# PLANTS AS ALTERNATIVE HOSTS FOR HUMAN AND ANIMAL PATHOGENS, VOLUME 2

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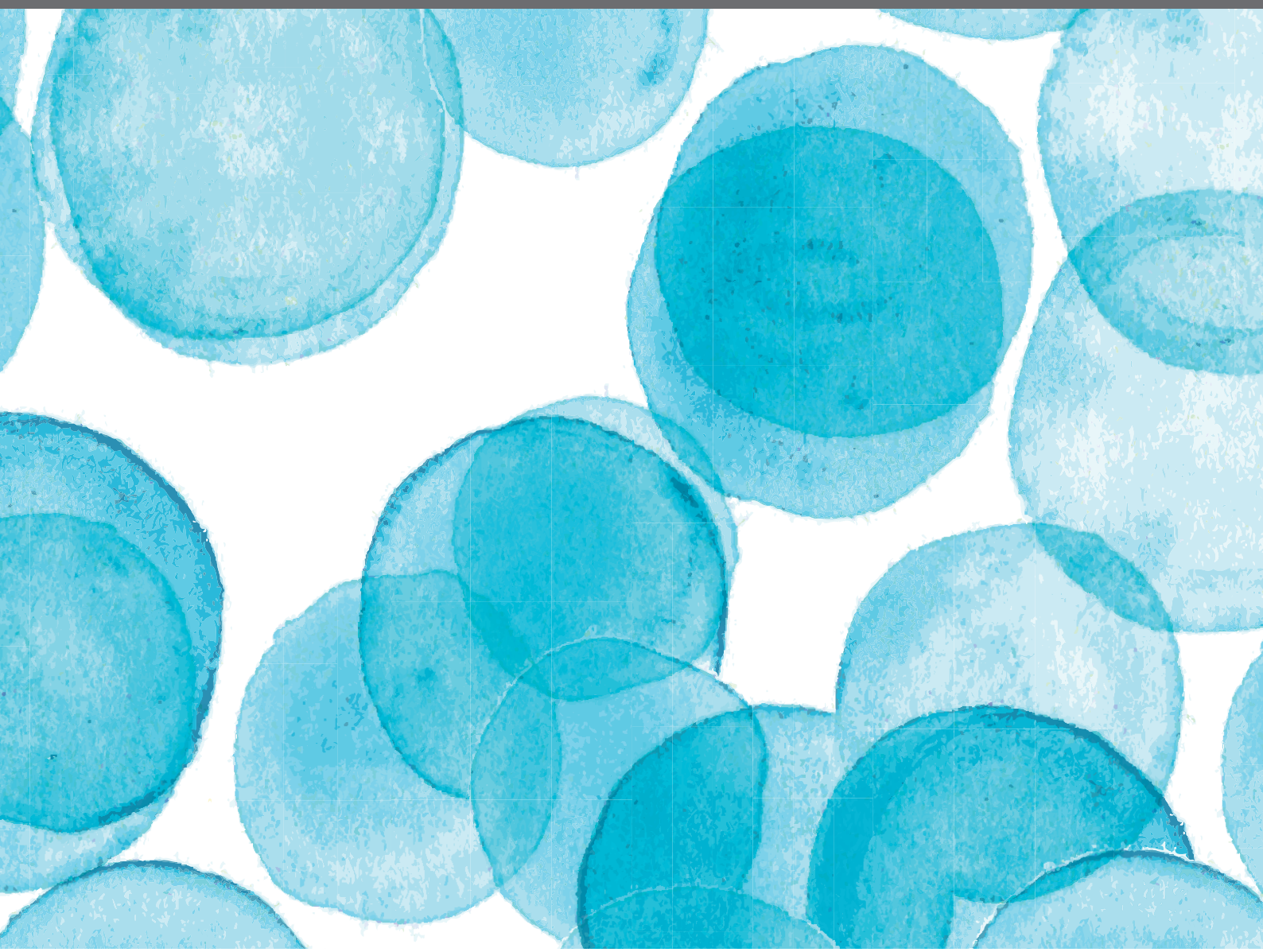

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# PLANTS AS ALTERNATIVE HOSTS FOR HUMAN AND ANIMAL PATHOGENS, VOLUME 2

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EDITORIAL
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# Editorial: Plants as Alternative Hosts for Human and Animal Pathogens – Second Edition

*Adam Schikora*[1]*, *Robert Wilson Jackson*[2], *Leo Van Overbeek*[3] and *Nicola Holden*[4]

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**Keywords: human pathogens on plants, *Salmonella*, *E. coil*, fresh produce, crop plants**

## Editorial on the Research Topic

## Plants as Alternative Hosts for Human and Animal Pathogens – Second Edition

Outbreaks of food-borne diseases are often associated with minimally processed fruits and vegetables. Interactions between human pathogens and plants are not incidental, transient or passive. For many, there is a defined and specific molecular basis. Both partners, the microbe and plant, play active roles in this interaction. Our understanding on how human pathogens colonize plants, especially in the face of microbial competition, is far from clear. Since these pathogens pose a health risk, important aspects include detection and surveillance, inevitably related to epidemiology, risk analysis, and therefore relevant to food safety, whether in the field or in the factory. This eBook second edition provides new discoveries in the field and incorporates a collection of articles focusing on several aspects of interactions between diverse human pathogens and crop plants. Those range from comprehensive approaches in food safety, through adaptation to the plant environment to shifts in the structure of microbial communities during the change in global climate.

Requirement for safe produce is of utmost importance for consumers and producers. Since it is not possible to remove microbes from leafy green vegetable production, avoidance of contamination with human pathogens such as Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella enterica, Shigella, Yersinia enterocolitica,* or *Listeria monocytogenes*, is crucial. These have the potential to lower the risk of contamination. How such critical points can be combined to decrease risks and improve microbial stability, nutritional properties and quality of leafy vegetables, is a key question. A toolbox with different physical, physiochemical, and microbial possibilities could be an option (Mogren et al.). The authors present mechanisms, which could be used for leafy greens and other produce. To enter food production systems, human pathogens need to adapt and establish in an environment, which is not optimal for their survival. Those environments, for example soil or plant tissues, have a significant impact on their physiology and influence additionally the virulence toward the plant host. The effects of soil type, organic fertilization, and plant species on the survival of *S. enterica* in soil as well as the colonization of plants are very substantial (Jechalke et al.). Authors revealed that different strains of *S. enterica* were able to persist in soil for several weeks and colonize leaves of lettuce and corn salad. Moreover, *S.* Typhimurium 14028s responded to lettuce or lettuce root exudates showing an upregulation of genes associated with biofilm formation and virulence. In addition to *Salmonella*, *E. coli* O157:H7 responds to physicochemical stresses in cut lettuce and lettuce lysates by upregulation of several stress response pathways. Damaged lettuce leaf tissue supplied *E. coli* with substrates for proliferation, and also provides pathogenic bacteria with choline for osmotic protection (Scott et al.). Internalization of bacterial cells into stomata enables human pathogens to evade the hostile environment of the

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leaf surface and reside in a protected, nutrient-rich niche. However, growth condition, different preadaptation, and temperature have an impact on the internalization abilities of *Salmonella enterica* (Kroupitski et al.). The authors further emphasized the function of universal stress-related genes in leaf internalization, highlighting the complexity of the bacterial internalization process. The above studies clearly demonstrated the ability of human pathogenic bacteria to adapt to the plant environment. How this adaptation differs from the adaptation to animal hosts is a very intriguing question. Comparison of the metabolic pathways, induced in those different environments, revealed that biosynthesis of amino acids, purines, vitamins, and the catabolism of glycerol and glucose are important (Kwan et al.). Nevertheless, whereas biosynthesis and degradation of fatty acid contributed to *S. enterica* animal colonization, it seems that only fatty acid biosynthesis was required during plant colonization. A genome-wide mutant screen comparing the plant-associated *S.* Newport with the animal-associated *S.* Typhimurium emphasized the importance of amino acid biosynthetic pathways and iron acquisition during the persistence in tomatoes (de Moraes et al.). Besides, a newly discovered gene, *papA*, which is unique to *S.* Newport contributes to its fitness in tomatoes. Homologs of *papA* are present in bacteria that colonize both plants and animals. Human pathogens do not restrict themselves to reaction and adaptation to a plant environment. They produce plant hormones, which may influence their vegetal host. The *Salmonella ipdC* gene, responsible for production of auxin via the IPyA pathway, is expressed on root surfaces, increasing the formation of lateral roots. Very interestingly, the *ipdC* mutant is less virulent in a murine model, because of a defect in its ability to cross the intestinal barrier (Cox et al.). Yet another aspect important in plant production systems is the occurrence of insects. The presence of leafhoppers results in longer persistence of *S. enterica* in plants. The reason could be the activation of contrary signaling pathways in the plants, whereby leafhopper infestation activated jasmonic acid defense responses while *S. enterica* colonization triggered salicylic acid responses. Such a situation hinders the defense ability of the plant (Cowles et al.).

In addition to bacteria, also pathogenic fungi inhabit plant production systems. Species from the *Fusarium solani* complex are frequently isolated from soils. They are one of the major opportunistic human pathogenic filamentous fungi, responsible, for example, for mycotic keratitis. Unfortunately, first-generation treatments, like triazoles, fluconazole, or itraconazole, are already ineffective. However, despite the intensive use of azoles, fusaria have not developed resistance against imidazole, leaving a treatment opportunity (Homa et al.). Global warming represents another challenge for the quality and safety of produce. Long-term analysis of moderate surface warming revealed a reduction of beneficial bacteria and an increased presence of potential pathogenic bacteria in the phyllosphere of plants. Such changes bear the risk for transmission of both plant and human pathogens in plant production systems (Aydogan et al.).

It is clear that interactions between human pathogens and plants are still not fully understood. Therefore, a collective effort is needed in order to develop new approaches and strategies in plant and consumer protection.

This Research Topic aligns closely with the networking partnership of the European COST Action 16110 "Control of Human Pathogenic Micro-organisms in Plant Production Systems" (www.huplantcontrol.igzev.de).

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

**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.

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ORIGINAL RESEARCH
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# *Escherichia coli* O157:H7 Converts Plant-Derived Choline to Glycine Betaine for Osmoprotection during Pre- and Post-harvest Colonization of Injured Lettuce Leaves

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Plant injury is inherent to the production and processing of fruit and vegetables. The opportunistic colonization of damaged plant tissue by human enteric pathogens may contribute to the occurrence of outbreaks of foodborne illness linked to produce. *Escherichia coli* O157:H7 (EcO157) responds to physicochemical stresses in cut lettuce and lettuce lysates by upregulation of several stress response pathways. We investigated the tolerance of EcO157 to osmotic stress imposed by the leakage of osmolytes from injured lettuce leaf tissue. LC-MS analysis of bacterial osmoprotectants in lettuce leaf lysates and wound washes indicated an abundant natural pool of choline, but sparse quantities of glycine betaine and proline. Glycine betaine was a more effective osmoprotectant than choline in EcO157 under osmotic stress conditions *in vitro*. An EcO157 mutant with a deletion of the *betTIBA* genes, which are required for biosynthesis of glycine betaine from imported choline, achieved population sizes twofold lower than those of the parental strain ($P < 0.05$) over the first hour of colonization of cut lettuce in modified atmosphere packaging (MAP). The cell concentrations of the *betTIBA* mutant also were 12-fold lower than those of the parental strain ($P < 0.01$) when grown in hypertonic lettuce lysate, indicating that lettuce leaf cellular contents provide choline for osmoprotection of EcO157. To demonstrate the utilization of available choline by EcO157 for osmoadaptation in injured leaf tissue, deuterated (D-9) choline was introduced to wound sites in MAP lettuce; LC-MS analysis revealed the conversion of D9-choline to D-9 glycine betaine in the parental strain, but no significant amounts were observed in the *betTIBA* mutant. The EcO157 Δ*betTIBA*-Δ*otsBA* double mutant, which is additionally deficient in *de novo* synthesis of the compatible solute trehalose, was significantly less fit than the parental strain after their co-inoculation onto injured lettuce leaves and MAP cut lettuce. However, its competitive fitness followed a different time-dependent trend in MAP lettuce, likely due to differences in $O_2$ content, which modulates *betTIBA* expression. Our study demonstrates that damaged lettuce leaf tissue does not merely supply EcO157 with substrates for proliferation, but also provides the pathogen with choline for its survival to osmotic stress experienced at the site of injury.

