of the mutant strains. This procedure, which was readily available in a time- and cost-effective matter, allowed us to predict the genotype and the functional units missing in each mutant using the available STm 14028s and LT2 genome annotations (**Table 1** and **Supplementary Table S1**) (McClelland et al., 2001; Jarvik et al., 2010). The genomic regions deleted in all ten selected mutants were also confirmed by PCR (**Supplementary Figure S2**). Furthermore, each mutant, except Mut5, was able to swim and swarm (**Figure 3**), confirming the predicted genotype of Mut5 is missing genes involved in flagellar biosynthesis and chemotaxis (**Supplementary Table S3**). Movement and chemotaxis have previously been associated with STm SL1344 internalization through the stomatal pore (Kroupitski et al., 2009). Thus, the identification of Mut5 during the *Salmonella* genetic screening validates our procedure, which identified known and novel features associated with bacterial epiphytic behavior. The 10

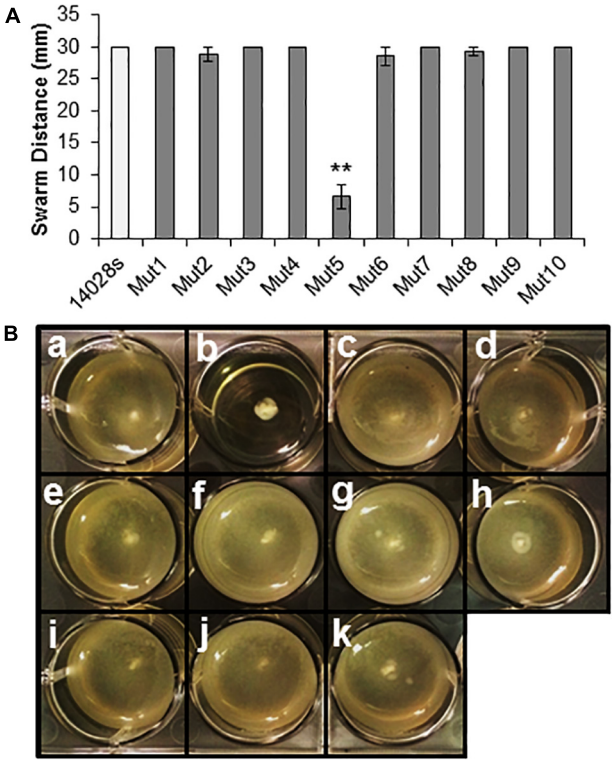


FIGURE 3 | Motility assays using the ten selected MGD mutants. **(A)** Swarming motility was assayed on soft agar by inoculating a freshly grown bacterial colony on top of the agar and incubating the culture under high RH (95 ± 5%) for 24 h. Single colony inocula were of similar size and age. Results are shown as the mean (n = 8) from eight independent plates assessed in two separate assays. Note that some error bars are too small to appear on the graph scale. Statistical analysis (STm 14028s vs. mutant) was performed using a Student's t-test (**$p < 0.01$). **(B)** Swimming motility assay on soft agar inoculated with 1 uL of bacterial culture (1×10^8 CFU/mL) within the agar and observed at 24 hpi. Diffuse turbidity indicates movement of cells from the point of inoculation (center of the well) and confirmed swimming capability in STm 14028s (a), Mut4 (c), Mut2 (d), Mut9 (e), Mut8 (f), Mut7 (g), Mut1 (h), Mut3 (i), Mut10 (j), and Mut6 (k). Lack of movement from the point of inoculation was observed for mutant Mut5 (b). The assay was performed three times with three replicates per assay, with consistent results.

MGD strains were further tested for phenotypic traits required for colonization of leaves as described below.

MGD Mutants Have Variable Apoplastic Persistence

As all selected mutants were unable to stimulate stomatal re-opening at 4 hpi (**Figure 1C**), we sought to determine whether each mutation also affected the population dynamics in the lettuce leaf apoplast. To characterize each mutant's ability to survive within the apoplast, leaves were infiltrated with bacterial inoculum. This allowed for direct analysis of population titer changes due to apoplastic interactions and eliminated confounding factors, such as failure to survive on the leaf surface and/or lack of internalization through stomata. Apoplastic titers were followed over a 3-week period to capture the dynamics of population growth.

The wild type bacterium STm 14028s population declined significantly ($p < 0.05$) in lettuce leaves (from an average of 3×10^4 CFU/cm^2 at day 0 to an average of 4.7×10^3 CFU/cm^2 at day 21), while variable population titers were observed among the mutants (**Figure 4**). For instance, seven mutants, Mut1/2/4/5/7/8/10, had significantly ($p < 0.05$) greater population titers (between 0.5 and 1 log increase) than that of the wild type bacterium at 21 dpi, whereas Mut3 and Mut6 apoplastic persistence did not differ from that of the wild type at 21 dpi (**Figure 4**).

Interestingly, only Mut9 showed significantly ($p < 0.05$) impaired endophytic survival (**Figure 4**). This finding indicates that genes missing in this mutant, including the SPI-2 and the *suf*, *ynh*, *lpp*, and *ttr* operons (**Supplementary Table S3**), may be required for the bacterium to cope with or overcome plant defenses and/or the ability of the bacterium to obtain nutrients from the apoplastic environment necessary to maintain its population. To test for these possibilities, we performed a callose deposit assay and a bacterial growth rate assay using lettuce apoplastic wash fluid (AWF).

STm 14028s Does Not Suppress Callose Deposition Through SPI2 or SPI1

Callose deposition is a hallmark plant defense response that is induced upon biotic stress (Hauck et al., 2003). Thus, we determined the average number of callose deposits in lettuce leaves inoculated with STm 14028s, Mut3, and Mut9. We observed that all three bacteria induced similar numbers of callose deposits that were significantly higher than those seen in the water control (**Figure 5A**). Because all three STm strains induced a relatively low number of callose deposits (average of 7–10 deposits/mm^2), we also inoculated Arabidopsis with the virulent phytopathogen Pst DC3000 for comparison with this well-established system. As previously reported by Hauck et al. (2003), Pst DC3000 did not induce strong callose deposition on its Arabidopsis plant host (approximately 15 deposits/mm^2), although callose deposit frequency was significantly higher than in the water control (**Figure 5B**). However, the Pst DC3000 type-three secretion system (TTSS) mutant (*hrc*C$^-$) induced 2.5 times more callose deposits than the wild type Pst DC3000 in Arabidopsis leaves (**Figure 5B**).

Altogether, our findings suggest that STm 14028s can induce a weak defense in lettuce leaves, similar to that of Pst DC3000 in Arabidopsis leaves. A major function of the SPI genomic region is to assemble the TTSS apparatus and encode effector proteins that could potentially suppress plant defenses. However, we observed that, unlike in the Arabidopsis-Pst DC3000 pathosystem where the TTSS is involved in suppressing plant immune response such as callose (Hauck et al., 2003), the SPI-1 and SPI-2 regions of STm 14028s are not involved in this process in the lettuce system.

Mut9 Growth Is Impaired in Lettuce AWF

Growth rates (generation/hour) of STm 14028s, Mut3, and Mut9 in AWF and LSLB were determined during the log-phase of

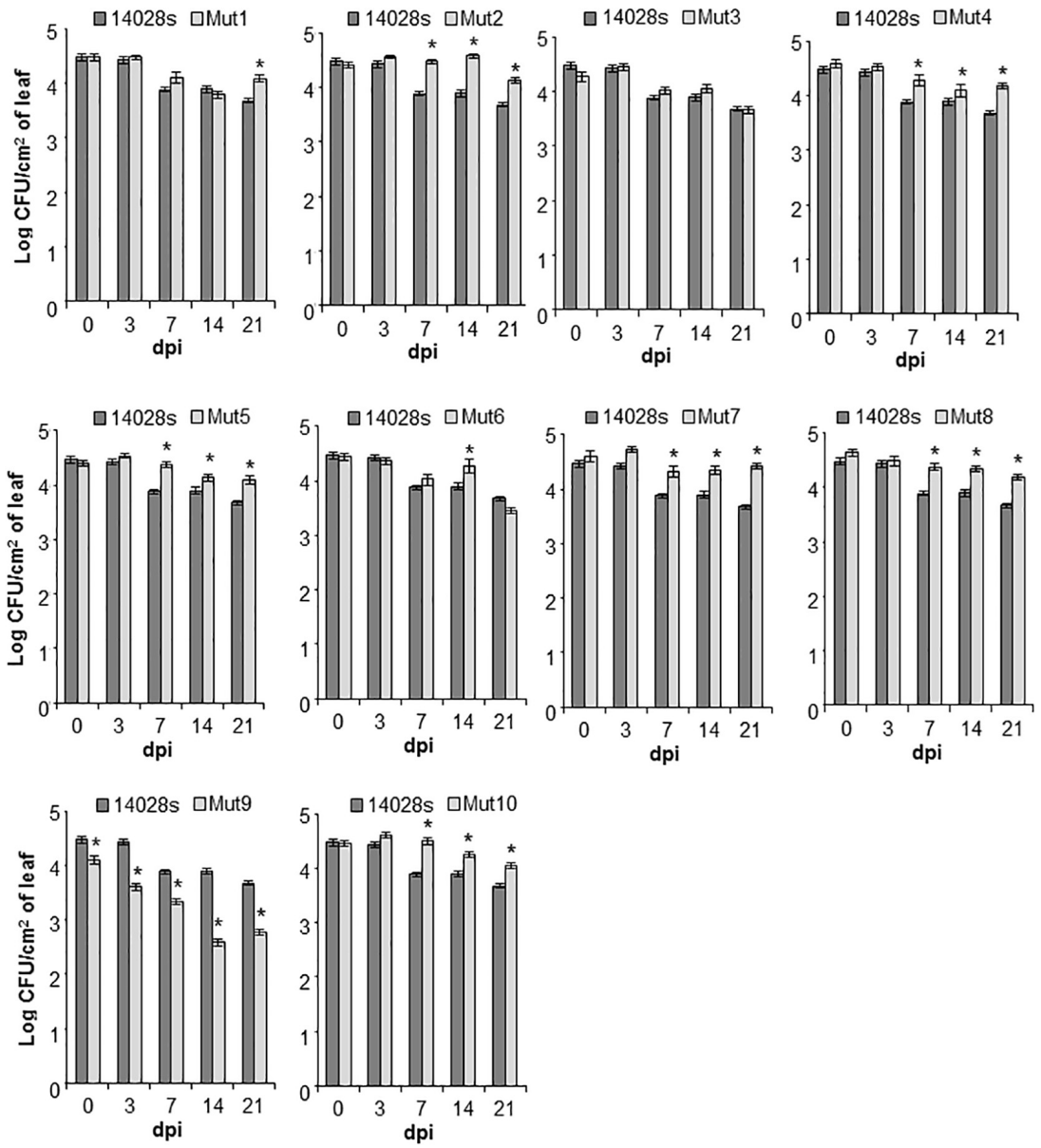


FIGURE 4 | Bacterial persistence in the apoplast of lettuce. Leaves of intact *L. sativa* cv. Salinas plants were syringe-infiltrated with bacterial inoculum (1×10^6 CFU/mL). Bacterial titers were followed over a 3-week period and enumerated at 0, 3, 7, 14, and 21 days post-inoculation (dpi) using a serial-dilution plating assay. Results are shown as mean ($n = 24 \pm$ SE) obtained from four technical replicates and three biological replicates (i.e., three leaves) repeated twice. The statistical significance between the means (STm 14028s vs. mutant at each time point) was determined using a Student's *t*-test (*$p < 0.05$). Lack of a star on top of the bar indicates no statistical difference.

bacterial growth. Water was used to identify growth rates under a no-nutrient condition. As expected, there was minimal bacterial growth in water (**Figure 6**), indicating that residual nutrients in the inoculum were not transferred to LSLB or AWF to enhance growth. In an attempt to correlate the ability of the bacterium to survive within the apoplast (**Figure 4**) with the ability to utilize apoplastic nutrients for growth, we included in this analysis Mut3 that contains a deletion of SPI-1 and adjacent genes (**Supplementary Table S3**) and shows apoplastic persistence similar to the wild type STm 14028s (**Figure 4**). When grown on LSLB, both Mut3 and Mut9 had statistically significant ($p < 0.05$) lower growth rates than STm 14028s. Mut3, Mut9, and STm 14028s had growth rates of 2.78, 2.18, and 3.53 generations/hour, respectively (**Figure 6**). When grown in lettuce AWF, Mut3 and STm 14028s had similar growth rates, while the Mut9 growth rate was significantly lower (**Figure 6**). This finding suggests that the STm's ability to persist in the apoplast may be linked to nutrient acquisition or the overall bacterial fitness in this niche that is dependent on yet-to-be determined gene(s) and operon(s) deleted in Mut9.

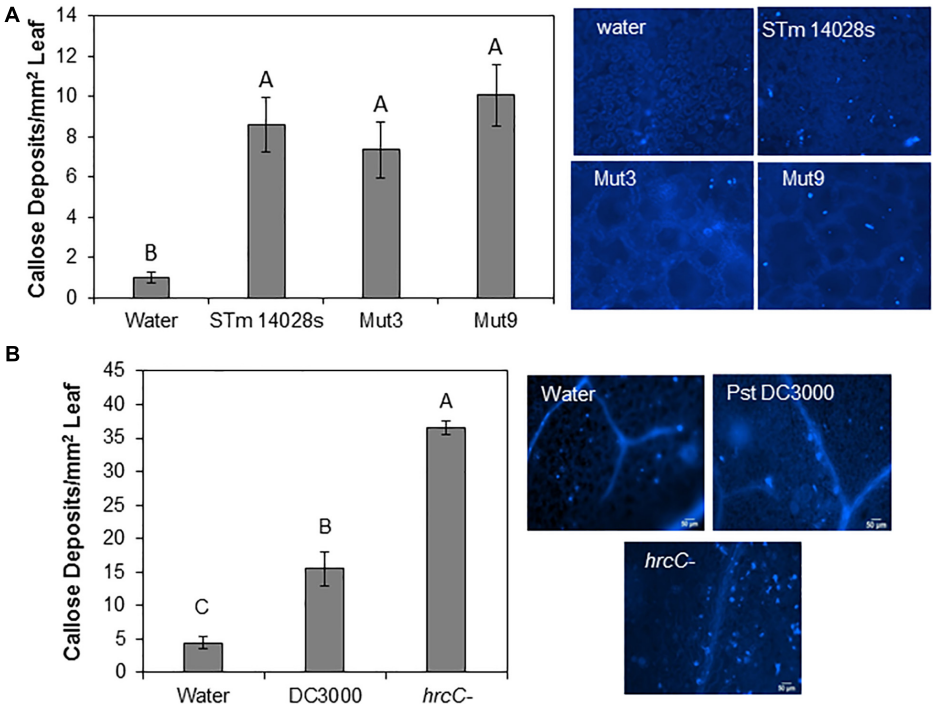


FIGURE 5 | Callose deposition assay. 4–5-week-old lettuce **(A)** or Arabidopsis **(B)** plants were syringe-infiltrated with a 1×10^8 CFU/mL bacterial inoculum (*S. enterica* or *P. syringae*) or water. Callose deposits were enumerated and bars represent the mean ($n = 12 \pm$ SE) number of deposits per mm^2 of leaf obtained from four different leaves and the assay was repeated three times. Statistical significance among the means was determined by one-way ANOVA with *post hoc* Tukey HSD ($p < 0.05$). Different letters above the bars indicate statistically significant differences. Images on the right show typical deposit patterns after each treatment.

DISCUSSION

The importance of foodborne illness caused by contamination of produce by *Salmonella* spp. and the prevalence of contamination associated with leafy greens (DeWaal et al., 2008) led us to investigate the molecular mechanisms allowing *Salmonella* spp. to use this alternate host for survival. As apoplastic populations of human pathogenic bacteria in lettuce are a potential risk for foodborne illnesses due to persistence from production to consumption, we directed our focus on the bacterial internalization into leaves through stomata and endophytic survival. *S. enterica* internalization of leaves can occur through the stomatal pore (Kroupitski et al., 2009; Roy et al., 2013).

We were able to identify ten regions in the STm 14028s genome that may directly or indirectly contribute to the bacterium's ability to open the stomatal pore facilitating its entry into the apoplast. Although it is not obvious which genes in those regions are specifically responsible for the observed phenotype on the leaf surface, the major metabolic functions of these regions are associated with sensing the environment, bacterium chemotaxis and movement, membrane transporters, and biosynthesis of surface appendices (**Supplementary Table S3**). Previously, these functions have been found to be associated with epiphytic fitness of bacterial phytopathogens (reviewed by Melotto and Kunkel, 2013 and Pfeilmeier et al., 2016). Furthermore, Kroupitski et al. (2009) observed that STm SL1344 aggregates near open stomata and uses chemotaxis and motility for internalization through lettuce stomata. Additionally, darkness prevents STm SL1344's ability to re-open the stomatal pore (Roy et al., 2013) and internalization into the leaves possibly due to the lack of chemoattractant leaching through closed stomata (Kroupitski et al., 2009). These findings suggest that close proximity to stomata may be required for *Salmonella* to induce opening of the pore. Therefore, STm invasion of the apoplast may be a consequence of a combined behavior of the bacterium on the phylloplane that can be modulated by plant-derived cues and,

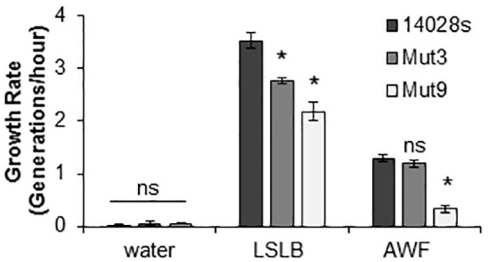


FIGURE 6 | Bacterial growth in different media. Generation times in the log-phase of growth was determined for STm 14028s, Mut3, and Mut9. Results are shown as mean growth rate ($n = 9 \pm$ SE). This experiment was performed three times with three technical replicated each time. Statistical significance between the means (STm 14028s vs. mutant in each medium) was determined using a Student's *t*-test (*$p < 0.05$). ns = no statistical difference, LSLB, low-salt Luria-Bertani; AWF, apoplastic wash fluid.

with this study, we have defined key genomic regions involved in this complex process.

Not all the genomic regions required for initiation of the leaf colonization (i.e., epiphytic behavior and tissue penetration) are essential for continuing bacterial survival as an endophyte (**Figure 4**). For instance, genes deleted from Mut3 (encoding SPI-1) and Mut6 [encoding unspecified membrane proteins, the PhoP/Q two-component system, SopE2 (an effector involved in mammalian infection) (Gong et al., 2009), phage genes, a transcriptional repressor (KdgR), and some unspecified transporters] do not contribute to endophytic survival. Thus, these regions missing in Mut3/6 are potential targets for disrupting leaf surface colonization, but not endophytic persistence. This observation is not entirely surprising as the phylloplane and the apoplast environments are unique and they pose different challenges for bacterial survival in these niches. STm seems to have metabolic plasticity for adaptation to varying conditions in the leaf. For instance, STm SL1344 can shift its metabolism to utilize nutrients available in decaying lettuce and cilantro leaves (Goudeau et al., 2013) and STm 14028s uses distinct metabolism strategies to colonize tomatoes and animal infection (de Moraes et al., 2017).

We also observed that seven regions of the STm 14028s genome have opposite effects on the different phases of colonization. Mut1/2/4/5/7/8/10 seem to lack the ability to promote penetration into the leaf (**Figure 1C**), but they show better fitness than that of the wild type strain in the apoplast (**Figure 4**). One hypothesis is that the increased bacterial population titers are due to lack of energy expenditure for maintaining large genomic segments that are not essential for survival as an endophyte, so that the excess energy can be spent on survival. However, this indirect effect of the deletion may not be valid for Mut4/10, where only small genomic regions are missing (**Supplementary Table S3**). Alternatively, these regions might encode for proteins that negatively affect bacterial survival in leaves. This interesting observation is worth future investigation.

Intriguingly, we found that genes deleted in Mut9 are important for re-opening the stomatal pore and successful endophytic survival. This deletion includes SPI-2 that functions in the production of the TTSS-2 apparatus, effectors, and a two-component regulatory system of this island (Coombes et al., 2004), which are important for the virulence of STm in animal systems (Waterman and Holden, 2003). The contribution of the TTSS-2 apparatus and effectors to the bacterium's ability to colonize the phyllosphere has been studied in several laboratories and it is largely dependent on the plant species analyzed (reviewed by Garcia and Hirt, 2014 and Melotto et al., 2014). Nonetheless, so far there is no evidence for the ability of STm to inject TTSS effectors inside plant cells (Chalupowicz et al., 2018). Furthermore, the STm 14028s *ssaV*-structural mutant, that cannot form the TTSS-2 apparatus (Vishwakarma et al., 2014), survives in the lettuce cv. Romit 936 to the same extent as the wild type bacterium after surface inoculation (Chalupowicz et al., 2018). Our data also support the notion that the TTSS-2 is not involved in STm ability to induce or subvert defenses, such as callose deposition in lettuce cv. Salinas (**Figure 5**). While studies in other plant systems have suggested that TTSS and encoded effectors may contribute to bacterial survival in the plant environment (Schikora et al., 2011; Shirron and Yaron, 2011) or in some cases are detrimental for bacterial colonization of plant tissues (Iniguez et al., 2005), it has become evident that the TTSS-2 within the SPI-2 region is not relevant in the STm 14028s-lettuce leaf interaction.

It is important to note that SPI-2 is a genomic segment of roughly 40 kb with 42 open reading frames arranged into 17 operons (**Supplementary Table S3**) (Hensel, 2000). It is present in all pathogenic serovars and strains of *S. enterica*, but only partially present in species of a more distant common ancestor, such as *S. bongori* (Hensel, 2000). Besides encoding structural and regulatory components of the TTSS-2 (Coombes et al., 2004), SPI2 also carries genes coding for a tetrathionate reductase complex, a cysteine desulfurase enzyme complex, membrane transport proteins, murein transpeptidases, as well as genes with still uncharacterized functions (NCBI Resource Coordinators, 2017). Thus, it is possible that genes and operons, other than the ones associated with TTSS-2, may have a function in the bacterium colonization of the lettuce leaf.

To date, it has not been demonstrated whether STm 14028s can access and utilize nutrients from the apoplast of intact lettuce leaves. Although nutrients in the apoplast might be limiting (Lindow and Brandl, 2003), it has been hypothesized that *Salmonella* may scavenge nutrients to persist in the plant environment (Teplitski and de Moraes, 2018) and/or adjust its metabolism to synthesize compounds that are not readily available at the colonization site. For instance, a mutant screen analysis indicated that STm 14028s requires genes for biosynthesis of nucleotides, lipopolysaccharide, and amino acids during colonization of tomato fruits (de Moraes et al., 2017). Moreover, plants might secrete antimicrobial compounds into the apoplast as a plant defense mechanism, imposing a stressful condition to the microbial invader (reviewed by Doehlemann and Hemetsberger, 2013). Therefore, considering that subversion of plant defenses is not a function of the TTSS-2 in the apoplast of lettuce (**Figure 5**), it is possible that the Mut9 population reduces 20 fold over 21 days (**Figure 4**) due to its inability to obtain nutrients from this niche and/or to cope with plant defenses. Although Mut9 shows reduced growth on lettuce leaf AWF (**Figure 6**), additional experimentation is required to distinguish between these two possibilities. It is tempting to speculate, however, that the tetrathionate reductase gene cluster (*ttrRSBCA*) within SPI-2 or the sulfur mobilization (SUF) operon deleted in Mut9 (**Supplementary Table S3**) might be involved in this process. Particular to the ttr operon, TtrAB forms the enzyme complex, TtrC anchors the enzyme to the membrane, whereas TtrS and TtrR are the sensor kinase and DNA-binding response regulator, respectively (James et al., 2013). The reduction of tetrathionate by this membrane-localized enzyme is part of the *Salmonella*'s anaerobic respiration (Hensel et al., 1999). Intriguingly, the use of tetrathionate as an electron acceptor during propanediol and ethanolamine utilization by the bacterium (Price-Carter et al., 2001) has been suggested to occur in macerated leaf tissue (Goudeau et al., 2013). A significant number of genes involved in the PDU (propanediol utilization),

EUT (ethanolamine), and cobalamin pathways as well as the *ttrC* gene are upregulated in STm SL1344 when co-inoculated with the soft rot pathogen *Dickeya dadantii* onto cilantro and lettuce leaf cuts (Goudeau et al., 2013). Altogether, these findings suggest that these biochemical pathways may occur in both soft rot contaminated and healthy leaves.

Considering that the encounter of the plant with a pathogenic bacterium triggers molecular action and reaction in both organisms overtime, it is not surprising that multiple regions of the STm 14028s genome may be required for lettuce leaf colonization. For instance, Goudeau et al. (2013) reported that 718 (16.4%) genes of the STm SL1344 genome were transcriptionally regulated upon exposure to degrading lettuce cell wall. In any case, further studies using single-gene mutants are still required to identify the specific genes and functions within each MGD mutant that are involved in the interaction between STm 14028s and lettuce cultivar Salinas.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

MMe conceived the research. JM and MMe designed the research and wrote the manuscript. JM, GR, JT, and SP performed the experiments. JM, GR, and MMe analyzed the data. SS, MMc, and MMe provided materials and discussed the project in detail. All authors read and approved the manuscript.

FUNDING

This research was supported by grants from the U.S. Department of Agriculture – National Institute of Food and Agriculture (NIFA; 2015-67017-23360) and NIFA Hatch grant (CA-D-PLS-2327-H) to MMe, a NIFA-BARD award (2017-67017-26180) to SS, MMc, and MMe. JM and GR were supported by a Graduate Research Fellowship award from the Department of Plant Sciences, UC Davis.

ACKNOWLEDGMENTS

The authors thank Dr. Shweta Panchal and Cristián Jacob for gathering preliminary data for the callose assays and Dr. R. Michelmore for the lettuce seeds and guidance on growing healthy lettuce plants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00006/full#supplementary-material

FIGURE S1 | Multi-gene deletion (MGD) mutant strain screen design. **(A)** 3-week old *L. sativa* cv. Salinas plants grown in Peat Pellets. Leaves from plants of this stage were used in leaf float stomatal assays. **(B)** Leaf sections of the *L. sativa* cv. Salinas floated, abaxial side down, on bacterial inoculum (1×10^8 CFU/mL) or water control were used for stomatal assays according to Montano and Melotto (2017). **(C)** Schematic of three-layer screen to identify ten mutants for detailed characterization.

FIGURE S2 | Genotype confirmation of selected MGD mutants. **(A)** Diagram showing primer locations. Shaded areas represent wild type sequence that is present in both the wild type and the mutant strains. White areas represent sequence that is unique to either the wild type or the mutant. WTFP, Wild Type Forward Primer; WTRP, Wild Type Reverse Primer; MFP, Mutant Forward Primer; MRP, Mutant Reverse Primer. **(B)** Agarose gel electrophoresis of PCR reactions containing the indicated gDNA and primer sets. The far left lanes contain the molecular weight marker with band sizes indicated in Kb. No amplification was expected in the samples loaded onto Lanes 2 and 4.

TABLE S1 | List of 303 MGD mutant strains screened for the inability to re-open the stomatal pore. Plate-well numbers are based on previously published data (Porwollik et al., 2014).

TABLE S2 | Functional unit overview for 51 MGD mutant strains selected for a confirmation screen. Plate-well number and predicted mutation site are based on previously published data (Porwollik et al., 2014). The ability of each strain to re-open stomata (yes, no, or partial) was determined by comparisons with the wild type STm 14028s (yes) and Mut9 (no) phenotypes using Student's *t*-test ($p < 0.01$). MGD mutant strains classified as "partial" for the stomatal re-opening ability indicates that the phenotype of those strains was intermediate between STm 14028s and Mut9. Functional annotation was inferred by BLAST analysis using the NCBI database. Strains in bold letters were chosen for further analysis.

TABLE S3 | Genes deleted in each MGD mutant strain and their corresponding annotation based on the published genomes of the STm strains 14028s and LT2.

TABLE S4 | Primers sequences and target genomic regions to validate the mutation site of each MGD strain.

REFERENCES

Barak, J., and Schroeder, B. (2012). Interrelations of food safety and plant pathology: the life cycle of human pathogens on plants. *Ann. Rev. Phytopath.* 50, 241–266. doi: 10.1146/annurev-phyto-081211-172936

Bender, C., Alarcón-Chaidez, F., and Gross, D. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthases. *Microbiol. Mol. Biol. Rev.* 63, 266–292. doi: 10.1128/mmbr.63.2.266-292.1999

Borodovsky, M., and McIninch, J. (1993). GENMARK: parallel gene recognition for both DNA strands. *Comput. Chem.* 17, 123–133. doi: 10.1016/0097-8485(93)85004-v

Brandl, M., and Amudson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *App. Environm. Microbiol.* 74, 2298–2306. doi: 10.1128/AEM.02459-07

Chalupowicz, L., Nissan, G., Brandl, M. T., McClelland, M., Sessa, G., Popov, G., et al. (2018). Assessing the ability of *Salmonella enterica* to translocate type III effectors into plant cells. *Mol. Plant Microb. Interact.* 2, 233–239. doi: 10.1094/MPMI-07-17-0166-R

Chitrakar, R., and Melotto, M. (2010). Assessing stomatal response to live bacterial cells using whole leaf imaging. *J. Vis. Exp.* 44:2185. doi: 10.3791/2185

Coombes, B. K., Brown, N. F., Valdez, Y., Brumell, J. H., and Finlay, B. B. (2004). Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. *J. Biol. Chem.* 279, 49804–49815. doi: 10.1074/jbc.m404299200

Critzer, F. J., and Doyle, M. P. (2010). Microbial ecology of foodborne pathogens associated with produce. *Curr. Opin. Biotech.* 21, 125–130. doi: 10.1016/j.copbio.2010.01.006

de Moraes, M. H., Desai, P., Porwollik, S., Canals, R., Perez, D. R., Chu, W., et al. (2017). *Salmonella* persistence in tomatoes requires a distinct set of metabolic functions identified by transposon insertion sequencing. *App. Environm. Microbiol.* 83:e003028-16. doi: 10.1128/AEM.03028-3016

Delcher, A. L., Harmon, D., Kasif, S., White, O., and Salzberg, S. L. (1999). Improved microbial gene identification with GLIMMER. *Nucleic Acids Res.* 27, 4636–4641. doi: 10.1093/nar/27.23.4636

DeWaal, C. S., Tian, X., and Bhuiya, F. (2008). *Outbreak Alert! 2008: Closing the Gaps in Our Federal Food-Safety Net*. Washington, DC: Center for Science in the Public Interest (CSPI).

Dewey-Mattia, D., Manikonda, K., and Vieira, A. (2016). *Surveillance for foodborne disease outbreaks, United States, 2014: Annual Report. US Department of Health and Human Services, CDC Atlanta, Georgia*. Available at: https://www.cdc.gov/foodsafety/pdfs/foodborne-outbreaks-annual-report-2014-508.pdf

Doehlemann, G., and Hemetsberger, C. (2013). Apoplastic immunity and its suppression by filamentous plant pathogens. *New Phytol.* 198, 1001–1016. doi: 10.1111/nph.12277

Fischer, N., Bourne, A., and Plunkett, D. (2015). *Outbreak Alert! 2015: A Review of Foodborne Illness in the U.S. From 2004-2013*, 17th Edn, Washington, DC: Center for Science in the Public Interest (CSPI).

Garcia, A. V., and Hirt, H. (2014). *Salmonella enterica* induces and subverts the plant immune system. *Front. Microbiol.* 5:141. doi: 10.3389/fmicb.2014.00141

Gil, M. I., Selma, M. V., López-Gálvez, F., and Allende, A. (2009). Fresh-cut product sanitation and wash water disinfection: problems and solutions. *Int. J. Food Microb.* 134, 37–45. doi: 10.1016/j.ijfoodmicro.2009.05.021

Gong, H., Su, J., Bai, Y., Miao, L., Kim, K., Yang, Y., et al. (2009). Characterization of the expression of *Salmonella* Type III secretion system factors PrgI, SipA, SipB, SopE2, SpaO, and SptP in cultures and in mice. *BMC Microb.* 9:73. doi: 10.1186/1471-2180-9-73

Goodburn, C., and Wallace, C. A. (2013). The microbiological efficacy of decontamination methodologies for fresh produce: a review. *Food Control* 32, 418–427. doi: 10.1016/j.foodcont.2012.12.012

Goudeau, D. M., Parker, C. T., Zhou, Y., Sela, S., Kroupitski, Y., and Brandl, M. T. (2013). The *Salmonella* transcriptome in lettuce and cilantro soft rot reveals a niche overlap with the animal host intestine. *Appl. Environ. Microbiol.* 79, 250–262. doi: 10.1128/AEM.02290-12

Hauck, P., Thilmony, R., and He, S. Y. (2003). A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8577–8582. doi: 10.1073/pnas.1431173100

Heaton, J., and Jones, K. (2008). Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *J. App. Microb.* 104, 613–626. doi: 10.1111/j.1365-2672.2007.03587.x

Hensel, M. (2000). *Salmonella* pathogenicity island 2. *Mol. Microbiol.* 36, 1015–1023. doi: 10.1046/j.1365-2958.2000.01935.x

Hensel, M., Hinsley, A. P., Nikolaus, T., Sawers, G., and Berks, B. C. (1999). The genetic basis of tetrathionate respiration in *Salmonella typhimurium*. *Mol. Microbiol.* 32, 275–287. doi: 10.1046/j.1365-2958.1999.01345.x

Hirano, S. S., and Upper, C. D. (1983). Ecology and epidemiology of foliar bacterial plant pathogens. *Ann. Rev. Phytopathol.* 21, 243–270. doi: 10.1111/j.1364-3703.2012.00816.x

Hoiseth, S. K., and Stocker, B. A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 291, 238–239. doi: 10.1038/291238a0

Iniguez, A. L., Dong, Y., Carter, H. D., Ahmer, B. M., Stone, J. M., and Triplett, E. W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant Microb. Interact.* 18, 169–178. doi: 10.1094/mpmi-18-0169

Jacob, C., Panchal, S., and Melotto, M. (2017). Surface inoculation and quantification of *Pseudomonas syringae* population in the Arabidopsis leaf apoplast. *Bio Protoc.* 7:e2167. doi: 10.21769/BioProtoc.2167

James, M. J., Coulthurst, S. J., Palmer, T., and Sargent, F. (2013). Signal peptide etiquette during assembly of a complex respiratory enzyme. *Mol. Microbiol.* 90, 400–414. doi: 10.1111/mmi.12373

Jarvik, T., Smillie, C., Groisman, E. A., and Ochman, H. (2010). Short-term signatures of evolutionary change in the *Salmonella enterica* serovar typhimurium 14028 genome. *J. Bacteriol.* 192, 560–567. doi: 10.1128/JB.01233-09

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acid Res.* 45, D353–D361. doi: 10.1093/nar/gkw1092

Katagiri, F., Thilmony, R., and He, S. Y. (2002). The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *Arabidopsis Book* 1:e0039. doi: 10.1199/tab.0039

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., et al. (2009). Internalization of *Salmonella* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75, 6076–6086. doi: 10.1128/AEM.01084-09

Lindow, S. E., and Brandl, M. T. (2003). Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* 69, 1875–1883. doi: 10.1128/aem.69.4.1875-1883.2003

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., et al. (2001). Complete genome sequence of *Salmonella enterica Serovar typhimurium* LT2. *Nature* 413, 852–856.

Melotto, M., and Kunkel, B. N. (2013). "Virulence strategies of plant pathogenic bacteria," in *The Prokaryotes – Prokaryotic Physiology and Biochemistry*, 4th Edn, eds E. Rosenberg, E. Stackebrand, E. F. DeLong, F. Thompson, and S. Lory, (Berlin: Springer-Verlag), 61–82. doi: 10-1007/978-3-642-30141-4-62

Melotto, M., Panchal, S., and Roy, D. (2014). Plant innate immunity against human bacterial pathogens. *Front. Microbiol.* 5:411. doi: 10.3389/fmicb.2014.00411

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980. doi: 10.1016/j.cell.2006.06.054

Montano, J., and Melotto, M. (2017). "Stomatal bioassay to characterize bacterial-stimulated PTI at the pre-invasion phase of infection," in *Plant Pattern Recognition Receptors. Methods in Molecular Biology*, Vol. 1578, eds L. Shan, and P. He, (New York, NY: Humana Press), 233–241. doi: 10.1007/978-1-4939-6859-6-19

NCBI Resource Coordinators, (2017). Database resources of the national center for biotechnology information. *Nucleic Acid Res.* 45, D12–D17.

Newman, M. A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139

Nguyen, H., Chakravarthy, S., Velasquez, A., McLane, H., Zeng, L., Nakayashiki, H., et al. (2010). Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant Microb. Inter.* 23, 991–999. doi: 10.1094/MPMI-23-8-0991

Nicaise, V., Roux, M., and Zipfel, C. (2009). Recent advances in PAMP-triggered immunity against bacteria: patter recognition receptors watch over and raise the alarm. *Plant Physiol.* 150, 1638–1647. doi: 10.1104/pp.109.139709

O'Leary, B. M., Rico, A., McCraw, S., Fones, H. N., and Preston, G. M. (2014). The Infiltration-centrifugation technique for extraction of apoplastic fluid from plant leaves using *Phaseolus vulgaris* as an example. *J. Vis. Exp.* 94:e52113. doi: 10.3791/52113

O'May, C., and Tufenkji, N. (2011). The swarming motility of *Pseudomonas aeruginosa* is blocked by cranberry proanthocyanidins and other tannin-containing materials. *App. Environm. Microbiol.* 77, 3061–3067. doi: 10.1128/AEM.02677-10

Pfeilmeier, S., Caly, D. L., and Malone, J. G. (2016). Bacterial pathogenesis of plants: future challenges from a microbial perspective. *Mol. Plant Pathol.* 17, 1298–1313. doi: 10.1111/mpp.12427

Porwollik, S., Santiviago, C. A., Cheng, P., Long, F., Desai, P., Fredlund, J., et al. (2014). Defined single-gene and multi-gene deletion mutant collections in *Salmonella enterica* sv *typhimurium*. *PLoS One* 9:e99820. doi: 10.1371/journal.pone.0099820

Price-Carter, M., Tingey, J., Bobik, T. A., and Roth, J. R. (2001). The alternative electron acceptor tetrathionate supports B12-dependent anaerobic growth of

Salmonella enterica Serovar typhimurium on ethanolamine or 1,2-propanediol. *J. Bacteriol.* 183, 2463–2475. doi: 10.1128/jb.183.8.2463-2475.2001

Roy, D., Panchal, S., Rosa, B., and Melotto, M. (2013). *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica typhimurium* SL1344. *Phytopathology* 4, 326–332. doi: 10.109/PHYTO-09-12-0230-FI

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrier, A., et al. (2011). Conservation of *Salmonella* infection mechanisms in plants and animals. *PLoS One* 6:e24112. doi: 10.1371/journal.pone.0024112

Shirron, N., and Yaron, S. (2011). Active suppression of early immune response in tobacco by the human pathogen *Salmonella typhimurium*. *PLoS One* 6:e18855. doi: 10.1371/journal.pone.0018855

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., et al. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* 44, 6614–6624. doi: 10.1093/nar/gkw569

Tatusova, T. A., and Madden, T. L. (1999). BLAST 2 sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* 174, 247–250. doi: 10.1111/j.1574-6968.1999.tb13575.x

Teplitski, M., and de Moraes, M. (2018). Of mice and men and plants: comparative genomics of the dual lifestyles of enteric pathogens. *Trends Microbiol.* 9, 748–754. doi: 10.1016/j.tim.2018.02.008

Todar, K. (2012). "The growth of bacterial populations," in *Todar's Online Textbook of Bacteriology* (Madison, WI: Department of Bacteriology). Available at: www.textbookofbacteriology.net.

Vishwakarma, V., Pati, N. B., Ray, S., Das, S., and Suar, M. (2014). TTSS2-deficient *hha* mutant of *Salmonella typhimurium* exhibits significant systemic attenuation in immunocompromised hosts. *Virulence* 5, 311–320. doi: 10.4161/viru.27605

Waterman, S. R., and Holden, D. W. (2003). Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell. Microbiol.* 5, 501–511. doi: 10.1046/j.1462-5822.2003.00294.x

Conflict of 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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 January 2020
doi: 10.3389/fpls.2019.01769

Human Pathogen Colonization of Lettuce Dependent Upon Plant Genotype and Defense Response Activation

Cristián Jacob[1,2] *and Maeli Melotto*[1]*

[1] Department of Plant Sciences, University of California, Davis, Davis, CA, United States, [2] Horticulture and Agronomy Graduate Group, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Monica Fernandez-Aparicio,
Institute for Sustainable Agriculture,
CSIC, Spain

Reviewed by:
Jacqueline Fletcher,
Oklahoma State University,
United States
Jianbin Su,
University of Missouri,
United States
Adam Schikora,
Julius Kühn-Institut – Braunschweig,
Germany

***Correspondence:**
Maeli Melotto
melotto@ucdavis.edu

Specialty section:
This article was submitted to
Plant Breeding,
a section of the journal
Frontiers in Plant Science

Received: 15 August 2019
Accepted: 18 December 2019
Published: 30 January 2020

Citation:
Jacob C and Melotto M (2020) Human Pathogen Colonization of Lettuce Dependent Upon Plant Genotype and Defense Response Activation.
Front. Plant Sci. 10:1769.
doi: 10.3389/fpls.2019.01769

Fresh produce contaminated with human pathogens may result in foodborne disease outbreaks that cause a significant number of illnesses, hospitalizations, and death episodes affecting both public health and the agribusiness every year. The ability of these pathogens to survive throughout the food production chain is remarkable. Using a genetic approach, we observed that leaf colonization by *Salmonella enterica* serovar Typhimurium 14028s (*S.* Typhimurium 14028s) and *Escherichia coli* O157:H7 was significantly affected by genetic diversity of lettuce (*Lactuca sativa* L. and *L. serriola* L.). In particular, there was a significant variation among 11 lettuce genotypes in bacterial attachment, internalization, and apoplastic persistence after surface- and syringe-inoculation methods. We observed a significant correlation of the bacterial leaf internalization rate with stomatal pore traits (width and area). Moreover, bacterial apoplastic populations significantly decreased in 9 out of 11 lettuce genotypes after 10 days of surface inoculation. However, after syringe infiltration, populations of *E. coli* O157:H7 and *S.* Typhimurium 14028s showed positive, neutral, or negative net growth in a 10-day experimental period among seedlings of different lettuce types. The relative ability of the bacteria to persist in the apoplast of lettuce genotypes after syringe inoculation was minimally altered when assessed during a longer period (20 days) using 3.5- to 4-week-old plants. Interestingly, contrasting bacterial persistence in the lettuce genotypes Red Tide and Lollo Rossa was positively correlated with significant differences in the level of reactive oxygen species burst and callose deposition against *S.* Typhimurium 14028s and *E. coli* O157:H7 which are related to plant defense responses. Overall, we characterized the genetic diversity in the interaction between lettuce genotypes and enterobacteria *S.* Typhimurium 14028s and *E. coli* O157:H7 and discovered that this genetic diversity is linked to variations in plant immune responses towards these bacteria. These results provide opportunities to capitalize on plant genetics to reduce pathogen contamination of leaves.

Keywords: lettuce genotypes, fresh produce, genetic diversity, bacterial persistence, human pathogen, *Escherichia coli*, *Salmonella enterica*, disease outbreak

INTRODUCTION

During the last two decades, the number, severity, and distribution of outbreaks of human diseases linked to the consumption of fresh produce have attracted the attention of farmers, the food industry, consumers, politicians, and scientists. According to data reported to the U.S. Centers for Disease Control and Prevention's Foodborne Disease Outbreak Surveillance System from 1998 and 2013, there were 972 raw produce-associated outbreaks reported, which accounted for 34,674 illnesses, 2,315 hospitalizations, and 72 deaths in the U.S. (Bennett et al., 2018). The most common etiologic agents identified were norovirus (54% of outbreaks), *Salmonella enterica* (21%), and Shiga toxin-producing *Escherichia coli* (10%) (Bennett et al., 2018). This is concerning considering the current upward trend and the steady promotion of fresh produce consumption. In the case of lettuce (*Lactuca sativa* L.), the major ingredient of leafy salads, the U.S. per capita consumption is relatively high at an average of 12.0 kg per person per year in the last decade (ERS-USDA (Economic Research Service - United States Department of Agriculture), 2018). Moreover, the yearly sales of bagged salads have been growing in the U.S., reaching $3.7 billion in 2015 (Cook, 2016), which represents an important change in the consumers' behavior towards purchasing ready-to-eat and/or minimally processed salads.

Fresh produce is susceptible to contamination by human pathogens from diverse sources during field production, storage, transport, packaging, and processing (Barak and Schroeder, 2012; Sapers and Doyle, 2014). During vegetable production, the major vehicles for bacterial contamination are irrigation water, manure soil amendments, and wild animal intrusion (Jay-Russell, 2013; Allende and Monaghan, 2015; Jiang et al., 2015). For successful phyllosphere colonization, bacteria require the ability to attach, form aggregates, and/or produce biofilms to survive epiphytically. Both *Salmonella* and *E. coli* are able to modulate their metabolism upon leaf contact towards the production of molecules involved in attachment and biofilm matrix formation (Yaron and Römling, 2014). Phylloplane settlement processes are followed and/or accompanied by the bacterial movement toward and through the stomatal pore. Studies have demonstrated that both *Salmonella* and *E. coli* are able to reach the leaf intercellular space through the stomatal pore (Seo and Frank, 1999; Kroupitski et al., 2009; Saldaña et al., 2011; Roy et al., 2013). Plant cell recognition of Microbe-Associated Molecular Patterns (MAMPs) of human bacterial pathogens can trigger the production of Pattern-Triggered Immunity (PTI)-associated defense responses (Garcia et al., 2014), including a decrease in stomatal aperture width (Melotto et al., 2006; Roy et al., 2013). On the other hand, bacteria might counter-attack the plant responses by subverting the stomatal closure defense (Roy et al., 2013) or activating genes associated with oxidative stress tolerance and antimicrobial resistance (Van der Linden et al., 2016).

The overall outcome of the interaction between plants and human bacterial pathogens on/in the leaf is the persistence of the microorganisms for few days to several weeks (Solomon et al., 2003; Islam et al., 2004a; Islam et al., 2004b; Fonseca et al., 2011; Kisluk and Yaron, 2012). The ability of bacteria to survive in the phyllosphere is largely dependent upon the plant species and specific genotypes of each species (Klerks et al., 2007; Mitra et al., 2009; Barak et al., 2011; Golberg et al., 2011; Quilliam et al., 2012; Macarisin et al., 2013; Hunter et al., 2015; Crozier et al., 2016; Erickson et al., 2018; Roy and Melotto, 2019). Certain leaf traits have been associated with intraspecific and interspecific differences in plant colonization, together with variation between and within plant tissues. For instance, Macarisin et al. (2013) found differential persistence of *E. coli* O157:H7 on the leaves of spinach cultivars, which was influenced by leaf blade roughness and stomatal density. Other leaf surface factors, such as vein density, hydrophobicity, and level of epicuticular wax, were associated with cultivar-specific differences in *S. enterica* ser. Senftenberg attachment on iceberg and Batavia type lettuces (Hunter et al., 2015). In tomato, the level of *S. enterica* persistence in the phyllosphere after dip-inoculation with an eight-serovar cocktail (serovars Baildon, Cubana, Enteritidis, Havana, Mbandaka, Newport, Poona, and Schwarzengrund) also seems to be influenced by plant genotype (Barak et al., 2011). Furthermore, *S. enterica* seedling colonization of lettuce and tomato has been reported not only to be influenced by the plant species and cultivar, but also by the bacterial serovar and strain (Wong et al., 2019).

Although there is evidence indicating that plant genotypic diversity influences the colonization of the phyllosphere by human bacterial pathogens, phenotypes associated with the observed differences are limited to the morphological and chemical composition of the leaf surface. Molecular mechanisms and biological processes involved in the variation of bacterial survival in the phyllosphere of different plant genotypes are largely unknown. Moreover, variation in the interaction between plants and human pathogenic bacteria due to plant genetic diversity has been shown to be quantitative (Barak et al., 2011; Quilliam et al., 2012; Marvasi et al., 2014). Therefore, few to several genetic factors might be influencing variation in the resulting phenotype (Corwin and Kliebenstein, 2017). This complex scenario exposes the necessity to find robust phenotypic differences in a phyllosphere-human pathogenic bacterium system that enables an in-depth analysis of the underlying factors. In this study, we characterized the genetic diversity in the interaction between lettuce genotypes and the enterobacteria *S. enterica* Typhimurium 14028s and *E. coli* O157:H7. Furthermore, we discovered that this genetic diversity is linked to differences in the plant immune responses.

MATERIALS AND METHODS

Plant Material and Growth Conditions

A set of 11 lettuce genotypes was used to conduct this study (**Table 1**). Seeds were sown on water-soaked germination paper in square Petri dishes and incubated for 2 days at 20°C. Germinated seeds were transferred to either peat moss pellets (42 mm, Jiffy® 7, Canada) or to 7.62 cm^2 pots (Kord Products,

TABLE 1 | List of lettuce genotypes used to evaluate the natural genetic variability regarding the plant response to *Salmonella enterica* Typhimurium 14028s and *Escherichia coli* O157:H7.

Species	Genotype/ lettuce type	Accession number	Life cycle[1]	Plant disease traits			
				Resistance		Susceptibility	
				Pathogen	*Reference(s)*	*Pathogen*	*Reference(s)*
Lactuca sativa L. var. *crispa* L.	Salinas/Iceberg	14G1846-1	Long	Dieback (caused by two viruses from the family Tombusviridae)	Grube and Ochoa (2005); Simko et al. (2009)	Lettuce big-vein associated virus	Ryder and Robinson (1995)
				Fusarium oxysporum f.sp. *lactucae* race 1	McCreight et al. (2005)	*Sclerotinia minor*	Hayes et al. (2010)
				Fusarium oxysporum f.sp. *lactucae*	Scott et al. (2010)	*Verticillium dahliae*	Vallad and Subbarao (2008)
						Bremia lactucae	Grube and Ochoa (2005); Simko et al. (2014)
L. sativa L. var. *crispa* L.	Emperor/ Iceberg	14G11-1	Long				
L. sativa L. var. *crispa* L.	La Brillante/ Batavia	13G637-2	Short	Dieback	Simko et al. (2009)	.	
				Lettuce big-vein associated virus	Ryder and Robinson (1995)		
				Bremia lactucae	Simko et al. (2015)		
				Xanthomonas campestris pv. *vitians*	Hayes et al. (2014)		
				Verticillium dahliae	Vallad and Subbarao (2008)		
L. sativa L. var. *acephala* Dill.	Lollo Rossa/ Red loose leaf	10G11-2	Short	*Xanthomonas campestris* pv. *vitians*	Hayes et al. (2014)	Dieback	Simko et al. (2009)
				Fusarium oxysporum f.sp. *lactucae*	Scott et al. (2010)		
L. sativa L. var. *acephala* Dill.	Red Tide/Red loose leaf	10G12-2	Short	*Sclerotinia sclerotiorum*	Hayes et al. (2010)	Dieback	Simko et al. (2009)
						Bremia lactucae	Simko et al. (2014)
L. sativa L. var. *acephala* Dill.	Grand Rapids/ Green loose leaf	13G1033-1	Short	Dieback	Grube et al. (2003)		
				Bremia lactucae	Grube and Ochoa (2005)		
L. sativa L. var. *longifolia* (Lam.) Janchen	Green Towers/ Romaine	14G388-2	Medium	*Sclerotinia sclerotiorum*	Hayes et al. (2010)	Dieback	Grube et al. (2003); Simko et al. (2009)
						Bremia lactucae	Grube and Ochoa (2005)
						Phoma exigua	Grube et al. (2003)
L. sativa L. var. *capitata* (L.) Janchen	Bibb/Butterhead	15G6-1	Medium	Dieback	Simko et al. (2009)	*Sclerotinia sclerotiorum*	Whipps et al. (2002)
L. sativa L.	PI 251246/ Oilseed	13G640-1	Very short	Dieback	Simko et al. (2009)		
				Sclerotinia sclerotiorum	Whipps et al. (2002); Hayes et al. (2010)		
L. serriola L.	Serriola I/Prickly lettuce	12G239-1	Short				
L. serriola L.	Serriola II/Prickly lettuce	UC23US96	Short			*Bremia lactucae*	Simko et al. (2014)

[1]*Relative differences in life cycle (from seed to seed) under the same environmental conditions. Long = 5–6 months, Medium = 4–5 months, Short = 3–4 months, and Very Short <3 months. Note that all* L. sativa *genotypes, except PI 251246/Oilseed, are commercial cultivars of lettuce.*

Toronto, Canada) containing a commercial soil mix (Sun Gro® Sunshine® #1 Grower Mix with RESiLIENCE™, MA, USA). Plants were grown under photosynthetic active light intensity of 240 ± 10 μmol m^{-2} sec^{-1} with a 12-hour photoperiod. Relative humidity (RH) and temperature were recorded every 15 min with a data logger (GSP-6, Elitech®, CA, USA). Day and night conditions were 19 ± 1°C and 75 ± 4% RH and 18 ± 1°C and 92 ± 2% RH, respectively. One liter of tap water was added to the tray two to three times per week depending on the developmental stage of the plants. At 10 days after germination, 0.05 g/plant of fertilizer (Multi-Purpose 19-11-21, Peters®Excel, OH, USA) was dissolved in the irrigation water.

Bacterial Strains and Preparation of Inoculum

The non-typhoid *S. enterica* subspecies *enterica* serovar Typhimurium strain 14028s (Porwollik et al., 2014) (hereafter *S.* Typhimurium 14028s) and the enterohemorrhagic *E. coli* serotype O157:H7 strain 86-24 (Sperandio et al., 2001) (hereafter *E. coli* O157:H7) were grown in Low Salt Luria-

Bertani (LSLB) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) at 28°C. Medium supplemented with 50 μg/mL of streptomycin was used to grow *E. coli* O157:H7. Bacterial culture for the preparation of inoculum was obtained by streaking cells from frozen glycerol stocks on to solid LSLB medium and incubating overnight. From this culture, a single colony was used to inoculate liquid LSLB medium, which was incubated until reaching an OD_{600} of 0.9 to 1. Bacterial cells were collected by centrifugation at 1,360 x *g* for 20 min at 20°C (Eppendorf Centrifuge 5810R, Rotor 157 A-4-81, Hamburg, Germany) and suspended in sterile distilled water (SDW) to obtain the desired inoculum concentration.

Bacterial Attachment Assay

Bacterial attachment to the lettuce leaf surface was assessed as described by Van der Linden et al. (2014) with some modifications. Specifically, the inoculum was prepared in 1 mM $MgCl_2$ to avoid bacterial osmotic stress during the incubation period and the bacterial concentration in the inoculum was 1 x 10^8 CFU/mL. For sampling consistency, the second, fully expanded leaf of three 2.5- to 3-week-old plants grown in peat moss pellets were excised from the base of the petiole. Each leaf was immersed in an open 50 mL tube containing 45 mL of inoculum, preventing contact between the cut zone and the inoculum. Tubes were incubated for 2 hours without agitation at room temperature and a photosynthetic active light intensity of 240 ± 10 μmol m^{-2} sec^{-1}. After incubation, leaves were rinsed twice in SDW for 1 min and then blotted on a paper towel. Then, the bacterial population was enumerated by serial-dilution plating as described by Jacob et al. (2017). Three leaves from each genotype were used for each treatment and the experiment was repeated four times (n = 12) with independent batches of plants.

Analysis of Leaf Surface Traits

Leaf surface traits were quantified using the Nikon Eclipse 80i fluorescent microscope and the NIS Elements Imaging Software version 4.13.04 (Nikon Corporations, Shinagawaku, Tokyo, Japan). All measurements were conducted on the abaxial side of the second leaf from three 2.5- to 3-week-old plants grown in peat moss pellets. Leaf pieces (~0.5 cm^2) were cut from each side of the midrib (six pieces of leaf per genotype) and immediately imaged under the microscope. Stomatal pore traits (aperture width and pore area) were quantified in 18 randomly chosen pictures taken at 6 hours after first light (n = 45 to 60 stomata). To calculate the stomatal density (number of stomata per mm^2 of leaf), four randomly chosen microscopic fields of view from each piece of leaf (n = 24) were used. All quantifications were carried out three times using independently grown batches of plants.

Leaf Surface Inoculation

Surface-inoculation was conducted to evaluate the bacterial internalization rate (IR) and subsequent bacterial survival in the leaf intercellular space of lettuce genotypes. The protocol was adapted from those previously described for the pathosystem *Arabidopsis thaliana–Pseudomonas syringae* (Katagiri et al., 2002; Jacob et al., 2017). Lettuce plants (2.5- to 3-week-old) grown in peat moss pellets were used. Surface inoculation consisted of dipping for 5 seconds the aerial part of the plants in 200 mL of inoculum (1 x 10^8 CFU/mL) containing 0.03% Silwet L-77 (Lehle Seeds Co., Round Rock, TX, USA). The second leaf of each plant was sampled to quantify the bacterial population at 0, 1, 5, and 10 days post inoculation (DPI). Four 0.2 cm^2 discs, punched with a cork-borer, were placed in a 1.7 mL centrifuge tube and ground in 100 μL of SDW. The bacterial population was enumerated by serial-dilution plating as described by Jacob et al. (2017). To quantify the bacterial IR and subsequent apoplastic persistence, bacterial enumeration on day 0 was conducted in non-surface sterilized leaves; for the rest of the sampling points, leaves were gently washed in 2% (v/v) sodium hypochlorite for 1 min, 70% (v/v) ethanol for 1 min, rinsed in SDW for 1 min, and blotted onto a paper towel. This surface-sterilization method was optimized to kill all *S.* Typhimurium 14028s and *E. coli* O157:H7 on the leaf surface. The IR was estimated as the ratio of CFU/cm^2 leaf at 1 DPI over that of at 0 DPI. Three leaves from three plants were used for each sample point per genotype and the experiment was conducted three times (n = 9) with independent batches of plants.

Leaf Apoplast Inoculation

Syringe-infiltration inoculation was conducted to evaluate the bacterial survival in the leaf intercellular space of the lettuce genotypes. Lettuce seedlings (2.5 to 3 weeks old) grown in peat moss pellets were subjected to syringe infiltration and sampled at 0 and 10 DPI; similarly, 3.5- to 4-week-old plants grown in pots were subjected to syringe infiltration and sampled at 0, 10, and 20 DPI. The inoculum (1 x 10^6 CFU/mL) was infiltrated into the apoplastic space using a needleless syringe according to Katagiri et al. (2002). The bacterial population was enumerated by serial-dilution plating as described by Jacob et al. (2017). Leaves were surface sterilized prior to bacteria enumeration by gently washing in 2% (v/v) sodium hypochlorite for 1 min, 70% (v/v) ethanol for 1 min, rinsed in SDW for 1 min, and blotted onto a paper towel. Three leaves were used for each sample point per genotype and the experiment was conducted three times (n = 9) with independent batches of plants.

ROS Burst Assay

Apoplastic reactive oxygen species (ROS) were quantified through a fast and robust bioassay, as described by Smith and Heese (2014). Leaf discs (0.2 cm^2) from the second leaf of 2.5- to 3-week-old plants were placed individually into wells of a 96-well microplate containing 200 μL of SDW and incubated overnight at constant light and 22°C to reduce the wounding response. After incubation, SDW was replaced with 100 μL of the elicitation solution composed of 5.38 units of Horseradish

Peroxidase (MilliporeSigma, Burlington, MA, USA) and 34 μg of Luminol (MilliporeSigma, Burlington, MA, USA) per mL of SDW with or without 5 x 10^8 CFU/mL of *E. coli* O157:H7 or *S.* Typhimurium 14028s. The elicitation solution containing bacteria was prepared with heat-killed bacterial suspensions (incubated at 100°C for 10 minutes) to avoid possible inhibition of ROS production by any unknown virulence factor produced by live bacteria in contact with leaf tissue. After adding the elicitation solution to the wells, plates were immediately inserted in a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, Biotek, Winooski, VT, USA) to measure luminescence and estimate ROS production every 2 minutes between 0 and 90 minutes. For each treatment, 24 leaf discs were collected from six different plants. The experiment was repeated five times with independent batches of plants.

Callose Deposition Assay

A callose deposition assay was performed according to the procedure described by Schenk and Schikora (2015). The second leaf of 2.5- to 3-week-old plants was syringe-infiltrated with either water (mock treatment) or 1 x 10^8 CFU/mL of bacterium inoculum. After 24 hours, leaves were harvested, and chlorophyll was cleared by immersing the leaves into 95% (v/v) ethanol and kept at 37°C for 24 hours in a rotary shaker. Cleared leaves were rinsed in 50% (v/v) ethanol for 1 min, SDW for 1 min twice, 50 mM K_2HPO_4 for 3 min, followed by a 1 hour incubation in a 150 mM K_2HPO_4 SDW based solution containing 0.05% aniline blue. Leaves were imaged with a Nikon Eclipse 80i fluorescent microscope (Nikon Corporations, Shinagawaku, Tokyo, Japan) equipped with a DAPI (4',6-diamidino-2-phenylindole) filter, and the NIS Elements Imaging Software Version 4.13.04 was used to process images. Three leaves of each genotype were used per treatment and six images were randomly captured from each side of the midrib (12 pictures per leaf). Infiltrated zones, damaged areas, mid vein, and leaf edges were avoided for imaging to prevent false positive results. The total area of callose deposits (mm^2 per cm^2 of leaf) was quantified using the binary tool of the abovementioned software. The experiment was repeated four times with independent batches of plants.

Statistical Analysis

To assess the effect of lettuce genotype and bacterium species on bacterial leaf attachment, internalization rate, and apoplastic persistence after surface inoculation, the data was subjected to a two-way analysis of variance (ANOVA) followed by comparisons of multiple means using Tukey's test with a significance threshold of $\alpha = 0.05$. The statistical analysis of the bacterial persistence was conducted with the data transformed with the square root function as recommended for data where the variance is proportional to the mean, as often happens in variables that are measured as counts per area (Manikandan, 2010). However, the graphs showing bacterial enumeration were created with untransformed values. Averages of bacterial population after syringe inoculation (10 or 20 DPI versus 0 DPI) and averages of plant defense responses (Lollo Rossa versus Red Tide) were compared using a two-tailed Student's *t*-test. To evaluate the strength of the linear correlation between quantitative variables, bacterial leaf colonization traits and stomatal traits, a Pearson's correlation test was conducted. Statistical analysis was done using InfoStat/E software version 2016e (Agricultural College of the National University of Córdoba, Argentina) and R software version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/).

RESULTS

Attachment of *S.* Typhimurium 14028s and *E. coli* O157:H7 Onto Lettuce Leaves

Bacterial attachment to leaf surface is largely affected by various topographic traits (Crawford et al., 2012). In lettuce, leaf vein and stomatal densities, leaf surface hydrophobicity, soluble protein concentrations, and wax content have been reported as factors influencing differences in the attachment of *S. enterica* ser. Senftenberg among lettuce genotypes and leaves at different developmental stages (Hunter et al., 2015). Our results show significant variation ($p < 0.0001$) in the attachment of *E. coli* O157:H7 and *S.* Typhimurium 14028s among lettuce genotypes (**Figure 1**). Attachment of *E. coli* O157:H7 ranged from 5.4 x 10^4 ± 8.3 x 10^3 (mean ± standard error) to 5.8 x 10^5 ± 1.9 x 10^5 CFU/cm^2 leaf from Serriola I to Salinas, respectively (**Figure 1A**). In contrast, extension of variation of *S.* Typhimurium 14028s leaf surface attachment ranged from 1.9 x 10^6 ± 5.1 x 10^5 CFU/cm^2 on Oilseed leaves to 3.9 x 10^6 ± 5.1 x 10^5 CFU/cm^2 on Grand Rapids leaves (**Figure 1B**).

Considering the significant variation in the bacterial attachment onto leaves, we determined the stomatal density together with the stomatal aperture width and stomatal pore area of the lettuce genotypes to assess the potential correlation between these traits and bacterial attachment. Stomatal characteristics varied significantly ($p < 0.0001$) among the eleven lettuce genotypes (**Figure 2**). The two *L. serriola* genotypes, Serriola II and Serriola I, had the widest stomatal aperture width, averaging 6.1 ± 0.1 and 5.3 ± 0.1 μm, respectively, while Grand Rapids had the smallest stomatal aperture width (2.6 ± 0.1 μm; **Figure 2A**). Stomatal pore area also varied significantly among lettuce genotypes, from 98.6 ± 3.0 μm^2 in the genotype Serriola II to 15.6 ± 0.7 μm^2 in Grand Rapids (**Figure 2B**). Regarding stomatal density, this trait showed genotypic variation ranging from 76.0 ± 2.1 to 41.7 ± 1.0 stomata/mm^2 in the genotypes Bibb and Red Tide, respectively (**Figure 2C**). These stomatal traits (aperture width, pore area, and density) presented no significant correlation with *E. coli*

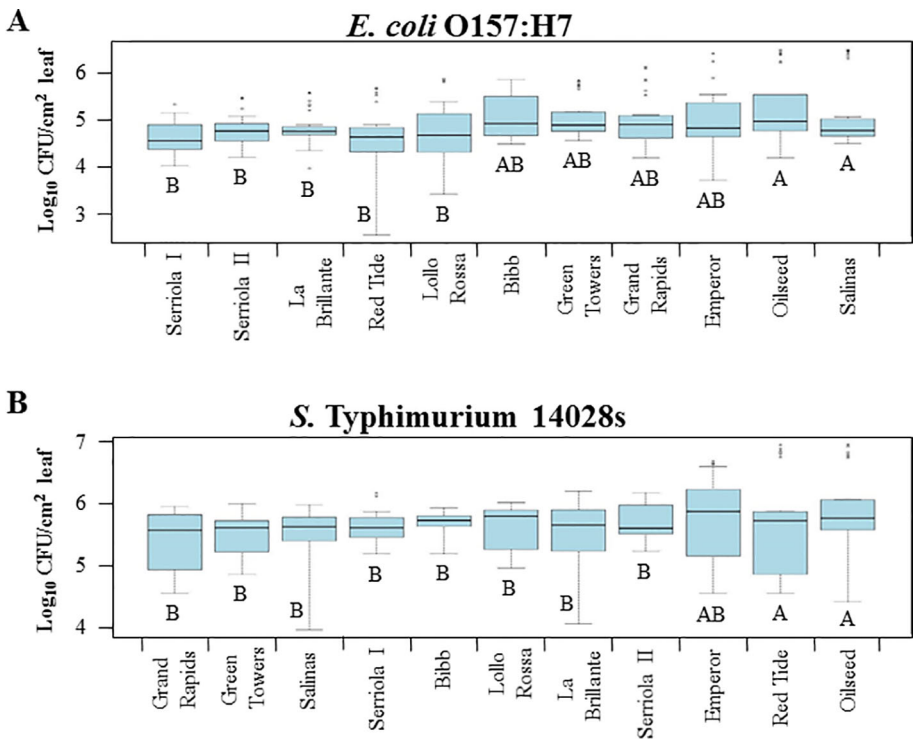


FIGURE 1 | *Escherichia coli* O157:H7 **(A)** and *Salmonella enterica* Typhimurium 14028s **(B)** attachment to the leaf surface varies among lettuce genotypes. The second leaf of 2.5- to 3-week-old lettuce plants was immersed in 1 x 10^8 CFU/mL bacterial inoculum for 2 hours at room temperature. After incubation, leaves were rinsed with sterile distilled water and bacterial population was enumerated by serial dilution plating. Plots show data from four independent experiments (n = 12). Different letters on the bottom of the boxes indicate significant statistical differences among the means, as calculated with ANOVA followed by Tukey's test ($\alpha = 0.05$).

O157:H7 or *S.* Typhimurium 14028s attachment onto leaves of the different lettuce genotypes (**Table 2**). Thus, these results suggest that bacterial attachment might be influenced by properties of the leaf surface and by specific bacterial traits (*e.g.*, motility, chemotaxis) on the phyllosphere.

Internalization Rate of *S.* Typhimurium 14028s and *E. coli* O157:H7

Previously, we determined that STm SL1344 and *E. coli* O157:H7 penetrate leaves through the stomatal pore (Roy et al., 2013). Here, we observed significant differences ($p < 0.0001$) in the internalization rate (IR; estimated as the ratio of CFU/cm^2 leaf at 1 DPI over that of at 0 DPI) of these bacteria among the lettuce genotypes (**Figure 3**). *E. coli* O157:H7 IR varied from 1.07 ± 0.17 in Serriola II to 0.28 ± 0.08 in Emperor, while *S.* Typhimurium 14028s IR ranged from 0.79 ± 0.15 to 0.13 ± 0.02 in Serriola II and Red Tide, respectively (**Figure 3**). Interestingly, we found a significant correlation ($p < 0.05$) between the IR of both human pathogenic bacteria and stomatal traits (aperture width and pore area) among the eleven lettuce genotypes (**Table 2**). No significant correlation between stomatal density and bacterial IR was detected (**Table 2**). These results suggest that, to a certain extent, morphological traits of the stomatal pore (width and area) contribute to bacterial penetration into the lettuce leaf.

E. coli O157:H7 and *S.* Typhimurium 14028s Persistence After Surface Inoculation

Previous studies have demonstrated that survival of *E. coli* O157:H7 and different *S. enterica* serovars in/on leaves is significantly affected by the plant genotype, such as in spinach and tomato (Mitra et al., 2009; Barak et al., 2011; Gutiérrez-Rodríguez et al., 2011; Macarisin et al., 2013; Han and Micallef, 2014). Thus, we sought to determine whether this phenomenon is also true for the lettuce system. Similarly, we observed that changes in internalized *E. coli* O157:H7 and *S.* Typhimurium 14028s populations throughout the experimental period (10 DPI) were significantly ($p < 0.0001$) influenced by lettuce genotype and the species of bacteria. With the exception of a few genotypes, bacterial populations in the apoplast decreased significantly from 1 DPI to 10 DPI in the different plant-bacterium combinations (**Figure 4**). The average log change in the *E. coli*

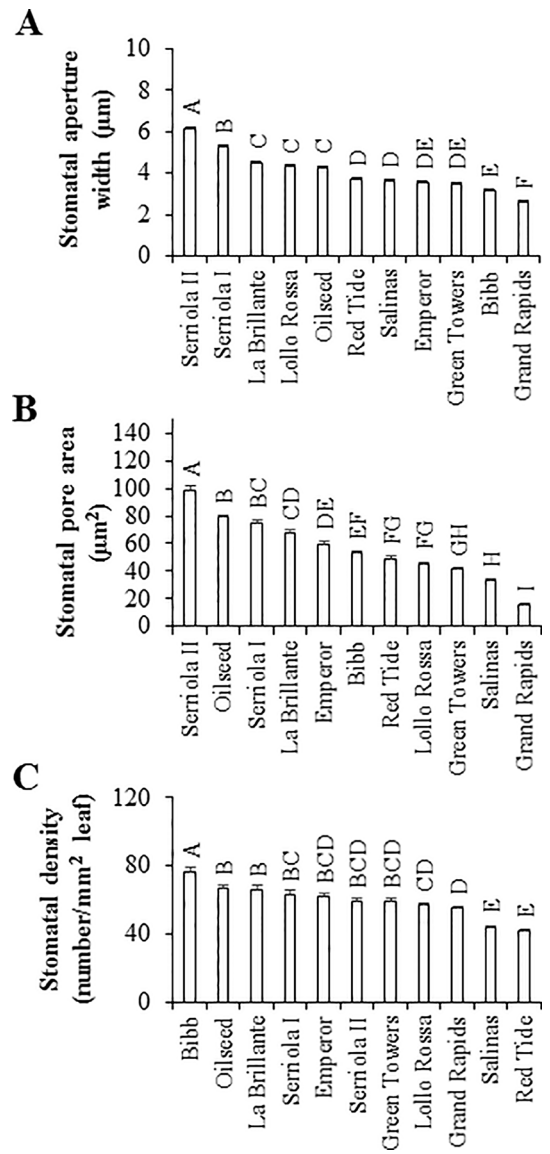


FIGURE 2 | Stomatal characteristics vary among the lettuce genotypes. Stomatal aperture width **(A)** and pore area **(B)** were measured in 45 to 60 stomata at 6 hours after first light. Stomatal density **(C)** was calculated in eight randomly chosen microscopic fields of view from three leaves (n = 24). All measurements were taken from the abaxial side of the second leaf from 2.5- to 3-week-old plants. Stomatal characteristic data are shown as average ± standard error from three experiments using different batches of plants. Different letters on the top of the bars indicate significant statistical differences among the means, as calculated with ANOVA followed by Tukey's test ($\alpha = 0.05$).

O157:H7 population varied from a non-significant 0.03 log increment in Serriola I to a significant 1.71 log reduction in Emperor (**Figure 4A**), while the *S.* Typhimurium 14028s population change ranged from a non-significant 0.09 log reduction in Red Tide to a significant 1.41 log reduction in La Brillante (**Figure 4B**). Although the population of both inoculated bacteria generally decreased with the duration of the experiment, the kinetics and the extent of the decrease were significantly different ($p < 0.0001$) among the lettuce genotypes. It is noteworthy that enumerating internalized population size after surface inoculation represents a combined effect of leaf surface features, bacterium IR, and endophytic persistence. The bacterial population at the end of the experimental period (10 DPI) exhibited a significant correlation ($p < 0.05$) with stomatal aperture width and pore area for *E. coli* O157:H7 but not for *S.* Typhimurium 14028s (**Table 2**). Therefore, these findings suggest that each lettuce genotype may have a combination of features both on the leaf surface and in the intercellular space, which hamper or facilitate the persistence of these two human pathogens.

TABLE 2 | Correlation (Pearson's coefficient and p-value) between *Escherichia coli* O157:H7 or *Salmonella enterica* Typhimurium 14028s leaf colonization traits [leaf attachment, internalization rate (IR), and bacterial population 10 days post surface inoculation (10 DPI)] and stomatal traits (stomatal aperture width, stomatal pore area, and stomatal density) among the 11 lettuce genotypes.

Bacteria	Bacterial colonization trait	Pearson correlation	Stomatal trait		
			Aperture width	Pore area	Density
***E. coli* O157:H7**	**Attachment**	Coefficient (*r*)	-0.41	-0.29	-0.19
		p-value	0.216	0.379	0.575
	IR	Coefficient (r)	0.77	0.71	0.36
		p-value	***0.005***	***0.015***	0.274
	10 DPI	Coefficient (r)	0.75	0.67	0.19
		p-value	***0.008***	***0.025***	0.570
***S.* Typhimurium 14028s**	**Attachment**	Coefficient (r)	0.06	0.28	-0.22
		p-value	0.860	0.405	0.516
	IR	Coefficient (r)	0.72	0.64	0.02
		p-value	***0.012***	***0.032***	0.954
	10 DPI	Coefficient (r)	0.48	0.49	-0.13
		p-value	0.139	0.128	0.704

Significant p-values (< 0.05) are in bold italic numbers.

S. Typhimurium 14028s and *E. coli* O157:H7 Persistence in 2.5- to 3-Week-Old Plants After Inoculum Infiltration

To assess the genotypic variation in bacterial persistence in the apoplast, a niche where the pathogen is shielded from commonly used sanitation procedures (Erickson, 2012), we directly infiltrated leaves with a needless syringe. This protocol allowed us to exclusively assess the capacity of bacterial persistence in the apoplast of each lettuce genotype, regardless of the bacterial behavior on the phylloplane or differences in leaf surface traits, which might influence bacterial attachment and internalization. Bacterial population kinetics after syringe inoculation differed significantly ($p < 0.0001$) depending on the bacterium and the lettuce genotype (**Figure 5**). To represent changes on bacterial

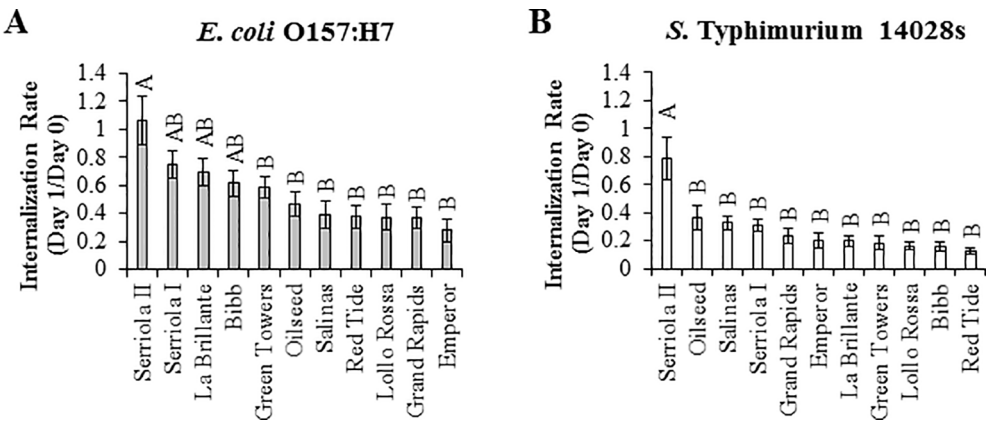


FIGURE 3 | The internalization rate of *Escherichia coli* O157:H7 **(A)** and *Salmonella enterica* Typhimurium 14028s **(B)** vary among the lettuce genotypes. Internalization rate was calculated as the ratio of bacterial concentration (CFU/cm^2 leaf) between day 1 and day 0 after surface inoculation (1 x 10^8 CFU/mL). Bacterial population was quantified in intact leaves for day 0 after inoculation and in leaves previously surface sterilized for day 1 after inoculation. Data shown is the average of three independent experiments (n = 9). Different letters on the top of the bars indicate significant statistical differences among the means, as calculated with ANOVA followed by Tukey's test (α = 0.05).

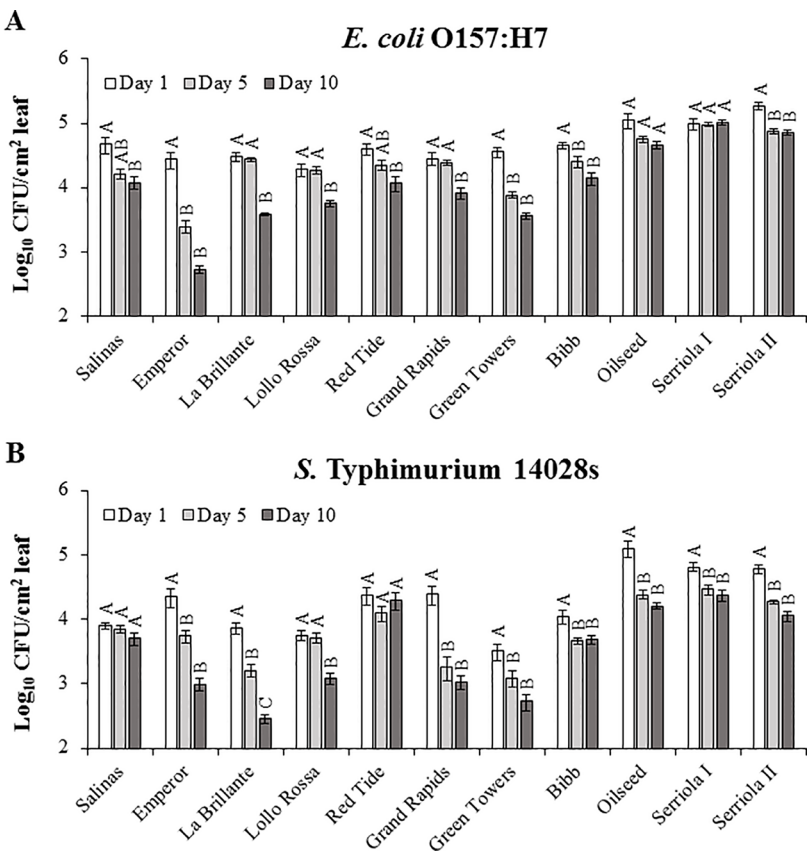


FIGURE 4 | Persistence of *Escherichia coli* O157:H7 **(A)** and *Salmonella enterica* Typhimurium 14028s **(B)** after surface inoculation of leaves varies with lettuce genotypes. Lettuce plants (2.5- to 3-week-old) were dipped into 1 x 10^8 CFU/mL bacterial inoculum. Leaves were surface sterilized prior to quantification of the bacterial population in the intercellular space. Results are shown as untransformed averages from three independent experiments (n = 9 ± standard error). Different letters on the top of adjacent bars (*i.e.*, within the plant genotype) indicate significant statistical differences among the means (transformed with the root square function), as calculated with ANOVA followed by Tukey's test (α = 0.05).

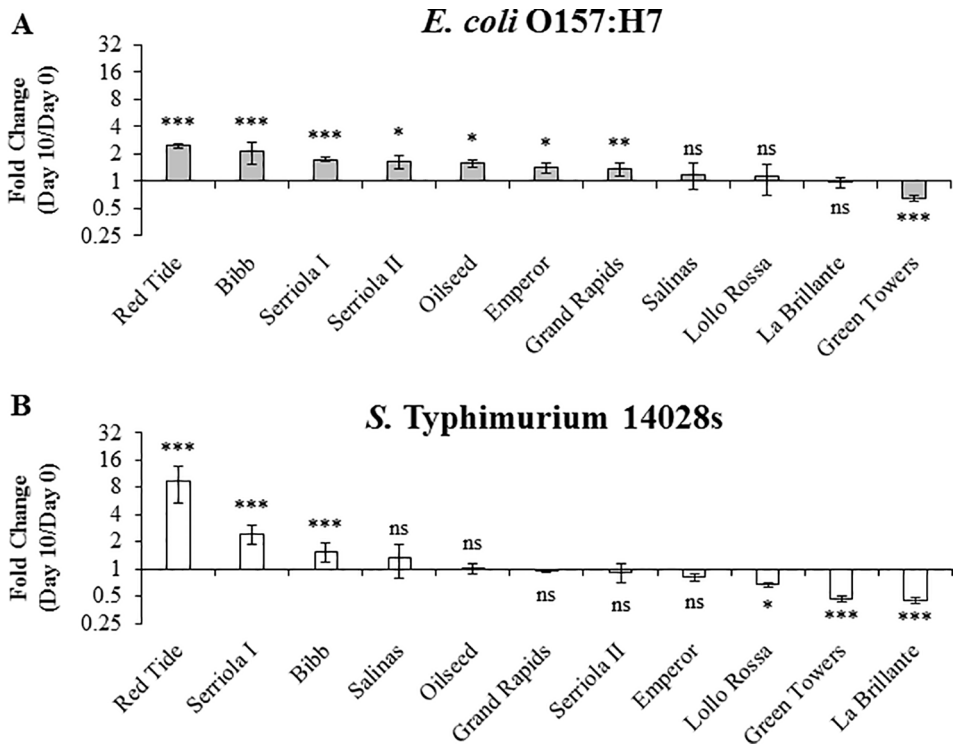


FIGURE 5 | Persistence of *Escherichia coli* O157:H7 **(A)** and *Salmonella enterica* Typhimurium 14028s **(B)** varies in 2.5- to 3-week-old plants of lettuce genotypes after syringe inoculation. The second leaf was infiltrated with 1 x 10^6 CFU/mL bacterial inoculum. Leaves were surface sterilized prior to quantification of the bacterial population in the intercellular space. Results are shown as the average of three independent experiments (n = 3 ± standard error). Pairwise mean comparison (bacterial population at day 0 versus day 10) was performed with two-tail Student's *t*-test (ns, not significant, *p < 0.05, **p < 0.01, and ***p < 0.001).

population dynamics, we calculated the net population growth overtime as fold change (FC) using the ratio 10 DPI/0 DPI (**Figure 5**). Three patterns of bacterial population growth were observed among the genotypes: positive, neutral, and negative growth. *E. coli* O157:H7 exhibited significant positive net growth in seven genotypes ranging from 1.4 ± 0.2 to 2.5 ± 0.1 FC, where the highest growth was observed in the genotypes Red Tide and Bibb (**Figure 5A**). Salinas, Lollo Rossa, and La Brillante showed no significant changes from 0 to 10 DPI and, interestingly, Green Towers showed a net decrease in its population of 0.65 ± 0.05 times (**Figure 5A**). The *S.* Typhimurium 14028s population showed significant positive net growth in only three lettuce genotypes, Red Tide, Serriola I, and Bibb (**Figure 5B**). In contrast, the apoplastic *S.* Typhimurium 14028s population decreased significantly in genotypes Lollo Rossa, Green Towers, and La Brillante by 0.67 ± 0.04, 0.47 ± 0.03, and 0.45 ± 0.03 times, respectively, whereas the *S.* Typhimurium 14028s population did not change significantly in the other five genotypes (**Figure 5B**). Overall, the *E. coli* O157:H7 apoplastic population showed a narrower variation (0.65 < FC < 2.47) inside this panel of lettuce genotypes than that of *S.* Typhimurium 14028s (0.45 < FC < 9.38) (**Figure 5**).

S. Typhimurium 14028s and *E. coli* O157:H7 Persistence in 3.5- to 4-Week-Old Plants After Inoculum Infiltration

Long term survival (≥3 weeks after inoculation) of *E. coli* O157:H7 and *S.* Typhimurium has been previously reported in leaves of species such as Arabidopsis and lettuce Islam et al., 2004a; Islam et al., 2004b; Roy et al., 2013). To explore the effect of plant genetic differences in the survival of human pathogenic bacteria over a longer period of time (i.e., 10 to 20 DPI), we used 3.5- to 4-week-old plants. During this period, no significant positive net population growth was observed for either bacteria in any of the lettuce genotypes (**Figures 6** and **7**). In fact, the population growth of *E. coli* O157:H7 was neutral in five of the lettuce genotypes, Oilseed, Red Tide, Bibb, Serriola II, and Serriola I, during the 20-day period (**Figure 7A**). *E. coli* O157:H7 net growth was negative in the rest of the genotypes, where the smallest FC values were observed in Green Towers, Lollo Rossa, and Salinas (**Figure 7A**). In contrast, Red Tide was the only genotype where *S.* Typhimurium 14028s bacterial population growth remained neutral from 0 to 20 DPI, while the net growth of this bacterium was negative in the other ten genotypes (**Figure 7B**). The genotypes with the smallest FC in the *S.* Typhimurium

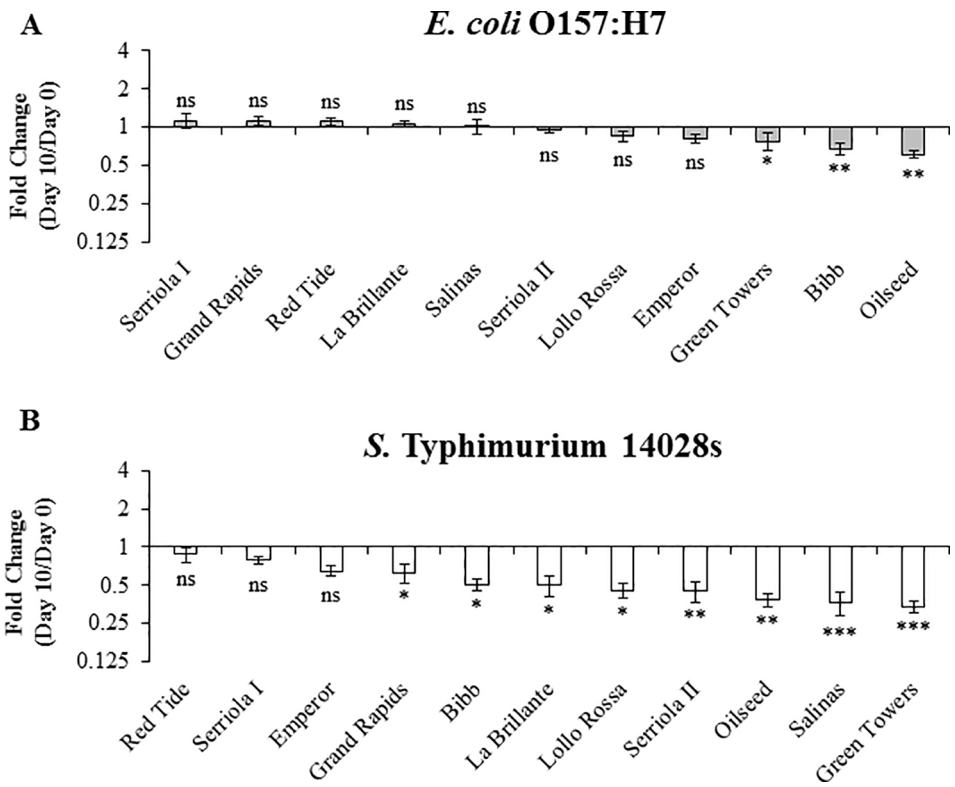


FIGURE 6 | *Escherichia coli* O157:H7 **(A)** and *Salmonella enterica* Typhimurium 14028s **(B)** persistence varies in 3.5- to 4-week-old plants of lettuce genotypes after syringe inoculation. Plants of the lettuce genotypes were grown in pots and then three leaves were infiltrated with bacterial inoculum (1 x 10^6 CFU/mL) with a needleless syringe. Leaves were surface sterilized after inoculation and serial dilution plating was conducted to quantify the bacterial population in the intercellular space. Three leaves were used for each sample point per genotype. Results are shown as the average of three independent experiments (n = 9 ± standard error). Pairwise mean comparison (bacterial population at day 0 versus day 10) was performed with a two-tail Student's *t*-test (ns, not significant, *p < 0.05, **p < 0.01, and ***p < 0.001).

14028s population were Lollo Rossa and Green Towers with values of 0.28 ± 0.05 and 0.18 ± 0.03, respectively (**Figure 7B**). Interestingly, the extreme bacterial persistence phenotypes were observed in the same genotypes at the two plant developmental stages (2.5- to 3- and 3.5- to 4-week-old plants) for each human pathogen. In particular, the genotype Red Tide appears to sustain the highest *S.* Typhimurium 14028s and *E. coli* O157:H7 titers, whereas bacterial populations consistently decrease in the genotypes Lollo Rossa, La Brillante, and Green Towers (**Figures 5** and **7**). These differences in apoplastic persistence suggest the existence of constitutive conditions in the apoplast environment and/or induced plant responses that vary among lettuce genotypes and influences bacterial survival.

S. Typhimurium 14028s and *E. coli* O157:H7 ROS Burst Induction in Lettuce Leaves

To correlate plant defense response levels with bacterial persistence after syringe infiltration inoculation, we chose two lettuce genotypes, Red Tide and Lollo Rossa, which support the two extreme bacterial titers, high and low, respectively (**Figures 5** and **7**), and are parents of a mapping population already characterized by genotyping-by-sequencing. Generation of ROS is among the earliest induced plant cell responses after the perception of MAMP (Yu et al., 2017) and has been reported to occur in Arabidopsis (Garcia et al., 2014) and tobacco (Shirron and Yaron, 2011) after exposure to *S.* Typhimurium 14028s. Consistently, we also observed peak ROS production at approximately 20 minutes after exposure to bacteria; however, the extent of the burst varied significantly between the two plant genotypes after exposure to *E. coli* O157:H7 ($p = 0.0117$) or *S.* Typhimurium 14028s ($p = 0.0001$). ROS burst in Lollo Rossa was greater than that of Red Tide after exposure to either *S.* Typhimurium 14028s or *E. coli* O157:H7 (**Figure 8**). The peak of relative light units (RLUs) of Lollo Rossa after treatment with *E. coli* O157:H7 reached 780 ± 156, while Red Tide showed a peak of 315 ± 66 RLUs (**Figure 8C**). After exposure to *S.* Typhimurium 14028s, the peak RLUs in Lollo Rossa was 1,125 ± 140, which was significantly higher than the peak of Red Tide (402 ± 50 RLUs; **Figure 8C**). These results indicate that the differences in bacterial persistence among these genotypes might in part be due to variation in the level of ROS-associated defense responses developed by the plant.

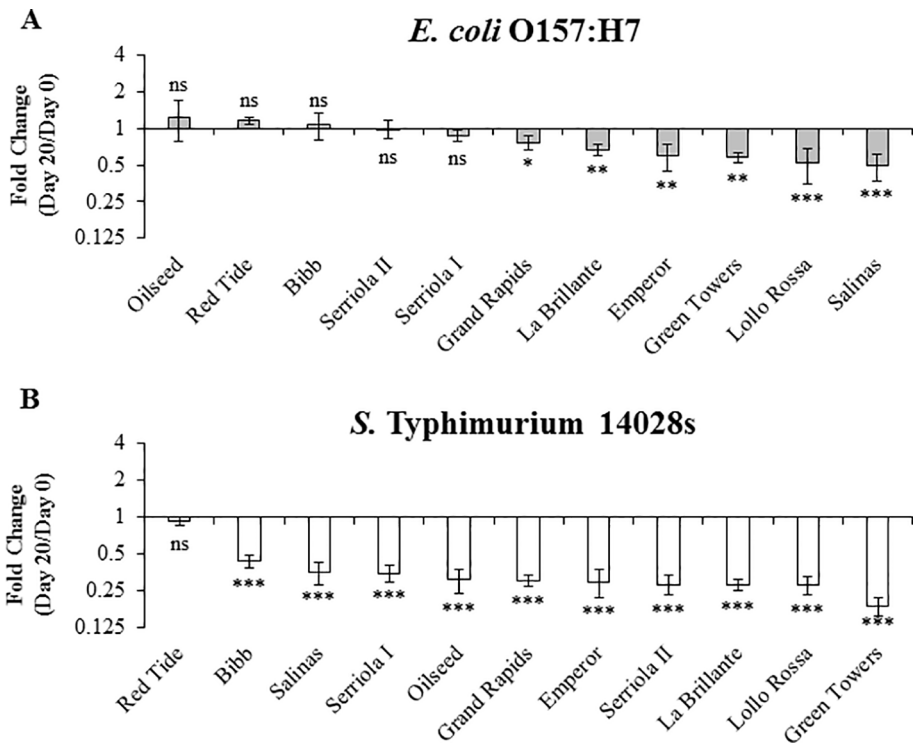


FIGURE 7 | *Escherichia coli* O157:H7 **(A)** and *Salmonella enterica* Typhimurium 14028s **(B)** persistence varies in 3.5- to 4-week-old plants of lettuce genotypes after syringe inoculation. Plants of the lettuce genotypes were grown in pots and then three leaves were infiltrated with bacterial inoculum (1 x 10^6 CFU/mL) with a needleless syringe. Leaves were surface sterilized after inoculation and serial dilution plating was conducted to quantify the bacterial population in the intercellular space. Three leaves were used for each sample point per genotype. Results are shown as the average of three independent experiments (n = 3 ± standard error). Pairwise mean comparison (bacterial population at day 0 versus day 20) was performed with a two-tail Student's *t*-test (ns, not significant, *p < 0.05, **p < 0.01, and ***p < 0.001).