**Keywords: foodborne pathogen, plants, produce, damage, osmotic stress response, compatible solute, osmoprotection**

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## INTRODUCTION

*Escherichia coli* serovar O157:H7 (EcO157) is a prevalent foodborne pathogen that has caused numerous outbreaks of human infection linked to the consumption of lettuce in the United States, Europe, and other industrialized countries (Franz and van Bruggen, 2008; Lynch et al., 2009; Mandrell, 2009; Pennington, 2010). While field studies have demonstrated the ability of EcO157 to persist on lettuce plants for a few days after inoculation or up to harvest, none have demonstrated an overall increase in the population size of the pathogen on lettuce leaves under field conditions (Islam et al., 2004; Barker-Reid et al., 2009; Fonseca et al., 2011; Moyne et al., 2011; Bezanson et al., 2012; Oliveira et al., 2012). Given the occurrence of outbreaks associated with lettuce, it is reasonable to hypothesize that EcO157 must encounter microsites that allow for its multiplication to an infectious dose on/in the plant tissue during the production and processing of lettuce.

We have reported previously that plant lesions promoted high multiplication rates of EcO157 on lettuce leaves (Brandl, 2008), and field studies by others showed that EcO157 (Aruscavage et al., 2008) and non-pathogenic *E. coli* (Barker-Reid et al., 2009; Harapas et al., 2010) survived at greater rates on mechanically injured than intact lettuce. Additionally, we have demonstrated previously that downy mildew infection sites on lettuce supported greater multiplication and survival rates of EcO157 than healthy leaf tissue under wet and dry conditions in the phyllosphere, respectively (Simko et al., 2015). Tip burn lesions, caused by a physiological disease of lettuce also hosted high densities of EcO157 (Brandl, 2008). In lettuce microbiome studies, infection with *Rhizoctonia solani* enriched for the presence of members of the Enterobacteriaceae in the phyllosphere, including a significant upshift in abundance of *Enterobacter* spp. within that community (Erlacher et al., 2014, 2015). Hence, compromised lettuce leaf tissue resulting from various insults provides new favorable niches for colonization and persistence of enteric pathogens and related species.

Although human pathogens may benefit from the release of substrates from damaged cells in plant lesions (Aruscavage et al., 2010; Kyle et al., 2010; Goudeau et al., 2013; George et al., 2016), physicochemical conditions resulting from the injury *per se* and from the plant defense response to wounding or infection may also contribute to the outcome of a contamination event at the site of injury. Our transcriptomic studies previously revealed that in addition to responding to oxidative and antimicrobial stress in romaine lettuce leaf lysate and in cut leaf tissue, EcO157 mounted a response to osmotic stress by upregulation of the *betA* and *betB* genes for GB synthesis while the *otsBA* genes for production of the osmoprotectant trehalose were strongly downregulated (Kyle et al., 2010). RNAseq data from an ongoing study in our laboratory corroborated these findings by revealing also an increased expression of the *betTIBA* genes and concomitant repression of *otsBA* in EcO157 during colonization of shredded Iceberg lettuce compared with that of intact lettuce (unpublished data). Correlations between the osmotic stress tolerance of human pathogens and their ability to colonize their animal hosts have been documented (Kunin et al., 1992; Wargo, 2013a). However, the role of the osmotic stress response in the survival and proliferation of human foodborne pathogens in the plant habitat has not been investigated to date and most of our understanding of the osmoprotectants used by EcO157 in the environment outside of its primary hosts comes from soil studies (Burgess et al., 2016).

Bacteria may scavenge osmoprotectants, such as GB and proline directly from their environment, whereas others are synthesized *de novo* (trehalose) or from environmental precursors, as is the case for choline uptake and its conversion to GB (Kempf and Bremer, 1998). The transport of choline and GB in *E. coli* is regulated by osmotic stress (Lamark et al., 1996) and mediated by BetT, ProP, and ProU transmembrane transporters (Ly et al., 2004). Once imported into the cell, choline may be converted by the choline dehydrogenase BetA and the betaine aldehyde dehydrogenase BetB to produce GB, which confers a high level of osmotolerance in *E. coli* (Landfald and Strøm, 1986). In response to osmotic stress, *E. coli* accumulates GB to a range of intracellular levels proportionally to the environmental water potential (Perroud and Le Rudulier, 1985; Larsen et al., 1987; Wood et al., 2001). While *otsBA* also is positively regulated by osmotic stress (via RpoS), the *otsA* gene product is suppressed post-translationally by high intracellular levels of GB (Giaever et al., 1988).

Choline is commonly found in plants, where it is formed in the cytosol and is incorporated into membranes as the polar head group of phosphatidyl choline (Summers and Weretilnyk, 1993), a major phospholipid component of plant membranes (Whitman and Travis, 1985; Vu et al., 2014). Among other quaternary compounds, choline may serve for production of compatible osmolytes to facilitate plant adaptation to saline and drought conditions (Rhodes and Hanson, 1993). Plants suffering from damaged tissue also face desiccation as water is lost from plant cells, therefore necessitating repair (Boerjan et al., 2003). Upon plant injury, chemical wound signals are produced by enzymatic reactions with membrane phospholipids; phosphatidic acid is a jasmonic acid precursor and itself a prominent signaling molecule generated when wounding triggers the membrane localization and activation of various phospholipases (Wang et al., 2000).

Given our previous observations of the increased expression of *bet* genes in EcO157 in two experimental model systems for mechanically injured lettuce leaves, we hypothesized that wounded lettuce leaf tissue inflicts hyperosmotic stress on EcO157 and that choline is likely available to the pathogen in plant wounds to serve as a precursor to the BetTIBA pathway. We therefore assessed the presence of the precursor choline and end product GB in both the bacterium and plant host wounds by quantification of these compounds using LC-MS analysis and an enzymatic assay. Furthermore, we determined the osmotolerance of a single BetTIBA mutant and a double BetTIBA-OtsBA mutant *in vitro* as well as

their competitive fitness in various models of injured plant tissue.

# MATERIALS AND METHODS

## Bacterial Strains and Culture Conditions

All strains and plasmids used in this study are listed in **Table 1**. *E. coli* O157:H7 strain TW14588, a clinical isolate from a 2006 outbreak attributed to Iceberg lettuce served at Taco John (Manning et al., 2008), was kindly provided by the Thomas Whittam STEC Center, MI and used for our studies. The genome sequence of this strain is available in GenBank (Acc. No. ABKY02000001- ABKY02000010). Unless mentioned otherwise, all strains were cultured in Luria-Bertani half-salt (5 g NaCl/L) (LBHS) broth and incubated at 28°C on a roller drum. LBHS broth was amended as appropriate with kanamycin (Km) (30 μg/ml), gentamicin (Gm) (15 μg/ml), chloramphenicol (Cm) (20 μg/ml), or carbenicillin (Cb) (50 μg/ml).

Lettuce leaf lysate was used immediately after preparation, and when appropriate, was amended with a NaCl solution to a final concentration of 550-, 650-, and 750 mM, or with DDI $H_2O$ at 10% volume in the control. The leaf lysate then was dispensed at 500 μl per well into 48-well plates prior to inoculation at $2 \times 10^7$ cells/ml. The cultures in lysate were incubated at 28°C with constant shaking in a BIOTEK Epoch II plate incubator and reader. Inoculum suspensions were prepared from overnight cultures in the stationary phase of growth. Cells were centrifuged, washed twice in 10 mM $KPO_4$ (KP) buffer (pH 7), and resuspended in 1 mM KP buffer at the desired cell concentration based on $OD_{600}$.

## Strain Construction

The EcO157 TW14588 WT strain was marked with a gentamicin- or chloramphenicol resistance cassette introduced on the chromosome at the *att*Tn*7* site using the mini-Tn*7* procedure described by Crépin et al. (2012a) with minor modifications. Plasmids were kindly provided by Dr. Charles Dozois (INRS-Institut Armand-Frappier, Laval, QC, Canada). The helper plasmid pSTNSK was introduced into the WT strain by electroporation, which was transformed again with pGP-Tn*7*-Gm or pGP-Tn*7*-Cm to generate TW14588::*aacC1* and TW14588::*cat*, respectively. The transformants were selected on LBHS agar with the appropriate antibiotic and incubation at

**TABLE 1 |** Strains and plasmids used in this study.

| | Relevant features | Source or reference |
|---|---|---|
| **Strains** | | |
| ***E. coli* O157:H7** | | |
| TW14588 | "Taco John" outbreak clinical strain from the Thomas Whittam STEC Center; genome-sequenced strain | Manning et al., 2008 |
| TW14588::*aacC1* | TW14588 *att*Tn*7*::*aacC1,* $Gm^r$ | This study |
| TW14588::*cat* | TW14588 *att*Tn*7*::*cat,* $Cm^r$ | This study |
| TW14588::*cat*/pGT-Kan | TW14588::*cat* with pGT-KAN, $Cm^r$, $Gm^r$ | This study |
| TW14588::*cat* /pMBotsBA | TW14588::*cat* with pBBR1-MCS-5-*otsBA*, $Cm^r$, $Gm^r$ | This study |
| TW14588::*cat*/pMBbetTIBA | TW14588::*cat* with pBBR1-MCS5-*betTIBA*, $Cm^r$, $Gm^r$ | This study |
| TW14588 Δ*betTIBA* | TW14588 Δ*betTIBA*::*cat*, $Cm^r$ | This study |
| TW14588 Δ*otsBA* | TW14588 Δ*otsBA*::*kan*, $Km^r$ | This study |
| TW14588 Δ*otsBA* Δ*betTIBA* | TW14588 Δ*otsBA*::*kan* Δ*betTIBA*::*cat*, $Km^r$ $Cm^r$ | This study |
| TW14588 Δ*otsBA* Δ*betTIBA*/pMBbetTIBA | TW14588 Δ*otsBA*::*kan* Δ*betTIBA*::*cat* with pMBbetTIBA, $Km^r$, $Cm^r$, $Gm^r$ | This study |
| TW14588 Δ*otsBA* Δ*betTIBA*/pMBotsBA | TW14588 Δ*otsBA*::*kan* Δ*betTIBA*::*cat* with pMBotsBA, $Km^r$, $Cm^r$, $Gm^r$ | This study |
| TW14588 Δ*otsBA*/pMBotsBA | TW14588 Δ*otsBA*::*kan* with pMBOtsBA, $Km^r$, $Gm^r$ | This study |
| TW14588 Δ*betTIBA*/pMBbetTIBA | TW14588 Δ*betTIBA*::*cat* with pMBBetTIBA, $Cm^r$, $Gm^r$ | This study |
| ***Pseudomonas* spp.** | | |
| *Pseudomonas aeruginosa* PAO1 | Wild type *P. aeruginosa* | Holloway, 1955 |
| *Pseudomonas syringae* pv. syringae B728A | Wild type *P. syringae* | Loper, 1987 |
| *Pseudomonas syringae* pv. tomato DC3000 | Wild type *P. syringae* | Cuppels, 1986 |
| **Plasmids** | | |
| pSTNSK | pST76-K::*tnsABCD*; $Km^r$ | Crépin et al., 2012a |
| pGP-Tn7-Gm | pGP704::Tn*7*-*aacC1*; $Cb^r$ $Cm^r$ | Crépin et al., 2012a |
| pGP-Tn7-Cm | pGP-Tn*7*-FRT::*cat*; $Cb^r$ $Cm^r$ | Crépin et al., 2012b |
| pBBR1MCS-5 | Broad host range cloning vector, $Gm^r$ | Kovach et al., 1995 |
| pMBotsBA | pBBR1MCS-5 with *otsBA* from EcOI157 TW14588 cloned into SpeI-SacI; $Gm^r$ | This study |
| pMBbetTIBA | pBBR1MCS-5 with *betTIBA* from EcO157 TW14588 cloned into SpeI-SacI; $Gm^r$ | This study |
| pGT-Kan | pPROBE-GT with $P_{kan}$-*gfp;* $Gm^r$ | Brandl et al., 2005 |

*$Km^r$, kanamycin resistant; $Cm^r$, chloramphenicol resistant; $Gm^r$, gentamicin resistant, $Cb^r$, carbenicillin resistant.*

37°C. Colonies were restreaked also onto LBHS containing carbenicillin to confirm loss of pSTNSK, and insertion of the cassette at the *att*Tn*7* site was verified by PCR.

Deletion mutants were generated via lambda Red-mediated recombination using pKD4 or pKD3 (Datsenko and Wanner, 2000) and replacing *otsBA* or *betTIBA* with a kanamycin- and chloramphenicol resistance cassette, respectively. The double deletion mutant was constructed by deletion of *betTIBA* in the *otsBA* mutant, resulting in a strain marked with both kanamycin and chloramphenicol resistance. The deletion of *otsBA* and *betTIBA* was verified by PCR. PCR primers used for mutagenesis and verification of deleted target genes in the mutants are listed in Supplementary Table S1. The *otsBA* and *betTIBA* complementation plasmids were constructed by PCR cloning and ligation into SpeI and SacI restriction sites of pBBR1MCS-5 (Kovach et al., 1995) to generate pMBotsBA and pMBbetTIBA, respectively, using primers described in Supplementary Table S1. Complemented strains were generated by transformation of the mutants via electroporation.

## Plant Material and Growth Conditions

Iceberg Lettuce cv. Salinas seeds were kindly provided by Ivan Simko (USDA, Agricultural Research Service, Salinas, CA, United States). Plants were grown in Supersoil potting mix with approximately 150 mg of Osmocote Plus (15-9-12) at 22°C with a 14-h photoperiod for 6–8 weeks in a plant growth chamber (Percival Scientific, Inc.), and fertilized weekly with 20-20-20 (N-P-K) liquid fertilizer past the five expanded leaf-growth stage. For experiments with cut lettuce in modified atmosphere packaging (MAP) bags, mature Iceberg lettuce heads were acquired from local commercial sources, trimmed of outer leaves, and the leaves shredded into 2-mm wide strips with a sharp knife.

Lettuce leaf lysate was prepared as described previously with modifications (Kyle et al., 2010). Iceberg lettuce heads were obtained commercially and all portions except the outermost leaves and inner-most achlorophyllous heart were used. The leaves were homogenized in a Omega juicer (Omega Model No. 8003), with the homogenate kept on ice during production and immediately centrifuged at 7,000 rcf at 4°C for 10 min to pellet chloroplasts and plant debris. The supernatant was sterilized by passage through 0.45 μm- then through 0.2 μm pore-size filters.

## Plant Inoculations and Recovery of EcO157 from Wounds

For single inoculations of MAP lettuce, the WT strain (TW14588::*cat*) and the single *betTIBA* mutant (Δ*betTIBA*::*cat*) were inoculated individually onto the bagged cut leaves. In competitive fitness studies, the WT strain (TW14588::*aacC1*) was mixed with the double mutant Δ*otsBA*::*kan* Δ*betTIBA*::*cat* in a 1:1 ratio prior to co-inoculation.

For inoculation of cut (shredded) lettuce, square bags (24 cm × 24 cm) were made with MAP film commercially used for processed Iceberg lettuce and filled with 226 g lettuce, keeping bag size to product weight ratio as per commercial standards. Each bag received 2 ml inoculum at $1 \times 10^5$ cells/ml that was distributed by gently shaking the bag. Bags were flushed three times with $N_2$ gas, completely sealed and incubated at 24°C. Atmospheric $O_2$ and $CO_2$ contents in the bags were determined with a MOCON PAC CKECK O2/CO2 Analyzer (Model 650 EC). For bacterial cell recovery, the leaf material from each bag was transferred to Filtra-Bags (LabPlas, SCT07012A) and processed with 200 ml 10 mM KP buffer in a stomacher for 2 min. The resulting suspension was dilution-plated onto LBHS agar with appropriate antibiotics for CFU determinations of each strain.

For inoculation of leaf wounds on potted lettuce plants, 12″ straight dressing forceps with a 35 mm × 3 mm serrated surface area were immersed at the tip into inoculum suspensions of $1 \times 10^7$ cells/ml in 1 mM KP buffer, twice blotted on Whatman #42 filter paper and used to generate one wound in the middle of the leaf blade and away from the midvein. This resulted in an initial inoculum level of approximately $5 \times 10^4$ CFU/wound. Plants were incubated at 22–25°C and under 90–100% RH to promote microbial growth, but free water was absent macroscopically from the plant surface. Wounded tissue was sampled immediately after inoculation by wounding, and thereafter at indicated times, by cutting out a 7 mm diameter disk spanning initial wound striations. The disks were homogenized in 1 ml 10 mM KP buffer with 12 glass beads (Fisher Scientific, 11-312A), at 4°C for 2 min in a Mini-Bead beater 96+ 1HP (11.2A @115 VAC) (Biospec products). Five and ten disks were sampled at random from replicate leaves and replicate plants at the time of inoculation, and at each sampling time thereafter, respectively. The disk homogenate was dilution-plated onto LBHS agar containing appropriate antibiotics for CFU determinations of each strain.

## Compatible Solute Quantification by LC-MS

For analysis of uninoculated lettuce lysates, lysates were sampled by mixing 1:1 (v:v) with ice cold HPLC-grade 50% MeOH, centrifugation at 15,000 RCF and 4°C, and 500 μl supernatant dried in a SpeedVac, reconstituted in a smaller volume prior to filtration using 10K molecular weight cutoff microcentrifuge columns, and used for LC-MS. For quantification of osmoprotectants in EcO157 cells cultured in lettuce lysate, bacterial cells were recovered from the lysate by centrifugation and the pellets washed twice with ice-cold 1 M NaCl to prevent spontaneous expulsion/export of intracellular osmoprotectants, then frozen at −20°C prior to lysis (Fagerquist et al., 2014) with zirconia silica beads in 300 μl Nuclease-Free $H_2O$ by bead beating as described above. Cell lysates were centrifuged and the supernatant transferred to MeOH 1:1, then centrifuged again and the supernatant used for mass spectrometry. Choline, glycine betaine, and proline present in lettuce lysates and cells grown in lettuce lysates were analyzed by injection of samples into a nanoflow Reprocsil-PUR C18-AQ column (New Objective, Woburn, MA, United States) with a Thermo EZ-nano HPLC followed by detection on a Thermo Orbitrap Elite ion mass spectrometer (Thermo Fisher, Waltham, MA, United States). The Orbitrap was operated in positive ion mode, with the mass resolution set to 30,000. MS data were collected from 95 to 200 m/z. Analytes were minimally

retained and eluted with isocratic flow (400 nL/min) of 2% MeCN in HOH with 0.1%formic acid (Optima LC-MS grade, Fisher Scientific).

For D9 tracing experiments, EcO157 cells were inoculated onto MAP lettuce with a 1 ml suspension of $1 \times 10^9$ cells/ml containing [1 M] D9-choline, and incubated as described above, with the exception of non-MAP bags sealed using ambient atmosphere, and uninoculated bags which were MAP-sealed but received no inoculum or D9-choline. At 6 h post-inoculation, MAP, non-MAP, and uninoculated lettuce samples were processed by washing with 50 ml 1 M ice cold NaCl to recover cells, dilute away extracellular osmoprotectants, and retain intracellular osmoprotectants, and a portion retained for dilution plating. Cells were then centrifuged at 26,640 *g*, and lysed as described above for EcO157 cultured in lettuce lysates. Quantification was performed by comparison of relative peak intensity of the monoisotopic molecular ions in spectra generated by averaging scans across the analytes elution off the column with D9-choline and D11-betaine (Cambridge Isotope Laboratories, Tewksbury, MA, United States) co-injected as internal standards.

Analysis of D9-GB in the lysates was carried out on a Thermo Orbitrap Elite ion mass spectrometer fitted with an Agilent 1100 HPLC. A Thermo HESI source was used with the electrospray voltage set to 4 kV, sheath gas set to 10, auxiliary gas at 5, and capillary inlet temperature at 295°C. The MS was operated at 15000 resolution and mass spectra were collected in SIM mode monitoring five mass windows: 117.6–118.6, 128.7–129.7, 103.6–104.6, and 112.7–113.7 Da, and 126.6–127.6 for D9-Choline, D11-GB, native Choline, native GB, and D9-GB, respectively. Data were processed with Thermo Xcalibur Quant Browser using Genesis peak integration with 15 smoothing points.

Normal phase chromatography prior to MS analysis was carried out with a 15 cm × 2.1 mm Diamond Hydride column coupled with a 70000 HG5 Diamond Hydride guard column (MicroSolve, Leland, NC, 28541). The HPLC was operated at 400 μL/min with Fisher Optima LC-MS grade solvents (Fisher Scientific). The gradient of A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) was used as follows: 92% B for 1 min, to 20%B at time 15 min, hold for 3 min, then return to 92%B at time 20 min. The divert valve was programmed to divert to waste from time 0 to 4 min, to the source from 4 to 14 min, and to waste from 14 to 20 min. Prior to injection, samples were mixed 1:1 with D9-Choline and D9-Betaine (Cambridge Isotopes, Tewksbury, MA 01876, United States) to a final concentration of 20 pM/μL deuterated standards with the exception of runs designed to detect D9-GB from bacterial cell conversion of D9-choline in inoculated MAP lettuce, in which case only D11-GB was added to the samples in the same manner as D-9 Choline had been added previously for those experiments.

## Choline Quantification by Enzyme Assay

For quantification of choline in MAP cut lettuce, leaves were processed and bagged as described above. Immediately after bagging the cut leaves, and at various time after incubation in MAP, 20 ml of ice-cold $H_2O$ was used to wash off choline from the lettuce pieces by gently moving the $H_2O$ over the plant material in the bag. The resulting wash water was then mixed 1:1 with ice cold HPLC-grade MeOH and stored at −20°C. Choline was quantified with a choline assay kit (BioVision, Inc.) according to the manufacturer's instructions with modifications. Samples in MeOH:$H_2O$ were dried in wells of 96-well plates in a SpeedVac and reconstituted in 20 μL choline assay buffer. Diluted choline probe was added rapidly to reagents in control wells and the plate read in a BioTek plate reader (exc. 530 nm, emm. 590 nm) to establish background. Diluted enzyme mix was then immediately added to each well. The reactions were monitored at 28°C until $OD_{590}$ reached a maximum, and absorbance data from test samples were interpreted relative to internal choline standards (1 to 100 pmoles reaction$^{-1}$).