S. Typhimurium 14028s and *E. coli* O157:H7 Differentially Induce Callose Deposition Dependent Upon Lettuce Genotype

Another hallmark of plant defense against biotic stressors is callose deposition that occurs within hours after the perception of the microbe (Yu et al., 2017). In lettuce, the induction of callose deposition has been reported as a defense response against phytopathogens such as *Plasmopara lactucae-radicis* (Stanghellini et al., 1993) and *Bremia lactucae* (Cohen et al., 2010). Consistent with the ROS burst response levels, Lollo Rossa exhibited a significantly higher amount of callose deposition than Red Tide after inoculation with either *E. coli* O157:H7 ($p = 0.0001$) or *S.* Typhimurium 14028s ($p < 0.0001$; **Figure 9**). Specifically, the average area of callose deposition (mm^2 callose deposits/cm^2 leaf) in Lollo Rossa leaves after *E. coli* O157:H7 exposure was 5.2 times greater than Red Tide, and callose deposition after exposure to *S.* Typhimurium 14028s was 6.7 times greater than Red Tide (**Figure 9**). These results suggest that Lollo Rossa is able to generate significantly stronger plant defense responses against *E. coli* O157:H7 and *S.* Typhimurium 14028s than Red Tide, which correlates with the level of bacterial population in the apoplast of these plants.

DISCUSSION

Despite significant progress achieved in the understanding of the ecology of human pathogens along the food chain environments, there are still relevant unanswered questions regarding molecular mechanisms underlying human pathogen–plant interactions (Barak and Schroeder, 2012; Melotto et al., 2014). The discovery that plant genetic diversity affects the interaction between the phyllosphere and human pathogenic bacteria provided an unprecedented opportunity to dissect the plant components associated with bacterial colonization and persistence phenotypes. However, it is crucial to define significant and robust variables in a genetically tractable system of economic and social importance. Therefore, we conducted a systematic approach to uncover lettuce traits associated with enterobacterium attachment, internalization, and apoplastic persistence.

Lettuce genotypes have extensive variations in leaf traits including the content of surface phenolics, proteins, wax, and sugars; contact angle; and stomatal density (Hunter et al., 2015). These properties have been shown to facilitate or hamper the leaf attachment of human pathogenic bacteria (Golberg et al., 2011; Kroupitski et al., 2011; Hunter et al., 2015). In this study, we

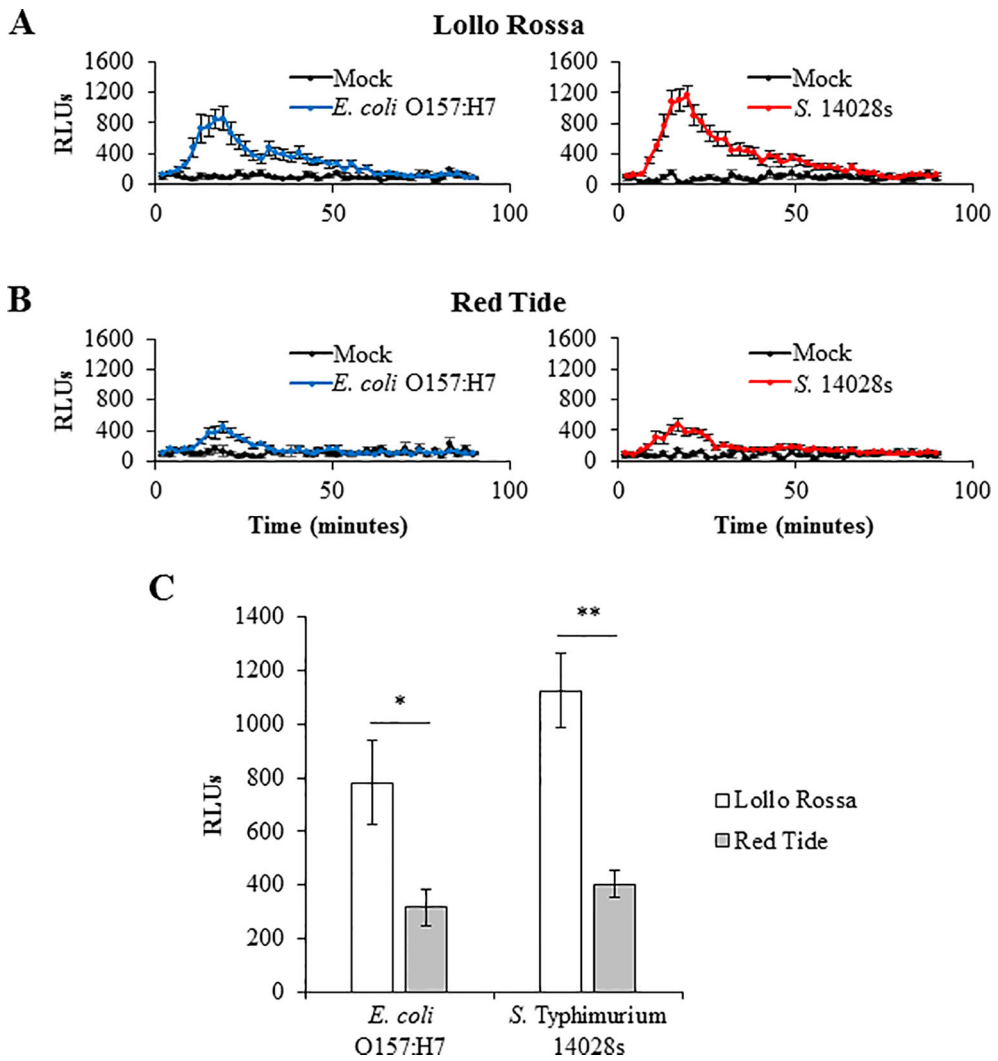


FIGURE 8 | Reactive oxygen species (ROS) burst induced by *Escherichia coli* O157:H7 and *Salmonella enterica* Typhimurium 14028s varies among the lettuce genotypes. The second leaf of Lollo Rossa **(A)** and Red Tide **(B)** plants (2.5- to 3-week-old) was used for the ROS burst assay. Graphs show ROS production after mock- or bacterium-elicitation overtime **(A**, **B)**. ROS production was quantified as relative light units (RLUs). The curve peak (approximately 20 minutes after elicitation) was used to assess statistical significance among the genotypes **(C)**. Results are shown as the RLU peak value (n = 24 ± standard error) of bacterium-treated samples normalized by the mock-treated samples. Data from one out of five independent experiments with similar results are shown. Pairwise mean comparisons (ROS produced by Lollo Rossa versus Red Tide) were performed with a two-tail Student's *t*-test (*$p < 0.05$ and **$p < 0.001$).

observed a significant variation in bacterial attachment among the lettuce genotypes and the differences were dependent upon the bacterial species (**Figure 1**). Our results showed that *E. coli* O157:H7 and *S.* Typhimurium 14028s attachment differs by 1.03 and 0.69 log, respectively, between the extreme genotypes (**Figure 1**). Hunter et al. (2015) reported a 0.22 log difference on leaf attachment of *S. enterica* ser. Senftenberg strain 070885 between *L. sativa* cv. Saladin and *L. serriola* (US96UC23) on the fifth/sixth leaves of 6-week-old plants, but no differences were observed on the first leaf. Furthermore, these authors found a significant correlation between bacterial attachment to leaves and various surface traits, whereas older leaves showed significantly higher levels of bacterial attachment and lower stomatal density than young leaves on the three lettuce genotypes tested (Hunter et al., 2015). Although we found significant differences in the stomatal traits among the lettuce genotypes (**Figure 2**), no significant correlation between these and the attachment level of *E. coli* O157:H7 or *S.* Typhimurium 14028s was detected (**Table 2**). The lack of correlation between these variables might be due to the low number of genotypes used in our study. Nevertheless, Kroupitski et al. (2011) also observed no relationship between differential attachment of *S.* Typhimurium SL1344 on abaxial and adaxial leaf surfaces and the level of stomatal density in romaine lettuce. Overall, the

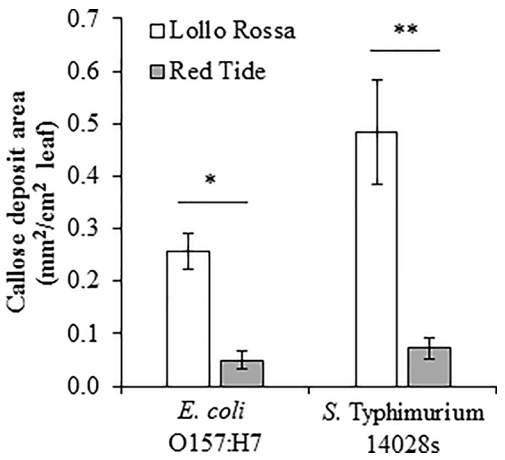


FIGURE 9 | Callose deposition induced by *Escherichia coli* O157:H7 and *Salmonella enterica* Typhimurium 14028s varies among the lettuce genotypes Lollo Rossa and Red Tide. The second leaf of 2.5- to 3-week-old plants was infiltrated with either 1 x 10^8 CFU/mL bacterial inoculum or water as a control. The area of callose deposition was measured in three plants for each treatment and the experiment was conducted independently four times. Results are shown as mean (n = 12 ± standard error) area of callose deposition normalized with the corresponding mock treatment value. Pairwise mean comparisons (callose deposits produced by Lollo Rossa versus Red Tide) were performed with a two-tail Student's *t*-test (*< 0.001 and **p < 0.0001).

extent of bacterial attachment is the outcome of microbial (Saldaña et al., 2011) and plant (Crawford et al., 2012) physicochemical and biological properties; our results agree with the complex scenario of interactions of each bacterium-lettuce genotype combination.

Successful leaf colonization not only depends on bacterial attachment and epiphytic survival, but also on the ability of the bacteria to penetrate the leaf tissue and occupy the apoplast. Using both microscopy and microbial enumeration tools, several studies have revealed internalization by enteric pathogens through stomata during preharvest plant growth (Erickson, 2012). Leaf penetration of human pathogenic bacteria can be significantly influenced by plant genotype variations (Golberg et al., 2011) and by the tissue site (*i.e.*, different regions of the leaf; Erickson et al., 2010). Using confocal laser scanning microscopy, Golberg et al. (2011) observed significant effects of plant genetic diversity on attachment and internalization of GFP-tagged *S.* Typhimurium, after 2 hours of suspension in bacterial inoculum (1 x 10^8 CFU/mL). The incidence of internalized bacteria varied among iceberg (81%), romaine (16%), and red (20%) lettuce types (Golberg et al., 2011). Similarly, we have also observed significant variation in the bacterial internalization level among the 11 lettuce genotypes, where the extent of variation between the extreme genotypes was 3.82 times for *E. coli* O157:H7 and 6.08 times for *S.* Typhimurium 14028s (**Figure 3**). Interestingly, taking into consideration the low number of lettuce genotypes used in our study, we were able to detect a significant correlation ($p < 0.05$) between the IRs of *S.* Typhimurium 14028s or *E. coli* O157:H7 and the stomatal pore traits (**Table 2**). Erickson et al. (2010) suggested that their findings of higher *E. coli* O157:H7 internalization in abaxial versus adaxial-sprayed leaves (1 x 10^8 CFU/mL inoculum) could be related to higher stomatal density in the leaf abaxial side. Nevertheless, we did not observe a significant correlation between *E. coli* O157:H7 or *S.* Typhimurium 14028s IR and stomatal density (**Table 2**). It is well known that the stomatal immune response also affects the ability of human and plant pathogenic bacteria to internalize leaf tissues (Melotto et al., 2006), and this response is bacterium- and plant-dependent (Roy et al., 2013; Roy and Melotto, 2019). Thus, it is likely that the bacterium internalization of leaves might be influenced not only by certain stomatal morphological traits, such as stomatal aperture width and pore area, but also by stomatal physiological traits and bacterial population dynamics on/in the leaf.

Bacterial access into and survival within the lettuce leaf apoplast pose a risk to consumers and threatens food safety because of the protection that this microenvironment provides to the bacterium from routine sanitization and cleaning treatments (Seo and Frank, 1999; Erickson et al., 2010 ; Golberg et al., 2011; Tomás-Callejas et al., 2011; Ge et al., 2013). Previous studies showing the influence of the plant genotype on bacterial persistence in the phyllosphere considered the total bacterial population (*i.e.*, epiphytic and endophytic populations), as leaves were not surface sterilized prior to bacterium enumeration (Barak et al., 2011; Macarisin et al., 2013). Therefore, the individual contribution of these two distinct niches (*i.e.,* leaf surface and apoplast) to bacterial persistence is not evident. Here, we conducted bacterial persistence assays through surface- and syringe-inoculation methods to distinguish the ability of the bacteria to survive in the leaf apoplast after epiphytic colonization and to persist in the intercellular space regardless of their fitness in the leaf surface, respectively. We found significant differences in the apoplastic persistence of *E. coli* O157:H7 and *S.* Typhimurium 14028s after surface- and syringe-inoculation methods (**Figures 4**–**7**). Although the persistence of inoculated bacteria might have been influenced by a possibly variable sensitivity of each lettuce genotype to the infiltration technique, the inoculation method clearly affected the overall ability of the bacteria to survive in the leaf apoplast of 2.5- to 3-week-old lettuce plants. The capacity of both *E. coli* O157:H7 and *S.* Typhimurium 14028s to persist in the leaf intercellular space was greater after syringe inoculation than after surface inoculation (**Figures 4** and **5**). Lang et al. (2004) reported that significantly higher *E. coli* O157:H7 or *S. enterica* populations were recovered from iceberg lettuce and curly parsley (*Petroselinum crispum*) after dip inoculation compared to spray- or spot-inoculation methods. Higher bacterial adherence after dip inoculation was associated with the detected differences (Lang et al., 2004). Moreover, we observed that bacterial survival during the 10-day experimental period was substantially affected by the inoculation method in certain lettuce genotypes (**Figures 4** and **5**). For example, the lettuce genotype Emperor exhibited

the most drastic decline of the *E. coli* O157:H7 population after surface inoculation; however, the *E. coli* O157:H7 population significantly increased after syringe inoculation in this genotype (**Figures 4** and **5**). In contrast, the lettuce genotypes Serriola I, Oilseed, Serriola II, and Bibb presented the highest *E. coli* O157:H7 population titers after both inoculation methods (**Figures 4** and **5**). We hypothesize that the initial epiphytic *S.* Typhimurium 14028s and *E. coli* O157:H7 populations after surface inoculation may either induce plant defense responses and/or be subjected to stress that affect the apoplastic survival and that the extent of these processes might vary according to each bacterium–lettuce genotype combination. For instance, in the case of *E. coli* O157:H7, the stomatal aperture width and pore area showed a significant correlation with the bacterial persistence after surface inoculation (**Table 2**). Therefore, to a certain extent, larger stomatal pores facilitate the leaf penetration of *E. coli* O157:H7 and enhances its apoplastic persistence. This may be due to higher initial internalized bacterial populations. Overall, bacterial persistence after surface inoculation is a complex phenotype, where plant and bacterial factors interact in every step of the colonization.

After syringe inoculations, the net apoplastic growth of *E. coli* O157:H7 and *S.* Typhimurium 14028s was generally higher in 2.5- to 3-week-old plants than in 3.5- to 4-week-old plants at 10 DPI (**Figures 5** and **6**). These results agree with those reported by Brandl and Amundson (2008), where the persistence of *E. coli* O157:H7 and *S. enterica* ser. Thompson strain RM1987 in the romaine lettuce (cultivar Parris Island) phyllosphere was higher in young leaves compared to older leaves, which was associated with the richer total nitrogen and carbon exudates from young leaves. Moreover, the relative bacterial persistence after syringe inoculation between the lettuce genotypes was slightly affected by the developmental stage of the lettuce plants (**Figures 5**–**7**). For instance, *E. coli* O157:H7 and *S.* Typhimurium 14028s exhibited the highest levels of apoplastic survival in the genotype Red Tide after syringe inoculation of 2.5- to 3-week-old plants (**Figure 5**) and 3.5- to 4-week-old plants (**Figures 6** and **7**). Likewise, the lowest levels of bacterial persistence in the leaves of Green Towers were observed in plants of the two developmental stages used in our study (**Figures 5**–**7**). Thus, factors determining bacterial apoplastic survival after syringe inoculation might change proportionally over time depending on the lettuce genotypes.

It has been previously shown that plant immune responses may be activated by human pathogens (Barak and Schroeder, 2012; Roy et al., 2013; Melotto et al., 2014; Jo and Park, 2019). However, plant immune responses have not been correlated with the level of *E. coli* O157:H7 and *S.* Typhimurium 14028s population growth. Therefore, we proceeded to assess hallmark PTI responses (Yu et al., 2017) with the lettuce genotypes Lollo Rossa and Red Tide that showed contrasting phenotypes in bacterial persistence after bacterial infiltration. The lettuce genotype Lollo Rossa, which exhibited one of the lowest bacterial apoplastic persistence levels after syringe inoculation, showed a significantly higher ROS burst and callose deposition than Red Tide (**Figures 8** and **9**). ROS burst and callose deposition were also reported to be generated by Arabidopsis plants after exposure to 1 μM of the flagellin epitope flg22 of *S.* Typhimurium 14028s (Garcia et al., 2014). Additionally, in lettuce, the induction of callose deposition has been observed as a defense response against lettuce phytopathogens (Stanghellini et al., 1993; Cohen et al., 2010), while oxidative stress has been associated with the non-host hypersensitive reaction against the bacteria *Pseudomonas syringae* pv. *phaseolicola* (Bestwick et al., 1998; Bestwick et al., 2001). These results strongly suggest that the differences in the ability of *E. coli* O157:H7 and *S.* Typhimurium 14028s to survive in the leaf apoplast of the genotypes Lollo Rossa and Red Tide are influenced by the variation in the level of defense responses activated against these bacteria. Although the contribution of the type III secretion systems and type III effectors in the colonization of plants by human pathogenic bacteria remains controversial (Schikora et al., 2011; Shirron and Yaron, 2011; Melotto et al., 2014; Chalupowicz et al., 2018; Montano et al., 2020), it is possible that the lettuce genotypes differ in their ability to recognize type III effectors of *S.* Typhimurium 14028s and *E. coli* O157:H7 resulting in variation in effector-triggered immunity against these human pathogenic bacteria. Interestingly, Lollo Rossa has been reported as resistant to the disease bacterial leaf spot of lettuce caused by *Xanthomonas campestris* pv. *vitians* (**Table 1**; Hayes et al., 2014), which suggests that this lettuce genotype might have a strong basal immune system to a wide range of bacteria. In tomato leaves, Potnis et al. (2015) observed that induction of water-soaked lesions by *X. euvesicatoria* or *X. gardneri* (causal agents of tomato bacterial spot disease) promoted *S. enterica* (serovars Enteritidis and Baildon cocktail) growth. Taking this into consideration, it is possible that the *X. campestris* pv. *vitians* interaction with lettuce could also provide a conducive environment for the growth of *Salmonella*. Therefore, Lollo Rossa could potentially possess traits that might contribute directly and indirectly to the prevention of human pathogenic bacterial survival in the phyllosphere. In addition, it has been shown that the composition of natural microbiota in the lettuce phyllosphere can be significantly influenced by leaf properties (Hunter et al., 2010) and affect the leaf colonization by human pathogenic bacteria (Lima et al., 2013). Possibly, differences in indigenous microbial communities among the lettuce genotypes used in our study might have contributed to the observed variation on *E. coli* O157:H7 and *S.* Typhimurium 14028s leaf colonization. The apoplastic survival of *E. coli* O157:H7 after syringe inoculation was in general higher than the apoplastic survival of *S.* Typhimurium 14028s in Lollo Rossa and the other lettuce genotypes (**Figures 5**–**7**). This agrees with the overall stronger immune responses of Lollo Rossa to *S.* Typhimurium 14028s compared to *E. coli* O157:H7 (**Figures 8** and **9**). This variation resembles quantitative resistance, where the phenotype is polygenically controlled and the predominant mechanisms extend beyond differences in pathogen recognition to variation in defense-related outputs such as strengthening of the cell wall

or defense compound biosynthesis (Corwin and Kliebenstein, 2017). Determining the genetic bases of this phenotype is key for the potential incorporation into lettuce breeding programs towards enhanced food safety.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

MM conceived research. CJ performed the experiments. CJ and MM designed the research, analyzed the data, and wrote the manuscript. All authors read and approved the manuscript.

FUNDING

This research was supported by grants from the U.S. Department of Agriculture – National Institute of Food and Agriculture (NIFA; 2015-67017-23360 and 2017-67017-26180) and NIFA Hatch grant (CA-D-PLS-2327-H) to MM. CJ was supported by a BECAS-Chile (CONICYT) fellowship and Horticulture and Agronomy Graduate Group fellowship from the University of California, Davis.

ACKNOWLEDGMENTS

The authors thank Dr. R. Michelmore for providing the lettuce seeds and guidance on the growth of healthy lettuce plants. We thank Dr. Paula Oblessuc and Logan DeMott for their helpful revisions of the manuscript.

REFERENCES

Allende, A., and Monaghan, J. (2015). Irrigation water quality for leafy crops: a perspective of risks and potential solutions. *Int. J. Environ. Res. Public Health* 12, 7457–7477. doi: 10.3390/ijerph120707457

Barak, J. D., and Schroeder, B. K. (2012). Interrelationships of food safety and plant pathology: the life cycle of human pathogens on plants. *Annu. Rev. Phytopathol.* 50, 241–266. doi: 10.1146/annurev-phyto-081211-172936

Barak, J. D., Kramer, L. C., and Hao, L. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/AEM.01661-10

Bennett, S. D., Sodha, S. V., Ayers, T. L., Lynch, M. F., Gould, L. H., and Tauxe, R. V. (2018). Produce-associated foodborne disease outbreaks, USA, 1998–2013. *Epidemiol. Infect.* 11, 1397–1406. doi: 10.1017/S0950268818001620

Bestwick, C. S., Brown, I. R., and Mansfield, J. W. (1998). Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. *Plant Physiol.* 118, 1067–1078. doi: 10.1104/pp.118.3.1067

Bestwick, C. S., Adam, A. L., Puri, N., and Mansfield, J. W. (2001). Characterisation of and changes to pro- and anti-oxidant enzyme activities during the hypersensitive reaction in lettuce (*Lactuca sativa* L.). *Plant Sci.* 161, 497–506. doi: 10.1016/S0168-9452(01)00427-7

Brandl, M. T., and Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Appl. Environ. Microbiol.* 74, 2298–2306. doi: 10.1128/AEM.02459-07

Chalupowicz, L., Nissan, G., Brandl, M. T., McClelland, M., Sessa, G., Popov, ,. G., et al. (2018). Assessing the ability of *Salmonella enterica* to translocate type III effectors into plant cells. *Mol. Plant Microbe In.* 31, 233–239. doi: 10.1094/MPMI-07-17-0166-R

Cohen, Y., Rubin, A. E., and Kilfin, G. (2010). Mechanisms of induced resistance in lettuce against *Bremia lactucae* by DL-β-amino-butyric acid (BABA). *Eur. J. Plant Pathol.* 126, 553–573. doi: 10.1007/s10658-009-9564-6

Cook, R. (2016). *Fresh-cut/value-added produce marketing trends. UC Davis fresh-cut products workshop: maintaining quality and safety* (Davis, CA, USA). Retrieved April, 11, 2019 from: https://arefiles.ucdavis.edu/uploads/filer_public/fb/7b/fb7b6380-cdf9-4db5-b5d2-993640bcc1e6/freshcut2016cook20160926final.pdf.

Corwin, J. A., and Kliebenstein, D. J. (2017). Quantitative resistance: more than just perception of a pathogen. *Plant Cell* 29, 655–665. doi: 10.1105/tpc.16.00915

Crawford, R. J., Webb, H. K., Truong, V. K., Hasan, J., and Ivanova, E. P. (2012). Surface topographical factors influencing bacterial attachment. *Adv. Colloid Interface Sci.* 179–182, 142–149. doi: 10.1016/j.cis.2012.06.015

Crozier, L., Hedley, P. E., Morris, J., Wagstaff, C., Andrews, S. C., Toth, I., et al. (2016). Whole-Transcriptome analysis of verocytotoxigenic *Escherichia coli* O157:H7 (Sakai) suggests plant-species-specific metabolic responses on exposure to spinach and lettuce extracts. *Front. Microbiol.* 7, 1088. doi: 10.3389/fmicb.2016.01088

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, ,. L., et al. (2010). 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. doi: 10.1146/annurev-food-022811-101211

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., Den Bakker, H. C., Bautista, J., et al. (2018). Fate of enteric pathogens in different spinach cultivars cultivated in growth chamber and field systems. *Food Quality Safety* 2, 221–228. doi: 10.1093/fqsafe/fyy020

Erickson, M. C. (2012). Internalization of fresh produce by foodborne pathogens. *Annu. Rev. Food Sci. Technol.* 3, 283–310. doi: 10.1146/annurev-food-022811-101211

ERS-USDA (Economic Research Service - United States Department of Agriculture) (2018). Vegetables and pulses yearbook data. U.S. per capita use of fresh and processing vegetables, dry pulse crops, and potatoes; cash receipts; U.S. vegetable trade. Retrieved April, 11, 2019 from: https://www.ers.usda.gov/data-products/vegetables-and-pulses-data/vegetables-and-pulses-yearbook-tables/.

Fonseca, J. M., Fallon, S. D., Sanchez, C. A., and Nolte, K. D. (2011). *Escherichia coli* survival in lettuce fields following its introduction through different irrigation systems. *J. Appl. Microbiol.* 110, 893–902. doi: 10.1111/j.1365-2672.2011.04942.x

Garcia, A. V., Charrier, A., Schikora, A., Bigeardm, J., Pateyron, S., de Tauzia-Moreau, M. L., et al. (2014). *Salmonella enterica* flagellin is recognized *via* FLS2 and activates PAMP-Triggered immunity in *Arabidopsis thaliana*. *Mol. Plant* 7, 657–674. doi: 10.1093/mp/sst145

Ge, C., Bohrerova, Z., and Lee, J. (2013). Inactivation of internalized *Salmonella* Typhimurium in lettuce and green onion using ultraviolet C irradiation and chemical sanitizers. *J. Appl. Microbiol.* 114, 1415–1424. doi: 10.1111/jam.12154

Golberg, D., Kroupitski, Y., Belausov, E., Pinto, R., and Sela, S. (2011). *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. *Int. J. Food Microbiol.* 145, 250–257. doi: 10.1016/j.ijfoodmicro.2010.12.031

Grube, R. C., and Ochoa, O. E. (2005). Comparative genetic analysis of field resistance to downy mildew in the lettuce cultivars 'Grand Rapids' and 'Iceberg'. *Euphytica* 142, 205–215. doi: 10.1007/s10681-005-1683-3

Grube, R. C., Ryder, E. J., Koike, S. T., McCreight, J. D., and Wintermantel, W. M. (2003). "Breeding for resistance to new and emerging lettuce diseases in California," in *Eucarpia Leafy Vegetables 2003*. Eds. Th,, J. L. van Hintum, A. Lebeda, D. Pink and J. W. Schut, 37–42. © CGN Wageningen, Netherlands.

Gutiérrez-Rodríguez, E., Gundersen, A., Sbodio, A. O., and Suslow, T. V. (2011). Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *J. Appl. Microbiol.* 112, 109–118. doi: 10.1111/j.1365-2672.2011.05184.x

Han, S., and Micallef, S. A. (2014). *Salmonella* Newport and Typhimurium colonization of fruit differs from leaves in various tomato cultivars. *J. Food Prot.* 77, 1844–185. doi: 10.4315/0362-028X.JFP-13-562

Hayes, R. J., Wu, B. M., Pryor, B. M., Chitrampalam, P., and Subbarao, K. V. (2010). Assessment of resistance in lettuce (*Lactuca sativa* l.) to mycelial and ascospore infection by *Sclerotinia minor* Jagger and *S. sclerotiorum* (Lib.) de Bary. *HortScience* 45, 333–341. doi: 10.21273/HORTSCI.45.3.333

Hayes, R. J., Trent, M. A., Mou, B., Simko, I., Gebben, S. J., and Bull, C. T. (2014). Baby leaf lettuce germplasm enhancement: developing diverse populations with resistance to bacterial leaf spot caused by *Xanthomonas campestris* pv. *vitians*. *HortScience* 49, 18–24. doi: 10.21273/HORTSCI.49.1.18

Hunter, P. J., Hand, P., Pink, D., Whipps, J. M., and Bending, G. D. (2010). Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* Species) phyllosphere. *Appl. Environ. Microbiol.* 76, 8117–8125. doi: 10.1128/AEM.01321-10

Hunter, P. J., Shaw, R. K., Berger, C. N., Frankel, G., Pink, D., and 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. doi: 10.1093/femsle/fnv077

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., and Jiang, X. (2004a). Persistence of *Salmonella enterica* Serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. *Foodborne Pathog. Dis.* 1, 27–35. doi: 10.1089/153531404772914437

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., and Jiang, X. (2004b). Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *J. Food Prot.* 67, 1365–1370. doi: 10.4315/0362-028x-67.7.1365

Jacob, C., Panchal, S., and Melotto, M. (2017). Surface inoculation and quantification of *Pseudomonas syringae* population in the Arabidopsis leaf apoplast. *Bio-protocol* 7 (5), e2167. doi: 10.21769/BioProtoc.2167

Jay-Russell, M. T. (2013). What is the risk from wild animals in food-borne pathogen contamination of plants? *CAB Reviews: Perspectives Agriculture Veterinary Science Nutrition Natural Resources* 8, 1–6. doi: 10.1079/PAVSNNR20138040

Jiang, X., Chen, Z., and Dharmasena, M. (2015). "The role of animal manure in the contamination of fresh food, Chapter 13, pp 312-350," in *Advances in microbial food safety, Volume 2*. Ed. J. Sofos (Oxford, UK: Woodhead Publishing-Elsevier Limited), 405.

Jo, S. H., and Park, J. M. (2019). The dark side of organic vegetables: interactions of human enteropathogenic bacteria with plants. *Plant Biotechnology Reports* 13, 105–110. doi: 10.1007/s11816-019-00536-1

Katagiri, F., Thilmony, R., and He, S. Y. (2002). The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *Arabidopsis Book.* 1, e0039. doi: 10.1199/tab.0039

Kisluk, G., and Yaron, S. (2012). Presence and persistence of *Salmonella enterica* serotype Typhimurium in the phyllosphere and rhizosphere of spray-irrigated parsley. *Appl. Environ. Microbiol.* 78, 4030–4036. doi: 10.1128/AEM.00087-12

Klerks, M. M., Franz, E., van Gent-Pelzer, M., Zijlstra, C., and 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. doi: 10.1038/ismej.2007.82

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., et al. (2009). Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75, 6076–6086. doi: 10.1128/AEM.01084-09

Kroupitski, Y., Pinto, R., Belausov, E., and Sela, S. (2011). Distribution of *Salmonella typhimurium* in romaine lettuce leaves. *Food Microbiol.* 28, 990–997. doi: 10.1016/j.fm.2011.01.007

Lang, M. M., Harris, L. J., and Beuchat, L. R. (2004). Survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. *J. Food Prot.* 67, 1092–1103. doi: 10.4315/0362-028X-67.6.1092

Lima, P. M., São José, J. F. B., Andrade, N. J., Pires, A. C. S., and Ferreira, S. O. (2013). Interaction between natural microbiota and physicochemical characteristics of lettuce surfaces can influence the attachment of *Salmonella* Enteritidis. *Food Control* 30, 157–161. doi: 10.1016/j.foodcont.2012.06.039

Macarisin, D., Patel, J., Bauchan, G., Giron, J. A., and Ravishankar, S. (2013). Effect of spinach cultivar and bacterial adherence factors on survival of *Escherichia coli* O157:H7 on spinach leaves. *J. Food Prot.* 76, 1829–1837. doi: 10.4315/0362-028X.JFP-12-556

Manikandan, S. (2010). Data transformation. *J. Pharmacol. Pharmacother.* 1, 126–127. doi: 10.4103/0976-500X.72373

Marvasi, M., Noel, T., George, A. S., Farias, M. A., Jenkins, K. T., Hochmuth, G., et al. (2014). Ethylene signaling affects susceptibility of tomatoes to *Salmonella*. *Microb. Biotechnol.* 7, 545–555. doi: 10.1111/1751-7915.12130

McCreight, J. D., Matheron, M. E., Tickes, B. R., and Platts, B. (2005). Fusarium wilt race 1 on lettuce. *HortScience* 40, 529–531. doi: 10.21273/HORTSCI.40.3.529

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980. doi: 10.1016/j.cell.2006.06.05

Melotto, M., Panchal, S., and Roy, D. (2014). Plant innate immunity against human bacterial pathogens. *Front. Microbiol.* 5, 1–12. doi: 10.3389/fmicb.2014.00411

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliland, S., and Fletcher, J. (2009). Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157:H7 in spinach. *J. Food Prot.* 72, 1521–1530. doi: 10.4315/0362-028x-72.7.1521

Montano, J., Rossidivito, G., Torreano, J., Porwollik, S., Sela, S., McClelland, M., et al. (2020). *Salmonella enterica* serovar Typhimurium 14028s genomic regions required for colonization of lettuce leaves. *Front. Microbiol.* (in press). doi: 10.3389/fmicb.2020.00006

Porwollik, S., Santiviago, S. A., Cheng, P., Long, F., Desai, P., Fredlund, J., et al. (2014). Defined single-gene and multi-gene deletion mutant collections in *Salmonella enterica* sv Typhimurium. *PLoS ONE* 9, e99820. doi: 10.1371/journal.pone.0099820

Potnis, N., Colee, J., Jones, J. B., and Barak, J. D. (2015). Plant pathogen-induced water-soaking promotes *Salmonella enterica* growth on tomato leaves. *Appl. Environ. Microbiol.* 81, 8126–8134. doi: 10.1128/AEM.01926-15

Quilliam, R. S., Williams, A. P., and Jones, D. L. (2012). Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157:H7. *PLoS ONE* 7, e33842. doi: 10.1371/journal.pone.0033842

Roy, D., and Melotto, M. (2019). Stomatal response and human pathogen persistence in leafy greens under preharvest and postharvest environmental conditions. *Postharvest Biol. Technol.* 148, 76–82. doi: 10.1016/j.postharvbio.2018.10.013

Roy, D., Panchal, S., Rosa, B. A., and Melotto, M. (2013). *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. *Phytopathology* 103, 326–332. doi: 10.1094/PHYTO-09-12-0230-FI

Ryder, E. J., and Robinson, B. J. (1995). Big-vein resistance in lettuce: identifying, selecting, and testing resistant cultivars and breeding lines. *J. Amer. Soc. Hort. Sci.* 120, 741–746. doi: 10.21273/JASHS.120.5.741

Saldaña, Z., Sánchez, E., Xicohtencatl-Cortes, J., Puente, J. L., and Girón, J. A. (2011). Surface structures involved in plant stomata and leaf colonization by Shiga-toxigenic *Escherichia coli* O157:H7. *Front. Microbiol.* 27, 119. doi: 10.3389/fmicb.2011.00119

Sapers, G. M., and Doyle, M. P. (2014). "Chapter 1 - Scope of the produce contamination problem. p. 3-20," in *The produce contamination problem: causes and solutions. Second Edition*. Eds. K. R. Matthews, G. M. Sapers and C. P. Gerba (Amsterdam, Netherlands: Elsevier), 467.

Schenk, S. T., and Schikora, A. (2015). Staining of callose depositions in root and leaf tissues. *Bio-protocol* 5, e1429. doi: 10.21769/BioProtoc.1429

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrieret, A., et al. (2011). Conservation of *Salmonella* infection mechanisms in plants and animals. *PLoS ONE* 6, e24112. doi: 10.1371/journal.pone.0024

Scott, J. C., Kirkpatrick, S. C., and Gordon, T. R. (2010). Variation in susceptibility of lettuce cultivars to fusarium wilt caused by *Fusarium oxysporum* f.sp. *lactucae*. *Plant Pathol.* 59, 139–146. doi: 10.1111/j.1365-3059.2009.02179.x

Seo, K. H., and Frank, J. F. (1999). Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 62, 3–9. doi: 10.4315/0362-028x-62.1.3

Shirron, N., and Yaron, S. (2011). Active suppression of early immune response in tobacco by the human pathogen *Salmonella* Typhimurium. *PLoS ONE* 6, e18855. doi: 10.1371/journal.pone.0018855

Simko, I., Pechenick, D. A., McHale, L. K., Truco, M. J., Ochoa, O. E., Michelmore, R. W., et al. (2009). Association mapping and marker-assisted selection of the lettuce dieback resistance gene Tvr1. *BMC Plant Biol.* 9, 135. doi: 10.1186/1471-2229-9-135

Simko, I., Rauscher, G., Sideman, R. G., McCreight, J. D., and Hayes, R. J. (2014). Evaluation and QTL mapping of resistance to powdery mildew in lettuce. *Plant Pathol.* 63, 344–353. doi: 10.1111/ppa.12087

Simko, I., Ochoa, O. E., Pel, M. A., Tsuchida, C., Font, C., Forcada, I., et al. (2015). Resistance to downy mildew in lettuce 'La Brillante' is conferred by Dm50 gene and multiple QTL. *Phytopathology* 105, 1220–1228. doi: 10.1094/PHYTO-02-15-0057-R

Smith, J. M., and Heese, A. (2014). Rapid bioassay to measure early reactive oxygen species production in Arabidopsis leave tissue in response to living *Pseudomonas syringae*. *Plant Methods* 10, 6. doi: 10.1186/1746-4811-10-6

Solomon, E. B., Pang, H. J., and Matthews, K. R. (2003). Persistence of *Escherichia coli* O157:H7 on lettuce plants following spray irrigation with contaminated water. *J. Food Prot.* 66, 2198–2202. doi: 10.4315/0362-028X-66.12.2198

Sperandio, V., Torres, A. G., Girón, J. A., and Kaper, J. B. (2001). Quorum sensing is a global regulatory mechanism in entrohemorrhagic *Escherichia coli* O157:H7. *J. Bac.* 183, 5187–5197. doi: 10.1128/JB.183.17.5187-5197.2001

Stanghellini, M. E., Rasmussen, S. L., and Vandemark, G. J. (1993). Relationship of callose deposition to resistance of lettuce to *Plasmopara lactucae-radicis*. *Phytopathology* 83, 1498–1501. doi: 10.1094/Phyto-83-1498

Tomás-Callejas, A., López-Velasco, G., Camacho, A. B., Artés, F., Artés-Hernández, F., and Suslow, T. V. (2011). Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. *Int. J. Food Microbiol.* 151, 216–222. doi: 10.1016/j.ijfoodmicro.2011.08.027

Vallad, G. E., and Subbarao, K. V. (2008). Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. *Phytopathology* 98, 871–885. doi: 10.1094/PHYTO-98-8-0871

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., and Heyndrickx, M. (2014). Evaluation of an attachment assay on lettuce leaves with temperature- and starvation-stressed *Escherichia coli* O157:H7 MB3885. *J. Food Prot.* 77, 549–557. doi: 10.4315/0362-028X.JFP-13-332

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Heyndrickx, M., Maes, M., et al. (2016). Microarray-based screening of differentially expressed genes of *E. coli* O157:H7 Sakai during preharvest survival on butterhead lettuce. *Agriculture* 6, 6. doi: 10.3390/agriculture6010006

Whipps, J. M., Budge, S. P., McClement, S., and Pink, D. A. C. (2002). A glasshouse cropping method for screening lettuce lines for resistance to *Sclerotinia sclerotiorum*. *Eur. J. Plant Pathol.* 108, 373–378. doi: 10.1023/A:1015637018474

Wong, C. W. Y., Wang, S., Lévesque, R. C., Goodridge, L., and Delaquis, P. (2019). Fate of 43 *Salmonella* strains on lettuce and tomato seedlings. *J. Food Prot.* 82, 1045–1051. doi: 10.4315/0362-028X.JFP-18-435

Yaron, S., and Römling, U. (2014). Biofilm formation by enteric pathogens and its role in plant colonization and persistence. *Microb. Biotechnol.* 7, 496–516. doi: 10.1111/1751-7915.12186

Yu, X., Feng, B., He, P., and Shan, L. (2017). From chaos to harmony: responses and signaling upon microbial pattern recognition. *Annu. Rev. Phytopathol.* 55, 109–137. doi: 10.1146/annurev-phyto-080516-035649

Conflict of 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 March 2020
doi: 10.3389/fmicb.2020.00391

Salmonella enterica Elicits and Is Restricted by Nitric Oxide and Reactive Oxygen Species on Tomato

*Angela Marie C. Ferelli[1], Samantha Bolten[1†], Brooke Szczesny[1] and Shirley A. Micallef[1,2]**

[1] Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, United States,
[2] Centre for Food Safety and Security Systems, University of Maryland, College Park, MD, United States

OPEN ACCESS

Edited by:
Max Teplitski,
University of Florida, United States

Reviewed by:
Juan Manuel Cevallos-Cevallos, ESPOL Polytechnic University, Ecuador
Neha Potnis, Auburn University, United States
Andree George, Western Regional Research Center (USDA-ARS), United States

***Correspondence:**
Shirley A. Micallef
smicall@umd.edu

†Present address:
Samantha Bolten, Environmental Microbial and Food Safety Laboratory, United States Department of Agriculture – Agricultural Research Service, Beltsville, MD, United States

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *25 November 2019*
Accepted: *25 February 2020*
Published: *13 March 2020*

Citation:
Ferelli AMC, Bolten S, Szczesny B and Micallef SA (2020) Salmonella enterica Elicits and Is Restricted by Nitric Oxide and Reactive Oxygen Species on Tomato. Front. Microbiol. 11:391. doi: 10.3389/fmicb.2020.00391

The enteric pathogen *Salmonella enterica* can interact with parts of the plant immune system despite not being a phytopathogen. Previous transcriptomic profiling of *S. enterica* associating with tomato suggested that *Salmonella* was responding to oxidative and nitrosative stress in the plant niche. We aimed to investigate whether *Salmonella* was eliciting generation of reactive oxygen species (ROS) and nitric oxide (NO), two components of the microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) of plants. We also sought to determine whether this interaction had any measurable effects on *Salmonella* colonization of plants. Biochemical, gene expression and on-plant challenge assays of tomato vegetative and fruit organs were conducted to assess the elicitation of ROS and NO in response to *Salmonella* Newport association. The counter bacterial response and the effect of NO and ROS on *Salmonella* colonization was also investigated. We detected H_2O_2 in leaves and fruit following challenge with live *S.* Newport ($p < 0.05$). Conversely, NO was detected on leaves but not on fruit in response to *S.* Newport ($p < 0.05$). We found no evidence of plant defense attenuation by live *S.* Newport. Bacterial gene expression of *S.* Newport associating with leaves and fruit were indicative of adaptation to biotic stress in the plant niche. The nitrosative stress response genes *hmpA* and *yoaG* were significantly up-regulated in *S.* Newport on leaves and fruit tissue compared to tissue scavenged of NO or ROS ($p < 0.05$). Chemical modulation of these molecules in the plant had a restrictive effect on bacterial populations. Significantly higher *S.* Newport titers were retrieved from H_2O_2 scavenged leaves and fruit surfaces compared to controls ($p < 0.05$). Similarly, *S.* Newport counts recovered from NO-scavenged leaves, but not fruit, were higher compared to control ($p < 0.05$), and significantly lower on leaves pre-elicited to produce endogenous NO. We present evidence of *Salmonella* elicitation of ROS and NO in tomato, which appear to have a restricting effect on the pathogen. Moreover, bacterial recognition of ROS and NO stress was detected. This work shows that tomato has mechanisms to restrict *Salmonella* populations and ROS and NO detoxification may play an important role in *Salmonella* adaptation to the plant niche.

Keywords: human pathogens on plants, nitrosative stress, oxidative stress, food safety, *Salmonella*–tomato interaction, nitric oxide, ROS

INTRODUCTION

Non-typhoidal *Salmonella enterica* is a leading cause of foodborne illness transmitted by fresh fruit and vegetables (Callejón et al., 2015). Traceback investigations of several salmonellosis outbreaks implicating fresh produce have pointed to contamination sources in crop production areas (Greene et al., 2008; Bennett et al., 2015). *S. enterica* is frequently isolated from water and soil in agricultural settings (Micallef et al., 2012; Bell et al., 2015; Callahan et al., 2019), suggesting that this enteric pathogen is able to cycle through various ecological niches and become established in the plant phyllosphere.

Salmonella enterica can survive and multiply on plants (Brandl and Mandrell, 2002), the success of which is influenced by multiple factors (Brandl et al., 2013), including plant genotype and organ (Barak et al., 2011; Han and Micallef, 2014), age (Brandl and Amundson, 2008; Zheng et al., 2013), surface metabolite profiles (Han and Micallef, 2016), as well as resident epiphytes (Poza-Carrion et al., 2013). Studies on *S. enterica* colonizing tomato fruit wounds, lettuce soft rot lesions and sprouts have identified specific sets of genes expressed under these conditions, including genes involved in amino acid biosynthesis, fatty acid metabolism, iron acquisition, attachment and stress response (Goudeau et al., 2013; Salazar et al., 2013; Tan et al., 2016; de Moraes et al., 2017, 2018). Research within our group investigating gene expression in *S. enterica* epiphytically colonizing tomato shoots and roots detected several genes involved in nitrosative and oxidative stress mitigation and multiple *Salmonella* pathogenicity island-2-encoded type III secretion system genes (T3SS-2) (Han et al., unpublished). These findings pointed to interplay between the enteric pathogen and the plant, as a result of tomato plant recognition and immune response.

Plants recognize potential microbial pathogens via MAMP interaction with pathogen recognition receptors (PRRs) (Jones and Dangl, 2006). This recognition initiates several strong yet transient signaling events to occur, beginning with an influx of calcium ions into the cell (Ranf et al., 2011) which induces a burst of reactive oxygen species (ROS) (Doke, 1983) and nitric oxide (NO) (Ma et al., 2008; Rasul et al., 2012). The generation of ROS and the more recently identified NO serve multiple purposes for the plant. The ROS burst can directly control the potential pathogen threat and, together with NO, may activate mitogen associated protein kinases (MAPKs) and signal the up-regulation of transcription factors that initiate transient defense responses. These include salicylic acid (Tsuda et al., 2008) and ethylene biosynthesis (Liu and Zhang, 2004) which in part comprise MAMP- triggered immunity (MTI) (Meng and Zhang, 2013). The non-plant pathogen *S.* Typhimurium and its flagellin 22 (flg22) have been shown to induce an ROS burst in tobacco and tomato leaf disks, respectively (Shirron and Yaron, 2011; Meng et al., 2013). Flagellin 22 from *S.* Typhimurium was recognized by tobacco and *Arabidopsis thaliana* through the FLS2 receptor, inducing MTI which was effective in restricting *S. enterica* and the plant pathogen *Pseudomonas syringae* pv. *tomato* (Meng et al., 2013; Garcia et al., 2014). The role of NO in plant defense is less understood but it was generated in *Arabidopsis* in response to lipopolysaccharide (LPS) challenge (Zeidler et al., 2004) and also required for abscisic acid-induced stomatal closure (Neill et al., 2002). Melotto et al. (2006) showed that *Arabidopsis* guard cells generated NO in response to flg22 and LPS, which was followed by stomatal closure. They also reported that *S. enterica* was able to trigger stomatal closure in *Arabidopsis*. Furthermore, flagellar mutants of *S.* Typhimurium were shown to better colonize wheat, alfalfa and *Arabidopsis*, suggesting that attenuation of MAMPs favored bacterial colonization (Iniguez et al., 2005). Finally, some studies have suggested that *S. enterica* may have the ability to suppress MTI in *Arabidopsis* and tobacco and the role of effector proteins has been invoked (Shirron and Yaron, 2011; Garcia et al., 2014; Neumann et al., 2014).

The current state of knowledge and our finding that *S. enterica* expresses oxidative and nitrosative stress genes, and T3SS-2 genes when colonizing tomato shoot and root surfaces (Han et al., unpublished), implied that *S. enterica* recognition by tomato induces the generation of both ROS and NO, and that *S. enterica* attempts to attenuate the plant immune response. We set out to test this hypothesis in leaves, but also more relevantly, on tomato fruit, in relation to both live and killed *S. enterica* to assess potential plant defense suppression. MTI induction in tomato leaves and fruit in response to *S. enterica* Newport (*Se*N) association was investigated by measuring NO and ROS generation. Moreover, we sought to describe the effect of this plant response on *S. enterica* populations. The reciprocal bacterial response and the effect of surface modulation of NO and ROS on *S. enterica* colonization of tomato leaves and fruit was also evaluated.

MATERIALS AND METHODS

Cultivation of Plant Material

Tomato seeds cv. 'Heinz-1706' were obtained from the Tomato Genetics Resource Center (TGRC) from the University of California, Davis. After pre-treatment in 30% w/v polyethylene glycol solution at room temperature with shaking for 72 h, seeds were germinated in potting media (Sunshine LC1; Sungro Horticulture, Canada) at 25°C. Germinated seeds were transferred to fresh potting media supplemented with fertilizer (Osmocote controlled release fertilizer 18–6–12:nitrogen–phosphate–potash, The Scotts Company LLC., Marysville, OH, United States) and subjected to a 16 h-light/8 h-dark photoperiod and 26°C day temperature/18°C night temperature with 70% humidity (RH) at the University of Maryland Research Greenhouse. Plants were drip irrigated. Tomato seedlings were grown to five true leaves before experimentation, unless otherwise noted. Seedlings for experimentation were transported to a BSL-2 growth chamber (16 h-light/8 h-dark photoperiod and 23°C constant temperature with 70–80% RH) at least 5 days prior to inoculation and flood irrigated to a depth of 5 cm in trays every 4 days. Water was withheld 3 h before inoculating leaves and for the duration of experiments. For fruit, plants were either grown in the field (summer) at the Wye Research and Education Centre, Queenstown, MD, United States, or transplanted into 6 L pots to be grown in the greenhouse (winter)

once they reached the 5-leaf stage. In the greenhouse, plants were fertilized once a week and treated with non-organophosphate containing pesticide once every 2 weeks for aphid and white fly management. Once plants reached maturity, red, ripe fruit was collected immediately prior to experimentation, rinsed with sterile water and air dried, unless otherwise stated.

Bacterial Strains

The *Salmonella enterica* Newport strain used was an environmental isolate collected from an irrigation pond that matched a recurring tomato outbreak strain (Greene et al., 2008). *Se*N had been previously adapted to rifampicin (rif) and was therefore maintained at −80°C in Brucella Broth (BD, Sparks, MD, United States) containing 15% glycerol and 50 μg/mL rifampicin (rif, Tokyo Chemical Industry, Portland, OR, United States). For each experiment, cultures of *Se*N were grown overnight on Trypticase Soy Agar (TSA; BD) + rif at 35°C. A single colony was selected, suspended in sterile water, and diluted to OD_{600} = 0.34 – approximately 8.5 log CFU/mL. Serial dilutions were made in sterile water for inoculum preparation and in 0.1% peptone water for bacterial quantification (BD Difco, Sparks, MD, United States). Cells were enumerated on TSArif. *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*), a relative to *P. syringae* pv. *tomato* with similar virulence and in the same genospecies clade (genospecies III) (Preston, 2000) was grown in TSA at 25°C, and inoculum prepared as described above.

Detection of ROS in Leaves and Fruit

To detect the amount of H_2O_2 produced in leaves following *Se*N challenge, 3,3′-diaminobenzidine (DAB) staining was adapted from Bindschedler et al. (2006). Briefly, a third emerged leaflet from freshly watered, 5-leaved 'Heinz-1706' plants was syringe infiltrated into the abaxial surface with 500 uL of either *Se*N in sterile water at 8 log CFU/mL, heat-killed *Se*N, or sterile water (control) (N = 6 plants per treatment). Positive controls were conducted with *Psm*. Inoculated plants were incubated in a BSL-2 growth chamber. All experiments were performed in a complete randomized design (CRD) and repeated at least twice. At 0.1 and 24 hours post-inoculation (hpi), inoculated leaflets were excised and submerged in 5 mL DAB solution (1 mg/mL aqueous DAB (Alfa Aesar, Ward Hill, MA, United States), 200 mM Na_2HPO_4 (VWR, West Chester, PA, United States), 0.05% Tween 20 (Amresco, Solon, OH, United States) and 100 μL 3 N HCl). Samples were vacuum-infiltrated in a vacuum desiccator attached to the laboratory vacuum system for 4 min, then incubated in the dark at 23°C with shaking at 50 rpm for 4 h. At the end of staining, decolorizer solution was added (3:1:1 95% ethanol, glycerol, glacial acetic acid) and samples were incubated in a boiling water bath for 15 min. Decolorized leaflets were fixed to paper and imaged with an Epson V330 photo scanner. The stain, corresponding to H_2O_2 production, was analyzed for intensity via ImageJ2 FIJI package (Schindelin et al., 2012). Optical density in leaves was calculated using the formula

$$OD = \log_{10}(\max intensity \div mean\ intensity\ of\ leaf\ area).$$

To detect a range of ROS produced from *Se*N challenge on fruit, staining with 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Invitrogen, Molecular Probes Inc., Eugene, OR, United States) was adapted from Shin and Schachtman (2004). Briefly, 3 mm × 3 mm sections of ripe tomato exocarp per fruit were excised with a sterile razor and placed in separate black 96-well plates to serve as technical replicates for one fruit (Corning, Nazareth, PA, United States). Aliquots of 150 μL deionized water were delivered to the sample wells and incubated overnight in the dark at 4°C to allow for dissipation of any ROS production due to injury. Immediately before experimentation, wells were washed with 100 μL sterile water. One hundred micro-liters of 8.0 log CFU/mL *Se*N, heat killed *Se*N (14 h only) or sterile water were delivered to sample wells (N = 4 fruit per treatment, with three technical replicates per fruit). Samples were vacuum-infiltrated in a vacuum desiccator for 5 min, then shaken at 100 rpm at 27°C for 3 or 14 hpi. At the time of sampling, 25 μM CM-H_2DCFDA in water was added to each well. The fluorophore was allowed to react for 30 min at 23°C in the dark with shaking at 50 rpm before being imaged with a Synergy HTX Microplate reader (BioTek, Winooski, VT, United States) at 485 nm excitation, 520 nm emission with 50 gain.

Detection of NO in Leaves and Fruit

To measure amounts of NO release from tomato when challenged with *Se*N, 4,5-diaminofluorescein diacetate (DAF-2 DA; Fisher Scientific, Hampton, NH, United States) was used for its ability to complex intercellular NO as well as NO in solution (Rasul et al., 2012). For measurements on leaves, leaflets of mature 'Heinz-1706' plants grown in the research greenhouse were punched three times with a 3 mm hole puncher and cut tissue pieces were placed in separate wells in a black 96-well plate, to serve as technical replicates for each leaf (Corning, Nazareth, PA, United States) (N = 5 leaves per treatment, with three technical replicates per leaf). For measurements on fruit, 3 mm × 3 mm sections of ripe tomato exocarp were excised with a sterile razor (N = 5 fruit per treatment, with three technical replicates each). For both experiments, 150 μL deionized water was placed in the sample wells and plates were incubated overnight in the dark at 4°C to allow for the dissipation of injury related NO signal. All experiments were performed in a CRD and repeated at least twice. Prior to inoculation, tissues were washed twice with sterile water, then challenged with 100 μL of 8 log CFU/mL *Se*N, heat killed *Se*N, sterile water or 8 log CFU/mL *Psm*. Samples were vacuum-infiltrated for 5 min, then shaken at 100 rpm at 27°C. At 0.1, 1, and 3 h a final concentration of 15 μM DAF-2 DA in 50 mM Tris HCl pH 7.5 was delivered to the wells. Plates were incubated in the dark for 30 min at 27°C with shaking at 50 rpm and immediately read on the Synergy HTX (BioTek) at 485 nm excitation, 520 nm emission with 50 gain.

Targeted q-RT-PCR of *Se*N Genes Colonizing Leaf and Fruit Surfaces

To evaluate genes involved in nitrosative and oxidative stress responses in *Se*N colonizing the tomato phyllosphere,

3-leaf tomato seedlings cv. 'Heinz-1706' were pre-treated with water (native environment), 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; NO-limiting environment) or $CaCl_2$ (excess NO environment), then challenged with *Se*N. To achieve this, 48 plants grown in autoclaved LC-1 potting media (Sunshine LC1) were separated into three groups and aerosol-sprayed with either 1 mL 0.5% $CaCl_2$, ddH_2O or 0.2 mM cPTIO. The plants were allowed to air-dry for 30 min. The second emerged leaf was challenged with 7 log CFU/mL *Se*N, delivered as ten 2-μL spots onto the leaf surface. Plants were incubated in the BSL-2 growth chamber, as previously described. At 6 hpi, inoculated leaves of three plants were pooled to comprise one composite sample (N = 4 composite samples per treatment). Samples were immediately fixed in 2:1 RNAProtect Bacteria Reagent (Qiagen, Germantown, MD, United States):ddH_2O. Samples were sonicated on a 8510 Branson Sonicator at full strength for 2 min to dislodge surface attached bacteria. The wash solution containing *Se*N was transferred to a fresh tube and processed as described below.

To evaluate the role of genes involved in colonization of tomato fruit, cv. 'Heinz-1706' mature red fruit were washed with 200 ppm sodium hypochlorite and triple rinsed with ddH_2O. Fruit were then syringe-injected at the calyx with either 500 μL ddH_2O or 0.25 mM ascorbic acid. Seven log CFU/mL *Se*N was delivered as five 20-μL spots on the fruit surface. Fruit were incubated in the BSL-2 growth chamber in identical conditions as seedlings. At 6 hpi, five fruit from each treatment were pooled to comprise one composite sample (N = 4 composite samples per treatment). Samples were placed in RNA*later* Stabilization Solution (Invitrogen, Carlsbad, CA, United States). Fruit were vigorously vortexed for 3 min to dislodge attached cells. The wash solution containing *Se*N was transferred to a fresh tube and processed as described below.

In both experiments, 0.5 mL *Se*N inoculum in water was immediately fixed with RNAProtect Bacteria Reagent or RNA*later* Stabilization Solution to serve as the baseline for gene expression. All samples were centrifuged at 5,000 × g for 30 min. Total RNA was extracted from the resultant pellet using the Qiagen RNeasy Mini kit (leaves) (Qiagen) or the Purelink RNA Isolation kit (fruit) (Invitrogen) with 45 min on-column DNA digestion (Invitrogen). Resulting total RNA was evaluated on the Nanodrop 1000 (Thermo Fisher) for quality. PCR of target genes was performed using 1 μL total RNA sample template to ensure depletion of gDNA. cDNA was synthesized with Verso cDNA kit (Thermo Scientific, Waltham, MA, United States) and 1 ng samples were subjected to q-PCR of genes using primers listed in **Table 1**. Primers were used at 100 nM concentration and verified to be 90–105% efficient. Plant material was verified to produce no off-target amplification before experimentation. In a series of experiments using TSB amended with treatment reagents, *Se*N

TABLE 1 | List of genes, their qPCR primers and efficiencies used to examine *S.* Newport gene expression on tomato leaves and fruits.

Functional category	Gene	Function	5′–3′ sequence	qPCR primer source	Efficiency
ROS response	*ahpC*	Peroxiredoxin	TCGCTTCGCCTTCTTTCCAT GACCTTTGTTGTTGACCCGC	This study	101%
	katG	Catalase	GACTCACCGACACCCTGAAG CACGGTCTCTTCGTCGTTCA	This study	102%
NO response and detoxification	*hmpA*	Flavohemoglobin, nitric oxide dioxygenase activity	GAACATTTCGTCCAGCGTCG ATCAGCGTGAAGCCCTGTTT	This study	95%
	yoaG	Cytoplasmic protein in NsrR regulon	ATAGCAACGGCGTCTCTGTG GGTATCGTAGGAACGCACGG	This study	101%
Virulence	*phoP*	Virulence regulator	CGACTTTATCCTGCCAGCCT GCCTTTCCTTAATACGCCGC	This study	91%
	phoQ	Virulence regulator, membrane-bound sensor kinase	TATGGTGTGGAGCTGGTTCG CGGCGATCCACAGTAAAGGA	This study	91%
	sdiA	Virulence regulator, quorum-sensing regulator	GATGAGGTCTTCCCTTCCGC TACGCTGCTCCTCGTTTACC	This study	90%
Environmental fitness	*marA*	DNA-binding transcriptional activator for antibiotics resistance operon MarRAB	TACGGCTGCGGATGTATTGG CGAGGATAACCTGGAGTCGC	This study	105%
	nmpC	Outer membrane porin protein, cell wall biogenesis	GTCCGTCCATCGCTTACCTG GCTTTGGTGAAGTCGCTGTC	This study	94%
	trpE	Tryptophan biosynthesis protein	CGCTTTTTCACCAGGTCTGC AACGCCTGAATGGTGACAGT	This study	102%
Housekeeping	*rpoD*	RNA polymerase sigma factor	GTGAAATGGGCACTGTTGAACTG TTCCAGCAGATAGGTAATGGCTTC	Karlinsey et al. (2012)	101%

gene expression was confirmed to be reflective of epiphytic habit on tomato surface and not an artifact of interaction with elicitor or scavenger (**Supplementary Figure 1**). Amplification was conducted on an ABI Step-One Plus (Applied Biosystems, Foster City, CA, United States) with SYBR as a reporter (PowerUP™ SYBR Green Master Mix, Thermo Fisher Scientific, Austin, TX, United States) using the following parameters: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 59°C for 30 s. Melt curve analysis was included to ensure product specificity. The cutoff Ct was set to 36.5 cycles. Data were analyzed on the ABI Step One Plus instrument with the ΔΔCt method (Pfaffl, 2001) using RNA polymerase sigma factor *rpoD* as the endogenous control. Relative gene expression was compared to expression in *Se*N inoculum after internal normalization to *rpoD* expression.

Modulation of Endogenous Hydrogen Peroxide and Nitric Oxide Levels, and Plant Colonization Assays

To investigate the effect of plant derived ROS and NO on *Se*N survival on tomato surfaces, the third emerged leaf on 5-leaved 'Heinz-1706' seedlings or mature fruit were treated with reagents to either scavenge surface ROS (Bradley et al., 1992; Lee et al., 1999) or NO (Małolepsza and Różalska, 2005; Keshavarz-Tohid et al., 2016), or elicit production of NO (Chakraborty et al., 2016), then subsequently inoculated with *Se*N. The reagents employed, concentrations and application methods for leaves and fruit are detailed in **Table 2**. All experiments were performed in a CRD and repeated at least twice. After application of cPTIO and $CaCl_2$, fruit (N = 10 for each treatment) and leaves (N = 18 for cPTIO experiments, N = 4 for $CaCl_2$ experiments) were left to air-dry for 4 h at room temperature. Ascorbic acid-treated leaves (N = 3 per treatment) were left to dry for 2 h and fruit (N = 11 per treatment) were left to dry for 30 min prior to *Se*N inoculation. Following pretreatment, a suspension of 5.5 log CFU/mL *Se*N in water was applied to the surface of leaflets or fruit in ten 2-μL spots. Samples were incubated at 23°C at 75% RH for 12 h. To retrieve viable *Se*N, inoculated leaflets or fruit exocarp were cut and excised, respectively, as previously described, and diluted in 0.1% peptone water. Leaflets were hand-massaged and sonicated and fruit were hand-massaged and vortexed for 2 min before serially plating dilutions onto TSArif and incubating at 35°C for 20 h.

Statistical Analysis

Statistical analysis was conducted in JMP version 14.1.0. The degree of NO and ROS elicitation was analyzed for significance via Student's t-test in pairwise comparisons, or ANOVA and orthogonal contrasts for *a priori* comparisons excluding the positive control ($\alpha = 0.05$). Targeted gene expression data was analyzed for significance using ANOVA and *post hoc* via Dunnett's test (inoculum control versus on-plant gene expression) or Tukey's Honestly Significant Difference (cPTIO versus $CaCl_2$ versus control on leaves), and Student's t-test (ascorbic acid versus control on fruit) all at $\alpha = 0.05$. For on-plant challenge assays, Student's t-test was employed to compare treatment to water control ($\alpha = 0.05$).

RESULTS

Salmonella Newport Elicits H_2O_2 Production in Tomato Leaves and Fruit

A dark brown precipitate indicative of H_2O_2 production was detected immediately following inoculation of leaves with *Se*N (0.1 hpi) (**Figures 1A,C**). At 0.1 hpi, the brown precipitate deposited in *Se*N-challenged leaves (0.47 ± 0.10; $p < 0.05$) and heat-killed *Se*N-challenged leaves (0.45 ± 0.08; $p < 0.05$) was significantly darker than the negative control. Twenty-four hpi, heat-killed *Se*N (0.20 ± 0.05) and water control (0.23 ± 0.08) had comparable measurements, while leaves treated with live *Se*N exhibited darker staining (0.35 ± 0.18; $p < 0.05$) compared to control. The staining obtained with *Se*N was diffuse, as opposed to localized in spots as was observed for the virulent *Psm* pathogen (**Figure 1C**). Overall, we found no evidence of live *Se*N suppression of ROS generation.

In fruit, at 3 h post-challenge, more ROS was detected in live *Se*N-treated exocarp than in water-treated exocarp ($p < 0.01$; **Figure 1B**). At 14 hpi, significantly more ROS was detected in exocarp samples treated with heat-killed (98.18 ± 51.0 Arbitrary fluoresence units, Au; $p < 0.01$) and live *Se*N (82.82 ± 17.0 Au; $p < 0.05$) compared to controls (51.41 ± 15.5 Au). Conversely to leaves, heat-killed S*e*N produced a similar signal to live S*e*N in fruit at the later timepoint. Taken together, these measurements suggest that live *Se*N can induce ROS generation in both tomato leaf and fruit exocarp tissue. Further, the differential ROS levels detected in tomato leaves inoculated with live or heat-killed *Se*N showed that ROS generation was more prolonged in response to live cells, potentially triggered by *Salmonella* activity in the leaf niche, and not solely from interaction with microbial surface cellular components.

NO Production Was Detected in Leaves but Not in Fruit Exocarp Challenged With SeN

Following leaf surface inoculation with *Se*N, NO was detected in all treatments (**Figure 2**). The tomato pathogen *Psm*-treated leaf sections served as a positive control and, as expected, induced significantly more NO than the water control at all sampling times ($p < 0.001$). At each timepoint, live *Se*N induced a stronger signal in leaves compared to water (87.67 ± 13.24, 100.6 ± 13.4 and 112.9 ± 18.8 Au, respectively; all $p < 0.01$; **Figure 2A**). Heat-killed *Se*N also induced NO at 3 hpi ($p < 0.01$). We did not observe any evidence of live *Se*N suppression of NO generation.

Fruit tissue fluorescence for all treatments decreased over time by an average of 100.1 Au, with the smallest average decrease observed in live *Se*N-treated fruit tissue and the largest change in *Psm* treated leaf sections (**Figure 2B**). *Psm* produced significantly more NO than the water control at 0.1 hpi ($p < 0.001$) but not at 3 hpi. No significant exocarp production of NO was detected in *Se*N-treated fruit exocarp compared to the water control, either at 0.1 or 3 hpi.

Overall, fruit exocarp tissue produced a stronger NO signal than leaf tissue ($p < 0.05$) regardless of treatment. If NO was produced in relation to SeN, it was not strong enough to be

TABLE 2 | Chemicals and application methodology for modulation of tomato leaf and fruit NO, ROS levels.

Pretreatment purpose	Tissue	Chemical	Source	Application method
H_2O_2 scavenger	Fruit	0.25 mM ascorbic acid	Sigma, St. Louis, MO, United States	Calyx syringe injection
	Leaves	2.5 mM ascorbic acid	Sigma, St. Louis, MO, United States	Abaxial syringe infiltration
NO scavenger	Leaves and fruit	0.22 mM 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)	Enzo Life Sciences, Farmingdale, NY, United States	Adaxial aerosol spray
NO elicitor	Leaves and fruit	0.5% Calcium Chloride ($CaCl_2$)	Sigma, St. Louis, MO, United States	Adaxial aerosol spray

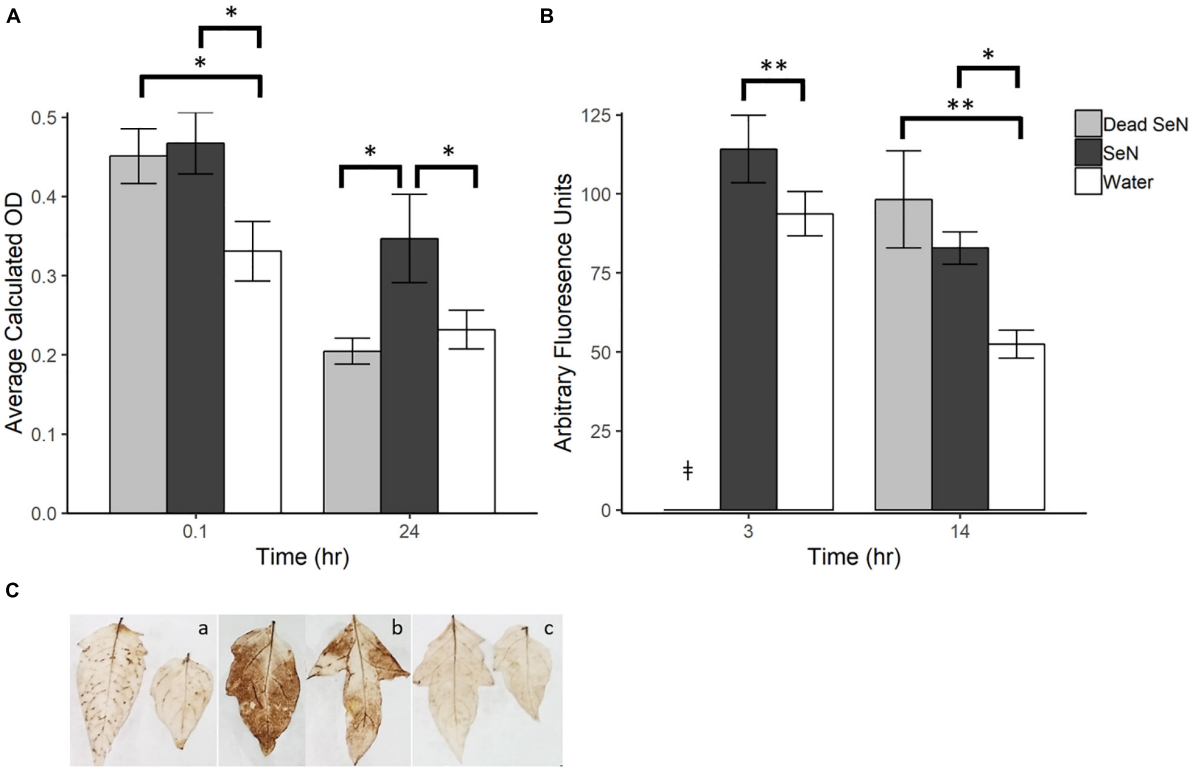


FIGURE 1 | Plant derived ROS produced following challenge with sterile water (H_2O), heat killed *S.* Newport (dead *Se*N) or live *S.* Newport. **(A)** Optical density showing degree of DAB stain in tomato leaves following *Se*N challenge via syringe infiltration. **(B)** CM-H_2DCFDA fluorescence from tomato fruit peel challenged with *Se*N. **(C)** DAB staining of leaflets 24 h post-inoculation with (a) *Pseudomonas syringae* (*Psm*), (b) *Se*N, and (c) water. Error bars represent standard error of the mean and ** $p < 0.01$; * $p \leq 0.05$. ‡denotes treatment was not included in that timepoint.

detected with the method employed over the background NO being generated. Further, exocarp measurements exhibited a larger variation with a coefficient of variation (CV) of 0.52 compared to 0.36 for leaf sections. Taken together, these data suggest that live *Se*N can induce NO generation in tomato leaves, but not fruit.

Expression of ROS and NO Detoxification Genes Was Detected in *Se*N Colonizing Leaf Surfaces

To investigate specific bacterial responses to the observed elicited H_2O_2 and NO on tomato leaves, a targeted gene expression analysis was conducted on *Se*N cells inoculated onto native leaves, or leaves pre-treated to enhance or limit NO. We assayed genes responsible for NO detoxification, ROS mitigation and other environmental fitness factors (**Table 1**) that were previously found to be expressed in *S. enterica* Typhimurium colonizing tomato shoots and roots (Han et al., unpublished). On leaves, 78% of *Se*N samples displayed $\geq|2|$ -fold change in gene expression compared to the inoculum. The flavohemoglobin *hmpA*, a main detoxifier of NO in oxygenated environments (Crawford and Goldberg, 1998) and *yoaG*, a cytoplasmic protein in the NsrR regulon (Lin et al., 2007), were shown to be differentially up-regulated in NO-excess and native leaves compared to the inoculum and to NO-limiting treatments ($p < 0.05$) (**Figure 3**).

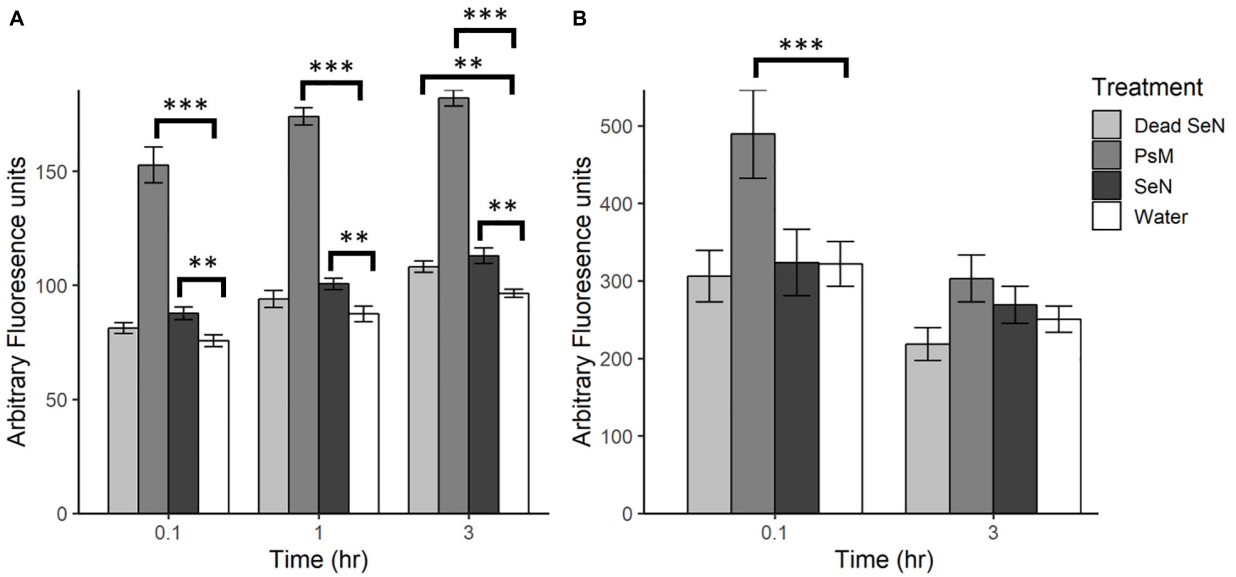


FIGURE 2 | Plant derived NO produced following challenge with heat killed *S.* Newport (dead *Se*N), *Pseudomonas syringae* (*Psm*) or *S.* Newport (*Se*N) on **(A)** tomato leaves and **(B)** tomato fruit, measured with DAF-2 DA. Error bars represent standard error of the mean and $^{**}p < 0.01$; $^{***}p \leq 0.001$.

Compared to the inoculum, expression of *hmpA* increased threefold ($p < 0.05$) in *Se*N associating with native leaves and NO-excess leaves. Additionally, the *yoaG* gene showed an almost threefold increase on native leaves and a fourfold increase on NO-excess leaves ($p < 0.05$) compared to inoculum expression levels. The similar increases in gene expression in both NO-excess and native leaves, relative to the inoculum, suggest that *Se*N itself is serving as a strong NO elicitor on leaves. Finally, gene expression of both *hmpA* and *yoaG* in NO-limiting environments was comparable to expression levels in the inoculum. Taken together, these findings suggest that *Se*N may be countering NO and/or NO-regulated plant responses.

The gene *ahpC* encodes an enzyme alkyl hydroperoxide reductase (Ahp) that protects cells from oxidative stress by catalyzing the reduction of hydrogen peroxide and organic peroxides (Seaver and Imlay, 2001). This gene was up-regulated in all treatments compared to inoculum ($p < 0.05$), but was not found to be differentially expressed among treatments. Expression of the catalase gene *katG* (Morgan et al., 1986) was not different from the inoculum, but differed between *Se*N on native versus cPTIO-treated leaves ($p = 0.053$). Overall, *Se*N appeared to be responding to oxidative stress on leaves.

The virulence factors *phoQ* two component system (Monsieurs et al., 2005) and the quorum sensing gene *sdiA* (Ahmer et al., 1998) both exhibited an increase in expression on native and NO-excess environments. The transcription of *sdiA* on $CaCl_2$-treated leaves and of *phoQ* on cPTIO-treated leaves was higher and lower, respectively, than transcription in the inoculum but weakly statistically supported ($p = 0.06$). Compared to expression on NO-limiting environments, *sdiA* expression increased fourfold on NO-excess leaves and *phoQ* expression increasing threefold on native leaves (**Figure 3**). Transcription levels of *sdiA* on NO-excess and native environments were significantly different ($p = 0.05$). The multiple antibiotic resistance transcriptional regulator *marA* (Lee et al., 2015) was also significantly up-regulated in NO-excess and native plant environments compared to expression in the inoculum. This gene expression pattern suggests these genes may be important for leaf colonization. The outer membrane porin *nmpC* involved in H_2O_2 and other small molecule diffusion across cell membranes (Calderón et al., 2011) and *trpE,* a component of tryptophan biosynthesis, displayed uniform significant up-regulation in all treatments compared to inoculum ($p < 0.05$), indicating they may not be directly affected by plant derived NO stress.

Expression of NO and ROS Detoxification Genes Was Detected in *Se*N Colonizing Fruit Surface

Gene expression was also investigated in *Se*N associating with the surface of tomato fruit. Ascorbic acid was employed as an ROS scavenger (ROS-limiting environment) before challenging fruit with *Se*N and comparing expression profiles to *Se*N on water treated tomato fruit (native environment). In total, 72% of *Se*N on fruit samples displayed a $\geq|2|$ -fold change in gene expression compared to the inoculum (**Figure 4**). *Se*N on native fruit exhibited significant up-regulation of *hmpA* and *yoaG* compared to the inoculum ($p < 0.05$). These genes were also up-regulated in the native fruit environment compared to the ROS-limiting environment ($p < 0.05$). Transcription of the oxidative stress gene *ahpC* was several log fold-change higher on native versus ascorbic-acid treated fruit ($p < 0.05$). The findings suggest *Se*N on fruit was countering plant derived NO stress and provides evidence that NO induction by *Se*N may be affected by ROS activity.

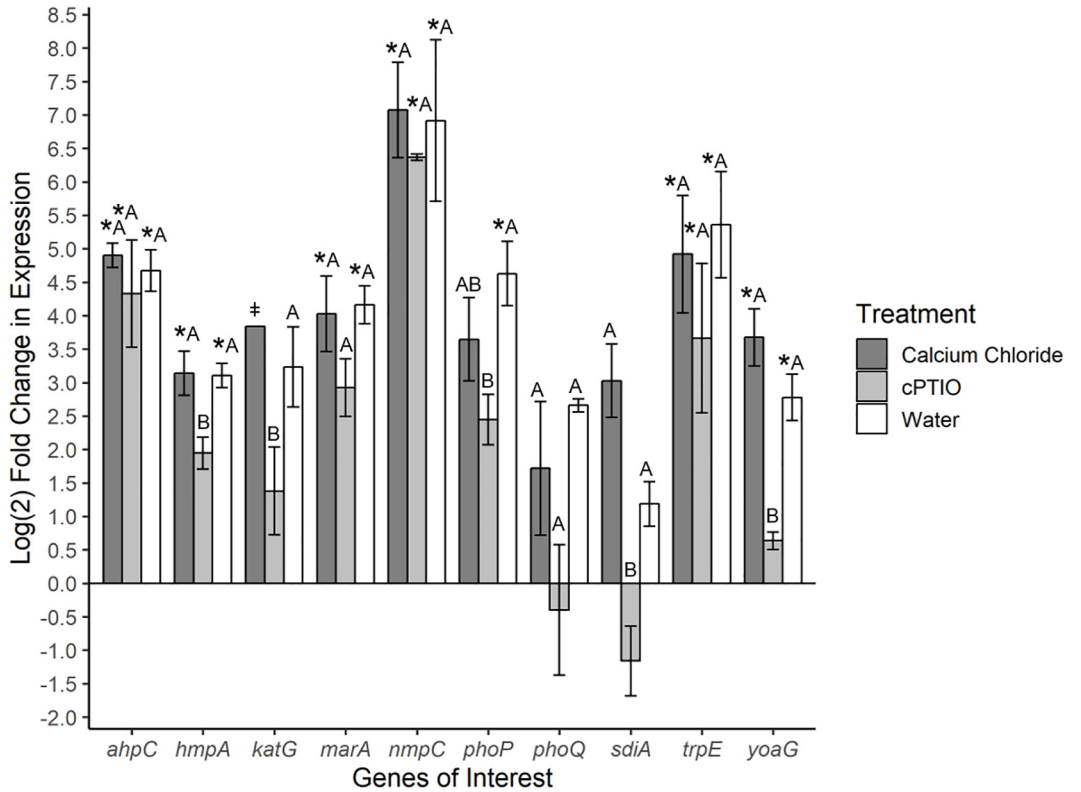


FIGURE 3 | Log_2-fold change in expression of *S.* Newport genes compared to expression in inoculum. $\Delta\Delta$Ct results of *S.* Newport colonizing 3-leaf tomato seedlings pretreated to reflect the native environment (H_2O), NO-limiting (cPTIO) or NO-excess ($CaCl_2$) environments normalized against expression of the sigma factor *rpoD. N* = 4 groups of three pooled plants per treatment. Error bars represent the standard error of the mean (SEM). ‡ denotes insufficient data to generate (SEM). Asterisks denote significance in gene transcription compared to inoculum according to Dunnett's test ($\alpha = 0.05$). Letters denote significant differences in gene transcription among treatments for each target gene using Tukey's Honestly Significant Difference ($\alpha = 0.05$).

As seen in *Se*N on leaves, transcription levels of *marA* were higher in *Se*N on native and ROS-scavenged fruit ($p < 0.05$) compared to inoculum and appeared unaffected by modulation of ROS. Down-regulation of *nmpC* was detected in *Se*N on both fruit treatments ($p < 0.05$), compared to inoculum.

Modulating Tomato Surface NO Levels Significantly Affected *Se*N Colonization of Leaves, but Not Fruit

To evaluate whether *Se*N colonization of tomato surfaces was significantly affected by modulated levels of plant-derived H_2O_2 and NO, a series of 12 h on-plant *Se*N challenge assays were conducted on leaves and fruit. *Se*N counts recovered from leaves pre-elicited to produce endogenous NO were almost 2 log lower at 12 hpi ($p < 0.001$), measured at 3.32 ± 0.2 log CFU/leaflet, compared to 5.15 ± 0.3 log CFU/leaflet recovered from mock-treated leaves (**Figure 5A**). Conversely, *Se*N counts recovered from NO-scavenged leaves were higher (4.85 ± 0.5 log CFU/leaflet) compared to sterile water treated leaves ($p < 0.05$; **Figure 5B**). This effect was not observed on fruit. Regardless of pre-treatment, *Se*N was retrieved at higher titers with smaller coefficients of variation (CV), on leaf compared to fruit samples, in both NO scavenged tissue ($CV_{fruit} = 0.39$ and $CV_{leaves} = 0.10$) and NO elicited tissue ($CV_{fruit} = 0.42$ and $CV_{leaves} = 0.24$).

Scavenging H_2O_2 on Tomato Leaves and Fruit Favors *Se*N

When ROS was scavenged from plant leaf and fruit samples with ascorbic acid, significantly higher *Se*N counts were retrieved from scavenged leaves (4.56 ± 0.10 log CFU/leaflet) and fruit surfaces (3.27 ± 0.47 log CFU/fruit) compared to control ($p < 0.05$; **Figure 5C**). On fruit, regardless of treatment, *Se*N retrieval was ∼0.8 log CFU/unit lower than on leaves, with a larger coefficient of variation (CV) in retrieval from fruit compared to leaves ($CV_{fruit} = 0.20$ and $CV_{leaves} = 0.07$).

DISCUSSION

In the present study, biochemical, gene expression and on-plant colonization assays of both vegetative tissue and ripe mature fruit provide evidence to support tomato plant recognition and response to *Salmonella* Newport. Tomato leaves and fruit generated ROS and leaves also generated NO in response to the enteric pathogen. In turn, *Salmonella* interpreted the mounted

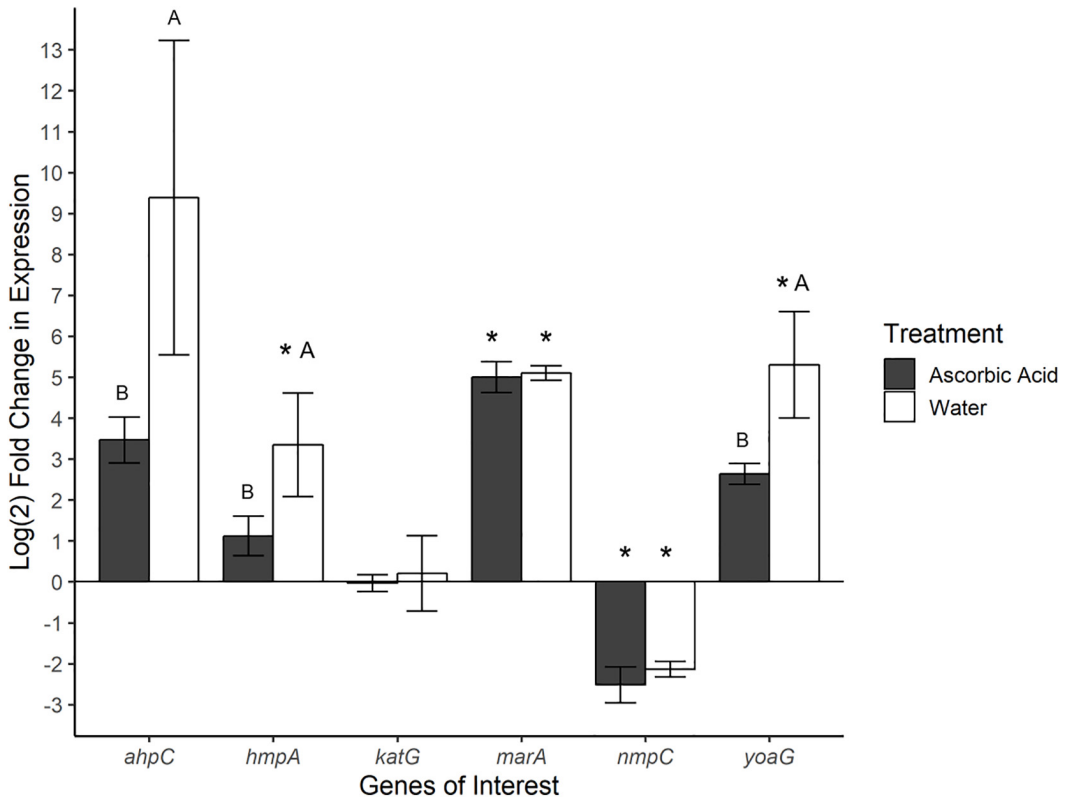


FIGURE 4 | Log_2-fold change in gene expression of *S.* Newport genes compared to expression in inoculum. ΔΔCt results of *S.* Newport colonizing tomato fruit pretreated to reflect the native fruit environment (H_2O) or ROS-limiting (AscA) environments normalized against expression of sigma factor *rpoD.* Error bars represent the standard error of the mean. Asterisks denote significance in gene transcription compared to inoculum via Dunnett's test (α = 0.05). Letters denote significant differences in expression between treatments for each target gene (Student's *t*-test, α = 0.05).

plant response as a stress, evidenced by differential ROS and NO detoxification gene up-regulation in *Salmonella* colonizing plant surfaces. *Salmonella*, therefore, may need to respond to plant-derived stimuli to ensure successful epiphytic colonization. Importantly, this study shows that tomato plants possess mechanisms capable of restricting *Salmonella* populations on leaf and fruit surfaces. ROS was detected in both leaf and fruit samples, and the reciprocal bacterial response was consistent with ROS negatively impacting *Salmonella* colonization, restricting bacterial counts. NO induction and an adverse effect of NO on *Salmonella* colonization was also detected on leaves, but not fruit. The ROS and NO bursts induced *Salmonella* to express genes needed for ROS and NO detoxification on leaves and NO detoxification on fruit. Despite no detected NO in fruit tissue subsequent to *Salmonella* challenge, and no effect of NO on bacterial restriction on fruit, bacterial gene expression results suggested *Salmonella* perception of NO on fruit. This work provides evidence that plant-derived NO is generated in response to *S. enterica* recognition.

Nitric oxide is required for PAMP-induced stomatal closure (Melotto et al., 2006). The production of NO in tomato leaves in response to *Salmonella* could therefore be signaling this innate immune response. This may also explain why NO was detected on leaves but not fruit, as tomato fruit lack stomata (Rančić et al., 2010). Low levels of NO, however, may have been present even on fruit, as transcription of NsR regulon genes was detected in *Salmonella* on both leaves and fruit. Up-regulation of NO detoxification gene *hmpA* during tomato surface colonization indicated that *S.* Newport perceived plant-derived NO as a stressor, corroborated by lower bacterial counts of *S.* Newport on NO-elicited seedling leaves. The NsrR regulon, controlled by the nitric oxide sensing transcriptional repressor NsrR, plays an important role in nitrosative stress resistance during infection. Within this regulon, the flavohemoglobin HmpA is identified as the main protein responsible for NO detoxification activities in the presence of an oxygenated environment (Karlinsey et al., 2012). A transcriptomic study of *S.* Typhimurium on tomato leaves and roots identified multiple up-regulated NsrR regulon genes, *ygbA*, *ytfE*, *yoaG* STM1808 and *yfhH* (Han et al., unpublished), which in *E. coli* is known to offer an NsrR binding site (Partridge et al., 2009). Supporting this, we assayed two genes in the NsrR regulon, *hmpA* and *yoaG*, both of which were differentially up-regulated compared to all native fruit and leaf environments. Han et al. (unpublished) measured gene expression in cells that had been colonizing plants for multiple days and did not detect *hmpA*. In our study, gene expression was assayed following 6 h of plant association, to capture *Se*N responses to early MTI. In fact, *hmpA* expression

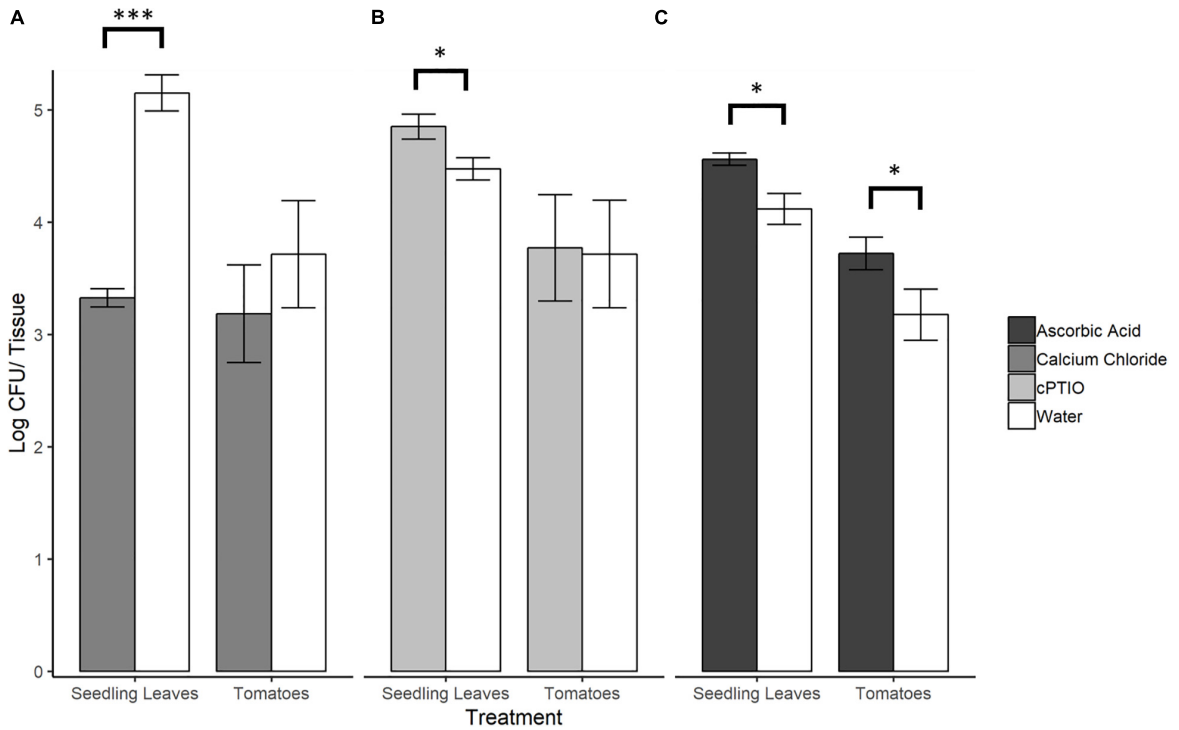


FIGURE 5 | *S.* Newport counts retrieved from tomato seedling leaves and fruit after pre-treatment of plant tissue with **(A)** NO elicitor, **(B)** NO scavenger, or **(C)** H_2O_2 scavenger. Error bars represent standard error of the mean. Asterisks denote statistically significant difference by Student's *t*-test between treatments and water control ($\alpha = 0.05$) with $^{***}p < 0.001$ and $^{*}p \leq 0.05$.

implies an early release of NO by the plant, and the need for *S.* Newport to mitigate its immediate effects. The NO detoxification-associated genes *hmpA, nfrA* and *ygbA* were also found to be up-regulated in soft rot macerated cilantro and lettuce leaf tissue caused by the plant pathogen *Dickeya dadantii* (Goudeau et al., 2013). In the present study, *Salmonella* NO detoxification was reported in the absence of a plant pathogen or tissue injury. Tomato plants produced NO upon perception of *Salmonella* which, in turn, led the bacteria to switch on reactive nitrogen species (RNS) detoxification machinery. This action may be necessary in order for *Salmonella* to successfully persist on some plant surfaces.