## Microscopy

For visualization of the pathogen in inoculated leaf wounds, EcO157 TW14588 was transformed with pGT-Kan (Brandl et al., 2005), which is stably maintained in *E. coli* and allows for the constitutive expression of the green florescent protein (GFP) gene. Leaves of young lettuce plants were wound-inoculated with forceps as described above, and the plants incubated at 28°C and 90–100% RH. Small disks were sampled from the wounded tissue immediately and 6 h after wounding/inoculation. The GFP-labeled bacteria on/in the tissue were visualized with a Leica confocal microscope TCS SP5 (Leica Microsystems, Germany) and pseudo-3D images were constructed by projected series of multiple optical scans in the *z* plane.

## Statistical Analysis

Statistical analysis of the data was performed with GraphPad Prism version 7.03 (GraphPad Software, San Diego, CA, United States[1]). Direct comparison of the log-transformed population size of an EcO157 mutant with that of the WT strain was done with the unpaired Student's *t*-test when appropriate. Percentage values obtained for representation of the mutant strain in the total EcO157 population in competitive fitness experiments were transformed to arcsin(squareroot %) before analysis. One-way ANOVA was used for comparison of more than two groups, followed by Tukey's or Dunnett's multiple comparison test. Differences were considered significant at $P < 0.05$.

# RESULTS

## Contributions of Osmoprotectants and Osmotic Stress Resistance Genes in Hypertonic Minimal Medium

Lettuce leaf wound colonization by EcO157 TW14588 occurred rapidly at temperatures conducive to multiplication of the pathogen. As illustrated in **Figure 1**, the population densities of GFP-labeled TW14588 evolved from single cells at distant locations to cell assemblages along the site of injury only 6 h after inoculation of the leaves of potted lettuce plants by

[1]http://www.graphpad.com

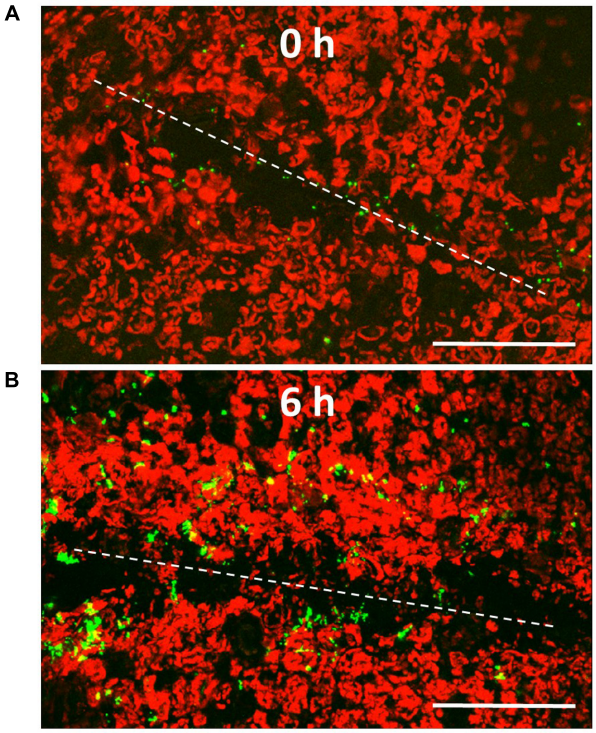



**FIGURE 1 |** Pseudo-3D confocal scanning laser microscopy images of GFP-EcO157 in wounded lettuce leaf tissue. Green fluorescent EcO157 cells in the leaf wounds **(A)** immediately after inoculation by wounding (0 h), and **(B)** 6 h after inoculation and wounding. White dotted lines delineate the wound generated by a single forcep striation. The red signal originates from the autofluorescence of the chloroplasts present in the leaf cells. Scale bars: 100 μm.

wounding the leaf blade with contaminated tweezers. Given that the *betTIBA* genes were induced in lettuce leaf lysates (Kyle et al., 2010), and in cut vs. intact lettuce under MAP conditions (unpublished data), we hypothesized that the presence of choline, the precursor of the BetTIBA pathway, could convey a growth advantage to EcO157 under osmotic stress conditions *in planta*. We first investigated the role of this pathway under osmotic stress conditions *in vitro* and observed that the growth of EcO157 in M9-glucose minimal medium amended with 500 mM NaCl was enhanced by amendment with choline, as well as with the pathway end-product GB (**Figure 2A**).

A marked site-directed deletion mutant of the *betTIBA* region was constructed by the λ Red recombinase approach (Datsenko and Wanner, 2000) (**Table 1**). The resulting strain MB1141 (TW14588Δ*betTIBA*::*cat*) exhibited growth similar to that of WT in M9 medium in the absence of osmotic stress (data not shown), as well as under osmotic stress in the absence of osmoprotectants (control) (**Figures 2A,B**). Consistent with the requirement of the *bet* genes for utilization of choline for osmoprotection, the *betTIBA* mutant did not receive a growth advantage under osmotic stress by addition of choline (**Figure 2B**). However, addition of GB restored growth of the *betTIBA* mutant to the same levels as those of the WT grown in the presence of choline or GB under these conditions (**Figures 2A,B**).

Because growth still occurred in the *betTIBA* mutant at high osmolyte concentrations (**Figure 2B**), the compensatory role of the *otsBA* operon in adaptation of EcO157 to osmotic stress was assessed in a WT and in a *betTIBA* mutant background. A mutant with a deletion in *otsBA* was constructed via λ Red recombinase by replacement of this operon with a kanamycin resistance cassette in the WT and in the *betTIBA* mutant background, resulting in the single mutant, strain MB962 (TW14588Δ*otsBA*::*kan)* and the double mutant, strain MB1145 (TW14588Δ*betTIBA*::*cat*-Δ*otsBA*::*kan*), respectively (**Table 1**). The WT, and *otsBA* and *betTIBA-otsBA* mutants displayed similar growth in M9 medium without additional osmoticum (data not shown), but the *otsBA* and *betTIBA-otsBA* mutants equally failed to grow in M9 medium containing 500 mM NaCl and lacking choline (**Figure 2**). Whereas the single *otsBA* mutant benefited from the presence of choline for growth under osmotic stress (**Figure 2C**), the double *betTIBA-otsBA* was not rescued by the addition of choline, although it displayed growth similar to that of the WT and both single *otsBA* and *betTIBA* mutants in the presence of GB (**Figure 2D**).

Tolerance of the *betTIBA*, *otsBA*, and double *betTIBA-otsBA* mutants to osmotic stress was restored genetically by complementation with expression of the respective operons driven by their native promoter (**Figure 3**). When choline was added to hypertonic M9 medium, the presence of pMBbetTIBA in the *betTIBA* mutant and in the *betTIBA-otsBA* mutant enhanced their growth to at least that achieved by the WT strain (**Figures 3A,C**, respectively). When the *otsBA* and *betTIBA-otsBA* mutants were transformed with pMBotsBA, they displayed greater growth rates than the WT in hypertonic M9 medium without choline, which was not provided in order to remove the osmoprotective effect via BetTIBA (**Figures 3B,D**). All mutants and their complemented derivatives grew similarly to WT in M9 without osmotic stress (data not shown).

## MS Analysis of Osmoprotectants in EcO157 Cultured in Lettuce Lysate

Lettuce leaf lysate first was used as a proxy for the chemical environment that EcO157 cells would experience when exposed to the contents of broken leaf cells, as we described previously (Kyle et al., 2010). Growth in lettuce lysate (amended with $H_2O$ at 10% final to adjust the volume in this control for addition of NaCl under hypertonic conditions) was similar in the WT and *betTIBA* single mutant, as well as in lysate with NaCl at 550 mM. However, upon addition of NaCl to 650 and 750 mM final concentration, the end-point absorbance of the *betTIBA* mutant cultures in lysate decreased sharply and was significantly different than that of the WT (Student's *t*-test, $P < 0.01$) this was equivalent to a 2.25 and 12.14× difference in cell concentration (CFU/ml), respectively (**Figure 4A**).

To further test a model wherein choline is abundant in plant wounds and imported into EcO157 to be converted into GB, the relative intracellular abundance of these compounds was measured in WT and *betTIBA* mutant cells cultured in lettuce

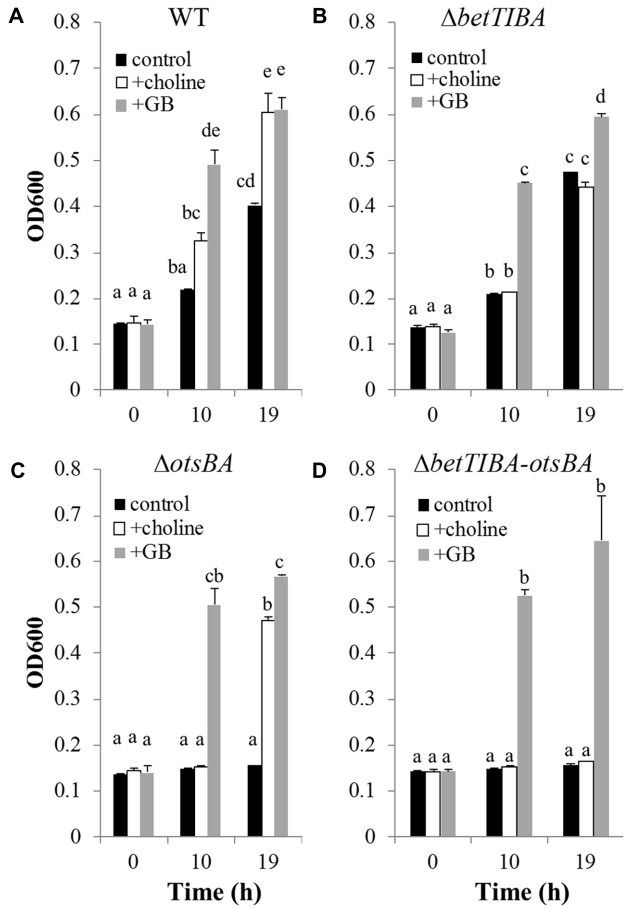



**FIGURE 2 |** Chemical complementation of EcO157 WT strain and osmotic solute accumulation mutants. Growth of **(A)** WT, **(B)** *betTIBA*-, **(C)** *otsBA*-, and **(D)** *betTIBA-otsBA* mutants in M9-glucose minimal medium amended with 500 mM NaCl, and with 1 mM choline (+choline), or 1 mM glycine betaine (+GB), or an equivalent volume of $H_2O$ (control). Each bar represents the mean of determinations from two replicate cultures in wells of a 48-well plate, and standard error of the mean. Within each panel, bars marked with a same letter indicate that means are not significantly different based on Tukey's multiple comparison test ($P > 0.05$).