Evidence of ROS elicitation in tomato colonized with *S.* Newport was also strong, consistent with other reports of MTI induction in plants associating with this enteropathogen (Schikora et al., 2008; Shirron and Yaron, 2011; Meng et al., 2013; Garcia et al., 2014). The response we detected on leaves was diffuse across the leaf tissue and similar to staining reported for avirulent pathogens, as opposed to virulent pathogens (Großkinsky et al., 2012). Although *Salmonella* is not known to enter the apoplastic space (Potnis et al., 2014), the assay we used required infiltration, such that the response may have been more pronounced than what occurs when *Salmonella* is associating with the leaf epiphytically or residing in sub-stomatal chambers. In any case, ROS had a restrictive effect on *Salmonella* populations on the surface of both leaves and fruit and evidence of bacterial detoxification of ROS stress while colonizing leaf surfaces was detected via up-regulation of *S. enterica ahpC*. By contrast, we did not detect expression of the catalase *katG*. AhpC and the catalase encoded by *katG* are both known to scavenge H_2O_2 and organic hydroperoxides, but we only detected consistent expression of *ahpC* in *Salmonella* associated with leaves. In macrophages, *ahpC* expression is stronger than *katG* expression (Hébrard et al., 2009). Ahp was reported to be a more efficient scavenger at low concentrations of H_2O_2 (below 20 μM) than catalase, which becomes the primary active enzyme at higher H_2O_2 concentrations (Seaver and Imlay, 2001). From our findings, therefore, we infer that levels of H_2O_2 reaching *Salmonella* on the tomato plant surface were in concentrations insufficient to induce catalase activity.

Modulation of NO on leaves did not impact *ahpC* expression, but the use of an ROS scavenger on fruit reduced transcription of *ahpC*. Transcription of the NsrR-regulated *hmpA* and *yoaG* genes in *Salmonella* on fruit was also higher when ROS was not attenuated, suggesting that higher levels of ROS may be related to NO levels. A large degree of interconnectivity exists between NO and ROS signaling in plant tissue (Romero-Puertas and Sandalio, 2016). On native and ascorbic acid-treated fruit tissue, *Salmonella* would have been responding to NO levels induced by the enteropathogen itself. However, NO has been shown to regulate ascorbate peroxidase (APX) (Yang et al., 2015), the enzyme that uses ascorbate as an electron donor to reduce H_2O_2 to H_2O. On ascorbic acid-treated fruit, therefore, the depleted H_2O_2 environment could have signaled the attenuation of NO,

such that *Salmonella* would have been responding to lower NO levels reflected in reduced *hmpA* and *yoaG* transcription.

Other than NO and ROS stress, *Se*N gene expression on leaves and fruit was indicative of adaptation to a novel environment. In leaves, *trpE*, a gene in the tryptophan biosynthesis pathway which has been associated with biofilm development (Hamilton et al., 2009), was up-regulated in all environments. Biofilm formation is known to enhance the capacity of pathogenic bacteria to survive stresses in the environment and during host infection. Thus, the present work provides more evidence to the growing body of work which defines attachment as paramount to survival in the phyllosphere (Barak et al., 2005, 2007). Also up-regulated in all leaf and fruit environments was the gene *marA*. In addition to their importance as regulators of xenobiotic efflux, *marRAB* may have indirect effects on expression of iron metabolism genes, membrane composition genes, and the stress related sigma factor *rpoS* (Lee et al., 2015). In *S. enterica* transcriptomic surveys (Wang et al., 2010; Brankatschk et al., 2014), the use of such machinery was found to be pertinent to survival in stress-inducing environments. In our study, *nmpC* (*ompD*, STM1572) displayed differential expression among plant tissue types, up-regulated compared to inoculum in *Salmonella* on leaf surfaces but down-regulated on fruit surfaces. NmpC is one of the most abundant outer membrane porins of *S. enterica*, used to transport molecules, including H_2O_2, into the cell and toxins out of the cell (Calderón et al., 2011). Expression of *nmpC* was down-regulated in *S.* Typhimurium exposed to H_2O_2 (Calderón et al., 2011). Additionally, NmpC may be needed for adherence and recognition of *S.* Typhimurium to human macrophages and epithelial cells during the initial stages of infection (Hara-Kaonga and Pistole, 2004) and is also involved in host cell recognition in mammalian models (Ipinza et al., 2014; van der Heijden et al., 2016). In our study, we observed up-regulation of both *nmpC* and ROS detoxification gene *ahpC* on leaves. However, *nmpC* was down-regulated in *Se*N on native fruit where *ahpC* expression was more variable. These findings suggest an additional function of this porin, perhaps engaging in efflux activity of other xenobiotics. Taken together, targeted gene expression on both leaves and fruit provide evidence that *S. enterica* attaches to and recognizes various stressors on plant surfaces.

Working with NO modulation on fruit proved challenging. While up-regulation of bacterial NO detoxification genes was detected on fruit, we could not easily measure NO released from fruit tissue challenge with various biotic treatments. *Salmonella* produced a fluorescent response in fruit exocarp greater than that of leaves but was not consistent. It is possible that exocarp excision generated high levels of NO, even in the negative control, and masking the weaker signal elicited by the pathogen. This could also explain why DAF-2 DA fluorescence diminished over time, even in the positive control. Further, ascorbic acid significantly affected *S. enterica* counts on fruit whereas NO modulation did not. This could be attributed to NO and ROS endogenous levels in mature red fruit at the time of study. Ripe red fruit are known to have lower concentrations of nitric oxide compared to mature green fruit, as NO is involved in regulating ethylene production and thus facilitating the ripening process (Ya'acov et al., 1998). Most NO modulation studies in fruit responding to plant pathogens are routinely conducted with mature green fruit (Lai et al., 2011; Zheng et al., 2011a,b; Zhu and Tian, 2012). The transition from green to red fruit is marked by accruement of high ROS concentrations (Kumar et al., 2016). Petrasch et al. (2019) investigated ROS detoxification by fungal pathogens in red ripe fruit. Thus, the ascorbic acid injected in our plant colonization assays could have been targeting ripening-related ROS, hence providing a more hospitable environment for colonizing *S. enterica*. Mature ripe fruit tissue also has lower levels of pathogen recognition response capacity compared to vegetative tissue, perhaps due in part to the breakdown of cellular wall components during ripening (Cantu et al., 2009). *S. enterica* studies on ripe and unripe tomato fruit have found the organism proliferates more readily in ripe red compared to mature green tomato fruit (Barak et al., 2011). Taken together, these data imply mechanisms of *S. enterica* restriction are plant tissue-specific and may be facilitated or confounded by the fruit ripening process. Regardless, more research is needed to evaluate the interconnectivity between ripening and pathogen defense, both in the contexts of plant and human pathogens.

Overall, higher titers of *S.* Newport were consistently retrieved from leaves compared to fruit, an observation which has been reported elsewhere (Barak et al., 2011; Han and Micallef, 2014; Gu et al., 2018). Supporting evidence can be found in *Salmonella* field sampling studies. For example in a multi-year field study, sampling tomato leaves and fruit for wild *S. enterica* colonization found only leaves returned positive *S. enterica* result, never fruit (Gu et al., 2018). This tissue-specific variability in *Salmonella* carrying capacity could be due to relative abundance and composition of nutrients on the surface of the different plant organs, higher relative humidity on leaves as a result of transpiration, and a higher and more rugged surface area or attachment on leaves compared to fruit. Higher proportions of fatty acids have been seen in fruit washes of tomato cv. 'Heinz-1706' compared to seedling shoot or mature leaf washes, and this correlated negatively with *S. enterica* growth (Han and Micallef, 2016). While the leaves of the tomato are not eaten, in the field leaves and tomato fruit are in constant contact with one another, serving as a contamination source for fruit. Vegetative matter is commonly picked up during harvest of tomato fruit and could lead to widespread contamination if appropriate Good Agricultural Practices are not followed. Cross-contamination of fruit from contaminated vegetative matter is a potential risk when using recirculating water to wash tomatoes without the appropriate concentration of sanitizer (Bolten et al., 2020).

We noted that *Se*N retrieval from leaves was less variable than fruit, suggesting a potential heterogeneity of response to unique stressors present on the latter plant organ. Diversification of stress response or "bet hedging" has been documented in intercellular *S. enterica* interaction with ROS and other stress agents (Helaine and Holden, 2013; Burton et al., 2014; Helaine et al., 2014). Tomato fruit may be a uniquely harsh plant niche for *S. enterica* for which "bet hedging" may be a significant strategy to ensure long term survival. Understanding tissue specific enteropathogen–plant interactions therefore can help devise

strategies to minimize fruit contamination. Yet, enteropathogen–plant interaction data relevant to agricultural situations, which relate directly to salmonellosis outbreak-causing *S. enterica* strains, remain limited.

Salmonella enterica mitigation of host-derived NO and ROS is crucial for successful invasion in animal host models. These processes have been well documented (Vazquez-Torres and Fang, 2001; Zheng et al., 2011a; Spector and Kenyon, 2012; van der Heijden et al., 2015). Presence of RNS in the mammalian gut increased overall colonization fitness of *S. enterica*, possibly because it can outcompete some resident microflora (Stecher et al., 2007). This compounds the importance of investigating the presence of analogous interactions in *S. enterica*–plant associations. Perception and response to NO and ROS, which can be short-term restricting agents, together with other important stress and host adaptation responses, may lead to long-term persistence in the field. As ROS and RNS may be present in multiple scenarios in the agricultural setting (Diaz and Plummer, 2018), mitigating these compounds may be a key factor for *S. enterica* persistence in between entry into an animal host. Future work should investigate the ability of *Salmonella* to mitigate these stresses on plants and whether they are shared by all serovars, or specific to serovars that are regularly implicated in produce outbreaks. This is essential to continue to elucidate *Salmonella* adaptation to non-animal host environments.

Deciphering the highly nuanced and complex plant-associated lifestyle of this enteric pathogen is imperative to inform strategies to minimize successful *Salmonella* contamination and persistence in an agricultural setting and help in identifying plant traits or cultivars that are unfavorable for *Salmonella* colonization. Furthermore, how non-plant pathogenic microorganisms, including enteric pathogens, interact with the plant immune system to colonize the phyllosphere is poorly understood. The MAMPs recognized by plants that result in MTI are highly conserved among microbes. It has recently been suggested that plants cannot differentiate between pathogens and commensals, and micro-organisms must evade or attenuate plant immunity to colonize plants (Teixeira et al., 2019). Untangling the role that plant immunity plays in the establishment of human pathogens on plants would add to knowledge about microbiome assembly, while also elucidating mechanisms that can lead to enhanced food safety.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SM and AF conceptualized the study and designed the experiments. AF conducted the experiments. AF, SB, and BS designed and conducted q-RT-PCR experiments. AF and SM analyzed and interpreted the data and wrote the manuscript. SB and BS reviewed and edited the manuscript. SM administered the project.

ACKNOWLEDGMENTS

We thank Dr. Shunyuan Xiao for generously providing *Pseudomonas syringae* pv. *maculicola* ES4326. This project was completed using UMD start-up funds to SM. We also thank Megan Holbert and Sydney Wallace at the University of Maryland research greenhouse and Michael Newell at the Central Maryland Wye Research and Education Centre, Queenstown Maryland for assistance in tomato cultivation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00391/full#supplementary-material

REFERENCES

Ahmer, B. M. M., Van Reeuwijk, J., Timmers, C. D., Valentine, P. J., and Heffron, F. (1998). *Salmonella* Typhimurium encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *J. Bacteriol.* 180, 1185–1193. doi: 10.1128/jb.180.5.1185-1193.1998

Barak, J. D., Gorski, L., Naraghi-Arani, P., and Charkowski, A. O. (2005). *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. *Appl. Environ. Microbiol.* 71, 5685–5691. doi: 10.1128/aem.71.10.5685-5691.2005

Barak, J. D., Jahn, C. E., Gibson, D. L., and Charkowski, A. O. (2007). The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. *Mol. Plant Microbe Interact.* 20, 1083–1091. doi: 10.1094/mpmi-20-9-1083

Barak, J. D., Kramer, L. C., and Hao, L. (2011). Colonization of tomato plants by *Salmonella enterica* Is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/AEM.01661-10

Bell, R. L., Zheng, J., Burrows, E., Allard, S., Wang, C. Y., Keys, C. E., et al. (2015). Ecological prevalence, genetic diversity, and epidemiological aspects of *Salmonella* isolated from tomato agricultural regions of the Virginia Eastern Shore. *Front. Microbiol.* 6:415. doi: 10.3389/fmicb.2015.00415

Bennett, S. D., Littrell, K. W., Hill, T. A., Mahovic, M., and Behravesh, C. B. (2015). Multistate foodborne disease outbreaks associated with raw tomatoes, United States, 1990–2010: a recurring public health problem. *Epidemiol. Infect.* 143, 1352–1359. doi: 10.1017/s0950268814002167

Bindschedler, L. V., Dewdney, J., Blee, K. A., Stone, J. M., Asai, T., Plotnikov, J., et al. (2006). Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. *Plant J.* 47, 851–863. doi: 10.1111/j.1365-313X.2006.02837.x

Bolten, S., Gu, G., Luo, Y., Van Haute, S., Zhou, B., Millner, P., et al. (2020). *Salmonella* inactivation and cross-contamination on cherry and grape tomatoes under simulated wash conditions. *Food Microbiol.* 87:103359. doi: 10.1016/j.fm.2019.103359

Bradley, D. J., Kjellbom, P., and Lamb, C. J. (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70, 21–30. doi: 10.1016/0092-8674(92)90530-P

Brandl, M. T., and Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157: H7 and *Salmonella enterica*. *Appl. Environ. Microbiol.* 74, 2298–2306. doi: 10.1128/aem.02459-07

Brandl, M. T., Cox, C. E., and Teplitski, M. (2013). *Salmonella* interactions with plants and their associated microbiota. *Phytopathology* 103, 316–325. doi: 10.1094/phyto-11-12-0295-rvw

Brandl, M. T., and Mandrell, R. E. (2002). Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl. Environ. Microbiol.* 68, 3614–3621. doi: 10.1128/aem.68.7.3614-3621.2002

Brankatschk, K., Kamber, T., Pothier, J. F., Duffy, B., and Smits, T. H. M. (2014). Transcriptional profile of *Salmonella enterica* subsp enterica serovar Weltevreden during alfalfa sprout colonization. *Microb. Biotechnol.* 7, 528–544. doi: 10.1111/1751-7915.12104

Burton, N. A., Schürmann, N., Casse, O., Steeb, A. K., Claudi, B., Zankl, J., et al. (2014). Disparate impact of oxidative host defenses determines the fate of *Salmonella* during systemic infection in mice. *Cell Host Microbe* 15, 72–83. doi: 10.1016/j.chom.2013.12.006

Calderón, I. L., Morales, E., Caro, N. J., Chahúan, C. A., Collao, B., Gil, F., et al. (2011). Response regulator ArcA of *Salmonella enterica* serovar Typhimurium downregulates expression of OmpD, a porin facilitating uptake of hydrogen peroxide. *Res. Microbiol.* 162, 214–222. doi: 10.1016/j.resmic.2010.11.001

Callahan, M. T., Van Kessel, J. A., and Micallef, S. A. (2019). *Salmonella enterica* recovery from river waters of the Maryland Eastern Shore reveals high serotype diversity and some multidrug resistance. *Environ. Res.* 168, 7–13. doi: 10.1016/j.envres.2018.09.012

Callejón, R. M., Rodríguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C., and 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. doi: 10.1089/fpd.2014.1821

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B., and Powell, A. L. T. (2009). Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires NOR but Not RIN or ethylene. *Plant Physiol.* 150, 1434–1449. doi: 10.1104/pp.109.138701

Chakraborty, N., Ghosh, S., Chandra, S., Sengupta, S., and Acharya, K. (2016). Abiotic elicitors mediated elicitation of innate immunity in tomato: an *ex vivo* comparison. *Physiol. Mol. Biol. Plants* 22, 307–320. doi: 10.1007/s12298-016-0373-z

Crawford, M. J., and Goldberg, D. E. (1998). Regulation of the *Salmonella* Typhimurium flavohemoglobin gene - A new pathway for bacterial gene expression in response to nitric oxide. *J. Biol. Chem.* 273, 34028–34032. doi: 10.1074/jbc.273.51.34028

de Moraes, M. H., Soto, E. B., Salas González, I., Desai, P., Chu, W., Porwollik, S., et al. (2018). Genome-wide comparative functional analyses reveal adaptations of Salmonella sv. Newport to a Plant Colonization Lifestyle. *Front. Microbiol.* 9:877. doi: 10.3389/fmicb.2018.00877

de Moraes, M. H., Desai, P., Porwollik, S., Canals, R., Perez, D. R., Chu, W., et al. (2017). *Salmonella* persistence in tomatoes requires a distinct set of metabolic functions identified by transposon insertion sequencing. *Appl. Environ. Microbiol.* 83:0e3028-e16. doi: 10.1128/AEM.03028-16

Diaz, J. M., and Plummer, S. (2018). Production of extracellular reactive oxygen species by phytoplankton: past and future directions. *J. Plankton Res.* 40, 655–666. doi: 10.1093/plankt/fby039

Doke, N. (1983). Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiol. Plant Pathol.* 23, 345–357. doi: 10.1016/0048-4059(83)90019-X

Garcia, A. V., Charrier, A., Schikora, A., Bigeard, J., Pateyron, S., de Tauzia-Moreau, M.-L., et al. (2014). Salmonella enterica flagellin is recognized via FLS2 and activates PAMP-Triggered Immunity in *Arabidopsis thaliana*. *Mol. Plant* 7, 657–674. doi: 10.1093/mp/sst145

Goudeau, D. M., Parker, C. T., Zhou, Y. G., Sela, S., Kroupitski, Y., and Brandl, M. T. (2013). The *Salmonella* transcriptome in lettuce and cilantro soft rot reveals a niche overlap with the animal host intestine. *Appl. Environ. Microbiol.* 79, 250–262. doi: 10.1128/aem.02290-12

Greene, S. K., Daly, E. R., Talbot, E. A., Demma, L. J., Holzbauer, S., Patel, N. J., et al. (2008). Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiol. Infect.* 136, 157–165. doi: 10.1017/s095026880700859x

Großkinsky, D. K., Koffler, B. E., Roitsch, T., Maier, R., and Zechmann, B. (2012). Compartment-specific antioxidative defense in *Arabidopsis* against virulent and avirulent *Pseudomonas syringae*. *Phytopathol* 102, 662–673. doi: 10.1094/PHYTO-02-12-0022-R

Gu, G., Strawn, L., Oryang, D., Zheng, J., Reed, E., Ottesen, A., et al. (2018). Agricultural practices influence *Salmonella* contamination and survival in pre-harvest tomato production. *Front. Microbiol.* 9:2451. doi: 10.3389/fmicb.2018.02451

Hamilton, S., Bongaerts, R. J. M., Mulholland, F., Cochrane, B., Porter, J., Lucchini, S., et al. (2009). The transcriptional programme of *Salmonella enterica* serovar Typhimurium reveals a key role for tryptophan metabolism in biofilms. *BMC Genomics* 10:599. doi: 10.1186/1471-2164-10-599

Han, S., and Micallef, S. A. (2014). *Salmonella* Newport and Typhimurium colonization of fruit differs from leaves in various tomato cultivars. *J. Food Prot.* 77, 1844–1850. doi: 10.4315/0362-028x.jfp-13-562

Han, S., and Micallef, S. A. (2016). Environmental Metabolomics of the Tomato Plant Surface Provides Insights on *Salmonella enterica* Colonization. *Appl. Environ. Microbiol.* 82, 3131–3142. doi: 10.1128/aem.00435-16

Hara-Kaonga, B., and Pistole, T. G. (2004). OmpD but not OmpC is involved in adherence of *Salmonella enterica* serovar Typhimurium to human cells. *Can. J. Microbiol.* 50, 719–727. doi: 10.1139/W04-056

Hébrard, M., Viala, J. P. M., Méresse, S., Barras, F., and Aussel, L. (2009). Redundant hydrogen peroxide scavengers contribute to *Salmonella* virulence and oxidative stress resistance. *J. Bacteriol.* 191, 4605–4614. doi: 10.1128/JB.00144-09

Helaine, S., Cheverton, A. M., Watson, K. G., Faure, L. M., Matthews, S. A., and Holden, D. W. (2014). Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* 343, 204–208. doi: 10.1126/science.1244705

Helaine, S., and Holden, D. W. (2013). Heterogeneity of intracellular replication of bacterial pathogens. *Curr. Opin. Microbiol.* 16, 184–191. doi: 10.1016/j.mib.2012.12.004

Iniguez, A. L., Dong, Y. M., Carter, H. D., Ahmer, B. M. M., Stone, J. M., and Triplett, E. W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant Microb. Interact.* 18, 169–178. doi: 10.1094/MPMI-18-0169

Ipinza, F., Collao, B., Monsalva, D., Bustamante, V. H., Luraschi, R., Alegría-Arcos, M., et al. (2014). Participation of the *Salmonella* OmpD Porin in the Infection of RAW264.7 *Macrophages and BALB/c Mice*. *PLoS One* 9:e111062. doi: 10.1371/journal.pone.0111062

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Karlinsey, J. E., Bang, I.-S., Becker, L. A., Frawley, E. R., Porwollik, S., Robbins, H. F., et al. (2012). The NsrR regulon in nitrosative stress resistance of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* 85, 1179–1193. doi: 10.1111/j.1365-2958.2012.08167.x

Keshavarz-Tohid, V., Taheri, P., Taghavi, S. M., and Tarighi, S. (2016). The role of nitric oxide in basal and induced resistance in relation with hydrogen peroxide and antioxidant enzymes. *J. Plant Physiol.* 199, 29–38. doi: 10.1016/j.jplph.2016.05.005

Kumar, V., Irfan, M., Ghosh, S., Chakraborty, N., Chakraborty, S., and Datta, A. (2016). Fruit ripening mutants reveal cell metabolism and redox state during ripening. *Protoplasma* 253, 581–594. doi: 10.1007/s00709-015-0836-z

Lai, T., Wang, Y., Li, B., Qin, G., and Tian, S. (2011). Defense responses of tomato fruit to exogenous nitric oxide during postharvest storage. *Postharvest Biol. Technol.* 62, 127–132. doi: 10.1016/j.postharvbio.2011.05.011

Lee, J.-J., Hsuan, S.-L., Kuo, C.-J., Wu, Y.-C., and Chen, T.-H. (2015). MarA and RamA regulate virulence in *Salmonella enterica* serovar Choleraesuis. *Vet. Microbiol.* 181, 323–327. doi: 10.1016/j.vetmic.2015.09.006

Lee, S., Choi, H., Suh, S., Doo, I.-S., Oh, K.-Y., Jeong Choi, E., et al. (1999). Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of Tomato and *Commelina communis*. *Plant Physiol.* 121, 147–152. doi: 10.1104/pp.121.1.147

Lin, H. Y., Bledsoe, P. J., and Stewart, V. (2007). Activation of yeaR-yoaG operon transcription by the nitrate-responsive regulator NarL is independent

of oxygen-responsive regulator Fnr in *Escherichia coli* K-12. *J. Bacteriol.* 189, 7539–7548. doi: 10.1128/JB.00953-07

Liu, Y., and Zhang, S. (2004). Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. 16, 3386–3399. *Plant Cell* doi: 10.1105/tpc.104.026609

Ma, W., Smigel, A., Tsai, Y. C., Braam, J., and Berkowitz, G. A. (2008). Innate immunity signaling: cytosolic Ca2+ elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. *Plant Physiol.* 48, 818–828. doi: 10.1104/pp.108.125104

Małolepsza, U., and Różalska, S. (2005). Nitric oxide and hydrogen peroxide in tomato resistance: nitric oxide modulates hydrogen peroxide level in o-hydroxyethylorutin-induced resistance to *Botrytis cinerea* in tomato. *Plant Physiol. Biochem.* 43, 623–635. doi: 10.1016/j.plaphy.2005.04.002

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant Stomata Function in Innate Immunity against Bacterial Invasion. *Cell* 126, 969–980. doi: 10.1016/j.cell.2006.06.054

Meng, F., Altier, C., and Martin, G. B. (2013). *Salmonella* colonization activates the plant immune system and benefits from association with plant pathogenic bacteria. *Environ. Microbiol.* 15, 2418–2430. doi: 10.1111/1462-2920.12113

Meng, X., and Zhang, S. (2013). MAPK cascades in plant disease resistance signaling. *Annu. Rev. Phytopathol.* 51, 245–266. doi: 10.1146/annurev-phyto-082712-102314

Micallef, S. A., Rosenberg Goldstein, R. E., George, A., Kleinfelter, L., Boyer, M. S., McLaughlin, C. R., et al. (2012). Occurrence and antibiotic resistance of multiple *Salmonella* serotypes recovered from water, sediment and soil on mid-Atlantic tomato farms. *Environ. Res.* 114, 31–39. doi: 10.1016/j.envres.2012.02.005

Monsieurs, P., De Keersmaecker, S., Navarre, W. W., Bader, M. W., De Smet, F., McClelland, M., et al. (2005). Comparison of the PhoPQ regulon in *Escherichia coli* and *Salmonella* Typhimurium. *J. Mol. Evol* 60, 462–474. doi: 10.1007/s00239-004-0212-7

Morgan, R. W., Christman, M. F., Jacobson, F. S., Storz, G., and Ames, B. N. (1986). Hydrogen peroxide-inducible proteins in *Salmonella* Typhimurium overlap with heat shock and other stress proteins. *Proc. Natl. Acad. Sci. U.S.A.* 83, 8059–8063. doi: 10.1073/pnas.83.21.8059

Neill, S. J., Desikan, R., Clarke, A., and Hancock, J. T. (2002). Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. *Plant Physiol.* 128, 13–16. doi: 10.1104/pp.010707

Neumann, C., Fraiture, M., Hernàndez-Reyes, C., Akum, F. N., Virlogeux-Payant, I., Chen, Y., et al. (2014). The *Salmonella* effector protein SpvC, a phosphothreonine lyase is functional in plant cells. *Front. Microbiol.* 5:548. doi: 10.3389/fmicb.2014.00548

Partridge, J. D., Bodenmiller, D. M., Humphrys, M. S., and Spiro, S. (2009). NsrR targets in the *Escherichia coli* genome: new insights into DNA sequence requirements for binding and a role for NsrR in the regulation of motility. *Mo. Microbiol.* 73, 680–694. doi: 10.1111/j.1365-2958.2009.06799.x

Petrasch, S., Silva, C. J., Mesquida-Pesci, S. D., Gallegos, K., van den Abeele, C., Papin, V., et al. (2019). Infection strategies deployed by *Botrytis cinerea*, *Fusarium acuminatum*, and *Rhizopus stolonifer* as a function of tomato fruit ripening stage. *Front. Plant Sci.* 10:223. doi: 10.3389/fpls.2019.00223

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Potnis, N., Soto-Arias, J. P., Cowles, K. N., van Bruggen, A. H. C., Jones, J. B., and Barak, J. D. (2014). *Xanthomonas perforans* colonization influences *Salmonella enterica* in the tomato phyllosphere. *Appl. Environ. Microbiol.* 80, 3173–3180. doi: 10.1128/AEM.00345-14

Poza-Carrion, C., Suslow, T., and Lindow, S. (2013). Resident Bacteria on Leaves Enhance Survival of Immigrant Cells of *Salmonella enterica*. *Phytopathol* 103, 341–351. doi: 10.1094/PHYTO-09-12-0221-FI

Preston, G. M. (2000). Pseudomonas syringae pv. tomato: the right pathogen, of the right plant, at the right time. *Mol. Plant Pathol.* 1, 263–275. doi: 10.1046/j.1364-3703.2000.00036.x

Rančić, D., Quarrie, S. P., and Pećinar, I. (2010). "Anatomy of tomato fruit and fruit pedicel during fruit development," in *Microscopy: Science, Technology, Applications and Education*, eds A. Méndez-Vilas, and J. Díaz, (Badajoz: Formatex), 851–861.

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J., and Scheel, D. (2011). Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J.* 68, 100–113. doi: 10.1111/j.1365-313X.2011.04671.x

Rasul, S., Dubreuil-Maurizi, C., Lamotte, O., Koen, E., Poinssot, B., Alcaraz, G., et al. (2012). Nitric oxide production mediates oligogalacturonide-triggered immunity and resistance to Botrytis cinerea in Arabidopsis thaliana. *Plant Cell Environ.* 35, 1483–1499. doi: 10.1111/j.1365-3040.2012.02505.x

Romero-Puertas, M. C., and Sandalio, L. M. (2016). Nitric oxide level is self-regulating and also regulates its ROS partners. *Front. Plant Sci.* 7:316. doi: 10.3389/fpls.2016.00316

Salazar, J. K., Deng, K. P., Tortorello, M. L., Brandl, M. T., Wang, H., and Zhang, W. (2013). Genes ycfR, sirA and yigG contribute to the surface attachment of *Salmonella enterica* Typhimurium and Saintpaul to Fresh Produce. *PLoS One* 8:e57272. doi: 10.1371/journal.pone.0057272

Schikora, A., Carreri, A., Charpentier, E., and Hirt, H. (2008). The dark side of the salad: *Salmonella typhimurium* overcomes the innate immune response of *Arabidopsis thaliana* and shows an endopathogenic lifestyle. *PLoS One*. 3:e2279. doi: 10.1371/journal.pone.0002279

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji - an Open Source platform for biological image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Seaver, L. C., and Imlay, J. A. (2001). Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* 183, 7173–7181. doi: 10.1128/JB.183.24.7173-7181.2001

Shin, R., and Schachtman, D. P. (2004). Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8827–8832. doi: 10.1073/pnas.0401707101

Shirron, N., and Yaron, S. (2011). Active suppression of early immune response in tobacco by the human pathogen *Salmonella* Typhimurium. *PLoS One* 6:e18855. doi: 10.1371/journal.pone.0018855

Spector, M. P., and Kenyon, W. J. (2012). Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Res. Int.* 45, 455–481. doi: 10.1016/j.foodres.2011.06.056

Stecher, B., Robbiani, R., Walker, A. W., Westendorf, A. M., Barthel, M., Kremer, M., et al. (2007). *Salmonella enterica* serovar Typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol.* 5:e244. doi: 10.1371/journal.pbio.0050244

Tan, M. S. F., White, A. P., Rahman, S., and Dykes, G. A. (2016). Role of Fimbriae, Flagella and Cellulose on the Attachment of *Salmonella* Typhimurium ATCC 14028 to Plant Cell Wall Models. *PLoS One* 11:e0158311. doi: 10.1371/journal.pone.0158311

Teixeira, P. J. P. L., Colaianni, N. R., Fitzpatrick, C. R., and Dangl, J. L. (2019). Beyond pathogens: microbiota interactions with the plant immune system. *Curr. Opinion Microbiol.* 49, 7–17. doi: 10.1016/j.mib.2019.08.003

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* 53, 763–775. doi: 10.1111/j.1365-313X.2007.03369.x

van der Heijden, J., Bosman, E. S., Reynolds, L. A., and Finlay, B. B. (2015). Direct measurement of oxidative and nitrosative stress dynamics in *Salmonella* inside macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 112, 560–565. doi: 10.1073/pnas.1414569112

van der Heijden, J., Reynolds, L. A., Deng, W., Mills, A., Scholz, R., Imami, K., et al. (2016). *Salmonella* rapidly regulates membrane permeability to survive oxidative stress. *mBio* 7:e01238-16. doi: 10.1128/mBio.01238-16

Vazquez-Torres, A., and Fang, F. C. (2001). Oxygen-dependent anti-*Salmonella* activity of macrophages. *Trends Microbiol.* 9, 29–33. doi: 10.1016/s0966-842x(00)01897-7

Wang, S., Phillippy, A. M., Deng, K., Rui, X., Li, Z., Tortorello, M. L., et al. (2010). Transcriptomic Responses of *Salmonella enterica* serovars enteritidis and typhimurium to chlorine-based oxidative stress. *Appl. Environ. Microbiol.* 76, 5013–5024. doi: 10.1128/AEM.00823-10

Ya'acov, Y. L., Wills, R. B. H., and Ku, V. V.-V. (1998). Evidence for the function of the free radical gas—nitric oxide (NO)—as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiol. Biochem.* 36, 825–833. doi: 10.1016/s0981-9428(99)80020-5

Yang, H., Mu, J., Chen, L., Feng, J., Hu, J., Li, L., et al. (2015). S-nitrosylation positively regulates ascorbate peroxidase activity during plant stress responses. *Plant Physiol.* 167, 1604–1615. doi: 10.1104/pp.114.255216

Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., et al. (2004). Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15811–15816. doi: 10.1073/pnas.0404536101

Zheng, J., Allard, S., Reynolds, S., Millner, P., Arce, G., Blodgett, R. J., et al. (2013). Colonization and Internalization of *Salmonella enterica* in Tomato Plants. *Appl. Environ. Microbiol.* 79, 2494–2502. doi: 10.1128/AEM.03704-12

Zheng, Y., Shen, L., Yu, M., Fan, B., Zhao, D., Liu, L., et al. (2011a). Nitric oxide synthase as a postharvest response in pathogen resistance of tomato fruit. *Postharvest Biol. Technol.* 60, 38–46. doi: 10.1016/j.postharvbio.2010.12.003

Zheng, Y., Sheng, J., Zhao, R., Zhang, J., Lv, S., Liu, L., et al. (2011b). Preharvest l-Arginine treatment induced postharvest disease resistance to *Botrysis cinerea* in Tomato Fruits. *J. Agric. Food Chem.* 59, 6543–6549. doi: 10.1021/jf2000053

Zhu, Z., and Tian, S. (2012). Resistant responses of tomato fruit treated with exogenous methyl jasmonate to *Botrytis cinerea* infection. *Sci. Hortic.* 142, 38–43. doi: 10.1016/j.scienta.2012.05.002

Conflict of 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 April 2020
doi: 10.3389/fmicb.2020.00500

Evasion of Plant Innate Defense Response by *Salmonella* on Lettuce

Nicholas Johnson[1,2], Pushpinder K. Litt[3], Kalmia E. Kniel[3] and Harsh Bais[1,2]**

[1] Department of Plant and Soil Sciences, University of Delaware, Newark, DE, United States, [2] Delaware Biotechnology Institute, University of Delaware, Newark, DE, United States, [3] Department of Animal and Food Sciences, University of Delaware, Newark, DE, United States

To establish host association, the innate immune system, which is one of the first lines of defense against infectious disease, must be circumvented. Plants encounter enteric foodborne bacterial pathogens under both pre- and post-harvest conditions. Human enteric foodborne pathogens can use plants as temporary hosts. This unique interaction may result in recalls and illness outbreaks associated with raw agricultural commodities. The purpose of this study was to determine if *Salmonella enterica* Typhimurium applied to lettuce leaves can suppress the innate stomatal defense in lettuce and utilization of UD1022 as a biocontrol against this ingression. Lettuce leaves were spot inoculated with *S.* Typhimurium wild type and its mutants. Bacterial culture and confocal microscopy analysis of stomatal apertures were used to support findings of differences in *S.* Typhimurium mutants compared to wild type. The persistence and internalization of these strains on lettuce was compared over a 7-day trial. *S.* Typhimurium may bypass the innate stomatal closure defense response in lettuce. Interestingly, a few key T3SS components in *S.* Typhimurium were involved in overriding stomatal defense response in lettuce for ingression. We also show that the T3SS in *S.* Typhimurium plays a critical role in persistence of *S.* Typhimurium *in planta*. *Salmonella* populations were significantly reduced in all UD1022 groups by day 7 with the exception of *fljB* and *invA* mutants. *Salmonella* internalization was not detected in plants after UD1022 treatment and had significantly higher stomatal closure rates (aperture width = 2.34 μm) by day 1 compared to controls (8.5 μm). *S.* Typhimurium SPI1 and SPI2 mutants showed inability to reopen stomates in lettuce suggesting the involvement of key T3SS components in suppression of innate response in plants. These findings impact issues of contamination related to plant performance and innate defense responses for plants.

Keywords: *S. enterica*, *L. monocytogenes*, T3SS, stomata, innate defense response, food safety, pattern triggered immunity, hormonal response

OPEN ACCESS

Edited by:
Max Teplitski,
University of Florida, United States

Reviewed by:
Massimiliano Marvasi,
University of Florence, Italy
Marcos H. De Moraes,
University of Washington, United States

****Correspondence:***
Kalmia E. Kniel
kniel@udel.edu
Harsh Bais
hbais@udel.edu;
bais@dbi.udel.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *22 October 2019*
Accepted: *09 March 2020*
Published: *03 April 2020*

Citation:
Johnson N, Litt PK, Kniel KE and Bais H (2020) Evasion of Plant Innate Defense Response by Salmonella on Lettuce. Front. Microbiol. 11:500. doi: 10.3389/fmicb.2020.00500

INTRODUCTION

Salmonella remains a critical foodborne pathogen given its ability to persist in various environments and hosts (plant, animal, human). Microbial contamination can originate from countless areas along the farm to fork continuum. At greatest risk are those aspects of contamination that can occur within the pre-harvest environment, whereby microbial

Abbreviations: ABA, abscisic acid; CFL, culture filtrate; CFU, colony forming units; h post inoculation or hpi, hours post inoculation; LPS, lipopolysaccharide; SA, salicylic acid; SPI1, SPI2, salmonella pathogenesis islands One or Two; T3SS, type three secretion system.

contamination can come in contact with plant foliar tissues from water, soil amendments, wind, birds, insects, animals, and other fomites including food contact surfaces on-farm and at packaging facilities (Beuchat, 2002; Brandl, 2006; Berger et al., 2010). Risk of contamination of plants in the field likely occurs *via* direct and indirect mechanisms (Monaghan and Hutchison, 2012). Laboratory studies suggest that bacterial pathogens on plants in the field decrease quickly over the first 3 days, but low numbers continue to persist for several weeks, which may cause human health issues (Erickson, 2012). This study explores potential host-microbe ingression between plants and plant associated bacteria, which may be involved in plant contamination (Schikora et al., 2008; Kumar et al., 2012; Hsu and Micallef, 2017).

The Centers for Disease Control and Prevention (CDC) attributed 46% of illnesses to fresh produce and particularly leafy greens (Painter et al., 2013). Indicating that greater and more sophisticated efforts are needed to prevent contamination on these commodities that cause foodborne illness. Plants grow in close association with large communities of microbes, yet comparatively little is known about the diversity of microbes that associate with plants, and their interactions and effects on performance, crop yields and plant protection. The fields of plant science and food microbiology have been merging over the past few years in the best interest of produce safety, however, critical data gaps remain. Importantly, there is limited knowledge about the modes of entry human bacterial pathogens may utilize for plant ingression, which may occur through several routes, including openings in roots, on the cortex, or epidermis (Deering et al., 2012; Erickson, 2012; Hirneisen et al., 2012).

Reports have shown that human pathogens that infect mammals and other higher animals are able to infect plants (Bishop and Davis, 1990; Plotnikova et al., 2000; Rahme et al., 2000). Studies using *Pseudomonas aeruginosa* showed that few human pathogens use conserved virulence factors to infect multiple hosts including plants (Rahme et al., 2000). Like *P. aeruginosa*, *Salmonella enterica* serovar Typhimurium also uses plants as alternative hosts to humans and other animals. It is shown that these bacteria adhere to plant surfaces and actively infect the interior of plants. Using *Arabidopsis thaliana* as a model system, it was shown that, *S. enterica* serovar Typhimurium suppresses plant defense responses mediated by a type III secretion mechanism (Schikora et al., 2008). In addition, it was also shown that several *S. enterica* serovars exhibit variations in pathogenicity, on different plant species revealing different innate defense response toward these bacteria (Schikora et al., 2011, 2012). An important feature of *Salmonella* infections in plants is its ability to adhere to plant surface. Interestingly, it was shown that several *S. enterica* serovars adhere to plant surfaces better then pathogenic *E. coli* strain O157:H7 (Barak et al., 2002). A mutant screen identified over 20 mutants impaired in surface attachment in *S. enterica* serovars on *Medicago sativa* (alfalfa) sprouts (Barak et al., 2005). Majority of genes involved in attachment of *Salmonella* to alfalfa were related to surface-exposed aggregative fimbria nucleator curli (*agfB*) and for the global stress regulator *rpoS* which regulates the production of curli, cellulose and other adhesins (Barak et al., 2005). It is shown that the involvement of biofilm operons and swarming genes modulate attachment to plant surface, in the phyllosphere and light sensing leading to modification in stomatal aperture (Barak et al., 2009; Kroupitski et al., 2009). Contrastingly, not much is known about how *Salmonella* spp. infect and ingress in leafy green plants. In mammalian systems, *S. enterica* serovar Typhimurium uses both pathogenicity islands 1 and 2 (SPI1, SPI2) to cause virulence (Coburn et al., 2007). Primary function of the SPI1 is cellular invasion, while the SPI2 is required for cellular survival and reproduction in the *Salmonella* containing vacuole (SCV) (Ohl and Miller, 2001; Löber et al., 2006). SPI1 virulence island is controlled via several genes; *hilC*, *hilD*, and *sirA*/*barA* that have been identified as regulators of *hilA* and downstream products in the SPI1 (Ellermeier et al., 2005). Regulation of *hilA* controls all SPI1 functions, including the T3SS. It is thus evident that the genetic equipment of *Salmonella*, previously thought to be animal-infection specific, plays an important role in the infection of animals and plants alike (Ellermeier et al., 2005). Within SPI2 SseB is a chaperone protein required for functional T3SS, mutations effect phase two pathogenicity that occurs within the *Salmonella* containing vacuole within mammalian cells (Ellermeier et al., 2005).

Foodborne pathogens can contaminate at any part of the farm to fork continuum, most often it occurs during preharvest through non-specific sources or irrigation water. A recent multistate outbreak in the United States was linked to a contaminated water source on a farm in California, and no farm is immune to accidental contamination via feces from wildlife or presence of native soil pathogens such as *Listeria* spp. (CDC, 2018, 2012). It is known that various beneficial microbes induce defense response in plants (Rosier et al., 2018). It was shown that root association of a beneficial microbe, *Bacillus subtilis* strain UD1022 (hereafter UD1022) protects *Arabidopsis* plants from aerial pathogens by modulating stomatal apertures (Kumar et al., 2012). The stomatal aperture reduction by UD1022 didn't modify the gaseous exchange process in plants and was transient in nature (Kumar et al., 2012). The stomatal closure by UD1022 in *Arabidopsis* was dependent on ABA and SA pathways (Kumar et al., 2012). We also showed that the reduction of stomatal aperture by UD1022 may be translated to leafy greens such as lettuce and spinach plants (Markland et al., 2015).

Here, we developed a model patho-system with lettuce as a plant system to evaluate the ingression and persistence of *S. enterica* serovar Typhimurium 14028 (henceforth *S.* Typhimurium) *in planta*. We observed that *S.* Typhimurium may bypass the early stomatal closure defense response in lettuce. Our observation shows that *S.* Typhimurium subverts the immune system and prevents stomatal closure at times when it normally would be closed as part of the innate immune response. Interestingly, few key T3SS components in *S.* Typhimurium were involved in overriding stomatal defense response in lettuce for ingression. We also show that the T3SS in *S.* Typhimurium plays a critical role in persistence of *S.* Typhimurium *in planta*. Gene expression analysis shows that *S.* Typhimurium may perturb the ABA biosynthesis pathway to subvert stomatal defense. We also showed that root application of a UD1022 reduces stomatal aperture leading

to reduced ingression by *S.* Typhimurium. Interestingly, co-inoculation of UD1022 with *S.* Typhimurium overrides ability of *S.* Typhimurium to reopen stomates in lettuce. Our findings show a development of patho-system involving leafy green specie with *S.* Typhimurium. The model system provides many possibilities of understanding molecular, biochemical and physiological networks that underpin this unique plant-human pathogen interaction.

MATERIALS AND METHODS

Plant Growth Methods

Lactuca sativa (Family: Asteraceae) var. Black Seeded Simpson was purchased from Johnny's Select Seed. Before cultivation all seeds were stratified for 48 h on a damp paper towel in a conical tube at 4°C, the seeds were then soaked in 50% bleach for 8 min in the same 50 mL conical tube before being washed thrice with a minimum of 25 mL sterile water each time. This cleaning method resulted in only one plant in 2 years that had a mold presence and no loss of seed viability or germination rate. The clean seeds were placed on MS agar with 1% sucrose and grown under a 1750 (PAR = 200–230) lumen grow light grid at room temperature (25 ± 3°C) for 2 weeks with a 12 h photoperiod or placed in sterilized magenta boxes with autoclaved hydroponic clay and a modified Sonneveld Solution (Mattson and Peters, 2014). For persistence internalization assays: *Lactuca sativa* var. Black Seeded Simpson was planted in a sterile pro-mix made up of 85% Canadian sphagnum peat moss with perlite, vermiculite, dolomitic and calcitic lime, a wetting agent, and mycorrhizae (Premier Tech Horticulture, Quakertown, PA, United States) in a seed tray with holes at the bottom (4 cm × 3.5 cm × 4.5 cm in dimension; T.O. Plastics, ON, Canada) and placed in another plastic container. Trays were maintained in Biosafety Level 2 growth chamber (Percival Scientific, Boon, IA, United States) at 20°C with 12 h photoperiod and at a constant relative humidity of 60% (Markland et al., 2015). Plants were irrigated by pouring water into the bottom plastic container to saturation of the soil.

Bacterial Culture Preparation

All bacterial strains were kept at −80°C freezer in 20% glycerol for long term storage. Prior to use each bacterial strain was streaked onto a complex-media (Tryptic Soy or Luria-Bertani) containing necessary antibiotics where applicable (**Supplementary Table S1**). Each plate was incubated for 16–24 h at 30°C, and re-streaked from the glycerol stocks as needed. Before experimental use, a single colony from solid media was moved into liquid media via sterile loop technique and incubated at 30°C overnight on an orbital shaker at 200 rpm. Following incubation, liquid bacterial cultures were aliquoted into conical tubes and centrifuged for 15 min at 4000 rpm and washed twice with 25 mL PBS buffered to a pH of 7.4, followed by a final suspension in PBS. The optical density was measured at 600 nm with a Bio-Rad SmartSpec + spectrophotometer (Bio-Rad Inc.) and adjusted to the working concentration of 10^7 CFU mL^{-1} in sterile DI water. For all experiments utilizing live cells, bacterial cultures were prepared in the manner as described above.

Bacterial Culture Filtrate (CFL) Preparation

Salmonella Typhimurium, *sseB*, or *hilD* were grown in 50 mL M9 media with 2% dextrose for 24 h at 30°C, the resulting suspension was measured with a spectrophotometer to check for similar cell density. The culture was then centrifuged for 15 min at 4000 rpm and filter sterilized through a 0.22 μm filter. Heat treated CFL was prepared in an identical fashion followed by 3 h incubation in a 65°C water bath before use. Contamination was checked for via plating 100 μL onto LB agar; no contamination was ever observed.

Stomatal Assay

Light adapted 2-week-old lettuce plants grown on MS agar were brushed with sterilized water, a suspension of 10^7 cfu mL^{-1} of the bacteria listed in **Supplementary Table S1**, various MAMPs [S. *typhimurium* LPS 10 μg mL^{-1}, *Pseudomonas aeruginosa* LPS 10 μg mL^{-1}, FLG22 peptide at 10 μg mL^{-1}], or plant growth hormones [5 μM SA, or 20 μM ABA]. Co-inoculation of bacteria and a plant growth hormone [5 μM SA, or 20 μM ABA] occurred by brushing the plants (Markland et al., 2015) first with ABA or SA followed by bacteria with a separate sterilized brush to avoid cross contamination. Following inoculation, the plants were incubated at room temperature under the previously described grow light grid for 3–12 h, stomatal aperture were monitored at 3 h post inoculation or 3, 6, and 12 h post inoculation (Markland et al., 2015). For image analysis inoculated leaves were excised with alcohol sterilized forceps. From the excised leaf a small circle was removed with a potato corer which was stained with propidium iodide (PI) for 8 min followed by a light rinse with deionized water and placed abaxial side up under a glass block in chambered cover glass (NUNC/VWR). Directly after staining plant samples were imaged with a Zeiss 710 Confocal Microscope.

B. subtilis (UD1022) Treatment for Modulation of Stomatal Apertures

A suspension of *B. subtilis* strain UD1022 (henceforth UD1022) was made as previously described, final suspension in sterile nanowater water was to a concentration of 10^5 CFU mL^{-1}. Lettuce grown in magenta boxes received 10 mL of this dilute culture for a total of 10^6 cells per magenta box. The boxes were then returned to the growth chamber for a 48 h incubation with a 24 h photoperiod after the first 24 h. Following light adaptation *S.* Typhimurium WT, *hilD, sseB,* or water inoculations (control) were completed as described above after the 48 h incubation and imaged at 3 and 6 h post inoculation. Alternatively, UD1022 treated plants were taken for imaging directly after UD1022 inoculation, with the remaining plants placed in the growth chamber. Samples were imaged at 0, 4, 8, 24, 36, 48, and 60 h post inoculation. At identical time points using different plants a calibrated porometer was utilized to gather 3–4 measurements from three plants per time point.

Confocal Imaging Parameters

Imaging was performed using a Zeiss710 Inverted Laser Scanning Microscope located in the University of Delaware Bioimaging

Core, samples were imaged at 0, 3, 6, and 12 h post inoculation. PI stain was excited with a 561 nm laser through a 488/561 bandpass filter with the emission spectra set to 580–640 nm. Images were captured with 2048 × 2048 pixels per frame and 20× magnification +1× digital zoom for 425.1 μm per frame. Settings were consistent over all samples with exception to, digital gain, and aperture width. In addition to PI fluorescent bands, the phase image in grayscale was retrieved for each micrograph.

Cryo-SEM Imaging

Light adapted 2-week-old lettuce was brushed with a suspension of either *S.* Typhimurium WT, *hilD*, *sseB*, or left un inoculated. To prepare leaves for SEM small holes were removed from each sample leaf with a potato corer (1 per leaf) to retrieve symmetrical circles for imaging, each leaf circle was placed adaxial side up on a gold block with tissue mounting fluid and carbon-black. After mounting, the leaves were flash frozen by being plunged into liquid nitrogen and contained under a vacuum, the block was then transferred to the cryo-SEM chamber and brought to −120°C, the leaves were then sublimated at −90°C to remove ice-films on the surface, and finally sputter coated with gold and palladium before imaging took place at −120°C. Leaves treated with *S.* Typhimurium WT, *sseB*, *hilD* were imaged at 3 and 6 h post inoculation, with a references plant sample left uninoculated (0 h post inoculation). Each leaf subsample was imaged at a magnification of 500X. All images were processed in the same fashion as those from confocal based leaf assays, albeit with an adjusted scale.

Gene Expression Studies

Light adapted 2-week-old lettuce was brushed with either water or *S.* Typhimurium in the identical fashion to the above stomatal assay. At 0 h post inoculation, 3 h post inoculation, and 6 h post inoculation samples were flash frozen in liquid nitrogen followed by RNA extraction following the Qiagen RNeasy protocol. In order to quantify the expression levels within *S.* Typhimurium treated lettuce the RNA was converted to complementary DNA via reverse transcription PCR. Rt-PCR was completed with Multiscribe® Reverse Transcriptase (Thermo-Fischer), for each reaction 1000 ng of RNA was used. The master mix was made as follows; 2 μL RT-Buffer, 2 μL RT-random Primers, 0.8 μL dNTP-Nucleotides, 1 μL Multiscribe Reverse Transcriptase, 1000 ng RNA, and enough nuclease free water to bring the reaction volume to 20 μl. PCR parameters were as follows for every sample: 25°C for 10 min, 37°C for 2 h, 85°C for 5 min, and 4°C until samples were moved to a −20°C freezer for long term storage. A semi-quantitative PCR technique using a DreamTaq Green Polymerase and protocol (Thermofischer) was employed to check cDNA viability and relative expression of genes. The primers and PCR conditions used can be found in **Supplementary Tables 2A,B**, **3**. Following PCR gels were imaged using a Biorad Gel Dock, individual bands were measured using Image J and data added to Microsoft Excel. Relative expression of each primer was averaged and normalized against Actin. Significant differences were found using a Student's *t*-test at a significance level of $p < 0.05$.

Persistence and Internalization of *S.* Typhimurium Mutants Lacking Type-III Secretion System (T3SS) Virulence Factors and Flagellar Genes in Lettuce Grown Under Greenhouse Conditions

Two-week-old lettuce plant leaves were spot-inoculated randomly with 120 μl (6 droplets) of respective *Salmonella* culture on the leaf surface. Inoculated leaves were placed in the growth chamber for 2 h, to facilitate bacterial attachment. Leaf samples were collected for each treatment group separately and processed to enumerate surviving *Salmonella* populations on day 0, 1, 3, 5, and 7. A sample of 6 plants (total of 12 leaves in each) was collected on each sampling day and split into two equal sections for bacterial enumeration and pathogen internalization assay. To enumerate, leaf samples (6 leaves from plant) were weighed in individual Whirl-Pak™ bags (Nasco, Fort Atkison, WI, United States) and submerged in 0.1% buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, England) in a 1:9 ratio. The sample was mixed for 2 min at 230 rpm in a stomacher (BagMixer® 400 S, Interscience), the resulting mix was serially diluted in 0.1% BPW and plated on TSA with antibiotic (**Supplementary Table S2**), or by a mini-MPN method (Sharma et al., 2016). *Salmonella* colonies were counted after 22–24 h of incubation at 37°C.

Bacillus subtilis UD1022 Colonization of *S.* Typhimurium Mutants Lacking Flagellar and Type-III Secretion System (T3SS) Virulence Factors

Roots of 14 days old plant were inoculated with live culture of *B. subtilis* UD1022 (∼10^8 CFU/ml) by adding culture to irrigation water to a saturation. Plants for maintained in bio-chamber for 48 h at 20°C with a 12 h photoperiod and at a constant relative humidity. After 48 h, lettuce leaves were spot inoculated randomly with respective *Salmonella* mutant culture on the leaf surface and leave samples were processed as explained above. Treatment groups also included, negative control, *B. subtilis* UD1022 only control.

Internalization Assay

To detect pathogen internalization in lettuce, lettuce leaves were spot inoculated with 120 μl (6 droplets) of respective *Salmonella* culture on the leaf surface. Inoculated leaves were placed in the growth chamber for 2 h, to facilitate bacterial attachment. Leave samples were collected on day 0, 1, 3, 5, and 7. Sampled leaves were surface disinfected by immersing them in 80% ethanol for 10 s, followed by 10 min dip in 0.1% mercuric chloride (Markland et al., 2015 and Erickson et al., 2010). Leaves were washed with 10 mL sterile water and grounded using a rubber mallet. Subsequently, leaves were transferred to a fresh Whirl-Pak bags and re-suspended in 0.1% BPW. Re-suspended leaves were homogenized for 2 min at 230 rpm in a stomacher and 1 mL of the resulting mix was transferred to 9 mL TSB. Inoculated TSB was incubated for 18–24 h at 37°C. After incubation, a loop full sample from each tube was streaked onto TSA plates

and incubated for 18 h at 37°C. Plates were observed for *Salmonella* colonies.

Statistical Analysis

All the experiments were repeated in triplicate per treatment. Bacterial populations, recovered at each sampling point, were converted to log10 CFU/g or MPN/g, and the mean values of the three replicates were obtained. The limit of detection was 1 log CFU/g. Stomatal width at each time point was captured with confocal microscopy and measured using ImageJ. Data were analyzed using a one-way ANOVA to determine the effect of treatments, and student's *t*-test was performed to compare the means of bacterial populations, stomatal width, and relative gene expression over time by using JMP software (JMP v.14; SAS Institute Inc., Cary, NC) at a significance level of $P < 0.05$.

RESULTS

S. Typhimurium Treatment Modifies Stomatal Aperture in Lettuce

Previous studies have shown that stomata act as an entry points for various plant and human pathogens to ingress in plants (Kumar et al., 2012; Markland et al., 2015). To determine if different opportunistic pathogens modulate stomatal aperture in lettuce, in this study lettuce leaves were (2-weeks-old) inoculated with *S.* Typhimurium or *L. monocytogenes*. This study evaluated two methods to measure stomatal aperture: confocal microscopy of PI stained leaf subsamples and cryo-scanning electron microscopy (cryo-SEM) of the frozen leaf subsamples (Van Gardingen et al., 1989). Cryo-SEM was chosen as the validation method because it was possible to examine and capture the effect of the signals generated during the early time points of tritrophic interactions in the context of the entire plant. *S.* Typhimurium leaf inoculation did not change or reduce the average stomatal aperture size at both 3 and 12 h post inoculation compared to the mock-inoculated control, referred to as mock from here forward (**Figure 1A** and **Supplementary Figures S1A,B**). Both confocal microscopy and cryo-SEM showed similar aperture profile under *S.* Typhimurium treatment. Using images obtained through the confocal imaging and cryo-SEM technique the stomatal width taken at the widest point was averaged for 40–60 stomata per leaf after which the average of all samples in a set were averaged ($n = 3$) (**Figure 1A** and **Supplementary Figure S2**). Treatment of both *S.* Typhimurium and *L. monocytogenes* showed stomatal aperture reduction at 6 h post inoculation, but only *L. monocytogenes* treatments induced a smaller stomatal phenotype at 12 h post inoculation compared to the control and *S.* Typhimurium treatment (**Figure 1A** and **Supplementary Figure S1A**). *S.* Typhimurium presented a phenotype like that of the mock after 3 h and was not different from the mock at 12 h post inoculation as well. Both the control and *S.* Typhimurium were significantly different ($p < 0.05$) from the reduced stomatal aperture observed in *L. monocytogenes* treated plants. A visual comparison shows that *L. monocytogenes* causes visibly smaller apertures while water treatments and *S.* Typhimurium treatments are visually similar (micrographs not shown). Besides altering the aperture sizes, *L. monocytogenes* leaf inoculation significantly increased the percentage of closed stomata on the abaxial leaf surface at both 3 and 6 h post inoculation compared to the control and *S.* Typhimurium treatments (**Supplementary Figure S1B**). Taken together, these data showed that leaf inoculation of *S.* Typhimurium can modulate stomatal aperture to keep stomates open post inoculation.

S. Typhimurium Culture Filtrate (CFL) and Microbe-Associated Molecular Patterns (MAMPs) Modifies Stomatal Aperture in Lettuce

Salmonella Typhimurium treatments negated noticeable stomatal changes in lettuce plants. It is hypothesized that the cell-derived products from *S.* Typhimurium may be causing modulations in in plant innate responses. To facilitate this a filter sterilized culture filtrate (CFL) was prepared from *S.* Typhimurium, *sseB*, or *hilD* grown in a minimal media, these CFLs were used in a stomatal leaf assay. Each prepared CFL led to closure of stomates compared to the untreated control and *S.* Typhimurium post 3 h inoculation (**Figure 1B**). A heat-treated CFL prepared from *S.* Typhimurium showed a stomatal phenotype similar to live *S.* Typhimurium treated lettuce, suggesting that produced components from *S.* Typhimurium may trigger plant innate defense response to close stomates and these products were subsequently destroyed during a heating step (**Figure 1B**). Other known stomata modulating components such as lipopolysaccharides and flg22 were also evaluated for stomatal closure post 3 h inoculation. It is known that MAMPs and other cellular components derived from bacterial plant pathogens trigger plant innate defense response to close stomata (Melotto et al., 2006; Kumar et al., 2012). To test our results against purified *S.* Typhimurium specific LPS, a non-specific LPS (*P. aeruginosa*) and flg22 (10 $\mu g\ mL^{-1}$) were applied to the stomatal leaf assay. Each of these cellular components caused stomatal closure statistically similar to *S.* Typhimurium CFL-challenged lettuce, except flg22 which caused 50% more closure than *S.* Typhimurium CFL (**Figure 1B**). The stomatal closure induced by these cellular components was 60 to 25% greater than the aperture means gathered in the control and live *S.* Typhimurium trials. The data suggests that *S.* Typhimurium but not the secreted CFL from *S.* Typhimurium can regulate stomatal apertures by suppressing the plant innate immune response of lettuce.

S. Typhimurium Mutants Lacking Type-III Secretion System (T3SS) Virulence Factors Show Reduced Suppression of Stomatal Defense Response

Various studies have shown that *S.* Typhimurium possesses components that are recognized in plants (Shirron and Yaron, 2011; Garcia and Hirt, 2014). The experiments involving CFL from *S.* Typhimurium showed that plants recognize secreted components from *S.* Typhimurium to launch a PAMP triggered immunity to close stomata. Furthermore, inoculation

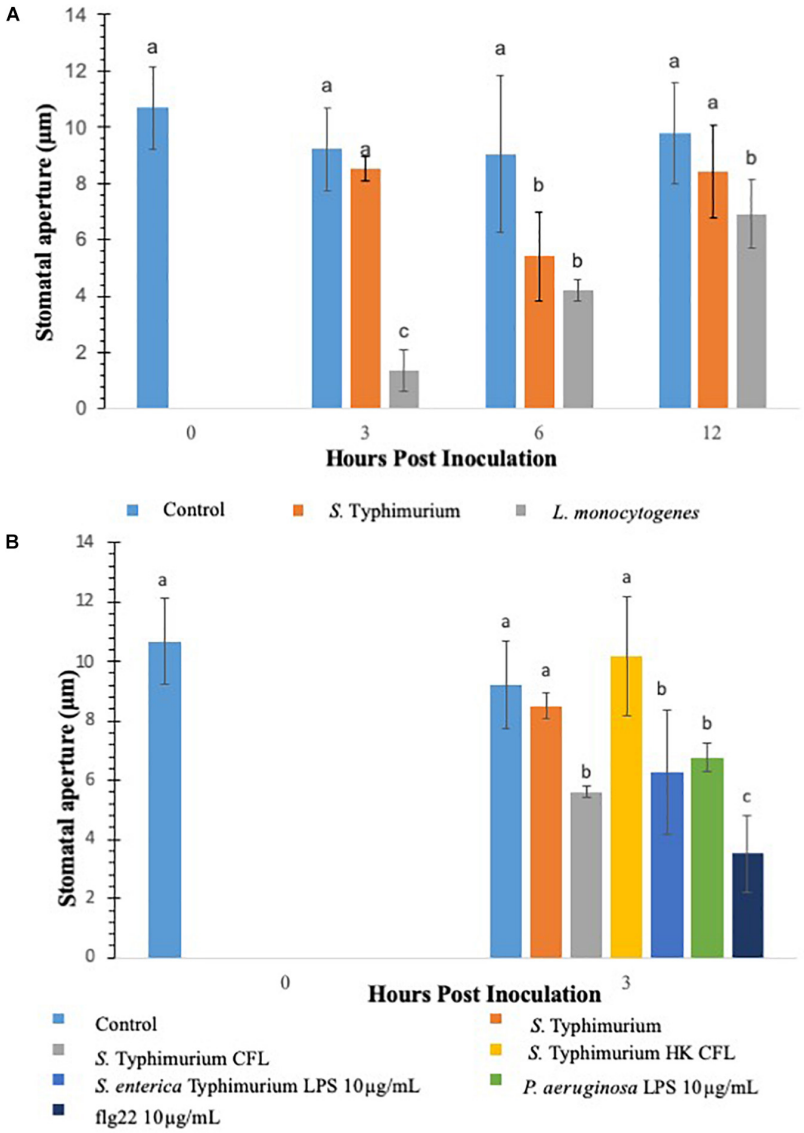


FIGURE 1 | Comparison of innate immune response in terms of stomatal closure by *L. monocytogenes* and *S. enterica* Typhimurium on lettuce. Aperture changes at 0 h remains unchanged, and 0 h represents only the water control. Different letters signify a difference at $p < 0.05$. For each time point the average was found from three plants with $n = 40–60$ stomata imaged per plant **(A)**. Comparison of innate immune response in terms of stomatal closure by *S. enterica* Typhimurium and MAMPs on lettuce **(B)**. Aperture changes at 0 h remains unchanged, and 0 h represents only the water control. Different letters signify a difference at $p < 0.05$. For each time point the average was found from three plants with $n = 40–60$ stomata imaged per plant.

of *S.* Typhimurium serovars to *Arabidopsis thaliana* (Family: Brassicaceae) seedlings triggered MAPK activation and defense gene expression to a similar extent as that provoked by *P. syringae* inoculation (Schikora et al., 2008, 2011; Garcia and Hirt, 2014). We also showed that classical MAMPs such as flg22 and LPS induce PTI-stomatal closure in lettuce. We hypothesized that factors governed through the T3SS system in *S.* Typhimurium may modify stomatal defense response differently in lettuce. To this end, we used various T3SS mutants belonging to both SP1 and SP2 system in *S.* Typhimurium for modification of stomatal defense response in lettuce. Recently, *Salmonella* T3SSs and effectors were proposed to contribute to the plant colonization process (Garcia and Hirt, 2014). *S.* Typhimurium mutants in T3SS-1 and T3SS-2 induced stronger cell death and chlorosis symptoms and proliferated to lower levels in *Arabidopsis* leaves (Schikora et al., 2011). We tested all the *S.* Typhimurium 14028 mutants (*fljB*, *fliC*, *hilD*, *sseB*) by applying them to light adapted lettuce leaves and the stomatal aperture at 3 h post inoculation was recorded (**Figure 2**). Of the nine mutants applied to the stomatal assay only two resulted in consistent stomatal closure like that seen on plants treated with *S.* Typhimurium CFL (**Figure 2**). A majority of T3SS mutants caused no apparent stomatal closure at 3 h post inoculation. Most *Salmonella* serovars carry two flagellin-encoding genes, *fliC* and *fljB* (Silverman and

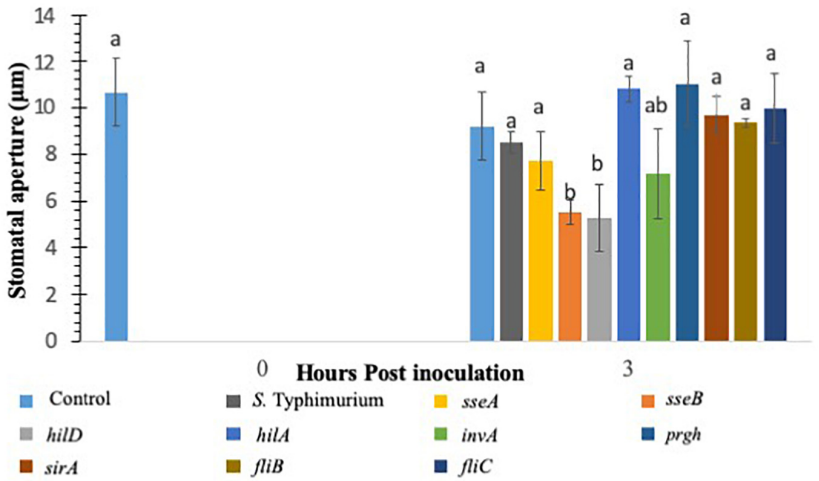


FIGURE 2 | Stomatal closure in lettuce plants treated with *S.* Typhimurium and mutants of SPI1, SPI2, regulatory proteins controlling T3SS, Flagellin B, and Flagellin C. Aperture changes at 0 h remains unchanged, and 0 h represents only the water control. Different letters signify a difference at $p < 0.05$. For each time point the average was found from three plants with n = 40–60 stomata imaged per plant.

Simon, 1980). The mutants lacking flagellar genes (*fljB* and *fliC*) in *S.* Typhimurium also showed lack of stomatal closure (**Figure 2**). It is shown that *S.* Typhimurium flagellin mutants triggered reduced defense responses in *Arabidopsis* and tomato (Iniguez et al., 2005). The T3SS mutants *hilD* and *sseB* caused stomatal closure that was significantly different ($p < 0.05$) then the mock or live *S.* Typhimurium (**Figure 2**). *hilD* is a transcription regulator for the master control of SPI1 and *sseB* lacks a functional T3SS in SPI2 (Ruiz-Albert et al., 2003; Ellermeier et al., 2005). Validation of *hilD* and *sseB* stomatal phenotype was completed using cryo-SEM; stomatal apertures of plants treated with *sseB* showed significant closure compared to *S.* Typhimurium at both 3 and 6 h post inoculation. *hilD*-challenged plants showed a significant change in stomatal aperture at only 6 h post inoculation (**Supplementary Figures S2A,B**). We also evaluated if host sensing by lettuce is mediated through a T3SS, to this end, the CFL from the two T3SS mutants (*hilD* and *sseB*) were added to lettuce leaves and compared with live *sseB* and *hilD* for stomatal apertures (**Supplementary Figure S3**). It should be noted that, both *hilD* and *sseB* challenged lettuce leaves led to greater stomatal closure compared to the WT. The CFL from both T3SS mutants led to stomatal closure as seen with WT CFL and live *sseB*/*hilD* cells, suggesting that the host sensing by lettuce is independent of T3SS and T3SS may be required to bypass stomatal defense in plants.

Persistence and Internalization of *S.* Typhimurium Mutants Lacking Type-III Secretion System (T3SS) Virulence Factors and Flagellar Genes in Lettuce Grown Under Greenhouse Conditions

Salmonella populations were significantly ($p < 0.05$) reduced in plants inoculated with T3SS (*hilD, sseB* and *invA*), and phase-1 flagellin (*fliC*) mutants by day 3 compared to wild type control (*S.* Typhimurium) except phase-2 flagellin (*fljB*) mutant (**Figure 3**). Mutants *hilD, sseB, invA,* and *fliC* populations were reduced between 2.3 and 3.3 MPN logs g^{-1} on day 3 compared to day 0 (4.1 and 7.2 log CFU g^{-1}). *Salmonella* populations were further reduced to undetectable levels for *hilD invA, fliC,* and to 0.7 log MPN g^{-1} for *sseB* mutants, by day 5. The *fljB* mutants (4.3 to 5.8 CFU MPN logs g^{-1}) and wild type control (5.0 to 6.0 logs CFU MPN logs g^{-1}) showed similar survival throughout the 7-day trial. Similarly, in *S.* Newport inoculated plants, no significant ($p < 0.05$) change in pathogen populations (5.3–6.3 logs CFU MPNg^{-1}) was observed on the lettuce surface, during the 7-day trial. Internalization assay results showed that *Salmonella* internalization was not detected in plants inoculated with *hilD* and *sseB* mutants, suggesting that these genes are vital for *Salmonella* internalization in lettuce (**Supplementary Table S4**). In *fliC* and *S.* Newport inoculated plants internalized *Salmonella* was observed until day 5 and in *invA* plants until day 1, of the trial. However, pathogen internalization was observed in *fljB* and wild type throughout the 7-day trial. It has been shown that *Salmonella* T3SS genes play different roles during plant-bacteria interactions based on plant species (Brandl et al., 2013). Iniguez et al. (2005) observed hyper-colonization of *Salmonella* T3SS SPI mutants than the wild type in alfalfa sprouts and *Arabidopsis thaliana,* On the contrary, in the current study, significantly low colonization was recorded in SPI mutants compared to the wild type. It is noteworthy that a previous study tested SPI- structural gene (*sipB*) mutant, while the current study analyzed the effects of SPI-1 transcriptional regulator (*hilD*), SPI-2 (*sseB*), and SPI-1 (*invA*) mutants on survival. This could explain the differences in survival and internalization of mutants and suggests that these genes could affect *Salmonella* survival and persistence in lettuce leaves. Additionally, in the current study flagellar mutants showed similar internalization as wild type. This means that in the absence of either phase 1 or 2 flagellin genes, it could express flagella genes of the other phase (Olsen et al., 2012). This

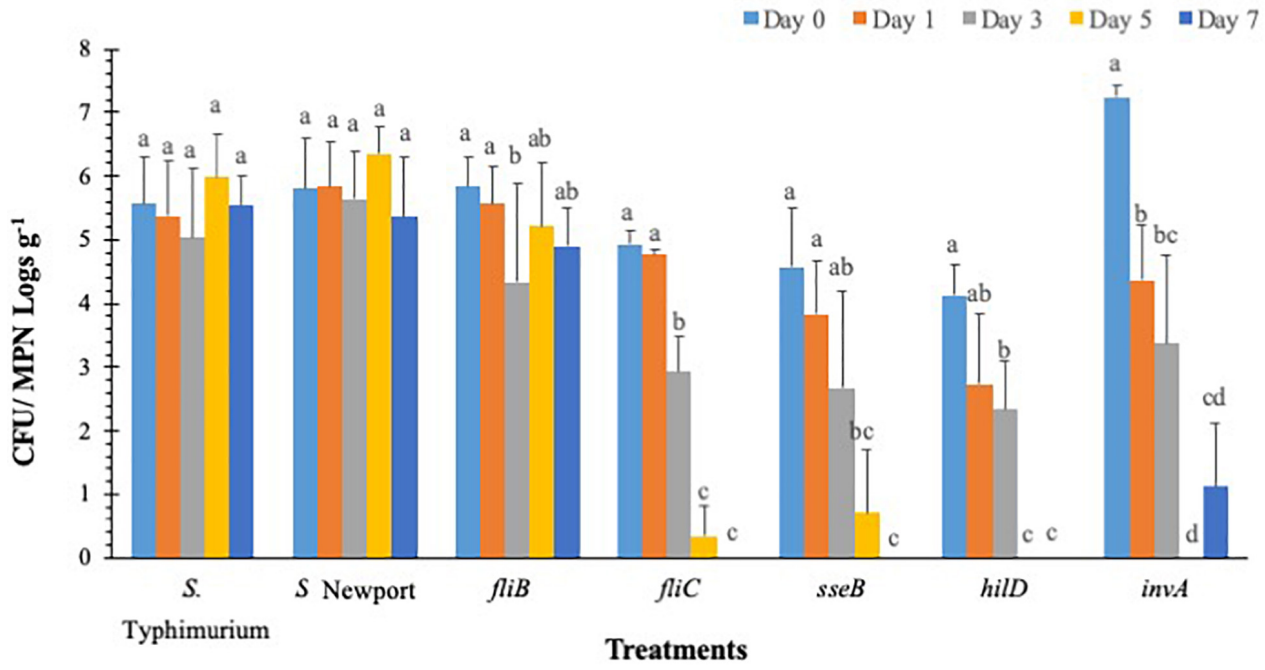


FIGURE 3 | *S.* Typhimurium populations on lettuce over a 7-day period. Values represent the average of three replications. Standard deviation (±) for surviving *S.* Typhimurium population (Log_{10} CFU or MPN g^{-1}) follows mean value. Letters *a, b, c* shows statistically significant differences ($p < 0.05$) between sampling days for the same strain.

could explain similarities in survival and internalization pattern between flagellar gene mutants and wild type strain.

Exogenous Hormones SA and ABA Induce Differing Stomatal Phenotypes in Lettuce Treated With *S.* Typhimurium

Previous studies have shown that defense hormones play a critical role in the interaction between *S.* Typhimurium and plants (Garcia and Hirt, 2014.) Growth regulators such as SA, JA, and ET signaling pathways regulate *Salmonella* colonization in plants (Iniguez et al., 2005; Schikora et al., 2008). Studies have also shown that plants treated with *S.* Typhimurium biosynthesize SA and the induction of several marker genes of the SA pathway (Garcia et al., 2014). To this end, we evaluated if addition of exogenous SA and ABA, another key growth regulator which plays a critical role in stomatal physiology (Kumar et al., 2012) plays a role in co-inoculation with *Salmonella* in lettuce. Plants were inoculated with SA/ABA alone or co-inoculated with live *S.* Typhimurium and stomatal apertures were measured post 3–12 h post inoculation. When compared to other PTI induced plants like those treated with CFL and flg22, SA was similar in percentage and duration (**Figure 4A**). At 3 h post inoculation stomatal closure was apparent and different from the control treatment, and by 12 h post inoculation SA-challenged plants had returned to a normal stomata phenotype (**Figure 4A**). Exogenous SA in co-inoculation assays caused stomata to remain closed for longer than when just SA was applied. In fact, the stomatal stays closed twice as long (out to 12 hpi) when both SA and *S.* Typhimurium was present. The reasoning for this is that SA triggered a PTI that was not affected by *S.* Typhimurium's phytopathogenic behavior therefore the presence of exogenous SA triggered PTI independently from *S.* Typhimurium. Despite these interesting observations, no hypersensitive response (patterned cell death) was observed on any plants, which may only be due to the 12 h length of the experiments. Patterned cell death was observed in research with *S.* Typhimurium applied to *Tabacum* and *Lycopersicum* (Family: Solanaceae) (Meng et al., 2013).

This study also evaluated the role of ABA in co-inoculation with *S.* Typhimurium in lettuce. It is shown that ABA plays a critical role in stomatal physiology under various biotic and abiotic conditions and is shown to be both synergistic and antagonistic with other plant hormones (Anderson et al., 2004; Merilo et al., 2015). When ABA was exogenously applied to the lettuce leaves, stomatal closure was observed just like on SA-challenged, plants (**Figure 4B**). Unlike SA, ABA caused closure at 3 h post inoculation and 6 h post inoculation but at 12 h post inoculation, when most plants in previous tests had reopened stomata, almost 100% of the stomata had closed (**Figure 4B**). The ABA induced closure significantly different ($p < 0.01$) from both SA and SA co-inoculated tests at 12 h post inoculation (**Figures 4A,B**). The co-inoculation with ABA and *S.* Typhimurium was set up by exogenously adding ABA (20 μM) and *S.* Typhimurium together. The stomatal apertures using confocal imaging were performed at 3, 6, and 12 h post inoculation. ABA co-inoculation with *S.* Typhimurium was performed with identical parameters and concentrations.

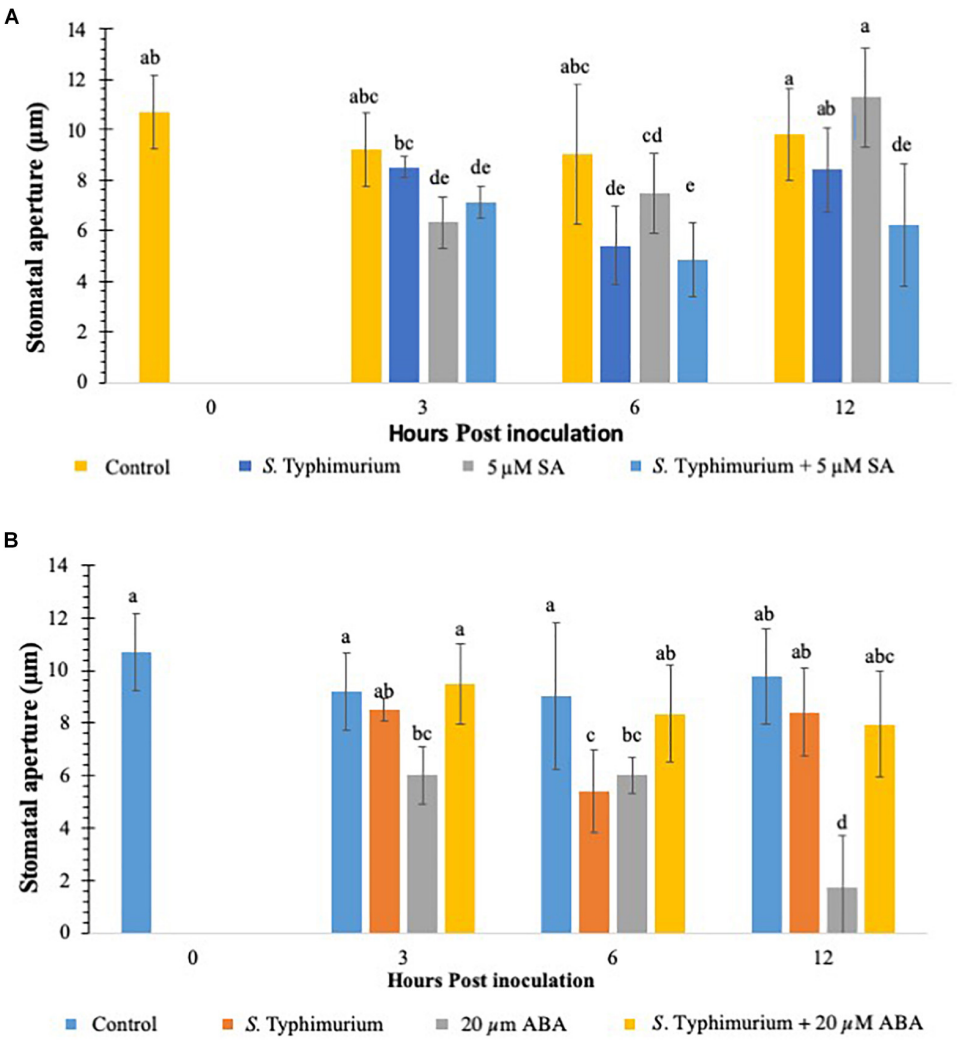


FIGURE 4 | Stomatal aperture modulation in lettuce treated with *S.* Typhimurium co-inoculated with salicylic acid (SA) **(A)** or abscisic acid (ABA) **(B)**. Aperture changes at 0 h remains unchanged, and 0 h represents only the water control. Different letters signify a difference at $p < 0.05$. For each time point the average was found from three plants with $n = 40–60$ stomata imaged per plant.

The expected results with the co-inoculation of ABA with *S.* Typhimurium were to see stomatal closure from 3 until 12 h post inoculation as before; oddly enough the resulting stomatal phenotype was most similar to *S.* Typhimurium - challenged plants (**Figures 1A,B**). The data clearly showed that *S.* Typhimurium mediated stomatal opening was not actively closed by ABA treatment under co-inoculation, suggesting that *S.* Typhimurium may disrupt ABA biosynthesis and signaling *in planta* as a roundabout way of preventing stomatal closure. *SseB* and *hilD* were also tested in a co-inoculation experiment with ABA. At 3 h post inoculation, *sseB* showed stomatal closures similar to ABA treatment (**Supplementary Figure S4**). Co-inoculation of ABA with *sseB* led to stomatal reopening after 3 h post inoculation compared to *hilD* which at first had a phenotype more like that of *S.* Typhimurium but by 12 h post inoculation it had closed to that of the 20 µM ABA treated plants, a similar trend was observed with subsequent tests (**Supplementary Figure S4**). The later time point (6 and 12 h post inoculation) showed no reopening of stomates under a co-inoculation experiment with ABA and *hilD*, suggesting, the involvement of T3SS in suppressing ABA-mediated stomatal defense may occur early in plants interaction with *S.* Typhimurium.

To analyze if addition of ABA later to *S.* Typhimurium supplementation in lettuce leaves changes stomatal physiology, ABA was added 3 h post inoculation of *S.* Typhimurium supplementation. As shown previously, supplementation of ABA rapidly closes stomates in multiple plants (Kriedemann et al., 1972; Melotto et al., 2006). In comparison to co-inoculation experiment when ABA was added as a delayed application (3 h post inoculation), post *S.* Typhimurium treatment, stomatal re-opening with *S.* Typhimurium was observed (12 h post inoculation time point); though the reopening of stomates was not

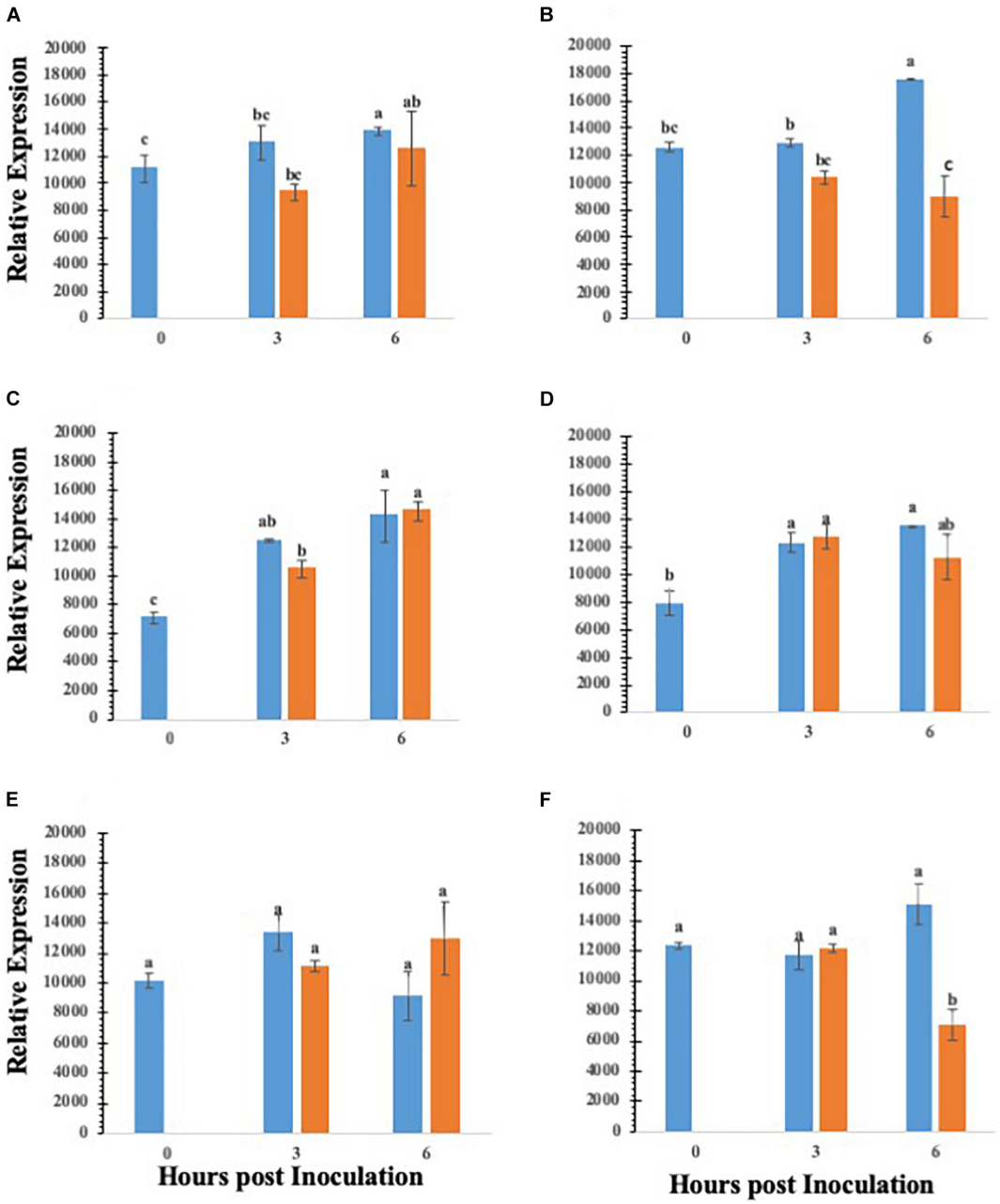


FIGURE 5 | Gene expression analysis of various ABA biosynthesis genes in roots and leaves of lettuce plants treated with water (blue bar) or *S. enterica* Typhimurium (orange bar): *ABA3* leaf **(A)**; *ABA3* root **(B)**; *ZEP1* leaf **(C)**; *ZEP1* root **(D)**; *NCED3* leaf **(E)**; *NCED3* root **(F)**. Aperture changes at 0 h remains unchanged, and 0 h represents only the water control. Different letters signify a difference at $p < 0.05$.

as prominent as that in the co-inoculation experiment (**Supplementary Figure S5**).

S. Typhimurium Affects Genes in the ABA Biosynthesis and Translocation Pathway

Abscisic acid is a central regulator of stomatal closure (Acharya and Assmann, 2009), and our data showing that co-inoculation of ABA with *S.* Typhimurium led to reopening of stomates, we further analyzed the key ABA biosynthetic genes in lettuce treated with *S.* Typhimurium. The precise mechanism by *S.* Typhimurium for suppressing or blocking ABA effects on stomates remains to be shown. We analyzed transcript levels of ABA biosynthetic genes (*LsZEP1*, *LsNCED3* and *LsABA3*) in lettuce plants treated with *S.* Typhimurium. The data shows that transcript levels of ABA biosynthetic genes (*LsABA3* and *LsNCED3*) in roots and leaves of lettuce reduced post treatment with *S.* Typhimurium (**Figures 5A–F**). Treatment of

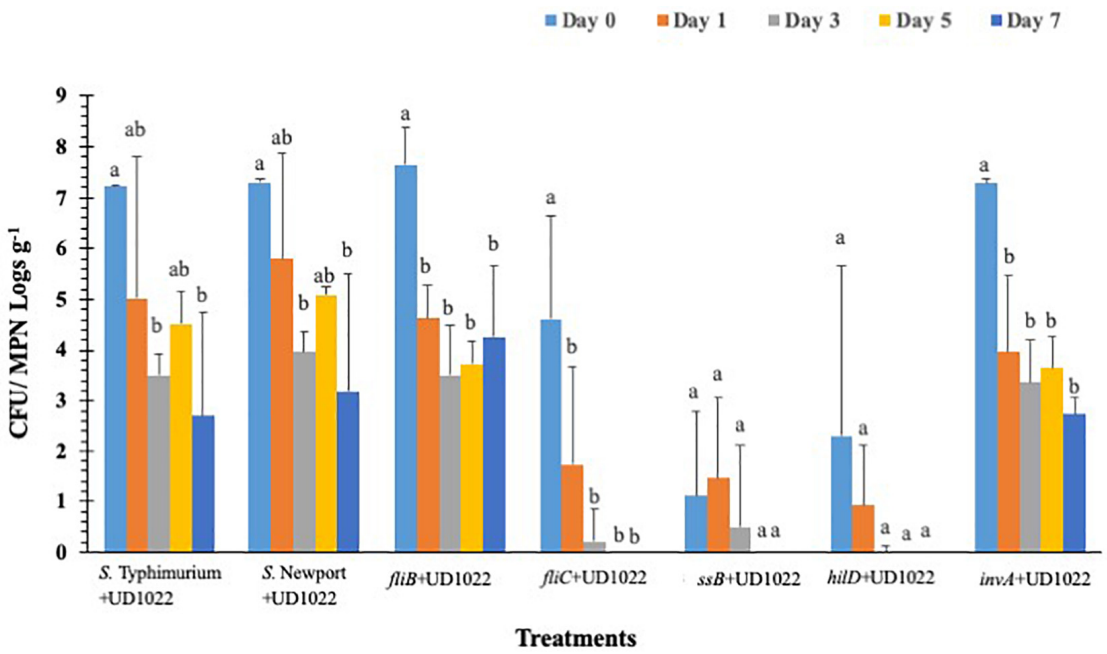


FIGURE 6 | Surviving *S.* Typhimurium populations on lettuce surface with *B. subtilis* UD1022 under greenhouse conditions. Values represent the average of three replications. Standard deviation (±) for surviving *S.* Typhimurium population (Log_{10} CFU or MPN g^{-1}) follows mean value. Letters *a, b, c* show statistically significant differences ($p < 0.05$) between sampling days for the same strain.

S. Typhimurium to lettuce plants did not lead to changes in other transcript levels of genes from classical plant defense pathways (**Supplementary Figure S6**).