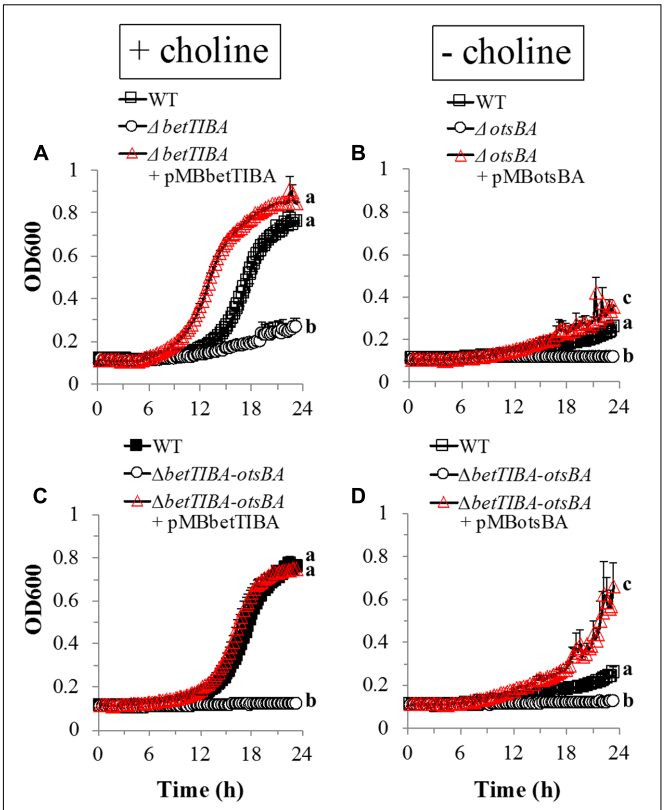
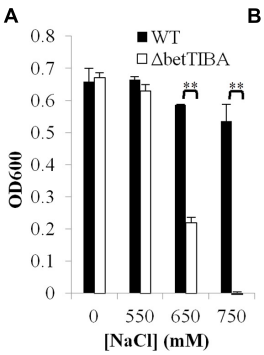



**FIGURE 3 |** Genetic complementation of EcO157 mutants with a deletion in osmoprotectant accumulation operons. The *betTIBA*-, *otsBA*-, and *betTIBA-otsBA* mutants were complemented with **(A,C)** pMBbetTIBA or with **(B,D)** pMBotsBA. Strains were grown in M9-glucose minimal medium with 500 mM NaCl and with **(A,C)** or without **(B,D)** 1 mM choline. Each point represents the mean of determinations from two replicate cultures in wells of a 48-well plate, and standard error of the mean. Within each panel, data series marked with different letters indicate that the mean of the last three $OD_{600}$ values in the time course for any given strain are significantly different based on Tukey's multiple comparison test ($P < 0.05$).

lysate using LC-MS analysis. As per relative ion intensity, both choline and GB accumulated in the control lysate (amended with $H_2O$ only) to lower levels than in lysate amended with 650 mM NaCl (**Figure 4B**). Under the latter hypertonic condition, the *betTIBA* mutant accumulated 31-fold greater quantities of the precursor choline than the WT, which in turn produced 216-fold more of the pathway end product GB than the mutant. Very low levels of proline, which can serve as an important osmoprotectant in *E. coli* (Kempf and Bremer, 1998), were detected in both strains even in the presence of high NaCl concentrations. Furthermore, quantities of this osmoprotectant were only 0.7% those of choline in the uninoculated sterile lettuce leaf lysate; GB levels were similarly low, at only 1.3% those of choline. Of note, while we observed that choline and GB serve in EcO157 TW14588 and EDL933 as osmoprotectants, these strains do not utilize choline or GB as sole carbon sources as certain *Pseudomonas* strains do (Chen and Beattie, 2008; Wargo et al., 2008) (Supplementary Figure S1).

## D9-Choline Tracing in EcO157 and Fitness of the *betTIBA* Mutant in Lettuce Wounds

In order to determine the activity of the BetTIBA pathway in EcO157 during colonization of injured lettuce leaves, deuterated choline (D9-choline) was fed to the TW14588 WT and *betTIBA* mutant by its addition to cell suspensions inoculated onto cut (shredded) lettuce. LC-MS analysis revealed that after 6 h of incubation under MAP conditions, D9-GB accumulated to 170 ± 47 μmoles $L^{-1}$ cell volume in the WT whereas accumulation was minimal in the *betTIBA* mutant and 10.8-fold lower than in the WT cells (Student's *t*-test, $P < 0.01$) (**Figure 5A**, upper panel). D9-GB levels in the WT in cut lettuce packaged under ambient atmosphere were 156 ± 45 μmoles $L^{-1}$ cell volume and similar to those in MAP. D-9 GB was not detected




**Intracellular Compound Levels**

| | | | Relative Ion Intensity [1] Choline | Glycine Betaine | Proline |
|---|---|---|---|---|---|
| Lysate-cultured cells | No NaCl | TW14588 | 11 (2) | 2 (1) | 2 (1) |
| | | *ΔbetTIBA* | 8 (2) | 3 (1) | 1 (1) |
| | With NaCl | TW14588 | 12 (10) | 1,945 (188) | 2 (0) |
| | | *ΔbetTIBA* | 376 (71) | 9 (1) | 7 (4) |
| Sterile Lysate (control) | | | 25,863 (5,088) | 347 (79) | 206 (30) |

[1] Numbers represent the mean relative ion intensity of three replicate cultures; SEM, in parentheses.

**FIGURE 4 |** Growth and osmoprotectant accumulation in EcO157 WT and *betTIBA* mutant cells in lettuce lysates. **(A)** Growth after 24 h incubation in lettuce lysate amended with 550, 650, or 750 mM NaCl, or an equivalent volume of $H_2O$ (0 mM NaCl). Each point represents the mean of determinations from two replicate cultures in wells of a 48-well plate, and standard error of the mean. ** indicates significant difference by Student's *t*-test ($P < 0.01$). **(B)** Relative intracellular levels of choline, glycine betaine, and proline in WT and *betTIBA* mutant cells recovered after 24-h growth in lettuce lysates containing 0 or 650 mM NaCl. Levels of same compounds in sterile lettuce lysates incubated under the same conditions are also shown. Numbers represent the mean relative ion intensity of three replicate cultures, with standard error of the mean provided in parentheses, as determined by MS analysis.

in pellets recovered from uninoculated MAP cut lettuce (control) incubated for 6 h (**Figure 5A**, lower panel).

Given the above evidence that the BetTIBA pathway was active in TW14588 on injured leaf tissue, the WT and *betTIBA* mutant were compared for their colonization of cut lettuce leaves. Both strains multiplied rapidly on cut leaves under MAP conditions, but the population sizes of the WT strain were significantly greater than those of the mutant (Student's *t*-test, $P < 0.05$) by 2.0- and 1.4-fold after 1- and 6 h incubation, respectively (**Figure 5B**).

## Comparative Growth of WT, Single *otsBA* Mutant and Double *betTIBA-otsBA* Mutant in Hypertonic Lettuce Lysate

With the knowledge that EcO157:H7 has several redundant pathways to respond to osmotic stress and that free GB and proline as available compatible solutes were detected in low amounts in plant cells (**Figure 4B**), a double mutant in which *de novo* trehalose synthesis could not serve a compensatory role in the absence of BetTIBA was investigated as well. The deletion of *otsBA* was important also because the product of the BetTIBA pathway, GB, is known to repress OtsBA in a post-translational manner (Giaever et al., 1988) and therefore, the deletion of *betTIBA* may have resulted in the re-activation of OtsBA in this single mutant. The *betTIBA-otsBA* double mutant was severely impaired in growth in lettuce lysate amended with NaCl, compared with the WT and with the single *otsBA* mutant (**Figure 6A**) despite that the latter likely benefited from choline availability and the activity of the BetTIBA pathway under these conditions, as shown in **Figures 4**, **5**. Addition of choline to the lettuce lysate did not increase the initial growth rate of the *otsBA* mutant, but promoted slightly greater cell concentrations in late exponential phase, likely because the natural pool of choline in the lysate provided osmoprotection in the early phase of growth (**Figure 6B**). However, addition of GB to the lysate strongly enhanced the growth of the double mutant since it curtailed the requirement of the BetTIBA pathway for use of GB as an osmoprotectant (**Figure 6C**). This observation suggests that although GB is present in significantly lower amounts in leaf lysate than choline (**Figure 4**), its uptake in damaged leaf tissue may offer some osmotic protection when the OtsBA and BetTIBA pathways are not present or active in EcO157.

## Competitive Fitness of a Double *betTIBA-otsBA* Mutant in Lettuce Wounds

Population dynamics following co-inoculation of the *betTIBA-otsBA* mutant and WT strains in a ratio of 1:1 onto shredded MAP lettuce showed that the total EcO157 population increased 5-log over 48 h, and that the proportion of the mutant decreased significantly from 51.1 to 42.6% over the first 6 h of incubation [arcsin(squareroot %)-transformed data; Student's *t*-test, $P < 0.05$], but increased thereafter (**Figure 7A**). This trend in the competitive fitness of the mutant was concomitant with a sharp drop in $O_2$ content from 1.3 to 0.4% in the bag atmosphere during the first 6 h to remain constant thereafter, and a steady increase in $CO_2$ content throughout incubation due to plant respiration (**Figure 7B**). Choline was quantified in the washes of MAP shredded lettuce in order to assess its availability for uptake and transformation into GB in EcO157 cells in the injured tissue. Choline that leaked from the damaged tissue was abundant at the time of wounding the leaves by cutting (shredding) and decreased by 21% within 24 h after incubation under MAP conditions (**Figure 7C**).

The competitive fitness of the mutant followed a different trend in wounds on leaves of whole lettuce plants since its proportion decreased throughout the entire incubation to reach 40.8% over 72 h, which was significantly different than that of 52.0% at the time of inoculation [arcsin(squareroot %)-transformed data; ANOVA, $P < 0.01$; Dunnett's multiple comparison test, $P < 0.005$] (**Figure 8**). Application of exogenous GB to the wounds by its addition to the inoculum suspension

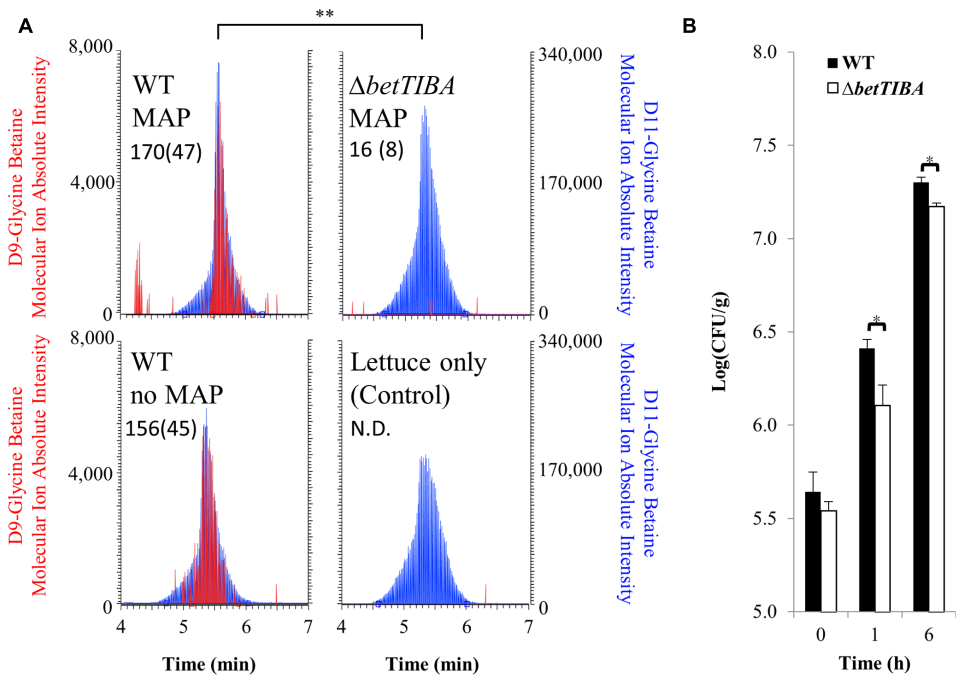



**FIGURE 5 |** HPLC-MS quantification of D9-choline conversion to D9-GB in EcO157 WT and *betTIBA* mutant, and their growth in MAP cut lettuce. **(A)** Representative MS ion traces of D9-GB (left axis, red trace) from quantitative analysis in WT and *betTIBA* mutants cells recovered 6 h after single inoculation of their suspension amended with D9-choline onto cut lettuce incubated in bags under MAP (upper traces), or at room atmosphere (no MAP) (lower left trace). The lower right trace illustrates the lack of detection of D9-GB in uninoculated lettuce spiked with D9-choline and incubated for 6 h in MAP (control). The trace for D11-GB, which served as an internal standard is also shown (right axis, blue trace). Left and right axes indicate relative molecular ion intensity for representative peaks obtained for each treatment. Each trace is also shown with the amount of D9-GB produced by the strains; values are expressed as μmoles detected per L cell volume assessed in each bag (inferred from CFU determinations and assumption of 1 cubic μm volume per cell). Each value represents the mean from three replicate injections of samples from three replicate bags, with standard error of the mean provided in parenthesis. **(B)** Population dynamics of the WT and *betTIBA* mutant after their individual inoculation onto MAP cut lettuce. Each bar represents the mean population size per g cut lettuce from three replicate bags; error bars are standard error of the mean. ND, not detected. * and ** indicate significant differences by Student's *t*-test with $P < 0.05$ and $P < 0.01$, respectively.

did not appear to affect these competition dynamics (data not shown).