B. subtilis UD1022 Colonization of *S.* Typhimurium Mutants Lacking Type-III Secretion System (T3SS) Virulence Factors and Flagellar Genes in Lettuce Grown Under Greenhouse Conditions

Overall *S.* Typhimurium populations were significantly ($p < 0.05$) reduced in all UD1022 treated groups by day 3 except *fljB* and *invA*, compared to the wild type (**Figure 6**). The *fljB* and *invA* showed significant ($p < 0.05$) decrease in pathogen survival on day 1 (4.6 and 4.0 logs CFU g^{-1}, respectively) compared to day 0 (7.7 and 7.2 CFU g^{-1}, respectively). Other mutant's *fliC, hilD,* and *sseB* were reduced to undetectable levels in UD1022 treated plants compared to wild type strain (3.5 logs CFU g^{-1}), by day 3. It is noteworthy that in *hilD,* and *sseB* groups had significantly ($p < 0.05$) lower (2.3-1.1 logs CFU g^{-1}) levels of mutant's colonization compared to the wild type (7.2 logs CFU g^{-1}) strain, on day 0. Whereas the same mutants (*hilD,* and *sseB*) showed higher colonization on day 0 in the lettuce plants which were not treated with UD1022, suggesting an interaction between UD1022, these mutants and plant itself through a signal exchange to prevent bacterial attachment and internalization. In control groups, *S.* Typhimurium and *S.* Newport populations decreased significantly ($p < 0.05$) to 3.5 and 3.9 logs CFU g^{-1}, respectively, by day 3, compared to day 0 (7.2 and 7.3 logs CFU g^{-1}, respectively). *Salmonella* internalization was not detected in plants inoculated with mutants after UD1022 treatment, during the 7-day trial, except for *invA*, *S.* Typhimurium and *S.* Newport groups on day 0 (**Supplementary Table S4**).

DISCUSSION

It has been shown previously that few plant pathogens have an ability to modify stomatal physiology to ingress and cause infection (Melotto et al., 2006). Bacterial pathogens such as *P. syringae* DC3000 exploit a polyketide (coronatine) to reopen stomata in *Arabidopsis thaliana* to cause infection (Melotto et al., 2006; Kumar et al., 2012). On the contrary, plants get exposed to various human pathogens such as *P. aeruginosa* and *Enterococcus faecalis* and show classical signs of infection as seen previously with plant pathogens (He et al., 2004; Jha et al., 2005). Along the same lines, opportunistic human pathogens such as *E. coli* strains, *L. monocytogenes* and *Salmonella* serovars interact with plants and cause ingression and contamination (Gorski et al., 2009; Deering et al., 2012). The mechanisms by which opportunistic human pathogens, such as *Salmonella* spp., demonstrate plant ingression are not well understood. It is often speculated that opportunistic human pathogens such as *Salmonella* may use natural entry points (stomata) or mechanical injuries to ingress *in planta* (Melotto et al., 2006; Barak et al., 2011; Deering et al., 2012; Zheng et al., 2013). *Salmonella* can adapt well and is able to proliferate in some plant organs such as tomato fruit, more over there is

overlap of plant pathogen and *Salmonella* mechanisms which shows the versatility and repurposing abilities of *Salmonella* (de Moraes et al., 2017). In this study, we report that *S.* Typhimurium 14028 may bypass the plant innate immune response to suppress stomatal defense for ingression. We also show that *S.* Typhimurium uses key T3SS factors to overcome stomatal defense for ingression. Rapid cryo-immobilization, cryo-SEM and confocal imaging were employed to capture the complex interaction between lettuce and *S.* Typhimurium in intact leaves with high accuracy and reproducibility. This approach revealed that plants treated with other opportunistic pathogens such as *L. monocytogenes* can induce stomatal closure, however, only the *S.* Typhimurium treatment showed ability to keep stomates open bypassing innate stomatal defense. We also showed that ability of *S.* Typhimurium to overcome stomatal defense was T3SS dependent. In addition, the SPI1 and SPI2 mutants of T3SS mutants revealed poor fitness and persistence under realistic growth conditions in lettuce. Using exogenous application of SA and ABA, we showed that *S.* Typhimurium may suppress ABA response in lettuce to keep the stomates open for longer durations. Using the gene expressions for ABA specific biosynthetic genes, we discovered that the addition of the *S.* Typhimurium, mediated stomatal non-closure mainly through a suppression of ABA biosynthetic pathway. This study suggests that pathogenic bacteria may associate with plants leading to development of key strategies by *Salmonella* to invade plants resulting in contamination.

S. Typhimurium Live Cells and Cell Free Lysate Treatment Indicate the Critical Role of Stomatal Defense in Identifying Microbial-Derived Factors Regulating Innate Response

Previous studies showed that application of bacteria to isolated epidermal peels from *Arabidopsis thaliana* induced a MAMP-triggered immune response that closed stomata (Melotto et al., 2006; Zeng and He, 2010). Our data show that leaf inoculation of bacteria such as *L. monocytogenes* closed stomata at 3 h post inoculation; in contrast, *S.* Typhimurium treatments kept the stomata open until 12 h post inoculation. The rapid closure of stomata with *L. monocytogenes*, a classical innate defense response, suggests the response is most likely related to a MAMP-triggered immunity (Zeng and He, 2010). A similar response was observed when known MAMPs, such as LPS and flg22 were added to the lettuce leaves. Melotto et al. (2006) and Roy et al. (2013) showed that flg22 and LPS close stomata in the isolated leaf epidermal peels of Col-0 and lettuce plants, similarly, our data showed that supplementation of MAMPs such as LPS and flg22 closed stomata in lettuce. The stomatal closure by MAMPs was contrastingly opposite in case of *S.* Typhimurium treatment wherein no stomatal closure was observed post 3 and 12 h post inoculation. The data shown by Roy and Melotto (2019) showed treatment by *S.* Typhimurium SL1344 negated stomatal closure in Romaine at 4 h post inoculation under both 65 and 95% relative humidity. In contrast, cell free lysate (CFL) treatment to lettuce leaves from *S.* Typhimurium showed a classical MAMP based stomatal closure compared to live cell treatments, suggesting that cell free or secreted components from *S.* Typhimurium are recognized by plants to trigger innate response. The host sensing of cell free lysate from *S.* Typhimurium SL1344 was monitored and shown with human cells, wherein cell free lysates from *S.* Typhimurium SL1344 were recognized by the hosts leading to caspase-1-mediated proteolytic cell death contributing to pathogen clearance (Shivcharan et al., 2018). Interestingly, the heat-killed CFL treatment showed similar stomatal aperture as seen previously with live *S.* Typhimurium cells, suggesting that host sensing for innate stomatal defense requires secreted exo-metabolites. A recent study has shown that under high relative humidity conditions *S.* Typhimurium SL1344 but not *E. coli* O157:H7 suppresses stomatal defense response in Romaine compared to spinach, *E. coli* O157:H7 has also been shown to induce a higher degree of immune response from *Arabidopsis* and lettuce (Roy et al., 2013; Roy and Melotto, 2019). However, through the current study it is evident that host defense suppression and host sensing are two different layers of innate immune response that follow invasion of *S.* Typhimurium in lettuce.

S. Typhimurium T3SS Components Regulate Both Host Sensing and Innate Stomatal Defense Response in Lettuce

To further test the hypothesis that *S.* Typhimurium can modulate the plant innate defense response several T3SS mutants of *S.* Typhimurium were tested with the stomatal assay. Our results showed the dependency of both SPI1 and the T3SS of SPI2 (*hilD* and *sseB*) in suppressing stomatal defense response at early time points of the exposure of *S.* Typhimurium. The mutants (*hilD* and *sseB*) are defective in SPI1, and the T3SS of SPI2, respectively. A lack of *hilD* lead to stomatal closure at time points consistent with that observed in *L. monocytogenes*-treated plants with confocal microscopy. *SseB* normally functions as a structural component of the SPI2 T3SS, and removal disrupted SPI2 functions (Ruiz-Albert et al., 2003). A strong stomatal closure was observed with lettuce leaves treated with *sseB* at 3 h post inoculation. The invasion protein mutant *invA* was able to partially suspend stomatal closure, resulting in a phenotype similar to both *S.* Typhimurium and *hilD, sseB* phenotypes.

The data shows that *S.* Typhimurium may need both SPI1 and SPI2 to fully subvert the basal immune response of *L. sativa* but may have more dependence on SPI2 rather than SPI1. Internalization assays showed that while *S.* Typhimurium was capable of internalizing and surviving on the plant surface concurrently: *fliC*, *hilD* and *sseB* did not persist on the leaf and no internalized cells were isolated within the 7-day long trial. As seen in stomatal assays *invA* was slightly better able to survive, on the surface and was isolated from the apoplast when *hilD* and *sseB* were not recovered at any time point from inside leaf samples. We concluded that *invA* lacking an essential invasion protein was partially competent as a plant pathogen and that InvA is a possible effector responsible for plant immune suppression.

Intercellular transportation and colonization by *Salmonella* is a pathogenic trait that allows rapid spread and infection in mammalian hosts and is mediated by SPI2 (Löber et al., 2006*)*. Previous work has shown that *Salmonella* ingress in plant tissues using natural openings as stomata, trichomes or injured roots (Barak et al., 2002, 2011; Deering et al., 2012). Our studies and that of others showed *S.* Typhimurium T3SS mutants have high morbidity rates on and in plants, suggesting that both SPI-1- and SPI-2-encoded apparatuses are necessary to establish robust proliferation and ingression in plants (Schikora et al., 2011). Using a brush inoculation technique, we did not observe any hypersensitive response or lesion formation with both *S.* Typhimurium and T3SS mutants in plants. Schikora et al. (2011), showed a classical HR and lesion formation followed with chlorosis using *S.* Typhimurium and T3SS mutants by using blunt injection technique. It should be mentioned that a blunt injection technique may bypass both leaf and stomatal defense response in plants. A similar study used tobacco as a model system and showed that *S.* Typhimurium, but not the T3SS mutant *invA*$^{-}$, were able to suppress the oxidative burst and the increase of extracellular pH after inoculation, suggesting that *Salmonella* actively suppresses plant defense mechanisms using the SPI-1 encoded T3SS (Shirron and Yaron, 2011). Chalupowicz et al. (2017) found no evidence that *Salmonella* can translocate effectors from plant pathogenic origin, and no HR response was generated by *Salmonella*. It does appear that *Salmonella* can influence plant physiology but is dependent on experimental protocols and does not always follow previously published doctrine.

Our data showed that treatment with *S.* Typhimurium may keep the stomates open for possible ingression, similarly, Roy et al. (2013), revealed that *S.* Typhimurium treatment of *Arabidopsis* and lettuce leaves triggered reduced stomatal closure as compared with *E. coli* (Roy et al., 2013). Similar to the results presented here, *Salmonella* treated leaves showed stronger stomatal reopening 4 h after bacterial inoculation (Roy et al., 2013; Roy and Melotto, 2019). Our studies show that *Salmonella* may keep the stomates open for longer duration and the suppression of stomatal defense is partly dependent on SPI1 triggered T3SS during the early onset of *Salmonella* exposure with lettuce plants.

ABA Has a Central Role in *Salmonella's* Suppression of Stomatal Defense for Ingression

Stomatal regulation and function are affected by abiotic, biotic and hormonal interactions, and typically, ABA plays an overriding role during stomatal closure (Acharya and Assmann, 2009). Previously, it was shown that beneficial soil microbes may mediate stomatal closure mediated through ABA (Kumar et al., 2012). Suppression of ABA biosynthetic pathway was critical for *Salmonella* mediated suppression of stomatal defense. Supplementation with ABA in a co-inoculation experiment showed that *S.* Typhimurium may override ABA's effect to close stomates. In addition, *S.* Typhimurium inoculated plants showed the downregulation of transcript levels of ABA biosynthetic genes. Specifically, biosynthetic genes such as LsNCED3, and LsABA3 were all downregulated post *S.* Typhimurium treatment at 6 h post inoculation. Both LsZEP1 and LsNCED3 are involved in early steps of ABA biosynthesis (Xiong and Zhu, 2003). LsZEP1 regulates the conversion of zeaxanthin to neoxanthin and is reported to be regulated by circadian rhythm (Taylor et al., 2000) in contrast, the regulation of LsZEP in roots is regulated under drought conditions (Taylor et al., 2000). Similarly, the expression of LsNCED3 is highly regulated under both biotic and abiotic stress regimes (Xiong and Zhu, 2003). It is also shown that the expression of LsNCED3 was early under abiotic/biotic stress regime (Qin and Zeevaart, 1999). The *S.* Typhimurium treatment to leaves of lettuce led to downregulation of LsNCED3, but not LsZEP1, post 3 and 6 h post inoculation in roots suggesting the inducible nature of some ABA biosynthetic genes, previous work has not used lettuce as a model organism when observing ABA expression following a biotic stressor. Frey et al. (2012), remarks that a slight change in ABA levels due to mutation of NCED3 can lead to reduced vegetative growth and that other NCED genes only partially contribute to ABA production if NCED3 is removed. Our data showed that *S.* Typhimurium reduced expression of NCED3 in both the roots and leaves, suggesting that both ABA biosynthesis and translocation can be modulated by *S.* Typhimurium treatment causing a change in key ABA biosynthesis genes. The ABA modulation by *S.* Typhimurium was independent of T3SS apparatus. Study with an ABA overproducer transgenic line in tomato (Sp5) showed that the decline rate of *Salmonella* on the leaf surface of Sp5 was significantly higher than that of its wild type "Ailsa Craig" (Gu et al., 2013). In contrast, there was no significant difference for the internal decline rate of Salmonella in between Sp5 and the parental line (Gu et al., 2013). How *S.* Typhimurium modulates temporal production and translocation of ABA *in planta* needs to be elucidated. There is a tempting possibility that ABA directly may impact the growth of *Salmonella in planta*, which needs to be evaluated.

B. subtilis UD1022 Prevents *S.* Typhimurium Ingression and Persistence

It was previously shown that *B. subtilis* UD1022 promotes biomass, drought tolerance, and pathogen resistance in many plant species (Kumar et al., 2012; Bais et al., 2014; Zheng et al., 2018). Recently, it was demonstrated that the addition of the rhizobacteria, *B. subtilis* UD1022 to the roots of the *A. thaliana* plants restricted the entry of the foliar pathogen *Pst*DC3000 through the stomata (Kumar et al., 2012). The root-inoculation apparently triggered an *in planta* signal that resulted in the closure of the guard cells, which was more pronounced in the presence of the foliar pathogen *Pst*DC3000 (Kumar et al., 2012). Both abscisic acid (ABA) and SA were shown to be involved in the early closure of the stomata thus minimizing the pathogen entry points on the leaf surface (Kumar et al., 2012). The influence on the stomatal closure was observed as a general phenomenon with the entire *Bacillus* species tested, which indicates that members of this

genus can modulate the stomatal phenotype. A recent study involving *B. amyloliquefaciens* FZB42 showed stomatal closure and protection in *Nicotiana benthamiana* caused by *Phytophthora nicotianae* (Wu et al., 2018). Both the studies (Kumar et al., 2012 and Wu et al., 2018) *per se* portrays the involvement of the primary signaling components, mainly SA and ABA, during the beneficial interaction of *Bacillus* spp. with the plant root and its effect on the stomatal behavior.

In this article UD1022 was utilized to prevent a foodborne pathogen from both internalizing and setting up a persisting population within lettuce. We proved that UD1022 after associating with lettuce for at least 48 h induced no adverse stress which was also observed in Kumar et al. (2012) but on *A. thaliana*. UD1022 primed lettuce showed a quick response to *S.* Typhimurium in that stomata quickly closed and remained closed for at least 12 h, while water treated plants did not exhibit a synonymous response. By observing successfully closed stomata it was determined that UD1022 had effectively induced system resistance (ISR), and work could continue to examine the actual amount of ingression, if any, and persistence of *S.* Typhimurium on UD1022 inoculated plants. Initial persistence assays showed that *S.* Typhimurium and *S.* Newport is very capable at getting into and surviving in the plant as well as surviving on the surface while T3SS mutants were not. The same was not observed on UD1022 primed plants, where *S.* Typhimurium maintained a small external population but did not internalize or perhaps survive inside the plant long enough to be detected. We hypothesized that UD1022 effectively induces ISR, which returns defense to immune limited plants, in this case lettuce. We do know that UD1022 is not outrightly toxic to *Salmonella* as shown in Markland et al. (2015). It is probable that an increase in defense related genes such as PR-1 leads to induction of ROS generation which is both a method for raising the alarm in plants and can be antagonistic to many bacteria. Evidence that UD1022 provides defense against foodborne pathogen contamination has been proven in the lab but is yet be shown in a field grown setting, although the methods used in the paper do illustrate a potential use in hydroponic systems which needs be evaluated with a more complex production system. Studies have shown that rhizobacteria could act a biocontrol against plant pathogen through enhanced plant defense response (Srivastava et al., 2016; Backer et al., 2018). A study showed that plant growth promoting rhizobacteria *B. amyloliquefaciens* (SN13) can effectively control a pathogenic plant fungus i.e., *Rhizoctonia solani in vitro* and in rice (Srivastava et al., 2016). Authors suggested that the rhizobacteria can elicit immune response in plant by modulating metabolic and biochemical pathways, which could play an important role in protecting plant against infection and pathogens. Similarly, in the current study, plants primed/inoculated with UD1022 displayed decreased internalization incidence in *Salmonella* mutants and controls, suggesting that rhizobacteria could be of importance to plant against human pathogen. Moreover, low levels of bacterial colonization observed in SPI mutants (*hilD,* and *sseB*) inoculated plants along with UD1022 indicates that these genes may play a critical role to override signal transmission in plant against *Salmonella*. In the absence of these genes, the plant growth promoting rhizobacteria UD1022 was able to express signals and plant immune response with decreased internalization and surface colonization. The peculiar response in colonization observed in *invA* further supports the notion that *invA* is involved in immune suppression but does not act alone, and UD1022's ISR is able to overcome these effects through multiple pathways. A study by Hsu and Micallef (2017) showed a similar result, where the plant growth promoting rhizobacteria i.e., *Pseudomonas* strains S2 and S4 significantly reduced *S.* Newport population on spinach, lettuce and tomato surface. The authors evaluated the wild type strain and suggested that increased leaf nitrogen content might have limited pathogen survival on the surface. Whereas, the current study revealed the effect of *S.* Typhimurium virulence genes and their interactions with rhizobacteria on its survival and persistence on lettuce surface. Furthermore, UD1022 treatments caused only transient stomatal closure which corrects by 48 h post inoculation to stomatal apertures observed on control plants (**Supplementary Figure S7**). Little change in stomatal conductance was observed on dark adapted or light adapted UD1022 treated plants compared to control plants (**Supplementary Figure S7**). The photosynthetic efficiency of UD1022 treated plants compared to those left untreated was not perturbed over a 60 h time series (data not shown). The data related to transpiration and stomatal conductance showed that application of UD1022 may not affect plants physiologically and may act as an effective biocontrol mechanism against foodborne pathogens.

CONCLUDING REMARKS

Taken together, these observations suggest that *S.* Typhimurium uses a T3SS-delivered effector protein to suppress the immune stomatal defense system. The two-tier system of stomatal innate defense response and host sensing was shown using a CFL from *S.* Typhimurium. The study suggests that exo-metabolites from *Salmonella* may be sensed by plants to trigger stomatal defense response. How *Salmonella* achieves the delivery of effectors across plant cell walls and plant plasma membranes remains unclear. However, numerous phytopathogenic bacteria (like *Pseudomonas*, *Erwinia* and *Xanthomonas* spp.) are known to utilize T3SS to deliver effector proteins across plant cell wall, indicating that the plant cell wall is not a sufficient barrier to prevent bacteria from effector delivery (Schikora et al., 2011). *Salmonella* remains an important foodborne pathogen and food safety hazard for both pre- and post-harvest conditions. Knowledge pertaining to non-host survival in the external environment is useful in development of novel strategies to mitigate risks of contamination. The use of PGPRs may elucidate a viable and safe method to affect plant-pathogen interactions.

DATA AVAILABILITY STATEMENT

The dataset is available and uploaded through dryad. NJ, HB, KK, PL (2020), Evasion of plant innate defense response by

Salmonella on Lettuce, v2, Dryad, Dataset, https://doi.org/10.5061/dryad.bg79cnp76.

AUTHOR CONTRIBUTIONS

All authors designed the experiments, edited and contributed to the final manuscript, and approved for submission. NJ completed lab work pertaining to microscopy and genetic expression. PL designed and completed persistence, internalization assays and *Bacillus subtills* efficacy trials.

FUNDING

HB and KK acknowledge the support from CANR seed Grant and from USDA ARS NACA # 58-8042-7-034.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00500/full#supplementary-material

REFERENCES

Acharya, B. R., and Assmann, S. M. (2009). Hormone interactions in stomatal function. *Plant Mol. Biol.* 69, 451–462. doi: 10.1007/s11103-008-9427-0

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* 16, 3460–3479. doi: 10.1105/tpc.104.025833

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1473. doi: 10.3389/fpls.2018.01473

Bais, H., Sherrier, D. J., and Lakshmanan, V. (2014). Compositions and methods for increasing biomass, iron concentration, and tolerance to pathogens in plants. *Cell* 2011, 1–32.

Barak, J. D., Gorski, L., Liang, A. S., and Narm, K. (2009). Previously uncharacterized *Salmonella enterica* genes required for swarming play a role in seedling colonization. *Microbiology* 155, 3701–3709. doi: 10.1099/mic.0.032029-0

Barak, J. D., Gorski, L., Naraghi-Arani, P., and Charkowski, A. O. (2005). *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. *Appl. Environ. Microbiol.* 71, 5685–5691. doi: 10.1128/aem.71.10.5685-5691.2005

Barak, J. D., Kramer, L. C., and Hao, L. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/AEM.01661-10

Barak, J. D., Whitehand, L. C., and Charkowski, A. O. (2002). Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157: H7 to alfalfa sprouts. *Appl. Environ. Microbiol.* 68, 4758–4763. doi: 10.1128/aem.68.10.4758-4763.2002

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., et al. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ. Microbiol.* 12, 2385–2397. doi: 10.1111/j.1462-2920.2010.02297.x

Beuchat, L. R. (2002). Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microb. Infect.* 4, 413–423. doi: 10.1016/s1286-4579(02)01555-1

Bishop, A. L., and Davis, R. M. (1990). Internal decay of onions caused by *Enterobacter cloacae*. *Plant Dis.* 74, 692–694. doi: 10.1094/PDIS-05-11-0375

Brandl, M. T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.* 44, 367–392. doi: 10.1146/annurev.phyto.44.070505.143359

Brandl, M. T., Cox, C. E., and Teplitski, M. (2013). *Salmonella* interactions with plants and their associated microbiota. *Phytopathology* 103, 316–325. doi: 10.1094/PHYTO-11-12-0295-RVW

CDC (2012). *Multistate Outbreak Of Listeriosis Linked to Whole Cantaloupes From Jensen Farms, Colorado*. Atlanta: CDC.

CDC (2018). *Multistate ouTbreak of Coli, E., O157:H7 Infections Linked To Romaine Lettuce*. Atlanta: CDC.

Chalupowicz, L., Nissan, G., Brandl, M. T., McClelland, M., Sessa, G., and Popov, G. (2017). Assessing the ability of *Salmonella enterica* to translocate type III effectors into plant cells. *Mol. Plant Microb. Interact* 31, 233–239. doi: 10.1094/MPMI-07-17-0166-R

Coburn, B., Grassl, G. A., and Finlay, B. B. (2007). *Salmonella*, the host and disease: a brief review. *Immunol. Cell Biol.* 85, 112–118. doi: 10.1038/sj.icb.7100007

de Moraes, M. H., Desai, P., Porwollik, S., Canals, R., Perez, D. R., and Chu, W. (2017). *Salmonella* persistence in tomatoes requires a distinct set of metabolic functions identified by transposon insertion sequencing. *Appl. Environ. Microbiol.* 83:3028. doi: 10.1128/AEM.03028-16

Deering, A. J., Mauer, L. J., and Pruitt, R. E. (2012). Internalization of coli, E., O157: H7 and Salmonella spp. in plants: a review. *Food Res. Int.* 45, 567–575. doi: 10.1016/j.foodres.2011.06.058

Ellermeier, C. D., Ellermeier, J. R., and Slauch, J. M. (2005). HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in *Salmonella enterica Serovar typhimuriu*. *Mol. Microbiol.* 57, 691–705. doi: 10.1111/j.1365-2958.2005.04737.x

Erickson, M. C. (2012). Internalization of fresh produce by foodborne pathogens. *Annu. Rev. Food Sci. Technol.* 3, 283–310. doi: 10.1146/annurev-food-022811-101211

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., and Davey, L. (2010). Surface and internalized *Escherichia coli* O157: H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. *J. Food Protect.* 73, 1023–1029. doi: 10.4315/0362-028x-73.6.1023

Frey, A., Effroy, D., Lefebvre, V., Seo, M., Perreau, F., Berger, A., et al. (2012). Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. *Plant J.* 70, 501–512. doi: 10.1111/j.1365-313X.2011.04887.x

Garcia, A. V., Charrier, A., Schikora, A., Bigeard, J., Pateyron, S., and Tauzia-Moreau, D. M. (2014). Salmonella enterica flagellin is recognized via FLS2 and activates PAMP-triggered immunity in *Arabidopsis thaliana*. *Mol. Plant* 7, 657–674. doi: 10.1093/mp/sst145

Garcia, A. V., and Hirt, H. (2014). *Salmonella enterica* induces and subverts the plant immune system. *Front. Microbiol.* 5:141. doi: 10.3389/fmicb.2014.00141

Gorski, L., Duhé, J. M., and Flaherty, D. (2009). The use of flagella and motility for plant colonization and fitness by different strains of the foodborne pathogen *Listeria monocytogenes*. *PLoS One* 4:e5142. doi: 10.1371/journal.pone.0005142

Gu, G., Cevallos-Cevallos, J. M., and van Bruggen, A. H. (2013). Ingress of *Salmonella enterica Typhimurium* into tomato leaves through hydathodes. *PLoS One* 8:e53470. doi: 10.1371/journal.pone.0053470

He, J., Baldini, R. L., Déziel, E., Saucier, M., Zhang, Q., and Liberati, N. T. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2530–2535. doi: 10.1073/pnas.0304622101

Hirneisen, K. A., Sharma, M., and Kniel, K. E. (2012). Human enteric pathogen internalization by root uptake into food crops. *Foodborn. Pathog. Dis.* 9, 396–405. doi: 10.1089/fpd.2011.1044

Hsu, C., and Micallef, S. A. (2017). Plant-mediated restriction of *Salmonella enterica* on tomato and spinach leaves colonized with *Pseudomonas* plant growth-promoting rhizobacteria. *Int. J. Food Microbiol.* 259, 1–6. doi: 10.1016/j.ijfoodmicro.2017.07.012

Iniguez, A. L., Dong, Y., Carter, H. D., Ahmer, B. M., Stone, J. M., and Triplett, E. W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant Microb. Interact* 18, 169–178. doi: 10.1094/mpmi-18-0169

Jha, A. K., Bais, H. P., and Vivanco, J. M. (2005). *Enterococcus faecalis* mammalian virulence-related factors exhibit potent pathogenicity in the *Arabidopsis thaliana* plant model. *Infect. Immun.* 73, 464–475. doi: 10.1128/iai.73.1.464-475.2005

Kriedemann, P. E., Loveys, B. R., Fuller, G. L., and Leopold, A. C. (1972). Abscisic acid and stomatal regulation. *Plant Physiol.* 49, 842–847.

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., et al. (2009). Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75, 6076–6086. doi: 10.1128/AEM.01084-09

Kumar, A. S., Lakshmanan, V., Caplan, J. L., Powell, D., Czymmek, K. J., Levia, D. F., et al. (2012). Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomat. *Plant J.* 72, 694–706. doi: 10.1111/j.1365-313x.2012.05116.x

Löber, S., Jäckel, D., Kaiser, N., and Hensel, M. (2006). Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. *Intern. J. Med. Microbiol.* 296, 435–447. doi: 10.1016/j.ijmm.2006.05.001

Markland, S. M., Ferelli, A. M., Craighead, S. A., Bais, H., and Kniel, K. E. (2015). Application of *Bacillus subtilis* to the roots of leafy greens, in the presence of *Listeria innocua* and *Salmonella* Newport, induces closure of stomata. *Foodborn. Pathog. Dis.* 12, 828–835. doi: 10.1089/fpd.2015.1952

Mattson, N. S., and Peters, C. (2014). A recipe for hydroponic success. *Inside Grower* 2014, 16–19.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980. doi: 10.1016/j.cell.2006.06.054

Meng, F., Altier, C., and Martin, G. B. (2013). *Salmonella* colonization activates the plant immune system and benefits from association with plant pathogenic bacteria. *Environ. Microbiol.* 15, 2418–2430. doi: 10.1111/1462-2920.12113

Merilo, E., Jalakas, P., Laanemets, K., Mohammadi, O., Hõrak, H., Kollist, H., et al. (2015). Abscisic acid transport and homeostasis in the context of stomatal regulation. *Mol. Plant* 8, 1321–1333. doi: 10.1016/j.molp.2015.06.006

Monaghan, J. M., and Hutchison, M. L. (2012). Distribution and decline of human pathogenic bacteria in soil after application in irrigation water and the potential for soil-splash-mediated dispersal onto fresh produce. *J. Appl. Microbiol.* 112, 1007–1019. doi: 10.1111/j.1365-2672.2012.05269.x

Ohl, M. E., and Miller, S. I. (2001). *Salmonella*: a model for bacterial pathogenesis. *Annu. Rev. Med.* 52, 259–274. doi: 10.1146/annurev.med.52.1.259

Olsen, J. E., Hoegh-Andersen, K. H., Casades, J., and Thomsen, L. E. (2012). The importance of motility and chemotaxis for extra-animal survival of *Salmonella enterica* serovar *Typhimurium* and Dublin. *J. Appl. Microbiol.* 113, 560–568. doi: 10.1111/j.1365-2672.2012.05363.x

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., et al. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerg. Infect. Dis.* 19:407. doi: 10.3201/eid1903.111866

Plotnikova, J. M., Rahme, L. G., and Ausubel, F. M. (2000). Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in *Arabidopsis*. *Plant Physiol.* 124, 1766–1774. doi: 10.1104/pp.124.4.1766

Qin, X., and Zeevaart, J. A. (1999). The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc. Natl. Acad. Sci. U.S.A.* 96, 15354–15361. doi: 10.1073/pnas.96.26.15354

Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., and Lau, G. W. (2000). Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8815–8821. doi: 10.1073/pnas.97.16.8815

Rosier, A., Medeiros, F. H., and Bais, H. P. (2018). Defining plant growth promoting rhizobacteria molecular and biochemical networks in beneficial plant-microbe interactions. *Plant Soil* 428, 35–55. doi: 10.1007/s11104-018-3679-5

Roy, D., and Melotto, M. (2019). Stomatal response and human pathogen persistence in leafy greens under preharvest and postharvest environmental conditions. *Postharvest. Biol. Technol.* 148, 76–82. doi: 10.1016/j.postharvbio.2018.10.013

Roy, D., Panchal, S., Rosa, B. A., and Melotto, M. (2013). *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. *Phytopathology* 103, 326–332. doi: 10.1094/PHYTO-09-12-0230-FI

Ruiz-Albert, J., Mundy, R., Yu, X., Beuzon, C. R., and Holden, D. W. (2003). SseA is a chaperone for the SseB and SseD translocon components of the *Salmonella* pathogenicity-island-2-encoded type III secretion system. *Microbiology* 149, 1103–1111. doi: 10.1099/mic.0.26190-0

Schikora, A., Carreri, A., Charpentier, E., and Hirt, H. (2008). The dark side of the salad: *Salmonella Typhimurium* overcomes the innate immune response of *Arabidopsis thaliana* and shows an endopathogenic lifestyle. *PLoS One* 3:e2279. doi: 10.1371/journal.pone.0002279

Schikora, A., Garcia, A. V., and Hirt, H. (2012). Plants as alternative hosts for *Salmonella*. *Trends Plant Sci.* 17, 245–249. doi: 10.1016/j.tplants.2012.03.007

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., and Charrier, A. (2011). Conservation of *Salmonella* infection mechanisms in plants and animals. *PLoS One* 6:e24112. doi: 10.1371/journal.pone.0024112

Sharma, M., Millner, P. D., Hashem, F., Camp, M., Whyte, C., Graham, L., et al. (2016). Survival and persistence of nonpathogenic *Escherichia coli* and attenuated *Escherichia coli* O157:H7 in soils amended with animal manure in a greenhouse environment. *J. Food Prot.* 79, 913–921. doi: 10.4315/0362-028X.JFP-15-421

Shirron, N., and Yaron, S. (2011). Active suppression of early immune response in tobacco by the human pathogen *Salmonella Typhimurium*. *PLoS One* 6:e18855. doi: 10.1371/journal.pone.0018855

Shivcharan, S., Yadav, J., and Qadri, A. (2018). Host lipid sensing promotes invasion of cells with pathogenic *Salmonella*. *Sci. Rep.* 8:15501. doi: 10.1038/s41598-018-33319-9

Silverman, M., and Simon, M. (1980). Phase variation: genetic analysis of switching mutants. *Cell* 19, 845–854. doi: 10.1016/0092-8674(80)90075-6

Srivastava, S., Bist, V., Srivastava, S., Singh, P. C., Trivedi, P. K., Asif, M. H., et al. (2016). Unraveling aspects of *Bacillus amyloliquefaciens* mediated enhanced production of rice under biotic stress of *Rhizoctonia solani*. *Front. Plant Sci.* 7:587. doi: 10.3389/fpls.2016.00587

Taylor, I. B., Burbidge, A., and Thompson, A. J. (2000). Control of abscisic acid synthesis. *J. Exp. Bot.* 51, 1563–1574. doi: 10.1093/jexbot/51.350.1563

Van Gardingen, P. R., Jeffree, C. E., and Grace, J. (1989). Variation in stomatal aperture in leaves of Avena fatua observed, L., by low-temperature scanning electron microscopy. *Plant Cell Environ.* 12, 887–898. doi: 10.1111/j.1365-3040.1989.tb01968.x

Wu, L., Huang, Z., Li, X., Ma, L., Gu, Q., Wu, H., et al. (2018). Stomatal closure and SA-, JA/ET-signaling pathways are essential for *Bacillus amyloliquefaciens* FZB42 to restrict leaf disease caused by *Phytophthora nicotianae* in *Nicotiana benthamiana*. *Front. Microbiol.* 9:847. doi: 10.3389/fmicb.2018.00847

Xiong, L., and Zhu, J. (2003). Regulation of abscisic acid biosynthesis. *Plant Physiol.* 133, 29–36. doi: 10.1104/pp.103.025395

Zeng, W., and He, S. Y. (2010). A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to *Pseudomonas syringae* pv tomato DC3000 in *Arabidopsis*. *Plant Physiol.* 153, 1188–1198. doi: 10.1104/pp.110.157016

Zheng, J., Allard, S., Reynolds, S., Millner, P., Arce, G., Blodgett, R. J., et al. (2013). Colonization and internalization of *Salmonella enterica* in tomato plants. *Appl. Environ. Microbiol.* 79, 2494–2502. doi: 10.1128/AEM.03704-12

Zheng, W., Zeng, S., Bais, H., LaManna, J. M., Hussey, D. S., Jacobson, D. L., et al. (2018). Plant growth-promoting rhizobacteria (PGPR) reduce evaporation and increase soil water retenti. *Water Resour. Res.* 54, 3673–3687. doi: 10.1029/2018wr022656

Conflict of 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.

frontiers in Plant Science

REVIEW
published: 15 April 2020
doi: 10.3389/fpls.2020.00428

Breeding Crops for Enhanced Food Safety

Maeli Melotto[1], Maria T. Brandl[2], Cristián Jacob[1], Michele T. Jay-Russell[3], Shirley A. Micallef[4], Marilyn L. Warburton[5] and Allen Van Deynze[6]*

[1] Department of Plant Sciences, University of California, Davis, Davis, CA, United States, [2] United States Department of Agriculture-Agricultural Research Service, Produce Safety and Microbiology Research, Albany, CA, United States, [3] Western Center for Food Safety, University of California, Davis, Davis, CA, United States, [4] Department of Plant Science and Landscape Architecture, Center for Food Safety and Security Systems, University of Maryland, College Park, MD, United States, [5] United States Department of Agriculture-Agricultural Research Service, Corn Host Plant Research Resistance Unit Mississippi State, Starkville, MS, United States, [6] Plant Breeding Center, Department of Plant Sciences, University of California, Davis, Davis, CA, United States

An increasing global population demands a continuous supply of nutritious and safe food. Edible products can be contaminated with biological (*e.g.*, bacteria, virus, protozoa), chemical (*e.g.*, heavy metals, mycotoxins), and physical hazards during production, storage, transport, processing, and/or meal preparation. The substantial impact of foodborne disease outbreaks on public health and the economy has led to multidisciplinary research aimed to understand the biology underlying the different contamination processes and how to mitigate food hazards. Here we review the knowledge, opportunities, and challenges of plant breeding as a tool to enhance the food safety of plant-based food products. First, we discuss the significant effect of plant genotypic and phenotypic variation in the contamination of plants by heavy metals, mycotoxin-producing fungi, and human pathogenic bacteria. In addition, we discuss the various factors (*i.e.*, temperature, relative humidity, soil, microbiota, cultural practices, and plant developmental stage) that can influence the interaction between plant genetic diversity and contaminant. This exposes the necessity of a multidisciplinary approach to understand plant genotype × environment × microbe × management interactions. Moreover, we show that the numerous possibilities of crop/hazard combinations make the definition and identification of high-risk pairs, such as *Salmonella*-tomato and *Escherichia coli*-lettuce, imperative for breeding programs geared toward improving microbial safety of produce. Finally, we discuss research on developing effective assays and approaches for selecting desirable breeding germplasm. Overall, it is recognized that although breeding programs for some human pathogen/toxin systems are ongoing (*e.g., Fusarium* in wheat), it would be premature to start breeding when targets and testing systems are not well defined. Nevertheless, current research is paving the way toward this goal and this review highlights advances in the field and critical points for the success of this initiative that were discussed during the Breeding Crops for Enhanced Food Safety workshop held 5–6 June 2019 at University of California, Davis.

Keywords: food safety, crop improvement, plant breeding, enterobacterium, mycotoxins, heavy metals, human pathogens on plants, allergens

OPEN ACCESS

Edited by:
Soren K. Rasmussen, University of Copenhagen, Denmark

Reviewed by:
Martha Vaughan, United States Department of Agriculture (USDA), United States
Zerihun Tadele, University of Bern, Switzerland

****Correspondence:***
Maeli Melotto
melotto@ucdavis.edu

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *27 January 2020*
Accepted: *24 March 2020*
Published: *15 April 2020*

Citation:
Melotto M, Brandl MT, Jacob C, Jay-Russell MT, Micallef SA, Warburton ML and Van Deynze A (2020) Breeding Crops for Enhanced Food Safety. Front. Plant Sci. 11:428. doi: 10.3389/fpls.2020.00428

INTRODUCTION

The demand for nutritious and safe food will increase as the human population is expected to reach between 9.4 and 10.1 billion in 2050 and between 9.4 and 12.7 billion in 2100 (United Nations [UN], Department of Economic, and Social Affairs, Population Division., 2019a), along with increasing urbanization and standards of living (United Nations [UN], Department of Economic, and Social Affairs, Population Division, 2019b). Healthy consumption of grains, oilseeds, nuts, and fresh fruits and vegetables is part of an integrated strategy to decrease the risk for diet-related chronic diseases, such as cardiovascular disease, type 2 diabetes, some types of cancer, and obesity (U.S. Department of Health and Human Services-U.S. Department of Agriculture [HHS-USDA], 2015). However, the World Health Organization (WHO) report shows that at global level, 31 hazards caused 600 million foodborne illnesses and 420,000 deaths in 2010 (World Health Organization [WHO], 2015). Health concerns exist due to the consumption of mycotoxins produced by fungi that frequently infect grain, oilseed, and nut crops (Binder et al., 2007; Marin et al., 2013). The health burdens placed on consumers and economic burdens placed on farmers and processors by the presence of these toxins can be severe (Schaafsma, 2002; Wu, 2007; Zain, 2011). Furthermore, heavy metals (*e.g.,* cadmium and arsenic), allergens (*e.g.*, actinidin and Ara h proteins), and accumulations of natural molecules and compounds (*e.g.*, nitrates, cyanoglycosides, vicine and convicine, gluten, and Kunitz trypsin inhibitor) may be detrimental to human health.

The fresh market has resulted in a wide variety of fresh fruits and vegetables available throughout the year (Huang, 2013; Siegel et al., 2014). At the same time, the number of foodborne disease outbreaks related to consumption of contaminated fresh or minimally processed produce has been increasing (Nguyen et al., 2015; Bennett et al., 2018; Turner et al., 2019). In the United States, 48 million illnesses and 3000 deaths associated with food-borne diseases occur annually, with approximately one half associated with crops (Painter et al., 2013). In the European Union, during the period 2004–2012, there were 198 outbreaks linked to the consumption of fresh produce (Callejón et al., 2015). Beyond the burden on public health, foodborne illness outbreaks negatively affect the economics of the industry. It is estimated that the overall cost of food safety incidences for the economy of the United States is $7 billion per year, which comes from notifying consumers, removing food from shelves, and paying damages from lawsuits (Hussain and Dawson, 2013). Furthermore, a single produce-borne disease outbreak can trigger a sharp decrease in the market of the affected crop for years (Calvin et al., 2004; Arnade et al., 2009; Ribera et al., 2012).

Following a number of large multistate foodborne disease outbreaks linked to contaminated fresh produce[1], the American Phytopathological Society-Public Policy Board (APS-PPB) convened the first formal activity in 2007 in a symposium titled "Cross Domain Bacteria: Emerging Threats to Plants, Humans, and Our Food Supply"[2]. A working group on "Human Pathogens on Plants" was assembled to create solutions for this problem and has since convened as a satellite meeting during annual APS meetings. Similar activities have been conducted in Europe through the COST Action on "Control of Human Pathogenic Micro-organisms in Plant Production Systems"[3].

Leafy greens are annually involved in food safety incidents in the United States. From 1996 to 2016, 134 confirmed incidents, including 46 outbreaks, were identified to be linked to products from California (Turner et al., 2019) that provides one-third of the vegetables and two-thirds of the fruit and nuts in the United States according to the California Department of Agriculture (CDFA), California Agricultural Production Statistics[4]. During this period, lettuce and spinach were reported as the main vehicles of food safety incidents (39 and 26%, respectively; Turner et al., 2019). After three major outbreaks in 2006, the leafy green industries in Arizona and California created the Leafy Green Marketing Agreement (LGMA) with evidence-based food safety metrics that are updated to incorporate the most current state-of-the-science[5]. Likewise, the U.S. Food and Drug Administration (FDA) subsequently implemented the Food Safety Modernization Act (FSMA) to address the significant public health burden of preventable foodborne diseases. Under FSMA, the Produce Safety Rule established, for the first time, science-based minimum standards that include on-farm regulation of fresh fruits and vegetables grown for human consumption[6].

Food safety is a complex issue that requires a concerted effort among scientists, regulators, seed/nursery industry, processors, retailers, and other stakeholders from diverse disciplines and research fields who do not often have the opportunity to meet and discuss global, comprehensive, and objective solutions. On 5–6 June 2019, the University of California, Davis hosted the first workshop on Breeding Crops for Enhanced Food Safety[7] to identify knowledge gaps and research priorities in this emerging field to inform the USDA-NIFA and other agencies for funding and research priorities. This workshop connected plant scientists, plant breeders, extension specialists, microbiologists, and food safety experts from industry and academia to discuss collaborative efforts and multidisciplinary approaches geared toward preventing the occurrence of hazardous microbes, mycotoxins, elements, and allergens in crop and food production systems. Together, these pivotal steps by academia, industry, and government groups have laid out the opportunities to enhance food safety with

[1]https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html

[2]http://www.apsnet.org/members/outreach/ppb/Pages/FoodSafety.aspx

[3]http://www.cost.eu/COST_Actions/ca/CA16110?

[4]https://www.cdfa.ca.gov/statistics/

[5]https://lgma.ca.gov/food-safety-program

[6]https://www.fda.gov/food/food-safety-modernization-act-fsma/fsma-final-rule-produce-safety

[7]https://melotto.ucdavis.edu/nifa-workshop-2019

plant breeding and created avenues for unique collaborative efforts and new research directions, which formed the basis for this review.

BREEDING RESEARCH TO UNDERSTAND THE OPPORTUNITIES TO REDUCE FOOD SAFETY ISSUES ASSOCIATED WITH CROPS

The presence of mycotoxins, elements, and allergens in affected food crops has a high potential for mitigation via plant breeding (Zhou et al., 2013; Warburton and Williams, 2016; Arias et al., 2018; Gaikpa and Miedaner, 2019). These substances, produced by the fungus, the plant itself, or taken up by the plant from the environment, are generally not defense compounds, but can be severely detrimental to the health of humans and animals who consume the crop in which the substances have accumulated. Crop varieties that do not support growth of the fungi that produce mycotoxins have been created in some cases (*i.e.*, aflatoxin resistant maize; Williams and Windham, 2012) and heritability is sufficiently high for genetic gain in others (*e.g.*, *Fusarium* resistant wheat; Petersen et al., 2016). Additionally, it may be possible to create host plants that do not allow or create the need for the fungi to produce mycotoxins. The level of allergens in crop plants can also be reduced in some cases via plant breeding, or in others, via genetic engineering or gene editing (*e.g.*, removal of peanut allergens via transformation; Sáiz et al., 2013) and breeding for wheat varieties that do not accumulate heavy metals (Liu et al., 2019). Furthermore, breeding efforts are conducted for the reduction of antinutritional compounds, such as vicine and convicine in faba bean (Hazaei et al., 2019) and the acrylamide-forming potential of potatoes (Bethke, 2018).

Mounting evidence suggests that zoonotic bacterial pathogens of humans (*e.g.*, non-typhoidal *Salmonella enterica* and *Escherichia coli* O157:H7) may have adapted to both animal and plant hosts, enabling them to survive in the food production chain (Brandl, 2006; Barak and Schroeder, 2012; Sapers and Doyle, 2014; Wiedemann et al., 2015). For example, romaine lettuce and other leafy greens continue to be linked to *E. coli* O157:H7 outbreaks traced to major leafy green production regions in Arizona and California despite widespread implementation of LGMA food safety practices; moreover, traceback and environmental assessments suggest that contamination is occurring at the pre-harvest level, but root causes remain elusive (California Department of Public Health, Emergency Response Unit [CDPH], 2010, 2014; Centers for Disease Control and Prevention [CDC], 2018; U.S. Food and Drug Administration [FDA], 2018, 2020). A few research groups have discovered phenotypic variability in the interaction between these pathogens and fresh produce, suggesting that plant genetic traits may affect plant susceptibility or tolerance to human pathogen colonization (**Table 1**). A complete description of the methods used in each study is listed in **Supplementary Table S1**. Similarly to the examples of breeding strategies described above, these reports support the basis for breeding (*i.e.*, genetic variability) for decreased microbial hazards in several systems.

PLANT BREEDING TO ADDRESS THE PRE-HARVEST COMPONENT OF A SYSTEMS APPROACH REQUIRED TO MANAGE FOOD SAFETY

Host plant resistance to the fungi that produce mycotoxins can be a synergistic part of a systems approach to reducing mycotoxins in crop plants (Grace et al., 2015). The method is economical for the farmer because it requires no additional equipment or supplies and is integral to the seed itself. It works well with other methods for controlling mycotoxins, including the use of biocontrol agents that farmers can buy and apply to the field, and proper handling and environmental conditions during harvest, drying, and storage, which can also help prevent the growth of the fungi. There has been a long history of breeding wheat for resistance to *Fusarium graminearum* that produces deoxynivalenol (DON; Moghimi et al., 2019), although complete resistance remains elusive. Significant progress has also been made, for example, in pre-breeding germplasm in maize that does not support the production of aflatoxin or significantly reduces it compared to conventional maize varieties. These traits are being introgressed into U.S. maize inbreds by Paul Williams and Marilyn Warburton at USDA–ARS, Mississippi, and Seth Murray and Wenwei Xu at Texas A&M University. Heritable plant traits that reduce the numbers of harmful human pathogen cells on the edible portions of the plant may also be incorporated into a system designed to reduce risk from these microorganisms without negatively influencing the other components of the system. Similarly, Charlie Brummer and Allen Van Deynze, with support from Richard Smith (University of California, Davis) have identified and are breeding lines of spinach that have reduced accumulation of cadmium, a heavy metal found in some soils in California that can have chronic health effects, especially in children. Wheat varieties that accumulate low levels of cadmium are being developed using the latest genomic and phenotyping technologies (Liu et al., 2019).

The fresh produce industry faces several major challenges related to controlling risks from in-field contamination of crops by zoonotic enteric pathogens (Beuchat, 1996; Cooley et al., 2007; Jay-Russell, 2013; Jeamsripong et al., 2019). First, zoonotic fecal-borne pathogens may be widespread in the environment, but rarely detected in field crop, thus making it difficult to precisely define the most important direct and indirect routes of contamination (e.g., agriculture water, animal intrusion, bioaerosols, soil amendments, etc.). Second, if bacterial contamination occurs in the field, there is no subsequent "kill step" for many popular produce items such as salad greens that are consumed raw or minimally processed. Third, the infectious dose for these pathogens may be low, especially among vulnerable populations such as young children (Tuttle et al., 1999). Although it may seem improbable that a low level of in-field contamination could result in large numbers of human

TABLE 1 | A comprehensive list of studies focused on the effect of plant genotypic variation in the interaction between plants and human pathogenic bacteria.

Classification	Plant genotypes	Pathogen genotype(s)	References
Seeds	Alfalfa, fenugreek, lettuce (cultivar Iceberg), spinach, and tomato (cultivar Roma)	*Escherichia coli* serotypes O157:H7 (strains F4546, K4499, and H1730) and O104:H4 (strain BAA-2326). *Salmonella enterica* serovars Baildon, Cubana, Montevideo, and Stanley	Cui et al., 2017
Sprouts and seedlings	Alfalfa, fenugreek, lettuce (cultivar Iceberg), spinach, and tomato (cultivar Roma)	*E. coli* serotypes O157:H7 (strains F4546, K4499, and H1730) and O104:H4 (strain BAA-2326). *S. enterica* serovars Baildon, Cubana, Montevideo, and Stanley	Cui et al., 2018
	Broccoli, carrot, cilantro, endive, lettuce (cultivars Balady Aswan, Salinas 88, Little Gem, PI251246, Pavane, Valmaine, Iceberg, La Brillante, Paris Island, and Parade, Calmar), tomato (cultivars Brandywine, Amish Paste, Money Maker, Rose, Soldacki, Stupice, Green Grape, San Marzano, Nyarous, and Yellow Pear), parsley, radicchio, radish, spinach, and turnip	*S. enterica* serovars Baildon, Cubana, Eteritidis, Havana, Mbandaka, Newport, Poona, and Schwarzengrund; eight strains cocktail	Barak et al., 2008
	Lettuce (cultivars Vaila-Winter Gem, Lobjoits Green, Marshall, Little Gem, Dazzle, Unrivaled, Rosseta, Lakeland, Regina dei Ghiacci, Webbs Wonderful, Set, and Lollo Rossa)	*E. coli* serotype O157:H7 (bioluminescent strain Tn5 luxCDABE)	Quilliam et al., 2012
	Lettuce (cultivars Tamburo, Nelly, and Cancan)	*S. enterica* serovars Dublin, Typhimurium, Enteritidis, Newport, and Montevideo	Klerks et al., 2007
	Tomato (cultivars, CA Red Cherry, Heinz-1706, Moneymaker, Nyagous, Micro-Tom, Florida 91VFF, Rutgers Select, Rutgers VFA, Virginia Sweets, Plum Dandy VF. Genotypes LA4013, Movione, and Mobox)	*S. enterica* serovars Newport and Typhimurium	Han and Micallef, 2014
	Tomato (cultivars H7996, Yellow Pear, and Nyagous) and *Solanum pimpinellifolium* (cultivar WVa700)	*S. enterica* serovars Baildon, Cubana, Eteritidis, Havana, Mbandaka, Newport, Poona, and Schwarzengrund; eight strains cocktail	Barak et al., 2011
Mature leaves	Arugula, basil, lettuce (iceberg and romaine types and cultivar Ruby Red), parsley, tomato (cultivar MP1)	*S. enterica* serovar Typhimurium (strain SL1344 expressing green fluorescent protein)	Golberg et al., 2011
	Basil, cilantro, lettuce (butterhead and romaine types), and spinach	*E. coli* serotype O157:H7 (strain 86-24). *S. enterica* serovar Typhimurium (strain SL1344)	Roy and Melotto, 2019
	Cabbage (red type), lettuce (green leafy), and spinach	*S. enterica* serovars Enteritidis (strain ME18), Newport (strain 11590), and Typhimurium (strain χ3985 Δcrp-11)	Erickson and Liao, 2019
	Corn salad (cultivar Verte à coeur plein 2) and lettuce (cultivar Tizian) and	*S. enterica* serovar Typhimurium (strain 14028s)	Jechalke et al., 2019
	Lettuce (cultivars Saladin and Iceberg) and *Lactuca serriola* (accession US96UC23)	*S. enterica* serovar Senftenberg (strain 070885)	Hunter et al., 2015
	Lettuce (romaine types line RH08-0464 and cultivar Triple Threat)	*E. coli* serotype O157:H7 (strain ATCC43888)	Simko et al., 2015
	Lettuce (cultivars Gabriella, Green Star, Muir, New Red Fire, Coastal Star, Starfighter, Tropicana, and Two Star)	*E. coli* serotype O157:H7 (USDA 5, MD56, and MD58). *S. enterica* serovars Enteritidis (strain ME 18), Newport (strain 11590K), and Typhimurium (strains χ3985 Δcrp-11 and Δcya-12)	Erickson et al., 2019
	Lettuce (cultivars Salinas, Emperor, La Brillante, Lollo Rossa, Red Tide, Grand Rapids, Green Towers, and Bibb and accession 13G640-1) and *L. serriola* (accessions 12G239-1 and UC23US96)	*E. coli* serotype O157:H7 (strain 86-24). *S. enterica* serovar Typhimurium (strain 14028s)	Jacob and Melotto, 2020
	Spinach (cultivars Tyee, Space, and Bordeaux)	*E. coli* serotype O157:H7 (strains ATCC 43888, EO122, K3995, K4492, and F4546); five strains cocktail	Mitra et al., 2009
	Spinach (cultivars Emilia, Waitiki, Lazio, and Space)	*E. coli* serotype O157:H7 (strain EDL933)	Macarisin et al., 2013

(Continued)

TABLE 1 | Continued

Classification	Plant genotypes	Pathogen genotype(s)	References
	Spinach (cultivars Whale, Shasta, Barbosa, and Avenger)	*E. coli* (generic strains TVS 353, 354, and 355); individually and three strains cocktail. *E. coli* serotype O157:H7 (strains ATCC 700728 and ATCC 43888); two strains cocktail	Gutiérrez-Rodríguez et al., 2011
	Tomato (cultivars Florida Lanai, Crown Jewel, and Alisa Craig)	*S. enterica* serovar Typhimurium (strain MAE110)	Gu et al., 2013
Fruits	Cucumber (cultivars Marketmore 97, Patio Snacker, Corinto, Bella, Pepinex, and Summer Dance)	*S. enterica* serovars Newport (strain MDD 314) and Javiana (strain ATCC BAA-1593)	Callahan and Micallef, 2019
	Melon (cultivars Arava, Athena, Dulce Nectar, Jaune de Canaries, and Sivan)	*S. enterica* serovar Newport	Korir et al., 2019
	Melon (cultivars Burpee's Ambrosia, Hale's Best, Hearts of Gold, Israel Old Original, and Sweet 'n Early)	*S. enterica* serovar Thompson. *E. coli* serotype O157:H7	Duffy et al., 2008
	Melon (cultivars Oro Rico, Top Mark, and Summer Dew)	*S. enterica* serovar Typhimurium (strain aPTVS150)	Lopez-Velasco et al., 2012a
	Melon and watermelon	*E. coli* serotype O157:H7 (strains 04P,30lC, 505B, and 5753-35); four strain cocktail	Del Rosario and Beuchat, 1995
	Melon (types cantaloupe and honeydew) and watermelon	*S. enterica* serovars Anatum, Chester, Havana, Poona, and Senftenberg); five strain cocktail	Golden et al., 1993
	Tomato (cultivars Bonny Best, Florida-47, and Solar Fire)	*S. enterica* serovar Typhimurium (strain 14028); individually. *S. enterica* serovars Javiana (strain ATCC BAA-1593), Montevideo (strain LJH519), Newport (strain C6.3), and Braenderup (strains 04E01347, 04E00783, and 04E01556); six strain cocktail	Devleesschauwer et al., 2017
	Tomato (cultivars Bonny Best, Florida-47, and Solar Fire)	*S. enterica* serovar Typhimurium (strain 14028); individually. *S. enterica* serovars Javiana (strain ATCC BAA-1593), Montevideo (strain LJH519), Newport (strain C6.3), and Braenderup (strains 04E01347, 04E00783, and 04E01556); six strain cocktail	Marvasi et al., 2013
	Tomato (cultivars, CA Red Cherry, Heinz-1706, Moneymaker, Nyagous, Micro-Tom, Florida 91VFF, Rutgers Select, Rutgers VFA, Virginia Sweets, Plum Dandy VF. Genotypes LA4013, Movione, and Mobox)	*S. enterica* serovar Typhimurium (strain LT2)	Han and Micallef, 2016
	Tomato (cultivars Alisa Craig, Amish Salad, Beefsteak, Bloody Butcher, Bonny Best, Brown Berry, Campari, Celebrity, Cocktails on Vine, Early Wonder, Florida74, Florino, Glacier, Hawaii 7997, HP/HP, John Baer, Kumato, Large Red Cherry, Mariana, Marmande, Money Maker, Never Ripe, Pearson, Red Calabash, Sebring, Snow White, Solar Fire, Solar Set, Success, Sun Gold, Tasti-Lee, and Tommy Toe)	*S. enterica* serovar Typhimurium (strain 14028); individually. *S. enterica* serovars Javiana (strain ATCC BAA-1593), Montevideo (strain LJH519), Newport (strain C6.3), and Braenderup (strains 04E01347, 04E00783, and 04E01556); six strain cocktail	Marvasi et al., 2014b
	Tomato (cultivars Bonny Best, Florida-47, and Solar Fire) and pepper (cultivar Aristotle)	*S. enterica* serovar Typhimurium (strain 14028); individually. *S. enterica* serovars Javiana (strain ATCC BAA-1593), Montevideo (strain LJH519), Newport (strain C6.3), and Braenderup (strains 04E01347, 04E00783, and 04E01556); six strain cocktail	Marvasi et al., 2015
	Tomato (cultivars Campari, Hawaii 7997, and Bonny Best)	*S. enterica* serovar Typhimurium (strain 14028)	Noel et al., 2010
	Tomato (cultivars Solar Fire and Sebring)	*S. enterica* serovar Typhimurium (strain 14028); individually. *S. enterica* serovars Javiana (strain ATCC BAA-1593), Montevideo (strain LJH519), Newport (strain C6.3), and Braenderup (strains 04E01347, 04E00783, and 04E01556); six strain cocktail	Marvasi et al., 2014a

foodborne illnesses, Danyluk and Schaffner (2011) developed a quantitative risk assessment model that predicted that exposure to levels of *E. coli* O157:H7 in the field—as low as -1 log CFU/g and 0.1% prevalence—could result in a nationwide outbreak in combination with postharvest contributing factors such as cross-contamination during the washing process.

These challenges underscore the critical need to identify novel approaches to prevent or reduce the public health risk from pre-harvest microbial contamination of fresh produce. Although to date, no breeding program has adopted strategies to control human pathogens on fresh produce, a few studies have taken steps in this direction. For instance, Shirley Micallef (University of Maryland) is exploring cultivar variability in fatty acid content in tomato fruit as a means to reduce the favorability of tomato fruit for *Salmonella* (Han and Micallef, 2016). Maeli Melotto (University of California, Davis) is screening lettuce germplasm for susceptibility or tolerance to *E. coli* O157:H7 and *S. enterica* to define the genetic basis for the persistence of these pathogens in leafy vegetables (Jacob and Melotto, 2020). Additionally, in collaborative studies with USDA-ARS, Salinas, CA, United States and FDA-CFSAN, Laurel, MD, Maria Brandl (USDA-ARS, Albany, CA, United States) has been investigating lettuce cultivars in relation to basal plant defense responses to plant pathogen infection and to processing for their role in enteric pathogen colonization (Simko et al., 2015; Leonard et al., unpublished).

RESEARCH TO DEFINE AND FOCUS ON HIGH-RISK CROP–HAZARD PAIRS

Given the complexity of produce safety issues and the need to prioritize efforts for the highest impact, a logical step would be to identify the crop–hazard pairs (*e.g.*, human pathogen–fresh produce) that create the largest burden on public health and the economy. Typically, the severity of an outbreak is estimated by the number of illnesses, hospitalizations, and deaths. With a hazard × occurrence (probability of infection or accumulation of toxin) risk model, one can begin classifying crop/hazard pairs. Although these are relevant metrics, it is very difficult to calculate the relative risk of each crop–hazard pair due to the low re-occurrence of particular pairs associated with outbreak events and the need to accumulate a substantial amount of data over extended periods of time (sometimes decades). Nonetheless, potential targets for plant breeding that are being identified may be the basis of future research to reduce human pathogens, mycotoxins, heavy metals, toxic elements, and allergens in foods.

Currently, the National Outbreak Reporting System (NORS[8]) of the Centers for Disease Control and Prevention reports disease outbreaks in the United States and maintains a comprehensive searchable database with information spanning from 1998 to 2017. Using this resource, we have generated a heatmap illustrating the relative importance of the major fresh produce in combination with reported etiological agents of outbreaks (**Figure 1** and accompanying raw data in **Supplementary Table S2**). Hierarchical clustering analysis (R heatmap.2 package) of the etiological agents revealed *Salmonella*, Norovirus, and *Escherichia* as the three most important biological hazards based on the number of outbreaks, illnesses, hospitalizations, and deaths (**Figure 1**). In addition, the compilation of these data has enabled the identification of high priority pairs (*i.e.*, lettuce–*E. coli* O157:H7, tomato–*Salmonella*, melon–*Salmonella*, and melon–*Listeria*) for breeding programs geared toward improving microbial safety of produce (**Figure 1**). These systems have been studied at the genetic level by Jeri Barak (University of Wisconsin-Madison), Maria Brandl (USDA, ARS, Albany, CA, United States), Maeli Melotto (University of California, Davis), and Shirley Micallef (University of Maryland, College Park). For instance, it has been discovered that certain varieties of tomato (Marvasi et al., 2013, 2014b; Han and Micallef, 2014), lettuce (Klerks et al., 2007; Quilliam et al., 2012; Simko et al., 2015; Jacob and Melotto, 2020), cucumbers (Callahan and Micallef, 2019), and melons (Korir et al., 2019) are less likely to support pathogen populations than others, suggesting a plant genetic component underlying these traits (**Table 1** and **Supplementary Table S1**). Bacterial serotype and strain specificities to plants have also been uncovered (Klerks et al., 2007; Cui et al., 2018; Erickson and Liao, 2019; Wong et al., 2019). Identifying the molecular mechanisms underlying these interactions can point to promising plant traits to further explore and integrate in plant breeding programs. Encouraging commercial production of plant varieties that carry relevant traits without compromising other aspects of plant productivity and product marketing might help reduce illness from produce.

In the area of mycotoxin contamination, *Fusarium* in wheat is an annual occurrence with prevalence determined by local weather at crop maturity (GIPSA, 2006). Aflatoxin in maize is regional and limited to more hot and humid regions, but remains relatively low in the main U.S. corn belt. However, on a global scale, up to 80% of maize seed lots can be contaminated in tropical areas such as Sub-Saharan Africa and India (GIPSA, 2006). Peanuts have similar occurrence of aflatoxin in areas such as East Africa. Heavy metals are predicted to continue to be a problem as arable land becomes increasingly scarce due to desertification and urbanization, and lands or irrigation water with heavy metals are more extensively used (Arora et al., 2008). These hazards can also be prioritized and paired with the crops in which the highest occurrence makes them the greatest human health hazards (**Figure 1**).

MULTIDISCIPLINARY APPROACH TO UNDERSTANDING PLANT GENOTYPE × ENVIRONMENT × MICROBE × MANAGEMENT INTERACTIONS

A multidisciplinary approach will be necessary to develop plant breeding research programs since the occurrence of a contamination event depends on the interaction of several factors such as plant genotype, environmental conditions, the microbe and its community, and plant management practices. Together,

[8]https://www.cdc.gov/nors/

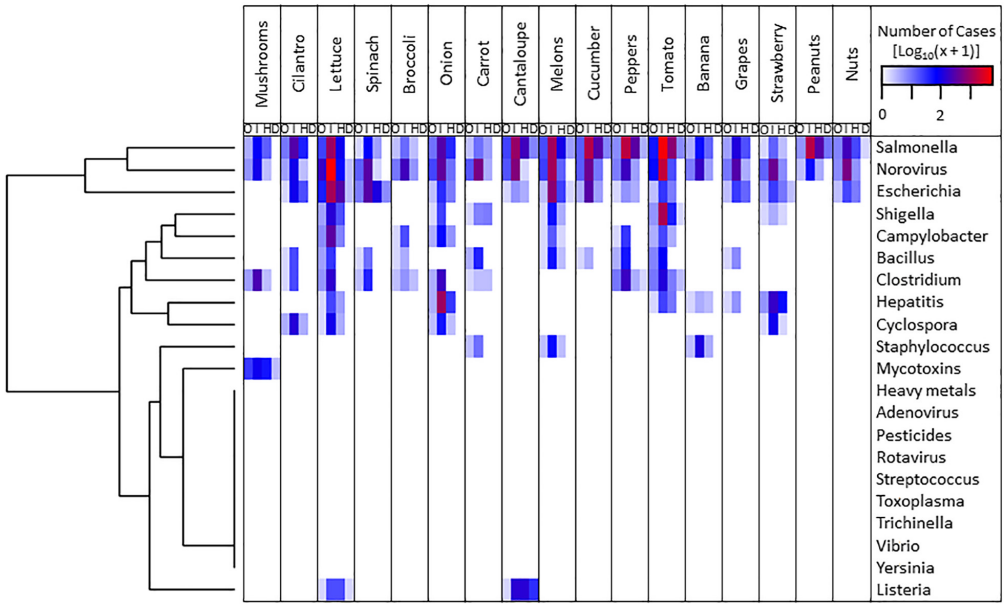


FIGURE 1 | Number of outbreak (O), illness (I), hospitalization (H), and death (D) episodes of human diseases caused by the consumption of fresh produce contaminated with different etiological agents between 1998 and 2017 in the United States, according to the National Outbreak Reporting System database (https://www.cdc.gov/nors/index.html). Data were transformed with the $Log_{10}(x + 1)$ function. The plot was constructed with the heatmap.2 package of R using hierarchical clustering analysis for etiological agents.

these variables may create "The Perfect Storm." Interactions between enteric pathogens and plants affect all mitigation strategies aimed at inhibiting pathogen growth and survival on crops to improve their microbial safety. Below, we discuss various hurdles and important aspects of these interactions that must be considered to ensure the success of a plant breeding program for enhanced crop safety.

One of the most significant challenges in breeding crops to decrease the risk of contamination with enteric pathogens is that they have lower fitness on plants than most well-characterized plant commensal and pathogenic bacterial species. Nevertheless, given the recurrence of food-borne illness outbreaks linked to produce (**Figure 1**), the ability of enteric pathogens to multiply and survive as epiphytes and endophytes implies that particular plant phenotypes and genotypes can affect their fitness in the plant habitat (**Table 1**). For example, the composition of substrates available on fruit and leaf surfaces as well as in their internal tissue (Brandl and Amundson, 2008; Marvasi et al., 2014b; Crozier et al., 2016; Han and Micallef, 2016); the density of trichomes, stomata, and veins (Barak et al., 2011; Kroupitski et al., 2011; Macarisin et al., 2013; Jacob and Melotto, 2020), which harbor larger pools of substrates than other areas of leaves; and the physical and chemical composition of the cuticle layer on various parts of the plant (Lima et al., 2013; Hunter et al., 2015), which affects water dispersal and hence, water and nutrient availability to microbial inhabitants (Marcell and Beattie, 2002), may all be relevant traits to investigate in plant breeding efforts for their effect on enteric pathogen colonization.

Temperature and humidity conditions, and the presence of free water, are important in the multiplication and survival of enteric pathogens (Brandl and Mandrell, 2002; Brandl et al., 2004; Stine et al., 2005; Fonseca et al., 2011; Deblais et al., 2019; Roy and Melotto, 2019) and must be investigated simultaneously with the role of other plant traits. This includes consideration of agricultural practices, such as irrigation type and frequency (Fonseca et al., 2011; Williams et al., 2013; Castro-Ibáñez et al., 2015; Moyne et al., 2019), which may greatly affect the success of any breeding strategy aimed at reducing surface and internal plant colonization by food-borne pathogens. It is also clear that physicochemical stressors in the plant environment (*e.g.*, desiccation and UV radiation) (Jacobs and Sundin, 2001; Lindow and Brandl, 2003; Poza-Carrion et al., 2013) may overshadow other factors in their inhibitory effect on enteric pathogens. Therefore, the role of certain heritable plant traits at microsites that shield the bacterial cells from such fatal stressors should be investigated at the microscopic level as well as the plant or tissue level.

Fully elucidating the interaction between food safety-relevant microbes and crops necessitates the consideration of the entire plant microbiome below and above ground. Plant microbiota are complex and strongly driven by plant genetics, plant age, plant anatomical structure, and environmental factors (Lopez-Velasco et al., 2011; Rastogi et al., 2012; Ottesen et al., 2013; Lima et al., 2013; Williams et al., 2013; Yu et al., 2018). Identifying conditions that select for members of the plant microbiota able to competitively exclude enteric pathogens, which in general exhibit reduced fitness in the plant niche, can form an important component of this phytobiome approach (Cooley et al., 2003, 2006; Lopez-Velasco et al., 2012b; Williams et al., 2013; López-Gálvez et al., 2018). In addition, rhizosphere

and phyllosphere microbial communities can comprise epiphytes (including pathogenic species) known to affect plant colonization by enteric pathogens or toxigenic fungi either antagonistically through biocontrol strategies or favorably by supporting survival and growth. For instance, phytopathogens that actively degrade plant tissue or trigger plant chlorosis and necrosis may cause changes in pH and nutrient levels that favor the establishment and proliferation of enteric pathogens (Brandl, 2008; Goudeau et al., 2013; Potnis et al., 2014, 2015; Simko et al., 2015; George et al., 2018). Adjustment of management practices and environmental conditions to modulate and exploit microbe–microbe interactions should be actively investigated as part of a holistic approach to inhibit or prevent the colonization of enteric pathogens on/in plants.

Certain plant phenotypes may have independent as well as co-dependent effects with other plant features so that their role may only be fully revealed by actively investigating and/or selecting for both traits simultaneously. For example, entry of enteric pathogens into the plant tissue, where they are shielded from external environmental stressors, is thought to increase their survival in the plant habitat (Kroupitski et al., 2009; Erickson, 2012; Roy et al., 2013; Roy and Melotto, 2019). Thus, selecting for genotypes with lower stomatal density and stomatal pore size (Jacob and Melotto, 2020) may prove to be effective in reducing the probability of pathogen survival on plants in the field, provided that plant productivity is not impacted by the selection of that trait. Furthermore, basal plant defense responses to the presence of human pathogens (Thilmony et al., 2006; Schikora et al., 2011; Garcia et al., 2014), which can only take place upon exposure of plant cells to, and close interaction with, microbial cells in the plant apoplast, require entry of the enteric pathogen cells into the substomatal space of the tissue. Consequently, the full potential of breeding for a cultivar that is less hospitable to the endophytic lifestyle of an enteric pathogen may require consideration of both plant traits, *i.e.*, traits that affect the entry of the pathogen cells into the plant (Oblessuc et al., 2019) and those that affect the plant response once the cells have gained entry (Jacob and Melotto, 2020; Oblessuc et al., 2020).

The role of the physiological state of plants in their interaction with enteric pathogens cannot be understated. Plant defense responses may vary depending on the age of the plant tissue, the overall plant age, challenge history, and association with other microbes such as plant growth promoting rhizobacteria and plant pathogens (Simko et al., 2015; Bernstein et al., 2017; Hsu and Micallef, 2017). The carrying capacity of plant tissue for enteric pathogens depends on plant species and cultivar, leaf age, fruit ripeness, and root age given that structure and opening density via cracks at the secondary root emergence sites change over time (**Table 1**). Evidence is increasing that changes in temperature and rainfall caused by climate change may affect plant physiological and anatomical responses. These include stomatal conductance and density, leaf area and cuticle thickness, plant morphology, and plant nutrient cycling (Wang et al., 2008; Fraser et al., 2009; Cornelissen and Makoto, 2014). The level of relative humidity can significantly influence stomatal movement that can affect colonization of the leaf interior by human pathogenic bacteria (Roy and Melotto, 2019). It is clear that if these are targets of breeding programs for improving food safety, these traits will have to be resilient under long-term shift in weather patterns. Enteric pathogens vary broadly in their fitness as epiphytes and endophytes in a species-specific manner, and even based on variation at the inter- and intra-strain level (Klerks et al., 2007; Cui et al., 2018; Erickson and Liao, 2019; Wong et al., 2019). In particular, surface appendages, such as different types of fimbriae and adhesins that act as important plant attachment factors or flagella and other surface molecules that may trigger defense signaling cascades, vary among and within enteric species and strains (Lapidot and Yaron, 2009; Macarisin et al., 2012; Seo and Matthews, 2012; Roy et al., 2013; Garcia et al., 2014; Carter et al., 2018). Preferential bacterial pathogenic species and even serotype-commodity pairs are not uncommon and the basis for this specificity is still poorly understood. Clearly, phenotypic and genotypic variation among food-borne pathogen targets must also be taken into account while selecting for plant targets to enhance microbial crop safety.

Domestication of several crops has resulted in desirable agronomic and organoleptic traits such as shape, color, and prolonged shelf-life, with the unintended loss of other traits (Gepts, 2014; Purugganan, 2019). The resulting loss in genetic variation may have reduced the ability of some crops to cope with fluctuating environmental conditions and biotic challenges (Chen et al., 2015; Brisson et al., 2019). Despite this, genetic diversity could still reside in germplasm that is not commercially grown (such as traditional varieties, landraces, and crop wild relatives), allowing for the possibility of reintroducing genotypic and phenotypic traits that restore lost properties or establish new ones (Tanksley and McCouch, 1997; Smale and Day-Rubenstein, 2002). The underlying genetic basis for traits that enhance food safety are largely unknown, but as more research uncovers the interactions between plant, pathogen, and the environment, opportunities for identifying these traits will increase.

Traits that confer enhanced food safety are likely complex and controlled by multiple genes, presenting challenges to breeding efforts, especially for human pathogen–plant interactions. A starting point could be genome-wide association studies followed by metabolic pathway analysis (Li et al., 2019; Thrash et al., 2020) or functional analysis of mapped intervals (Korte and Farlow, 2013; Bartoli and Roux, 2017). For instance, one could predict various biochemical pathways needed for the synthesis of secondary metabolites with antioxidant and antimicrobial properties that could influence plant-microbe interactions and plant responses to associated microbiota. These interactions may be extremely important in food safety and should be a major focus of pre-breeding efforts.

Given the overall challenge of considering numerous aspects of plant genotype × environment × microbe × management interactions, a concerted effort to focus on given pathogen–crop models may be necessary to make headway in utilizing plant breeding as a feasible strategy to enhance produce safety. For effective genetic gain, a systems approach that maximizes consistency and differentiation of the desired phenotypes is essential. These traits must be considered with major traits of crop yield, quality, and resistance to abiotic and biotic stresses.

RESEARCH ON DEVELOPING EFFECTIVE ASSAYS AND APPROACHES FOR SELECTING DESIRABLE BREEDING GERMPLASM

Microbial food safety issues are rare events and tracking the source of disease outbreaks is extremely complex, making it difficult to predict or determine their cause (Slayton et al., 2011; Taylor et al., 2013; Hu et al., 2016). Thus, the best way to minimize these events is to perform risk assessment analyses (Uyttendaele et al., 2016; Pang et al., 2017). As discussed above, it has become evident that the plant is not a passive vehicle for microbial food hazards, hence providing opportunities to breed crops for enhanced food safety. The challenge remains to identify effective traits and genetic variability useful for breeding.

It has long been possible to breed plant germplasm that is resistant to plant pathogens. For example, the *Fusarium* pathogen synthesizes toxic DON and/or fumonisins and reduces seed set and fill in wheat; *Aspergillus flavus* can cause ear rots of maize in environmental conditions suitable for fungal growth. In both cases, these fungi can reduce plant yield and germplasm resistant to these pathogens is available (Petersen et al., 2016; Pekar et al., 2019). However, in cases where the fitness of the plant is not as directly reduced by the presence of the pathogen, traits that could potentially increase food safety may be harder to find and may require indirect or more creative solutions. They also compete with priorities for crop production and quality in breeding programs.

Edible plants carrying human pathogens generally do not show visual symptoms as they would when infected with plant pathogens, particularly when they occur at low levels[9]. This creates a challenge in developing screening assays to identify phenotypes with useful variation to support breeding efforts. Unlike the challenges associated with microbial hazards, detection of elements such as nitrates or heavy metals (*e.g.*, cadmium) is relatively easy with standard tissue analysis. Allergens can often be detected by routine (but still somewhat costly) assays (Senyuva et al., 2019). However, for human pathogens, rapid and cost-effective assays still need to be developed for routine screening of breeding populations, although some efforts have been made in this direction (Jacob and Melotto, 2020)[9]. These assays will allow large scale assessment of germplasm to find the best expression of useful traits and their introgression into cultivated varieties. Despite the challenges, variations in human pathogen colonization of lettuce, tomato, and spinach genotypes have already been determined.

An additional hurdle comes from the fact that microbial colonization is a complex behavior influenced by the plant host–pathogen combination and crop management practice such as irrigation type and crop fertilization (Fonseca et al., 2011; Marvasi et al., 2014a). Human pathogen–plant models should be developed for the purpose of breeding efforts to enhance food safety based on enteric pathogen strain–plant commodity variety pairs identified from prominent or recurring foodborne illness outbreaks. At the same time, plant genetic resources that may facilitate genome-wide association studies should not be excluded. Furthermore, the use of human pathogens in routine assays requires highly trained personnel and laboratory/greenhouse biosafety conditions according to NIH guidelines, in addition to considerable costs associated with the handling of microbial hazards in contained facilities. These approaches will require collaborative efforts among food safety experts, plant–microbe interaction biologists, microbiologists, and crop breeders for successful advancements in the field.

[9]Oblessuc, P. R., and Melotto, M. (under review). A simple assay to assess *Salmonella enterica* persistence in lettuce leaves after a low inoculation dose. *Front. Plant Sci.*

CONCLUSION AND RECOMMENDATIONS

It is generally recognized that although breeding programs for certain human pathogen/toxin systems are ongoing (*e.g.*, *Fusarium* in wheat), it would be premature to engage in plant breeding for other aspects of food safety for which targets and testing systems are not yet well defined. Nevertheless, current research is paving the way toward this goal. To ensure advances in the field, the following points are critical to the success of this initiative:

(1) Continue foundational research to generate crucial knowledge of plant interactions with human pathogens and contamination of food with microbes, mycotoxins, elements, and allergens.
(2) Initiate pre-breeding screening strategies to characterize genetic variability, heritability, and efficacy of target traits.
(3) Support breeding programs where genetic variation and efficacy is established (*e.g.,* breeding lines that accumulate less aflatoxins or heavy metals).

Ultimately, in combination with agricultural practices and interventions, the recognition that plant breeding for enhanced food safety can be another layer in our fight to reduce foodborne illness associated with crops necessitates research goals and funding prioritization to enable advances in this area.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing of the review.

FUNDING

The workshop Breeding Crops for Enhanced Food Safety was sponsored by United States Department of Agriculture – National Institute of Food and Agriculture (USDA-NIFA, Grant No. 2019-67013-29005) to MM, MJ-R, and AV. Additional support was provided by the California Leafy Greens Research Program, the Department of Plant Sciences and Plant Breeding Center

at the University of California, Davis. Any opinions, findings, conclusions, or recommendations expressed in this review are those of the authors and do not necessarily represent the official views, opinions, or policies of the funding agencies.

ACKNOWLEDGMENTS

We thank the 65 workshop participants who contributed to the breakout session discussions that formed the basis for this review.

We would like to thank Dr. Ed Kaleikau, Dr. Isabel Walls, and Dr. Max Teplitski, national program leaders at USDA-NIFA for their perspective, support, and input.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00428/full#supplementary-material

REFERENCES

Arias, R. S., Sobolev, V. S., Massa, A. N., Orner, V. A., Walk, T. E., Ballard, L. L., et al. (2018). New tools to screen wild peanut species for aflatoxin accumulation and genetic fingerprinting. *BMC Plant Biol.* 18:170. doi: 10.1186/s12870-018-1355-9

Arnade, C., Calvin, L., and Kuchler, F. (2009). Consumer response to a food safety shock: the 2006 food-borne illness outbreak of *E. coli* O157:H7 linked to spinach. *Rev. Agric. Econ.* 31, 734–750. doi: 10.1111/j.1467-9353.2009.01464.x

Arora, M., Kiran, B., Rani, S., Rani, A., Kaur, B., and Mittal, N. (2008). Heavy metal accumulation in vegetables irrigated with water from different sources. *Food Chem.* 111, 811–815. doi: 10.1016/j.foodchem.2008.04.049

Barak, J. D., Kramer, L. C., and Hao, L. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/AEM.01661-10

Barak, J. D., Liang, A., and Narm, K. E. (2008). Differential attachment to and subsequent contamination of agricultural crops by *Salmonella enterica*. *Appl. Environ. Microbiol.* 74, 5568–5570. doi: 10.1128/AEM.01077-08

Barak, J. D., and Schroeder, B. K. (2012). Interrelationships of food safety and plant pathology: the life cycle of human pathogens on plants. *Annu. Rev. Phytopathol.* 50, 241–266. doi: 10.1146/annurev-phyto-081211-172936

Bartoli, C., and Roux, F. (2017). Genome-wide association studies in plant pathosystems: toward an ecological genomics approach. *Front. Plant Sci.* 8:763. doi: 10.3389/fpls.2017.00763

Bennett, S. D., Sodha, S. V., Ayers, T. L., Lynch, M. F., Gould, L. H., and Tauxe, R. V. (2018). Produce-associated foodborne disease outbreaks, USA, 1998–2013. *Epidemiol. Infect.* 11, 1397–1406. doi: 10.1017/S0950268818001620

Bernstein, N., Sela Saldinger, S., Dudai, N., and Gorbatsevich, E. (2017). Salinity stress does not affect root uptake, dissemination and persistence of *Salmonella* in sweet-basil (*Ocimum basilicum*). *Front. Plant Sci.* 8:675. doi: 10.3389/fpls.2017.00675

Bethke, P. C. (2018). Progress and successes of the specialty crop research initiative on acrylamide reduction in processed potato products. *Am. J. Potato Res.* 95, 328–337. doi: 10.1007/s12230-018-9660-2

Beuchat, L. R. (1996). Pathogenic microorganisms associated with fresh produce. *J. Food Prot.* 59, 204–216. doi: 10.4315/0362-028X-59.2.204

Binder, E. M., Tan, L. M., Chin, L. J., Handl, J., and Richard, J. (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Anim. Feed Sci. Technol.* 137, 265–282. doi: 10.1016/j.anifeedsci.2007.06.005

Brandl, M. T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.* 44, 367–392. doi: 10.1146/annurev.phyto.44.070505.143359

Brandl, M. T. (2008). Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on postharvest lettuce. *Appl. Environ. Microbiol.* 74, 5285–5289. doi: 10.1128/AEM.01073-08

Brandl, M. T., and Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Appl. Environ. Microbiol.* 74, 2298–2306. doi: 10.1128/AEM.02459-07

Brandl, M. T., Haxo, A. F., Bates, A. H., and Mandrell, R. E. (2004). Comparison of survival of *Campylobacter jejuni* in the phyllosphere with that in the rhizosphere of spinach and radish plants. *Appl. Environ. Microbiol.* 70, 1182–1189. doi: 10.1128/aem.70.2.1182-1189.2004

Brandl, M. T., and Mandrell, R. E. (2002). Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl. Environ. Microbiol.* 68, 3614–3621. doi: 10.1128/AEM.68.7.3614-3621.2002

Brisson, V. L., Schmidt, J. E., Northen, T. R., Vogel, J. P., and Gaudin, A. C. M. (2019). Impacts of maize domestication and breeding on rhizosphere microbial community recruitment from a nutrient depleted agricultural soil. *Sci. Rep.* 9:15611. doi: 10.1038/s41598-019-52148-y

California Department of Public Health, Emergency Response Unit [CDPH] (2010). *Investigation of the Escherichia coli O157:H7 Outbreak Associated with Iceberg Lettuce*. Sacramento, CA: California Department of Public Health, Food and Drug Branch.

California Department of Public Health, Emergency Response Unit [CDPH] (2014). *Environmental Investigation of Escherichia coli O157:H7 Outbreak in October 2013 Associated with Pre-Packaged Salads*. Sacramento, CA: California Department of Public Health, Food and Drug Branch.

Callahan, M. T., and Micallef, S. A. (2019). Waxing and cultivar affect *Salmonella enterica* persistence on cucumber (*Cucumis sativus* L.) fruit. *Int. J. Food Microbiol.* 310:108359. doi: 10.1016/j.ijfoodmicro.2019.108359

Callejón, R. M., Rodríguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C., and 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. doi: 10.1089/fpd.2014.1821

Calvin, L., Avendaño, B., and Schwentesius, R. (2004). *The Economics of Food Safety: The Case of Green Onions and Hepatitis A Outbreaks. Economic Research Service/USDA VGS-305-01*. Available online at: https://www.ers.usda.gov/publications/vgs/nov04/VGS30501/VGS30501.pdf (accessed November 20, 2012).

Carter, M. Q., Brandl, M. T., Kudva, I. T., Katani, R., Moreau, M. R., and Kapur, V. (2018). Conditional function of autoaggregative protein *Cah* and common *cah* mutations in Shiga toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 84:e01739-17. doi: 10.1128/AEM.01739-17

Castro-Ibáñez, I., Gil, M. I., Tudela, J. A., Ivanek, R., and Allende, A. (2015). Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain. *Food Microbiol.* 49, 173–181. doi: 10.1016/j.fm.2015.02.004

Centers for Disease Control and Prevention [CDC] (2018). *Multistate outbreak of E. coli O157:H7 Infections Linked To Romaine Lettuce (final Update)*. Available online at: https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html (accessed January 24, 2020).

Chen, Y. J., Gols, R., and Benrey, B. (2015). Crop domestication and its impact on naturally selected trophic interactions. *Rev. Entomol.* 60, 35–58. doi: 10.1146/annurev-ento-010814-020601

Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M. T., Myers, C., Rose, C., et al. (2007). Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. *PLoS One* 2:e1159. doi: 10.1371/journal.pone.0001159

Cooley, M. B., Chao, D., and Mandrell, R. E. (2006). *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *J. Food Prot.* 69, 2329–2335. doi: 10.4315/0362-028x-69.10.2329

Cooley, M. B., Miller, W. G., and Mandrell, R. E. (2003). Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. *Appl. Environ. Microbiol.* 69, 4915–4926. doi: 10.1128/AEM.69.8.4915-4926.2003

Cornelissen, J. H. C., and Makoto, K. (2014). Winter climate change, plant traits and nutrient and carbon cycling in cold biomes. *Ecol. Res.* 29, 517–527. doi: 10.1007/s11284-013-1106-1

Crozier, L., Hedley, P. E., Morris, J., Wagstaff, C., Andrews, S. C., Toth, I., et al. (2016). Whole-Transcriptome analysis of verocytotoxigenic *Escherichia coli* O157:H7 (Sakai) suggests plant-species-specific metabolic responses on exposure to spinach and lettuce extracts. *Front. Microbiol.* 7:1088. doi: 10.3389/fmicb.2016.01088

Cui, Y., Liu, D., and Chen, J. (2018). Fate of various *Salmonella enterica* and enterohemorrhagic *Escherichia coli* cells attached to alfalfa, fenugreek, lettuce, and tomato seeds during germination. *Food Control* 88, 229–235. doi: 10.1016/j.foodcont.2018.01.011

Cui, Y., Walcott, R., and Chen, J. (2017). Differential attachment of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* to alfalfa, fenugreek, lettuce, and tomato seeds. *Appl. Environ. Microbiol.* 83:AEM.03170-16. doi: 10.1128/AEM.03170-16

Danyluk, M. D., and Schaffner, D. W. (2011). Quantitative assessment of the microbial risk of leafy greens from farm to consumption: preliminary framework, data, and risk estimates. *J. Food Prot.* 74, 700–708. doi: 10.4315/0362-028X.JFP-10-373

Deblais, L., Helmy, Y. A., Testen, A., Vrisman, C., Jimenez Madrid, A. M., and Kathayat, D. (2019). Specific environmental temperature and relative humidity conditions and grafting affect the persistence and dissemination of *Salmonella enterica* subsp. *enterica* serotype Typhimurium in tomato plant tissues. *Appl Environ. Microbiol.* 85:e00403-19. doi: 10.1128/AEM.00403-19

Del Rosario, B. A., and Beuchat, L. R. (1995). Survival and growth of enterohemorrhagic *Escherichia coli* 0157:H7 in cantaloupe and watermelon. *J. Food Prot.* 58, 105–107. doi: 10.4315/0362-028X-58.1.105

Devleesschauwer, B., Marvasi, M., Giurcanu, M. C., Hochmuth, G. J., Speybroeck, N., Havelaar, A. H., et al. (2017). High relative humidity pre-harvest reduces post-harvest proliferation of *Salmonella* in tomatoes. *Food Microbiol.* 66, 55–63. doi: 10.1016/j.fm.2017.04.003

Duffy, B., Ravva, S., and Stanker, L. (2008). Cantaloupe cultivar differences as opportunistic hosts for human pathogenic *Escherichia coli* O157:H7 and *Salmonella*. *Eur. J. Hort. Sci.* 73, 73–75.

Erickson, M. C. (2012). Internalization of fresh produce by foodborne pathogens. *Annu. Rev. Food Sci. Technol.* 3, 283–310. doi: 10.1146/annurev-food-022811-101211

Erickson, M. C., and Liao, J. Y. (2019). Variation in recovery of *Salmonella* strains extracted from leafy greens. *LWT Food Sci. Technol.* 107, 185–190. doi: 10.1016/j.lwt.2019.02.078

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., Den Bakker, H. C., Bautista, J., et al. (2019). Pre-harvest internalization and surface survival of *Salmonella* and *Escherichia coli* O157:H7 sprayed onto different lettuce cultivars under field and growth chamber conditions. *Int. J. Food Microbiol.* 16, 197–204. doi: 10.1016/j.ijfoodmicro.2018.12.001

Fonseca, J. M., Fallon, S. D., Sanchez, C. A., and Nolte, K. D. (2011). *Escherichia coli* survival in lettuce fields following its introduction through different irrigation systems. *J. Appl. Microbiol.* 110, 893–902. doi: 10.1111/j.1365-2672.2011.04942.x

Fraser, L. H., Greenall, A., Carlyle, C., Turkington, R., and Friedman, C. R. (2009). Adaptive phenotypic plasticity of *Pseudoroegneria spicata*: response of stomatal density, leaf area and biomass to changes in water supply and increased temperature. *Ann. Bot.* 103, 769–775. doi: 10.1093/aob/mcn252

Gaikpa, D. S., and Miedaner, T. (2019). Genomics-assisted breeding for ear rot resistances and reduced mycotoxin contamination in maize: methods, advances and prospects. *Theor. Appl. Genet.* 132, 2721–2739. doi: 10.1007/s00122-019-03412-2

Garcia, A. V., Charrier, A., Schikora, A., Bigeardm, J., Pateyron, S., de Tauzia-Moreau, M. L., et al. (2014). Salmonella enterica flagellin is recognized via FLS2 and activates PAMP-Triggered Immunity in *Arabidopsis thaliana*. *Mol. Plant* 7, 657–674. doi: 10.1093/mp/sst145

George, A. S., Cox, C. E., Desai, P., Porwollik, S., Chu, W., de Moraes, M. H., et al. (2018). Interactions of *Salmonella enterica* serovar Typhimurium and *Pectobacterium carotovorum* within a tomato soft rot. *Appl. Environ. Microbiol.* 84:e01913-17. doi: 10.1128/AEM.01913-17

Gepts, P. (2014). The contribution of genetic and genomic approaches to plant domestication studies. *Curr. Opin. Plant Biol.* 18, 51–59. doi: 10.1016/j.pbi.2014.02.001

Gipsa. (2006). *Grain Fungal Diseases & Mycotoxin Reference*, 2nd Edn. Kansas City: USDA.