## DISCUSSION

Plant injury allows for breaching of the natural physical barrier to infection and offers new opportunities for colonization by members of its microbiota, as is common in eukaryotic organisms at large. Human enteric pathogens have emerged as the causal agents of numerous outbreaks of foodborne illness associated with fruit and vegetables. These pathogens have the ability to exploit mechanically damaged or diseased tissue to enhance their growth and survival on plants (Aruscavage et al., 2006, 2008; Brandl, 2008; Goudeau et al., 2013; Simko et al., 2015), which are otherwise not as hospitable to enteric pathogens as the intestinal milieu (Brandl, 2006; Wiedemann et al., 2015). Epidemiological trends showing that a large proportion of outbreaks of EcO157 infection linked to leafy vegetables is associated with minimally processed product (Mandrell, 2009), which inherently harbors wounded tissue, indeed point to an important role of plant lesions in this emergence.

Bacterial colonists may benefit from increased nutrient availability in wounded plant tissue, but also must contend with the physico-chemical stresses resulting from plant cell leakage and the plant innate defense response to injury, as we demonstrated previously (Kyle et al., 2010). A close examination of the EcO157 transcriptome in lettuce lysates in the latter study, and in MAP shredded lettuce in a recent RNAseq-based study (unpublished data) revealed that the *betTIBA* genes for the import of choline and its catabolism to GB were upregulated, indicating that the human pathogen responds to low water potential in wounded lettuce leaves by production of this osmoprotective compound.

Studies by Beattie and co-workers revealed that the plant colonist *P. syringae* experiences water limitation during colonization of the leaf apoplast and responds by *de novo* synthesis of the compatible solutes trehalose and *N*-acetylglutaminylglutamine amide, and by uptake of quaternary ammonium compounds, including choline (Wright and Beattie, 2004; Chen et al., 2013; Yu et al., 2013). Free choline, choline-containing compounds, and GB have been measured in various amounts in the leaf tissue of Iceberg and romaine lettuce, cabbage, and spinach (Zeisel et al., 2003). Additionally, evidence for the extracellular presence of choline compounds and their

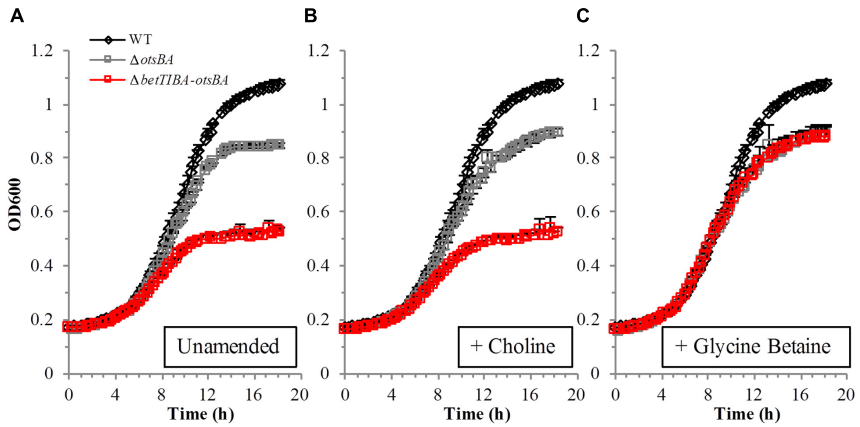


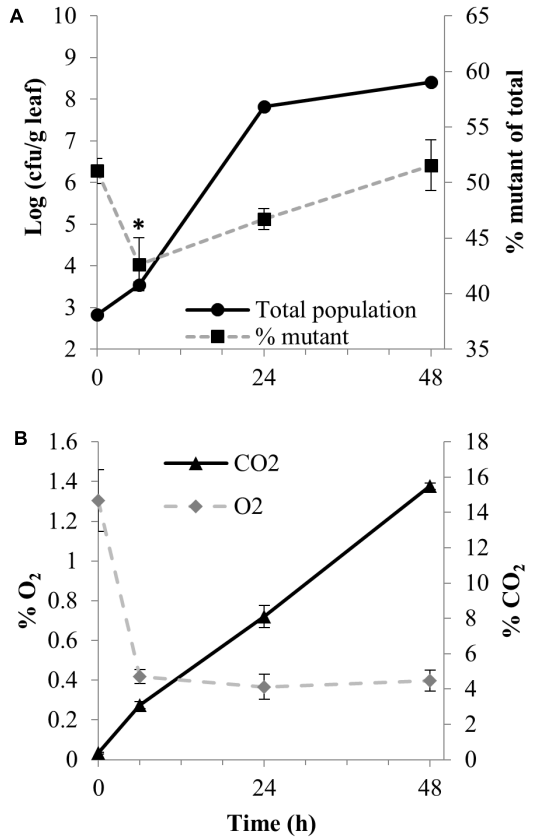

**FIGURE 6 |** Growth of EcO157 WT, and *otsBA* and *betTIBA-otsBA* mutants in hypertonic lettuce lysates. Cells were cultured in lysates amended with 800 mM NaCl, and with **(A)** $H_2O$ (control), **(B)** 1 mM choline, and **(C)** 1 mM GB. Each point represents the mean of determinations from two replicate cultures in wells of a 48-well plate, and standard error of the mean.

availability to *P. syringae* in bean leaves and germinating seeds was obtained using whole-cell bacterial reporters (Chen et al., 2013). Using LC-MS analysis, we demonstrate here that (1) choline, but not GB, nor proline, is present in large amounts in Iceberg lettuce leaves; and (2) during growth in Iceberg leaf lysates with enhanced osmotic strength, EcO157 TW14588 WT strain accumulated high intracellular levels of GB and was more depleted in choline than its *betTIBA* mutant. In contrast, the *betTIBA* mutant contained little GB but accumulated choline in high concentration. Additionally, the *betTIBA* mutant was significantly impaired in growth compared with the WT in lettuce lysate under low water potential conditions. Hence, choline is sufficiently abundant in lettuce tissue contents to be imported and used via the BetTIBA pathway in EcO157 for osmoprotection in that environment. We observed also that in contrast to pseudomonads (Chen et al., 2013; Wargo, 2013b), EcO157 does not utilize choline and GB as substrates for growth, thus making these solutes highly available to regulate intracellular osmotic potential. Despite the upregulation of *betTIBA* in lettuce lysate and the indication that EcO157 uses this pathway to produce GB to respond to osmotic stress, the similar growth of the *betTIBA* mutant and WT strains in lettuce homogenates with 550 mM NaCl (Student's *t*-test, $P > 0.05$) suggests that the mutant may have relied also on other compatible solutes under these conditions. Indeed, transcriptomic studies by Kocharunchitt et al. (2012, 2014) revealed that several osmoprotective pathways other than the Bet pathway could be induced in EcO157 Sakai by low water activity in complex culture medium that, similarly to lettuce lysate, may have provided a range of osmoprotective compounds.

Further evidence of the role of the BetTIBA pathway in the fitness of EcO157 in injured plant tissue was obtained by monitoring population dynamics after single inoculations of the WT and *betTIBA* mutant onto cut/shredded lettuce leaves under MAP conditions, a commodity with which outbreaks of EcO157 infections linked to produce have been predominantly associated (Mandrell, 2009). The population sizes of the *betTIBA* mutant were significantly lower than those of the WT after 1 and 6 h of colonization of MAP Iceberg lettuce (Student's *t*-test, $P < 0.05$), indicating that first, a natural choline pool is available to EcO157 in injured lettuce tissue and second, deficiency in choline import and its conversion to GB impacts the ability of EcO157 to tolerate osmotic stress on cut leaves and fully exploit that habitat.

Close examination of the fate of choline leaked from injured lettuce cells using tracing experiments and LC-MS analysis revealed that the supplementation of the EcO157 inoculum suspension with D9-choline resulted in the presence of D9-GB in the WT cells 6 h after colonization of MAP lettuce whereas this compound was present in the *betTIBA* mutant cells only at levels near the detection limit of the system. Uninoculated lettuce leaves to which D9-choline was added in the same experimental set up did not yield any D9-GB above the detection limit, corroborating the results of our LC-MS analysis of lettuce homogenates and that of others (Zeisel et al., 2003), which showed that lettuce is not a high GB-producing plant species. This also indicated that the indigenous microflora on lettuce produced little, if any, GB in cut lettuce under MAP conditions.

Consistent with previous reports that trehalose must be synthesized *de novo* for trehalose-mediated osmoprotection in *E. coli* (Klein et al., 1991), addition of this solute to minimal medium amended with high NaCl concentrations did not rescue growth of the WT, nor that of the single *otsBA* and *betTIBA* mutants (data not shown). In our previous transcriptomic studies, *otsBA* was downregulated in EcO157 in leaf lysates (Kyle et al., 2010) and cut lettuce leaves (unpublished), consistent with the high activity of the GB biosynthetic pathway in the pathogen in these two environments and the previously reported post-translational repression of OtsA by high intracellular levels of GB (Giaever et al., 1988). Indeed, in the absence of GB as a compatible solute in minimal medium with high salt, the *betTIBA* mutant grew similarly to the WT in our study, presumably because a lack of GB allowed for the activity of the trehalose biosynthetic pathway in the two strains. In order to avoid such compensation in osmoprotection by *de novo* trehalose synthesis in the *betTIBA*




C

| Time (h) | Choline (μmole/g leaf) |
|---|---|
| 0 | 7.38 (0.36) |
| 3 | 7.29 (0.46) |
| 6 | 5.99 (0.17) |
| 24 | 5.83 (0.28) |

**FIGURE 7 |** Competitive fitness of EcO157 WT and *betTIBA-otsBA* mutant in MAP cut lettuce, and chemical environment determinations. **(A)** Total EcO157 population size per g cut lettuce (●) and percentage of the *betTIBA-otsBA* mutant in the total EcO157 population (■) after their co-inoculation onto MAP cut lettuce. **(B)** Atmospheric $O_2$ and $CO_2$ content in the MAP bags from which the EcO157 strains were recovered. **(C)** Choline content in washes of MAP cut lettuce over time as measured via enzyme assay. All values represent mean measurements from three replicate bags. Standard error of the mean given as error bars or in parentheses. * indicates a significant difference in the % of mutant over total EcO157 cells at 6 h compared with the initial % at the time of inoculation (0 h), based on Student's *t*-test on transformed % data ($P < 0.05$).