Golberg, D., Kroupitski, Y., Belausov, E., Pinto, R., and Sela, S. (2011). *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. *Int. J. Food Microbiol.* 145, 250–257. doi: 10.1016/j.ijfoodmicro.2010.12.031

Golden, D. A., Rhodehamel, E. F., and Kautter, D. A. (1993). Growth of *Salmonella* spp. in cantaloupe, watermelon, and honeydew melons. *J. Food Prot.* 56, 194–196. doi: 10.4315/0362-028X-56.3.194

Goudeau, D. M., Parker, C. T., Zhou, Y., Sela, S., Kroupitski, Y., and Brandl, M. T. (2013). The *Salmonella* transcriptome in lettuce and cilantro soft rot reveals a niche overlap with the animal host intestine. *Appl. Environ. Microbiol.* 79, 250–262. doi: 10.1128/AEM.02290-12

Grace, D., Mahuku, G., Hoffmann, V., Atherstone, C., Upadhyaya, H. D., and Bandyopadhyay, R. (2015). International agricultural research to reduce food risks: case studies on aflatoxins. *Food Sec.* 7, 569–582. doi: 10.1007/s12571-015-0469-2

Gu, G., Cevallos-Cevallos, J. M., and van Bruggen, A. H. C. (2013). Ingress of *Salmonella enterica* Typhimurium into tomato leaves through hydathodes. *PLoS One* 8:e53470. doi: 10.1371/journal.pone.0053470

Gutiérrez-Rodríguez, E., Gundersen, A., Sbodio, A. O., and Suslow, T. V. (2011). Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *J. Appl. Microbiol.* 112, 109–118. doi: 10.1111/j.1365-2672.2011.05184.x

Han, S., and Micallef, S. A. (2014). *Salmonella* Newport and Typhimurium colonization of fruit differs from leaves in various tomato cultivars. *J. Food Prot.* 77, 1844–1850. doi: 10.4315/0362-028X.JFP-13-562

Han, S., and Micallef, S. A. (2016). Environmental metabolomics of the tomato plant surface provides insights on *Salmonella enterica* colonization. *Appl. Environ. Microbiol.* 82, 3131–3142. doi: 10.1128/AEM.00435-16

Hazaei, H., Purves, R. W., Hughes, J., Link, W., O'Sullivan, D. M., Schulman, A. H., et al. (2019). Eliminating vicine and convicine, the main anti-nutritional factors restricting faba bean usage. *Trends Food Sci. Technol.* 91, 549–556. doi: 10.1016/j.tifs.2019.07.051

Hsu, C. K., and Micallef, S. A. (2017). Plant-mediated restriction of *Salmonella enterica* on tomato and spinach leaves colonized with *Pseudomonas* plant growth-promoting rhizobacteria. *Int. J. Food Microbiol.* 16, 1–6. doi: 10.1016/j.ijfoodmicro.2017.07.012

Hu, K., Renly, S., Edlund, S., Davis, M., and Kaufman, J. (2016). A modeling framework to accelerate food-borne outbreak investigations. *Food Control* 59, 53–58. doi: 10.1016/j.foodcont.2015.05.017

Huang, S. W. (2013). *Imports Contribute to Year-Round Fresh Fruit Availability*. Washington, DC: USDA Economic Research Service.

Hunter, P. J., Shaw, R. K., Berger, C. N., Frankel, G., Pink, D., and 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. doi: 10.1093/femsle/fnv077

Hussain, M. A., and Dawson, C. O. (2013). Economic impact of food safety outbreaks on food businesses. *Foods* 2, 585–589. doi: 10.3390/foods2040585

Jacob, C., and Melotto, M. (2020). Human pathogen colonization of lettuce dependent upon plant genotype and defense response activation. *Front. Plant Sci.* 10:1769. doi: 10.3389/fpls.2019.01769

Jacobs, J. L., and Sundin, G. W. (2001). Effect of solar UV-B radiation on a phyllosphere bacterial community. *Appl. Environ. Microbiol.* 67, 5488–5496. doi: 10.1128/AEM.67.12.5488-5496.2001

Jay-Russell, M. T. (2013). What is the risk from wild animals in food-borne pathogen contamination of plants? *CAB Rev.* 8, 1–16. doi: 10.1079/PAVSNNR20138040

Jeamsripong, S., Chase, J. A., Jay-Russell, M. T., Buchanan, R. L., and Atwill, E. R. (2019). Experimental in-field transfer and survival of *Escherichia coli* from animal feces to romaine lettuce in Salinas Valley, California. *Microorganisms* 7:408. doi: 10.3390/microorganisms7100408

Jechalke, S., Schierstaedt, J., Becker, M., Flemer, B., Grosch, R., Smalla, K., et al. (2019). *Salmonella* establishment in agricultural soil and colonization of crop

plants depend on soil type and plant species. *Front. Microbiol.* 10:967. doi: 10.3389/fmicb.2019.00967

Klerks, M. M., Franz, E., van Gent-Pelzer, M., Zijlstra, C., and 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. doi: 10.1038/ismej.2007.82

Korir, R. C., Everts, K. L., and Micallef, S. A. (2019). Interactions between *Salmonella enterica* Newport, *Fusarium* spp., and melon cultivars. *Foodborne Pathog. Dis.* [Epub ahead of print].

Korte, A., and Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods* 9:29. doi: 10.1186/1746-4811-9-29

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., et al. (2009). Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75, 6076–6086. doi: 10.1128/AEM.01084-09

Kroupitski, Y., Pinto, R., Belausov, E., and Sela, S. (2011). Distribution of *Salmonella typhimurium* in romaine lettuce leaves. *Food Microbiol.* 28, 990–997. doi: 10.1016/j.fm.2011.01.007

Lapidot, A., and Yaron, S. (2009). Transfer of *Salmonella enterica* serovar Typhimurium from contaminated irrigation water to parsley is dependent on curli and cellulose, the biofilm matrix components. *J. Food Protect.* 72, 618–623. doi: 10.4315/0362-028X-72.3.618

Li, H., Thrash, A., Tang, J. D., He, L., Yan, J., and Warburton, M. L. (2019). Leveraging GWAS data to identify metabolic pathways and networks involved in maize lipid biosynthesis. *Plant J.* 98, 853–863. doi: 10.1111/tpj.14282

Lima, P. M., São José, J. F. B., Andrade, N. J., Pires, A. C. S., and Ferreira, S. O. (2013). Interaction between natural microbiota and physicochemical characteristics of lettuce surfaces can influence the attachment of *Salmonella* Enteritidis. *Food Control* 30, 157–161. doi: 10.1016/j.foodcont.2012.06.039

Lindow, S. E., and Brandl, M. T. (2003). Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* 69, 1875–1883. doi: 10.1128/AEM.69.4.1875-1883.2003

Liu, C., Guttieri, M. J., Waters, B. M., Eskridge, K. M., and Baenziger, P. S. (2019). Selection of bread wheat for low grain cadmium concentration at the seedling stage using hydroponics versus molecular markers. *Crop Sci.* 59, 945–956. doi: 10.2135/cropsci2018.08.0484

López-Gálvez, F., Gil, M. I., and 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. doi: 10.1016/j.fm.2017.09.014

Lopez-Velasco, G., Sbodio, A., Tomás-Callejas, A., Wei, P., Tan, K. H., and Suslow, T. V. (2012a). Assessment of root uptake and systemic vine-transport of *Salmonella enterica* sv. Typhimurium by melon (*Cucumis melo*) during field production. *Int. J. Food Microbiol.* 158, 65–72. doi: 10.1016/j.ijfoodmicro.2012.07.005

Lopez-Velasco, G., Tydingsa, H. A., Boyera, R. R., Falkinham, J. O., and Ponder, M. A. (2012b). Characterization of interactions between *Escherichia coli* O157:H7 with epiphytic bacteria *in vitro* and on spinach leaf surfaces. *Int. J. Food Microbiol.* 153, 351–357. doi: 10.1016/j.ijfoodmicro.2011.11.026

Lopez-Velasco, G., Welbaum, G. E., Falkinham, J. O., and Ponder, M. A. (2011). Phyllosphere bacterial community structure of spinach (*Spinacia oleracea*) as affected by cultivar and environmental conditions at time of harvest. *Diversity* 3, 721–738. doi: 10.3390/d3040721

Macarisin, D., Patel, J., Bauchan, G., Giron, J. A., and Ravishankar, S. (2013). Effect of spinach cultivar and bacterial adherence factors on survival of *Escherichia coli* O157:H7 on spinach leaves. *J. Food Prot.* 76, 1829–1837. doi: 10.4315/0362-028X.JFP-12-556

Macarisin, D., Patel, J., Bauchan, G., Giron, J. A., and Sharma, V. K. (2012). Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. *Foodborne Pathog. Dis.* 9, 160–167. doi: 10.1089/fpd.2011.102

Marcell, L. M., and Beattie, G. A. (2002). Effect of leaf surface waxes on leaf colonization by *Pantoea agglomerans* and *Clavibacter michiganensis*. *Mol. Plant Microbe Interact.* 15, 1236–1244. doi: 10.1094/MPMI.2002.15.12.1236

Marin, S., Ramos, A. J., Cano-Sancho, G., and Sanchis, V. (2013). Mycotoxins: occurrence, toxicology, and exposure assessment. *Food Chem. Toxicol.* 60, 218–237. doi: 10.1016/j.fct.2013.07.047

Marvasi, M., George, A. S., Giurcanu, M., Hochmuth, G. J., Noel, J. T., Gause, E., et al. (2014a). Effects of nitrogen and potassium fertilization on the susceptibility of tomatoes to post-harvest proliferation of *Salmonella enterica*. *Food Microbiol.* 43, 20–27. doi: 10.1016/j.fm.2014.03.017

Marvasi, M., George, A. S., Giurcanu, M. C., Hochmuth, G. J., Noel, J. T., and Teplitski, M. (2015). Effect of the irrigation regime on the susceptibility of pepper and tomato to post-harvest proliferation of *Salmonella enterica*. *Food Microbiol.* 46, 139–144. doi: 10.1016/j.fm.2014.07.014

Marvasi, M., Hochmuth, G. J., Giurcanu, M. C., George, A. S., Noel, J. T., Bartz, J., et al. (2013). Factors that affect proliferation of *Salmonella* in tomatoes post-harvest: the roles of seasonal effects, irrigation regime, crop and pathogen genotype. *PLoS One* 8:e80871. doi: 10.1371/journal.pone.0080871

Marvasi, M., Noel, T., George, A. S., Farias, M. A., Jenkins, K. T., Hochmuth, G., et al. (2014b). Ethylene signaling affects susceptibility of tomatoes to *Salmonella*. *Microb. Biotechnol.* 7, 545–555. doi: 10.1111/1751-7915.12130

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliland, S., and Fletcher, J. (2009). Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157:H7 in spinach. *J. Food Prot.* 72, 1521–1530. doi: 10.4315/0362-028x-72.7.1521

Moghimi, A., Yang, C., Anderson, J. A., and Reynolds, S. K. (2019). "Selecting informative spectral bands using machine learning techniques to detect *Fusarium* head blight in wheat," in *Proceedings of the 2019 ASABE Annual International Meeting*, (St. Joseph, MI: ASABE).

Moyne, A. L., Blessington, T., Williams, T. R., Koike, S. T., Cahn, M. D., Marco, M. L., et al. (2019). Conditions at the time of inoculation influence survival of attenuated *Escherichia coli* O157:H7 on field-inoculated lettuce. *Food Microbiol.* 85:103274. doi: 10.1016/j.fm.2019.103274

Nguyen, V. D., Bennett, S. D., Mungai, E., Gieraltowski, L., Hise, K., and Gould, L. H. (2015). Increase in multistate foodborne disease outbreaks–United States, 1973–2010. *Foodborne Pathog. Dis.* 12, 867–872. doi: 10.1089/fpd.2014.1908

Noel, J. T., Arrach, N., Alagely, A., McClelland, M., and Teplitski, M. (2010). Specific responses of *Salmonella enterica* to tomato varieties and fruit ripeness identified by *in vivo* expression technology. *PLoS One* 5:e12406. doi: 10.1371/journal.pone.0012406

Oblessuc, P. R., Bisneta, M. V., and Melotto, M. (2019). Common and unique Arabidopsis proteins involved in stomatal susceptibility to *Salmonella enterica* and *Pseudomonas syringae*. *FEMS Microbiol. Lett.* 366:fnz197. doi: 10.1093/femsle/fnz197

Oblessuc, P. R., Matiolli, C. V., and Melotto, M. (2020). Novel molecular components involved in callose-mediated Arabidopsis defense against *Salmonella enterica* and *Escherichia coli* O157:H7. *BMC Plant Biol.* 20:16. doi: 10.1186/s12870-019-2232-x

Ottesen, A. R., González Peña, A., White, J. R., Pettengill, J. B., Li, C., Allard, S., et al. (2013). Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). *BMC Microbiol.* 13:114. doi: 10.1186/1471-2180-13-114

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., et al. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerg. Infect. Dis.* 19, 407–415. doi: 10.3201/eid1903.111866

Pang, H., Lambertini, E., Buchanan, R. L., Schaffner, D. W., and Pradhan, A. K. (2017). Quantitative microbial risk assessment for *Escherichia coli* O157:H7 in fresh-cut lettuce. *J. Food Prot.* 80, 302–311. doi: 10.4315/0362-028X.JFP-16-246

Pekar, J. J., Murray, S. C., Isakeit, T. S., Scully, B. T., Guo, B., Knoll, J. E., et al. (2019). Evaluation of elite maize inbred lines for reduced *Aspergillus flavus* infection, aflatoxin accumulation, and agronomic traits. *Crop Sci.* 59, 2562–2571. doi: 10.2135/cropsci2019.04.0206

Petersen, S., Lyerly, J. H., McKendry, A. L., Islan, M. S., Brown-Guedira, G., Cowger, C., et al. (2016). Validation of fusarium head blight resistance QTL in US winter wheat. *Crop Sci.* 57, 1–12. doi: 10.2135/cropsci2015.07.0415

Potnis, N., Colee, J., Jones, J. B., and Barak, J. D. (2015). Plant pathogen-induced water-soaking promotes *Salmonella enterica* growth on tomato leaves. *Appl. Environ. Microbiol.* 81, 8126–8134. doi: 10.1128/AEM.01926-15

Potnis, N., Soto-Arias, J. P., Cowles, K. N., van Bruggen, A. H. C., Jones, J. B., and Barak, J. D. (2014). *Xanthomonas perforans* colonization influences *Salmonella enterica* in the tomato phyllosphere. *Appl. Environ. Microbiol.* 80, 3173–3180. doi: 10.1128/AEM.00345-14

Poza-Carrion, C., Suslow, T., and Lindow, S. (2013). Resident bacteria on leaves enhance survival of immigrant cells of *Salmonella enterica*. *Phytopathology* 103, 341–351. doi: 10.1094/PHYTO-09-12-0221-FI

Purugganan, M. D. (2019). Evolutionary insights into the nature of plant domestication. *Curr. Biol.* 29, R705–R714. doi: 10.1016/j.cub.2019.05.053

Quilliam, R. S., Williams, A. P., and Jones, D. L. (2012). Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157:H7. *PLoS One* 7:e33842. doi: 10.1371/journal.pone.0033842

Rastogi, G., Sbodio, A., Tech, J. J., Suslow, T. V., Coaker, G. L., and Leveau, J. H. J. (2012). Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J.* 6, 1812–1822. doi: 10.1038/ismej.2012.32

Ribera, L. A., Palma, M. A., Paggi, M., Knutson, R., Masabni, J. G., and Anciso, J. (2012). Economic analysis of food safety compliance costs and foodborne illness outbreaks in the United States. *Horttechnology* 22, 150–156. doi: 10.21273/HORTTECH.22.2.150

Roy, D., and Melotto, M. (2019). Stomatal response and human pathogen persistence in leafy greens under preharvest and postharvest environmental conditions. *Postharvest Biol. Technol.* 148, 76–82. doi: 10.1016/j.postharvbio.2018.10.013

Roy, D., Panchal, S., Rosa, B. A., and Melotto, M. (2013). *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. *Phytopathology* 103, 326–332. doi: 10.1094/PHYTO-09-12-0230-FI

Sáiz, J., Montealegre, C., Marina, M., and García-Ruiz, C. (2013). Peanut allergens: an overview. *Crit. Rev. Food Sci. Nutr.* 53, 722–737. doi: 10.1080/10408398.2011.556758

Sapers, G. M., and Doyle, M. P. (2014). "Chapter 1 - Scope of the produce contamination problem," in *The Produce Contamination Problem: Causes and Solutions*, 2nd Edn, eds K. R. Matthews, G. M. Sapers, and C. P. Gerba (Amsterdam: Elsevier).

Schaafsma, A. W. (2002). "Economic changes imposed by mycotoxins in food grains: case study of deoxynivalenol in winter wheat," in *Mycotoxins and Food Safety Advances in Experimental Medicine and Biology*, Vol. 504, eds J. W. DeVries, M. W. Trucksess, and L. S. Jackson (Boston, MA: Springer).

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrier, A., et al. (2011). Conservation of *Salmonella* infection mechanisms in plants and animals. *PLoS One* 6:e24112. doi: 10.1371/journal.pone.0024112

Senyuva, H. Z., Baricevic Jones, I., Sykes, M., and Baumgartner, S. (2019). A critical review of the specifications and performance of antibody and DNA-based methods for detection and quantification of allergens in foods. *Food Addit. Contam. Part A* 36, 507–547. doi: 10.1080/19440049.2019.1579927

Seo, S., and Matthews, K. R. (2012). Influence of the plant defense response to *Escherichia coli* O157:H7 cell surface structures on survival of that enteric pathogen on plant surfaces. *Appl. Environ. Microbiol.* 78, 5882–5889. doi: 10.1128/AEM.01095-12

Siegel, K. R., Ali, M. K., Srinivasiah, A., Nugent, R. A., and Naraya, K. M. V. (2014). Do we produce enough fruits and vegetables to meet global health need? *PLoS One* 9:e104059. doi: 10.1371/journal.pone.0104059

Simko, I., Zhou, Y., and Brandl, M. T. (2015). Downy mildew disease promotes the colonization of romaine lettuce by *Escherichia coli* O157:H7 and *Salmonella enterica*. *BMC Microbiol.* 15:19. doi: 10.1186/s12866-015-0360-5

Slayton, R. B., Turabelidze, G., Bennett, S. D., Schwensohn, C. A., Yaffee, A. Q., Khan, F., et al. (2011). Outbreak of Shiga Toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. *PLoS One* 8:e55300. doi: 10.1371/journal.pone.0055300

Smale, M., and Day-Rubenstein, K. (2002). The demand for crop genetic resources: international use of the us national plant germplasm system. *World Dev.* 30, 1639–1655. doi: 10.1016/S0305-750X(02)00055-4

Stine, S. W., Song, I., Choi, C. Y., and Gerba, C. P. (2005). Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. *J. Food Prot.* 68, 1352–1358. doi: 10.4315/0362-028x-68.7.1352

Tanksley, S. D., and McCouch, S. R. (1997). Seed banks and molecular maps: unlocking genetic potential from the wild. *Science* 277, 1063–1066. doi: 10.1126/science.277.5329.1063

Taylor, E. V., Nguyen, T. A., Machesky, K. D., Koch, E., Sotir, M. J., Bohm, S. R., et al. (2013). Multistate outbreak of *Escherichia coli* O145 infections associated with romaine lettuce consumption, 2010. *J. Food Prot.* 76, 939–944. doi: 10.4315/0362-028X.JFP-12-503

Thilmony, R., Underwood, W., and He, S. Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* 46, 34–53. doi: 10.1111/j.1365-313X.2006.02725.x

Thrash, A., Tang, J. D., DeOrnellis, M., Peterson, D. G., and Warburton, M. L. (2020). PAST: the pathway association studies tool to infer biological meaning from GWAS datasets. *Plants* 9:58. doi: 10.3390/plants9010058

Turner, K., Moua, C. N., Hajmeer, M., Barnes, A., and Needham, M. (2019). Overview of leafy greens–related food safety incidents with a California link: 1996 to 2016. *J. Food Prot.* 82, 405–414. doi: 10.4315/0362-028X.JFP-18-316

Tuttle, J., Gomez, T., Doyle, M. P., Wells, J. G., Zhao, T., Tauxe, R. V., et al. (1999). Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect.* 122, 185–192. doi: 10.1017/s0950268898001976

U.S. Department of Health and Human Services-U.S. Department of Agriculture [HHS-USDA] (2015). *2015–2020 Dietary Guidelines for Americans*, 8th Edn. Washington, DC: U.S. Department of Health and Human Services.

U.S. Food and Drug Administration [FDA] (2018). *Environmental Assessment of Factors Potentially Contributing to the Contamination of Romaine Lettuce Implicated in a Multi-State Outbreak of E. coli O157:H7*. Available online at: https://www.fda.gov/food/outbreaks-foodborne-illness/environmental-assessment-factors-potentially-contributing-contamination-romaine-lettuce-implicated(accessed January 24, 2020).

U.S. Food and Drug Administration [FDA] (2020). *Statement on the Salinas-Linked Romaine Lettuce E. coli O157:H7 Outbreak and Status Update on Investigation*. Available online at: https://www.fda.gov/news-events/press-announcements/statement-salinas-linked-romaine-lettuce-e-coli-o157h7-outbreak-and-status-update-investigation (accessed January 24, 2020).

United Nations [UN], Department of Economic, and Social Affairs, Population Division. (2019a). *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423)*. Available online at: https://population.un.org/wpp/Publications/Files/WPP2019_Highlights.pdf (accessed January 24, 2020)

United Nations [UN], Department of Economic, and Social Affairs, Population Division (2019b). *World Urbanization Prospects 2018: Highlights (ST/ESA/SER.A/421)*. Available online at: https://population.un.org/wup/Publications/Files/WUP2018-Highlights.pdf (accessed January 24, 2020)

Uyttendaele, M., De Boeck, E., and Jacxsen, L. (2016). Challenges in food safety as part of food security: lessons learnt on food safety in a globalized world. *Procedia Food Sci.* 6, 16–22. doi: 10.1016/j.profoo.2016.02.003

Wang, X., Arora, R., Horner, H. T., and Krebs, S. L. (2008). Structural adaptations in overwintering leaves of thermonastic and nonthermonastic *Rhododendron* species. *J. Am. Soc. Hortic. Sci.* 133, 768–776. doi: 10.21273/JASHS.133.6.768

Warburton, M. L., and Williams, W. P. (2016). "Advances in mycotoxin-resistant maize varieties," in *Achieving sustainable maize cultivation: From improved varieties to local applications*, Vol. 1, ed. D. Watson (Sawston: Burleigh Dodds Science Publishing Ltd).

Wiedemann, A., Virlogeux-Payant, I., Chaussé, A. M., Schikora, A., and Velge, P. (2015). Interactions of *Salmonella* with animals and plants. *Front. Microbiol.* 5:791. doi: 10.3389/fmicb.2014.00791

Williams, T. R., Moyne, A. L., Harris, L. J., and Marco, M. L. (2013). Season, irrigation, leaf age, and *Escherichia coli* inoculation influence the bacterial diversity in the lettuce phyllosphere. *PLoS One* 8:e68642. doi: 10.1371/journal.pone.0068642

Williams, W. P., and Windham, G. L. (2012). Registration of Mp718 and Mp719 germplasm lines of maize. *J. Plant Regist.* 6, 200–202. doi: 10.3198/jpr2011.09.0489crg

Wong, C. W. Y., Wang, S., Lévesque, R. C., Goodridge, L., and Delaquis, P. (2019). Fate of 43 *Salmonella* strains on lettuce and tomato seedlings. *J. Food Prot.* 82, 1045–1051. doi: 10.4315/0362-028X.JFP-18-435

World Health Organization [WHO] (2015). *WHO Estimates of the Global Burden of Foodborne Diseases: Foodborne Disease Burden Epidemiology Reference Group 2007-2015*. Geneva: World Health Organization.

Wu, F. (2007). Measuring the economic impacts of *Fusarium* toxins in animal feeds. *Anim. Feed Sci. Technol.* 137, 363–374. doi: 10.1016/j.anifeedsci.2007.06.010

Yu, Y. C., Yum, S. J., Jeon, D. Y., and Jeong, H. G. (2018). Analysis of the microbiota on lettuce (*Lactuca sativa* L.) cultivated in South Korea to identify foodborne pathogens. *J. Microbiol. Biotechnol.* 28, 1318–1331. doi: 10.4014/jmb.1803.03007

Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *J. Saudi Chem. Soc.* 15, 129–144. doi: 10.1016/j.jscs.2010.06.006

Zhou, Y., Wang, J., Yang, X., Lin, D., Gao, Y., Su, Y., et al. (2013). Peanut allergy, allergen composition, and methods of reducing allergenicity: a review. *Int. J. Food Sci.* 2013:909140. doi: 10.1155/2013/909140

Conflict of 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.

frontiers in Microbiology

MINI REVIEW
published: 28 May 2020
doi: 10.3389/fmicb.2020.01087

Frontiers in Plant Breeding: Perspectives for the Selection of Vegetables Less Susceptible to Enteric Pathogens

*Tania Henriquez[1], Anna Lenzi[2], Ada Baldi[2] and Massimiliano Marvasi[3]**

[1] Biozentrum, Ludwig-Maximilians-Universität München, Munich, Germany, [2] Department of Agriculture, Food, Environment and Forestry (DAGRI), University of Florence, Florence, Italy, [3] Department of Biology, University of Florence, Florence, Italy

Fresh vegetables including baby greens, microgreens, and sprouts can host human pathogens without exhibiting any visible signs of spoilage. It is clear that the vast majority of foodborne disease outbreaks associated with vegetable produce are not simply a result of an oversight by a producer, as it was shown that zoonotic pathogens from *Enterobacteriaceae* can contaminate produce through various routes throughout the entire production cycle. In this context, phenotypic and genotypic signatures have been used since early ages in agriculture to obtain better produce, and can be used today as a strategy to reduce the risk of outbreaks through plant breeding. In this mini-review, we provide an updated view and perspectives on to what extent the selection of biological markers can be used to select safer cultivars of vegetable crops such as tomato (the most studied), leafy greens and cabbage. Once this knowledge will be better consolidated, these approaches should be integrated into the development of comprehensive farm-to-fork produce safety programs.

Keywords: plant breeding, enteric pathogens, biomarkers, cultivars, produce, food safety

OPEN ACCESS

Edited by:
Max Teplitski,
University of Florida, United States

Reviewed by:
Abani Kumar Pradhan,
University of Maryland, United States
Kalmia Kniel,
University of Delaware, United States

****Correspondence:***
Massimiliano Marvasi
massimiliano.marvasi@unifi.it

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *03 January 2020*
Accepted: *30 April 2020*
Published: *28 May 2020*

Citation:
Henriquez T, Lenzi A, Baldi A and Marvasi M (2020) Frontiers in Plant Breeding: Perspectives for the Selection of Vegetables Less Susceptible to Enteric Pathogens. Front. Microbiol. 11:1087. doi: 10.3389/fmicb.2020.01087

HUMAN PATHOGENS IN CROP PRODUCTION ENVIRONMENT

Outbreaks linked to the consumption of fresh fruits, vegetables, and sprouts suggest that human pathogens can contaminate produce pre- and/or post-harvest (Bartz et al., 2014). Human enteric pathogens, such as non-typhoidal *Salmonella* and Shiga toxin-producing *Escherichia coli* can survive in the crop production environment, causing recurrent outbreaks (U.S. FDA, 2019). The majority of outbreaks of gastrointestinal illnesses have been associated with fruits, lettuce, alfalfa sprouts, spinach and tomatoes (Dewey-Mattia et al., 2018; CDC, 2019). Based on these observations, it is reasonable to hypothesize that enteric pathogens interact differently with various crops (although a number of other hypotheses can be offered and tested). Such pathogens also exhibit different contamination in greenhouses or in the field (Barak et al., 2011; Barak and Schroeder, 2012). These pathogens can survive in soil and water for extended periods of time and surface irrigation water improperly treated has been commonly identified as a source of contamination (Lapidot and Yaron, 2009; Moorman et al., 2014). Good Agricultural Practices and agronomical operations, as well as intervention technologies optimized to manage plant pathogens and safety have been put in place to minimize the risk of produce contamination and the spread of the pathogens through the supply chain (Bartz et al., 2014; Marvasi et al., 2015a;

Murray et al., 2017). In addition to these tools, plant breeding has been recently suggested as another opportunity to enhance produce safety. This mini-review focuses on the feasibility of harnessing crop's genetic potential to improve produce safety, with the emphasis on enteric pathogenic bacteria, primarily non-typhoidal *Salmonella* and Shiga toxin-producing *E. coli*, which have been the primary culprits of a large number of outbreaks linked to vegetable produce. In the first section of this mini-review we show the intra-species variability in the susceptibility to contamination and proliferation of enteric pathogens. Such variability among varieties and cultivars can be the result of specific genomic traits that can be transferred to the offspring. In the second section, we explore cases when potential biomarkers are identified and tentatively associated with the response to enteric pathogens (**Figure 1**).

CULTIVARS OF VEGETABLE CROPS DIFFER IN THEIR SUSCEPTIBILITY TO ENTERIC PATHOGENS

Tomato

Tomato is by far the most studied species, probably due to the fact that it is the most important vegetable crop worldwide, and also often involved in enteric pathogens outbreaks. A greenhouse screening of 31 cultivars with characteristics that could conceivably affect how conducive tomatoes are to *Salmonella* proliferation has been carried out (Marvasi et al., 2014a). From this screening, tomato cultivar 'Sun Gold' and 'Snow White' were shown to be less prone to support *Salmonella* proliferation. This study also showed that cherry tomatoes (small size) were generally less conducive to proliferation of *Salmonella* when compared with tomato cultivars with a regular size (Marvasi et al., 2014a). A few studies were also carried out under field conditions. The post-harvest proliferation of *Salmonella* in the post-harvest tomato cultivars 'Bonny Best,' 'Florida-47,' and 'Solar Fire' grown under a variety of irrigation regimes showed that 'Bonny Best' was significantly less prone to *Salmonella* proliferation when compared with 'Solar Fire' but not when compared with 'Florida-47' (considering all maturity stages) (Marvasi et al., 2013a). Interestingly, in the greenhouse study, unripe 'Bonny Best' tomato fruits were less susceptible to *Salmonella* also when compared with 'Florida-47' (Marvasi et al., 2014a). This suggests that caution should be taken against directly translating the results of greenhouse studies to field conditions. The cultivar 'Solar Fire' was not significantly different from 'Sebring' when subjected to different fertilization regimes (Marvasi et al., 2013a, 2014b).

In another field study, 13 tomato cultivars selected based on a range of distinct fruit phenotypes, including morphology, pigmentation and resistance to phytopathogens were tested for susceptibility to *Salmonella* surface colonization (Han and Micallef, 2014). The study demonstrated that fruits and leaves of the same cultivar differed in their ability to suppress or support *Salmonella* growth arguing for the important role of tissue differentiation (Han and Micallef, 2014). Fruits of cultivar 'Heinz-1706' were the least colonized by *Salmonella* Newport, while the highest populations were observed on 'Nyagous.' By contrast, seedlings of the cultivar 'Florida-91' supported lowest populations of *Salmonella* Newport while the cherry variety 'Virginia Sweets' supported the highest. For seedling leaves infected with *Salmonella* Typhimurium the lowest susceptible was the cultivar 'Nyagous' and the highest were the cultivars 'Heinz-1706' and 'Moneymaker' (Han and Micallef, 2014). The comparative genomics of the pathogenic strains and in particular different serovars could help to determine why they behave differently during infection (e.g., *Salmonella* Typhimurium versus Newport) (Teplitski and de Moraes, 2018). Although tomato leaves are not edible, the information about *Salmonella* attachment and susceptibility to leaf colonization are relevant since *Salmonella* residing on leaves can be transmitted to fruits (Han and Micallef, 2014).

Cabbage

Response of cabbage cultivars to internalization or surface survival of *Salmonella* and *Escherichia coli* O157:H7 was studied by Erickson et al. (2019a; 2019b). In a growth chamber study, internalized *Salmonella* was detected in cabbage within 24 h with prevalence ranging from 62% plants for the cultivar 'Super Red 80' to 92% for 'Red Dynasty.' The study showed that surface survival of both *Salmonella* and *Escherichia coli* O157:H7 on small cabbage plants over nine days was significantly affected by cultivars: pathogens survived the most on the cultivar "Farao" (Erickson et al., 2019b). In a field study which compared medium-sized cabbage heads, 'Red Dinasty' was more likely to be positive for *Salmonella* and *E. coli* O157:H7 (3.0 and 11.5 times, respectively) on day 5 post-inoculation when compared with the cultivar 'Bravo F1' (Erickson et al., 2019a).

Leafy Greens

When seeds of cilantro, parsley, radicchio, endive, lettuce and spinach were sown in the same pots containing contaminated soil, radicchio and endive had a significantly higher contamination index (CI) than lettuce (Barak et al., 2008). Since radicchio, endive, and lettuce all belong to the *Asteraceae*, these results revealed different response to enteric pathogens within the same Family, suggesting a possible effect of the genotype, at least under these greenhouse conditions (Barak et al., 2008). It must be mentioned that over the last decade, lettuce was instead associated with the highest number of outbreak investigations (U.S. FDA, 2019), but this could be due to the much higher acreage and consumption of lettuce versus other leafy crops (USDA - Statistics, 2019). The experiments described above were conducted in greenhouses and therefore may have missed some typical conditions of the field environment. Other field studies were carried out aiming to compare attachment of *Salmonella* on different lettuce cultivars. In this scenario prevalence of contamination was observed on leaves with the following order (from the less to the most): 'Muir' < 'Gabriella' < 'Green Star' = 'New Red Fire' < 'Coastal Star' (Erickson et al., 2019c).

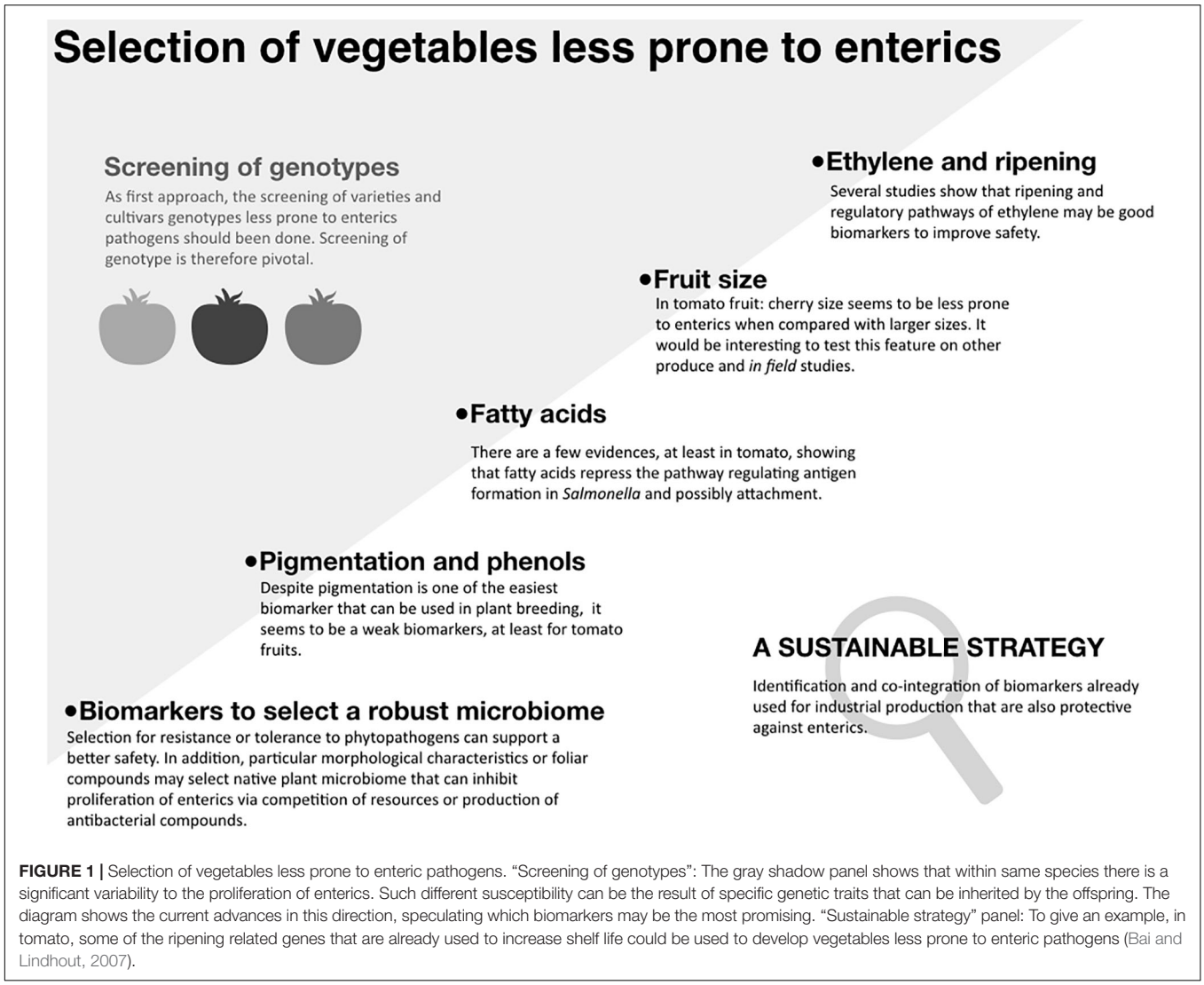


FIGURE 1 | Selection of vegetables less prone to enteric pathogens. "Screening of genotypes": The gray shadow panel shows that within same species there is a significant variability to the proliferation of enterics. Such different susceptibility can be the result of specific genetic traits that can be inherited by the offspring. The diagram shows the current advances in this direction, speculating which biomarkers may be the most promising. "Sustainable strategy" panel: To give an example, in tomato, some of the ripening related genes that are already used to increase shelf life could be used to develop vegetables less prone to enteric pathogens (Bai and Lindhout, 2007).

POTENTIAL BIOMARKERS THAT CAN CONTRIBUTE TO PRODUCE SAFETY

We have previously seen that cultivars differ in their ability to support proliferation of enteric pathogens, therefore a number of potential biomarkers to be used in breeding programs can be obtained from studying such differences. Tomato is mainly used as a model plant for this purpose, but limited literature is also available on other raw eaten crops.

Flavonoids, Carotenoids, and Phenolics

Pigmentation, due to the presence of flavonoids and carotenoids, is the easiest biomarker that can be used in plant breeding programs. A recent study tested whether pigmentation can serve as an appropriate indicator of plant susceptibility to human pathogens. Thirty-one different tomato cultivars, including those that show different pigmentations have been screened. Despite color differences, pigmentation *per se* did not appear to account for the increased proliferation of *Salmonella* (Marvasi et al., 2014a). Follow-up studies demonstrated that tomato phenolics rutin, quercetin and kaempferol did not impact growth of *Salmonella* in rich laboratory media. Therefore, these three phenolics may not be useful as biomarkers for selection either (Marvasi et al., 2014a). The discovery of any association of pigmentation or other phenotypic characteristics (for example flavors) with enteric pathogens conductivity would be an easy phenotypic trait to be used. Less susceptible leafy greens or cabbage cultivars may be also potentially screened for pigments or phenolic compounds. To our knowledge no research has been carried out on these species.

Fatty Acids

The presence of fatty acids has been proposed as a possible indicator of susceptibility to enteric pathogens. Linolenic and linoleic acids are unsaturated fatty acids present in tomato and are precursors of hexanal which contributes to the fruity flavor (Jadhav et al., 1972). Linoleic acid seems to be involved in proliferation of *Salmonella* (Noel et al., 2010; Marvasi et al.,

2013b). Linoleic acid is a polyunsaturated omega-6 fatty acid and its accumulation is progressively reduced in mature tomato fruits (Carrari et al., 2006). Linoleic acid has been demonstrated to play a role in the regulation of *yihT* gene which is involved in the synthesis of the O-antigen capsule in *Salmonella*. It is therefore reasonable to assume that capsule may be involved in attachment/persistence also outside the human host (Maruzani et al., 2019). Interestingly, deletion of *yihT* in *S. enterica* sv. Typhimurium reduced competitive fitness of the pathogen in immature but not in ripe tomatoes, regardless of their color at maturity. Linoleic acid is able to completely repress the *yihT* gene in *Salmonella* and the suppression of such gene could reduce the proliferation in immature tomatoes. Repression of the *yihT* reporter was stronger in tomato cultivars 'Kumato,' 'Snow White,' and 'Mariana' when compared with the variety 'Bloody Butcher' (Marvasi et al., 2013b). However, the response seemed to be generic to other fatty acids because decanoic and linolenic also affected the *yihT* gene reporter (Noel et al., 2010; Marvasi et al., 2013b). Other studies showed that linoleic acid was responsible for the upregulation of *Salmonella fadH* gene in green tomato fruit (Noel et al., 2010). The *fadH* gene encodes 2,4-dienoyl-CoA reductase, an iron-sulfur flavoenzyme required for metabolism of unsaturated fatty acids with double bonds at even carbon positions (Hubbard et al., 2003). More studies should be conducted to determine if fatty acids could serve as biomarkers due to their ability to repress key genes of enteric pathogens. As per phenolic, to our knowledge no additional literature is available to associate fatty acids and susceptibility to enteric pathogens in other crops, such as leafy greens or cabbage.

Ethylene

Ethylene is a plant hormone playing a key role in climacteric fruit ripening (Liu et al., 2015). *Salmonella* relies on a distinct set of metabolic and regulatory genes, which are differentially regulated *in planta* in response to the host genotype and during tomato fruit ripening (Noel et al., 2010). Because ethylene is involved in fruit ripening, it is clear that this metabolic pathway could be a good candidate to be further explored.

Fruits of three tomato cultivars, 'Bonny Best,' 'Solar Fire,' and 'Florida-47,' were harvested and infected with 100 CFU of *Salmonella* and incubated for a week at 22°C. In this experiment maturity stages of the fruits at the time of infection with *Salmonella* were assessed using the USDA tomato maturity chart. Maturity stages 5 and 6 at field harvest correspond to "ripe," fruits that were harvested at 4 and 3 stages were considered "partially ripe," and those that were harvested at 1 and 2 stages (and did not ripen beyond stage 5 during the experiment) were considered "unripe" (Marvasi et al., 2013a). The results showed that each ripening stage was significantly different in terms of *Salmonella* proliferation. The researchers found *Salmonella* counts to be at least 1 log higher in ripe tomatoes compared to the unripe tomatoes under the same growing conditions (Marvasi et al., 2013a). *Salmonella* ability to colonize bell peppers (cultivar 'Aristotle') at different maturity stages was also tested, showing that mature peppers were significantly more prone to support *Salmonella* when compared with the un-ripe ones (Marvasi et al., 2015b). The survival and/or proliferation of *Escherichia coli* O157:H7 and *Salmonella* on the surface of artificially bruised and unbruised tomatoes was tested at three ripeness stages (breaker, pink, and red) and two storage temperatures (10 and 20°C) (Tokarskyy et al., 2018). Tomatoes at the red ripeness stage showed a significant effect of bruising on *Salmonella* survival at both 10 and 20°C (Tokarskyy et al., 2018). Ripening is associated with tissue softening in tomato or bell peppers, therefore the proliferation of *Salmonella* during ripening may be associated with the ability of the bacterial cells to spread more easily into the softener tissue. In addition, a mature pericarp is also more prone to shallow wounds (Marvasi et al., 2014a). According with these results, it is reasonable to assume that testing a series of mutants in ethylene signaling could offer insights about the role of ethylene related genes and ripening during enteric pathogens proliferation. Results of the experiments with tomato mutants with specific defects in ethylene synthesis and perception suggested that ethylene signaling pathways mediated by RIN and NOR (MADS box and SPBP transcriptional factors, respectively) are more consequential that those that rely on the ethylene response sensor-like ethylene receptor NR (Fujisawa and Ito, 2013; Marvasi et al., 2014a). These mutants showed also changes in pigment production, being RIN and NOR not ripening (remaining green), while NR changing to dark red (Osorio et al., 2011; Liu et al., 2015). When post-harvest mature tomato fruits carrying RIN, NOR, and NR mutations were infected with *Salmonella*, reduced proliferation of *Salmonella* was observed when compared with the wild type parent 'Ailsa Craig' (Marvasi et al., 2014a). Some of these ripening related genes are already used in tomato breeding programs, therefore a systematic evaluation of the susceptibility of these cultivars to enteric pathogens can provide further information (Bai and Lindhout, 2007).

Another experiment to test the involvement of the ethylene cascade in the proliferations of enteric pathogens has been done by treating *Medicago truncatula* seedlings with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The treatment strongly reduced endophytic populations of *Salmonella* (Iniguez et al., 2005). Are therefore ethylene mutants/variants good indicators? It is too early to advise, but the ethylene pathway may be a good candidate for further studies. In addition, the selection of plants that differently respond to the application of post-harvest ethylene can be interesting for produce safety (such as the ethylene receptor NR in tomato).

BREEDING PLANTS TO SUPPORT A ROBUST MICROBIOME

Microbiome has been shown to contribute to the proliferation of *Salmonella* in tomato fruits (Marvasi et al., 2013a; Devleesschauwer et al., 2017). Tomato fruits hosting the native microbiome were less prone to *Salmonella* infection when compared with surface cleaned fruits (Marvasi et al., 2013a; Devleesschauwer et al., 2017). *Salmonella*, for example, seems to harbor traits allowing for its interaction with plants and its microbiota (Brandl et al., 2013). It is tempting to speculate

that the selection for leaf morphology, presence of exudates or waxes can stimulate the robust "healthy" microbiome and discourage establishment of enteric pathogens. There are no specific studies in this area, however, a few publications support the overall rationale. First, it is well established that leaf architecture, nutrients and secondary metabolites impact the composition of the plant epibiome (Singh et al., 2019). Second, recent studies demonstrated that certain members of the native plant microbiome can inhibit proliferation of *Salmonella* and pathogenic *E. coli* (Brandl et al., 2013).

For example, an antagonist epiphyte to *Salmonella*, *Paenibacillus alvei TS-15*, was isolated from different plants native to the Virginia Eastern Shore tomato-growing region (Allard et al., 2014). The strain TS-15 exhibited broad-range antimicrobial activity against both major foodborne pathogens and major bacterial phytopathogens of tomato. Survival of *Salmonella* after inoculation was measured for groups with and without the antagonist at days 0, 1, 2, and 3 and either day 5 on blossoms or day 6 for fruits and leaves. After *P. alvei* strain TS-15 was applied onto the fruits, leaves, and blossoms of tomato plants, the cell numbers of *Salmonella* Newport declined significantly compared with the controls. More than 90% of the plants tested had no detectable levels of *Salmonella* by day 5 for blossoms (Allard et al., 2014).

Bacterial phytopathogens also affect enteric pathogens proliferation in produce. Supermarket produce surveys showed that 60% of produce showing symptoms of soft rot also harbored presumptive *Salmonella* (Brandl et al., 2013). *Salmonella* cells inoculated in pre-existing aggregates of *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Erwinia* spp. had a greater probability of surviving dry conditions on lettuce and cilantro leaves than as solitary cells (Poza-Carrion et al., 2013). Within soft rots, *Salmonella* reached population densities 10- to 100-fold higher than within intact plants (Cox et al., 2013). The current view is that enteric pathogens appear to be successful secondary colonists in post-harvest produce, benefiting from the action of phytopathogens, e.g., suppression of plant defense and plant tissue damage (lesions, water soaking, and soft rots) (Brandl et al., 2013). Therefore, the selection of cultivars resistant to phytopathogens would be important not only to increase yield and quality but also to support produce safety (Brandl et al., 2013; Bettini et al., 2016).

CONCLUSION

The implementation of appropriate breeding practices could provide an additional important step toward produce safety. Currently the main picture is still in its infancy and it is difficult to provide directions on selecting safer cultivars or include them in risk assessments. Nevertheless, some data are useful: for example a study on tomato mutants showed that RIN gene, used to increase shelf life, has also showed some protection against enteric pathogens in 'Ailsa Craig' fruit. More studies must be done on different cultivars testing heterozygosity and alleles type (Marvasi et al., 2014a).

Excellent reviews also show that plants respond to *Salmonella* via defense pathways and that such genetic traits could also be used to achieve increase in produce safety (Teplitski et al., 2009; Brandl et al., 2013). At the same time, a better knowledge of the ecology of enteric pathogens in the environment, their interactions with plants and their persistence in wildlife are required to develop comprehensive solutions that build on the "One Health" concept.

Breeding practices have been used for a long time to reduce risk of plant pathogens. Can the same be done for pathogenic enteric pathogens? The question still remains extremely actual and open.

AUTHOR CONTRIBUTIONS

TH and MM focused on the microbiological component. AL and AB on the agronomical part. All authors wrote the manuscript.

FUNDING

This work was supported by the BioMentoring Program, Faculty of Biology, LMU Munich, Germany 2018–2020.

REFERENCES

Allard, S., Enurah, A., Strain, E., Millner, P., Rideout, S. L., Brown, E. W., et al. (2014). In situ evaluation of *Paenibacillus alve*i in reducing carriage of *Salmonella enterica* Serovar Newport on whole tomato plants. *Appl. Environ. Microbiol.* 80, 3842–3849. doi: 10.1128/AEM.00835-14

Bai, Y., and Lindhout, P. (2007). Domestication and breeding of tomatoes: what have we gained and what can we gain in the future? *Ann. Bot.* 100, 1085–1094. doi: 10.1093/aob/mcm150

Barak, J. D., Kramer, L. C., and Hao, L. Y. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/AEM.01661-10

Barak, J. D., Liang, A., and Narm, K. E. (2008). Differential attachment to and subsequent contamination of agricultural crops by *Salmonella enterica*. *Appl. Environ. Microbiol.* 74, 5568–5570. doi: 10.1128/AEM.01077-08

Barak, J. D., and Schroeder, B. K. (2012). Interrelationships of food safety and plant pathology: the life cycle of human pathogens on plants. *Annu. Rev. Phytopathol.* 50, 241–266. doi: 10.1146/annurev-phyto-081211-172936

Bartz, J. A., Marvasi, M., and Teplitsi, M. (2014). "*Salmonella* and tomatoes," in *The Produce Contamination Problem: Causes and Solution*, eds K. R. Matthews, G. M. Sapers, and C. P. Gerba (Cambridge, MA: Academic Press), 269–289.

Bettini, P. P., Marvasi, M., Fani, F., Lazzara, L., Cosi, E., Melani, L., et al. (2016). *Agrobacterium rhizogenes* rolB gene affects photosynthesis and chlorophyll content in transgenic tomato (*Solanum lycopersicum* L.) plants. *J. Plant Physiol.* 204, 27–35. doi: 10.1016/j.jplph.2016.07.010

Brandl, M. T., Cox, C. E., and Teplitski, M. (2013). *Salmonella* interactions with plants and their associated microbiota. *Phytopathology* 103, 316–325. doi: 10.1094/PHYTO-11-12-0295-RVW

Carrari, F., Baxter, C., Usadel, B., Urbanczyk-Wochniak, E., Zanor, M. I., Nunes-Nesi, A., et al. (2006). Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and

highlight regulatory aspects of metabolic network behavior. *Plant Physiol.* 142, 1380–1396. doi: 10.1104/pp.106.088534

CDC (2019). *Web site Centers for Disease Control*. Available online at: https://www.cdc.gov/foodsafety/outbreaks/index.html (accessed December 20, 2019).

Cox, C. E., McClelland, M., and Teplitski, M. (2013). Consequences of disrupting *Salmonella* AI-2 signaling on interactions within soft rots. *Phytopathology* 103, 352–361. doi: 10.1094/PHYTO-09-12-0237-FI

Devleesschauwer, B., Marvasi, M., Giurcanu, M. C., Hochmuth, G. J., Speybroeck, N., Havelaar, A. H., et al. (2017). High relative humidity pre-harvest reduces post-harvest proliferation of *Salmonella* in tomatoes. *Food Microbiol.* 66, 55–63. doi: 10.1016/j.fm.2017.04.003

Dewey-Mattia, D., Manikonda, K., Hall, A. J., Wise, M. E., and Crowe, S. J. (2018). Surveillance for foodborne disease outbreaks – United States, 2009–2015. *MMWR Surveill. Summ.* 67:1. doi: 10.15585/mmwr.ss6710a1

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., Den Bakker, H. C., Bautista, J., et al. (2019a). Survival of *Salmonella enterica* and *Escherichia coli* O157:H7 sprayed onto the foliage of field-grown cabbage plants. *J. Food Prot.* 82, 479–485. doi: 10.4315/0362-028X.JFP-18-326

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., Den Bakker, H. C., Bautista, J., et al. (2019c). Pre-harvest internalization and surface survival of *Salmonella* and *Escherichia coli* O157:H7 sprayed onto different lettuce cultivars under field and growth chamber conditions. *Int. J. Food Microbiol.* 291, 197–204. doi: 10.1016/j.ijfoodmicro.2018.12.001

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., and Ortega, Y. R. (2019b). Survival and internalization of *Salmonella* and *Escherichia coli* O157:H7 sprayed onto different cabbage cultivars during cultivation in growth chambers. *J. Sci. Food Agric.* 99, 3530–3537. doi: 10.1002/jsfa.9573

Fujisawa, M., and Ito, Y. (2013). The regulatory mechanism of fruit ripening revealed by analyses of direct targets of the tomato MADS-box transcription factor RIPENING INHIBITOR. *Plant Signal. Behav.* 8:e24357. doi: 10.4161/psb.24357

Han, S., and Micallef, S. A. (2014). *Salmonella* Newport and Typhimurium colonization of fruit differs from leaves in various tomato cultivars. *J. Food Prot.* 77, 1844–1850. doi: 10.4315/0362-028X.JFP-13-562

Hubbard, P. A., Liang, X., Schulz, H., and Kim, J. J. P. (2003). The crystal structure and reaction mechanism of *Escherichia coli* 2,4-dienoyl-CoA reductase. *J. Biol. Chem.* 278, 37553–37560. doi: 10.1074/jbc.M304642200

Iniguez, A. L., Dong, Y., Carter, H. D., Ahmer, B. M. M., Stone, J. M., and Triplett, E. W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant Microbe Interact.* 18, 169–178. doi: 10.1094/MPMI-18-0169

Jadhav, S., Singh, B., and Salunkhe, D. K. (1972). Metabolism of unsaturated fatty acids in tomato fruit: linoleic and linolenic acid as precursors of hexanal. *Plant Cell Physiol.* 13, 449–459. doi: 10.1093/oxfordjournals.pcp.a074758

Lapidot, A., and Yaron, S. (2009). Transfer of *Salmonella enterica* Serovar Typhimurium from contaminated irrigation water to parsley is dependent on curli and cellulose, the biofilm matrix components. *J. Food Prot.* 72, 618–623. doi: 10.4315/0362-028X-72.3.618

Liu, M., Pirrello, J., Chervin, C., Roustan, J. P., and Bouzayen, M. (2015). Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. *Plant Physiol.* 169, 2380–2390. doi: 10.1104/pp.15.01361

Maruzani, R., Sutton, G., Nocerino, P., and Marvasi, M. (2019). Exopolymeric substances (EPS) from *Salmonella enterica*: polymers, proteins and their interactions with plants and abiotic surfaces. *J. Microbiol.* 57, 1–8. doi: 10.1007/s12275-019-8353-y

Marvasi, M., Cox, C. E., Xu, Y., Noel, J. T., Giovannoni, J. J., and Teplitski, M. (2013b). Differential regulation of *Salmonella* Typhimurium genes involved in O-antigen capsule production and their role in persistence within tomato fruit. *Mol. Plant Microbe Interact.* 26, 793–800. doi: 10.1094/MPMI-09-12-0208-R

Marvasi, M., George, A. S., Giurcanu, M., Hochmuth, G. J., Noel, J. T., Gause, E., et al. (2014b). Effects of nitrogen and potassium fertilization on the susceptibility of tomatoes to post-harvest proliferation of *Salmonella enterica*. *Food Microbiol.* 43, 20–27. doi: 10.1016/j.fm.2014.03.017

Marvasi, M., George, A. S., Giurcanu, M. C., Hochmuth, G. J., Noel, J. T., and Teplitski, M. (2015b). Effect of the irrigation regime on the susceptibility of pepper and tomato to post-harvest proliferation of *Salmonella enterica*. *Food Microbiol.* 46, 139–144. doi: 10.1016/j.fm.2014.07.014

Marvasi, M., Hochmuth, G., and Teplitski, M. (2015a). The role of crop production practices and weather conditions in microbiological safety of tomatoes and peppers. *Food Microbiol.* 46, 139–144. doi: 10.13140/RG.2.2.20981.01762

Marvasi, M., Hochmuth, G. J., Giurcanu, M. C., George, A. S., Noel, J. T., Bartz, J., et al. (2013a). Factors that affect proliferation of *Salmonella* in tomatoes post-harvest: the roles of seasonal effects, irrigation regime, crop and pathogen genotype. *PLoS One* 8:e80871. doi: 10.1371/journal.pone.0080871

Marvasi, M., Noel, J. T., George, A. S., Farias, M. A., Jenkins, K. T., Hochmuth, G., et al. (2014a). Ethylene signalling affects susceptibility of tomatoes to *Salmonella*. *Microb. Biotechnol.* 7, 545–555. doi: 10.1111/1751-7915.12130

Moorman, G. W., Gevens, A. J., Granke, L. H., Hausbeck, M. K., Hendricks, K., Roberts, P. D., et al. (2014). "Sources and distribution systems of irrigation water and their potential risks for crop health," in *Biology, Detection, and Management of Plant Pathogens in Irrigation Water*, eds C. Hong, G. W. Moorman, W. Wohanka, and C. Buttner (St. Paul, MN: APS Press), 3–12. doi: 10.1094/9780890544914.002

Murray, K., Wu, F., Shi, J., Jun Xue, S., and Warriner, K. (2017). Challenges in the microbiological food safety of fresh produce: limitations of post-harvest washing and the need for alternative interventions. *Food Qual. Saf.* 1, 289–301. doi: 10.1093/fqsafe/fyx027

Noel, J. T., Arrach, N., Alagely, A., Mcclelland, M., and Teplitski, M. (2010). Specific responses of *Salmonella enterica* to tomato varieties and fruit ripeness identified by *In Vivo* expression technology. *PLoS One* 5:e12406. doi: 10.1371/journal.pone.0012406

Osorio, S., Alba, R., Damasceno, C. M. B., Lopez-Casado, G., Lohse, M., Zanor, M. I., et al. (2011). Systems biology of tomato fruit development: combined transcript, protein, and metabolite analysis of tomato transcription factor (nor, rin) and ethylene receptor (Nr) mutants reveals novel regulatory interactions. *Plant Physiol.* 157, 405–425. doi: 10.1104/pp.111.175463

Poza-Carrion, C., Suslow, T., and Lindow, S. (2013). Resident bacteria on leaves enhance survival of immigrant cells of *Salmonella enterica*. *Phytopathology* 103, 341–351. doi: 10.1094/PHYTO-09-12-0221-FI

Singh, P., Santoni, S., Weber, A., This, P., and Péros, J. P. (2019). Understanding the phyllosphere microbiome assemblage in grape species (*Vitaceae*) with amplicon sequence data structures. *Sci. Rep.* 9:14294. doi: 10.1038/s41598-019-50839-0

Teplitski, M., Barak, J. D., and Schneider, K. R. (2009). Human enteric pathogens in produce: un-answered ecological questions with direct implications for food safety. *Curr. Opin. Biotechnol.* 20, 166–171. doi: 10.1016/j.copbio.2009.03.002

Teplitski, M., and de Moraes, M. (2018). Of mice and men.and plants: comparative genomics of the dual lifestyles of enteric pathogens. *Trends Microbiol.* 26, 748–754. doi: 10.1016/j.tim.2018.02.008

Tokarskyy, O., De, J., Fatica, M. K., Brecht, J., and Schneider, K. R. (2018). Survival of *Escherichia coli* O157:H7 and *Salmonella* on bruised and unbruised tomatoes from three ripeness stages at two temperatures. *J. Food Protect.* 81, 2028–2033. doi: 10.4315/0362-028X.JFP-18-220

U.S. FDA (2019). *Investigation Summary: Factors Potentially Contributing to the Contamination of Romaine Lettuce Implicated in the Fall 2018 Multi-State Outbreak of E. coli* O157:H7. Silver Spring, ML: U.S. Food and Drug Administration.

USDA - Statistics (2019). *Annual Reports: Crop Acreage, Yields, Areas Harvested, and Other Production Information*. Available online at: https://www.usda.gov/topics/farming/crop-production (accessed December 21, 2019).

Conflict of 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 May 2020
doi: 10.3389/fmicb.2020.01135

Colonization and Internalization of *Salmonella enterica* and Its Prevalence in Cucumber Plants

Kellie P. Burris[1], Otto D. Simmons III[2], Hannah M. Webb[1], Lauren M. Deese[1], Robin Grant Moore[1], Lee-Ann Jaykus[1], Jie Zheng[3], Elizabeth Reed[3], Christina M. Ferreira[3], Eric W. Brown[3] and Rebecca L. Bell[3]*

[1] Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, NC, United States, [2] Department of Horticultural Science, North Carolina State University, Raleigh, NC, United States, [3] Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD, United States

Consumption of cucumbers (*Cucumis sativus* var. *sativus*) has been linked to several foodborne outbreaks involving *Salmonella enterica*. The purpose of this work was to investigate the efficiency of colonization and internalization of *S. enterica* into cucumber plants by various routes of contamination. Produce-associated outbreak strains of *Salmonella* (a cocktail of serovars Javiana, Montevideo, Newport, Poona, and Typhimurium) were introduced to three cultivars of cucumber plants (two slicing cultivars and one pickling) via blossoms (ca. 6.4 $\log_{10}$ CFU/blossom, 4.5 $\log_{10}$ CFU/blossom, or 2.5 $\log_{10}$ CFU/blossom) or soil (ca. 8.3 $\log_{10}$ CFU/root zone) and were analyzed for prevalence of *Salmonella* contamination (internal and external) and serovar predominance in fruit and stems. Of the total slicing fruit harvested from *Salmonella*-inoculated blossoms (ca. 6.4, 4.5, or 2.5 $\log_{10}$ CFU/blossom), 83.9% (47/56), 81.4% (48/59) or 71.2% (84/118) were found colonized and 67.9% (38/56), 35.6% (21/59) or 22.0% (26/118) had *Salmonella* internalized into the fruit, respectively. *S.* Poona was the most prevalent serovar isolated on or in cucumber fruits at all inoculation levels. When soil was inoculated at 1 day post-transplant (dpt), 8% (10/120) of the plants were shown to translocate *Salmonella* to the lower stem 7 days post-inoculation (dpi). Results identified blossoms as an important route by which *Salmonella* internalized at a high percentage into cucumbers, and *S.* Poona, the same strain isolated from the 2015 outbreak of cucumbers imported from Mexico, was shown to be well-adapted to the blossom niche.

Keywords: *Salmonella*, cucumber, fitness, internalization, blossom

OPEN ACCESS

Edited by:
Max Teplitski,
University of Florida, United States

Reviewed by:
Patricia Millner,
Agricultural Research Service (USDA), United States
Massimiliano Marvasi,
University of Florence, Italy
Manan Sharma,
United States Department of Agriculture (USDA), United States

****Correspondence:***
Kellie P. Burris
kpburris@ncsu.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *05 January 2020*
Accepted: *05 May 2020*
Published: *29 May 2020*

Citation:
Burris KP, Simmons OD III, Webb HM, Deese LM, Moore RG, Jaykus L-A, Zheng J, Reed E, Ferreira CM, Brown EW and Bell RL (2020) Colonization and Internalization of Salmonella enterica and Its Prevalence in Cucumber Plants. Front. Microbiol. 11:1135. doi: 10.3389/fmicb.2020.01135

INTRODUCTION

An increase in the incidence of foodborne disease outbreaks associated with consumption of fresh fruits and vegetables has been reported in the United States (DeWaal and Bhuiya, 2007). *Salmonella enterica* causes an estimated 1.2 million illnesses annually (Scallan et al., 2011) and is the primary bacterial etiological agent responsible for produce-related outbreaks in the U.S. (Hanning et al., 2009). Four recent multistate outbreaks, occurring between 2013 and 2016, were linked to the consumption of fresh cucumbers (Centers for Disease Control Prevention, 2013; Angelo et al., 2015; Bottichio et al., 2016; Kozyreva et al., 2016).

While contamination of produce can occur during postharvest processing, research has shown that *Salmonella* has been associated with the growing environment (Islam et al., 2004a,b; Barak and Liang, 2008; Gallegos-Robles et al., 2008; Hanning et al., 2009; Patchanee et al., 2010; Gorski et al., 2011; Bell et al., 2012; Micallef et al., 2012) and plants can serve as niche environments for such enteric pathogens (Brandl et al., 2013). The manner in which produce contamination occurs in the field is largely unknown; however, there has been a surge in research examining routes for *Salmonella* to contaminate produce. During crop production, plants may become contaminated with *Salmonella* through untreated biological amended soil of animal origin or water, especially if overhead irrigation with surface water is used (Jacobsen and Bech, 2012; Olaimat and Holley, 2012; Allende and Monaghan, 2015; Li et al., 2015). Environmental surveillance studies have revealed surface water and sediment as the most common sources of *Salmonella* (Gorski et al., 2011; Bell et al., 2012; Micallef et al., 2012; Strawn et al., 2013). *Salmonella* have been shown to spread throughout the field via natural events, such as rain splash (Cevallos-Cevallos et al., 2012). Once it reaches the plant via splash, *Salmonella* can internalize through roots, leaves, stems, and flowers. *Salmonella* also has been shown to translocate within tomato plants and colonize fruit following leaf inoculation without inducing any symptomatic plant response (i.e., wilting, necrotic lesions, or other hypersensitive response) (Gu et al., 2011). While irrigation water is a likely source of *Salmonella* contamination in the production environment, inconsistent results have been observed relative to the ability of *Salmonella* to colonize the root zone and translocate to other tissues (Jablasone et al., 2004; Miles et al., 2009; Hintz et al., 2010; Zheng et al., 2013). *Salmonella* has been shown to invade root tissue and translocate to the shoots (Cooley et al., 2003; Klerks et al., 2007; Barak et al., 2011) but the interaction with plants is likely *Salmonella* serovar (Klerks et al., 2007; Zheng et al., 2013) and plant cultivar dependent (Han and Micallef, 2014).

A direct route for pathogens to the fruit is through contamination of flowers. Several studies have investigated whether blossom inoculation with plant pathogens can result in subsequent fruit and seed contamination (Lessl et al., 2007; van der Wolf and van der Zouwen, 2010; Gautam et al., 2011; van der Wolf et al., 2013; Dutta et al., 2014). For instance, Gautam et al. (2011) demonstrated colonization of cantaloupe fruits by *Erwinia tracheiphila* through blossom inoculation. Eight of nine plants inoculated with *Erwinia* through the flowers demonstrated symptoms of bacterial wilt and the fruit of three of the plants developed lesions (Gautam et al., 2011). Many angiosperm flowers contain a nectary that provides pollinators a source of energy as well as a primary habitat for microbes by offering higher humidity and a reservoir of nutrients (Aleklett et al., 2014). Flowers have also been examined as a route of enteric pathogen contamination for several produce commodities, including melons (Gautam, 2012), tomatoes (Zhuang et al., 1995; Guo et al., 2001; Shi et al., 2007; Zheng et al., 2013) and most recently cucumbers (Erickson et al., 2018). *Salmonella* can travel to the ovule and colonize new fruits when inoculated onto the pistil of a tomato or cantaloupe flower (Guo et al., 2001; Gautam, 2012).

In addition to dispersal and colonization via water, *Salmonella* have been shown to survive for long periods of time in the environment (Kumar et al., 2018). For example, *Salmonella* was shown to survive in the soil environment over a period of 40 days (Kumar et al., 2018). They are able to transition between hosts and can survive and persist under harsh desiccant and temperature conditions and exposure to UV radiation, all of which are common to the agricultural environment (reviewed in Brandl, 2006; Fatica and Schneider, 2011). Survival in this harsh environment can be associated with both plant (cultivar as well as growth stage) and microbial (serovar) attributes (Klerks et al., 2007; Zheng et al., 2013; Han and Micallef, 2014).

The purpose of this work was to investigate the efficiency of colonization and internalization of *Salmonella* into cucumber plants by routes of contamination consistent with current agricultural practices. The first objective sought to assess the uptake of *Salmonella* via the stems of cucumber plants after inoculation into the soil, simulating what might happen upon exposure to contaminated drip irrigation water. The second objective focused on determining if blossoms served as a potential pathogen entry point into the fruit, representing an overhead irrigation water contamination scenario. Three cucumber cultivars ("Puccini," "Marketmore 76," and "Thunder") and five produce associated outbreak *S. enterica* serovars (Javiana, Montevideo, Newport, Poona, and Typhimurium) were included in the study to evaluate relative differences by plant cultivar or strain fitness. Quantitative data on prevalence and concentration of *S.* enterica in roots and on/in the fruit, and strain-specific predominance were also collected.

MATERIALS AND METHODS

Bacterial Cultures

Five *S. enterica* serovars were obtained from the stock culture collection of the Division of Microbiology, Center for Food Safety and Applied Nutrition (CFSAN), U.S. Food and Drug Administration (U.S. FDA, College Park, MD, United States) and were all isolated from cucumber or other produce-associated outbreaks or environmental samples: *S.* Javiana (CFSAN 037803, cucumber environmental isolate, serogroup D); *S.* Montevideo (CFSAN 001232, clinical strain associated with tomato consumption; serogroup C1); *S.* Newport (CFSAN 037836, cucumber environmental isolate; serogroup C2); *S.* Poona (CFSAN 038692, cucumber isolate from 2015 outbreak, Mexico, serogroup G); and *S.* Typhimurium (CFSAN 014512, loose leaf lettuce isolate from 2006 outbreak, serogroup B).

Inoculum Preparation

Stock cultures were stored in tryptic soy broth (TSB) (Difco, Becton, Dickinson and Company, Sparks, MD, United States) containing 25% glycerol (Acros Organics Fisher Scientific, Fair Lawn, NJ, United States) at -80°C. Cultures were plated onto tryptic soy agar (TSA) (Difco) and incubated at 37°C for 20 h. A single colony from each culture was transferred to 5 ml of TSB and incubated for 20 h at 37°C. Subsequently, each culture was harvested by centrifugation at 5,000 × g for 10 min and washed

with 0.01 M phosphate-buffered saline (PBS) (pH 7.2) (Life Technologies Corporation, Grand Island, NY, United States). Washing and subsequent centrifugation were performed three times. Bacterial cultures were resuspended in 5 ml PBS, which ˜9 $\log_{10}$ (CFU/ml). An equal volume of cell suspension of each serovar was combined to make up the inocula for cucumber plants. As precedent, there have been numerous studies that have used a cocktail consisting of multiple *S.* enterica serovars to identify serovar-specific responses in tomatoes (Guo et al., 2001, 2002; Zheng et al., 2013). The five-strain cocktail was further diluted in PBS to ca. 8.3 $\log_{10}$ (CFU/root zone) for the soil inoculation studies and to ca. 6.4 $\log_{10}$ (CFU/blossom), 4.5 $\log_{10}$ (CFU/blossom), and 2.5 $\log_{10}$ (CFU/blossom) for three sets of blossom experiments representing high, medium, and low levels of inoculum. Concentration of each individual serovar was confirmed to be equally distributed in the final suspension by plate count immediately before inoculation.

Plant Preparation

Cucumber seeds (*Cucumis sativus* var. *sativus*) of cultivars "Puccini" (pickling cucumber cultivar, treated with FarMore F1400 and film coated) and "Thunder" (slicing cucumber cultivar, treated with Thiram and film coated) were purchased from Stokes Seeds Inc. (Buffalo, NY, United States) and "Marketmore 76" (slicing cucumber cultivar, untreated) was purchased from Hummert Seed Co. (St. Joseph, MO, United States). Seeds were planted in 50:50 (w:w) mix substrate [50% Sunshine Redi-Earth Professional Growing Mix (Canadian Sphagnum peat moss 50–65%, vermiculate, dolomitic lime, 0.0001% silicon dioxide) and 50% cement sand] in standard 4.5" pots and maintained in the North Carolina State University (NCSU) Phytotron greenhouse at 26°C (day) and 22°C (night) without additional artificial lighting and grown for 3 weeks (wk). Authors have the necessary authorization to carry out the experiments within the BSL-3P greenhouse with BSL-2 class organisms. Plants for use in blossom studies were then transferred to 8" pots and planted in standard mixed substrate [33% Sunshine Redi-Earth Professional Growing Mix (Canadian Sphagnum peat moss 50–65%, vermiculite, dolomitic lime, 0.0001% silicon dioxide) and 66% pea gravel (w:w)] and were placed in the NCSU Biological Safety Level-3P Phytotron greenhouse at 26°C (day) and 22°C (night) under ambient lighting. Plants used for root inoculation studies were transplanted at 20-day old to 4.5" pots containing either the 50:50 mix substrate or the standard substrate. Plants were watered twice daily, a nutrient solution (106.23 ppm N, 10.41 ppm P, 111.03 ppm K, 54.40 ppm Ca, 12.40 ppm Mg, 5.00 ppm Fe, 13.19 ppm S, 0.113 ppm Mn, 0.24 ppm B, 0.013 ppm Zn, 0.005 ppm Cu, 0.00003 ppm Co, 0.005 ppm Mo, and 11.04 ppm Na) used in the morning and reverse osmosis (RO)-purified water in the afternoon. For the first 3 weeks, plants were limited to the nutrient solution three times a wk to prevent symptoms of nutrient toxicity. Plants used for blossom inoculations were grown on trellises.

Soil Inoculation With *S. enterica*

A total of 150 plants, ∼20 days post-planting and 1 day post-transplant (dpt), were divided into two treatment groups: a negative control group [inoculated with PBS (n = 30)] and an experimental group [inoculated with 4 ml of a five-strain cocktail of ca. 8 $\log_{10}$ CFU/root zone (n = 120)]. Plants were designated into four subgroups of two cultivars [Group 1 (control), Puccini (n = 15); Group 2 (treatment), Puccini (n = 60); Group 3 (control), Thunder (n = 15); and Group 4 (treatment), Thunder (n = 60)]. The "Marketmore 76" slicing cultivar was only used in blossom studies. Four milliliters of prepared cocktail inoculum or 4 ml PBS were directly injected into the 50:50 mix substrate at two locations. More specifically, a 10 ml serological pipet was used to make two holes (about 40-mm depth) around the rhizosphere area. Two milliliters of prepared inoculum [ca. 8.3 log (CFU/root zone)] or 2 ml PBS were applied in each hole. After absorption, holes were refilled with 50:50 mix substrate. Plants were watered in the trays to avoid splash from watering the soil surface and to ensure that no contact was made between the treatments during application and the rest of the plant to prevent cross-contamination.

Blossom Inoculation With *S. enterica*

A total of 184 plants at the blossom stage (n = 50 Puccini, n = 42 Marketmore 76, n = 92 Thunder) were divided and used in three separate experimental designs. The first used cultivars "Puccini" and "Thunder" and was comprised of two treatment groups: a negative control group [inoculated with PBS (n = 10)] and an experimental group [inoculated with 50 μl of a five-strain cocktail of ca. 6.4 $\log_{10}$ CFU/blossom (n = 40)]. The second experimental design included the same two cultivars and treatment groups with lower inoculation level of ca. 4.5 $\log_{10}$ CFU/blossom. The third design using two slicer varieties, i.e., "Thunder" and "Marketmore 76" with two treatment groups: a negative control group [inoculated with PBS (n = 12)] and an experimental group [inoculated with 50 μl of a five-strain cocktail of ca. 2.5 $\log_{10}$ CFU/blossom (n = 72)]. Each experiment was replicated one additional time, for a total of two replicates. For each experiment, greater than 380 blossoms (ca. 10 blossoms/plant) were inoculated with 50 μl of the five-strain cocktail inoculum, and ˜100 blossoms (ca. 5 blossoms/plant) with PBS (control).

Recovery of Endophytically Colonized *S. enterica* From Stems

At 7 days post-inoculation (dpi), each stem from 1 cm above the 50:50 mix substrate surface was aseptically removed from a plant using scissors sterilized between cutting stems, placed into Ziploc bags for transport to the laboratory and processed and sterilized as previously described (Franz et al., 2007; Zheng et al., 2013). Plants did not remain growing after stem sacrifice. Side branches were removed aseptically, and the main stem was immediately immersed in 70% ethanol for 1 min, 5% Clorox for 1 min, 70% ethanol for 1 min, and 1% silver nitrate for 20 min. Stems were then washed in sterile deionized water for 1 min to eliminate residual silver nitrate. They were divided into 0.5 cm long pieces using a sterile scalpel in positional order from apical to basal, discarding the last basal piece. Finally, stems were sectioned and each 0.5 cm stem piece was placed immediately after sectioning onto the surface of XLD agar

medium (Difco) arranged in positional order from apical to basal. Appearance of presumptively-positive *Salmonella* colonies was observed daily for up to 7 days at room temperature and stem position of presumptive positive stem pieces was recorded (Zheng et al., 2013). For those presumptively positive stem pieces, an aliquot of bacteria surrounding the stem piece was collected and re-streaked onto XLD agar with overnight incubation at 37°C for colony purification. Five colonies of differing morphology from each XLD-positive stem piece were randomly chosen for subsequent serological characterization, plated on TSA and incubated overnight at 37°C.

Fruit Sampling and Testing Procedures

For each experiment, more than 250 cucumber fruit were harvested between 10 and 42 dpi. Fruit was carefully removed from the plant using a Ziploc bag so as not to contaminate gloves or cucumbers. Two samples were recovered from each fruit harvested (termed surface and inside). One hundred milliliters of modified Buffered Peptone Water (mBPW) (Vassiliadis et al., 1978) were added to each Ziploc bag and the cucumber was massaged for 2 min to recover *Salmonella* from the surface (Zheng et al., 2013) (termed surface samples). Following massaging, cucumber fruit was carefully removed from the Ziploc bag and placed into a 70% ethanol bath for 20 min to surface sterilize, followed by submersion in a sterile water bath for 5 min (Zheng et al., 2013). The efficacy of the surface sterilization method was previously validated in our laboratory prior to initiation of experiments (data not shown). Fruit were then dried in a laminar flow hood and aseptically cut using a standard kitchen knife into ca. 2 cm^3 pieces. Up to ca. 200 g of cut fruit were aseptically placed into individual sterile Whirl-Pak filter bag (termed inside samples). Each bag was gently massaged and pre-enriched in 200 ml of mBPW with at least 1:1 test portion-to-broth ratio (weight to volume) at 37°C for 20–24 h. An aliquot of 0.1 ml from the incubated pre-enrichment was transferred to a culture tube containing 10 ml Rappaport-Vassiliadis medium (RV) (Rappaport et al., 1956; Vassiliadis et al., 1978) vortexed and incubated at 42°C for 20–24 h. Each incubated selective enrichment broth was streaked onto XLD agar plates (Difco) and incubated at 37°C overnight. Five colonies of differing morphology from each XLD-positive plate were randomly chosen for serological characterization, plated for purification on TSA and incubated overnight at 37°C.

Molecular Serotyping

A DNA template was prepared from each purified isolate by resuspending a single colony into 75 µl 1× Tris-EDTA (TE) pH 8 (Thermo Fisher Scientific, Vilnius, LT, United States) and heating at 100°C for 20 min. The 20 µl PCR mixture contained HotStart MasterMix (Qiagen, Germantown, MD, United States) and 1 µl DNA template. Amplification was conducted as described by Leader et al. (2009). Briefly, a single 16-plex PCR was performed in a thermal cycler (Bio-Rad, Hercules, CA, United States) with the following parameters: initial denaturation at 94°C for 5 min, then 25 cycles of 94°C for 30 s, 57°C for 60 s, 72°C for 30 s followed by a 72°C incubation for 5 min, then 15 cycles of 94°C for 30 s, 60°C for 60 s, 72°C for 30 s followed by 72°C incubation for 5 min. The Agilent 2100 Bioanalyzer was used to analyze PCR amplicons using the DNA 1000 Labchip® kit (Agilent Technologies, Santa Clara, CA, United States) (Leader et al., 2009). Unique banding patterns could distinguish each serovar from one another.

Statistical Analysis

Data was analyzed for prevalence of contamination, serovar predominance in fruits and stems, and cultivar differences. The Pearson Chi-Square Fisher's Exact test was used to determine significant differences in sample positivity (i.e., fruit colonization) obtained for inoculated fruit for all cultivars over all inoculum levels. For serological surveillance of *S. enterica* colonies isolated from stems, an estimated percentage was calculated for each of the serovars used in the studies. The estimated percent was calculated based on the number of isolates identified as a specific serovar divided by the total number of isolates (ca. 100 colonies per each sample type). Data for serological surveillance reflects percent colonization. This percentage was calculated as the number of cucumber fruits colonized by individual serovar, both surface and inside, divided by the total number of *Salmonella*-positive cucumbers for each inoculum level. Two-way analysis of variance (ANOVA) was performed to examine significance and interaction effects of inoculum level, cultivar, location of contamination (outer vs. inner), or serovar. The Tukey-Kramer honestly significant differences (HSD) test was used to identify significant differences in serovar fitness ($P < 0.05$). JMP (JMP Pro 13, SAS Institute Inc., Raleigh, NC, United States) software was used for all the statistical analyses.

RESULTS

Internalization and Migration of *S. enterica* in Cucumber Plants via Soil

A total of 120 stems were collected at 7 dpi from plants exposed to inoculated soil, with all stems inoculated at 1 dpt. Greater than 8% (10/120) of the plants contained endophytically colonized *Salmonella* based on direct plating. Of those 10 plants found positive for *Salmonella*, 6 were cultivar Puccini (pickling cucumber) (6/60, 10%) and 4 were cultivar Thunder (slicing cucumber) (4/60, 7%). Interestingly, *Salmonella* was recovered from inside the stem ca. 5–5.5 cm above the soil line within a week following inoculation, and serovars Montevideo and Newport were recovered from stem segments farthest from the soil line (**Figure 1**). Of those colonies isolated from stem segments that were positive for *Salmonella* (n = 135), serovars Javiana, Montevideo, Newport, and Poona each were identified at 20–30%. No stem segments collected from any of the control plants (n = 30) were positive for the presence of *Salmonella*.

S. enterica Contamination of Fruit via Blossoms

In total, 480 cucumbers [233 slicing (**Table 1**) and 147 pickling (**Supplementary Table S1**)] were harvested and analyzed for the presence of *Salmonella* on the surface and inside from blossoms

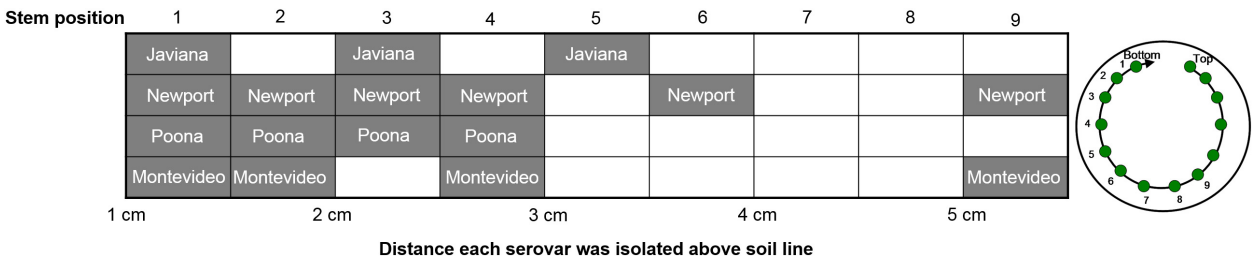


FIGURE 1 | Endophytic recovery of *S. enterica* serovars and the distance traveled into the vascular stem tissue from soil inoculated with a five-strain cocktail (*S.* Javiana, Newport, Poona, Montevideo, and Typhimurium) at ca 8.3 $\log_{10}$ CFU/root zone. All stems derived from inoculated soil in the experimental group were harvested aseptically at ca. 1 cm above the soil line, sterilized, cut into ca. 0.5 cm segments and screened for endophytic populations of *Salmonella*. Molecular serotyping was used to determine the serovar of isolated colonies for each *Salmonella*-positive stem segment. Serovar Typhimurium was not identified from any of the stem segments and therefore, is not included in this figure. Stem position 1 represents ca. 1.0–1.5 cm from soil line while position 9 is closest to the apical portion of the stem (ca. 5.0–5.5 cm from soil line).

TABLE 1 | Prevalence of *Salmonella enterica* for slicer cultivars (Thunder or Marketmore 76) challenged via the blossom route at low (2.5 $\log_{10}$ CFU/blossom), medium (4.5 $\log_{10}$ CFU/blossom), and high (6.4 $\log_{10}$ CFU/blossom) inoculum concentrations.

Cucumber Variety and Inoculation Status	Total # of cucumbers colonized/total # of cucumbers challenged (%)*	Proportion of *S. enterica*-positive cucumbers by colonization location: **	
		# Cucumbers colonized on surface only/total # *S. enterica*- positive cucumbers (%)	# Cucumbers colonized on surface and inside***/total # *S. enterica*-positive cucumbers (%)
HIGH INOCULUM (6.4 $\log_{10}$ CFU/BLOSSOM)			
Thunder			
Inoculated blossoms[a]	47/56 (83.9) a	9/47 (19.1) a	38/47 (80.9) a
Adjacent blossoms[b]	8/24 (33.3)	7/8 (87.5)	1/8 (12.5)
MEDIUM INOCULUM (4.5 $\log_{10}$ CFU/BLOSSOM)			
Thunder			
Inoculated blossoms	48/59 (81.4) a	27/48 (56.2) b	21/48 (43.8) b
Adjacent blossoms	3/32 (9.4)	3/3 (100.0)	0/3 (0.0)
LOW INOCULUM (2.5 LOG_{10} CFU/BLOSSOM)			
Thunder			
Inoculated blossoms	48/67 (71.6) a	35/48 (72.9) b	13/48 (27.1) c
Adjacent blossoms	1/54 (1.9)	1/1 (100.0)	0/1 (0.0)
Marketmore 76			
Inoculated blossoms	36/51 (70.6) a	23/36 (63.9) b	13/36 (36.1) c
Adjacent blossoms	0/50 (0.0)	0/0 (0.0)	0/0 (0.0)

*[a]Cucumbers collected from blossoms directly challenged with the S. enterica cocktail. [b]Cucumbers collected from blossoms that were in close proximity to S. enterica-inoculated blossoms. *The Pearson Chi-Square Fisher's Exact test was used to determine significant differences in sample positivity (i.e., fruit colonization) obtained after inoculating blossoms for all cultivars over all inocula concentrations (within each column). Different letters indicate statistically significant differences ($P<0.05$). **No cucumber fruit were found contaminated inside only. ***If a cucumber fruit was found positive for Salmonella inside the fruit, it also had surface contamination in every instance. External colonization with S. enterica was observed in a small number of the negative control cucumbers (5/167, 3.0%), i.e., those derived from uninoculated control plants.*

inoculated with high (6.4 $\log_{10}$ CFU/blossom), medium (4.5 $\log_{10}$ CFU/blossom), and low (2.5 $\log_{10}$ CFU/blossom) inoculum concentrations. No significant differences were observed in the colonization or internalization between inoculum levels for the pickling cultivar (**Supplementary Table S1**). Because of this observation and the importance of slicing cucumbers associated with *Salmonella* outbreaks, slicing cultivars were focused on for prevalence of contamination analysis. Of the total slicing cucumber fruits harvested from *Salmonella*-inoculated blossoms, there was no significant difference in surface colonization by cultivar (Thunder vs. Marketmore 76; $X^2 = 0.935$, $P = 0.3336$) or blossom inoculum level ($X^2 = 3.606$, $P = 0.1648$; **Table 1**). Even at the lowest level of inoculum tested (2.5 $\log_{10}$ CFU/blossom), ca. 71% (84/118) of cucumber fruits were externally colonized with *Salmonella* (**Table 1**). However, significant differences were observed in internalization between inoculum levels ($X^2 = 34.440$, $P < 0.0001$; **Table 1**). As levels of inoculum increased, the number of *Salmonella*-positive samples found inside the fruits increased (**Table 1**). However, even at the lowest level of inoculum tested, *Salmonella* was found to internalize in ca. 31% (26/84) of those cucumber fruits that were *Salmonella*-positive (**Table 1**).

In total 160 slicing cucumbers harvested from blossoms adjacent to *Salmonella*-inoculated blossoms were analyzed for

colonization and internalization (**Table 1**). As levels of inoculum increased, the number of *Salmonella-positive* samples obtained from fruits collected adjacent to inoculated blossoms increased significantly ($X^2 = 29.658$, $P < 0.0001$; **Table 1**). However, ca. 4% (1/24) adjacent cucumber fruits were positive for internalized *Salmonella* at the highest level of inoculum tested, and no significant differences were observed in internalization between inoculum levels ($X^2 = 5.702$, $P > 0.05$; **Table 1**). It is of note that we did observe external colonization of *Salmonella* in a small number of our control samples (5/167, 3%); however, none were observed to internalize *Salmonella*.

There were no significant differences in predominant serotype isolated by cucumber cultivar; therefore, pickling and slicing cultivars were combined in the subsequent analysis. *S.* Poona was the most prevalent serovar isolated from both the surface and inside of *Salmonella*-positive *c*ucumber fruits (**Figure 2**). Greater than 98% (99/101), 95% (117/123), and 80% (67/84) of *Salmonella*-positive cucumbers harbored *S.* Poona on the surface and greater than 77% (78/101), 52% (64/123), and 20% (17/84) were observed inside for each inoculum level, respectively (**Figure 2**). *S.* Montevideo was the second most prevalent serovar obtained from fruit produced from blossoms inoculated with high, medium and low inoculum levels, with greater than 75% (76/101), 72% (88/123), and 68% (57/84) found on surface for each inoculum level, respectively (**Figure 2**). *S.* Montevideo was also the second most prevalent serovar obtained from inside fruits produced from blossoms inoculated with high and medium inoculum levels at 49% (49/101) and 46% (57/123), respectively; however, for the lowest level of inoculum, the remaining serovars were not statistically different from each other for inside samples (**Figure 2**). For all three levels of inoculum, cucumbers were found to harbor more than one serovar on the surface and internally (**Figure 3**). *S.* Poona and *S.* Montevideo were shown to co-colonize on the surface and inside of *Salmonella*-positive cucumbers at the three inoculum levels (**Figure 3**). *S.* Poona was the most prevalent single serovar to colonize *Salmonella*-positive cucumbers on both the surface and inside (**Figure 3**).

DISCUSSION

Many studies have been conducted to examine the possible routes for *S. enterica* contamination of tomatoes, melons and leafy greens, but little to no work has been done on cucumbers. The goal of the present study was to evaluate the ability of *Salmonella* to colonize cucumber fruit via the inoculation of roots and blossoms during plant growth and fruit maturation. This study capitalized on access to a BSL-3P Phytotron, allowing direct work with agriculturally relevant plants and fully-virulent human outbreak strains of *Salmonella*, utilizing an increased study size as compared to growth chamber experiments and mimicking an environment relevant to field conditions.

While many studies have used relatively high concentrations of *S. enterica* to study fundamental questions about the fate of *Salmonella* in the environment, here we chose to use two different inoculation routes at different inoculum levels [ca. 8.3 $\log_{10}$ (CFU/root zone) and ca. 6.4, 4.5, and 2.5 $\log_{10}$ (CFU/blossom)] of *S. enterica* to examine its colonization and fitness in cucumber. These inoculum levels represent a range of contamination and ca. 2.5 $\log_{10}$ (CFU/blossom) is the lowest inoculum concentration examined for blossom contamination of any fruit to date. The higher inoculum levels examined would be representative of gross fecal contamination of agriculture environments in close proximity to animal operations, as naturally infected pigs may excrete *Salmonella* up to 10^6 CFU/g feces and >5.3 log MPN/g has been found in commercial poultry house litter (Santos et al., 2005; Pires et al., 2013). However, for blossom contamination, lower inoculation levels would be anticipated in natural environments (Fravalo et al., 2003).

While screening a diversity of cucumber lineages for their responses to *Salmonella* was not the main goal of this study, it is important to note that the ability of *Salmonella* to colonize was not dependent on cucumber cultivar. Both pickling and slicer cultivars were found to colonize and internalize *Salmonella*, similarly. Each cultivar varied in its type (gynoecious, monoecious, or parthenocarpic), days from seed to fruit (50–68 days), and disease reactions. All three cultivars were resistant to scab and cucumber mosaic virus, but varied in their resistance to powdery mildew, anthracnose, angular leaf spot, and zucchini yellow mosaic virus. Therefore, we focused our data analysis on the slicer cultivar as it is most relevant to observed outbreaks and is typically consumed raw. Blossom inoculations performed in this study showed that *Salmonella* can internalize readily to fruit, pointing to its ability to evade washing. Interestingly, we observed similar rates of colonization and internalization of fruit through blossoms with all three inoculum levels (**Table 1**), suggesting that *Salmonella* can survive in the blossom and fruit niche at least until harvest (ca. 10–40 dpi), even when applied at ca. 2.5 $\log_{10}$ (CFU/blossom). In effect, blossoms are a direct route for pathogens to the fruit. Flowers likely offer a source of nutrients and energy as well as a hospitable habitat for microbes (Aleklett et al., 2014) and its nectary, attract pollinators which may facilitate transfer of *Salmonella* contamination from plant to plant (Aleklett et al., 2014). During reproduction, pollen grains form a tube that grows through pistil tissues to the ovule, giving a direct route of *Salmonella* internalization of the edible cucumber fruit.