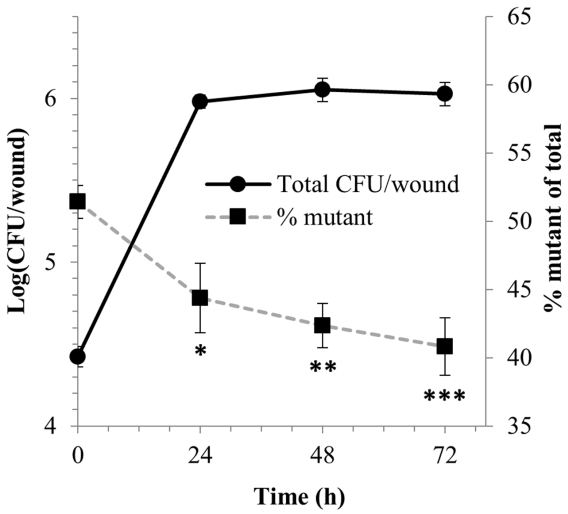



**FIGURE 8 |** Competitive fitness of EcO157 WT and *betTIBA-otsBA* mutant in leaf wounds on live lettuce plants. Total EcO157 population size per single wound per leaf (●) and percentage of the *betTIBA-otsBA* mutant in the total EcO157 population (■) after their co-inoculation during wounding of leaves on whole potted lettuce plants. All values represent mean determinations from five (immediately after inoculation) and ten (during incubation) leaf wound disks sampled from random leaves and random plants. *, **, and *** indicate significant difference in the % of mutant over total EcO157 cells at 24, 48, and 72 h compared with the initial % at the time of inoculation (0 h), as per Dunnett's multiple comparison test on transformed % data, with $P < 0.05$, 0.01, and 0.005, respectively. Error bars, standard error of the mean.

mutant, we also deleted the *otsBA* operon in the EcO157 Δ*betTIBA* background. This deletion of *otsBA* caused complete growth inhibition of the resulting Δ*betTIBA*-Δ*otsBA* double mutant compared with the WT in minimal medium amended with high NaCl concentrations. Moreover, transformation of the single *otsBA* mutant or double *betTIBA-otsBA* mutant with complementation plasmid pMBotsBA enabled greater growth of the complemented mutants than of the WT, further emphasizing the effectiveness of *de novo* produced trehalose in osmoadaptation of EcO157.

Competitive fitness studies in MAP cut lettuce and leaf lesions of whole live plants revealed that the *betTIBA-otsBA* mutant had significantly less adaptability in the injured plant tissue than the parental strain. In both study systems, the representation of the double mutant in the total EcO157 population decreased following inoculation at a ratio of 1:1 with the WT strain, indicating that the human pathogen coped more effectively with the conditions experienced in wounded leaf tissue when capable of mounting a full osmotic stress response. Similarly, a *P. syringae* mutant defective in BetT and two other major transporters of compatible solutes displayed reduced epiphytic fitness on bean and soybean leaves (Chen et al., 2013). In our study, the competitive fitness of the *betTIBA-otsBA* mutant showed a constant decline over 72 h after co-inoculation in the leaf lesions of whole lettuce plants. This is in contrast to MAP cut lettuce, in which the mutant had rebounded by 24 h post-inoculation, as indicated by the increase of the percentage of the mutant in the total EcO157 population. This increase in competitive fitness followed an adaptation phase (characterized by only a slight population size increase of 0.7-log within 6 h after inoculation). The period of recovery in competitive fitness of the mutant after initial adjustment to the environmental conditions of cut leaf tissue under MAP conditions correlated with the

period when the MAP bag atmosphere was near depletion in $O_2$ content. It is possible that by this time, the *betTIBA* mutant had already shifted its strategy to other osmoprotective mechanisms due to a lack of BetTIBA activity and thus was better adapted to colonizing the cut tissue, whereas the WT strain needed to readjust its osmoresponse once *betTIBA* expression was inhibited by these low levels of $O_2$ (Lamark et al., 1996) after 6 h of incubation. A lack of choline availability in the leaked contents of MAP lettuce was unlikely to have caused this change in population dynamics between the mutant and the WT since ample choline was still available to both strains even after 24 h of incubation.

Despite its significant proportional loss in the total EcO157 population in injured lettuce leaf tissue, the mutant retained considerable overall fitness. This may be expected considering the redundancy in osmotic stress response pathways in *E. coli* (Kempf and Bremer, 1998) and the range of compatible solutes that may be present in lettuce cell contents. Additionally, the heterogeneity in environmental conditions in damaged lettuce would also imply that not every single EcO157 encountered osmotic stress or mounted the same physiological response. More specifically in our study system, heterogeneous conditions experienced by EcO157 cells would first result at a larger scale from the presence of both intact and injured tissues. At the microscale, environmental heterogeneity as experienced by bacterial colonists in the phyllosphere has been observed in numerous studies (Joyner and Lindow, 2000; Brandl et al., 2001; Miller et al., 2001; Axtell and Beattie, 2002; Remus-Emsermann et al., 2012; Parangan-Smith and Lindow, 2013; Ryffel et al., 2016). Heterogeneity in water availability as sensed by individual *P. syringae* cells has been demonstrated on intact bean leaf surfaces using whole cell reporters (Axtell and Beattie, 2002). In damaged leaf tissue, heterogeneity may prevail at the microscale due to variations in water potential and in choline abundance driven by differences in the extent of injury among plant cells and hence, cell leakage across microsites colonized by the human pathogen, as well as due to variations in $O_2$-regulated *betTIBA* expression. Additionally, levels of free choline in damaged tissue are modulated by the plant defense response to injury, which involves rapid reincorporation of choline into plant membrane phospholipid phosphatidyl choline for cell repair in order to avoid water stress (Tasseva et al., 2004). This choline sink may have contributed to the overall decrease in choline concentration that we measured overtime in washes of cut lettuce and of leaf lesions of whole plants.

Consistent with EcO157 cellular accumulation of GB and not choline by the WT in lettuce lysate, amendment of hypertonic M9 medium with GB enhanced the growth rate of the WT more effectively than amendment with choline. This suggested that unlike in *P. syringae* (Chen and Beattie, 2008), GB uptake, which proceeds in *E. coli* via the ProU and ProP systems, or OmpC porins (Kempf and Bremer, 1998), is more efficient than choline import and its conversion to GB (in the presence of BetTIBA) under high osmolality. It is noteworthy, however, that chemical analyses of lettuce leaf contents showed that GB was present at low levels and that the related osmoprotectant proline betaine was undetectable (de Zwart et al., 2003; Zeisel et al., 2003). It will be valuable to determine if GB import would be a preferred osmoprotective strategy in EcO157 when GB is available in high abundance such as in the tissue of spinach leaves, which contain unusually high levels of this compound compared with other plant species (de Zwart et al., 2003; Zeisel et al., 2003). EcO157 contamination of minimally processed baby spinach caused a large outbreak in the United States in 2006 (Wendel et al., 2009), and differences in the ability of EcO157 to colonize damaged leaves of spinach and romaine lettuce have been reported (Khalil and Frank, 2010).

Given that leaf injury is inherent to agricultural pre-harvest practices and fresh-cut processing of leafy vegetables, it is critical to fully understand the physiology and behavior of enteric pathogens in this important ecological niche that provides a nutritional environment highly favorable to their proliferation. Our study demonstrates that the human pathogen EcO157 rapidly experiences osmotic stress in mechanical lesions of lettuce leaves and draws from an abundant pool of plant-derived choline to synthesize GB as a compatible solute for osmoadaptation. This opportune use of choline by EcO157 while other preferred osmoprotectants, such as proline, proline betaine and GB, are naturally scarce in lettuce tissue, illustrates the remarkable adaptability of enteric pathogens to a range of physicochemical conditions and stresses on plants.

## AUTHOR CONTRIBUTIONS

MB, RS, and RT designed experiments. RS, LH, YZ, and MB performed experiments. RS and MB analyzed data and wrote the manuscript. RT and LH edited the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02436/full#supplementary-material

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# Production of the Plant Hormone Auxin by *Salmonella* and Its Role in the Interactions with Plants and Animals

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The ability of human enteric pathogens to colonize plants and use them as alternate hosts is now well established. *Salmonella,* similarly to phytobacteria, appears to be capable of producing the plant hormone auxin via an indole-3-pyruvate decarboxylase (IpdC), a key enzyme of the IPyA pathway. A deletion of the *Salmonella ipdC* significantly reduced auxin synthesis in laboratory culture. The *Salmonella ipdC* gene was expressed on root surfaces of *Medicago truncatula*. *M. truncatula* auxin-responsive *GH3::GUS* reporter was activated by the wild type *Salmonella,* and not but the *ipdC* mutant, implying that the bacterially produced IAA (Indole Acetic Acid) was detected by the seedlings. Seedling infections with the wild type *Salmonella* caused an increase in secondary root formation, which was not observed in the *ipdC* mutant. The wild type *Salmonella* cells were detected as aggregates at the sites of lateral root emergence, whereas the *ipdC* mutant cells were evenly distributed in the rhizosphere. However, both strains appeared to colonize seedlings well in growth pouch experiments. The *ipdC* mutant was also less virulent in a murine model of infection. When mice were infected by oral gavage, the *ipdC* mutant was as proficient as the wild type strain in colonization of the intestine, but it was defective in the ability to cross the intestinal barrier. Fewer cells of the *ipdC* mutant, compared with the wild type strain, were detected in Peyer's patches, spleen and in the liver. Orthologs of *ipdC* are found in all *Salmonella* genomes and are distributed among many animal pathogens and plant-associated bacteria of the *Enterobacteriaceae*, suggesting a broad ecological role of the IpdC-catalyzed pathway.

**Keywords: indole acetic acid, auxin, RIVET, sprouts, produce safety, *Medicago truncatula,* enteric-plant interactions, tryptophan**

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## INTRODUCTION

Recurrent outbreaks of gastroenteritis caused by non-typhoidal *Salmonella* and shigatoxigenic *E. coli* and linked to the consumption of sprouts, vegetables, fruits and nuts led to the hypothesis that these enteric pathogens use plants as alternate hosts (Brandl, 2006; Brandl et al., 2013; Hernández-Reyes and Schikora, 2013; Walsh et al., 2014). Both *Salmonella* and *E. coli* can persist in manure, soil and water for at least several weeks or months (Brandl, 2006; Brandl et al., 2013; Martínez-Vaz et al., 2014; Wiedemann et al., 2014; Ongeng et al., 2015), from which, they can

get established on plants. *Salmonella* and *E. coli* colonized seedlings from contaminated manure, and following the feeding of the infected plants to snails and mice, these bacteria were detected within animals and shed in their feces (Franz et al., 2008; Semenov et al., 2010). While these studies establish that enteric pathogens can use plants as alternate hosts or vectors, less is known about the mechanisms by which they interact with plants, despite the significant progress in the characterization of interactions between enteric pathogens and plants (Klerks et al., 2007a,b; Hernández-Reyes and Schikora, 2013; Wiedemann et al., 2014; Han and Micallef, 2016; de Moraes et al., 2017). Plants are colonized with diverse assemblages of epiphytes and endophytes, which interact with each other and their plant hosts in a variety of ways. Epiphytes often congregate at preferred sites, such as hydathodes, stomata, lesions, or breaks in the epidermis caused by lateral root emergence, which offer more nutrients and potential routes for internalization (Leveau and Lindow, 2001; Lindow and Brandl, 2003). *Salmonella* has been shown to seek similar niches during plant colonization, as it can establish colonies on plant surfaces and may enter internal tissues under conducive conditions (Brandl and Mandrell, 2002; Dong et al., 2003; Klerks et al., 2007a; Golberg et al., 2011; Gu et al., 2013a). However, *Salmonella* likely faces considerable competition for the colonization of these preferred sites and may benefit from mechanisms that allow it to manipulate plant hosts to increase the availability of growth-conducive sites.

Production of plant hormones, and especially indole-3-acetic acid (IAA), is one of the mechanisms by which plant-associated bacteria and fungi manipulate plant growth and development. Microbial production of IAA alters root architecture to increase availability of nutrients to microbes capable of producing this plant hormone. Microbially derived auxins also contribute to the formation of new organs (such as nodules and galls), suppress plant defenses and regulate virulence in phytobacteria (Lindow and Brandl, 2003). The recognition of the roles auxin signaling in plant-microbial interactions led to the hypothesis that it functions as a reciprocal signal in these relationships (Duca et al., 2014).

The synthesis of auxin is widespread among phytobacteria (Duca et al., 2014; Ludwig-Müller, 2015). At least five bacterial IAA biosynthesic pathways exist. Of these IAA biosynthetic pathways, those proceeding via indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) are the two most widespread and best characterized. Tryptophan is the general precursor of IAA biosynthesis (Patten et al., 2013). The IAM pathway is a two-step process, conserved in three Kingdoms of Life. In the IAM pathway, tryptophan is first converted by tryptophan-2-monooxygenase (encoded by *iaaM*) into IAM, and then to IAA by IAM hydrolase (encoded by *iaaH*) (Lehmann et al., 2010). The IPyA pathway in bacteria is a three-step process mediated by the conversion of indole-3-pyruvate into indole-3-acetaldehyde by indole-3-pyruvate decarboxylase (encoded by *ipdC*) (Brandl and Lindow, 1996; Brandl et al., 1996). Indole-3-acetaldehyde is then converted into IAA. Production of IAA via IpyA in the common bacterial plant colonist *Pantoea agglomerans* contributes to its epiphytic fitness (Brandl and Lindow, 1996, 1998). A given phytobacterial species may commonly possess more than one pathway, suggesting a high degree of redundancy, which is likely indicative of an important phenotype (Patten et al., 2013). For example, the plant pathogen *E. herbicola* pv. *gypsophilae* harbors both pathways, which appear to function in controlling divergent behaviors. The deletion of *ipdC* reduced the epiphytic fitness of this pathogen during active growth on bean leaves, whereas a mutation in *iaaH* was linked to decreased virulence in *Gypsophila paniculata* (Manulis et al., 1998).

Genomic analysis demonstrates that all salmonellae have a copy of the *ipdC* gene, similar to that found in other γ-proteobacteria. Although the presence of the *ipdC* gene in *Salmonella* genomes has been previously detected (Spaepen et al., 2007), its function in *Salmonella* remains unclear. The production of IAA by *Salmonella*, therefore, could enable epiphytic survival in a manner similar to other phytobacteria and could represent a conserved strategy for colonization of plants as a typical part of its life cycle. In this study, we tested the hypothesis that *S. enterica* produces IAA through the IPyA pathway and that the *ipdC*-mediated IAA production impacts the outcomes of the interactions of *Salmonella* with a model plant, *Medicago truncatula*.

# MATERIALS AND METHODS

## Culture Conditions and Strain Construction

All strains used in this study are listed in **Table 1**, and primers used for the strain construction are in **Table 2**. Unless otherwise specified, strains were routinely grown in LB medium (Miller, 1972). Minimal A medium was prepared as in Ludwig-Müller (2015). A kanamycin marked *ipdC* mutant was constructed using Datsenko and Wanner mutagenesis (Datsenko and Wanner,

**TABLE 1 |** Strains used in this study.

| Strain | Relevant genotype | Source |
|---|---|---|
| 14028 | Wild-type *S. enterica* serovar Typhimurium | American type culture collection |
| JS246 | 14028 *yje*P8103::*res*1-*tetAR-res*1 | Merighi et al., 2005 |
| CEC1002 | 14028 Δ*ipdC*::FRT-*kanR*-FRT | This Study |
| CEC2002 | 14028 Δ*ipdC* | This Study |
| CEC5002 | JS246 $P_{ipdC}$-*tnpR-lacZY* Δ*ipdC* | This Study |
| CEC8002 | JS246 *ipdC-tnpR-lacZY* | This Study |
| Plasmids | Relevant feature(s) | Source |
| pGFP-ON | pGFP *dppA*-GFP | Noel et al., 2010 |
| pKD4 | *oriR6K bla rgnB* FRT-*kanR*-FRT (kanR) | Datsenko and Wanner, 2000 |
| pKD46 | *repA101ts oriR101 araC* $P_{araB}$-λ*Red(γ-β-exo)-tL3*) (ampR) | Datsenko and Wanner, 2000 |
| pCP20 | *repA101ts* $\lambda_{pR}$-Flp *ci857 (*ampR, kanR*)* | Cherepanov and Wackernagel, 1995 |
| pCE70 | *oriR6K* FRT-promoterless *tnpR-lacZYα* (kanR) | Merighi et al., 2005 |
| pCE71 | *oriR6K* FRT-promoterless *tnpR-lacZYα* (kanR) | Merighi et al., 2005 |

**TABLE 2 |** Primers used in this study.

| Primer | Sequence | Use |
|---|---|---|
| BA184 | CAAAAAGTCGCATAAAAATTTATCC | RIVET confirmation |
| K2 | CGGTGCCCTGAATGAACTGC | FRT-*kanR*-FRT confirmation Datsenko and Wanner, 2000 |
| CEC134 | TCCCCCTGTGGCGTGAAT | *ipdC* confirmation |
| CEC135 | CCTGGCTATTGCTGGCGG | *ipdC* confirmation |
| CEC207 | GCATTCCTTAATACTCAACATAATAT CAACGTCAGAAGGAAAGCTGTCt gaggctggagctgctt | Δ*ipdC* construction |
| CEC208 | TTACTGCGTACCGTGACCCGGGCG CTGGAAGCCCGCAACGGGGGATA Atgtaggctggagctgctt | *ipdC-tnpR-lacZY* construction |
| CEC209 | TGGCCCCCGCTGCGCCGGATTAG GGTTCGTGACGGTTGGCGGCCAG CAcatatgaatatcctccttag | ipdC_LR |
| CEC210 | GGACAGCCAGTGCGGATT | *ipdC* confrimation |

*Merighi et al. (2005), Noel et al. (2010), Datsenko and Wanner (2000), Cherepanov and Wackernagel (1995).*

2000) with primers CEC207 and CEC209. The deletion was confirmed with primers BA505 and CEC135. An unmarked mutant was constructed by removing the FRT-*kanR*-FRT cassette with plasmid pCP20 as in Datsenko and Wanner (2000). The deletion was confirmed with primers CEC134 and CEC135. RIVET reporters were constructed as in Merighi et al. (2005) by placing *tnpR* immediately downstream of the *ipdC* stop codon or by placing *tnpR* immediately downstream of the promoter region in a stream where *ipdC* has been removed between the start and stop codons. Constructs were confirmed with primers CEC134 and BA184 or CEC210 and BA184. Activation of the RIVET reporter is scored as the loss of the tetracycline resistance marker in the recovered cells (this occurs because activation of the promoter of interest leads to the expression of the TnpR recombinase and the resulting excision of the tetracycline-resistance marker encoded within "res" sites that are substrates for TnpR; Merighi et al., 2005). A plasmid carrying constitutively expressed *gfp* reporter driven by the *Salmonella dppA* promoter (Noel et al., 2010) was introduced into strains as needed via electroporation. To ascertain that the genetic manipulations did not result in a growth deficiency, strains were grown in LB broth shake cultures at 22°C and 37°C for 10–24 h and OD600 measurements were taken periodically, at 30 min or 2 h intervals.

## Detection of IAA Using Salkowski Reagent

*Salmonella enterica* sv Typhimurium (*S.* Typhimurium thereafter) cultures were grown overnight from glycerol stocks at 37°C. 1 mL was washed 3x in PBS and was diluted 1,000x in 5 mL Minimal A medium with or without 1 mM tryptophan. Cultures were incubated at 22°C for 72 h. Supernatant was harvested from 1 mL of culture by centrifugation for 15 min at 7,000 × g. The supernatant was transferred to a 12 mm × 75 mm glass tube and 2 mL of fresh Salkowski reagent R1 (Glickmann and Dessaux, 1995) was added and vortexed to mix. The tubes were incubated for 30 min at 30°C in the dark, aliquots were then withdrawn and A540 was measured using the Shimadzu biospec-mini.

## IAA Extraction, Purification, and Identification

Hundred millliter of *Salmonella* culture filtrates were extracted twice with 150 and 100 mL of ethyl acetate. The extracts were rotary evaporated over a water bath at 40°C. The resulting yellow oily residue was brought up in 600 μl of methanol. The extracts and standards (IAA, tryptophol and IPyA) were subject to reverse phase ($C_{18}$) liquid chromatography using Waters Atlantis dC18-5 μM column using isocratic elution (72 water 7.2% acetic acid 20.8% methanol), as before (Brandl and Lindow, 1996; Brandl et al., 1996). Eluting substances were detected with a UV/VIS detector set to 230 and 280 nm (Brandl and Lindow, 1996; Brandl et al., 1996). Low Resolution Electrospray Ionization LCMS was carried in a Thermo Scientific LTQ LC-MS instrument using a Grace Vydac 218TP C18 (5 μ, 7.5 cm, 2.1 mm ID) column. LCMS was run using a gradient of 10% acetonitrile (with 0.1% formic acid) and 90% water (with 0.1% formic acid) to 100% acetonitrile (with 0.1% formic acid) from 0 to 15 min and then 100% acetonitrile with formic acid was continued till 21 min.

## Plant Infections

Seeds of *M. truncatula* A17 (wild type) and transgenic line containing the *GH3::GUS* reporter (van Noorden et al., 2007) were surface sterilized in ethanol and then in a diluted chlorox bleach solution as before (Mathesius et al., 2003) and stratified overnight at 4°C. Imbibed seeds were transferred into individual growth pouches and were left to germinate at room temperature overnight. Once radicles reached ∼1 cm, six seedlings (per treatment) were inoculated with 100 μl of the suspension containing ∼5,000–10,000 CFU of either CEC1000 (*S.* Typhimurium 14028 marked with a kanamycin cassette in a neutral site) or CEC1002. Plants were watered as needed with the N-free Hoagland solution. To detect activation of the *GH3::GUS* reporter, seedlings were withdrawn after 48 hrs and 6 days past infection, and stained with X-gluc (10 mg/ml) in 0.2 mM Phosphate buffer (pH 7.2) overnight. They were then washed 3 times with cold phosphate buffer to remove excess substrate and the seedlings were kept in 50% ethanol/phosphate buffer at 4C. They were imaged under a dissecting microscope Olympus MVX10 fit with a MicroFIRE camera (Optronics).

For the reporter assays, washed RIVET reporter suspensions (containing ∼10,000–100,000 CFU) were inoculated onto *M. truncatula* seedlings as described in Supplemental Information. At indicated time intervals, seedlings were excised from pouches, blended with a glass mortar and pestle and dilution plated onto XLD with kanamycin. Bacteria were patched from these onto LB with tetracycline.

To detect changes in lateral root formation, seeds of *M. truncatula* A17 were surface sterilized, imbibed and then transferred into growth pouches. Seedlings were inoculated with 100 μl of the washed bacterial suspension containing ∼5,000–10,000 CFU of either CEC1000 (*S.* Typhimurium 14028

marked with a kanamycin cassette in a neutral site) or CEC1002 (*ipdC::kan*).

## Mouse Infections

BALB/c mice were inoculated by oral gavage, which results in a systemic infection. Prior to the infections, *S. enterica* sv. Typhimurium 14028 and CEC1002 were streaked on LB agar and incubated at 37°C overnight, colonies were harvested and re-suspended in 1 ml of sterile PBS. Each suspension was adjusted to a final dilution of $\sim 10^8$ CFUs/ml. Groups ($n = 3$) of female BALB/c mice received three doses of *Salmonella* in 0.2 ml of PBS. Inoculum doses were confirmed by serial titration, and were found to be $10^6$, $10^4$, and $10^2$ CFU per injection dose for the wild type, and $2.2 \times 10^6$, $2.2 \times 10^4$, and $2.2 \times 10^2$ CFU per injection for the *ipdC* mutant. Animals were observed daily for 7 days after infection and distressed animals presenting signs of morbidity were euthanized, their organs were not sampled. The experiment was terminated at 7 days, and all surviving animals were sacrificed. Liver, spleen, large intestine, and Peyer's patches were harvested. Tissues were weighed, and homogenized