At a relatively high level of inoculum [ca. 8.3 $\log_{10}$ (CFU/root zone)], we observed translocation of serovars *S.* Javiana, *S.* Newport, *S.* Poona and *S.* Montevideo to the lower stems of cucumber plants (between 3 and 5 cm above the 50:50 mix substrate line) a week following inoculation of the root zone. Previous studies examining the ability of *Salmonella* to internalize tomato plants through the root system have been conflicting (Guo et al., 2001; Miles et al., 2009; Hintz et al., 2010; Zheng et al., 2013). A multitude of factors can be responsible for these discrepancies between studies, including but not limited to: soil type, the amount of time before transplant and inoculation, cultivar used, and serotype applied. In comparison to our study, similar migration of *Salmonella* via soil has been demonstrated in tomato plants where multiple serovars (i.e., *S.* Newport, *S.* Montevideo, and *S.* Saintpaul) were recovered up to 10 cm from the soil line within a week following inoculation (Zheng et al., 2013). Our findings support a hypothesis that

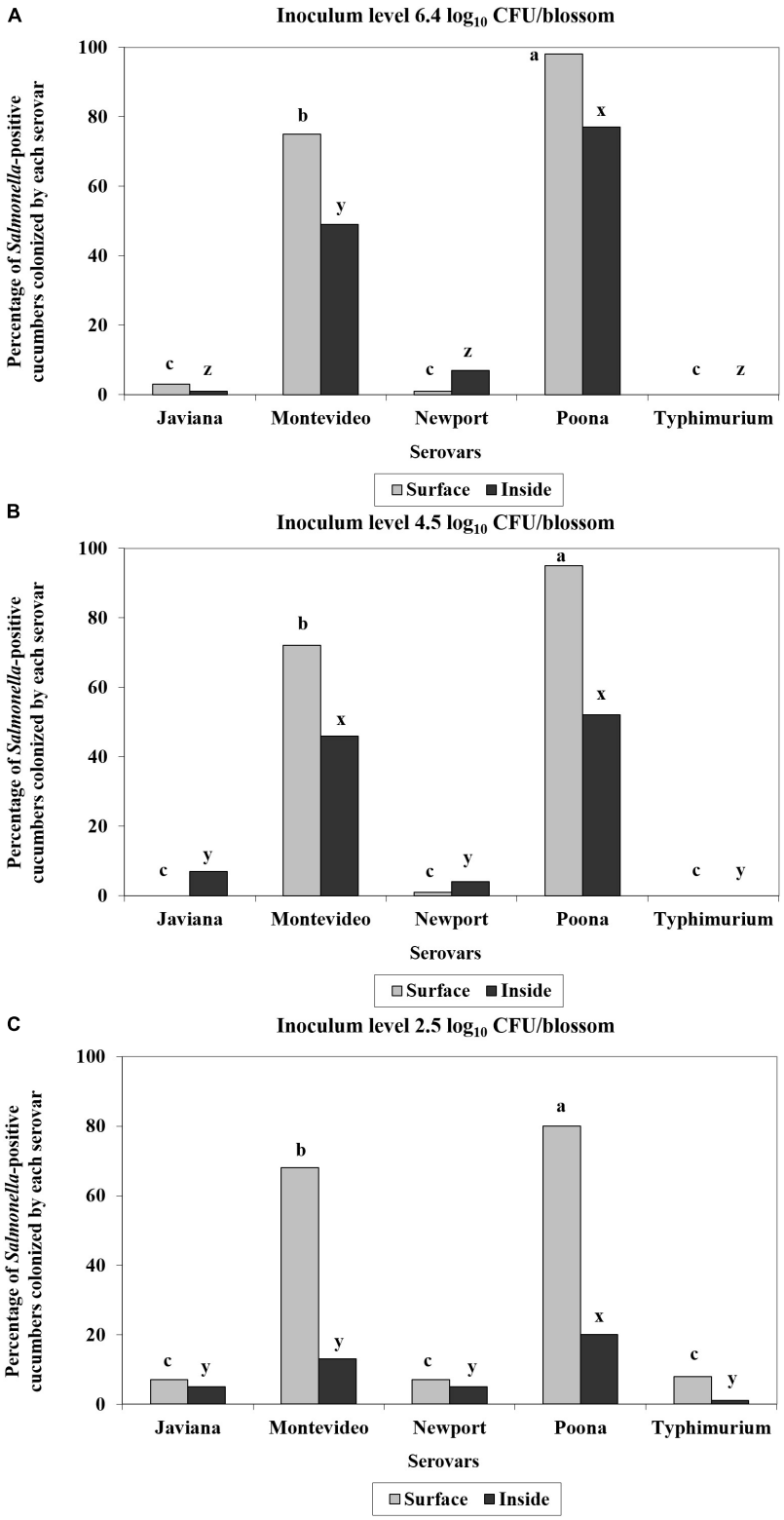


FIGURE 2 | Percentage of each of the five *S. enterica* serovars identified on the surface (gray) and inside (black) of *Salmonella*-positive fruits derived from inoculated blossoms. Inoculum levels were ca. 6.4 $\log_{10}$ CFU/blossom (total number of positive fruits, $N = 101$) **(A)**; ca. 4.5 $\log_{10}$ CFU/blossom ($N = 123$) **(B)**; or ca. 2.5 $\log_{10}$ CFU/blossom ($N = 84$) **(C)**. A five-strain cocktail (*S.* Javiana, Newport, Poona, Montevideo, and Typhimurium) was inoculated onto individually labeled blossoms of cucumber plants. All the cucumber fruits derived from inoculated flowers in the experimental group were harvested and screened for surface and internal

(Continued)

FIGURE 2 | Continued
populations of *Salmonella*. Five unique colonies from each XLD-positive plate were randomly chosen for molecular serotyping. Note: multiple serovars were isolated from some of the *Salmonella*-positive cucumbers. Because there were no significant differences in predominant serotype isolated by cucumber cultivar, all data were combined for statistical analysis. Serovars with different lowercase letters (a, b, c) denote significant differences ($P < 0.05$) for surface colonization (gray), while serovars with different lowercase letters (x, y, z) denote significant differences ($P < 0.05$) for inside contamination as determined by Tukey-Kramer honestly significant difference (HSD) testing on two-way analysis of variance (ANOVA) results.

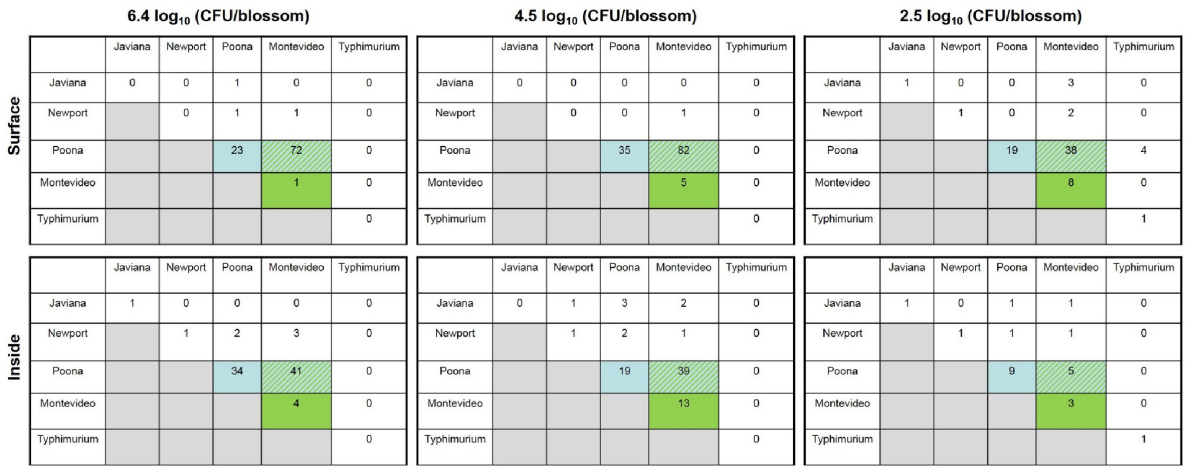


FIGURE 3 | Co-colonization of each *S. enterica* serovar on both the surface (top of figure) and inside (bottom of figure) of cucumber fruit derived from blossoms inoculated with ca. 6.4 $\log_{10}$ CFU/blossom, ca. 4.5 $\log_{10}$ CFU/blossom, or ca. 2.5 $\log_{10}$ CFU/blossom. Figure represents combined data from all cultivars tested ("Thunder," "Puccini," and "Marketmore 76") and all experimental replicates. A five-strain cocktail (*S.* Javiana, *S.* Newport, *S.* Poona, *S.* Montevideo, and *S.* Typhimurium) was inoculated onto individually labeled blossom of cucumber plants. All the cucumber fruits derived from inoculated flowers in the experimental group were harvested and screened for surface and internal populations of *Salmonella*. Five colonies of differing morphology from each XLD-positive plate were randomly chosen for molecular serotyping. Numbers in each box represent the number of positive cucumbers for which there is evidence of specific serovar colonization and co-colonization ($n = 84$, $n = 123$, $n = 101$ for 2.5 $\log_{10}$ CFU/blossom 4.5 $\log_{10}$ CFU/blossom and 6.4 $\log_{10}$ CFU/blossom, respectively) (light blue: *S.* Poona only; green: *S.* Montevideo only; green and blue stripe: *S.* Montevideo and *S.* Poona co-colonized). Note: triple serovars were isolated from 7 of a total of 308 *Salmonella*-positive cucumbers (*S.* Javiana, *S.* Montevideo, and *S.* Poona were isolated from five positive cucumbers – two from surface samples of fruit inoculated with 6.4 $\log_{10}$ CFU/blossom, one from surface sample of fruit inoculated with 2.5 $\log_{10}$ CFU/blossom, and two from internal samples of fruit inoculated with 4.5 $\log_{10}$ CFU/blossom; *S.* Montevideo, *S.* Newport, and *S.* Poona were isolated from one positive cucumber, the internal sample of fruit inoculated with 6.4 $\log_{10}$ CFU/blossom; *S.* Montevideo, *S.* Poona, and *S.* Typhimurium were isolated from one positive cucumber, the surface sample of fruit inoculated with 2.5 $\log_{10}$ CFU/blossom). Serovar Typhimurium was only identified from the *Salmonella*-positive cucumbers inoculated with 2.5 $\log_{10}$ CFU/blossom.

Salmonella can enter the cucumber plant through contaminated drip irrigation water and translocate up the stem, although we did not follow the plants long enough to determine the likelihood of internalization to the edible fruit. However, this method of contamination seems to be quite inefficient, at least when compared to the blossom route. While human pathogens cannot directly penetrate through root cells, previous research suggests that interior root colonization by enteric pathogens might occur passively through wounds in the roots that are damaged during transplantation or sites of lateral root emergence (Zheng et al., 2013). While *Salmonella* may be moving through the vasculature passively as one possibility, other means of active transport may exist. For example, in *Arabidopsis thaliana, Salmonella* was shown to colonize the entire plant following root inoculation and *Salmonella* was observed between epidermal cells and the vascular system (Cooley et al., 2003). The means by which *Salmonella* cells move in the vascular system needs further investigation. While many *Salmonella* serovars have been isolated from environmental samples such as surface water and sediment, only a few have been linked to multistate outbreaks associated with field and greenhouse grown cucumber – 2013 (Saintpaul), 2014 (Newport), 2015 (Poona), and 2016 (Oslo) (Centers for Disease Control Prevention, 2013; Angelo et al., 2015; Bottichio et al., 2016; Kozyreva et al., 2016). Studies that have used a cocktail of inocula containing multiple serovars of *Salmonella* have identified serovar-specific responses to tomato fruit (Guo et al., 2001; Shi et al., 2007; Zheng et al., 2013). For example, *S.* Dublin and *S.* Enteriditis were found to be less adapted to growth on or in tomato fruit than were *S.* Montevideo, *S.* Hadar, and *S.* Newport (Shi et al., 2007), and *S.* Montevideo was observed as the most prevalent serovar recovered from tomatoes following blossom inoculation (Guo et al., 2001; Shi et al., 2007; Zheng et al., 2013). Results from this study identified blossoms as an important route by which *Salmonella* internalized at a high percentage into cucumbers, and *S.* Poona, the same strain isolated from the 2015 outbreak of cucumbers imported from Mexico, was shown most adapted to the blossom niche, followed by *S.* Montevideo, another produce-outbreak associated strain. While competition between serovars can influence which species dominates within a specific plant niche (Shi et al.,

2007), we found that certain outbreak-associated strains (i.e., *S.* Poona and *S.* Montevideo) appeared to be better adapted for survival and persistence in cucumber plants than were those strains of environmental origin (i.e., *S.* Newport, *S.* Javiana, and *S.* Typhimurium). Interestingly, oftentimes *S.* Poona and *S.* Montevideo co-colonized, suggesting their robust fitness in the cucumber blossom and fruit niche. Additionally, no differences were noted among serovars recovered from stem samples in terms of prevalence except *S.* Typhimurium at 7 dpi via roots. However, it appeared that *S.* Newport and *S.* Montevideo were translocated the farthest up the stem compared to other serovars tested. Of note, previous research has shown that serovars of *Salmonella* that typically associate with poultry, i.e., *S.* Enteritidis, were less adapted and fit in tomatoes than was *S.* Montevideo (Shi et al., 2007). In a recent field study by Erickson et al. (2018), directly spraying attenuated *S.* Typhimurium contaminated irrigation water onto the cucumber flower resulted in 90–100% contamination of the ovary and flower at 0 day, but only 10–40% after 3 days, indicating the relative poor fitness of *S.* Typhimurium to the cucumber blossom niche.

One potential limitation of our study was the use of a *Salmonella* cocktail (*S.* Javiana, *S.* Montevideo, *S.* Newport, *S.* Poona, and *S.* Typhimurium) for inoculations, possibly introducing culture bias during enrichment. For example, *S.* Typhimurium was not observed to colonize blossoms at the higher inoculum levels, but was observed in fruit when introduced at lower levels. While enrichment bias may potentially be a problem, no other experimental methods exist, other than emerging metagenomic technologies that may eventually prove capable of overcoming this issue. Additionally, bias may also be attributed to the limited number of colonies (five for each sample) chosen for screening, which can be resolved by bioplexing the enrichment directly; however, this would require specialized equipment. We intentionally designed our experiments to examine serovar fitness simultaneously with colonization. Because we now know that colonization occurs at low levels of inoculum and that there is selective colonization, future work is in progress to examine individual serovar colonization at the lowest levels of contamination.

Even under the most stringent control using a BSL-3P Phytotron greenhouse, we observed a small degree of *Salmonella* cross-contamination to our negative controls. In an effort to identify the reservoir of this contamination, various samples were taken within the greenhouse. In those samples, we identified plant debris located on the floor near treatment plants as a potential source of *Salmonella.* In recent research, airborne soil particulates were identified as vehicles for *Salmonella* contamination of tomatoes through blossom inoculation (Kumar et al., 2017). In that study, tomato fruits or their associated calyces, developed after exposure to *Salmonella*-contaminated airborne soil particulates distributed onto blossoms, were found positive for *S.* Newport at 67 and 78%, respectively. Interestingly, *Salmonella* was also found internalized by the tomato fruit (Kumar et al., 2017). However, cross-contamination observed in our negative controls did not result in internalization.

Even when dealing with a crop that is typically drip irrigated, the risk of exposure of blossoms to *Salmonella* via spray could occur as a consequence of frost protection measures, fertilization or fumigation/pesticide applications, or by birds or animal intrusion. Overall, the ramifications of the blossom data are substantial as specific protection of blossoms, which is part of the edible reproductive body of a seed plant, and has been included in current recommendations for best practices in the fresh produce production environment. Current studies are examining alternative barriers to prevent the spread of pathogens through wind and water from dust and manure to produce near confined-animal feedlot operations (CAFOs) (reviewed in Gutierrez-Rodriguez and Adhikari, 2018).

This work clearly demonstrates the ease with which cucumber- and outbreak-associated *Salmonella* strains colonize and internalize healthy cucumber plants and fruit, particularly through the blossom route. We observed similar rates of colonization of fruit through blossoms with all inoculum levels tested, indicating that *Salmonella* can survive in the blossom and fruit niche at least until harvest (ca. 10–40 dpi), even when applied at levels as low as ca. 2.5 $\log_{10}$ CFU/blossom. Because *S.* Poona was isolated from an outbreak associated with cucumbers, it was not surprising it was the most prevalent serovar isolated from cucumber fruit. While this study was conducted in a greenhouse, it could be expected that a similar phenomenon would occur in open production environments. In light of these data, it may be important to further consider the potential role of current field applications encompassing overhead irrigation and fumigation in the subsequent downstream contamination of mature cucumber fruits.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KB, OS, L-AJ, JZ, ER, CF, EB, and RB designed the experiments. KB, HW, LD, and RM collected the data. KB analyzed the data and wrote the manuscript draft. KB, OS, HW, LD, RM, L-AJ, JZ, ER, CF, EB, and RB edited and approved the manuscript.

FUNDING

This research was funded by Lifesource Biomedical LLC and the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. The funder, Lifesource Biomedical LLC, was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We wish to thank the director, Dr. Carole Saravitz, of the NCSU Phytotron and her staff, especially Dr. Joseph Chiera,

David Dupree, Chuck Gibbs, and Samuel Cho, for their valuable assistance in greenhouse set up and plant care. We also wish to acknowledge C.H.E., E. Rios and Elsie Borden for their abundant energy and assistance during harvesting.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01135/full#supplementary-material

REFERENCES

Aleklett, K., Hart, M., and Shade, A. (2014). The microbial ecology of flowers: an emerging frontier in phyllosphere research. *Botany* 92, 253–266. doi: 10.1139/cjb-2013-0166

Allende, A., and Monaghan, J. (2015). Irrigation water quality for leafy crops: a perspective of risks and potential solutions. *Int. J. Environ. Res. Public Health* 12, 7457–7477. doi: 10.3390/ijerph120707457

Angelo, K. M., Chu, A., Anand, M., Nguyen, T.-A., Bottichio, L., Bell, R., et al. (2015). Outbreak of *Salmonella* Newport infections linked to cucumbers–United States, 2014. *MMWR Morb. Mortal. Wkly. Rep.* 64, 144–147.

Barak, J. D., Kramer, L. C., and Hao, L. Y. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/aem.01661-10

Barak, J. D., and Liang, A. S. (2008). Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. *PLoS One* 3:e1657. doi: 10.1371/journal.pone.0001657

Bell, R., Cao, G., Meng, J., Allard, M., Keys, C., Hill, T., et al. (2012). *Salmonella Newport Contamination of Produce: Ecological, Genetic, And Epidemiological Aspects. Salmonella: Classification, Genetics And Disease Outbreaks*. Hauppauge, NY: Nova Science Publishers, Inc.

Bottichio, L., Medus, C., Sorensen, A., Donovan, D., Sharma, R., Dowell, N., et al. (2016). Outbreak of *Salmonella* Oslo infections linked to Persian cucumbers — United States, 2016. *MMWR Morb. Mortal. Wkly. Rep.* 65, 1430–1433. doi: 10.15585/mmwr.mm655051a3

Brandl, M. T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.* 44, 367–392. doi: 10.1146/annurev.phyto.44.070505.143359

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

Centers for Disease Control Prevention (2013). *Multistate Outbreak Of Salmonella Saintpaul Infections Linked To Imported Cucumbers*. Atlanta: Centers for Disease Control Prevention.

Cevallos-Cevallos, J. M., Gu, G., Danyluk, M. D., Dufault, N. S., and van Bruggen, A. H. (2012). *Salmonella* can reach tomato fruits on plants exposed to aerosols formed by rain. *Int. J. Food Microbiol.* 158, 140–146. doi: 10.1016/j.ijfoodmicro.2012.07.009

Cooley, M. B., Miller, W. G., and Mandrell, R. E. (2003). Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter* asburiae. *Appl. Environ. Microbiol.* 69, 4915–4926. doi: 10.1128/aem.69.8.4915-4926.2003

DeWaal, C. S., and Bhuiya, F. (2007). "Outbreaks by the numbers: fruits and vegetables," in *Proceedings of the International Association for Food Protection 94th Annual Meeting*, Pittsburgh.

Dutta, B., Gitaitis, R., Sanders, H., Booth, C., Smith, S., and Langston, D. B. (2014). Role of blossom colonization in pepper seed infestation by *Xanthomonas euvesicatoria*. *Phytopathology* 104, 232–239. doi: 10.1094/phyto-05-13-0138-r

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., Bautista, J., and Diaz-Perez, J. C. (2018). Disposition of *Salmonella* and *Escherichia coli* O157:H7 following spraying of contaminated water on cucumber fruit and flowers in the field. *J. Food Prot.* 81, 2074–2081. doi: 10.4315/0362-028x.jfp-18-344

Fatica, M. K., and Schneider, K. R. (2011). *Salmonella* and produce: survival in the plant environment and implications in food safety. *Virulence* 2, 573–579. doi: 10.4161/viru.2.6.17880

Franz, E., Visser, A. A., Van Diepeningen, A. D., Klerks, M. M., Termorshuizen, A. J., and van Bruggen, A. H. (2007). Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. *Food Microbiol.* 24, 106–112. doi: 10.1016/j.fm.2006.03.002

Fravalo, P., Hascoet, Y., Le Fellic, M., Queguiner, S., Petton, J., and Salvat, G. (2003). Convenient method for rapid and quantitative assessment of *Salmonella enterica* contamination: the mini-MSRV MPN technique. *J. Rapid Method Autom. Microbiol.* 11, 81–88. doi: 10.1111/j.1745-4581.2003.tb00031.x

Gallegos-Robles, M. A., Morales-Loredo, A., Alvarez-Ojeda, G., Vega, P. A., Chew, M. Y., Velarde, S., et al. (2008). Identification of *Salmonella* serotypes isolated from cantaloupe and chile pepper production systems in Mexico by PCR-restriction fragment length polymorphism. *J. Food Prot.* 71, 2217–2222. doi: 10.4315/0362-028x-71.11.2217

Gautam, D. (2012). *Fate of Salmonella Introduced to Cantaloupe Through Natural Fruit Cracks and Flowers, Alone or in the Presence of the Plant Pathogen Erwinia tracheiphila*. Oklahoma: Oklahoma State University.

Gautam, D., Ma, L., Bruton, B., and Fletcher, J. (2011). *Erwinia tracheiphila* colonization of cantaloupe fruits through flower inoculation. *Phytopathology* 101:S59.

Gorski, L., Parker, C. T., Liang, A., Cooley, M. B., Jay-Russell, M. T., Gordus, A. G., et al. (2011). Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Appl. Environ. Microbiol.* 77, 2734–2748. doi: 10.1128/aem.02321-10

Gu, G., Hu, J., Cevallos-Cevallos, J. M., Richardson, S. M., Bartz, J. A., and van Bruggen, A. H. (2011). Internal colonization of *Salmonella enterica* serovar Typhimurium in tomato plants. *PLoS One* 6:e27340. doi: 10.1371/journal.pone.00027340

Guo, X., Chen, J., Brackett, R. E., and Beuchat, L. R. (2001). Survival of Salmonellae on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening. *Appl. Environ. Microbiol.* 67, 4760–4764. doi: 10.1128/aem.67.10.4760-4764.2001

Guo, X., Chen, J., Brackett, R. E., and Beuchat, L. R. (2002). Survival of *Salmonella* on tomatoes stored at high relative humidity, in soil, and on tomatoes in contact with soil. *J. Food Prot.* 65, 274–279. doi: 10.4315/0362-028x-65.2.274

Gutierrez-Rodriguez, E., and Adhikari, A. (2018). Preharvest farming practices impacting fresh produce safety. *Microbiol. Spect.* 6, FS–0022–2018.

Han, S., and Micallef, S. A. (2014). *Salmonella* Newport and Typhimurium colonization of fruit differs from leaves in various tomato cultivars. *J. Food Prot.* 77, 1844–1850. doi: 10.4315/0362-028x.jfp-13-562

Hanning, I. B., Nutt, J. D., and Ricke, S. C. (2009). Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathog. Dis.* 6, 635–648. doi: 10.1089/fpd.2008.0232

Hintz, L. D., Boyer, R. R., Ponder, M. A., Williams, R. C., and Rideout, S. L. (2010). Recovery of *Salmonella enterica* Newport introduced through irrigation water from tomato (*Lycopersicum esculentum*) fruit, roots, stems, and leaves. *Hortscience* 45, 675–678. doi: 10.21273/hortsci.45.4.675

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., and Jiang, X. (2004a). Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Appl. Environ. Microbiol.* 70, 2497–2502. doi: 10.1128/aem.70.4.2497-2502.2004

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., and Jiang, X. (2004b). Persistence of *Salmonella enterica* serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. *Foodborne Pathog. Dis.* 1, 27–35. doi: 10.1089/153531404772914437

Jablasone, J., Brovko, L. Y., and Griffiths, M. W. (2004). A research note: the potential for transfer of *Salmonella* from irrigation water to tomatoes. *J. Sci. Food Agric.* 84, 287–289. doi: 10.1002/jsfa.1646

Jacobsen, C. S., and Bech, T. B. (2012). Soil survival of *Salmonella* and transfer to freshwater and fresh produce. *Food Res. Int.* 45, 557–566. doi: 10.1016/j.foodres.2011.07.026

Klerks, M. M., van Gent-Pelzer, M., Franz, E., Zijlstra, C., and van Bruggen, A. H. (2007). Physiological and molecular responses of Lactuca sativa to colonization

by *Salmonella enterica* serovar Dublin. *Appl. Environ. Microbiol.* 73, 4905–4914. doi: 10.1128/aem.02522-06

Kozyreva, V. K., Crandall, J., Sabol, A., Poe, A., Zhang, P., Concepcion-Acevedo, J., et al. (2016). Laboratory investigation of *Salmonella enterica* serovar Poona outbreak in California: comparison of pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) results. *PLoS Curr.* 8:ecurrents.outbreaks.1bb3e36e74bd5779bc43ac3a8dae52e6. doi: 10.1371/currents.outbreaks.1bb3e36e74bd5779bc43ac3a8dae52e6

Kumar, G. D., Williams, R. C., Al Qublan, H. M., Sriranganathan, N., Boyer, R. R., and Eifert, J. D. (2017). Airborne soil particulates as vehicles for *Salmonella* contamination of tomatoes. *Int. J. Food Microbiol.* 243, 90–95. doi: 10.1016/j.ijfoodmicro.2016.12.006

Kumar, G. D., Williams, R. C., Sriranganathan, N., Boyer, R. R., and Eifert, J. D. (2018). Survival of tomato outbreak associated *Salmonella* serotypes in soil and water and the role of biofilms in abiotic surface attachment. *Foodborne Path Dis.* 15, 548–553. doi: 10.1089/fpd.2017.2416

Leader, B. T., Frye, J. G., Hu, J., Fedorka-Cray, P. J., and Boyle, D. S. (2009). High-throughput molecular determination of *Salmonella enterica* serovars by use of multiplex PCR and capillary electrophoresis analysis. *J. Clin. Microbiol.* 47, 1290–1299. doi: 10.1128/jcm.02095-08

Lessl, J. T., Fessehaie, A., and Walcott, R. R. (2007). Colonization of female watermelon blossoms by *Acidovorax avenae* ssp citrulli and the relationship between blossom inoculum dosage and seed infestation. *J. Phytopathol.* 155, 114–121. doi: 10.1111/j.1439-0434.2007.01204.x

Li, B., Jackson, S. A., Gangiredla, J., Wang, W., Liu, H., Tall, B. D., et al. (2015). Genomic evidence reveals numerous *Salmonella enterica* serovar Newport reintroduction events in Suwannee watershed irrigation ponds. *Appl. Environ. Microbiol.* 81, 8243–8253. doi: 10.1128/aem.02179-15

Micallef, S. A., Rosenberg Goldstein, R. E., George, A., Kleinfelter, L., Boyer, M. S., McLaughlin, C. R., et al. (2012). Occurrence and antibiotic resistance of multiple *Salmonella* serotypes recovered from water, sediment and soil on mid-Atlantic tomato farms. *Environ. Res.* 114, 31–39. doi: 10.1016/j.envres.2012.02.005

Miles, J. M., Sumner, S. S., Boyer, R. R., Williams, R. C., Latimer, J. G., and McKinney, J. M. (2009). Internalization of *Salmonella enterica* serovar Montevideo into greenhouse tomato plants through contaminated irrigation water or seed stock. *J. Food Prot.* 72, 849–852. doi: 10.4315/0362-028x-72.4.849

Olaimat, A. N., and Holley, R. A. (2012). Factors influencing the microbial safety of fresh produce: a review. *Food Microbiol.* 32, 1–19. doi: 10.1016/j.fm.2012.04.016

Patchanee, P., Molla, B., White, N., Line, D. E., and Gebreyes, W. A. (2010). Tracking *Salmonella* contamination in various watersheds and phenotypic and genotypic diversity. *Foodborne Pathog. Dis.* 7, 1113–1120. doi: 10.1089/fpd.2010.0572

Pires, A. F., Funk, J. A., Lim, A., and Bolin, S. R. (2013). Enumeration of *Salmonella* in feces of naturally infected pigs. *Foodborne Pathog. Dis.* 10, 933–937. doi: 10.1089/fpd.2013.1547

Rappaport, F., Konforti, N., and Navon, B. (1956). A new enrichment medium for certain salmonellae. *J. Clin. Pathol.* 9, 261–266. doi: 10.1136/jcp.9.3.261

Santos, F. B. O., Li, X., Payne, J. B., and Sheldon, B. W. (2005). Estimation of most probable number *Salmonella* populations on commercial North Carolina turkey farms. *J. Appl. Poultry Res.* 14, 700–708. doi: 10.1093/japr/14.4.700

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17, 7–15. doi: 10.3201/eid1701.p11101

Shi, X., Namvar, A., Kostrzynska, M., Hora, R., and Warriner, K. (2007). Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *J. Food Prot.* 70, 2725–2731. doi: 10.4315/0362-028x-70.12.2725

Strawn, L. K., Fortes, E. D., Bihn, E. A., Nightingale, K. K., Grohn, Y. T., Worobo, R. W., et al. (2013). Landscape and meteorological factors affecting prevalence of three food-borne pathogens in fruit and vegetable farms. *Appl. Environ. Microbiol.* 79, 588–600. doi: 10.1128/aem.02491-12

van der Wolf, J. M., and van der Zouwen, P. S. (2010). Colonization of cauliflower blossom (*Brassica oleracea*) by *Xanthomonas campestris* pv. campestris, via flies (*Calliphora vomitoria*) can result in seed infestation. *J. Phytopathol.* 158, 726–732. doi: 10.1111/j.1439-0434.2010.01690.x

van der Wolf, J. M., van der Zouwen, P. S., and van der Heijden, L. (2013). Flower infection of *Brassica oleracea* with *Xanthomonas campestris* pv. campestris results in high levels of seed infection. *Eur. J. Plant Pathol.* 136, 103–111. doi: 10.1007/s10658-012-0141-z

Vassiliadis, P., Trichopoulos, D., Kalandidi, A., and Xirouchaki, E. (1978). Isolation of salmonellae from sewage with a new procedure of enrichment. *J. Appl. Bacteriol.* 44, 233–239. doi: 10.1111/j.1365-2672.1978.tb00795.x

Zheng, J., Allard, S., Reynolds, S., Millner, P., Arce, G., Blodgett, R. J., et al. (2013). Colonization and internalization of *Salmonella enterica* in tomato plants. *Appl. Environ. Microbiol.* 79, 2494–2502. doi: 10.1128/aem.03704-12

Zhuang, R. Y., Beuchat, L. R., and Angulo, F. J. (1995). Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl. Environ. Microbiol.* 61, 2127–2131. doi: 10.1128/aem.61.6.2127-2131.1995

Conflict of Interest: The authors declare that this study received funding from Lifesource Biomedical LLC. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 June 2020
doi: 10.3389/fpls.2020.00778

Redomesticating Almond to Meet Emerging Food Safety Needs

*Thomas M. Gradziel**

Department of Plant Sciences, University of California, Davis, Davis, CA, United States

Almond is a desirable and high-quality food source where the presence of nut allergens and a vulnerability to aflatoxin and Salmonella contamination represent threats to consumer safety. In 2019, over 1 billion kg. of almonds, representing over 80% of the world total, were produced in California from a relatively few varieties with a very narrow genetic base. To address emerging needs mandated by cultural and climate changes, new germplasm has been introduced combining peach as well as wild peach and wild almond species. Advanced breeding selections incorporating exotic germplasm into a genetic background compatible with commercial production in California have demonstrated sizable reductions in level of kernel immunoreactivity as well as opportunities for improved control of aflatoxin and Salmonella. Breeding strategies employed include direct selection for reduced kernel immunoreactivity from an introgression enriched germplasm, the integration and pyramiding of resistance to multiple components of the aflatoxin disease-insect complex, and introduction of novel nut and tree traits to facilitate mechanized catch-frame field harvesting to avoid contamination with soil-borne pathogens such as Salmonella and *Escherichia coli*, as well as agrochemical residues.

Keywords: allergen, Salmonella, aflatoxin, domestication-bottleneck, germplasm, introgression, immunoreactivity

OPEN ACCESS

Edited by:
Max Teplitski,
University of Florida, United States

Reviewed by:
Pedro Martinez-Gomez,
Center for Edaphology and Applied Biology of Segura, Spanish National Research Council, Spain
Raquel Sánchez-Pérez,
University of Copenhagen, Denmark

***Correspondence:**
Thomas M. Gradziel
tmgradziel@ucdavis.edu

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *13 January 2020*
Accepted: *15 May 2020*
Published: *12 June 2020*

Citation:
Gradziel TM (2020) Redomesticating Almond to Meet Emerging Food Safety Needs. Front. Plant Sci. 11:778. doi: 10.3389/fpls.2020.00778

INTRODUCTION

The almond [*Prunus dulcis* (Miller D.A. Webb) syn. *Amygdalus dulcis* Mill., *Prunus amygdalus* (L.) Batsch, and *Amygdalus communis* L.] represents a nutritious, desirable, and relatively non-perishable food item as well as a durable propagation source for expanding plantings. These qualities made it commercially as well as horticulturally desirable, even in ancient times. The wild almonds traded and consumed by early civilizations were represented by over 30 species of diverse quality, morphology, and geographic origin (Zeinalabedini et al., 2010). Almond's widespread desirability and easy transportability appear to have made it an important commodity in prehistoric trade in Asia, North Africa, and Europe (Zohary et al., 2012), eventually leading to the establishment of an evolving commercial standard as well as a new species: the cultivated sweet almond (*Prunus dulcis*) probably selected by prehistoric societies from desirable interspecific hybridizations (Gradziel, 2017). Germplasm erosion resulted from domestication bottlenecks as well as subsequent regional planting practices. Almonds were originally planted as genetically diverse seedling orchards where the relatively common bitter seedlings would either be retained as a source of bitter almond extract for use in making marzipan, etc., or grafted to more desirable sweet varieties. In a major advancement for almond genomics, Sánchez-Pérez et al. (2019), after

successfully sequencing the almond genome, have identified the genetic mutation controlling kernel sweetness that allowed almond's domestication as a food crop. The higher value of established and so well-characterized sweet-kernel varieties led to their occupying a greater proportion of subsequent plantings, contributing to inbreeding and germplasm loss. In California, while over 100 geographically diverse varieties were grown in the early 1900s, the varieties "Nonpareil" and "Mission" dominated plantings by mid-century (Gradziel et al., 2017). Of the current plantings of approximately 540,000 hectares, "Nonpareil" remains the dominant variety with most of remaining pollenizer varieties (almond is self-sterile), having "Nonpareil" and "Mission" as direct parents (Gradziel and Martínez-Gómez, 2013).

In addition to domesticated almond, sweet kernels of apricots (*Prunus armeniaca* and *P. mandshurica*), plums (*Prunus domestica*), peaches (*Prunus persica*), and wild almond species would have been consumed in ancient times as they are to this day (Gradziel, 2011). The term "badam," which, when used alone refers to almond in a wide range of Asian languages can also refer to the edible kernels of other Prunus. For example, "tao'ze" badam refers to peach kernel in western China and "khasta badam" to peach or apricot kernel (sometimes called "poor man's almond") in India. Because of the absence of well-defined quality evaluation guidelines for almond and related nuts, most attempts to define kernel ideotypes consider only size, shape and kernel bitterness (R Socias i Company et al., 2008).

Due to its high nutritional and eating quality, almonds have become a valued component of many diets. Almond is an important source of macro-nutrients such as lipids, proteins, fiber and minerals, and is increasingly being recognized as an important source of the phytonutrients vitamin E (α-tocopherol), folate, and oleic acid. As with many other nut crops, almond are also important sources of food allergens and potential contamination with aflatoxins and human pathogens such as *Salmonella* and *Escherichia coli*, as well as agrochemical residues that can pose serious health risks for consumers.

In this study, previously published and unpublished data on allergenicity and susceptibility to aflatoxin and Salmonella contamination are evaluated in breeding germplasm derived from interspecies crosses in order to determine whether re-synthesized or redomesticated germplasms can be identified with improved nutritional and food safety qualities.

MATERIALS AND METHODS

A diverse germplasm, including 10 commercial varieties, seven related *Prunus* species and 47 inter-species hybrids and introgression lines from the University of California, Davis (UCD) genetic improvement program that had been selected for self-fertility and local adaptability but not kernel nutrient quality were evaluated for kernel and nut quality, soluble protein, and kernel immunoreactivity (**Table 1**). Commercial varieties evaluated originated in California, Spain, France and Italy, and include the recently released "Sweetheart" variety that originated from an intraspecific hybridization between "Mission" almond and "Lukens Honey" peach followed by three successive backcrosses to almond ("Mission" almond × *P. persica)BC3*. Evaluated germplasm included 6 additional introgression-derived selections newly released for grower testing. Commercial varieties and the introgression-derived "Sweetheart" variety were also evaluated for resistance to contamination by aflatoxin and *Salmonella* spp. The main commercial variety "Nonpareil" was included in all evaluations as the industry standard.

Seed Soluble Protein and Immunoreactivity

Whole seeds were ground to pass through a 20-mesh sieve. Soluble proteins were extracted in borate saline buffer (BSB) at flour: BSB = 1:10 (w/v). Flours were defatted and subjected to previously reported amandin cryoprecipitation methods (Su et al., 2015, 2017; Liu et al., 2017). Soluble protein was determined by Bradford and Lowry methods. Solubilized proteins were analyzed using electrophoresis and immunoassays employing mAbs 4C10 to assess conformational epitope immunoreactivity as described in Su et al. (2015).

Aflatoxin

Whole seeds were ground to a fine powder as described above. A mixture of 5% almond kernel powder and 1.5% agar in 40 mL water was autoclaved and 10 mL sterile solution poured into 60-mm Petri dishes. Each Petri dish was inoculated with 200 spores of *Aspergillus flavus* and incubated at 30°C for 7 days as described by Gradziel et al. (2000). Samples were then derivatized and analyzed for aflatoxin by high-performance liquid chromatography with fluorescence detection as described by Goodrich-Tanrikulu et al. (1995) with four Petri dish samples being evaluated for each genotype.

Oil Content and Composition

Total fat content and fatty-acid methyl esters (FAMEs) were determined according to the procedure of Garces and Mancha (1993). The FAMEs were identified based on retention times of known standards (Sigma, St. Louis). The presence of 17:0 as an internal standard allowed the calculation of the total lipids based on the area of the standard. Data were recorded on a dry-weight (DW) basis and analyzed using the SAS analysis of variance procedure for balanced data and the SAS REG procedure for regression analysis (SAS Institute, 1988) as previously described by Abdallah et al. (1998).

Navel Orangeworm (NOW) Infestation

Fruits were collected from UCD research plots at Winters, CA and inspected visually to ensure no previous infestation by navel Orangeworm (NOW). A total of 24 nuts of each selection were tested as exposed kernels (shells broken to expose kernels). Samples were placed in individual plastic containers with 15 NOW eggs added and incubated at 25°C for 90 days. Proportion of samples containing mature NOW moths at the end after 90 days were recorded.

TABLE 1 | Nut and kernel characteristics, including ELISA immunoreactivity values, for an intra- and interspecific almond breeding germplasm.

No.	Genotype	Origin	Expected Percent Almond	Kernel Length (mm.)	Kernel Width (mm.)	Kernel Breadth (mm.)	Kernel Mass (*g.*)	Nut Length (mm.)	Nut Width (mm.)	Nut Breadth (mm.)	Nut Mass (*g.*)	Soluble protein (*g./100g*)	ELISA
	Commercial varieties												
9	Sonora	Almond variety (United States)	100	27.7	13.1	7.8	1.52	37	18.9	12.7	2.25	22.07	0.74
10	Nonpareil	Almond variety (United States)	100	24.7	13.5	7.9	1.31	34.3	17	15	2.2	23.07	1.02
19	Mission	Almond variety (United States)	100	20.8	12.4	8.9	1.04	27.9	19.8	15.8	2.55	19.17	0.86
20	Chips	Almond variety (United States)	100	21.5	12.7	8.2	0.96	28.7	19.5	14.7	2.02	26.46	1.68
21	Kahl	Almond variety (United States)	100	26	12.1	8	1.2	34.3	17	15	2.2	26.29	1.22
22	Ferragnes	Almond variety (France)	100	26.8	14.2	8.3	1.48	36.4	23.1	17	4.09	19.37	1.56
24	Winters	Almond variety (United States)	100	26.3	11.9	8.1	1.21	36.4	19.3	14.1	2.09	22.37	1.05
25	Marcona	Almond variety (Spain)	100	22	17.3	8.8	1.55	29.4	25.8	19.6	5.55	22.22	0.88
26	Tuono	Almond variety (Italy)	94	26.4	16.3	8.2	1.58	38.4	27.7	18.3	5.45	17.14	0.52
	New releases												
23	Sweetheart	Almond variety (Peach × Almond)BC3	94	19.1	12.5	8.8	0.98	22.5	19	14.3	1.54	25.52	1.73
29	UCD,8–160	Nonpareil × 97,1–232	91	28.6	14.2	8.6	1.77	38.5	22.5	15.4	2.96	19.84	1.20
30	UCD,8–201	Nonpareil 97,1–232	91	24.1	13	8.1	1.26	32.1	21.5	14	2.06	15.81	1.67
41	UCD,2–3	[Almond × (*P. webbii* × *P. persica*)] (BC3)	94	23.9	11.6	9	1.17	31.8	22.4	14.6	4.74	19.89	1.93
42	UCD,8–27	[Almond × (*P. webbii* × *P. persica*)] (BC3)	94	24.3	12.1	8.6	1.2	30.4	20.9	14.2	3.36	23.92	0.55
54	UCD,2–240	(Nonpareil × *P. webbii*) BC3	94	23.8	12.6	9.5	1.28	30.3	24.3	14.3	5.62	22.22	0.40
64	UCD,3–40	[Almond × (*P. webbii* × *P. persica*)] (BC2)	88	33.3	15.1	8.7	2.08	39.2	29.6	18.8	9.21	25.31	0.90
	Related species												
15	40A–17	Peach (*P. persica*; bitter seed)	0	13.4	7.2	3.4	0.11	24.3	16.8	12.5	1.81	23.74	0.51
16	Andross	Peach (*P. persica*; bitter seed)	0	17.8	11.4	3.9	0.36	35.3	26.1	19.5	6.21	20.65	0.39
27	P11–58	*P. mira* (bitter seed)	0	14.5	9.9	4.3	0.29	26.6	17.8	12.8	2.48	23.39	0.53
43	A7–28	*P. webbii* (bitter seed)	0	18.4	9.1	6.3	0.49	25.7	14.1	10.2	1.39	21.04	0.88
55	A7–23	*P. argentea* (bitter seed)	0	13.4	9.7	6	0.37	19	15.3	12.1	1.47	17.28	0.61
61	A10–4	*P. bucharica* (bitter seed)	0	14.3	6.6	4.7	0.21	19.1	10.3	7.4	0.58	20.94	0.59
62	A2–11	*P. tangutica* (bitter seed)	0	13.4	10.3	8.3	0.49	16.5	15.2	12.4	1.34	25.44	0.70
63	A7–25	*P. webbii* (bitter seed)	0	20.4	11.8	7.3	0.82	29	18.3	13.7	2.93	19.09	0.51
	Interspecies hybrids												
1	F5,4–10	*P. webbii* × (Nonpareil × *P. persica*)	25	19.7	11.9	7.2	0.78	27.5	18.3	12.8	2.69	22.12	0.53
6	F5,20–42	Padre × F5,4–10	62	21.4	12.1	8.2	1	26.8	17.9	14	1.87	16.72	0.65
7	F8N,6–68	F5,4–10 × Solano	62	21.6	12.5	7.2	0.96	30.7	19.9	14.4	1.89	23.47	0.88
8	F8N,7–4	F5,4–10 × Sonora	62	22.7	10.7	6.2	0.76	32	16.1	10.7	1.17	19.52	0.65
12	8010–22	Nonpareil × F5,4–10	62	24.6	12.5	7.1	1.05	37.6	19.3	14.1	1.9	21.06	2.09
17	SB13,25–75	Nonpareil × F5,4–10	62	23.1	12.5	7.8	1.17	30	22.3	14.7	2.56	22.18	1.78

(Continued)

TABLE 1 | Continued

No.	Genotype	Origin	Expected Percent Almond	Kernel Length (mm.)	Kernel Width (mm.)	Kernel Breadth (mm.)	Kernel Mass (*g.*)	Nut Length (mm.)	Nut Width (mm.)	Nut Breadth (mm.)	Nut Mass (*g.*)	Soluble protein (*g./100g*)	ELISA
32	A13–1	*P. persica* × *P. davidiana* (bitter seed)	0	13.8	11.4	6.1	0.46	21.5	20.7	17.8	3.83	23.41	0.45
33	Hansen2	Almond × *P. persica* Rootstock	50	28	15.7	7.3	1.44	44.1	28.5	18.3	9.07	12.35	1.57
34	Hansen5	Almond × *P. persica*	50	23.8	13.9	7.5	1.12	34.5	24.6	18.9	7.44	21.06	0.66
35	Nickels	Almond × *P. persica*	50	23.9	16.4	8.8	1.53	36.9	28.7	20.9	9.18	13.79	0.75
39	F10D,1–26	Nonpareil × F5,4–10	62	23.1	14.2	6.9	1.11	30.8	24.8	15.8	3.88	17.64	1.61
	Interspecific introgressions												
2	F5,6–13	(Mission × *P. fenzliana*) BC1 × Sonora	88	22.1	10.8	6.7	0.84	32	17.3	10.5	1.66	25.6	0.95
3	F5,6–1	(Mission × *P. fenzliana*) BC2	88	23	14.6	7.4	1.33	33.8	23.7	16.8	5.08	25.88	0.92
4	F5,13–54	(Mission × *P. fenzliana*) BC1 × Sonora	88	23.7	11.9	8.3	1.05	37.2	19.5	16.7	2.94	16.28	0.70
5	F5,10–9	(Mission × *P. fenzliana*) BC1 × Sonora	88	21.1	12.2	7	0.82	27.3	18.8	14.2	3.08	18.11	0.61
11	SB13,54–39E	(Nonpareil × *P. persica*) BC3	94	16.9	10.2	8.2	0.7	26.2	15.8	12.3	1.05	21.51	1.96
13	F10C,12–28	(Nonpareil × *P. persica*) F2	50	20.2	13	9	1.08	35.1	23.9	18	4.96	19.32	1.76
14	F10C,20–51	(Nonpareil × *P. persica*) F2 (bitter seed)	50	25.1	12.6	7.3	1.1	35.1	21.3	15	2.43	23.87	0.56
18	F5,16–60	(Mission almond × *P. argentea*) F2	50	23.8	11.1	7.3	0.87	32.9	17.1	11.9	1.56	24.08	0.44
28	97,1–232	SB13,25–75 × Winters	81	23.6	13.4	8.2	1.29	31.3	20.4	13.5	2.27	20.61	2.06
31	2004,9–1	Nonpareil × 97,1–232	91	25	13.5	7.5	1.24	34.3	23.8	18.1	3.15	14.54	1.89
36	2005,20–192	(Nonpareil × *P. persica*) BC3	94	20.6	14.6	7.4	0.99	37.1	26.5	19.3	7.31	23.91	0.63
37	F10D,3–7	[Almond × (*P. webbii* × *P. persica*)] (BC1)	75	20.5	10.6	6.7	0.69	26.3	16.6	12.6	1.41	15.35	0.42
38	F10D,2–18	Nonpareil almond× *P. webbii* (BC1)	75	19	10.8	8.5	0.8	24.9	17.5	13.1	1.95	22.4	0.76
40	F10D,3–23	Padre almond × *P. webbii* (BC1)	75	20.4	11.9	7.7	0.84	27.5	19.8	13.4	2.32	14.48	1.49
44	F5,4–42	Almond × *P. webbii* (F2)	50	18.5	9.5	6.7	0.55	26.8	15	10.8	1.96	25.8	0.64
45	F10D,3–15	Almond × *P. webbii* (F2BC1)	75	24	12.9	7.2	0.96	33.3	21	14.6	4.1	18.58	0.33
46	F10D,1–22	Almond × *P. webbii* (F2BC1)	75	21.6	12.7	7.7	0.97	28.9	21.4	15.2	2.45	21.05	1.78
47	F10D,1–4	Almond × *P. webbii* (BC1)	75	23.1	11.9	7.6	0.95	30.8	18.1	13.3	1.94	20.5	1.32
48	F10D,1–2	Almond × *P. webbii* (BC1)	75	20.8	12.2	7.2	0.84	30	19.8	14.2	1.59	20.4	0.68
49	F10D,3–2	Almond × *P. webbii* (BC1)	75	19.7	11.1	7	0.77	30.6	17.8	13.6	1.53	17.84	0.66
50	F10D,2–5	Almond × *P. webbii* (BC1)	75	20.8	9.8	8.1	0.76	28.7	14.6	11.3	1.23	17.99	0.47
51	F10D,3–26	Almond × *P. webbii* (BC1)	75	24.1	11.4	7.5	0.93	33.6	20.3	14.4	3.23	21.17	1.06
52	F10D,3–13	Almond × *P. webbii* (BC1)	75	19.4	12	8	0.83	25.4	19.1	13.7	1.85	17.07	0.47
53	F10D,3–24	Almond × *P. webbii* (BC1)	75	19.3	13.2	6.1	0.71	25.7	19.5	13.3	2.66	13.39	1.27
56	F10D,3–3	Almond × *P. argentea* (BC1)	75	23.4	12.4	7	0.96	29.6	18.6	13.8	1.88	17.47	0.26
57	F10D,2–12	Almond × *P. fenzliana* (F2)	50	20.6	10.8	7	0.77	26.5	16.1	11.5	1.41	21.38	1.53
58	F10D,2–14	Almond × *P. fenzliana* (F2)	50	22.3	11.4	8.4	1.03	30.6	16.5	11.3	4.54	19.21	1.66
59	F10D,2–3	(Mission × *P. fenzliana*) BC1 × Sonora	88	21.8	13.2	8.9	1.13	27.6	20.1	16.3	3.24	20.71	1.56
60	F10D,3–50	Almond × *P. fenzliana* (BC1)	75	27.3	13.9	8.8	1.59	36.2	19.3	13.3	2.37	15.37	2.18

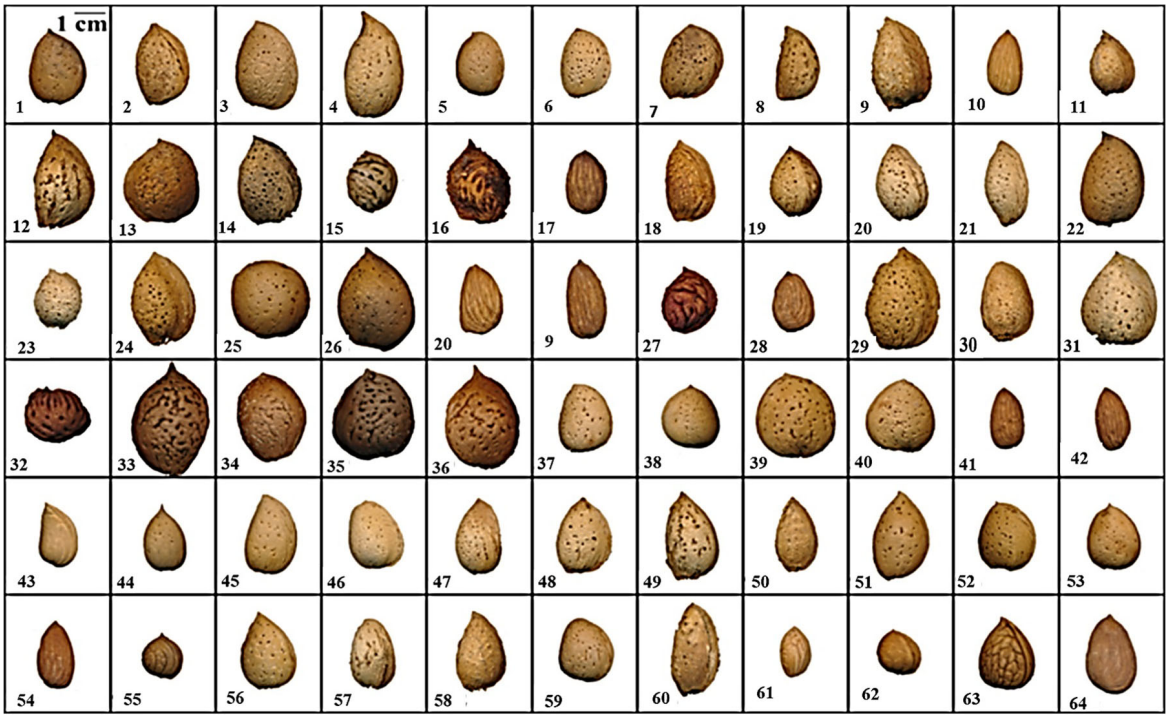


FIGURE 1 | Nut and kernel morphologies for an intra- and interspecific almond breeding germplasm. (Identifying numbers refer to the first column of **Table 1**).

Hull-Rot

Disease assessment was as described by Fresnedo-Ramírez et al. (2017). Fruit from each selection were harvested from UCD research plots at Winters, CA and stored at 4°C. Stored fruit were warmed to room temperature for 24 h prior to inoculation, surface sterilized for 30s by immersion in 10% bleach, rinsed in deionized water, and dried. A total of 24 unblemished hulls for each selection were placed in humidified plastic containers. Each fruit was inoculated with a 10 μL droplet containing conidia of *Monilinia fructicola*. (mixed field isolates) at a concentration of 2.5 × 104 spores per mL from 7 to 10-day-old cultures. Disease severity for each selection was calculated as the proportion of fruit with lesions greater than 3 mm. at 3 days after inoculation and incubation of the hulls in the humidified containers at room temperature.

RESULTS AND DISCUSSION

Seed Soluble Protein and Immunoreactivity

Seed soluble protein and kernel mass are uniformly high for all traditional varieties and new releases while immunoreactivity was moderate to high for traditional varieties but ranged from less than one-half to almost double the "Nonpareil" standard in the more genetically diverse new releases (**Table 1**). Strong breeding selection for self-fertility and local adaptability (which would have included kernel mass) thus does not appear to reduce variability for immunoreactivity, allowing subsequent selection within commercially adapted germplasm for reduced immunoreactivity risk.

Variability for all traits evaluated, including size, shape, soluble protein content and ELISA immunoreactivity was documented in this diverse germplasm (**Table 1** and **Figure 1**). Kernel mass, a critical commercial trait, ranges from 0.11 *g* to 2.08 *g*. All commercial varieties were approximately 1 *g* or greater, which has been shown to be an important threshold for optimizing orchard yield (Gradziel and Lampinen, 2013).

ELISA immunoreactivity values ranged from 0.26 to 2.18 times the level found in the "Nonpareil" standard, while soluble protein, an important trait in both processing and nutritional quality, ranges from 12.4 to 26.5 (*g*/100 *g*). The lower immunoreactivity scores were more strongly associated with interspecific hybridization lineages having peach or the wild almond species *P. argentea* or *P. webbii*. Velasco et al. (2016) have shown that while almond and peach are closely related and readily intercrossed, considerable trait differentiation has occurred between the species, suggesting fruit divergence long preceded domestication. The higher immunoreactivity scores were associated with hybridizations with *P. fenzliana,* which is generally considered to be one of the species from which cultivated almond was derived (Gradziel, 2011). No correlation was observed between almond seed size and either total soluble protein or amandin content. ELISA did show a general increase with increases in soluble protein content when only commercial varieties were analyzed. This positive association between amandin and immunoreactivity

is consistent with previous reports analyzing a broader range of commercial varieties that identified amandin, also known as almond major protein (AMP), prunin, 11S globulin, and Pru du 6, as the major storage protein in commercial almond seed (Sathe et al., 2001). This relation does not hold up, however, within the species, interspecies hybrids and introgressed germplasm. Of the 15 genotypes showing ELISA values of approximately one-half or less of the "Nonpareil" standard, four are found in commercially desirable selections having an average kernel mass of approximately 1 *g* or greater. In addition to sizable reductions in immunoreactivity, examples of increased immunoreactivity are also evident, as in the commercial varieties "Chips" and "Sweetheart," showing ELISA values of 1.68 and 1.73 of the "Nonpareil" standard, respectively.

All commercial varieties show ELISA values approaching or exceeding that of the "Nonpareil" standard with the exception of the Italian variety "Tuono." "Tuono" is unique among Mediterranean and California varieties in that it is self-compatible and so self-fertile. Recent molecular analysis has demonstrated the source of this self-fertility was a natural introgression from *P. webbii* which is native in the regions of southern Italy were "Tuono" originated (Gradziel and Martínez-Gómez, 2013). Similarly, the soluble protein content of 17.14 for "Tuono" is unusually low for a commercial cultivar, being well below the 20 *g*/100 *g* level desired for some forms of processing.

Several advanced introgression breeding selections combine the desirable characteristics of sweet kernels with high mass and high soluble protein content with low immunoreactivity. These include the new releases #42, UCD,8–27 [Almond × *(P. webbii* × *P. persica)]BC3* (i.e., three consecutive backcrosses to almond), and selection #54, UCD,2–240 ("Nonpareil" × *P. webbii)BC3*. Both intraspecific breeding selections are currently being considered for release as improved varieties based on their desirable nut and kernel characteristics (**Table 1** and **Figure 1**), self-fertility derived from peach and *P. webbii*, respectively, and high crop productivity.

Aflatoxin

A 77% reduction in aflatoxin was observed in the introgression-derived "Sweetheart" variety when compared to the "Nonpareil" standard (**Table 2**). Even higher levels of aflatoxin were observed in the other traditional varieties. Field suppression of aflatoxin contamination in "Sweetheart" is further enhanced through improved resistance to pest and mold damage associated with aflatoxin production.

"Sweetheart" is a UCD released commercial cultivar originating as a "Mission" almond by peach introgression line ("Mission" × *P. persica*)BC3 in an effort to transfer self-fertility from peach (Gradziel et al., 2001). While not expressing sufficiently high levels of self-fruitfulness to be commercially distinct, "Sweetheart" possesses an exceptionally high oil content as well as quality as demonstrated by its very high oleic acid content (**Table 2**) placing it in a premium roasting-quality category with the Spanish variety "Marcona" (Gradziel et al., 2013). "Sweetheart" is also exceptional in that, since its release in 2007, very few positive findings for aflatoxin contamination have been reported in commercial shipments. Early analysis by Gradziel et al. (2000) had shown significantly lower levels of aflatoxin production following inoculation under controlled laboratory conditions. More recent studies have shown that this

TABLE 2 | Kernel oil quantity and quality along with susceptibility to aflatoxin contamination, hull-rot disease and Navel-orangeworm (NOW) infestation for the almond variety *Sweetheart* compared to six commercial variety standards.

	Nonpareil	Sweetheart	Mission	Sonora	Chips	Kahl	Winters
Total oil (% dry weight)	38.8 (0.3)	47.3 (1.2)	43.4 (1.2)	43.8 (2.3)	38.4 (1.7)	44.7 (1.8)	43.4 (0.6)
Oleic acid (%)	66.8 (0.8)	73.0 (1.3)	71.9 (2.3)	69.3 (2.3)	66.0 (4.3)	67.3 (1.1)	66.9 (0.4)
Aflatoxin (ug *g*-1 dry wt.)	0.17 (0.02)	0.04 (0.003)	0.20 (0.04)	0.25 (0.05)	0.24 (0.05)	0.31 (0.02)	0.22 (0.05)
Hull rot (%)	97.3 (8.8)	23.1 (6.9)	64.5 (6.7)	83.7 (6.1)	72.8 (5.5)	86.5 (5.3)	55.4 (7.2)
NOW (%)	79.5 (5.3)	4.1 (0.8)	39.8 (4.7)	64.1 (6.3)	48.4 (5.1)	56.8 (7.1)	81.3.8 (3.5)

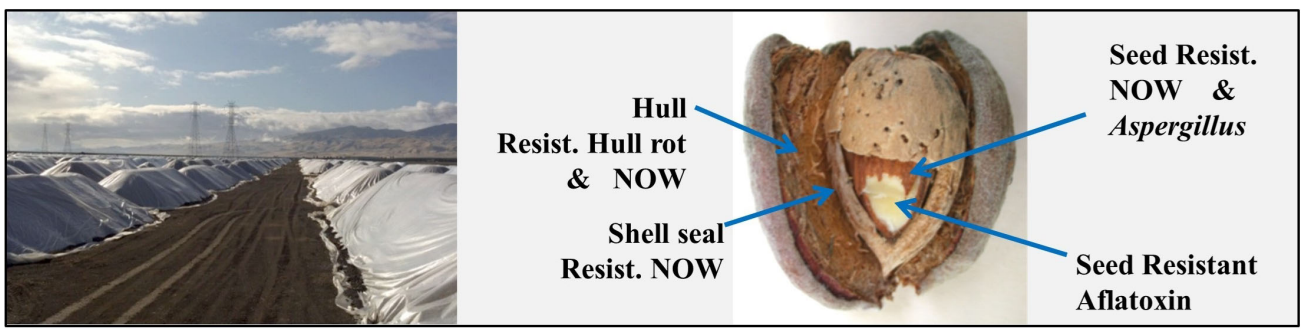


FIGURE 2 | In-field, bulk storage of almond fruit (hulls, shells, and kernels) under plastic tarps (left). Structure of the mature, dried almond fruit identifying the major components: hull, shell, and seed/seedcoat, along with associated resistances as identified in the "Sweetheart" variety (right).

variety also shows higher resistance to hull-rot as well as NOW infestation (**Table 2**).

Improved performance in unrelated traits is not unusual in interspecific introgressions because of the inherently higher genetic and so trait variability compared to the highly inbred (Gradziel, 2017) and so trait limited nature of traditional Californian varieties (Gradziel et al., 2001). In "Sweetheart," however, these traits appear to be complementary in reducing the overall risk of aflatoxin contamination. Under field conditions, *Aspergillus flavus* infection usually occurs following kernel damage by NOW, where infestation acts to inoculate the normally shell-protected kernel and where subsequent feeding creates a suitable environment for *A. flavus* growth and aflatoxin development (Hamby et al., 2011). Kernel infestation/infection can occur in the field from the time of fruit maturity (where the flesh or hull splits exposing the almond nut), to field harvest, and again during storage prior to hulling and shelling. In almond, as with other *Prunus* or "stone-fruit" species, the mesocarp develops into the hull or fruit flesh and the endocarp develops into the shell enclosing the nut/kernel, which is the seed with or without tegument/seed coat. Because of the size of the 1 billion kg. (kernel meat) crop, fruit are air-dried in the field and held in bulk storage for several months or more (**Figure 2**). When properly dried, nuts are relatively resistant to new NOW

FIGURE 3 | Longitudinal and cross-section images of shells of the wild almond species *P. webbii* (#63 in **Table 1** and **Figure 1**), the thin "paper"-shelled California "Mission" variety (#19), their interspecies hybrid, and the interspecies introgression selection UCD2-240 (#54) along with a shell cross-section of the Spanish variety "Marcona" for comparison (#25 in **Table 1** and **Figure 1**).

FIGURE 4 | Catch-frame harvesting of almonds in Spain using specialized equipment integrating tree-shakers and collection frames for catching harvested fruit (hulls plus nuts) before they hit the ground.

infestation because the 1st instar larvae are very delicate and particularly vulnerable to desiccation or starvation before it can access the nut kernel (Hamby et al., 2011). The occurrence of hull-rot during storage, however, acts to both macerate and hydrate hull tissue, making it much more vulnerable to NOW infestation. Under field conditions, the multiple barriers found in the "Sweetheart" almond, including increased resistance to NOW as well as hull-rot development, the reduced tendency for aflatoxin production and a highly sealed shell (**Figure 2**) have resulted in a high level of field resistance to this economically important insect-disease complex.

Soil-Born Contaminants

Soil-borne contaminants are an inevitable consequence of commercial off-ground harvest practices. Improved harvest methods, such as catch-frame harvesting, avoid the risk of soil-contamination but require novel fruit and nut traits in order to be commercially feasible. Required traits are available within the enriched, interspecies-introgressed breeding germplasm that are compatible with current and future harvest needs.

A problem with soil contaminants such as *Salmonella, E. coli* and pesticide residues is the difficulty in defining safe concentrations and so even detection of trace levels can lead to crop rejection. Avoiding contamination remains the most effective strategy for ensuring food safety. Like peach, the almond kernel is enclosed in a lignified endocarp or shell (**Figure 3**), which, if highly sealed, confers protection from insect infestation and mold infection. Unfortunately, an important post-harvest role of the shell is to facilitate the uptake of moisture for seed hydration/germination. Danyluk et al. (2008) have demonstrated that this moisture uptake pathway also provides a ready conduit for the entrance of bacteria and contaminated water.

A solution currently being pursued by the California almond industry is the use of catch-frame harvesting as currently practiced for pistachio in California and some orchards in Spain (**Figure 4**) because it avoids off-ground nut harvest with its high risk of soil contamination. In current practice, California almonds are shake-harvested to the orchard floor and allowed to dry in the Central Valley's warm, dry environment to kernel moisture levels of 7% or less to suppressed post-harvest disease. Dried fruit (hulls plus nuts) are then collected and bulk-stored until hull removal (hulling) and shelling in specialized industrial facilities. While off-site drying is feasible with the relatively limited production of California pistachio and Spanish almond, it presents huge technical challenges for the 4 billion kg. almond crop (2 billion kg. in hulls, 1 billion kg. in shells, and 1 billion kg. in kernel-meats). In-field hulling at harvest would reduce post-harvest handling by half and allow the vegetative hulls to be reincorporated into orchard soils in a more sustainable manner. Unlike Spanish almonds where the thick, highly lignified shells typically constitute about two thirds of the nut mass (**Figure 3**), California almonds have thin, "paper" shells with improved harvest index and shelling efficiency. The fragile nature of paper-shells results in unacceptable levels of nut and kernel damage with mechanically intensive in-field hulling, while the highly lignified Spanish-type shells dramatically reduce harvest efficiency and would require extensive retooling of industrial shelling equipment. Wild almond species such as *P. argentea, P. bucharica,* and *P. webbii* (#55, 61, and 63 in **Figure 1**) possess a thin, highly lignified shell that confers high structural strength while allowing a high kernel-to-nut "crack-out" ratio. This trait has proven highly heritable in certain *P. webbii* introgression lines allowing the development of California-adapted almonds possessing thin yet highly lignified *P. webbii*-type shells. An example can be seen in the previously discussed low-aflatoxin selection UCD,2-240 (#54 in **Figures 1**, **3** and **Table 1**). Combining good kernel size and quality with a durable, highly-sealed shell having a kernel-to-nut crack-out ratio of 70%, UCD,2-240 is currently undergoing grower field testing as a candidate for almond catch-frame harvest.

CONCLUSION

Selection during crop domestication for desirable traits such as large, non-bitter seed and uniform harvest to facilitate cultivation also results in a loss of genetic diversity and so trait variability. Results show that the absence of traits required for evolving market and agronomic needs are often the consequence of diminished genetic diversity and are not pleiotropic effects of the selection for specific commercial traits. For crops such as almond, possessing an extensive and diverse germplasm within its wild relatives and a relatively flexible characterization of crop-ideotype, a re-domestication process can develop commercial varieties with an enriched germplasm and so expanded opportunities for novel trait selection. These findings suggest that many of the relatively recently identified food safety threats may not be an inherent hazard of the crop but rather are a consequence of the limits imposed by the initial domestication events and later production practices. Where appropriate germplasm remains accessible, modern breeding programs can sometimes redo the domestication process, which, if properly focused, can provide a more effective selection for traits allowing agronomically viable solutions to modern food safety challenges.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

DEDICATION

This manuscript is dedicated to Shridhar K. Sathe (October 30, 1950–April 4, 2019), the Robert O. Lawton Distinguished

Professor and Hazel K. Stiebeling Professor in the College of Human Sciences, Florida State University, in recognition of his pioneering work on tree nut allergy identification and control.

ACKNOWLEDGMENTS

The author wishes to acknowledge the financial support of the Almond Board of California for portions of this research.

REFERENCES

Abdallah, A., Ahumada, M. H., and Gradziel, T. M. (1998). Oil content and fatty acid composition of almond kernels from different genotypes and California production regions. *J. Am. Soc. Horticult. Sci.* 123, 1029–1033. doi: 10.21273/jashs.123.6.1029

Danyluk, M. D., Brandl, A., and Harris, L. J. (2008). Migration of *salmonella* enteritidis phage Type 30 through almond hulls and shells. *J. Food Prot.* 71, 397–401. doi: 10.4315/0362-028x-71.2.397

Fresnedo-Ramírez, J., Famula, T. R., and Gradzie, L. T. M. (2017). Application of a Bayesian ordinal animal model for the estimation of breeding values for the resistance *to Monilinia fruticola* (G.Winter) honey in progenies of peach [*Prunus persica* (L.) Batsch). *Breed. Sci.* 67, 110–122. doi: 10.1270/jsbbs.16027

Garces, R., and Mancha, M. (1993). One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. *Anal. Biochem.* 211, 139–143. doi: 10.1006/abio.1993.1244

Goodrich-Tanrikulu, M., Mahoney, N. E., and Rodriguez, S. B. (1995). The plant growth regulator methyl jasmonate inhibits aflatoxin production by *Aspergillus flavus*. *Microbiol. Read.* 141, 2831–2837. doi: 10.1099/13500872-141-11-2831

Gradziel, T., Lampinen, B., Niederholzer, F., and Viveros, M. (2013). 'Sweetheart' almond: a fully cross-compatible pollenizer for the early 'Nonpareil' bloom that exhibits very high Marcona-type kernel quality. *HortScience* 48, 1320–1322. doi: 10.21273/hortsci.48.10.1320

Gradziel, T., Mahoney, N., and Abdallah, A. (2000). Aflatoxin production among almond genotypes is unrelated to either kernel oil composition or *Aspergillus flavus* growth rate. *HortScience* 35, 937–939. doi: 10.21273/hortsci.35.5.937

Gradziel, T. M. (2011). "Almond origin and domestication," in *Horticultural Reviews* Vol. 38, ed J. Janick (Hoboken, NJ: John Wiley & Sons), 23–82.

Gradziel, T. M. (2017). "History of cultivation," in *Almonds: Botany, Production and Uses*, eds R Socias i Company and T. Gradziel (Boston: CABI Press), 494.

Gradziel, T. M., Curtis, R., and R Socias i Company (2017). "Production and growing regions," in *Almonds: Botany, Production and Uses*, eds R Socias i Company and T. Gradziel (Boston: CABI Press), 70–86. doi: 10.1079/9781780643540.0070

Gradziel, T. M., and Lampinen, B. D. (2013). Defining the limits of almond productivity to facilitate marker assisted selection and orchard management. *Acta Hort.* 912, 33–39.

Gradziel, T. M., and Martínez-Gómez, P. (2013). Almond breeding. *Plant Breed. Rev.* 37, 207–258. doi: 10.1002/9781118497869.ch4

Gradziel, T. M., Martínez-Gómez, P., Dicenta, F., and Kester, D. E. (2001). The utilization of related almond species for almond variety improvement. *J. Amer. Pomol. Soc.* 55, 100–108.

Hamby, K., Gao, L. W., Lampinen, B., Gradziel, T., and Zalom, F. (2011). Hull split date and Shell seal in relation to navel orangeworm (*Lepidoptera*: *Pyralidae*) infestation of almonds. *J. Econ. Entom.* 104, 965–969. doi: 10.1603/ec10396

Liu, C., Chhabra, G. S., Zhao, J., Zaffran, V. D., Gupta, S., Roux, K. H., et al. (2017). Comparison of laboratory developed and commercial monoclonal antibody-based sandwich enzyme-linked immunosorbent assays for almond (*Prunus dulcis*) detection and quantification. *J. Food Sci.* 10, 2504–2515. doi: 10.1111/1750-3841.13829

R Socias i Company, Kodad, O., Alonso, J. M., and Gradziel, T. M. (2008). "Almond quality: a breeding perspective," in *Horticultural Reviews*, Vol. 34, ed. J. Janick (Hoboken, NJ: John Wiley & Sons), 197–238. doi: 10.1002/9780470380147.ch3

Sánchez-Pérez, R., Pavan, S., Mazzeo, R., Moldovan, C., Aiese Cigliano, R., Del Cueto, J., et al. (2019). Mutation of a bHLH transcription factor allowed almond domestication. *Science* 364, 1095–1098. doi: 10.1126/science.aav8197

Sathe, S. K., Teuber, S. S., Gradziel, T. M., and Roux, K. H. (2001). Electrophoretic and immunological analyses of almond (*Prunus dulcis* L.) genotypes and interspecies hybrids. *J. Agric. Food Chem.* 49, 2043–2052. doi: 10.1021/jf001303f

Su, M., Liu, C., Roux, K. H., Gradziel, T. M., and Sathe, S. K. (2017). Effects of processing and storage on almond (*Prunus dulcis* L.) amandin immunoreactivity. *Food Res. Int.* 100, 87–95. doi: 10.1016/j.foodres.2017.06.061

Su, M. N., Venkatachalam, M., Gradziel, T. M., Liu, C. Q., Zhang, Y., Roux, K. H., et al. (2015). Application of mouse monoclonal antibody (mAb) 4C10-based enzyme-linked immunosorbent assay (ELISA) for amandin detection in almond (*Prunus dulcis* L.) genotypes and hybrids. *LWT Food Sci. Technol.* 60, 535–543. doi: 10.1016/j.lwt.2014.08.042

Velasco, D., Hough, J., Aradhya, M., and Ross-Ibarra, J. (2016). Evolutionary genomics of peach and almond domestication. *G3-Genes/Genomes/Genet.* 6, 3985–3993. doi: 10.1534/g3.116.032672

Zeinalabedini, M., Khayam-Nekoui, M., Grigorian, V., Gradziel, T. M., and Martinez- Gomez, P. (2010). The origin and dissemination of the cultivated almond as determined by nuclear and chloroplast SSR marker analysis. *Sci. Horticult.* 125, 593–601. doi: 10.1016/j.scienta.2010.05.007

Zohary, D., Hopf, M., and Weiss, E. (2012). *Domestication of Plants in the Old World: The Origin and Spread of Domesticated Plants in Southwest Asia, Europe, and the Mediterranean Basin*. Oxford: Oxford University. Press, 237.

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

METHODS
published: 14 July 2020
doi: 10.3389/fmicb.2020.01516

A Simple Assay to Assess *Salmonella enterica* Persistence in Lettuce Leaves After Low Inoculation Dose

Paula Rodrigues Oblessuc† and Maeli Melotto**

Department of Plant Sciences, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Dario De Medici,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
Barbara Bertasi,
Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna (IZSLER), Italy
Ana Allende,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

***Correspondence:**
Paula Rodrigues Oblessuc
poblessuc@iplantprotect.pt
Maeli Melotto
melotto@ucdavis.edu

†Present address:
Paula Rodrigues Oblessuc,
Department of Protection of Specific Crops, InnovPlantProtect CoLab, Estrada de Gil Vaz, Elvas, Portugal

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *17 January 2020*
Accepted: *10 June 2020*
Published: *14 July 2020*

Citation:
Oblessuc PR and Melotto M (2020) A Simple Assay to Assess Salmonella enterica Persistence in Lettuce Leaves After Low Inoculation Dose. Front. Microbiol. 11:1516. doi: 10.3389/fmicb.2020.01516

Salmonella enterica is an enterobacterium associated with numerous foodborne illnesses worldwide. Leafy greens have been a common vehicle for disease outbreaks caused by *S. enterica*. This human pathogen can be introduced into crop fields and potentially contaminate fresh produce. Several studies have shown that *S. enterica* can survive for long periods in the plant tissues. Often, *S. enterica* population does not reach high titers in leaves; however, it is still relevant for food safety due to the low infective dose of the pathogen. Thus, laboratory procedures to study the survival of *S. enterica* in fresh vegetables should be adjusted accordingly. Here, we describe a protocol to assess the population dynamics of *S. enterica* serovar Typhimurium 14028s in the leaf apoplast of three cultivars of lettuce (*Lactuca sativa* L.). By comparing a range of inoculum concentrations, we showed that vacuum infiltration of a bacterium inoculum level in the range of 3.4 Log CFU ml^{-1} (with a recovery of approximately 170 cells per gram of fresh leaves 2 h post inoculation) allows for a robust assessment of bacterial persistence in three lettuce cultivars using serial dilution plating and qPCR methods. We anticipate that this method can be applied to other leaf–human pathogen combinations in an attempt to standardize the procedure for future efforts to screen for plant phenotypic variability, which is useful for breeding programs.

Keywords: lettuce, *Salmonella enterica*, apoplastic persistence, vacuum inoculation, bacterial population growth, fresh produce safety, lettuce cultivars

INTRODUCTION

Fruits and vegetables are known to have high nutrient content, making them the basis of a healthy diet. Many of these foods can be eaten raw, and although this represents a practical advantage, it also makes them notoriously relevant to foodborne illnesses. *Salmonella enterica* is one of the most common human pathogens found in fresh produce (Bennett et al., 2018; Melotto et al., 2020). Previously, plants were thought to be passive vectors for human pathogens, but recent studies showed that *S. enterica* can induce plant defense responses (Meng et al., 2013; Garcia and Hirt, 2014; Melotto et al., 2014; Oblessuc et al., 2020). Intriguingly, although the mechanism is not fully understood, this bacterium can overcome plant defense (Roy et al., 2013; Wahlig et al., 2019) and survive for weeks inside diverse plants species, including lettuce (*Lactuca sativa* L.) (Islam et al., 2004; Kroupitski et al., 2009, 2011; Jechalke et al., 2019; Roy and Melotto, 2019). These findings have prompted further research on the interaction between plants and human pathogens.

Artificial inoculation of plants is a common technique used to study plant interaction with phytopathogens (Katagiri et al., 2002; Jacob et al., 2017). Nevertheless, this approach has some technical limitations when studying plant interaction with enterobacteria, in particular *S. enterica* and enterohemorrhagic *Escherichia coli*, due to the relative low number of these bacteria inside the plant. In fact, recent studies have shown that *S. enterica* population can decrease with time in many plant species in an inoculum concentration-dependent manner (Deblais et al., 2019; Jechalke et al., 2019). Beyond that, the plant species and the inoculation procedure itself can affect bacterial population dynamics inside plants. For instance, tomato (*Solanum lycopersicum*) seedlings dip-inoculated with *S. enterica* at a concentration of 8 Log CFU ml^{-1} maintains the population size 1 day after inoculation (DAI) followed by a decrease after 14 DAI (Barak et al., 2011). Similarly, when adult lettuce leaves were dip-inoculated with 8 Log CFU ml^{-1} of *S. enterica*, the Log CFU cm^{-2} of leaf showed no alteration in bacterial population until three DAI, but a reduction in the population size after 7 DAI (Roy and Melotto, 2019). Nonetheless, when lower inoculum concentration of 4.7 Log CFU ml^{-1} of *S. enterica* was used to infiltrate small areas of fully expanded *Nicotiana benthamiana* leaves, a 100-fold increase in bacterial population was observed at three DAI (Meng et al., 2013). These findings indicate that the inoculation method and/or the initial concentration of the inoculum can influence the bacterial population dynamic in leaves.

In the field, plants can be exposed to variable amounts of pathogen load depending on the source of the inoculum. In a survey to quantify *Salmonella* in irrigation water, Antaki et al. (2016) found an average of 0.03 MPN (most probable number) of cells per 100 ml of water. Additionally, animals are reservoirs of bacterial pathogens of humans and might shed high level of inoculum in their feces. For instance, cattle feces can shed *E. coli* O157 at concentrations >4 Log CFU g^{-1} (Omisakin et al., 2003), whereas some animals such as mice are considered super-shedders of *S.* Typhimurium (Gopinath et al., 2012).

Once crops are exposed to these environmental inocula, bacterial cells can internalize into edible leaves through natural openings and wounds (Brandl, 2008; Kroupitski et al., 2009; Roy et al., 2013). Understanding human pathogen survival inside the leaf apoplast is very important as this niche protects the bacterium from common sanitation procedures of leafy vegetables (Pezzuto et al., 2016), posing a risk to reach the human host. Thus, we performed vacuum infiltration procedures using a range of low to high concentrations of bacterial inoculum (3–7 Log CFU ml^{-1}) to assess the effect of inoculation dose on bacterial survival and the detection limit of our procedure using contrasting lettuce cultivars over a period of 20 days. The findings of this study will assist with designing of plant phenotypic screening useful for breeding programs.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Approximately 15 lettuce seeds of each cultivar (Red Tide, Lollo Rossa, and Salinas) were germinated in water-soaked filter paper for 2 days at room temperature. Each germinated seed was transplanted to a peat pot pre-soaked with distilled water for 10–20 min. Ideally, sprouted seeds with approximately the same root size should be selected for transplanting. Pots were placed in trays covered with plastic dome, leaving a small space (around 4 cm) to avoid water condensation, and kept at 18 ± 2°C, 240 ± 10 μmol m^{-2} s^{-1} with a 12-h photoperiod, and 80 ± 10% of air relative humidity. One week after transplanting, seedlings were fertilized with 0.05 g of fertilizer per plant mixed with 30 ml of distilled water. Three- to 4-week-old plants were used for inoculation (**Figure 1**).

Bacterial Inoculum Preparation

S. enterica subsp. *enterica* serovar Typhimurium strain 14028s was streaked from frozen glycerol culture stock on low-salt Luria Bertani (LSLB) agar plate, supplemented with 60 μg ml^{-1} kanamycin, and incubated overnight at 28°C. Late in the afternoon of the day before the inoculation assay (around 5 pm), one single colony was placed in 100 ml of LSLB medium with 60 μg ml^{-1} kanamycin in a 125-ml Erlenmeyer flask. As a blank control, 5 ml of the LSLB plus antibiotic solution was placed into a clean culture tube. Bacterial and blank solutions were incubated in a rotary shaker at 28°C, 150 rpm, overnight (**Figure 1**).

In the morning of the next day, bacterial and blank solutions were removed from the incubator and the optical density at 600-nm wavelength (OD_{600}) was measured using a spectrophotometer. It is important to shake the culture flask before transferring 1 ml to a sterile cuvette to avoid errors during OD readings due to bacterial settling on the bottom of the flask. The OD_{600} should be between 0.8 and 1.0 to ensure that the bacterial growth is still in the log phase. A two-step bacterial dilution was used to prepare the final inoculum at the desired concentration. Step 1: the volume of the bacterial solution needed to obtain a bacterial OD_{600} of 0.2 was calculated using the formula C1 × V1 = C2 × V2, where C = concentration and V = volume. After transferring the desired bacterial solution volume (V2) to a 50-ml centrifuge tube, bacterial cells were harvested by centrifugation at 1,200 × *g* for 20 min at 22 ± 2°C. The supernatant was discarded, and the pellet was resuspended in sterile distilled water by vortexing. The centrifugation step was essential to remove the excess of LBLS media plus kanamycin, to avoid bacterial growth inhibition within the leaf due to the presence of the antibiotic, as well as to reduce the volume of *Salmonella* solution handled in the lab. Step 2: 0.0001, 0.01, 1, or 100 ml of the final solution from step 1 (OD_{600} = 0.2) was added to a new flask containing 1,000 ml of sterile distilled water to obtain the final inoculum concentration of OD_{600} 0.00000002 (1 Log CFU ml^{-1}), 0.000002 (3 Log CFU ml^{-1}), 0.0002 (5 Log CFU ml^{-1}), or 0.02 (7 Log CFU ml^{-1}). Finally, 0.1 ml of Silwet was added to the inoculum to obtain a final concentration of 0.01%. Inoculum was stirred with a magnetic bar.

Vacuum Inoculation of Lettuce Leaves

Three- to four-week-old lettuce plants (four plants per cultivar) were vacuum-infiltrated with the final bacterial solution of 1.8,

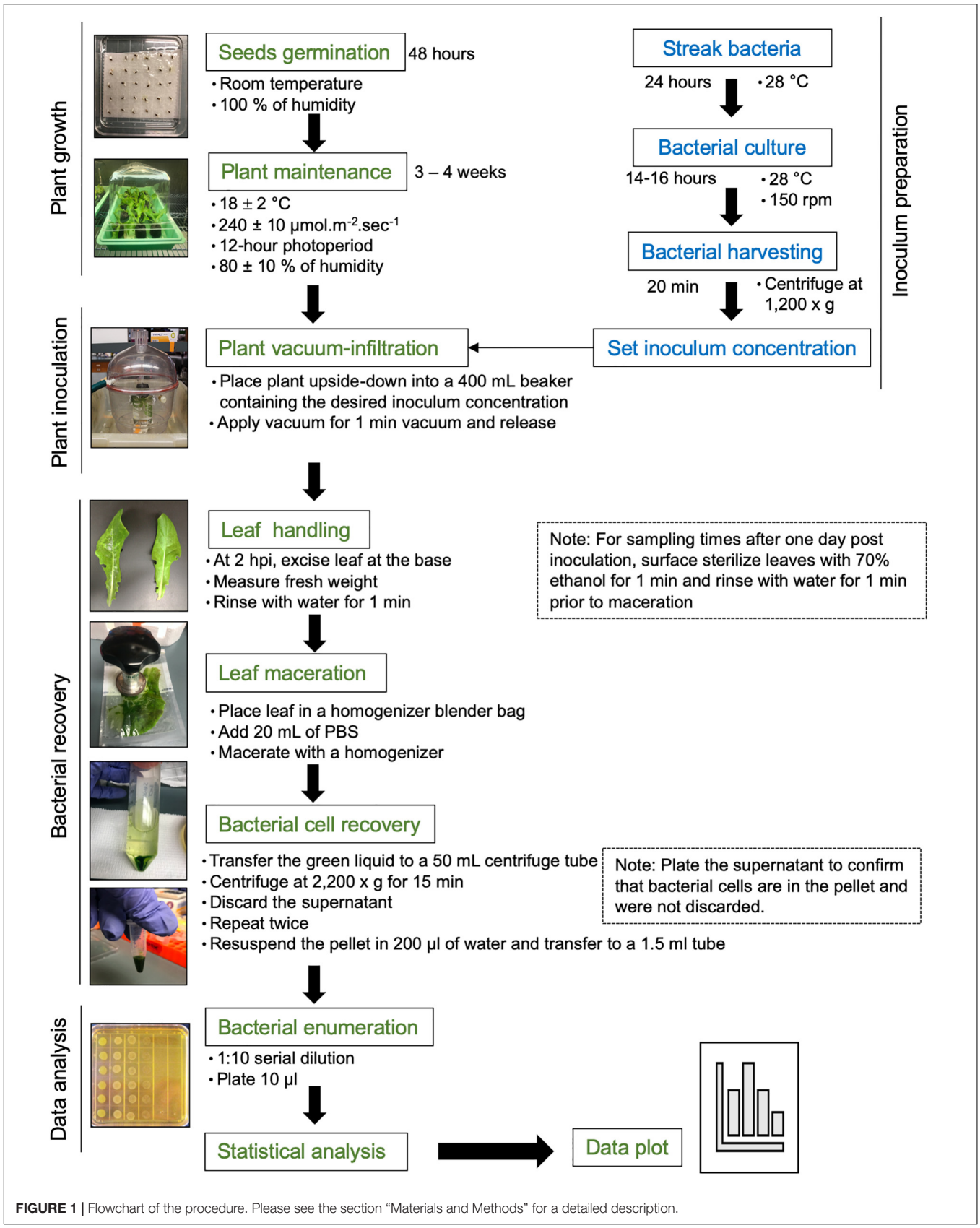


FIGURE 1 | Flowchart of the procedure. Please see the section "Materials and Methods" for a detailed description.

3.5, 5.4, or 7.7 Log CFU ml^{-1}. These concentration values were estimated by serial dilution plating of the inoculum. Each potted plant was placed upside-down into a 400-ml beaker containing enough inoculum to immerse the plant shoot completely. Aluminum foil was placed at the base of the plant to avoid the contact of soil with the inoculum. Submerged plants were placed in a vacuum chamber and vacuum was applied for 1 min. To enable a uniform filling of the leaf apoplast with inoculum, the vacuum was released quickly by disconnecting the suction tube to the vacuum chamber, allowing the chamber to depressurize. The leaves should become dark green due to inoculum infiltration (**Figure 1**). Fresh inoculum was added to the beaker to ensure total immersion of the inoculated leaves and after three plants were inoculated. Inoculated plants were placed back in the trays and partially covered with the plastic dome for the duration of the experiment.

Enumeration of Apoplastic Bacterium Population

Bacterial population size was estimated in the second true leaf of the inoculated plants at 2 h post inoculation (HPI), and 1, 10, and 20 DAI. After 2 HPI when the water soaking disappeared from the leaves, the second true leaf was excised at its base, the fresh weight (FW) was measured using an analytical balance, and the leaf was rinsed by immersion in sterile distilled water for 1 min. Sampling at this time point was crucial to determine the total number of bacteria inoculate on and in the plant. For the following time points, after FW measurement, the leaf was surface sterilized with 70% ethanol for 1 min and rinsed in water for 1 min.

For all time points, the excess water was removed from the leaves by gently blotting them on paper towel. Leaf was placed in a homogenizer blender bag containing 20 ml of phosphate-buffered saline (PBS) solution and macerated with a homogenizer until a green solution with very small leaf debris was obtained. The green solution, but not the leaf debris, was transferred to a 50-ml centrifuge tube and centrifuged at 2,200 × *g* for 15 min at 22 ± 2°C. The supernatant was removed, and fresh 20-ml PBS solution was added to the pellet containing bacterial cells, followed by centrifugation at 2,200 × *g* for 15 min (**Figure 1**). To confirm that no bacterium was present in the supernatant, this solution was plated on solid LSLB medium.

After the wash-centrifugation steps, 0.2 ml of sterile Milli-Q water was added to recover the bacterial pellet and transferred to a clean 1.7-ml tube. Please note that water was used as further DNA extraction was desired, but PBS buffer could be used if only serial dilution and plating would be performed. Immediately after bacterial recovery, 10 μl of the bacterial solution was added to 90 μl sterile water in another 1.7-ml microfuge tube, making a 1:10 dilution. This solution was diluted to 10^{-2} for the low inoculum dose and down to 10^{-8} for the highest inoculation dose. Including the most concentrated leaf sample, 10 μl of all dilutions was plated on LBLS agar with 60 μg ml^{-1} kanamycin (**Figure 1**; Jacob et al., 2017). Dilution plates were air-dried and subsequently incubated at 28°C overnight. The next day, bacterial colonies were counted at the dilution column that allowed for the visualization of individual colonies using a stereoscope.

Data Analysis

The number of single colony-forming units (CFU) was used to estimate the bacterial population per gram of fresh leaf tissue by multiplying the CFU counts by the dilution factor times 10, to account for the 10 μl out of the 100 μl used for plating. Data points represent the average of three biological replicates (three different plants) and two technical replicates during plating ($n = 6$). Average and the standard error (SE) were calculated using Microsoft Excel. Statistical significance among the different cultivars and time points was estimated by the analysis of variance (ANOVA) followed by Scott–Knott test with a significance threshold of $\alpha = 0.05$, using the square root of the means. This data transformation method is recommended when the variance is proportional to the mean (Manikandan, 2010). The graph was plotted with the Log CFU per gram of leaf FW over time using untransformed data.

Total DNA Extraction and qPCR Analysis

After the wash-centrifugation steps described above, 5 μl of the DNA extraction buffer was added to 50 μl of the recovered bacterial solution. After vortexing for 30 s, the solution was centrifuged for 1 min at 13,000 rpm at room temperature, and the supernatant was transferred to a clean tube. DNA in the supernatant was precipitated by adding 0.1 volumes of 5 M ammonium acetate and one volume of isopropanol, followed by vortexing and 1-h incubation at room temperature. Next, two washes were performed to remove excess salt by adding 1 ml of cold 70% ethanol, vortexing for 30 s, and spinning-down for 1 min at 13,000 rpm, room temperature. After each centrifugation, the ethanol solution was discarded. Finally, the DNA pellet was dried out on the bench for 15 min and resuspended in 30 μl of DNase-free water. Quantitative PCR (qPCR) was performed with 3 ng of DNA template, 200 nM of reverse and forward gene-specific primers, and 10 μl of iTaq Fast SYBR Green Supermix in a total reaction volume of 20 μl. Reactions were carried out in an Applied Biosystems 7300 thermocycler, using the following cycling parameter: 1 cycle of 95°C for 5 min, and 40 cycles of 95°C for 10 s and 60°C for 30 s. The dissociation curve was determined for every reaction to confirm the presence of a single amplicon and the lack of primer dimers and non-specific products.

The primer set efficiency was assessed using the standard curve method. The linear regression equation was plotted using the cycle threshold (CT) value and the Log of the DNA concentrations of 10-fold serial dilutions, using the Microsoft Excel software. The slope values were used to calculate the efficiency for each pair of primers tested (Kralik and Ricchi, 2017) and number of DNA copies (Brankatschk et al., 2012). *S. enterica*-specific primers (forward—TCGTCATTCCATTACCTACC and reverse—AAACGTTGAAAAACTGAGGA; Hoorfar et al., 2000) and the ribosomal 16S primers (forward—CCAGCAGCCGCGGTAAT and reverse—TTTACGCCCAGTAATTCCGATT; Choi et al., 2017) were selected for this assay. The number of DNA copies per gram of leaf tissue was calculated using the formula: number of DNA copies = (ng × 6.002 × 10^{23})/(length × 1 × 10^{9} × 650),

in which ng is the Log (CT – standard curve intercept/slope standard curve), 6.002×10^{23} is Avogadro's number, length is the size of the *S. enterica* strain 14028s genome (4,964,097 bases), 1×10^{9} is used to account for the ng unit conversion, and 650 is the molar mass in grams per mole of one single DNA base pair (Brankatschk et al., 2012). The number of DNA copies is equal to the number of cells per reaction for 1 µl of DNA sample, when using *S. enterica*-specific primers.

List of Materials

- Peat Pellets 42 mm (peat moss pellets) (Jiffy 7, catalog number: SO-JFPP).
- Plastic trays without holes (Hummert International, catalog number: 65-6963-2).
- Fertilizer (Peters Excel® pHLow® 19-11-21 Multi-Purpose, catalog number: G99001).
- Plastic domes (Hummert International, catalog number: 65-6964-1).
- Soil mix (Sun Gro® Sunshine® #1 Grower Mix with RESILIENCE™).
- 50-ml centrifuge tubes (Fisher Scientific, catalog number: 553860).
- 1.7-ml microcentrifuge tubes (VWR, catalog number: 87003-294).
- Culture Tubes, Plastic, with Dual-Position Caps (VWR, catalog number: 60818-703).
- 125-ml Erlenmeyer flasks (Pyrex®, catalog number: 4980-125).
- 250-ml and 1,000-ml beakers (VWR, catalog numbers: 10754-952 and 10754-960).
- Filter Whirl-Pak(R) homogenizer blender filter bag 207 ml (Millipore Sigma, catalog number: WPB01385WA-250EA).
- Sterile inoculating loops (VWR, catalog number: 82051-146).
- Magnetic stir bars (VWR, catalog number: 58948-988).
- Square petri dish with grid (VWR, catalog number: 60872-310).
- Round petri dishes, medium (100 × 15 mm) (VWR, catalog number: 25384-302).
- Disposable plastic cuvettes (VWR, catalog number: 97000-586).
- Micropipettes (Rainin Pipet-Life™).
- Tweezers (VWR, catalog number: 89259-984).
- Silwet L-77 (Lehle Seeds, catalog number: VIS-30).
- Agarose (VWR, catalog number: 97062-250).
- Tryptone (IBI Scientific, catalog number: 41116105).
- Yeast extract (US Biotech Sources, catalog number: Y01PD-500).
- Sodium chloride (Fisher Scientific, catalog number: S271-500).
- Bacteriological agar (IBI Scientific, catalog number: IB49171).
- LSLB medium (broth and agar; see Recipes).
- TRIS—tris(hydroxymethyl)aminomethane (VWR, catalog number: 33621.260).
- EDTA—ethylenediaminetetraacetic acid (VWR, catalog number: 20294.294).
- SDS—sodium dodecyl sulfate (VWR, catalog number: 1.13760.0100).
- Ammonium acetate (VWR, catalog number: 0103-500G).
- Kanamycin (GoldBio, catalog number: K-120).
- iTaq Fast SYBR Green Supermix (BioRad, Hercules, CA, United States).
- Sterile distilled water.
- Sterile Milli-Q water.
- Ethanol pure grade (Sigma-Aldrich, catalog number: 459836).
- PBS buffer (see Recipes).
- Lettuce cultivars (Red Tide, Lollo Rossa and Salinas, stored at 4°C).
- *S. enterica* stock cultures (stored in 20% glycerol at −80°C).

Required Equipment

- Plant growth chamber (Caron Products & Services, model: 6341-2).
- Shaker incubator (VWR, catalog number: 12620-946).
- Spectrophotometer (Thermo Fisher Scientific, model: Spectronic 20D + or equivalent).
- Centrifuge (Eppendorf, model: 5810).
- Homogenizer Hand Model (Bioreba, catalog number: 400010).
- Digital hygrometer (VWR, catalog number: 35519-047).
- Quantum meter (Apogee, catalog number: BQM).
- Vortex (BioExpress, GeneMate, catalog number: S-3200-1).
- Analytical Balance (VWR, catalog number: 10753-570).
- Magnetic stirrer (VWR, catalog number: 97042-642).
- Stereoscope (VWR, catalog number: 89404-502).
- Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA, United States).
- 20-, 200-, and 1,000-µl micropipettes and tips.
- Milli-Q filter (Millipore Sigma, catalog number: C85358).
- Autoclave.
- Biological safety cabinet level 2 (Labconco™ Purifier™ Axiom™ Class II, Type C1, Kansas City, MO, United States).

Solution Recipes

Low-Sodium Luria Bertani Medium

10 g L^{-1} Tryptone
5 g L^{-1} Yeast extract
5 g L^{-1} NaCl
15 g L^{-1} Agar (only for solid medium)
Autoclave medium at 15 psi, 120°C for 15 min.
Allow medium to cool down to about 55°C and add appropriate antibiotic if needed.

Phosphate-Buffered Saline Solution

8 g L^{-1} NaCl
0.2 g L^{-1} KCl
1.44 g L^{-1} Na_2HPO_4

0.24 g L^{-1} KH_2PO_4

DNA Extraction Buffer

200 mM Tris (pH 7.5)
250 mM NaCl
25 mM EDTA
0.5% SDS

RESULTS

Previously, it was reported that *S. enterica* persistence is dependent on the bacterial inoculum concentration (Deblais et al., 2019; Jechalke et al., 2019) and on the lettuce cultivar (Jacob and Melotto, 2020). Therefore, we tested whether our protocol was useful to reliably enumerate bacterial cells in lettuce leaves using four different concentrations of bacterial inoculum (1, 3, 5, or 7 Log CFU ml^{-1}) and three commercial cultivars of lettuce with contrasting bacterial growth patterns (Red Tide, Lollo Rossa, and Salinas). The lowest inoculum concentration (1 Log CFU ml^{-1}) is impractical to use as no live bacteria could be recovered at 2 HPI, i.e., no colonies grew on the medium after plating.

At the inoculum concentration of 3 Log CFU ml^{-1}, *S. enterica* grew in Red Tide leaves with a 2.3-fold increase in CFU observed between 2 HPI and 1 DAI, while in Lollo Rossa and Salinas, the bacterial titer showed a 1.8-fold or no increase in the same time

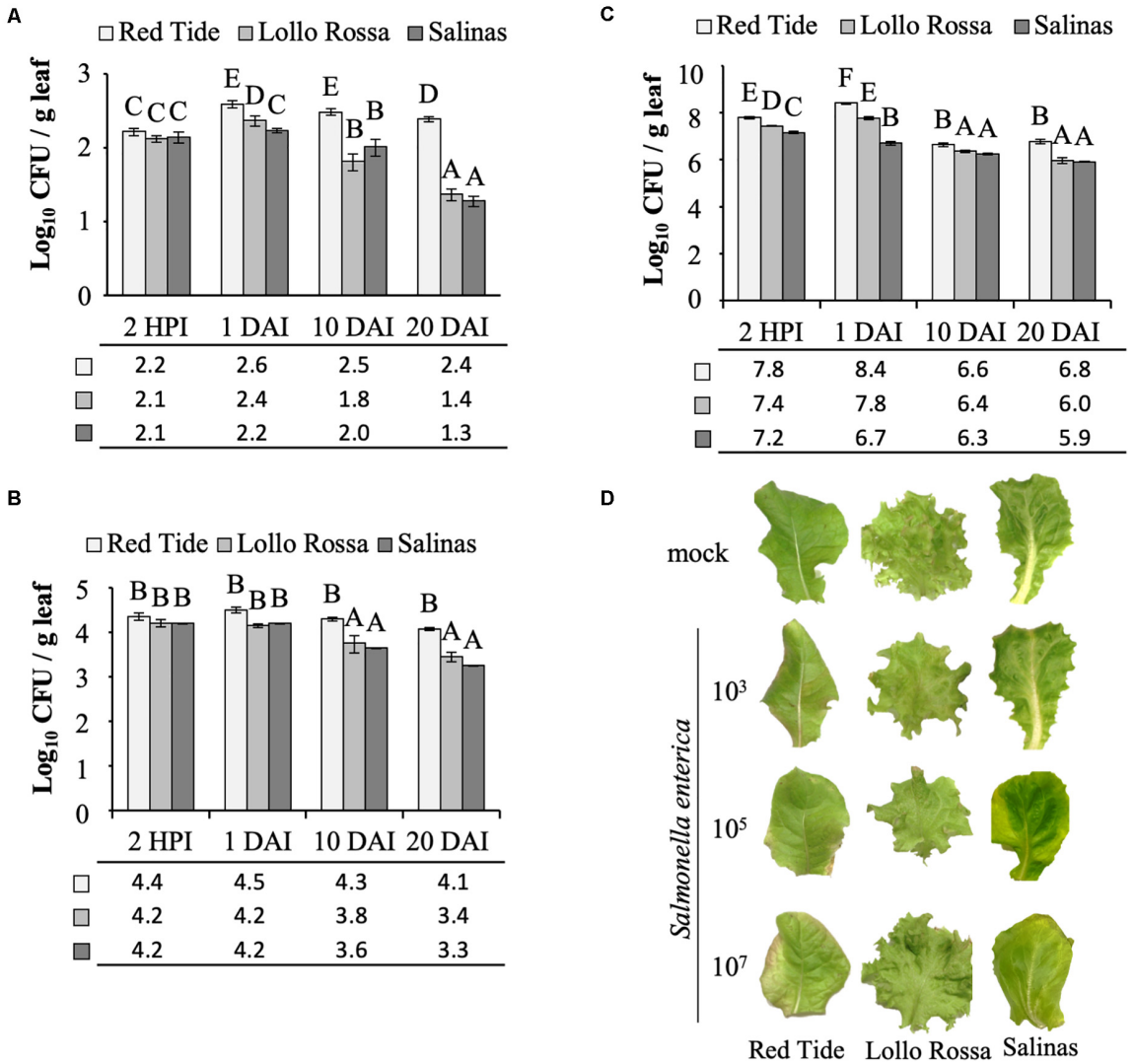


FIGURE 2 | *Salmonella enterica* persistence in the apoplast of lettuce leaves. Bacterial population size was estimated at 2 h post inoculation (HPI), and at 1, 10, and 20 days after vacuum inoculation (DAI) with the strain 14028s of *S. enterica* subsp. *enterica* serovar Typhimurium at a concentration of **(A)** 3.5 Log CFU ml^{-1}, **(B)** 5.4 Log CFU ml^{-1}, or **(C)** 7.7 Log CFU ml^{-1}. Results are shown as the average of three biological replicates and two technical replicates ($n = 6 \pm$ SE). Statistical difference among means was detected with ANOVA followed by the Scoot–Knott test ($\alpha = 0.05$). Different letters above the bars indicate significant statistical differences among cultivars across all time points. CFU = colony forming unit. g, grams. **(D)** Representative pictures of lettuce leaves at 20 DAI with each one of the bacterial inoculum dose used (3, 5, or 7 Log CFU ml^{-1}) or with the mock control.

period ($p < 0.05$), respectively (**Figure 2A**). From 1 to 20 DAI, the bacterial population decreased for all plant cultivars. However, the extent of bacterial population decrease was smaller in Red Tide (1.6-fold), whereas it decreased 10-fold in Lollo Rossa and 8.9-fold in Salinas ($p < 0.05$; **Figure 2A**).

When inoculated with 5 Log CFU ml^{-1}, a similar trend of higher bacterial population in Red Tide leaves as compared to Lollo Rosa and Salinas was observed throughout the experiment ($p < 0.05$). However, the bacterium CFU per gram of leaf remained constant in Red Tide until 20 DAI (**Figure 2B**). Bacterial population inside Lollo Rossa and Salinas leaves remained constant between 2 HPI and 1 DAI; however, the bacterial titers decreased 2.4- and 3.6-fold between 1 and 10 DAI in Lollo Rossa and Salinas, respectively ($p < 0.05$). By 20 DAI, *S. enterica* decreased further in Lollo Rossa and Salinas by 5.0- and 8.9-fold, respectively ($p < 0.05$; **Figure 2B**).

Remarkably, at 2 HPI with 7 Log CFU ml^{-1} of *S. enterica*, a significantly larger bacterial population in Red Tide leaves was observed in comparison to that in Lollo Rossa and Salinas (**Figure 2C**). The bacterial population further increased by 4.1-fold at 1 DAI in Red Tide leaves, but subsequently decreased by 56.8- and 40.9-fold at 10 and 20 DAI, respectively ($p < 0.05$; **Figure 2C**). Lollo Rossa also supported a higher number of *S. enterica* cells at 1 DAI, a 2.3-fold increase from 2 HPI, followed by a decrease of 26.5- and 64.9-fold between 1 and 10 DAI or 1 and 20 DAI, respectively ($p < 0.05$; **Figure 2C**). Bacterial growth inside Salinas leaves had a 2.9-fold decrease in population size between 2 HPI and 1 DAI, also decreasing the number of bacterial populations in its leaves by 2.8- and 6.3-fold at 10 and 20 DAI, respectively ($p < 0.05$; **Figure 2C**). These findings suggest that high levels of inoculum concentration lead to a higher *S. enterica* death rate inside the leaf apoplast, independently of the plant cultivar, considering that the second true leaf was fully expanded before inoculation and the role leaf was sampled. Although Red Tide supported larger bacterial populations at all times (**Figure 2C**).

Overall, our results showed that, independently of the inoculation dose, Red Tide supported higher bacterial population than Lollo Rossa and Salinas, in which the inoculum concentration of 3 Log CFU ml^{-1} enhanced these differences, mainly at later time points (**Figure 2**). No macroscopic symptoms such as chlorosis or necrosis were observed on mock-inoculated leaves or leaves inoculated with 3 Log CFU ml^{-1} of *S. enterica*, for all three cultivars tested. Red tide showed some chlorosis when inoculated with 5 and 7 Log CFU ml^{-1}, while no chlorosis was observed for Lollo Rossa or Salinas when these same concentrations of bacterial inoculum were used (**Figure 2D**).

To support the results of the serial dilution plating method in estimating the bacterial population size, we used qPCR as a second approach (Fu et al., 2006). This is a simple assay widely used in microbial community analysis, as well as it is quick and less labor-intensive than other methods (Brankatschk et al., 2012; Davis, 2014). To this end, we chose to test the sample from Red Tide leaves at 1 day post vacuum inoculation with 3 Log CFU ml^{-1} bacteria. Due to the small number of recovered bacterial cells (∼390 ± 41.6 cells per gram of leaf) (**Figure 2A**), technical error during plating could have occurred. Using the *S. enterica*-specific primers, we estimated that 612 ± 54.7 bacterial cells were present per gram of leaf, while no amplicon was detected in mock-inoculated leaves, the negative control (**Figure 3A**). To rule out the possibility that the lack of amplicon was due to the lack of DNA in the PCR reaction, the 16S primer set was used with the same DNA samples from bacterium- and mock-inoculated leaves. This primer set also aligns with the lettuce mitochondrion genome (NCBI reference NC_042756.1, e-value between 1×10^{-3} and 6×10^{-4}); thus, amplification of both plant and bacterium DNA was expected. Similar amount of DNA was recovered from both *S. enterica*- and mock-inoculated plants, indicating a consistent DNA extraction protocol, in which DNA from bacteria was precipitated together with the plant DNA (**Figure 3B**).

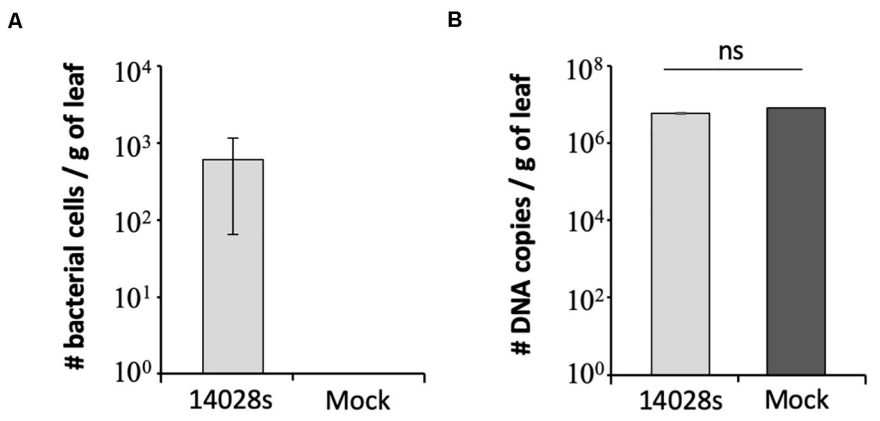


FIGURE 3 | Number of DNA copies estimated by qPCR with **(A)** *Salmonella enterica*-specific primers (Hoorfar et al., 2000) or **(B)** 16S primers (Choi et al., 2017) in Red Tide leaves. Bacterial population (*S. enterica* strain 14028s) was determined at 1 day post vacuum inoculation with 3.5 Log CFU ml^{-1} of bacterial cells or water + 0.01% Silwet as a mock control. Results are shown as the average of three biological replicates ($n = 3 \pm$ SE). Value of the mock treatment in graph **(A)** is zero and the error bars are very small to appear in graph **(B)**. CFU = colony forming unit, g, gram; ns, non-significant statistically.

DISCUSSION

Although *S. enterica* induces plant defense responses (Meng et al., 2013; Garcia and Hirt, 2014; Melotto et al., 2014; Oblessuc et al., 2020), it can still persist for long periods in the leaf apoplast depending on the bacterial strain and the plant genotype (Wong et al., 2019; Jacob and Melotto, 2020). Furthermore, recent studies have shown that variations in the *S. enterica* culturing conditions, such as temperature and nutrients in the medium (Kroupitski et al., 2019), and environmental conditions for the plant cultivation, such as temperature and humidity (Deblais et al., 2019; Jechalke et al., 2019; Roy and Melotto, 2019), can interfere mainly with the ability of *S. enterica* to internalize plant tissues. Nevertheless, variations in environmental conditions not only affect the bacterial internalization, which indeed is an important step during bacterial colonization of plants, but also interfere with the outcome of the plant–pathogen interactions and the persistence phenotype. These findings highlight the importance of establishing inoculation procedures and bacterial enumeration methods with controlled conditions that enable a realistic understanding of *S. enterica* survival in the plant, isolating the plant phenotype from environmental effects.

The method described here is simple and robust to assess *S. enterica* persistence in plant leaf apoplast and, in addition, to allow for comparisons among different inoculation doses and lettuce cultivars. We have determined that 3 Log CFU ml^{-1} is the minimum concentration of bacterial inoculum in which bacterial cells can be reliably recovered from leaves right after inoculation. This inoculum level also enabled us to follow the drastic decrease of bacterial population size in the cultivars Lollo Rossa and Salinas, when 23 $\pm$ 3.2 and 19 $\pm$ 3.2 cells per gram of leaf was detected at 20 DAI, respectively (**Figure 2A**). We used two methods to verify the results, in which the number of bacterial cells per gram of leaf tissue estimated by plating or qPCR was comparable. The plating method estimated the number of live bacterial cells only, whereas qPCR amplifies DNA from all cells present in the tissue, which might explain the lower cell number estimate by plating (390 $\pm$ 41.6 cells) as compared to qPCR (612 $\pm$ 54.7 cells). Plating also has the advantage of being cheaper and less labor-intensive than qPCR. However, if automation is an option, qPCR might be a better choice for larger screening procedure.

In addition to bacterial plating and qPCR techniques, other methods are known to be used to enumerate bacterial cells. Among these, treatment with propidium monoazide (PMA) alone or PMA + deoxycholate (DC) can be used before DNA extraction to detect bacterial cell death in the qPCR analysis. However, it may kill cells injured from experimental treatments that otherwise could have recovered (Laidlaw et al., 2019). Moreover, microscopy techniques, such as fluorescent *in situ* hybridization (FISH), and cell sorting techniques, such as flow cytometry (FC) and the specialized method of fluorescence-activated cell sorting (FACS), can also be used to access bacterial population (Davis, 2014), but these are expensive and labor-intensive. Hence, frequently conventical plating is qualified as the most robust and reliable method for cell quantification (Brankatschk et al., 2012; Laidlaw et al., 2019).

Understanding the various aspects of human bacterial pathogen interactions with plants is important to establish successful strategies to prevent, or at least reduce, contamination of fresh produce. We anticipate that this method will enable one to address questions related to the survival of human pathogens in leaves, such as the plant immune responses triggered by them, how human pathogens can affect the plant environment and its microbial community, and the mechanisms involved in the process. It is important to note that we chose vacuum infiltration in order to address bacterial survival in the leaf apoplast, but if internalization processes are the goal of future studies, we would indicate dip or spray inoculation followed by leaf surface sterilizing and print of the leaves in a petri dish with culture media, to confirm that the sterilization procedure was efficient. Ultimately, this procedure can be used to phenotype mapping populations to further identify genomic regions in the plant associated with defense against *S. enterica*, in addition to be useful for bacterial competition assays *in planta* to determine the relative fitness of various strains in this niche.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

PO and MM conceived the research, analyzed the data, and wrote the manuscript. PO performed the experiments. Both authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants from the U.S. Department of Agriculture–National Institute of Food and Agriculture (NIFA; 2015-67017-23360 and 2017-67017-26180) and NIFA Hatch grant (CA-D-PLS-2327-H) to MM.

REFERENCES

Antaki, E. M., Vellidis, G., Harris, C., Aminabadi, P., Levy, K., and Jay-Russell, M. T. (2016). Low concentration of *Salmonella enterica* and generic *Escherichia coli* in farm ponds and irrigation distribution systems used for mixed produce production in southern Georgia. *Foodborne Path. Dis.* 13, 551–558. doi: 10.1089/fpd.2016.2117

Barak, J. D., Kramer, L. C., and Hao, L. (2011). Plant cultivar alters *Salmonella enterica* colonization of tomato and type I trichomes are preferential colonization sites. *Appl. Environ. Microbiol.* 77, 498–504. doi: 10.1128/aem.01661-10

Bennett, S. D., Sodha, S. V., Ayers, T. L., Lynch, M. F., Gould, L. H., and Tauxe, R. V. (2018). Produce-associated foodborne disease outbreaks, USA, 1998-2013. *Epidemiol. Infect.* 146, 1397–1406. doi: 10.1017/s0950268818001620

Brandl, M. T. (2008). Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on postharvest lettuce. *App. Environ. Microbiol.* 74, 5285–5289. doi: 10.1128/AEM.01073-08

Brankatschk, R., Bodenhausen, N., Zeyer, J., and Bürgmann, H. (2012). Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. *Appl. Environ. Microbiol.* 78, 4481–4489. doi: 10.1128/aem.07878-11

Choi, E., Choi, S., Nam, D., Park, S., Han, Y., Lee, J. S., et al. (2017). Elongation factor P restricts *Salmonella*'s growth by controlling translation of a Mg2+ transporter gene during infection. *Sci. Rep.* 7:42098. doi: 10.1038/srep42098

Davis, C. (2014). Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. *J. Microbiol. Methods* 103, 9–17. doi: 10.1016/j.mimet.2014.04.012

Deblais, L., Helmy, Y. A., Testen, A., Vrisman, C., Madrid, A. M. J., Kathayat, D., et al. (2019). Specific environmental temperature and relative humidity conditions and grafting affect the persistence and dissemination of *Salmonella enterica* subsp. enterica serotype Typhimurium in tomato plant tissues. *App. Environ. Microbiol.* 85:e00403-19. doi: 10.1128/AEM.00403-19

Fu, C. J., Carter, J. N., Li, Y., Porter, J. H., and Kerley, M. S. (2006). Comparison of agar plate and real-time PCR on enumeration of *Lactobacillus*, *Clostridium perfringens* and total anaerobic bacteria in dog faeces. *Lett. Appl. Microbiol.* 42, 490–494. doi: 10.1111/j.1472-765x.2006.01893.x

Garcia, A. V., and Hirt, H. (2014). *Salmonella enterica* induces and subverts the plant immune system. *Front. Microbiol.* 5:141. doi: 10.3389/fmicb.2014.00141

Gopinath, S., Carden, S., and Monack, D. (2012). Shedding light on *Salmonella* carriers. *Trends Microbiol.* 20, 320–327. doi: 10.1016/j.tim.2012.04.004

Hoorfar, J., Ahrens, P., and Rådström, P. (2000). Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. *J. Clinical Microbiol.* 38, 3429–3435. doi: 10.1128/jcm.38.9.3429-3435.2000

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., and Jiang, X. (2004). Persistence of *Salmonella enterica* serovar typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. *Foodborne Pathog. Dis.* 1, 27–35. doi: 10.1089/153531404772914437

Jacob, C., and Melotto, M. (2020). Human pathogen colonization of lettuce dependent upon plant genotype and defense response activation. *Front. Plant Sci.* 10:1769. doi: 10.3389/fpls.2019.01769

Jacob, C., Panchal, S., and Melotto, M. (2017). Surface inoculation and quantification of *Pseudomonas* syringae population in the Arabidopsis leaf apoplast. *Bio-Protocol* 7:5. doi: 10.21769/BioProtoc.2167

Jechalke, S., Schierstaedt, J., Becker, M., Flemer, B., Grosch, R., Smalla, K., et al. (2019). *Salmonella* establishment in agricultural soil and colonization of crop plants depend on soil type and plant species. *Front. Microbiol.* 10:967. doi: 10.3389/fmicb.2019.00967

Katagiri, F., Thilmony, R., and He, S. Y. (2002). The Arabidopsis thaliana-*Pseudomonas* syringae interaction. *Arabidopsis Book* 1:e0039.

Kralik, P., and Ricchi, M. (2017). A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. *Front. Microbiol.* 8:108. doi: 10.3389/fmicb.2017.00108

Kroupitski, Y., Gollop, R., Belausov, E., Pinto, R., and Sela, S. (2019). *Salmonella enterica* growth conditions influence lettuce leaf internalization. *Front. Microbiol.* 10:639. doi: 10.3389/fmicb.2019.00639

Kroupitski, Y., Pinto, R., Belausov, E., and Sela, S. (2011). Distribution of *Salmonella* typhimurium in romaine lettuce leaves. *Food Microbiol.* 28, 990–997. doi: 10.1016/j.fm.2011.01.007

Kroupitski, Y., Pinto, R., Brandl, M. T., Belausov, E., and Sela, S. (2009). Interactions of *Salmonella enterica* with lettuce leaves. *J. App. Microbiol.* 106, 1876–1885.

Laidlaw, A. M., Gänzle, M. G., and Yang, X. (2019). Comparative assessment of qPCR enumeration methods that discriminate between live and dead *Escherichia coli* O157: H7 on beef. *Food Microbiol.* 79, 41–47. doi: 10.1016/j.fm.2018.11.002

Manikandan, S. (2010). Data transformation. *J. Pharmacol. Pharmacother.* 1, 126–127. doi: 10.4103/0976-500X.72373

Melotto, M., Brandl, M., Jacob, C., Jay-Russell, M., Micallef, S., Warburton, M., et al. (2020). Breeding crops for enhanced food safety. *Front. Plant Sci.* 11:428. doi: 10.3389/fpls.2020.00428

Melotto, M., Panchal, S., and Roy, D. (2014). Plant innate immunity against human bacterial pathogens. *Front. Microbiol.* 5:411. doi: 10.3389/fmicb.2014.00411

Meng, F., Altier, C., and Martin, G. B. (2013). *Salmonella* colonization activates the plant immune system and benefits from association with plant pathogenic bacteria. *Environ. Microbiol.* 15, 2418–2430. doi: 10.1111/1462-2920.12113

Oblessuc, P. R., Matiolli, C. M., and Melotto, M. (2020). Novel molecular components involved in callose-mediated defense against *Salmonella enterica* and *Escherichia coli* O157:H7. *BMC Biol.* 20:16.

Omisakin, F., MacRae, M., Ogden, I. D., and Strachan, N. J. (2003). Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. *App. Environ. Microbiol.* 69, 2444–2447. doi: 10.1128/aem.69.5.2444-2447.2003

Pezzuto, A., Belluco, S., Losasso, C., Patuzzi, I., Bordin, P., Piovesana, A., et al. (2016). Effectiveness of washing procedures in reducing *Salmonella enterica* and Listeria monocytogenes on a raw leafy green vegetable (*Eruca vesicaria*). *Front. Microbiol.* 7:1663. doi: 10.3389/fmicb.2016.01663

Roy, D., and Melotto, M. (2019). Stomatal response and human pathogen persistence in leafy greens under preharvest and postharvest environmental conditions. *Postharvest Biol. Technol.* 148, 76–82. doi: 10.1016/j.postharvbio.2018.10.013

Roy, D., Panchal, S., Rosa, B. A., and Melotto, M. (2013). *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. *Phytopathology* 103, 326–332. doi: 10.1094/PHYTO-09-12-0230-FI

Wahlig, T. A., Bixler, B. J., Valdés-López, O., Mysore, K. S., Wen, J., Ané, J. M., et al. (2019). *Salmonella enterica* serovar Typhimurium ATCC 14028S is tolerant to plant defenses triggered by the flagellin receptor FLS2. *FEMS Microbiol. Lett.* 366:fny296. doi: 10.1093/femsle/fny296

Wong, C. W., Wang, S., Levesque, R. C., Goodridge, L., and Delaquis, P. (2019). Fate of 43 *Salmonella* strains on lettuce and tomato seedlings. *J. Food Protect.* 82, 1045–1051. doi: 10.4315/0362-028x.jfp-18-435

Conflict of 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 17 July 2020
doi: 10.3389/fmicb.2020.01485

Leaf Surface Topography Contributes to the Ability of *Escherichia coli* on Leafy Greens to Resist Removal by Washing, Escape Disinfection With Chlorine, and Disperse Through Splash

*Hung K. Doan[1], María L. Antequera-Gómez[2], Atul N. Parikh[3,4] and Johan H. J. Leveau[1]**

[1] Department of Plant Pathology, University of California, Davis, Davis, CA, United States, [2] Departamento de Microbiología, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Universidad de Málaga, Málaga, Spain, [3] Department of Biomedical Engineering, University of California, Davis, Davis, CA, United States, [4] Department of Materials Science and Engineering, University of California, Davis, Davis, CA, United States

The attachment of foodborne pathogens to leaf surfaces is a complex process that involves multiple physical, chemical, and biological factors. Here, we report the results from a study designed to specifically determine the contribution of spinach leaf surface topography as it relates to leaf axis (abaxial and adaxial) and leaf age (15, 45, and 75 days old) to the ability of *Escherichia coli* to resist removal by surface wash, to avoid inactivation by chlorine, and to disperse through splash impact. We used fresh spinach leaves, as well as so-called "replicasts" of spinach leaf surfaces in the elastomer polydimethylsiloxane to show that leaf vein density correlated positively with the failure to recover *E. coli* from surfaces, not only using a simple water wash and rinse, but also a more stringent wash protocol involving a detergent. Such failure was more pronounced when *E. coli* was surface-incubated at 24°C compared to 4°C, and in the presence, rather than absence, of nutrients. Leaf venation also contributed to the ability of *E. coli* to survive a 50 ppm available chlorine wash and to laterally disperse by splash impact. Our findings suggest that the topographical properties of the leafy green surface, which vary by leaf age and axis, may need to be taken into consideration when developing prevention or intervention strategies to enhance the microbial safety of leafy greens.

Keywords: phyllosphere, phyllotelma, PDMS, topomimetic, leaf replicasting, food safety

OPEN ACCESS

Edited by:
Wei Zhang,
Illinois Institute of Technology, United States

Reviewed by:
Hao Feng,
University of Illinois at Urbana-Champaign, United States
Maria T. Brandl,
Agricultural Research Service (USDA), United States
Maria Isabel Gil,
Consejo Superior de Investigaciones Científicas (CSIC), Spain
Jitendra Patel,
Beltsville Agricultural Research Center (USDA-ARS), United States

****Correspondence:***
Johan H. J. Leveau
jleveau@ucdavis.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *11 January 2020*
Accepted: *08 June 2020*
Published: *17 July 2020*

Citation:
Doan HK, Antequera-Gómez ML, Parikh AN and Leveau JHJ (2020) Leaf Surface Topography Contributes to the Ability of Escherichia coli on Leafy Greens to Resist Removal by Washing, Escape Disinfection With Chlorine, and Disperse Through Splash. Front. Microbiol. 11:1485. doi: 10.3389/fmicb.2020.01485

INTRODUCTION

In recent years, fresh produce, in particular leafy greens such as lettuce and spinach, has been recognized as an important vehicle for the transmission of human pathogens that have traditionally been associated with foods of animal origin (Xicohtencatl-Cortes et al., 2009; Scallan et al., 2011). Among these causal agents, enterohemorrhagic *Escherichia coli* O157:H7 is of particular concern

as it causes illnesses ranging from diarrhea to life-threatening hemolytic uremic syndrome with as many as 63,000 cases in the United States annually (Scallan et al., 2011).

Contamination of leafy greens by bacterial pathogens of humans is a complex process that is influenced by multiple pre- and post-harvest factors. Some of these factors are well understood, which has spurred the development and implementation of science-based management strategies, such as protocols for produce sanitization, worker hygiene, and manure and irrigation application (Franz and Van Bruggen, 2008; Alegbeleye et al., 2018; Mahajan et al., 2018; Mogren et al., 2018; Riggio et al., 2019).

Other factors that influence pathogen establishment are less well appreciated, which currently prevent them from becoming points-of-departure for the development of practical approaches to pathogen exclusion or removal. One of these factors is the highly heterogeneous nature of the leaf surface, where spatial variation in nutrients (Joyner and Lindow, 2000; Leveau and Lindow, 2001b), water (Axtell and Beattie, 2002), and resident microbiota (Monier and Lindow, 2004) may differentially impact the fate of unwanted enteropathogens as they arrive on and colonize the leaf surface (Monier and Lindow, 2005). This spatial variation is often linked to leaf surface topography: for example, the availability of water and nutrients and the presence of microorganisms is often greater in association with leaf surface features such as veins (Beattie and Lindow, 1995; Beattie, 2011). This makes leaf surface topography a crucial topic of study for understanding the fate of enteropathogens on leafy greens.

To study *E. coli* on leafy greens, whole plants, detached leaves, leaf sections, and even isolated leaf cuticles have been used (Liao and Fett, 2001; Brandl, 2006; Brandl and Amundson, 2008). Studies have started to reveal a role for leaf age and leaf axis in the attachment, persistence, and survival of *E. coli* on leafy greens (Brandl and Amundson, 2008; Van Der Linden et al., 2013). Variation in the establishment and survival of *E. coli* O157:H7 on young vs. old leaves or abaxial vs. adaxial leaf surfaces may be explained by differences in nutrient availability (Brandl and Amundson, 2008; Van Der Linden et al., 2013) and other environmental (Brandl and Mandrell, 2002; Hora et al., 2005; Aruscavage et al., 2008; Lopez-Velasco et al., 2012; Moyne et al., 2013; Van Der Linden et al., 2013; Williams et al., 2013), biological (Tydings et al., 2011; Williams et al., 2013), and physical factors such as leaf roughness or venation (Wang et al., 2009; Macarisin et al., 2013; Hunter et al., 2015). Variation in leaf surface topography, for example between different plant species and cultivars (Tydings et al., 2011; Macarisin et al., 2013), in combination with other conditions such as temperature, relative humidity, and free surface water (Brandl and Mandrell, 2002; Hora et al., 2005; Aruscavage et al., 2008; Tydings et al., 2011) may also underlie the observed variability in effectiveness of chlorine treatments to inactivate *E. coli* (Seo and Frank, 1999; Behrsing et al., 2000; Takeuchi and Frank, 2000; Keskinen et al., 2009).

To deconstruct the complexity and interplay of physical, chemical, and biological processes that occur on leaves, researchers have started to use artificial surfaces that allow for a reductionist approach toward studying the success or failure of *E. coli* to attach, establish, and survive on leafy greens (Zhang et al., 2014; Doan and Leveau, 2015; Doan et al., 2020). Artificial polydimethylsiloxane (PDMS) surfaces consisting of patterned pillars, pits and channels have been used to show how micrometer-scale surface topography affects the dispersal, attachment, resistance to removal, biofilm formation, and survival of *E. coli* (Cao et al., 2006; Mitik-Dineva et al., 2009; Sirinutsomboon et al., 2011; Zhang et al., 2014; Gu et al., 2016, 2017). One disadvantage of such surfaces is that they are relatively poor approximations of the true landscapes of trichomes, stomata, grooves and other features that are present on fresh leaves. Therefore, several research labs have been using fresh leaves as templates in casting protocols to replicate (i.e., topomimetically) the micrometer-scale surface topography of leaves (Sun et al., 2005, 2019; Liu et al., 2007; McDonald, 2013; Zhang et al., 2014; Bernach et al., 2019; Soffe et al., 2019). Using such PDMS leaf replicasts (i.e., reproductions of plant leaf topography in polydimethylsiloxane), Zhang et al. (2014) demonstrated that attachment of *E. coli* cells to grooves between epidermal cells, replicated from PDMS onto agar, better protected the bacteria from biocide treatment than cells growing on flat agar surfaces. In our own work (Doan et al., 2020) we showed that leaf surfaces with greater topography, i.e., more venation, retained more *E. coli* cells than flatter surfaces after brief immersion in a bacterial suspension. Similarly, Sun et al. (2019) demonstrated that retention of spherical colloids on PDMS replicasts of lettuce, spinach, and tomato fruit was dependent on water retention, which was governed by surface roughness and hydrophobicity of the PDMS replica.

The objective of the study presented here was to utilize PDMS spinach leaf replicasts to investigate in more quantitative detail the impact of leaf surface topography as it relates to leaf axis (abaxial and adaxial) and leaf age (15, 45, and 75 day old) on the resistance to removal, escape from chlorine disinfection, and splash dispersal of *E. coli*. Such knowledge is potentially important from a food safety perspective with practical applications such as sanitization protocols and breeding leafy greens for leaf surface topographies that mitigate the attachment, establishment, and survival of *E. coli* on leafy greens.

MATERIALS AND METHODS

Fabrication of PDMS Replicasts

As a source of leaves for the fabrication of PDMS replicasts, we grew *Spinacia oleracea* L. (spinach, variety "Tyee," up to 75 days) from seed in Sunshine mix #1 (Sun Gro Horticulture, Bellevue, WA, United States) in the greenhouse with 10 h of supplemented light (provided by high-pressure sodium light bulbs), and at temperatures ranging from 27 to 30°C during the day and 18–21°C at night. Using fresh leaves from 15-, 45-, or 75-days-old spinach plants as templates (leaf ages correspond roughly to those of baby spinach, mature spinach, and freezer spinach, see Koike et al., 2011) PDMS leaf replicasts were prepared in a two-step molding process as described in detail previously (Doan et al., 2020). More specifically, the adaxial (top) and abaxial (bottom) sides of a fresh leaf were used to make a negative mold, and each one of those negatives was used to prepare four identical positive

PDMS leaf replicasts. From each leaf replicast (representative examples are shown in **Supplementary Figure S1**), we excised up to 13 circular sections called coupons, using a 24.3 mm inner diameter cork borer: one coupon from 15-days-old leaves; 3–4 coupons from 45-days-old leaves; and 7–13 coupons from 75-days-old leaves. We also prepared flat PDMS replicasts, i.e., lacking any topography, using a glass slide instead of a spinach leaf as template; from these flat PDMS surfaces, coupons were excised in the same way as for leaf replicasts.

Bacterial Strains and Growth Conditions

Escherichia coli ATCC 700728 (*Ec*700728), a non-toxigenic (confirmed lack of Shiga-toxin production genes) BSL-1 rifampicin-resistant derivative of a natural O157:H7 isolate was provided by Dr. Linda Harris (Department of Food Science and Technology, UC Davis) and transformed with plasmid pJBA28 (Andersen et al., 1998) to generate *Ec*700728::JBA28, which carries a chromosomal copy of the *gfp* gene for green fluorescent protein under the control of the $P_{A1/O4/O3}$ promoter and is resistant to kanamycin. *Pantoea agglomerans* 299R::JBA28 (*Pa*299R::JBA28) is a GFP-producing, kanamycin resistant derivative of the rifampicin-resistant phyllosphere model bacterium *P. agglomerans* (formerly known as *Erwinia herbicola*) 299R (Leveau and Lindow, 2001b). *Bacillus velezensis* FZB42 (*Bv*FZB42) was obtained from the Bacillus Genetic Stock Center[1]. All bacterial strains were stored at −80°C in 10% glycerol. In the "Results and Discussion" section, we refer to *Ec*700728::JBA28, *Pa*299R::JBA28, and *Bv*FZB42 as *E. coli*, *P. agglomerans*, and *B. velezensis*, respectively.

Strains *Ec*700728::JBA28 and *Pa*299R::JBA28 were routinely grown at 37° or 28°C on Lysogeny Broth (LB) agar or Tryptic Soy Agar (TSA), respectively, which was supplemented with kanamycin and/or rifampicin to a final concentration of 50 mg per liter. To prepare either strain for inoculation onto PDMS replicasts, a single bacterial colony was transferred from agar medium into LB or into M9 minimal medium supplemented with 0.4% glucose (M9+glucose) (Sambrook et al., 1989) plus appropriate antibiotic(s) and incubated on a rotary shaker, in the dark, at 250 rpm for 10 h at 37°C or 28°C, respectively. Two-hundred microliters of these cultures were transferred into 20 mL of fresh LB or M9+glucose containing Rif_{50} and Kan_{50} and incubated on a rotary shaker (250 rpm) for 6 h at 28°C in the dark to an optical density at 600 nm (OD_{600}) of 1–1.5. Bacterial cells were harvested by centrifugation at 2,500 × *g* for 10 min, rinsed twice with sterile water (Milli-Q; Millipore Corporation, Billerica, MA, United States) and resuspended in M9 containing Rif_{50} and Kan_{50} to a final concentration of 10^8 cells per mL.

As a control for the inoculation of PDMS replicasts with bacterial cells, we prepared a spore suspension of *Bv*FZB42 as follows. A single colony of *Bv*FZB42 was transferred into 100 mL Schaefer's sporulation medium (Schaeffer et al., 1965) and incubated on a rotatory shaker at 37°C, 200 rpm for 96 h. Endospores were collected by centrifugation at 8,000 rpm for 20 min and resuspended in 100 mL of Tris HCl (50 mM, pH 8.0) amended with 100 μL of a 10 mg/mL lysozyme (Sigma-Aldrich) solution and incubated on a rotary shaker at 200 rpm for 2 h at 37°C to lyse remaining vegetative cells. After centrifugation at 2,500 × g for 10 min, the supernatant was discarded, and the spore pellet was rinsed twice and resuspended in M9 to a final concentration of 10^8 spores per mL.

As an additional control, we prepared calcofluor-white stained yeast cell wall particles (YCWPblue) of *Saccharomyces cerevisiae* as described previously (Young et al., 2017). They were diluted in M9 to a concentration of 10^8 particles per mL, as determined by microscopy. We refer to YCWPblue as "yeast particles" in the "Results and Discussion" sections.

Inoculation and Incubation of Leaf Replicasts

The standard protocol that was used for inoculating PDMS coupons is shown in the left panels of **Figures 1A,B**. **Figure 1A** represents protocol A, which was used on coupons cut from flat PDMS surfaces or from PDMS spinach leaf replicasts (adaxial or abaxial side; 15, 45, or 75 days old). **Figure 1B** represents protocol B, which was used on coupons cut from flat PDMS surfaces, from PDMS replicasts of the abaxial side of 75-days-old spinach leaves, or from fresh spinach leaves (variety "Tyee," approximately 75 days old; adaxial or abaxial side). In both protocols A and B, inoculation involved pipetting five 10 μL drops on a single coupon in a quincunx pattern. Each drop contained per mL 10^8 cells (*Ec*700728::JBA28 or *Pa*299R::JBA28), spores (*Bv*FZB42), or yeast particles (YCWPblue) in M9 (protocol A) or 10^8 *Ec*700728::JBA28 cells in M9 or M9+glucose (protocol B) containing the appropriate antibiotic as described above. Inoculated coupons were placed in a parafilmed 100 × 15 mm diameter plastic Petri dish (VWR International) with one layer of germination paper (catalog number CDB 3 3/8″ Circle; Anchor Paper Co., Saint Paul, MN, United States) that was saturated with 4.5 mL of sterile deionized water (to create wat we refer to here as a high relative humidity). Petri dishes were incubated at 4 or 24°C: these temperatures are typical during harvest (24°C), processing (24°C/4°C), or storage (4°C) of leafy greens (Beuchat and Ryu, 1997; U.S. Food Drug Administration, 2010).

Processing of Leaf Replicasts

After 72 h incubation, coupons were processed as depicted in the right panels of **Figures 1A,B**. For protocol A, coupons were individually transferred into 5 mL of sterile phosphate buffer solution (PBS, 10 mM, pH 7.2) amended with 0.2% Tween 20 (Thermo Fisher Scientific, Waltham, MA, United States), vortexed vigorously for 30 sec, sonicated in a sonicator bath with adjustable power (Branson Ultrasonics Corp, St. Louis, MO, United States) at 250 W (frequency 40 kHz) for 5 min, and vortexed briefly again for 15 s. Dilutions of the resulting "stringent wash" were spread-plated in triplicate on LB Rif_{50} Kan_{50} (*Ec*700728::JBA28) or 0.1x TSA Rif_{50} Kan_{50} or without antibiotic (*Pa*299R::JBA28, *Bv*FZB42, respectively) using an Eddy Jet spiral plater (IUL, Barcelona, Spain). Surface washes containing yeast particles were not plated, but instead concentrated by centrifugation at 2500 × *g* for 10 min, resuspended in 1 mL of sterile water and enumerated with

[1]http://www.bgsc.org

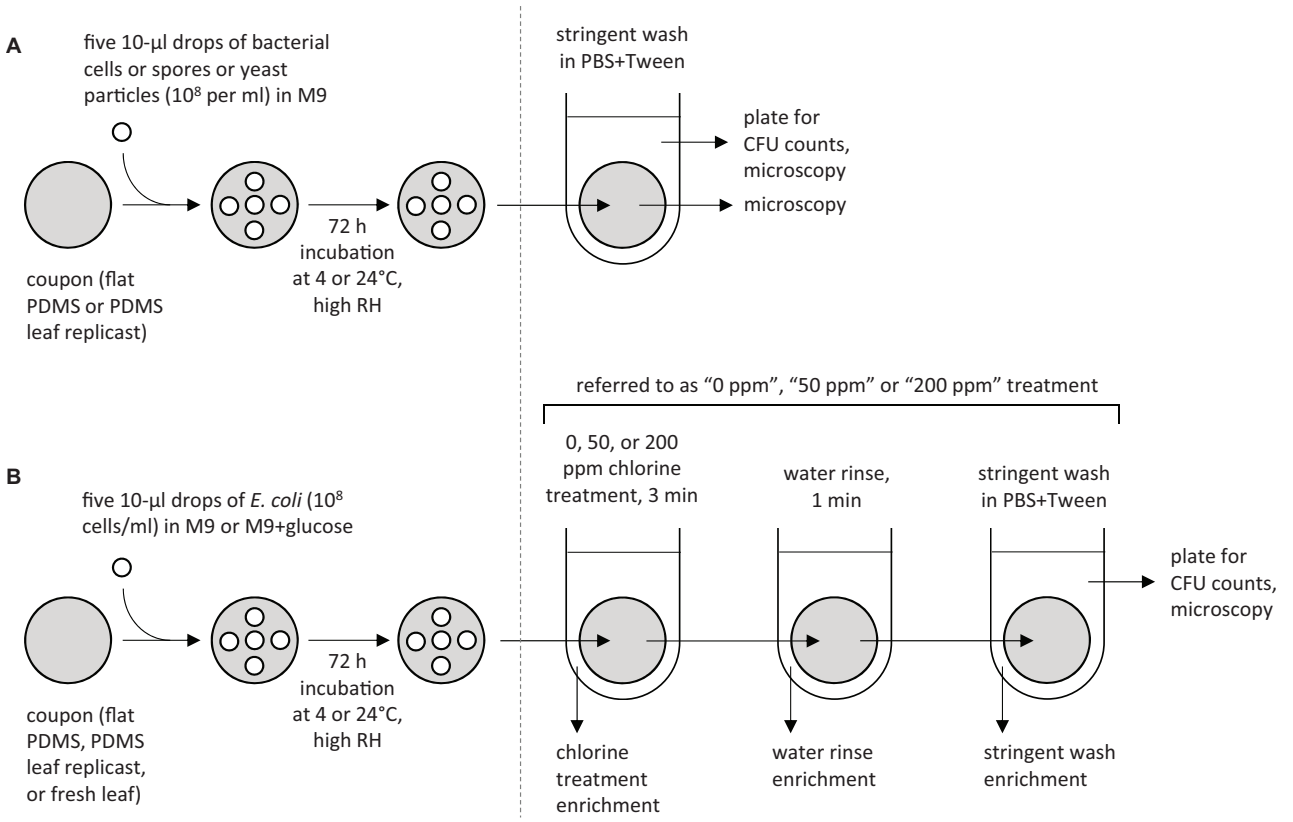


FIGURE 1 | Overview of experiments designed to assess the resistance of *E. coli* and other biomicroparticles to surface removal or chlorine treatment. For the first set of experiments **(A)**, coupons of flat PDMS surfaces or PDMS leaf replicasts (representing different spinach leaf axes and ages) were each inoculated with five 10 µL drops of a 10^8 per mL suspension of *E. coli* cells, *P. agglomerans* cells, *B. velezensis* spores, or yeast particles in M9 containing the appropriate antibiotic, then incubated and processed as described in detail in "Materials and Methods" section. For the second set of experiments **(B)**, *E. coli* cells were inoculated at 10^8 cells per mL in $M9_{Rif50Kan50}$ or $M9_{Rif50Kan50}$ +glucose onto coupons cut from flat PDMS surfaces, PDMS replicasts of spinach leaves, or fresh spinach leaves, incubated, and processed using "0 ppm," "50 ppm," or "200 ppm" chlorine treatments, as described in detail in "Materials and Methods" section. The resulting leaf washes were plated on $LB_{Rif50Kan50}$ to determine CFUs per coupon. We also performed *E. coli* enrichment protocols immediately after the chlorine wash, water rinse, and stringent wash, as described in the "Materials and Methods" section.

a hemocytometer. For protocol B, coupons were submerged in 10 mL of 0, 50, or 200 ppm available chlorine in water (PrimeSource Germicidal Ultra Bleach, Reliable Redistribution Resource, Fremont, CA, United States) for 3 min ("chlorine treatment"), followed by submersion in 20 mL of sterile water for 1 min ("rinse"), and transfer into 5 mL of PBS-Tween. Each coupon was then processed as above, resulting in a "stringent wash" solution that was dilution-plated in triplicate on LB agar containing Rif_{50} and Kan_{50}.

For both protocols A and B, the counts of colony-forming units (CFUs) or yeast particles recovered in the stringent wash from each coupon were ^{10}log-transformed, after replacing all zero values with LOD/2, where LOD is the limit of detection (Croghan and Egeghy, 2003) then averaged over the triplicate agar plates per coupon. The CFU count of each coupon was plotted as a function of the percentage of surface venation on that same coupon, as determined by microscopy using ImageJ[2]. We also averaged the count of bacterial cells/spores or yeast particles for all coupons cut from the same leaf, to obtain an average count per coupon for each leaf, of which there were three replicates for each type of leaf surfaces (i.e., 15, 45, or 75 days old, and abaxial or adaxial) plus three replicates of a flat surface.

In both protocols A and B, stringent wash solutions and the stringently washed coupons were examined for (residual) bacterial cells or spores or yeast particles by fluorescence microscopy using an Axio Imager M2 microscope (Zeiss, Jena, Germany). Furthermore, in protocol A, the surfaces of washed coupons were wiped with a sterile dampened cotton swab that was then streaked along the surface of LB agar containing Rif_{50} and Kan_{50} (*Ec*700728::JBA28) or 0.1x TSA containing Rif_{50} and Kan_{50} or without antibiotic (*Pa*299R::JBA28, *Bv*FZB42, respectively) to test for bacterial growth. In protocol B, we also checked each step in the protocol (i.e., "chlorine treatment," "water rinse," and "stringent wash") for viable *E. coli* by adding 1/10th-volume of 10x LB containing Rif_{500} and Kan_{500}, followed by incubation at 37°C for 24 h to enrich for *E. coli* growth. Samples were scored positive or negative for bacterial growth; positive growth was

[2]http://rsb.info.nih.gov/ij/

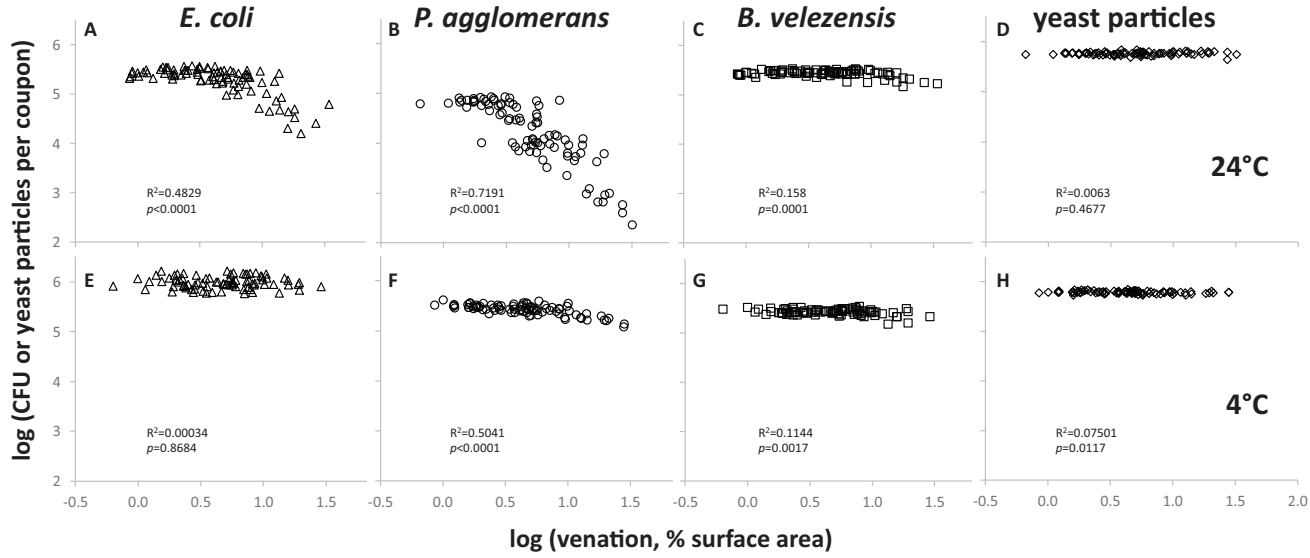


FIGURE 2 | Surface topography-dependent resistance of *E. coli* to removal from PDMS leaf replicasts by stringent washing. Each data point represents a single coupon that was inoculated with *E. coli* cells **(A,E)**, *P. agglomerans* cells **(B,F)**, *B. velezensis* spores **(C,G)** or yeast particles **(D,H)**, then incubated at 24°C **(A–D)** or 4°C **(E–H)**, and washed as depicted in **Figure 1A**. For each coupon, the count of bacterial CFUs (*E. coli*, *P. agglomerans*, *B. velezensis*) or yeast particles in the wash was plotted as a function of venation, i.e., the percentage of surface area covered by veins. Shown in each panel are between 84 and 88 coupons, representing PDMS leaf replicasts from the adaxial or abaxial surfaces of 15-, 45-, or 75-days-old spinach leaves, with three leaves per age per axis and between 1 (15-days-old plants) and 13 (75-days-old plants) coupons per leaf. In each panel, goodness of fit of the data points to a simple regression line (R^2) and *p*-values (is the slope of that line significantly different from zero?) are provided. These were calculated using a linear regression calculator (GraphPad).

confirmed to be *E. coli* using an Axio Imager M2 fluorescence microscope (Zeiss).

Dispersal of *E. coli* on Leaf Surfaces by Splash

To determine how leaf topography affected the short-distance, lateral dispersal of *E. coli* by splash, we placed single coupons from the abaxial side of 75-days-old fresh spinach leaves, from replicasts representing the abaxial side of 75-days-old fresh spinach leaves, or from flat replicasts, in the center of an LB Rif_{50} agar plate (100 × 15 mm diameter plastic Petri dish). A single 10-μL drop with 10^6 CFU of *Ec*700728 per ml was deposited onto the center of each coupon surface to create a source point. Immediately after, one 30-μL sterile deionized water droplet was dropped from a height of 30 cm onto the center of the coupon surface. Coupons were carefully removed from the agar surface, and the plates were incubated at 37°C for 24 h. For each plate, we recorded the number of colonies and the distance of each colony from the source point. For each coupon, the splash experiment was repeated two times, for a total of three replications per coupon. We calculated the average number of *E. coli* colonies and the average dispersal distance for three replicated experiments. From these data, we also averaged the dispersal distance of *E. coli* colonies for all coupons cut from the same leaf, to obtain an average count per coupon for each leaf, of which there were three different sets of leaves per experiment, with each set containing four identical PDMS flat/leaf replicasts or four fresh leaves each with 7–13 coupons.

Data Analysis

The data were analyzed using SAS version 9.4 (SAS Institute, Inc. Cary, NC, United States) or using R version 3.3.2 (The R Foundation for Statistical Computing, Vienna, Austria) with a general linear mixed effects model and analysis of variance (ANOVA) for least significant differences among the combinations of treatments ($P < 0.05$). Means were separated by Tukey's HSD at $P < 0.05$.

RESULTS

Recovery of *E. coli* as a Function of Leaf Topography, Axis, and Age

To quantify the impact of leaf surface topography on *E. coli*'s resistance to removal, we spot-inoculated coupons cut from PDMS replicasts of spinach leaves representing different leaf axes and ages, with five 10 μL drops of a 10^8 cell/mL suspension in M9, incubated the coupons at 24°C for 72 h at high relative humidity (as defined in "Materials and Methods" section), and then measured by spread-plating the number of CFUs that could be retrieved from the surface using a stringent wash protocol (**Figure 1A**). When these CFU counts were plotted for each coupon as a function of the percentage of surface covered by leaf veins, the recovery of *E. coli* cells was found to be lower from coupons with greatest venation (**Figure 2A**). An even more pronounced topography-dependent resistance to removal was observed with cells of a phyllosphere isolate of *P. agglomerans*, used here as a control (**Figure 2B**). When coupons inoculated

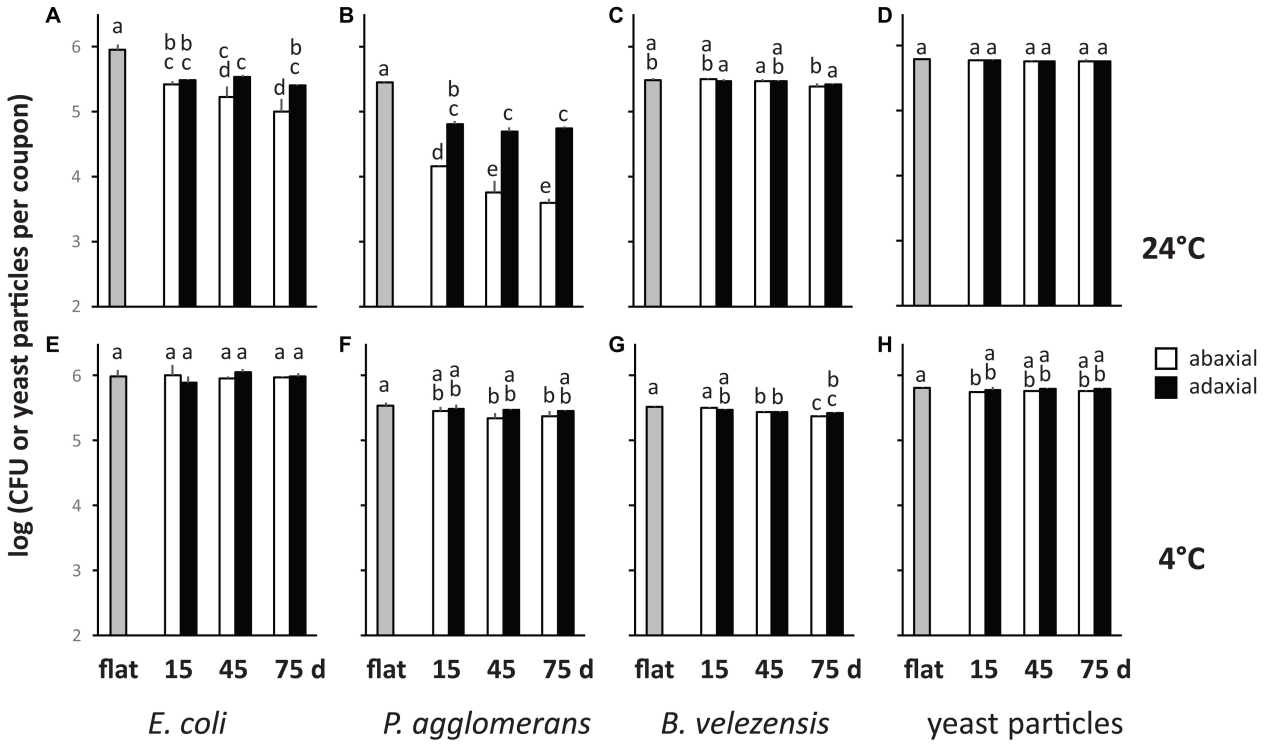


FIGURE 3 | Resistance of *E. coli* and *P. agglomerans* cells, *B. velezensis* spores, and yeast particles to removal by stringent washing from PDMS replicasts as a function of leaf axis and age, and temperature. These graphs were generated using the data shown in **Figure 2**, by averaging the counts of CFUs retrieved from coupons representing PDMS replicasts of the adaxial or abaxial side of three independent leaves from either 15-, 45-, or 75-days-old plants. Shown are the means and standard deviations across these three leaves. **(A–D)** Show data from coupons incubated at 24°C, **(E–H)** from coupons incubated at 4°C. White bars represent abaxial surfaces, black bars adaxial surfaces. Gray bars show the means and standard deviations for three sets of 4 coupons each cut from flat PDMS surfaces. Within each panel, bars with the same letter represent values that were not significantly different from each other.

with *E. coli* or *P. agglomerans* cells were incubated at 4°C instead of 24°C, the size of the effect of venation on recovery was diminished (**Figures 2E,F**, respectively). The impact of venation on recovery of two other controls, i.e., spores of *B. velezensis* (**Figures 2C,G**) and yeast particles (**Figures 2D,H**), either at 24°C (**Figures 2C,D**) or 4°C (**Figures 2G,H**), was minimal or non-significant.

Using fluorescence microscopy, we observed significant numbers of residual cells of *E. coli* and *P. agglomerans* on coupons from which fewer CFUs were recovered following stringent washes (**Supplementary Figure S2**). These bacterial cells were green fluorescent, indicating that they were viable. Many cells were found associated with leaf veins. Also, after surface-swabbing these same coupons and streaking the swab on agar medium, we observed colonies (not shown) suggesting that the bacteria that were protected from removal by stringent wash were viable and removable by physical force. No bacterial cells were observed on stringently washed coupons that were incubated at 4°C instead of 24°C.

When the CFU data were averaged by leaf axis and age, it became evident that after incubation at 24°C, *E. coli* cells were significantly more difficult to retrieve from PDMS replicasts of abaxial compared to adaxial leaf surfaces of 75-days-old spinach (**Figure 3A**). Such differences between abaxis and adaxis were not significant for younger leaves (**Figure 3A**). However, PDMS replicasts of both axes at all three plant developmental stages (15-, 45-, and 75-days-old) showed a significantly lower recovery rate than flat PDMS surfaces (**Figure 3A**). By contrast, we observed no significant differences in the retrieval of *E. coli* cells between flat PDMS surfaces and PDMS leaf replicasts when coupons were inoculated at 4°C (**Figure 3E**). Also, the numbers of *E. coli* cells that could be retrieved from flat surfaces after incubation at 4°C were not significantly different from the numbers of *E. coli* cells that could be retrieved from flat surfaces after incubation at 24°C ($p = 0.880$). This suggests that temperature did not impact the ability of *E. coli* cells to attach to PDMS.

Cells of *P. agglomerans* were significantly more difficult to retrieve, but only after incubation at 24°C, from (1) abaxial surfaces compared to adaxial surfaces (independent of leaf age), (2) the abaxial surfaces of 45- and 75-days-old leaves compared to 15-days-old leaves, and (3) any type of PDMS leaf replicast compared to flat PDMS surfaces (**Figure 3B**). At 4°C, retrieval was not significantly different between flat and adaxial surfaces, or between abaxial and adaxial surfaces (**Figure 3F**). For the other two controls (*B. velezensis* spores and yeast particles), differences in retrieval between flat and leaf-mimetic surfaces were either insignificant or significant but relatively small compared to what was observed for *E. coli* and

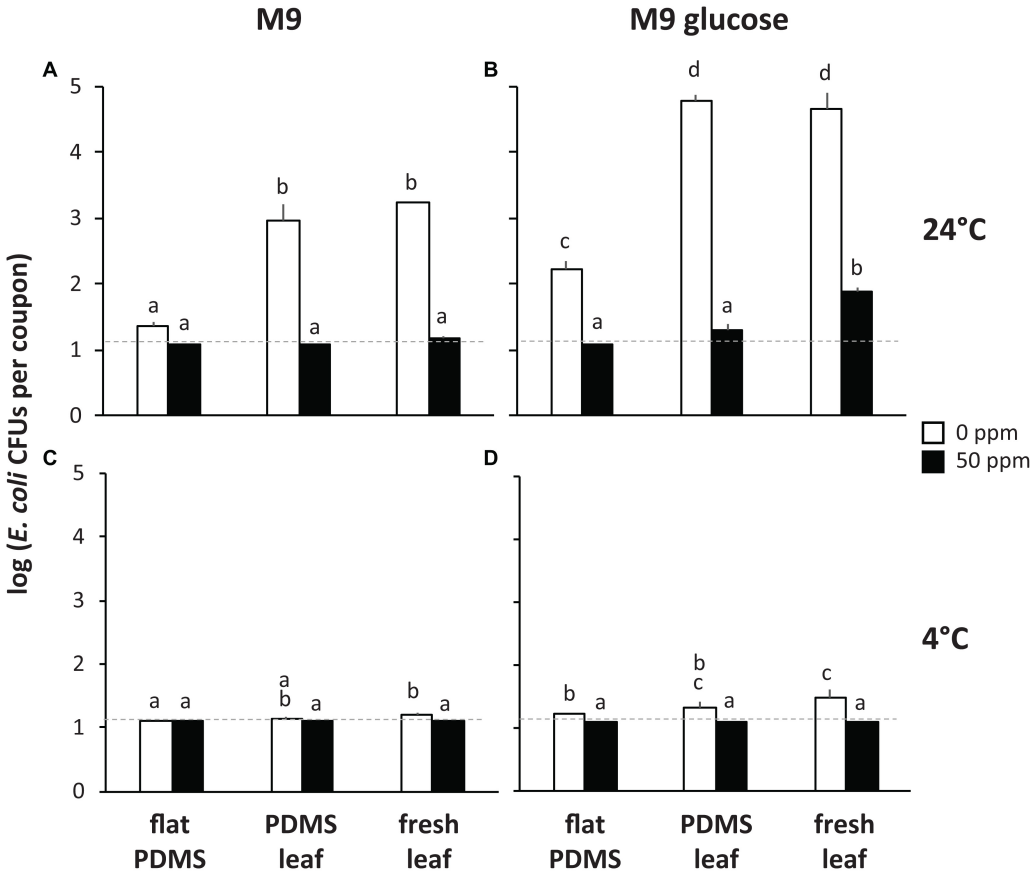


FIGURE 4 | Impact of surface topography, nutrient availability, temperature, and chlorine washes on the recovery of *E. coli* from leaf surfaces. For these experiments, coupons of flat PDMS surfaces ("PDMS flat"), PDMS replicasts of the abaxial side of 75-days-old spinach leaves ("PDMS leaf"), or abaxial side of 75-days-old fresh spinach leaves ("fresh leaf") were inoculated with *E. coli* in M9 (left panel) or M9+glucose (right panel), incubated, chlorine-treated, rinsed, and washed as depicted in **Figure 1B**. For each one of three independent leaves (PDMS or fresh) or three flat PDMS surfaces, the values of CFUs per coupon were averaged, and shown here are the means and standard deviations of coupons across these three leaves or flat surfaces. Black bars represent means from the "50 ppm" treatments, white bars from the "0 ppm" treatments. **(A,B)** Show data from coupons incubated at 24°C, **(C,D)** from coupons incubated at 4°C. Not shown are data for the "200 ppm" treatments (for which all values were below the limit of detection, which in these graphs is indicated by the stippled line). Within each panel, bars with the same letter represent values that are not significantly different from each other.

P. agglomerans, both at 24° (**Figures 3C,D**, respectively) and 4°C (**Figures 3G,H**, respectively).

Influence of Leaf Surface Topography on Resistance to Chlorine Treatment of *E. coli*

To assess the effect of leaf surface topography on the resistance of *E. coli* to foliar treatment with chlorine, we inoculated coupons cut from flat PDMS surfaces or from PDMS replicasts of the abaxial side of 75-days-old spinach leaves with five 10 μL drops of a 10^8 CFU per mL suspension of *E. coli* in M9 and then incubated these coupons at 4° or 24°C for 72 h at high relative humidity (as defined in "Materials and Methods" section), as described above. Instead of subjecting these coupons directly to a stringent wash protocol, we first immersed them in a solution of 0, 50, or 200 ppm available chlorine in water for 3 min, rinsed them in water for 1 min, and then measured by spread-plating the number of residual CFUs that could be retrieved from the surface using the stringent wash protocol (**Figure 1B**). We refer to these series of washes (3 min in 0, 50, or 200 ppm available chlorine, then 1 min water rinse, then a stringent wash) as the "0 ppm," "50 ppm," or 200 ppm" treatment, respectively.

The baseline for this experiment was the "0 ppm" treatment: it showed how many viable *E. coli* could still be retrieved from PDMS surfaces by stringent washing after first immersing and rinsing the same surfaces in water (i.e., a non-stringent wash). For flat PDMS surfaces incubated at 24°C, CFU counts were close to the limit of detection (about 10 CFUs per coupon), and significantly lower (about 2 orders of magnitude) than the counts of CFUs that were retrieved from PDMS leaf replicasts (**Figure 4A**). This suggests a topography-dependent resistance to surface removal by non-stringent immersion and rinse in water. In support of this notion, we observed a positive relationship between the level of venation of individual coupons cut from

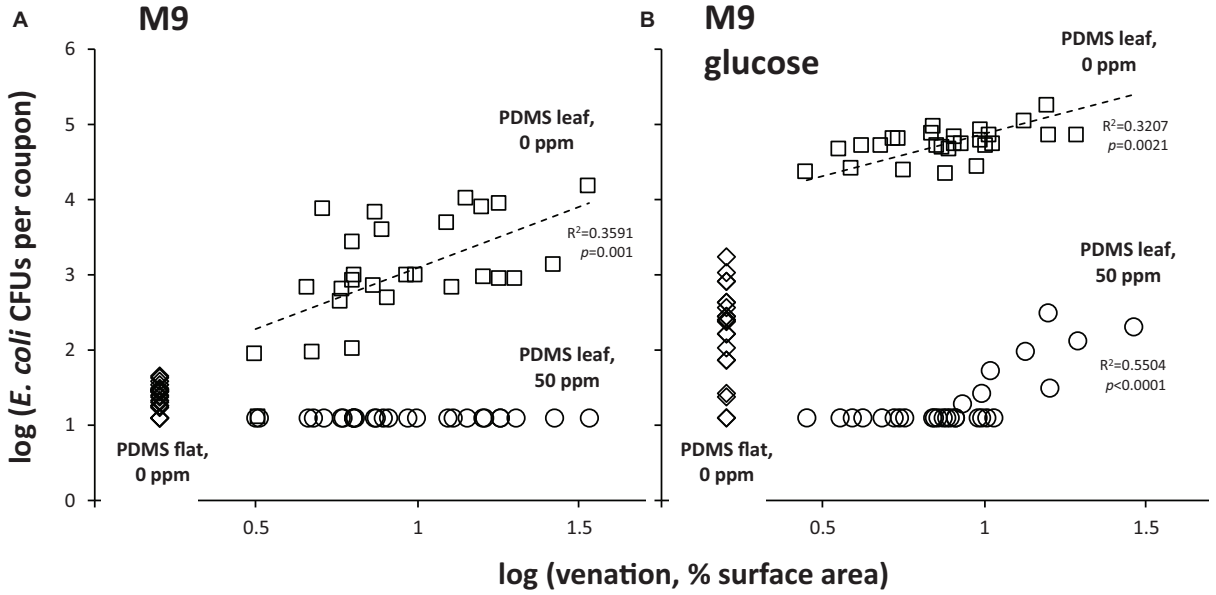


FIGURE 5 | Resistance of *E. coli* to chlorine and removal from PDMS surfaces as a function of leaf venation and nutrient availability. Shown are retrievable CFU data as a function of leaf venation for individual coupons from PDMS leaf replicast coupons that were subjected to the "0 ppm" (circles) or "50 ppm" (squares) treatment, as well as from flat PDMS surface coupons subjected to the "0 ppm" treatment (diamonds). Not shown are the data for the "50 ppm"-treated flat PDMS surface coupons (all below limit of detection). **(A)** Shows data from coupons that were inoculated with *E. coli* in M9, **(B)** from coupons inoculated with *E. coli* in M9+glucose. Shown are goodness of fit of data points to a simple regression line (R^2) and *p*-values (is the slope of that line significantly different from zero?). These were calculated using a linear regression calculator (GraphPad).

PDMS leaf replicasts and the number of CFUs that were retrieved from those same coupons (**Figure 5A**, squares).

When the same experiment was done with the "50 ppm" treatment, counts of retrievable *E. coli* CFUs were below the limit of detection, both for flat PDMS and PDMS leaf replicasts (**Figure 4A**), and independent of the degree of leaf venation (**Figure 5A**; circles). For flat surfaces, the impact of the "50 ppm" treatment was not significantly different from washing without chlorine (**Figure 4A**). However, with PDMS leaf replicasts, the same "50 ppm" treatment reduced *E. coli* counts significantly by two orders of magnitude on average (**Figure 4A**).

For comparison, we also performed the "0 ppm" and "50 ppm" treatments with coupons cut from the abaxial side of fresh leaves from 75-days-old spinach plants. The results were not significantly different from those obtained with PDMS leaf replicasts, although the average CFU count after the "50 ppm" treatment was slightly above the limit of detection (**Figure 4A**).

One important way in which fresh leaves and PDMS leaf replicasts differ is that the surfaces of the former harbor nutrients such as sugars, which can be used by *E. coli* and other bacteria to multiply. This led us to test the topography-dependent resistance of *E. coli* to surface removal and chlorine treatment under conditions of nutrient availability by inoculating coupons of flat PDMS and PDMS leaf replicasts with *E. coli* cells in glucose-supplemented M9. The availability of nutrients during the incubation period at 24°C increased the counts of retrievable CFUs from both flat and leaf PDMS surfaces by immersion and rinse in water (the "0 ppm" treatment) as would be expected. Importantly, however, as was observed in the absence of glucose (**Figure 4A**), there was a significant difference (about 2.5 orders of magnitude) in CFU counts retrieved between flat PDMS and PDMS leaf replicasts in the presence of glucose (**Figure 4B**). Consistent with this, we found a similar positive correlation with leaf venation (**Figure 5B**, squares).

With the "50 ppm" treatment, CFU counts from flat surfaces dropped about 10-fold compared to the "0 ppm" treatment, to below the limit of detection, whereas CFU counts from PDMS leaf replicast surfaces and fresh leaves dropped as much as 1,000–10,000-fold to an average just above the limit of detection (**Figure 4B**). The few PDMS leaf replicast coupons from which CFUs could be retrieved were coupons with a relatively high percentage of surface area covered by veins (**Figure 5B**, circles). To further assess the contribution of leaf surface venation on the resistance of *E. coli* to the "50 ppm" treatment, we subjected the "chlorine immersion," "water rinse" and "stringent wash" solutions for each coupon (**Figure 1B**) to an *E. coli* enrichment protocol and scored each coupon as either positive or negative for *E. coli*. While all "chlorine immersion" solutions scored negative for *E. coli* (not shown), those coupons that scored negative for *E. coli* both in the "water rinse" and "stringent wash" solutions showed a significantly lower degree of venation on average than coupons that scored (1) negative for *E. coli* in the "water rinse" but positive in the "stringent wash" solution, or (2) positive for *E. coli* both in the "water rinse" and "stringent wash" solutions (**Figure 6**). These findings are consistent with the notion that leaf surface topography, i.e.,

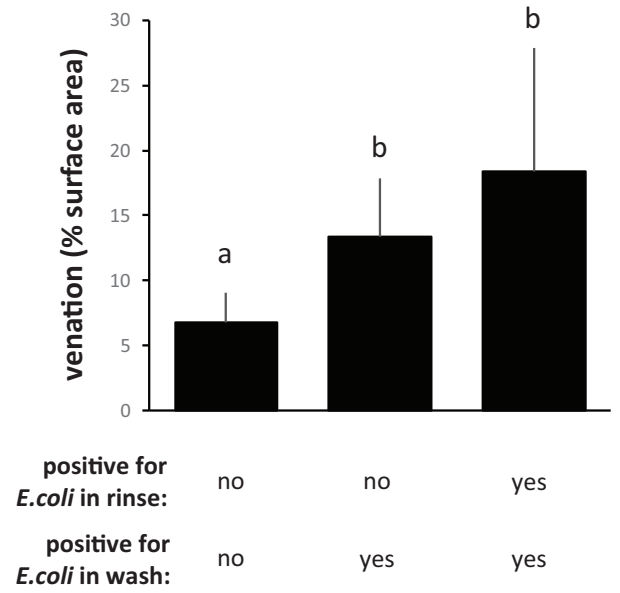


FIGURE 6 | Topography-dependent enrichment of *E. coli* in water rinses and stringent washes, following treatment with 50 ppm available chlorine. Shown are the averages and standard deviations of percentages of leaf surface venation for PDMS leaf replicast coupons shown in **Figure 1B** (circles) which scored negative for *E. coli* both in the water rinse and stringent wash, coupons that scored negative for *E. coli* in the rinse wash but positive in the stringent wash, and coupons that scored positive for *E. coli* both in the water rinse and stringent wash. Bars with the same letter represent values that were not significantly different from each other.

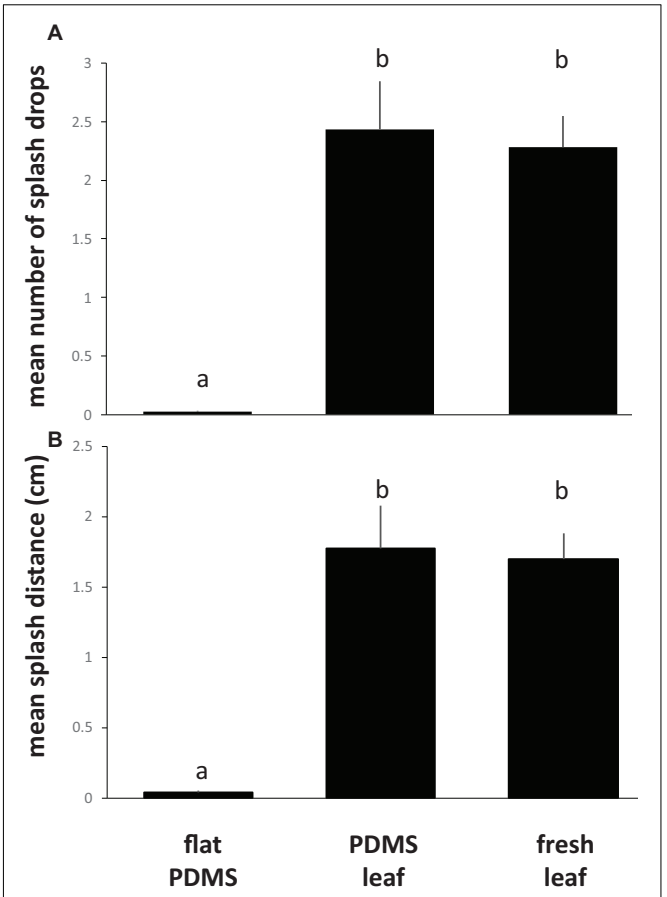


FIGURE 7 | Impact of leaf surface topography on the short-distance, lateral dispersal of *E. coli* through splash impact. For this experiment, *E. coli* cells were inoculated in the center of coupons cut from flat PDMS surfaces, from PDMS replicasts of the abaxial side of leaves from 75-days-old spinach plants, or the abaxial side of fresh leaves from 75-days-old spinach plants. Coupons were placed in the center of an LB agar plate, and a 30 μL drop of water was released from a height of 30 cm above the center of the coupon. Coupons were removed from the agar and the plates were incubated at 37°C to determine the amount of splash created by the impact of the drop **(A)** or the mean distance of splash drops from the center of the coupon **(B)**. Shown are the average and standard deviations for coupons from three independent leaves (PDMS or fresh) or flat surfaces. Bars with the same letter represent values that are not significantly different from each other.

venation, facilitates the escape of some *E. coli* cells from the "50 ppm" treatment.

When the "0 ppm" and "50 ppm" treatments were repeated at 4°C instead of 24°C, the difference in recovery of *E. coli* from flat PDMS and PDMS leaf replicast surfaces was much smaller overall, both in the absence (**Figure 4C**) and presence (**Figure 4D**) of glucose during incubation on the coupons. This confirms a role of temperature in the resistance of *E. coli* to removal from PDMS leaf replicasts. With the "200 ppm" treatment, we never recovered viable *E. coli* from any coupons (whether cut from PDMS surfaces or from fresh leaves), with or without glucose or whether at 4° or 24°C. This result highlights the efficacy of a surface wash with 200 ppm available chlorine to inactivate *E. coli*.

Topography-Dependent Dispersal of *E. coli* From Leaf Surfaces by Water Splash

To assess the effect of leaf topography on short-distance, lateral dispersal of *E. coli* by water splash, we spot-inoculated the center of coupons cut from flat PDMS surfaces, PDMS leaf replicasts (abaxial, spinach, 75 days old), or fresh leaves (abaxial, spinach, 75 days old) with a single, 10 μL drop of 10^6 cells of *E. coli* per mL, placed the inoculated coupon on an LB agar surface, then dropped a 30 μL drop of sterile water from a height of 30 cm onto the center of the coupon surface. Coupons were carefully removed from the agar surface, and plates were incubated to allow growth of *E. coli*. We measured the number of CFUs (equal to the number of splash drops) as well as the average distance of splash drops away from the center of the coupon. These measurements revealed a significant impact of leaf surface topography (whether on PDMS leaf replicasts or fresh leaves) on both the number of splash drops (**Figure 7A**) and the splash distance (**Figure 7B**) compared to flat PDMS surfaces. When broken down by individual coupons, there was a clear correlation between the percentage of surface covered by venation and the number of splash drops generated (**Figures 8A,B**) or the splash distance (**Figures 8C,D**). This was true both for PDMS leaf replicasts (**Figures 8A,C**) and fresh leaves (**Figures 8B,D**).

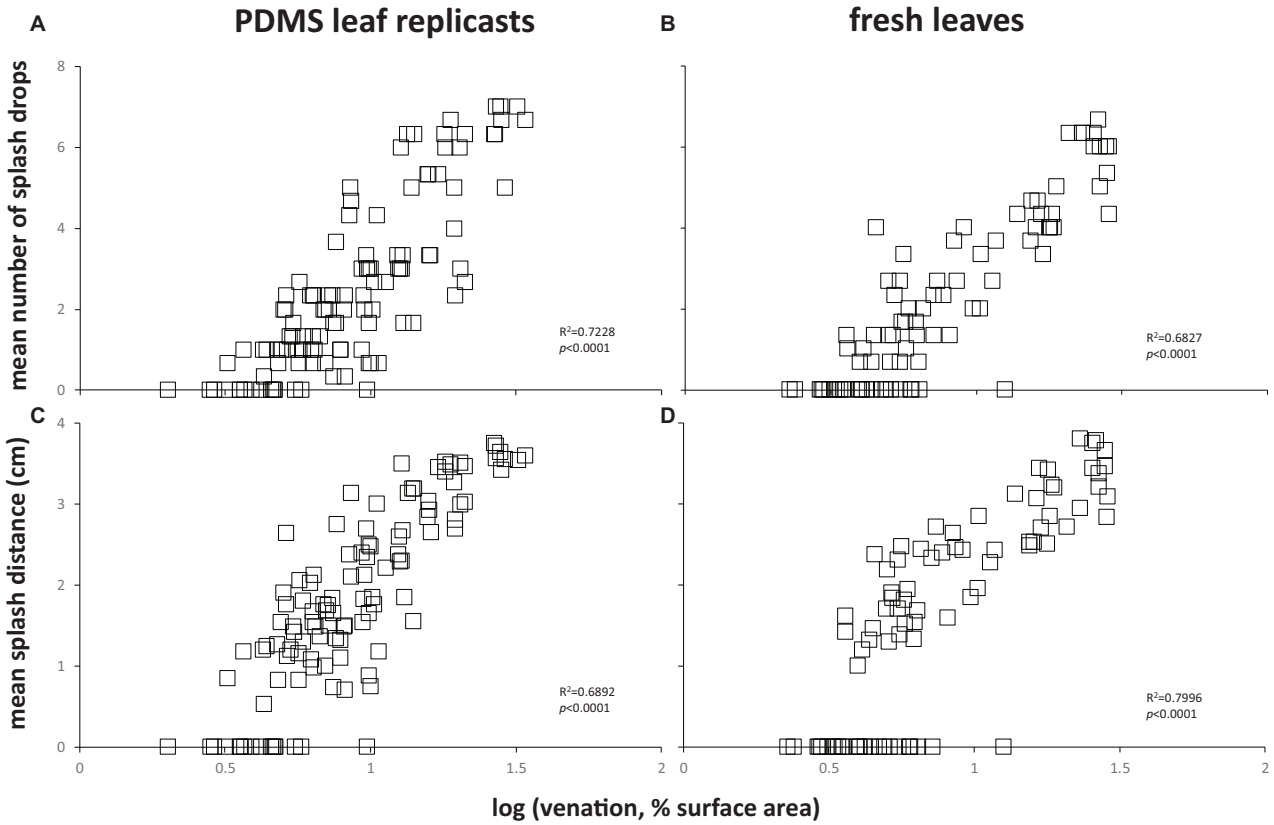


FIGURE 8 | Correlation between leaf venation and lateral dispersal of *E. coli* through splash impact. Each data point represents the amount of splash created by the impact of the drop **(A,B)** or the mean distance of splash drops from the center of the coupon **(C,D)** for a single coupon. These are the same data as shown in **Figure 7** for PDMS leaf replicasts or fresh leaves. In each panel, goodness of fit of the data points to a simple regression line (R^2) and *p*-values (is the slope of that line significantly different from zero?) are provided. These were calculated using a linear regression calculator (GraphPad).

DISCUSSION

The establishment and survival of foodborne pathogens such as *E. coli* O157:H7 on leafy greens are complex processes that involve interactions between many physical, chemical, and biological factors (Brandl and Amundson, 2008; Tydings et al., 2011; Macarisin et al., 2013; Van Der Linden et al., 2013). To deconstruct some of this complexity, PDMS replicasts of abaxial and adaxial surfaces of 15-, 45-, and 75-days-old spinach leaves were used in this study to quantitatively investigate the role of leaf surface topography on the resistance to removal, survival, and dispersal of *E. coli* on leafy greens. Leaves at different developmental stages were chosen for this study because fresh spinach is consumed at these developmental stages and leaf surface topographies are noticeably different at these stages. Under standardized conditions (i.e., incubated in the absence of nutrients and at 24°C), bacterial resistance to removal was a function of leaf topography, where the recovery of *E. coli* cells was lower from coupons with greater venation (**Figures 2A**, 5). Cells were significantly more difficult to recover from abaxial surfaces compared to adaxial surfaces, and from the abaxial surfaces of 75-days-old leaves compared to 15-days-old leaves (**Figure 3A**). Also, more cells of *E. coli* could be retrieved with stringent washing from not-stringently washed leaf topomimetic surfaces than with stringent washing from not-stringently washed flat surfaces (**Figure 4A**). Topography-dependent resistance to removal of *E. coli* is consistent with observations reported by Macarisin et al. (2013) using fresh spinach leaves. Similarly, Wang et al. (2009) found a positive correlation between surface topography of fruit surfaces and adhesion rate of *E. coli* (Wang et al., 2009). Hunter et al. (2015) reported that *Salmonella enterica* was more difficult to remove from older lettuce leaves than younger ones and that these differences were associated not only with leaf venation but stomatal densities, which for some (but not all) spinach varieties significantly differs between abaxial and adaxial surfaces (Lopez-Velasco et al., 2011). Important to note is that the above mentioned studies were done with fresh leaves, which makes it difficult to separate the contribution of leaf surface topography from other factors, such as leaf surface chemicals, hydrophobicity, and resident microbiota. Our experiments with PDMS leaf replicasts allow us to rule out such factors and to establish a contribution of leaf topography, in particular venation.

One possible explanation for the topography-dependent resistance to removal is the increased surface area that is available for adherence of *E. coli* cells, or of water bodies containing *E. coli* cells, to topomimetic PDMS surfaces compared to flat

PDMS surfaces. Such increased surface area has been proposed as an explanation for the difficulty of removing *E. coli* from rough surfaces (Wang et al., 2009; Macarisin et al., 2013; Hunter et al., 2015). Under the experimental conditions tested here, very few bacteria remained attached to flat PDMS surfaces after stringent washes (as observed by microscopy) suggesting that direct attachment to PDMS may not be a major factor in the observed resistance to removal. A more likely explanation is that *E. coli* cells got confined in spaces created by leaf surface features such as veins and maybe stomata, or within bodies of water associated with those leaf features such that they were protected from forces aimed to remove them during the stringent washing protocol (Characklis, 1981; Wang et al., 2009; Hunter et al., 2015). In our study, this is evidenced by the observation of residual bacterial cells near leaf venation with fluorescence microscopy after leaf washing (**Supplementary Figures S2B,D**). Such protection is absent from flat PDMS surfaces.

Chlorine is widely used by the leafy greens industry because of its ability, at low cost, to inactivate foodborne pathogens, and its minimal impact on the nutritional and aesthetic quality of produce. The United States Food and Drug Administration recommends 50–200 ppm of available chlorine at pH 6.0–7.5 and contact times of 1–2 min (U.S. Food Drug Administration, 1998). Many studies have revealed a broad range of chlorine efficacy in inactivation of *E. coli* on leafy greens (Seo and Frank, 1999; Behrsing et al., 2000; Takeuchi and Frank, 2000; Keskinen et al., 2009). Variation in efficacy may be attributed to differences in a number of factors, including leaf damage, internalization of *E. coli* cells, and the inactivation of chlorine by organic material associated with lettuce leaves (Seo and Frank, 1999; Behrsing et al., 2000; Takeuchi and Frank, 2000; Keskinen et al., 2009). The results we show here suggest that variation in leaf surface topography is another explanatory factor for differences in survival of *E. coli* on chlorine-treated leafy greens (**Figures 5**, **6**). Surface roughness of plant leaves has been implemented as a factor protecting *E. coli* cells from treatment with chlorine (Wang et al., 2009; Fransisca and Feng, 2012). This is also consistent with work by Zhang et al. (2014) who demonstrated that attachment of *E. coli* cells to grooves between epidermal cells, replicated from PDMS onto nutrient agar, better protected the bacteria from chlorine treatment at 200 ppm than cells growing on flat agar surfaces. Takeuchi and Frank (2000), similarly, found that lettuce leaf structures played an important role in the protection of *E. coli* O157:H7 cells from chlorine inactivation, as cells located near depressions in the cuticle survived chlorine treatments. Combined, these observations are consistent with a model in which leaf topography protects *E. coli* cells on the surface directly from getting into contact with chlorine and/or protects *E. coli* cells from being removed from the surface and indirectly from inactivation by chlorine in the wash water.

A topography-dependent resistance to removal was also observed with cells of *P. agglomerans* (**Figure 2B**). Cells of *P. agglomerans* were significantly harder to retrieve from the abaxial surface compared to adaxial surfaces, and from the abaxial surfaces of 45- and 75-days-old leaves compared to 15-days-old leaves (**Figure 3B**). The strain of *P. agglomerans* that we used in our study was originally isolated from plant foliage and is expected to be a better colonizer of plant surfaces than foodborne pathogens (Takeuchi et al., 2000; Brandl and Mandrell, 2002; Garrood et al., 2004; Brandl, 2006). We suspect that *P. agglomerans* has evolved features that maximize its retention on leaf surfaces and that such features are absent in less-phyllosphere-fit bacteria such as *E. coli*. No effect of topography was observed on the recovery of spores of *B. velezensis* (**Figures 2C,G**) and yeast particles (**Figures 2D,H**). A possible explanation for this observation lies in how bacterial spores and yeast particles differ in size and surface characteristics from bacterial cells, which is likely to differentially impact their interaction with leaf topographies and its associated water landscape.

In our experiments, the topography-dependent resistance to removal of *E. coli* or *P. agglomerans* cells was more pronounced when coupons were incubated at 24°C than at 4°C (**Figures 2E,F**, respectively). Many studies have reported the importance of temperature on the surface attachment of both foodborne pathogens and phyllosphere epiphytes (Maurelli et al., 1984; Brandl and Mandrell, 2002; Allwood et al., 2004; Barnhart and Chapman, 2006; Ells and Truelstrup Hansen, 2006; Xicohtencatl-Cortes et al., 2009; Dourou et al., 2011; Van Der Linden et al., 2014). However, seeing that in our experimental setup, there was no significant difference in *E. coli* removal from flat PDMS coupons that were incubated either at 4°C or at 24°C, the differences that we observed with leaf topomimetic PDMS surfaces at these two temperatures are most likely not due to differential attachment of *E. coli* cells to the PDMS surface. More likely, the effect of temperature is linked to interactions between the PDMS surface and the water drops, and/or interactions between the bacteria and the water drops. Perhaps temperature impacts the ability of bacteria to swim in the water landscape and to explore and end up in crevices and other features of the leaf topography that offer protection from later being washed off. This would be consistent with our observation that the effect of temperature was much less obvious or non-existent for *Bacillus* spores (**Figures 3C,G**) and yeast bioparticles (**Figures 3D,H**), neither of which possess the ability to swim.

Leaves harbor nutrients such as sugars that can be utilized by bacteria (Tukey and Tukey, 1966; Mercier and Lindow, 2000; Leveau and Lindow, 2001a; Brandl, 2006). *E. coli* O157:H7 can grow on several nutrients found on sliced cucumbers (Abdul-Raouf et al., 1993) shredded spinach and lettuce (Abdul-Raouf et al., 1993; Diaz and Hotchkiss, 1996; Cooley et al., 2006) and cantaloupe and watermelon cubes (Del Rosario and Beuchat, 1995) and in apple juice (Zhao et al., 1993). Nutrients have been shown to be a major factor in the attachment of *E. coli* to surfaces and its survival in the face of biocidal treatments (Herson et al., 1987; Beuchat, 1999; Li et al., 2001; Hassan and Frank, 2004; Ryu et al., 2004). In our experiments, glucose was used, which is one of the predominant sugars found on leaf surfaces (Mercier and Lindow, 2000; Cooley et al., 2006). Our findings support the notion that nutrient availability stacks the odds in favor of *E. coli* by giving it an opportunity to multiply, which increases its chances of persisting on leaf surfaces when challenged with a chlorine wash (**Figure 5B**).

It has been demonstrated that foliar irrigation can act as a vector for *E. coli* from soil (Mootian et al., 2009; Monaghan and Hutchison, 2012) fecal matter (Mootian et al., 2009; Atwill et al., 2015) or furrow water splash onto leafy greens (Mootian et al., 2009; Monaghan and Hutchison, 2012; Atwill et al., 2015). To the best of our knowledge, no studies have so far looked at the dispersal of *E. coli* by splash to other parts of the same leaf or to other nearby leaves of crops such as lettuce and spinach. In our study, we demonstrated a significant impact of leaf surface topography on the dispersal of *E. coli* by water splash, showing a higher number of splash drops as well as further splash distance from coupons with greater surface leaf topography, i.e., more venation. Our study suggests that surface topography may impact splash-driven cross-contamination of *E. coli* in pre- and post-harvest and food-processing environments. The effect of surface roughness on splash dispersal has been previously reported with fungal spores on leaves (Fitt and Lysandrou, 1984; Madden et al., 1996) fruit surfaces (Grove, 1985; Reynolds, 1989; Madden et al., 1996) and artificial leaf surfaces (Hörberg, 2002).

In summary, using experimentally amenable PDMS spinach leaf replicasts as a model surface, we were able to show that the resistance to removal, survival, and dispersal of *E. coli* O157:H7 on spinach is significantly affected by leaf surface topography (which varies by leaf axis and leaf age) in combination with temperature and nutrients. Leaf surface topography also was a contributing factor in the foliar survival of *E. coli* following chlorine treatment, with significantly greater numbers of viable cells on surfaces with more pronounced leaf venation (i.e., greater leaf roughness). Surface topography, as it relates to leaf age, may need to be taken into consideration by breeders and growers in selecting fresh-market spinach cultivars.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JL secured funding for this study. HD, JL, and AP did the experimental design. HD and MA-G performed the experiments. HD and JL wrote the draft of the manuscript with editing of relevant sections by other authors. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported in part by the USDA-NIFA award number 2014-67017-21695 and by the Dissertation Year Fellowship, the Bert & Nell Krantz Fellowship, the Graduate Research Mentorship Fellowship, the Golden International Agricultural Fellowship, from the University of California, Davis.

ACKNOWLEDGMENTS

We are grateful to Stephen Young and Nitin Nitin for their help with producing calcofluor-white stained yeast cell wall particles.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01485/full#supplementary-material

FIGURE S1 | Representative PDMS replicasts of abaxial and adaxial surfaces of spinach leaves of different ages. Each one of the replicasts is shown with the coupons that were cut from it using a 24.3 mm diameter corkborer (for the purpose of this figure, coupons were placed back into the cut-out).

FIGURE S2 | Fluorescence microscopy images of residual bacterial cells of *E. coli* O157:H7 **(A,B)** and *P. agglomerans* cells **(C,D)** on PDMS spinach leaf replicasts after coupons were washed with 5 mL of sterile PBS amended with 0.2% Tween 20, vortexed vigorously for 30 s and then sonicated.

REFERENCES

Abdul-Raouf, U. M., Beuchat, L. R., and Ammar, M. S. (1993). Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Appl. Environ. Microbiol.* 59, 1999–2006. doi: 10.1128/aem.59.7.1999-2006.1993

Alegbeleye, O. O., Singleton, I., and 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. doi: 10.1016/j.fm.2018.01.003

Allwood, P. B., Malik, Y. S., Hedberg, C. W., and Goyal, S. M. (2004). Effect of temperature and sanitizers on the survival of feline calicivirus, *Escherichia coli*, and F-specific coliphage MS2 on leafy salad vegetables. *J. Food Prot.* 67, 1451–1456. doi: 10.4315/0362-028x-67.7.1451

Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjørn, S. P., Givskov, M., and Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl. Environ. Microbiol.* 64, 2240–2246. doi: 10.1128/aem.64.6.2240-2246.1998

Aruscavage, D., Miller, S. A., Lewis Ivey, M. L., Lee, K. E. N., and LeJeune, J. T. (2008). Survival and dissemination of *Escherichia coli* O157:H7 on physically and biologically damaged lettuce plants. *J. Food Prot.* 71, 2384–2388. doi: 10.4315/0362-028x-71.12.2384

Atwill, E. R., Chase, J. A., Oryang, D., Bond, R. F., Koike, S. T., Cahn, M. D., et al. (2015). Transfer of *Escherichia coli* O157:H7 from simulated wildlife scat onto romaine lettuce during foliar irrigation. *J. Food Prot.* 78, 240–247. doi: 10.4315/0362-028x.jfp-14-277

Axtell, C. A., and Beattie, G. A. (2002). Construction and characterization of a proU-gfp transcriptional fusion that measures water availability in a microbial habitat. *Appl. Environ. Microbiol.* 68, 4604–4612. doi: 10.1128/aem.68.9.4604-4612.2002

Barnhart, M. M., and Chapman, M. R. (2006). Curli biogenesis and function. *Annu. Rev. Microbiol.* 60, 131–147. doi: 10.1146/annurev.micro.60.080805.142106

Beattie, G. A. (2011). Water relations in the interaction of foliar bacterial pathogens with plants. *Annu. Rev. Phytopathol.* 49, 533–555. doi: 10.1146/annurev-phyto-073009-114436

Beattie, G. A., and Lindow, S. E. (1995). The secret life of foliar bacterial pathogens on leaves. *Annu. Rev. Phytopathol.* 33, 145–172. doi: 10.1146/annurev.py.33.090195.001045

Behrsing, J., Winkler, S., Franz, P., and Premier, R. (2000). Efficacy of chlorine for inactivation of *Escherichia coli* on vegetables. *Postharvest Biol. Technol.* 19, 187–192. doi: 10.1016/s0925-5214(00)00092-2

Bernach, M., Soffe, R., Remus-Emsermann, M. N. P., and Nock, V. (2019). Micropatterning of hybrid polydimethylsiloxane for replica leaves. *Jpn. J. Appl. Phys.* 58:SDDK01.

Beuchat, L. R. (1999). Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *J. Food Prot.* 62, 845–849. doi: 10.4315/0362-028x-62.8.845

Beuchat, L. R., and Ryu, J. H. (1997). Produce handling and processing practices. *Emerg. Infect. Dis.* 3, 459–465. doi: 10.3201/eid0304.970407

Brandl, M. T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.* 44, 367–392. doi: 10.1146/annurev.phyto.44.070505.143359

Brandl, M. T., and Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Appl. Environ. Microbiol.* 74, 2298–2306. doi: 10.1128/aem.02459-07

Brandl, M. T., and Mandrell, R. E. (2002). Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl. Environ. Microbiol.* 68, 3614–3621. doi: 10.1128/aem.68.7.3614-3621.2002

Cao, T., Tang, H., Liang, X., Wang, A., Auner, G. W., Salley, S. O., et al. (2006). Nanoscale investigation on adhesion of *E. coli* to surface modified silicone using atomic force microscopy. *Biotechnol. Bioeng.* 94, 167–176. doi: 10.1002/bit.20841

Characklis, W. G. (1981). Fouling biofilm development: a process analysis. *Biotechnol. Bioeng.* 102, 310–347.

Cooley, M. B., Chao, D., and Mandrell, R. E. (2006). *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *J. Food Prot.* 69, 2329–2335. doi: 10.4315/0362-028x-69.10.2329

Croghan, C. W., and Egeghy, P. P. (2003). *Methods of Dealing with Values Below the Limit of Detection Using SAS*. Assam: SAS.

Del Rosario, B. A., and Beuchat, L. R. (1995). Survival and growth of enterohemorrhagic *Escherichia coli* O157:H7 in cantaloupe and watermelon. *J. Food Prot.* 58, 105–107. doi: 10.4315/0362-028x-58.1.105

Diaz, C., and Hotchkiss, J. H. (1996). Comparative growth of *Escherichia coli* 0157:H7, spoilage organisms and shelf-life of shredded iceberg lettuce stored under modified atmospheres. *J. Sci. Food Agric.* 70, 433–438. doi: 10.1002/(sici)1097-0010(199604)70:4<433::aid-jsfa518>3.0.co;2-q

Doan, H. K., and Leveau, J. H. J. (2015). Artificial surfaces in phyllosphere microbiology. *Phytopathology* 105, 1036–1042. doi: 10.1094/phyto-02-15-0050-rvw

Doan, H. K., Ngassam, V. N., Gilmore, S. F., Tecon, R., Parikh, A. N., and Leveau, J. H. J. (2020). Topography-driven shape, spread, and retention of leaf surface water impacts microbial dispersion and activity in the phyllosphere. *Phytobiomes J.* doi: 10.1094/PBIOMES-01-20-0006-R

Dourou, D., Beauchamp, C. S., Yoon, Y., Geornaras, I., Belk, K. E., Smith, G. C., et al. (2011). Attachment and biofilm formation by *Escherichia coli* O157:H7 at different temperatures, on various food-contact surfaces encountered in beef processing. *Int. J. Food Microbiol.* 149, 262–268. doi: 10.1016/j.ijfoodmicro.2011.07.004

Ells, T. C., and Truelstrup Hansen, L. (2006). Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage. *Int. J. Food Microbiol.* 111, 34–42. doi: 10.1016/j.ijfoodmicro.2006.04.033

Fitt, B. D. L., and Lysandrou, M. (1984). Studies on mechanisms of splash dispersal of spores, using *Pseudocercosporella herpotrichoides* spores. *Phytopathology* 111, 323–331. doi: 10.1111/j.1439-0434.1984.tb00777.x

Fransisca, L., and Feng, H. (2012). Effect of surface roughness on inactivation of *Escherichia coli* O157:H7 87-23 by new organic acid-surfactant combinations on alfalfa, broccoli, and radish seeds. *J. Food Prot.* 75, 261–269. doi: 10.4315/0362-028x.jfp-11-279

Franz, E., and Van Bruggen, A. H. C. (2008). Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. *Crit. Rev. Microbiol.* 34, 143–161.

Garrood, M. J., Wilson, P. D. G., and Brocklehurst, T. F. (2004). Modeling the rate of attachment of *Listeria monocytogenes*, *Pantoea agglomerans*, and *Pseudomonas fluorescens* to, and the probability of their detachment from, potato tissue at 10°C. *Appl. Environ. Microbiol.* 70, 3558–3565. doi: 10.1128/aem.70.6.3558-3565.2004

Grove, G. G. (1985). Splash dispersal of *Phytophthora cactorum* from infected strawberry fruit. *Phytopathology* 75, 611–615.

Gu, H., Chen, A., Song, X., Brasch, M. E., Henderson, J. H., and Ren, D. (2016). How *Escherichia coli* lands and forms cell clusters on a surface: a new role of surface topography. *Sci. Rep.* 6:29516.

Gu, H., Kolewe, K. W., and Ren, D. (2017). Conjugation in *Escherichia coli* biofilms on poly(dimethylsiloxane) surfaces with microtopographic patterns. *Langmuir* 33, 3142–3150. doi: 10.1021/acs.langmuir.6b04679

Hassan, A. N., and Frank, J. F. (2004). Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. *Int. J. Food Microbiol.* 96, 103–109. doi: 10.1016/s0168-1605(03)00160-0

Herson, D. S., McGonigle, B., Payer, M. A., and Baker, K. H. (1987). Attachment as a factor in the protection of *Enterobacter cloacae* from chlorination. *Appl. Environ. Microbiol.* 53, 1178–1180. doi: 10.1128/aem.53.5.1178-1180.1987

Hora, R., Warriner, K., Shelp, B. J., and Griffiths, M. W. (2005). Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *J. Food Prot.* 68, 2506–2509. doi: 10.4315/0362-028x-68.12.2506

Hörberg, H. M. (2002). Patterns of splash dispersed conidia of *Fusarium poae* and *Fusarium culmorum*. *Eur. J. Plant Pathol.* 108, 73–80.

Hunter, P. J., Shaw, R. K., Berger, C. N., Frankel, G., Pink, D., and 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.

Joyner, D. C., and Lindow, S. E. (2000). Heterogeneity of iron bioavailability on plants assessed with a whole-cell GFP-based bacterial biosensor. *Microbiology* 146, 2435–2445. doi: 10.1099/00221287-146-10-2435

Keskinen, L. A., Burke, A., and Annous, B. A. (2009). Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. *Int. J. Food Microbiol.* 132, 134–140. doi: 10.1016/j.ijfoodmicro.2009.04.006

Koike, S. T., Cahn, M., Cantwell, M., Fennimore, S., Lestrange, M., Natwick, E., et al. (2011). *Spinach Production in California*. Davis, CA: University of California Agriculture and Natural Resources.

Leveau, J. H. J., and Lindow, S. E. (2001a). Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3446–3453. doi: 10.1073/pnas.061629598

Leveau, J. H. J., and Lindow, S. E. (2001b). Predictive and interpretive simulation of green fluorescent protein expression in reporter bacteria. *J. Bacteriol.* 183, 6752–6762. doi: 10.1128/jb.183.23.6752-6762.2001

Li, Y., Brackett, R. E., Chen, J., and Beuchat, L. R. (2001). Survival and growth of *Escherichia coli* O157:H7 inoculated onto cut lettuce before or after heating in chlorinated water, followed by storage at 5 or 15°C. *J. Food Prot.* 64, 305–309. doi: 10.4315/0362-028x-64.3.305

Liao, C. H., and Fett, W. F. (2001). Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *J. Food Prot.* 64, 1110–1115. doi: 10.4315/0362-028x-64.8.1110

Liu, Y., Tang, J., Wang, R., Lu, H., Li, L., Kong, Y., et al. (2007). Artificial lotus leaf structures from assembling carbon nanotubes and their applications in hydrophobic textiles. *J. Mater. Chem.* 17, 1071–1078. doi: 10.1039/b613914k

Lopez-Velasco, G., Tydings, H. A., Boyer, R. R., Falkinham, J. O., and Ponder, M. A. (2012). Characterization of interactions between *Escherichia coli* O157:H7 with epiphytic bacteria in vitro and on spinach leaf surfaces. *Int. J. Food Microbiol.* 153, 351–357. doi: 10.1016/j.ijfoodmicro.2011.11.026

Lopez-Velasco, G., Welbaum, G. E., Falkinham, J. O. III, and Ponder, M. A. (2011). Phyllopshere bacterial community structure of spinach (*Spinacia oleracea*) as affected by cultivar and environmental conditions at time of harvest. *Diversity* 3, 721–738. doi: 10.3390/d3040721

Macarisin, D., Patel, J., Bauchan, G., Giron, J. A., and Ravishankar, S. (2013). Effect of spinach cultivar and bacterial adherence factors on survival of *Escherichia coli* O157:H7 on spinach leaves. *J. Food Prot.* 76, 1829–1837. doi: 10.4315/0362-028x.jfp-12-556

Madden, L. V., Yang, X., and Wilson, L. L. (1996). Effects of rain intensity on splash dispersal of *Colletotrichum acutatum*. *Phytopathology* 86, 864–874.

Mahajan, R., Chandel, S., and Goel, G. (2018). "A review on the implications of interaction between human pathogenic bacteria and the host on food quality and disease," in *Food Safety and Preservation Modern Biological Approaches*

to Improving Consumer Health, eds A. M. Grumezescu and A. M. Holban (London: Academic Press), 457–479. doi: 10.1016/b978-0-12-814956-0.00015-9

Maurelli, A. T., Blackmon, B., and Curtis, R. (1984). Temperature-dependent expression of virulence genes in *Shigella* species. *Infect. Immun.* 43, 195–201. doi: 10.1128/iai.43.1.195-201.1984

McDonald, B. (2013). *Pattern Transfer and Characterization of Biomimetic Micro-Structured Surfaces for Hydrophobic and Icephobic Applications*. Master's thesis, University of Waterloo, Ontario.

Mercier, J., and Lindow, S. E. (2000). Role of leaf surface sugars in colonization of plants by bacterial epiphytes. *Appl. Environ. Microbiol.* 66, 369–374. doi: 10.1128/aem.66.1.369-374.2000

Mitik-Dineva, N., Wang, J., Truong, V. K., Stoddart, P., Malherbe, F., Crawford, R. J., et al. (2009). *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* attachment patterns on glass surfaces with nanoscale roughness. *Curr. Microbiol.* 58, 268–273. doi: 10.1007/s00284-008-9320-8

Mogren, L., Windstam, S., Boqvist, S., Vågsholm, I., Söderqvist, K., Rosberg, A. K., et al. (2018). The hurdle approach-A holistic concept for controlling food safety risks associated with pathogenic bacterial contamination of leafy green vegetables. A review. *Front. Microbiol.* 9:1965. doi: 10.3389/fmicb.2018.01965

Monaghan, J. M., and Hutchison, M. L. (2012). Distribution and decline of human pathogenic bacteria in soil after application in irrigation water and the potential for soil-splash-mediated dispersal onto fresh produce. *J. Appl. Microbiol.* 112, 1007–1019. doi: 10.1111/j.1365-2672.2012.05269.x

Monier, J.-M., and Lindow, S. E. (2004). Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. *Appl. Environ. Microbiol.* 70, 346–355. doi: 10.1128/aem.70.1.346-355.2004

Monier, J.-M., and Lindow, S. E. (2005). Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. *Microb. Ecol.* 49, 343–352. doi: 10.1007/s00248-004-0007-9

Mootian, G., Wu, W. H., and Matthews, K. R. (2009). Transfer of *Escherichia coli* O157:H7 from soil, water, and manure contaminated with low numbers of the pathogen to lettuce plants. *J. Food Prot.* 72, 2308–2312. doi: 10.4315/0362-028x-72.11.2308

Moyne, A. L., Harris, L. J., and Marco, M. L. (2013). Assessments of total and viable *Escherichia coli* O157:H7 on field and laboratory grown lettuce. *PLoS One* 8:e70643. doi: 10.1371/journal.pone.0070643

Reynolds, K. M. (1989). Splash dispersal of *Phytophthora cactorum* from infected strawberry fruit by simulated canopy drip. *Phytopathology* 75, 611–615.

Riggio, G. M., Wang, Q., Kniel, K. E., and Gibson, K. E. (2019). Microgreens—A review of food safety considerations along the farm to fork continuum. *Int. J. Food Microbiol.* 290, 76–85. doi: 10.1016/j.ijfoodmicro.2018.09.027

Ryu, J. H., Kim, H., Frank, J. F., and Beuchat, L. R. (2004). Attachment and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. *Lett. Appl. Microbiol.* 39, 359–362. doi: 10.1111/j.1472-765x.2004.01591.x

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edn. New York, NY: Cold Spring Harbor Laboratory Press.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerg. Infect. Dis.* 17, 7–15. doi: 10.3201/eid1701.p11101

Schaeffer, P., Millet, J., and Aubert, J. P. (1965). Catabolic repression of bacterial sporulation. *Proc. Natl. Acad. Sci. U.S.A.* 54, 704–711. doi: 10.1073/pnas.54.3.704

Seo, K. H., and Frank, J. F. (1999). Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 62, 3–9. doi: 10.4315/0362-028x-62.1.3

Sirinutsomboon, B., Delwiche, M. J., and Young, G. M. (2011). Attachment of *Escherichia coli* on plant surface structures built by microfabrication. *Biosyst. Eng.* 108, 244–252. doi: 10.1016/j.biosystemseng.2010.12.007

Soffe, R., Altenhuber, N., Bernach, M., Remus-Emsermann, M. N. P., and Nock, V. (2019). Comparison of replica leaf surface materials for phyllosphere microbiology. *PLoS One* 14:e0218102. doi: 10.1371/journal.pone.0218102

Sun, M., Luo, C., Xu, L., Ji, H., Ouyang, Q., Yu, D., et al. (2005). Artificial lotus leaf by nanocasting. *Langmuir* 21, 8978–8981. doi: 10.1021/la050316q

Sun, T., Lazouskaya, V., and Jin, Y. (2019). Polydimethylsiloxane replicas efficacy for simulating fresh produce surfaces and application in mechanistic study of colloid retention. *J. Food Sci.* 84, 524–531. doi: 10.1111/1750-3841.14479

Takeuchi, K., and Frank, J. F. (2000). Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63, 434–440. doi: 10.4315/0362-028x-63.4.434

Takeuchi, K., Matute, C. M., Hassan, A. N., and Frank, J. F. (2000). Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Pseudomonas fluorescens* to lettuce leaves. *J. Food Prot.* 63, 1433–1437. doi: 10.4315/0362-028x-63.10.1433

Tukey, H. B. Jr., and Tukey, H. B. (1966). Leaching of metabolites from above-ground plant parts and its implications. *Bull. Torrey Bot. Club* 93, 385–401.

Tydings, H., Lopez-Velasco, G., Ponder, M., and Welbaum, G. (2011). Alterations to the phylloepiphytic bacterial community of spinach with leaf age. *Acta Hortic.* 917, 211–216. doi: 10.17660/actahortic.2011.917.27

U.S. Food Drug Administration (1998). *US Food and Drug Administration, 1998. Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*. College Park, MD: Center for Food Safety and Applied Nutrition.

U.S. Food Drug Administration (2010). *Program Information Manual Retail Food Protection: Recommendations for the Temperature Control of Cut Leafy Greens During Storage and Display in Retail Food Establishments*. College Park, MD: Center for Food Safety and Applied Nutrition.

Van Der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Heyndrickx, M., and Maes, M. (2013). Survival of enteric pathogens during butterhead lettuce growth: crop stage, leaf age, and irrigation. *Foodborne Pathog. Dis.* 10, 485–491. doi: 10.1089/fpd.2012.1386

Van Der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., and Heyndrickx, M. (2014). Evaluation of an attachment assay on lettuce leaves with temperature-and starvation-stressed *Escherichia coli* O157:H7 MB3885. *J. Food Prot.* 77, 549–557. doi: 10.4315/0362-028x.jfp-13-332

Wang, H., Feng, H., Liang, W., Luo, Y., and Malyarchuk, V. (2009). Effect of surface roughness on retention and removal of *Escherichia coli* O157:H7 on surfaces of selected fruits. *J. Food Sci.* 74, E8–E15.

Williams, T. R., Moyne, A. L., Harris, L. J., and Marco, M. L. (2013). Season, irrigation, leaf age, and *Escherichia coli* inoculation influence the bacterial diversity in the lettuce phyllosphere. *PLoS One* 8:e68642. doi: 10.1371/journal.pone.0068642

Xicohtencatl-Cortes, J., Chacón, E. S., Saldaña, Z., Freer, E., and Girón, J. A. (2009). Interaction of *Escherichia coli* O157:H7 with leafy green produce. *J. Food Prot.* 72, 1531–1537. doi: 10.4315/0362-028x-72.7.1531

Young, S., Dea, S., and Nitin, N. (2017). Vacuum facilitated infusion of bioactives into yeast microcarriers: evaluation of a novel encapsulation approach. *Food Res. Int.* 100, 100–112. doi: 10.1016/j.foodres.2017.07.067

Zhang, B., Luo, Y., Pearlstein, A. J., Aplin, J., Liu, Y., Bauchan, G. R., et al. (2014). Fabrication of biomimetically patterned surfaces and their application to probing plant–bacteria interactions. *ACS Appl. Mater. Interfaces* 6, 12467–12478. doi: 10.1021/am502384q

Zhao, T., Doyle, M. P., and Besser, R. E. (1993). Fate of enterohemorrhagic *Escherichia coli* O157:H7 in apple cider with and without preservatives. *Appl. Environ. Microbiol.* 59, 2526–2530. doi: 10.1128/aem.59.8.2526-2530.1993

Conflict of 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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 October 2021
doi: 10.3389/fmicb.2021.622068

Determination of *Salmonella enterica* Leaf Internalization Varies Substantially According to the Method and Conditions Used to Assess Bacterial Localization

Madhvi Chahar[1], Yulia Kroupitski[1†], Rachel Gollop[1†], Eduard Belausov[2], Maeli Melotto[3] and Shlomo Sela-Saldinger[1]*

[1]Department of Food Sciences, The Volcani Center, Institute for Postharvest and Food Sciences, Agriculture Research Organization, Rishon-LeZion, Israel, [2]Microscopy Unit, Plant Sciences, Ornamental Plants and Agricultural Biotechnology, The Volcani Center, Agriculture Research Organization, Rishon-LeZion, Israel, [3]Department of Plant Sciences, University of California, Davis, Davis, CA, United States

In a previous study, comparing the internalization of *S. enterica* serovar Typhimurium in various leaves by confocal microscopy, we have demonstrated that the pathogen failed to internalize tomato leaves. Numerous reasons may account for these findings, yet one such factor might be the methodology employed to quantify leaf internalization. To this end, we have systematically studied leaf localization of a Green-fluorescent protein-labeled *Salmonella* strain in tomato, lettuce, and *Arabidopsis* leaves by surface sterilization and enumeration of the surviving bacteria, side by side, with confocal microscopy observations. Leaf sterilization was performed using either sodium hypochlorite, silver nitrate, or ethanol for 1 to 7 min. The level of internalization varied according to the type of disinfectant used for surface sterilization and the treatment time. Treatment of tomato leaves with 70% ethanol for up to 7 min suggested possible internalization of *Salmonella*, while confocal microscopy showed no internalization. In the case of in lettuce and *Arabidopsis* leaves, both the plate-count technique and confocal microscopy demonstrated considerable *Salmonella* internalization thought different sterilization conditions resulted in variations in the internalization levels. Our findings highlighted the dependency of the internalization results on the specific disinfection protocol used to determine bacterial localization. The results underscore the importance of confocal microscopy in validating a particular surface sterilization protocol whenever a new pair of bacterial strain and plant cultivar is studied.

Keywords: tomato, lettuce, *Arabidopsis*, internalization, attachment, colonization, disinfection, fresh produce

OPEN ACCESS

Edited by:
Paula Teixeira, Catholic University of Portugal, Portugal

Reviewed by:
Ariena H.C. Van Bruggen, University of Florida, United States
Shabarinath Srikumar, United Arab Emirates University, United Arab Emirates

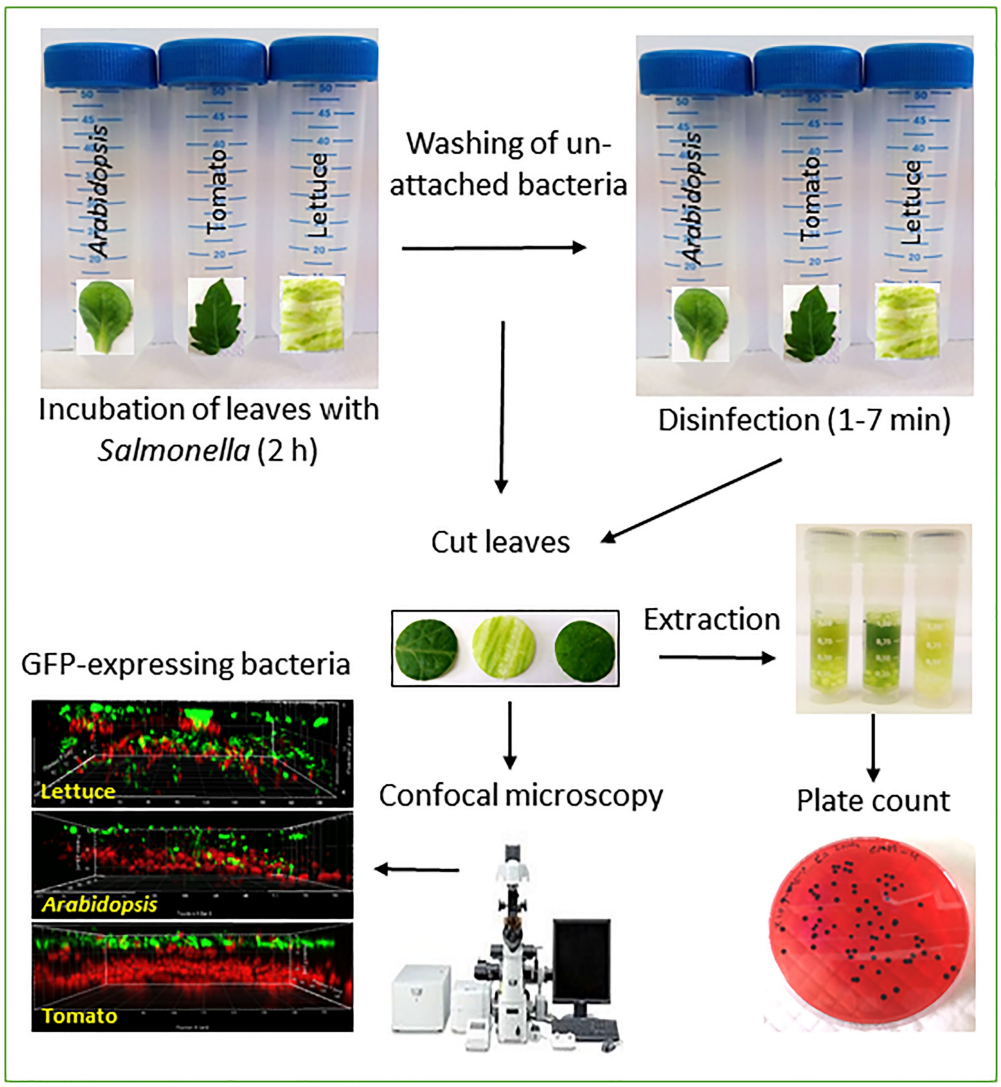
***Correspondence:**
Shlomo Sela-Saldinger
shlomos@volcani.agri.gov.il

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *27 October 2020*
Accepted: *27 August 2021*
Published: *04 October 2021*

Citation:
Chahar M, Kroupitski Y, Gollop R, Belausov E, Melotto M and Sela-Saldinger S (2021) Determination of Salmonella enterica Leaf Internalization Varies Substantially According to the Method and Conditions Used to Assess Bacterial Localization. Front. Microbiol. 12:622068. doi: 10.3389/fmicb.2021.622068



GRAPHICAL ABSTRACT | Outline of the methodologies used to assess leaf localization.

INTRODUCTION

Foodborne illness is one of the most serious health problems worldwide, affecting public health and development (Newman et al., 2015). *Salmonella* spp. and pathogenic *Escherichia coli* strains are two of the main bacterial pathogens causing foodborne diseases (Takkinen et al., 2005; Friesema et al., 2008; Söderström et al., 2008; Gajraj et al., 2012). Worldwide, it is estimated that *Salmonella* is responsible for 80.3 million cases of foodborne illness (Majowicz et al., 2010). Raw fruits and vegetables are increasingly recognized as an important source of foodborne disease outbreaks in many parts of the world (Hanning et al., 2009; Wendel et al., 2009; Gajraj et al., 2012; Mritunjay and Kumar, 2015). Leafy vegetables were identified as the fresh produce commodity group of most significant concern from a microbiological safety perspective (Callejón et al., 2015; Mogren et al., 2018; Carstens et al., 2019; Kintz et al., 2019). Consequently, recent studies have focused on understanding the interactions between human pathogens and plants (Gu et al., 2011, 2013b; Barak and Schroeder, 2012; Cevallos-Cevallos et al., 2012; Schikora et al., 2012; Brandl and Sundin, 2013; Fletcher et al., 2013; Lim et al., 2014; Pollard et al., 2014; Holden et al., 2015; Fornefeld et al., 2017; Jacob and Melotto, 2019). Leaf attachment and internalization enable bacteria to get a foothold on the leaf surface and potentially reach the leaf interior (Barak and Schroeder, 2012; Deering et al., 2012; Erickson, 2012). The ability of both plant and human pathogens to reach the leaf interior is considered an important virulent trait, as internalized bacteria gain access to the nutrient-rich milieu within the leaf tissue and are protected against external environmental stresses, such as desiccation, irradiation, starvation, competition, and predation.

Salmonella enterica and *E. coli* can internalize the plants through natural openings, such as hydathodes, stomata, lenticels, lateral

root emergence sites, or sites of biological or physical injury (Seo and Frank, 1999; Takeuchi and Frank, 2001; Solomon et al., 2002; Hora et al., 2005; Bernstein et al., 2007; Klerks et al., 2007; Underwood et al., 2007; Gomes et al., 2009; Kroupitski et al., 2009; Sharma et al., 2009; Barak and Schroeder, 2012; Deering et al., 2012; Gorbatsevich et al., 2013; Zheng et al., 2013; Gu et al., 2013a; Erickson et al., 2019; Riggio et al., 2019). A common method to assess leaf internalization is by taking advantage of the resistance of the internalized bacteria to surface disinfection (Deering et al., 2012; Erickson, 2012). Following inoculation of the pathogen of choice, surface-attached bacteria are killed by exposing the plants or plant's organ to disinfecting agents. The plant tissue is then macerated to release the internalized bacteria and the disinfectant-protected bacteria are then enumerated by viable count, e.g., plating the homogenate on appropriate agar media. The viable count technique is straightforward and easy to perform and, consequently, it was widely adopted in studies assessing leaf internalization by enteric pathogens (For example, Duffy et al., 2005, Franz et al., 2007, Hadjok et al., 2008, Zhang et al., 2009, Erickson et al., 2010a,b, Gu et al., 2011, 2013a, Ge et al., 2013, Fakruddin et al., 2017). However, a major caveat of this method is that the results depend on the conditions used for surface sterilization, e.g., type of disinfectant(s) and treatment duration, which require validation for each specific combination of bacterial strain and plant cultivar. A literature review showed that only a few studies had validated the complete inactivation of surface-attached enteric bacteria, while in most cases, surface sterilization conditions were based on previously reported protocols, or the validation data were not presented (**Table 1**). Another approach to assess leaf internalization by foodborne pathogens is confocal microscopy. This method utilizes fluorescence-tagged bacteria and enables direct and precise localization of the bacteria within the leaf tissue. Nevertheless, it is time-consuming and requires expensive equipment (confocal microscope) and expertise. Commonly, confocal microscopy provides supportive data to confirm the internal localization of the tested bacteria and validate complete inactivation of surface-attached bacteria (Takeuchi and Frank, 2001; Duffy et al., 2005; Gu et al., 2011, 2013a; Erickson, 2012). In some cases, confocal microscopy may also provide quantitative data regarding leaf internalization (Kroupitski et al., 2009; Golberg et al., 2011).

In a previous study, employing confocal microscopy, we compared the internalization of *S. enterica* serovar Typhimurium, through stomata, in various leaves and found that it efficiently internalizes lettuce leaves but virtually failed to internalize tomato leaves, based on visualization of at least 360 microscopic leaf fields obtained from three plants (Golberg et al., 2011). It should be noted that numerous factors, such as bacterial strain, plant cultivar, growing conditions, age, epiphytic and endophytic flora, mode of inoculation, and other experimental factors, might affect the level and quantification of leaf internalization (Deering et al., 2012; Erickson, 2012; Gu et al., 2013b); yet validated data regarding the efficacy of a given protocol to assess bacterial internalization in different plant models are scarce. In the present study, we have employed an *in vitro* model system to systematically examine *Salmonella* stomatal internalization in tomato, lettuce, and *Arabidopsis thaliana* leaves using a specific *Salmonella* strain with three surface sterilization protocols, side by side with confocal microscopy validation. While all three plant species differ in their leaf structure and topography, the first two were shown to support significant levels of *Salmonella* internalization. In contrast, nearly no internalization was shown in tomato leaves by confocal laser microscopy (Golberg et al., 2011), making these leaves an ideal control system for assessing potential misinterpretation when using surface sterilization and viable count.

MATERIALS AND METHODS

Bacterial Growth Conditions and Inoculum Preparation

Green-fluorescent protein (GFP)-labeled *S. enterica* serovar Typhimurium SL1334 strain (Kroupitski et al., 2009; Gu et al., 2013a) was used throughout the study. Bacterial culture was prepared and stored in Lysogeny broth (LB; Becton Dickinson, United States) supplemented with glycerol at −70°C, as described (Kroupitski et al., 2009). For each experiment, fresh culture was prepared by plating the bacteria on a new LB plate supplemented with 100 mg/ml streptomycin and 10 mg/ml gentamicin for 24 h at 37°C. Two to three single colonies were as inoculated into LB broth devoid of NaCl (LBNS) and grown at 37°C with shaking (150 rpm) for 18–20 h. Cultures were washed twice with sterile saline (0.85% NaCl) by centrifugation at 2,700 g for 10 min, and the final pellet was resuspended in sterile saline. Bacterial concentration was determined by plating × 10-fold serial dilutions on LB agar supplemented with the two antibiotics.

Preparation of Leaves

A. thaliana (Col-0) plants were grown in a potting mix containing (w/w) 70% peat, 30% perlite, supplemented with slow-release fertilizer (7,611, Even-Ari, Israel) under 10-h light / 14-h dark (short day) photoperiod, at 22°C with a relative humidity of 55–60% and light intensity of 130 $\mu mol\, m^{-2}\, S^{-1}$. Tomato plants (*Solanum Lycopersicon*), cultivar M82, were grown in Green quality soil mix, Tuff soil (Merom Golan, Israel) under 16-h light / 8-h dark, at 25°C. *Arabidopsis* and tomato leaves of 4- and 6-weeks old plants, respectively, were aseptically cut from the plants, and whole leaves or leaflets were used for the experiments. Fresh iceberg lettuce (*Lactuca sativa*) was obtained from a local retail store and used on the day of purchase or stored in the refrigerator for up to 12 h before use. The outermost leaves of the lettuce head were aseptically removed, and two or three inner leaves were taken for the experiments. The lettuce leaves were cut into *ca.* 3- by 3-cm pieces using a sterile scalpel, as described before (Kroupitski et al., 2009), and individual pieces were used for the experiments.

Inoculation of Leaves

Inoculation of leaves was performed, essentially as described before (Kroupitski et al., 2009, 2011, 2019; Golberg et al., 2011), except for the incubation temperature. Briefly, a single

TABLE 1 | List of selected reports on leaf internalization of human enteric pathogens and the method used to study bacterial localization.

Plant	Pathogen	Disinfectant	References	Source of protocol	Confocal microscopy
Parsley	GFP-tagged *Salmonella* serovars Javiana, Rubislaw, and Anatum	2,000 mg/liter sodium hypochlorite solution at 25°C for 3 min	Duffy et al., 2005	Buchanan et al., 1999; used for *E. coli* O157:H7 internalization in apples	Yes
Lettuce	*Escherichia coli* O157:H7, *S.* Typhimurium strain MAE 110	–1% $AgNO_3$ for 10 s followed by two washing steps of 10 S in water, –1% sodium hypochlorite for 5 s followed by 5 s in 70% EtOH and two washing steps	Franz et al., 2007	Franz et al., 2007	No
Lettuce	Five strains mixture of *E. coli* O157:H7 and 5 serovars of *Salmonella*	80% ethanol for 10 s followed by immersion in 0.1% $HgCl_2$ for 10 min followed by five washing steps with water	Zhang et al., 2009	Zhang et al., 2009; using leaf prints	No
Lettuce, Spinach, and Parsely	*E. coli* O157:H7	–80% ethanol for 10 s followed by immersion in 0.1% $HgCl_2$ for 10 min and washing with water, –1% $AgNO_3$ for 10 s followed by washing steps	Erickson et al., 2010a	Zhang et al., 2009; used for *E. coli* O157:H7 in lettuce (Franz et al., 2007); used for *E. coli* O157:H7 and S. Typhimurium in lettuce	No
Lettuce and Spinach	*E. coli* O157:H7	80% ethanol for 10 s followed by 0.1% $HgCl_2$ for 10 min and washing steps, 1% $AgNO_3$ for 10 s followed by two washing steps	Erickson et al., 2010b	Zhang et al., 2009; used for *E. coli* O157:H7 in lettuce (Franz et al., 2007); used for *E. coli* O157:H7 and *S.* Typhimurium in lettuce	No
Lettuce Green onion	GFP-labeled *S.* Typhimurium	80% ethanol for 10 s, 1% $AgNO_3$ for 5 min, washing with water	Ge et al., 2013	Franz et al., 2007; used for *E. coli* O157:H7 and *S.* Typhimurium in lettuce. Confirmed (data not shown)	No
Lettuce	*S.* Infantis	200 ppm NaClO solution for 1 min followed by washing steps	Zhang et al., 2016	FDA, 1998; Validated by comparing to the method of Zhang et al. (2009)	No
Tomato leaves	*S.* Montevideo	70% EtOH spray and allowed to dry under a flow hood until no visible solution remained	Miles et al., 2009	Not mentioned	no
Tomato plant	*S.* Typhimurium	70% alcohol for 20 s and then 0.6% sodium hypochlorite for 10 s followed by washing	Gu et al., 2011	Gu et al., 2011	Yes
Tomato leaves	*S.* Typhimurium SL1344 GFP-tagged	None	Golberg et al., 2011	Kroupitski et al., 2009	Yes
Tomato leaves	*S.* Typhimurium strain MAE110	70% alcohol for 15 s following by water rinsing	Gu et al., 2013a	Validated by the authors	Yes
Tomato leaves	*S.* Newport	70% ethanol until runoff	Pollard et al., 2014	Not mentioned	No
Tomato leaves	*S.* Typhimurium strain MAE110	70% alcohol for 15 s following by water rinsing	Gu et al., 2018	Not mentioned	Yes
Betel leaf	*S.* Enteritidis *S.* Typhimurium	80% ethanol for 10 s, 1% $AgNO_3$ for 5 min, rinsing with water	Fakruddin et al., 2017	Franz et al., 2007; used for *E. coli* O157:H7 and *S.* Typhimurium in lettuce	No
Cucumber	Five *Salmonella* serovars	70% ethanol bath for 20 min	Burris et al., 2020	Zheng et al., 2013; based on tomato leaf sterilization; validated in the lab; data not presented	No

tomato leaflet, *Arabidopsis* leaf, or lettuce piece were each submerged in a single 50-ml sterile polypropylene tube (Labcon, Petaluma, CA) containing 30-ml saline. The leaves were illuminated for 20 min under a light intensity of 150-μE m^{-2} s^{-1} at room temperature, and then, the saline was removed and replaced with a bacterial suspension containing *ca.* 10^8 *Salmonella*

CFU/ml saline. While this high inoculum does not represent real-life conditions, such high inocula were previously used to study *Salmonella* internalization *in vivo* (Gu et al., 2011, 2013a) and *in vitro* (Kroupitski et al., 2009, 2011, 2019; Golberg et al., 2011). The incubation proceeded for 2 h at 40°C, a temperature that increases stomatal openings in multiple species (Kostaki et al., 2020) to facilitate *Salmonella* internalization. The leaf samples were washed twice by dipping in fresh sterile saline for 1 min each time to remove unattached bacteria. *Salmonella* attachment to the leaf surface and internalization was analyzed by confocal microscopy and viable count, as described below. Each experiment included three leaves (repeats) of the same plant, each in a single tube and the three plants species were processed on the same day. The experiments were repeated twice for all plants on different days.

Determination of *Salmonella* Internalization Using Surface Disinfection

Surface disinfection was performed using one of the three disinfectants, 1% sodium hypochlorite (Bio-Lab Ltd., Jerusalem, Israel), 1% silver nitrate (Bio Basic Ltd. Toronto, Canada), and 70% ethanol (Gadot-Group, Netanya, Israel). Briefly, whole leaves of *Arabidopsis* and tomato or lettuce leaf samples were submerged in 20-ml disinfectant solution with gentle agitation for 7 min. Leaf samples were taken out after 1, 3, 5, and 7 min and washed extensively by dipping the leaves four times (1 min each) in 20-ml sterile double-distilled water (SDDW) to remove the residual disinfectant solution. In order to avoid interference by bacteria that may enter through the cut tissues, an internal leaf disks (2-cm^2 area) were excised from the three leaves (*Arabidopsis*, tomato, and iceberg lettuce) using a sterile cork-borer. The leaf disks were aseptically cut into two identical pieces with a sterile scalpel, one was taken for bacterial extraction and viable count, and the other was taken for confocal microscopy. A high-speed benchtop homogenizer Fast Prep®-24 (MP-Biomedicals, Solon Ohio, United States) was used for the homogenization of the leaf samples in 2-ml micro-tubes (MP-Biomedicals, Solon Ohio, United States) containing glass beads and 500 µl of buffer peptone water (BPW; Becton Dickinson, France, United States). Homogenization conditions were 4,000 rpm for 40 s at room temperature. Homogenate samples (100 µl) and 10× serial dilutions were spread plated into Xylose-Lysine-Desoxycolate (XLD; Becton Dickinson, France, United States) agar supplemented with streptomycin and gentamicin in order to enumerate internalized *Salmonella* cells that presumably survived the disinfection treatment. Inoculated leaves suspended for up to 7 min in SDDW without disinfection and then washed in fresh SDDW served as non-treated control to determine the initial number of leaf-associated bacteria. *Salmonella* counts of control and treated samples were converted to log CFU/cm^2.

Determination of *Salmonella* Internalization Using Confocal Microscopy

Fluorescently-labeled *Salmonella* cells were visualized using a confocal laser-scanning microscope (Olympus IX81; Olympus, Tokyo, Japan) with a 40X objective lens and a numerical aperture of 0.7. *Salmonella* localization of fluorescent bacteria on the leaf surface and in internal leaf tissues was determined in 30 randomly selected microscopic fields per leaf, as described before (Kroupitski et al., 2009). Briefly, quantification of the surface-attached and internalized bacteria was done by calculating the percentage of microscopic fields that harbor ≥1 internal or surface-attached *Salmonella* cells in 30 fields and is presented as the incidence (%) of *Salmonella* on the surface and internal tissues, as described previously (Kroupitski et al., 2009). The mean incidence of *Salmonella* was calculated based on two independent experiments, each containing three technical repeats.

Statistical Methods

All experiments were performed in triplicates (three different leaf samples) and repeated two times on different days. Statistical analysis was performed using the JMP software package version 14 (SAS Institute Inc., Cary, NC, United States). Incidence data were arcsine-transformed before analysis and residual data for logarithm of CFU/area after analysis were examined to determine normality and equality of variances. Two-way ANOVA was used to analyze the effect of disinfectant, time, and their interaction. After significant interaction was discovered, pairs of disinfectant-time means were compared by the Tukey-Kramer test (alpha = 0.05).

RESULTS

Determination of Leaf Internalization Using Surface Sterilization and Viable Count in Various Leaves

Leaf internalization was initially studied in lettuce and *Arabidopsis*, which were previously shown to support a high level of *Salmonella* internalization (Kroupitski et al., 2009; Golberg et al., 2011). Incubation of lettuce leaves with *S. enterica* serovar Typhimurium for 2 h resulted in a surface colonization density of 5.92 ± 0.15 log CFU/cm^2 (**Figure 1A**), representing both surface-attached and leaf-internalized bacteria. Treatment of the inoculated lettuce leaf with 1% NaHClO for 1, 3, 5, and 7 min reduced the number of viable *Salmonella* cells from 5.92 ± 0.15 log CFU/cm^2 to 5.11 ± 0.07, 3.92 ± 0.1, 3.11 ± 0.08, and 2.65 ± 0.11 log CFU/cm^2, respectively (**Figure 1A**).

After 70% ethanol treatment for 1, 3, 5, and 7 min, surviving *Salmonella* counts were reduced from 5.92 ± 0.15 log CFU/cm^2 to 5.18 ± 0.06, 5.17 ± 0.01, 4.97 ± 0.07, and 4.05 ± 0.08 log CFU/cm^2, respectively (**Figure 1B**). Unlike ethanol, surface disinfection with 1% $AgNO_3$ resulted in a higher killing rate. Treatment with 1% $AgNO_3$ for 1, 3, 5, and 7 min reduced the number of viable *Salmonella* cells from 5.92 ± 0.15 log CFU/cm^2 to 3.00 ± 0.01, 2.80 ± 0.04, 2.50 ± 0.10, and 2.20 ± 0.11 log CFU/cm^2, respectively (**Figure 1A**).

Incubation of *Arabidopsis* leaves with *Salmonella* for 2 h resulted in a surface colonization density of 5.91 ± 0.09 log CFU/cm^2 of leaf-associated bacteria (**Figure 1B**). Following surface disinfection with 1% NaHClO for 1, 3, 5, and 7 min,

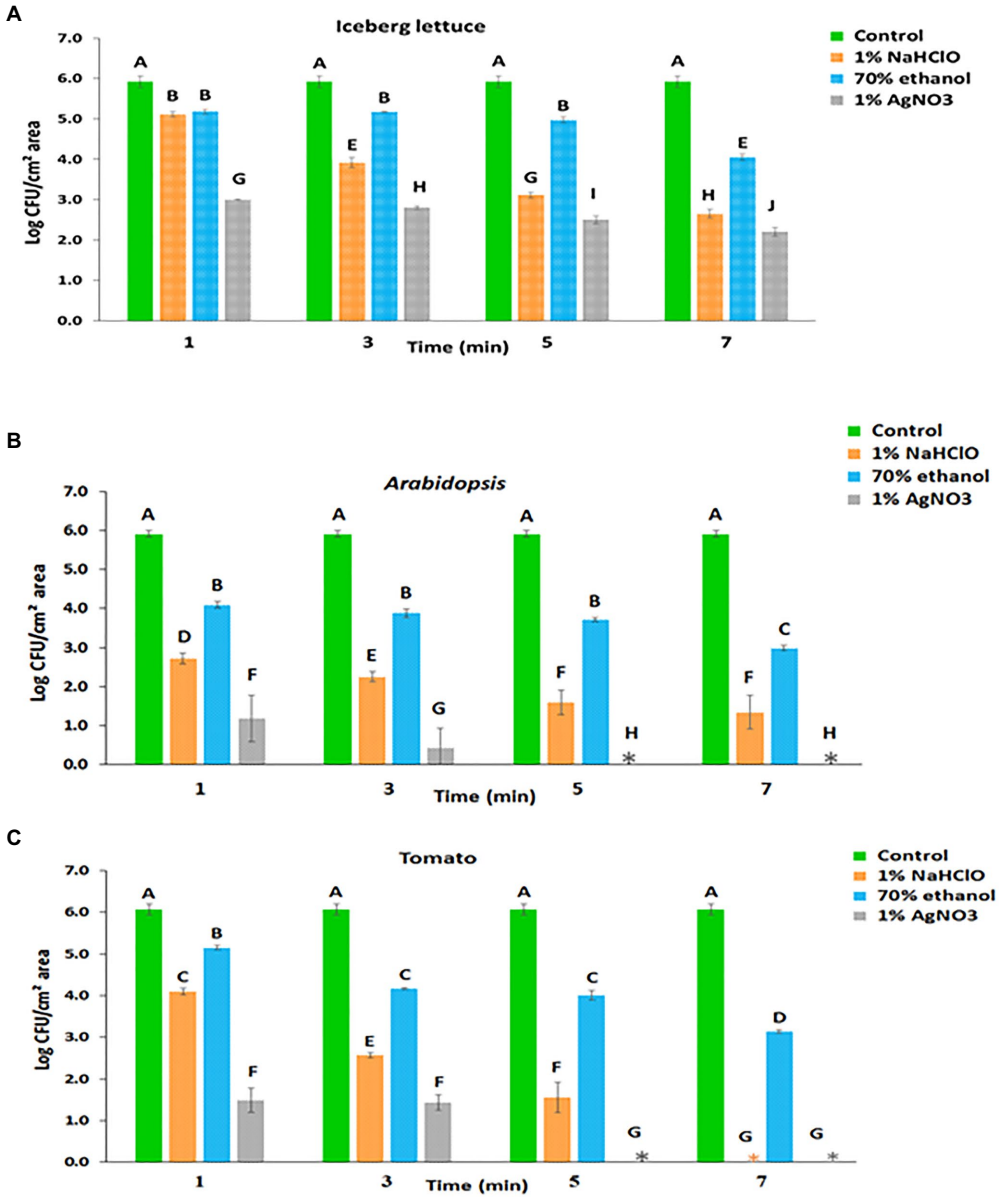


FIGURE 1 | Leaf internalization based on viable count following surface disinfection of **(A)** lettuce, **(B)** *Arabidopsis*, and **(C)** tomato. Mean and standard deviation values of two individual experiments each with three technical repeats are presented. Different letters indicate significant differences ($p < 0.05$) in control and treated leaf samples, for a given plant, according to the Tukey-Kramer multiple comparison test.

the counts were reduced to 2.72 ± 0.14, 2.25 ± 0.13, 1.60 ± 0.32, and 1.34 ± 0.43 log CFU/cm^2, respectively (**Figure 1B**). Surface disinfection with 70% ethanol for 1, 3, 5, and 7 min reduced the numbers of viable *Salmonella* cells to 4.09 ± 0.08, 3.88 ± 0.11, 3.72 ± 0.06, and 2.98 ± 0.07 log CFU/cm^2, respectively (**Figure 1B**). Disinfection with 1% $AgNO_3$ resulted in a higher inactivation of leaf-associated *Salmonella,* and after treatment for 1 and 3 min, the counts were reduced to 1.18 ± 0.60 and 0.43 ± 0.51 log CFU/cm^2, respectively. Longer incubation times resulted in the inactivation of all leaf-associated *Salmonella* cells (**Figure 1B**).

Incubation of tomato leaves with *Salmonella* suspension for 2 h resulted in a surface colonization density of 6.06 ± 0.14 log CFU/cm^2, representing the total number of leaf-associated *Salmonella* (**Figure 1C**). This value corresponds to the sum of surface-attached and internalized bacteria. The number of internalized *Salmonella* was assessed by the viable count technique following leaf disinfection. Treatment with 1% NaHClO for 1, 3, and 5 min duration resulted in the survival of 4.10 ± 0.08, 2.57 ± 0.06, and 1.56 ± 0.37 log CFU/cm^2 leaf area, respectively (**Figure 1C**), which presumably represent internalized bacteria. Treatment duration of 7 min resulted in complete *Salmonella*

eradication. Treatment with 70% ethanol for 1, 3, 5, and 7 min resulted in the survival of 5.15 ± 0.05, 4.17 ± 0.03, 4.01 ± 0.12, and 3.14 ± 0.04 log CFU/cm^2, respectively (**Figure 1C**). Finally, the treatment of inoculated tomato leaves with 1% $AgNO_3$ for 1 and 3 min resulted in 1.48 ± 0.29 and 1.43 ± 0.19 log CFU/cm^2, respectively; while longer incubation times of 5 and 7 min resulted in complete *Salmonella* inactivation (**Figure 1C**).

Determination of *Salmonella* Internalization by Confocal Microscopy

In parallel to the bacteriological studies, *Salmonella* internalization was studied by confocal microscopy using the other part of the same leaf piece used for assessing internalization by the viable count technique. Both non-treated and surface-disinfected leaf samples were utilized in these studies.

Confocal microscopy studies were performed with lettuce, *Arabidopsis*, and tomato leaves (**Figure 2** and **Table 2**). In lettuce leaves, *Salmonella* cells showed comparable distribution between the leaf surface and the leaf interior. The incidence of *Salmonella* cells on the leaf surface was 100%, while the incidence of *Salmonella* cells underneath the leaf surface was 92 ± 1% (**Table 2**). Treatment of the leaves with each of the three disinfectants for 1 or 7 min resulted in most cases in a substantial reduction in the incidence of fluorescent cells, both on the leaf surface and within the leaf interior. Ethanol treatment for 1 min reduced the incidence of fluorescent cells on the leaf surface to 58 ± 3%, yet it did not affect the incidence of endophytic *Salmonella*. The two other disinfectants reduced the incidence of fluorescent *Salmonella* both on the leaf surface as well as in the leaf interior during longer exposure times.

In the case of *Arabidopsis,* confocal microscopy of leaves incubated with *Salmonella* showed an incidence of *Salmonella* of 100% on the leaf surface and 42 ± 6% underneath the surface (**Figure 2** and **Table 2**). Surface disinfection with 1% NaHClO, 70% ethanol, and 1% $AgNO_3$ for 1 min resulted in a decrease in the incidence of surfaced-attached *Salmonella* from 100% to 41 ± 3.6, 55 ± 3.5 and 57 ± 7%, respectively, and a further reduction of surface-attached bacteria occurred after a longer exposure time (**Table 2**). However, fluorescent *Salmonella* cells were still observed on the leaf surface. Treatment of the leaves with 70% ethanol for 7 min reduced the incidence of endophytic *Salmonella* from 42 ± 6% to 31 ± 5.7%, while treatment with 1% NaHClO for 7 min and 1% $AgNO_3$ for 5 min resulted in complete loss of fluorescence, inferring *Salmonella* inactivation.

In contrast to the findings with lettuce and *Arabidopsis* leaves, imaging of tomato leaves following incubation with fluorescent *Salmonella* revealed no endophytic colonization. All leaf-associated *Salmonella* cells were confined to the leaf surface (**Table 2** and **Figure 2**). Disinfection with 1% NaHClO for 1 min resulted in reducing the incidence of surface-associated *Salmonella* from 100 to 35 ± 1.5%, while 7 min exposure resulted in the loss of fluorescence, inferring a complete inactivation of the pathogen (**Table 2**). Exposure of the leaf to 1% $AgNO_3$ for 1 and 5 min resulted in similar effects, while ethanol (70%) had a milder effect. It reduced the incidence of fluorescent *Salmonella* to 55 ± 1% after 1 min and to 11 ± 2% after 7 min.

DISCUSSION

Human pathogens can colonize plants and persist on and sometimes within various plant's tissues, and upon consumption may cause foodborne diseases (Barak and Schroeder, 2012; Deering et al., 2012; Erickson, 2012; Brandl and Sundin, 2013; Holden et al., 2015; Fornefeld et al., 2017; Jacob and Melotto, 2019; Roy and Melotto, 2019; Schierstaedt et al., 2019). Accordingly, accurate determination of the localization of human pathogen on or within leaves is vital for basic science as well as for developing new strategies for preventing and intervening to address the problem of fresh produce contamination.

The determination of bacterial internalization in a plant is a function of, among others, the method used to assess bacterial localization (Deering et al., 2012; Erickson, 2012). Ultimately, surface sterilization should completely inactivate external bacteria while leaving internalized bacteria intact. Still, only a few studies have systematically validated the efficacy of surface sterilization to kill surface-attached bacteria. In one such study, 13 disinfection conditions/methods were compared for their effectiveness in killing GFP-tagged *E. coli* O157:H7 on lettuce leaf surfaces using leaf imprints on agar media. Dipping in 80% ethanol for 10 s followed by immersion in 0.1% $HgCl_2$ for 10 min was reported to be the most effective disinfection method for inactivating both *E. coli* and *Salmonella* strains (Zhang et al., 2009). However, no confocal microscopy study corroborated the results. Many studies have adopted previously reported protocols to inactivate surface-attached bacteria, even when utilizing different plants and/or bacterial strains (see **Table 1**). Bacteria may vary in their intrinsic tolerance to disinfectants (Morente et al., 2013) and may preferentially reside at unique leaf-specific microsites (Beattie and Lindow, 1995; Erickson, 2012), which may facilitate the protection of the colonized bacteria against disinfection (Andrews and Harris, 2000; Erickson et al., 2010a; Erickson, 2012). Consequently, a disinfection protocol developed for inactivating a specific *Salmonella* strain on the leaves of a particular plant cultivar may not fit all. Evidently, when a partial inactivation is achieved, some surface-residing bacteria may be misclassified as internal bacteria, while truly internalized bacteria killed due to permeation of the disinfectant into the intact leaf tissue may be mistakenly regarded as surface-attached bacteria.

The present study provides data from a systematic comparison of leaf internalization through stomata by a GFP-tagged *Salmonella* Typhimurium strain in the leaves of the three plant species using surface sterilization and plate-count technique. The study did not compare leaf internalization among plants but rather the effect of the various disinfection protocols on leaf internalization in each plant species. We used three disinfectants (1% NaHClO, 1% $AgNO_3$, and 70% ethanol), commonly applied, alone or in combination with others, for sterilizing plant surfaces (Franz et al., 2007; Erickson et al., 2010b; Gu et al., 2011, 2013a; Erickson, 2012; Ge et al., 2013; Fakruddin et al., 2017). To simplify the comparison between the protocols, we used a single concentration of the disinfectants, each time, and compared the effect of the sterilization time (1 to 7 min) on quantifying viable bacteria, apparently

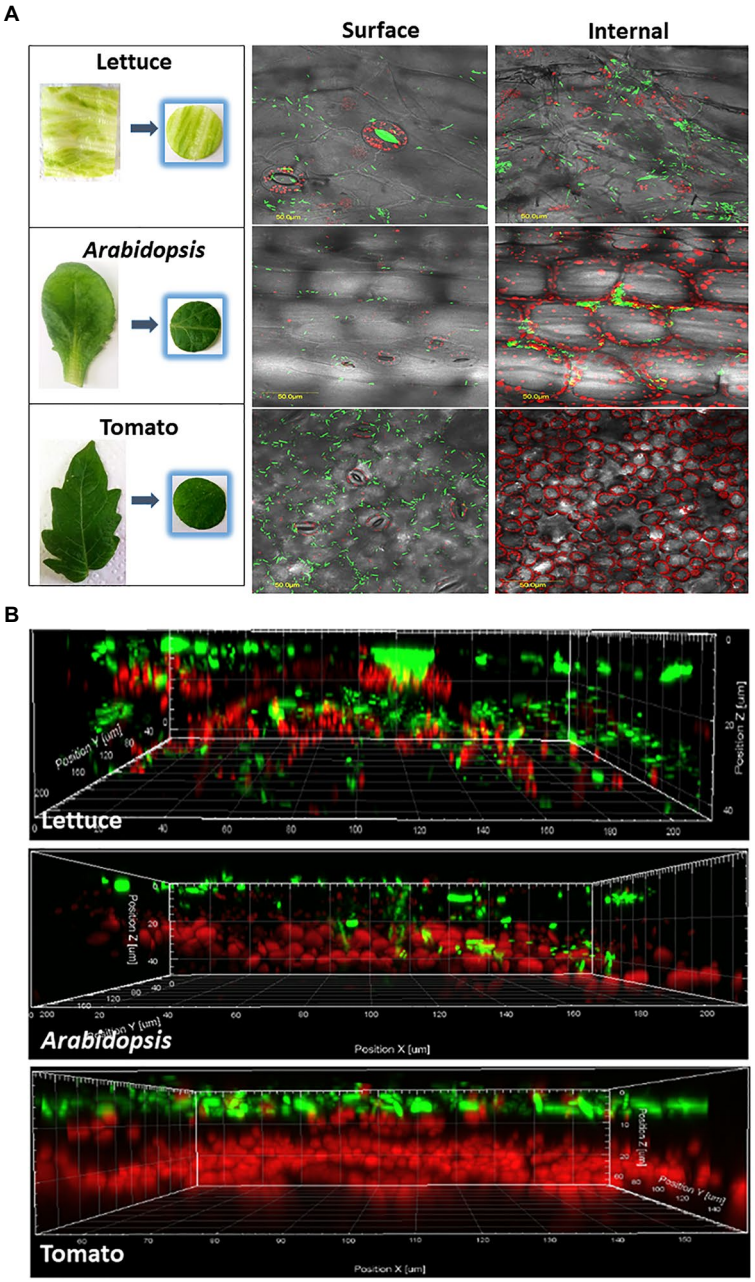


FIGURE 2 | Confocal microscopy visualization illustrating epiphytic and endophytic localization of GFP-labeled *Salmonella* cells in representative leaves of iceberg lettuce, *Arabidopsis*, and tomato. Panel **A** shows images taken from the surface of the leaves, and a stack of fluorescent images along a *z*-section taken every 1.2 μm to a depth of 100 μm below the surface. All images were overlaid with differential interference contrast (DIC) images taken from the same location in each leaf. Bar denotes 50 μm. Panel **B** shows a z-section model of the same leaves, demonstrating the location of bacteria (green) on and within the leaf tissues. Red fluorescence indicated autofluorescence of the chloroplasts.

representing internalized *Salmonella* cells. In parallel to the viable counts measurements, we utilized confocal microscopy to accurately assess bacterial localization on or within the leaf tissue.

We initially examined leaf internalization in iceberg lettuce leaves previously shown by confocal microscopy to support *Salmonella* internalization (Kroupitski et al., 2009; Golberg et al., 2011). Indeed, confocal microscopy confirmed a high incidence of internalization (92%) in non-disinfected leaves; however, surface disinfection with all three agents resulted in reducing fluorescence, suggesting that the disinfectants seemingly penetrated the leaf tissues to some degree and injured the cells (**Table 2**). A substantial decrease in the number of apparent internalized bacteria was observed using

TABLE 2 | Incidence of fluorescent *Salmonella* cells in various leaf localization using confocal microscopy.

		Incidence of *Salmonella* in leaf localization (%)			
		Surface		Internal	
	Treatment/ Time (min)	1	7	1	7
Iceberg lettuce	Control (water)	100^{A}	100^{A}	92 ± 1^{a}	92 ± 1^{a}
	1% NaHClO	50 ± 1.5^{D}	16 ± 2.5^{E}	63 ± 5^{c}	41 ± 2.5^{d}
	70% ethanol	58 ± 3^{B}	16 ± 4^{E}	91 ± 1.5^{a}	77 ± 2.6^{b}
	1% $AgNO_3$	59 ± 4^{B}	54 ± 1^{C}	67 ± 5^{bc}	66 ± 5^{bc}
Arabidopsis	Control (water)	100^{A}	100^{A}	42 ± 6^{a}	42 ± 6^{a}
	1% NaHClO	41 ± 3.6^{C}	12 ± 3.2^{D}	16 ± 3.5^{c}	0^{d}
	70% ethanol	55 ± 3.5^{BC}	14 ± 2.6^{D}	42 ± 2.6^{a}	31 ± 5.7^{b}
	1% $AgNO_3$	57 ± 7^{B}	15 ± 4.7^{D}	32 ± 5^{b}	0^{d}
Tomato	Control (water)	100^{A}	100^{A}	0^{a}	0^{a}
	1% NaHClO	35 ± 1.5^{C}	0^{E}	0^{a}	0^{a}
	70% ethanol	55 ± 1^{B}	11 ± 2^{D}	0^{a}	0^{a}
	1% $AgNO_3$	38 ± 7.6^{BC}	0^{E}	0^{a}	0^{a}

[a]*For each plant, means without a common uppercase letter or without a common lowercase letter differ significantly by the Tukey-Kramer multiple comparison test ($p < 0.05$) with regard to the incidence of Salmonella of surface-attached and internal Salmonella, respectively.*

the plate-count method following 1 to 7 min treatment (**Figure 1**). The determination of leaf internalization by surface disinfection and viable count showed wide variations in the number of apparently internalized bacteria in leaves of each plant species, depending upon the type of the disinfectant and the treatment duration. These differences are likely attributed to the increased killing of leaf-associated bacteria with time or to the transition of a portion of the *Salmonella* population to the viable but non-culturable (VBNC) state (Zhao et al., 2017). The observation of fluorescent cells on the leaf surface does not provide a clear indication regarding the presence of disinfection-tolerant bacteria, since the *Salmonella* strain carried a stable GFP (Kroupitski et al., 2009), which may continue to emit fluorescence in VBNC bacteria, as well as in dead cells with intact GFP. Further studies using methods that can discriminate between live and dead bacteria are needed to determine the physiological status of the treated fluorescent bacteria on the leaves' surface. Still, the possible entry of *Salmonella* into a VBNC state in the plant environment (Winfield and Groisman, 2003) may lead to underestimation of both attachment and internalization when using the viable count assay alone.

Based on the confocal microscopy studies, *Salmonella* displays a lower incidence of leaf internalization in *Arabidopsis* than in lettuce (**Table 2**). Likewise, the viable count method demonstrated lower numbers of viable bacteria during all treatment times (**Figure 1B,C**). All three agents displayed comparable surface disinfection effectiveness; however, they varied significantly in the apparent internalization (**Table 2**). A 7-min treatment with 1% NaHClO or 1% $AgNO_3$ resulted in the complete loss of fluorescent cells inside the leaf, suggesting that they efficiently penetrated the leaves and injured the internalized bacteria.

In a previous report, we were not able to show internalization of the same *Salmonella* strain in tomato leaves (Golberg et al., 2011). Consequently, the assessment of tomato leaf internalization, side by side, by the two methodologies provided a unique opportunity to assess the suitability of the tested disinfection conditions inactivate bacteria in the leaf surface. Evaluation of *Salmonella* internalization by confocal microscopy, with no surface sterilization, confirmed our inability to demonstrate the internalization of *Salmonella* in these tomato leaves with the techniques used. Usage of 1% NaHClO for 1 to 7 min resulted in different numbers of apparent internalized bacteria, ranging from 4 logs CFU/cm^2 to 0, respectively. Parallel confocal microscopy analysis of the treated leaf samples confirmed the lack of detection of leaf internalization, suggesting that only 7-min treatment resulted in sufficient killing of external bacteria in this model system. The use of 70% ethanol as a sole disinfectant for up to 7 min failed to inactivate all external bacteria, as determined by viable counts, thus mistakenly suggesting the internalization of about 3 log CFU/cm^2. Treatment with 1% $AgNO_3$ resulted in substantial inactivation of surface-attached bacteria in 1 and 3 min treatment, while treatment duration of 5 and 7 min was sufficient to kill all external bacteria, hence providing results comparable to those obtained by confocal microscopy. These findings indicate that non-validated surface sterilization conditions may lead to misinterpretation of the actual number of internalized bacterial cells. Notably, the apparent lack of leaf internalization of the tested *S. typhimurium* strain (SL 1344) in the tomato cultivar used in this study (*Solanum lycopersicon* cv. M82), as well as in *S. lycopersicon* cv. MP1, tested previously (Golberg et al., 2011), calls for further research. It is particularly interesting to examine whether the two cultivars are naturally resistant to leaf internalization of other *Salmonella* serovars and strains under more natural tomato growing conditions. Elucidation of the mechanisms involved in the inhibition of leaf internalization might prove important for understanding human pathogen-plant interactions and developing new mitigation strategies for *Salmonella* internalization.

Surface disinfection by treatment with 1% $AgNO_3$ was less effective in lettuce compared to tomato leaves. These differences are likely correlate to specific leaf features, such as surface morphology and/or physico-chemical properties known to impact leaf colonization (Beattie and Lindow, 1995; Andrews and Harris, 2000; Beuchat, 2002; Yadav et al., 2005; Heaton and Jones, 2008; Leveau, 2009; Cevallos-Cevallos et al., 2012). Previous studies have already noted that the attachment of bacteria to specific microenvironments on the leaf, such as cavities and crevices on the leaf surface, may favor the persistence

of surface-attached bacteria following disinfection (Gomes et al., 2009; Deering et al., 2012; Erickson, 2012).

Altogether, this is the first time a systematic study reported a comparison of three surface sterilization protocols in leaves of three plants, side by side, with a confocal microscopy study. While the selection of an optimal disinfection protocol for each of the three plants was beyond the scope of this study, we have demonstrated the dependency of the apparent bacterial internalization on the disinfection conditions and shown the impact of the quantification method on the extent of leaf internalization.

It should be noted that entry of bacterial pathogens into the leaf tissue might occur through stomata, hydathodes, and injured tissues or by transport through the roots and stem (Erickson, 2012; Gu et al., 2013a; Melotto et al., 2017). In the present study, we utilized specific *in vitro* inoculation and experimental conditions to compare the effect of three surface disinfection protocols on *Salmonella* internalization through stomata. The study was not designed to investigate other factors that might affect bacterial internalization nor the different mode of leaf internalization. Therefore, we suggest interpretation of our results with caution, especially when comparing to other studies that used different inoculation models and surface disinfection protocols.

Whole leaves or leaflets were used for inoculation in the case of *Arabidopsis* and tomato, respectively; however, in the case of lettuce, square leaf pieces were used, which potentially may enable direct access of bacteria into the apoplast through the injured tissue. However, previous confocal microscopy observations showed a limited penetration of *Salmonella* through the cut tissues (data not shown), which did not affect the internal leaf tissue used for bacterial enumeration.

While the use of confocal microscopy to determine bacterial localization is critical for confirming leaf internalization, this technique is limited to high concentrations of fluorescent cells, which may not represent natural contamination scenarios. Furthermore, unlike the bacteriological technique, quantification of internalization by confocal microscopy relies on a limited number of microscopic fields, which might bias the results. On the other hand, the viable count technique, but not confocal microscopy, may be prone to changes in the physiological status of the leaf-associated bacteria, such as transition into the VBNC state.

CONCLUSION

In conclusion, the data of the internalization model presented here emphasize the need for a careful examination and calibration of the surface sterilization protocol, including testing of different disinfectant's concentrations as well as combinations of disinfectant, particularly when a new plant system and bacterial strain are studied, where the sterilization conditions may need to be adjusted prior to further experimentation. Our findings may also be relevant to studies aimed at the isolation and characterization of endophytic microorganisms, which utilize an initial surface sterilization step to inactivate external plant microorganisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MC conducted the experiments, performed the data analysis, and drafted the manuscript. YK and RG assisted with the experiments and contributed to data analysis. EB performed the confocal microscopy studies. MM contributed to the discussion and reviewed the manuscript. SS-S conceived the study and wrote the manuscript. All authors read and approved the manuscript.

FUNDING

The study was partially supported by the NIFA-BARD Collaborative Research Program awarded to MM and SS-S. The research of SS-S was supported by the United States – Israel Binational Agricultural Research and Development Fund, NB-8316-16, and the research of MM was supported by the National Institute of Food and Agriculture – USDA Fund no. 2017–67017-26180.

ACKNOWLEDGMENTS

We are thankful to Gilor Kelly and Arye Harel (Institute of Plant Sciences, Vegetables and Field Crops Unit, Volcani Center, ARO) for providing the *Arabidopsis* and tomato plants used in this study. SS-S is a member of the EU COST Action 16110: Control of Human Pathogenic Microorganisms in Plant Production Systems (http://huplantcontrol.igzev.de/) and acknowledges the action. MC was supported by the ARO Postdoctoral Fellowship Fund under the Indo-Israeli Research and Development Program.

REFERENCES

Andrews, J. H., and Harris, R. F. (2000). The ecology and biogeography of microorganisms of plant surfaces. *Annu. Rev. Phytopathol.* 38, 145–180. doi: 10.1146/annurev.phyto.38.1.145

Barak, J. D., and Schroeder, B. K. (2012). Interrelationships of food safety and plant pathology: the life cycle of human pathogens on plants. *Annu. Rev. Phytopathol.* 50, 241–266. doi: 10.1146/annurev-phyto-081211-172936

Beattie, G. A., and Lindow, S. E. (1995). The secret life of foliar bacterial pathogens on leaves. *Annu. Rev. Phytopathol.* 33, 145–172. doi: 10.1146/annurev.py.33.090195.001045

Bernstein, N., Sela, S., and Neder-Lavon, S. (2007). Assessment of contamination potential of lettuce by *Salmonella enterica* serovar Newport added to the plant growing medium. *J. Food Protect.* 70, 1717–1722. doi: 10.4315/0362-028X-70.7.1717

Beuchat, L. R. (2002). Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect.* 4, 413–423. doi: 10.1016/S1286-4579(02)01555-1

Brandl, M. T., and Sundin, G. W. (2013). Focus on food safety: human pathogens on plants. *Phytopathology* 103, 304–305. doi: 10.1094/PHYTO-103-4-0304

Buchanan, R. L., Edelson, S. G., Miller, R. L., and Sapers, G. M. (1999). Contamination of intact apples after immersion in an aqueous environment containing *Escherichia coli* O157: H7. *J. Food Protect.* 62, 444–450. doi: 10.4315/0362-028X-62.5.444

Burris, K. P., Simmons, O. D., Webb, H. M., Deese, L. M., Moore, R. G., Jaykus, L.-A., et al. (2020). Colonization and internalization of *Salmonella enterica* and its prevalence in cucumber plants. *Front. Microbiol.* 11:1135. doi: 10.3389/fmicb.2020.01135

Callejón, R. M., Rodriguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C., and 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. doi: 10.1089/fpd.2014.1821

Carstens, C., Salazar, J. K., and Darkoh, C. (2019). Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010-2017. *Front. Microbiol.* 10:2667. doi: 10.3389/fmicb.2019.02667

Cevallos-Cevallos, J. M., Gu, G. Y., Danyluk, M. D., and van Bruggen, A. H. C. (2012). Adhesion and splash dispersal of *Salmonella enterica* Typhimurium on tomato leaflets: effects of rdar morphotype and trichome density. *Int. J. Food Microbiol.* 160, 58–64. doi: 10.1016/j.ijfoodmicro.2012.09.021

Deering, A. J., Mauer, L. J., and Pruitt, R. E. (2012). Internalization of *E. coli* O157: H7 and *Salmonella* spp. in plants: a review. *Food Res. Int.* 45, 567–575. doi: 10.1016/j.foodres.2011.06.058

Duffy, E., Cisneros-Zevallos, L., Castillo, A., Pillai, S., Ricke, S., and Acuff, G. (2005). Survival of salmonella transformed to express green fluorescent protein on Italian parsley as affected by processing and storage. *J. Food Protect.* 68, 687–695. doi: 10.4315/0362-028X-68.4.687

Erickson, M. C. (2012). Internalization of fresh produce by foodborne pathogens. *Annu. Rev. Food Sci. Technol.* 3, 283–310. doi: 10.1146/annurev-food-022811-101211

Erickson, M. C., Liao, J. Y., Payton, A. S., Cook, P. W., and Ortega, Y. R. (2019). Survival and internalization of *Salmonella* and *Escherichia coli* O157: H7 sprayed onto different cabbage cultivars during cultivation in growth chambers. *J. Sci. Food Agric.* 99, 3530–3537. doi: 10.1002/jsfa.9573

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, L., et al. (2010a). Infrequent internalization of *Escherichia coli* O157: H7 into field-grown leafy greens. *J. Food Protect.* 73, 500–506. doi: 10.4315/0362-028x-73.3.500

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, L., et al. (2010b). Surface and internalized *Escherichia coli* O157: H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. *J. Food Protect.* 73, 1023–1029. doi: 10.4315/0362-028x-73.6.1023

Fakruddin, M., Sultana, R., Hossain, M. N., Rahaman, M. M., Islam, M. K., and Ahmed, M. M. (2017). Occurrence of ingression of salmonella spp. In betel leaf (Piper betle L.). *Int. J. Food Contam.* 4:6. doi: 10.1186/s40550-017-0051-0

FDA (1998). *In: Guidance for Industry-Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*. Washington, DC: U.S. Department of Health and Human Services, Center for Food Safety and Applied Nutrition.

Fletcher, J., Leach, J. E., Eversole, K., and Tauxe, R. (2013). Human pathogens on plants: designing a multidisciplinary strategy for research. *Phytopathology* 103, 306–315. doi: 10.1094/PHYTO-09-12-0236-IA

Fornefeld, E., Schierstaedt, J., Jechalke, S., Grosch, R., Smalla, K., and Schikora, A. (2017). Interaction between *Salmonella* and plants: potential hosts and vectors for human infection. *Curr. Topics Salmonella Salmonellosis*. 10, 171–191. doi: 10.5772/67061

Franz, E., Visser, A. A., Van Diepeningen, A. D., Klerks, M. M., Termorshuizen, A. J., and van Bruggen, A. H. (2007). Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium. *Food Microbiol.* 24, 106–112. doi: 10.1016/j.fm.2006.03.002

Friesema, I., Sigmundsdottir, G., Van Der Zwaluw, K., Heuvelink, A., Schimmer, B., De Jager, C., et al. (2008). An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September–October 2007. *Eur. Secur.* 13:19065. doi: 10.2807/ese.13.50.19065-en

Gajraj, R., Pooransingh, S., Hawker, J. I., and Olowokure, B. (2012). Multiple outbreaks of *Salmonella* Braenderup associated with consumption of iceberg lettuce. *Int. J. Environ. Health Res.* 22, 150–155. doi: 10.1080/09603123.2011.613114

Ge, C., Bohrerova, Z., and Lee, J. (2013). Inactivation of internalized *Salmonella* Typhimurium in lettuce and green onion using ultraviolet C irradiation and chemical sanitizers. *J. Appl. Microbiol.* 114, 1415–1424. doi: 10.1111/jam.12154

Golberg, D., Kroupitski, Y., Belausov, E., Pinto, R., and Sela, S. (2011). *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. *Int. J. Food Microbiol.* 145, 250–257. doi: 10.1016/j.ijfoodmicro.2010.12.031

Gomes, C., Da Silva, P., Moreira, R. G., Castell-Perez, E., Ellis, E. A., and Pendleton, M. (2009). Understanding *E. coli* internalization in lettuce leaves for optimization of irradiation treatment. *Int. J. Food Microbiol.* 135, 238–247. doi: 10.1016/j.ijfoodmicro.2009.08.026

Gorbatsevich, E., Sela, S., Pinto, R., and Bernstein, N. (2013). Root internalization, transport and in-planta survival of *Salmonella enterica* serovar N ewport in sweet basil. *Environ. Microbiol. Rep.* 5, 151–159. doi: 10.1111/1758-2229.12008

Gu, G. Y., Cevallos-Cevallos, J. M., Lad, G. E. V., and van Bruggen, A. H. C. (2013b). Organically managed soils reduce internal colonization of tomato plants by *Salmonella enterica* Serovar Typhimurium. *Phytopathology* 103, 381–388. doi: 10.1094/phyto-04-12-0072-fi

Gu, G., Cevallos-Cevallos, J. M., and van Bruggen, A. H. (2013a). Ingress of *Salmonella enterica* Typhimurium into tomato leaves through hydathodes. *PLoS One* 8:e53470. doi: 10.1371/journal.pone.0053470

Gu, G., Hu, J., Cevallos-Cevallos, J. M., Richardson, S. M., Bartz, J. A., and Van Bruggen, A. H. (2011). Internal colonization of *Salmonella enterica* serovar Typhimurium in tomato plants. *PLoS One* 6:e27340. doi: 10.1371/journal.pone.0027340

Gu, G. Y., Strawn, L. K., Oryang, D. O., Zheng, J., Reed, E. A., Ottesen, A. R., et al. (2018). Agricultural practices influence *Salmonella* contamination and survival in pre-harvest tomato production. *Front. Microbiol.* 9:2451. doi: 10.3389/fmicb.2018.02451

Hadjok, C., Mittal, G., and Warriner, K. (2008). Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *J. Appl. Microbiol.* 104, 1014–1024. doi: 10.1111/j.1365-2672.2007.03624.x

Hanning, I. B., Nutt, J., and Ricke, S. C. (2009). Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathog. Dis.* 6, 635–648. doi: 10.1089/fpd.2008.0232

Heaton, J. C., and Jones, K. (2008). Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *J. Appl. Microbiol.* 104, 613–626. doi: 10.1111/j.1365-2672.2007.03587.x

Holden, N. J., Jackson, R. W., and Schikora, A. (2015). Editorial on plants as alternative hosts for human and animal pathogens. *Front. Microbiol.* 6:397. doi: 10.3389/fmicb.2015.00397

Hora, R., Warriner, K., Shelp, B. J., and Griffiths, M. W. (2005). Internalization of *Escherichia coli* O157: H7 following biological and mechanical disruption of growing spinach plants. *J. Food Protect.* 68, 2506–2509. doi: 10.4315/0362-028X-68.12.2506

Jacob, C., and Melotto, M. (2019). Human pathogen colonization of lettuce dependent upon plant genotype and defense response activation. *Front. Plant Sci.* 10:1769. doi: 10.3389/fpls.2019.01769

Kintz, E., Byrne, L., Jenkins, C., Mccarthy, N., Vivancos, R., and Hunter, P. (2019). Outbreaks of Shiga toxin-producing *Escherichia coli* linked to sprouted seeds, salad, and leafy greens: A systematic review. *J. Food Protect.* 82, 1950–1958. doi: 10.4315/0362-028X.JFP-19-014

Klerks, M. M., Franz, E., van Gent-Pelzer, M., Zijlstra, C., and Van Bruggen, A. H. (2007). Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *ISME J.* 1, 620–631. doi: 10.1038/ismej.2007.82

Kostaki, K. I., Coupel-Ledru, A., Bonnell, V. C., Gustavsson, M., Sun, P., McLaughlin, F. J., et al. (2020). Guard cells integrate light and temperature signals to control stomatal aperture. *Plant Physiol.* 182, 1404–1419. doi: 10.1104/pp.19.01528

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., et al. (2009). Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75, 6076–6086. doi: 10.1128/AEM.01084-09

Kroupitski, Y., Gollop, R., Belausov, E., Pinto, R., and Sela, S. (2019). *Salmonella enterica* growth conditions influence lettuce leaf internalization. *Front. Microbiol.* 10:639. doi: 10.3389/fmicb.2019.00639

Kroupitski, Y., Pinto, R., Belausov, E., and Sela, S. (2011). Distribution of *Salmonella* Typhimurium in romaine lettuce leaves. *Food Microbiol.* 28, 990–997. doi: 10.1016/j.fm.2011.01.007

Leveau, J. (2009). Life on leaves. *Nature* 461, 741–742. doi: 10.1038/461741a

Lim, J.-A., Lee, D. H., and Heu, S. (2014). The interaction of human enteric pathogens with plants. *Plant Pathol. J.* 30:109. doi: 10.5423/PPJ.RW.04.2014.0036

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., et al. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* 50, 882–889. doi: 10.1086/650733

Melotto, M., Zhang, L., Oblessuc, P. R., and He, S. Y. (2017). Stomatal Defense a Decade Later. *Plant Physiol.* 174, 561–571. doi: 10.1104/pp.16.01853

Miles, J. M., Sumner, S. S., Boyer, R. R., Williams, R. C., Latimer, J. G., and McKinney, J. M. (2009). Internalization of *Salmonella enterica* Serovar Montevideo into greenhouse tomato plants through contaminated irrigation water or seed stock. *J. Food Protect.* 72, 849–852. doi: 10.4315/0362-028X-72.4.849

Mogren, L., Windstam, S., Boqvist, S., Vågsholm, I., Söderqvist, K., Rosberg, A. K., et al. (2018). The hurdle approach–A holistic concept for controlling food safety risks associated with pathogenic bacterial contamination of leafy green vegetables A review. *Front. Microbiol.* 9:1965. doi: 10.3389/fmicb.2018.01965

Morente, E. O., Fernandez-Fuentes, M. A., Burgos, M. J. G., Abriouel, H., Pulido, R. P., Galvez, A., et al. (2013). Biocide tolerance in bacteria. *Int. J. Food Microbiol.* 162, 13–25. doi: 10.1016/j.ijfoodmicro.2012.12.028

Mritunjay, S. K., and Kumar, V. (2015). Fresh farm produce as a source of pathogens: a review. *Res. J. Environ. Toxicol.* 9, 59–70. doi: 10.3923/rjet.2015.59.70

Newman, K., Leon, J., Rebolledo, P., and Scallan, E. (2015). The impact of socioeconomic status on foodborne illness in high-income countries: a systematic review. *Epidemiol. Infec.* 143, 2473–2485. doi: 10.1017/S0950268814003847

Pollard, S., Barak, J., Boyer, R., Reiter, M., Gu, G. Y., and Rideout, S. (2014). Potential interactions between *Salmonella enterica* and Ralstonia solanacearum in tomato plants. *J. Food Protect.* 77, 320–324. doi: 10.4315/0362-028X.JFP-13-209

Riggio, G. M., Jones, S. L., and Gibson, K. E. (2019). Risk of human pathogen internalization in leafy vegetables during lab-scale hydroponic cultivation. *Horticulturae* 5:25. doi: 10.3390/horticulturae5010025

Roy, D., and Melotto, M. (2019). Stomatal response and human pathogen persistence in leafy greens under preharvest and postharvest environmental conditions. *Postharvest Biol. Technol.* 148, 76–82. doi: 10.1016/j.postharvbio.2018.10.013

Schierstaedt, J., Grosch, R., and Schikora, A. (2019). Agricultural production systems can serve as reservoir for human pathogens. *FEMS Microbiol. Lett.* 366:fnaa016. doi: 10.1093/femsle/fnaa016

Schikora, A., Garcia, A. V., and Hirt, H. (2012). Plants as alternative hosts for salmonella. *Trends Plant Sci.* 17, 245–249. doi: 10.1016/j.tplants.2012.03.007

Seo, K., and Frank, J. (1999). Attachment of *Escherichia coli* O157: H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Protect.* 62, 3–9. doi: 10.4315/0362-028X-62.1.3

Sharma, M., Ingram, D. T., Patel, J. R., Millner, P. D., Wang, X., Hull, A. E., et al. (2009). A novel approach to investigate the uptake and internalization of *Escherichia coli* O157: H7 in spinach cultivated in soil and hydroponic medium. *J. Food Protect.* 72, 1513–1520. doi: 10.4315/0362-028X-72.7.1513

Söderström, A., Österberg, P., Lindqvist, A., Jönsson, B., Lindberg, A., Blide Ulander, S., et al. (2008). A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog. Dis.* 5, 339–349. doi: 10.1089/fpd.2007.0065

Solomon, E. B., Yaron, S., and Matthews, K. R. (2002). Transmission of *Escherichia coli* O157: H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68, 397–400. doi: 10.1128/AEM.68.1.397-400.2002

Takeuchi, K., and Frank, J. F. (2001). Quantitative determination of the role of lettuce leaf structures in protecting *Escherichia coli* O157: H7 from chlorine disinfection. *J. Food Protect.* 64, 147–151. doi: 10.4315/0362-028X-64.2.147

Takkinen, J., Nakari, U.-M., Johansson, T., Niskanen, T., Siitonen, A., and Kuusi, M. (2005). A nationwide outbreak of multiresistant *Salmonella* Typhimurium in Finland due to contaminated lettuce from Spain, may 2005. *EuroSurveillance* 10:E050630. doi: 10.2807/esw.10.26.02734-en

Underwood, W., Melotto, M., and He, S. Y. (2007). Role of plant stomata in bacterial invasion. *Cell. Microbiol.* 9, 1621–1629. doi: 10.1111/j.1462-5822.2007.00938.x

Wendel, A. M., Johnson, D. H., Sharapov, U., Grant, J., Archer, J. R., Monson, T., et al. (2009). Multistate outbreak of *Escherichia coli* O157: H7 infection associated with consumption of packaged spinach, august–September 2006: the Wisconsin investigation. *Clin. Infect. Dis.* 48, 1079–1086. doi: 10.1086/597399

Winfield, M. D., and Groisman, E. A. (2003). Role of nonhost environments in the lifestyles of salmonella and *Escherichia coli*. *Appl. Environ. Microbiol.* 69, 3687–3694. doi: 10.1128/AEM.69.7.3687-3694.2003

Yadav, R., Karamanoli, K., and Vokou, D. (2005). Bacterial colonization of the phyllosphere of Mediterranean perennial species as influenced by leaf structural and chemical features. *Microb. Ecol.* 50, 185–196. doi: 10.1007/s00248-004-0171-y

Zhang, G., Ma, L., Beuchat, L. R., Erickson, M. C., Phelan, V. H., and Doyle, M. P. (2009). Evaluation of treatments for elimination of foodborne pathogens on the surface of leaves and roots of lettuce (Lactuca sativa L.). *J. Food Protect.* 72, 228–234. doi: 10.4315/0362-028X-72.2.228

Zhang, Y., Sallach, J. B., Hodges, L., Snow, D. D., Bartelt-Hunt, S. L., Eskridge, K. M., et al. (2016). Effects of soil texture and drought stress on the uptake of antibiotics and the internalization of *Salmonella* in lettuce following wastewater irrigation. *Environ. Pollut.* 208, 523–531. doi: 10.1016/j.envpol.2015.10.025

Zhao, X., Zhong, J., Wei, C., Lin, C.-W., and Ding, T. (2017). Current perspectives on viable but non-culturable sState in foodborne pathogens. *Front. Microbiol.* 8:580. doi: 10.3389/fmicb.2017.00580

Zheng, J., Allard, S., Reynolds, S., Millner, P., Arce, G., Blodgett, R. J., et al. (2013). Colonization and internalization of *Salmonella enterica* in tomato plants. *Appl. Environ. Microbiol.* 79, 2494–2502. doi: 10.1128/AEM.03704-12

Conflict of 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Frontiers in Microbiology

Explores the habitable world and the potential of microbial life

The largest and most cited microbiology journal which advances our understanding of the role microbes play in addressing global challenges such as healthcare, food security, and climate change.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact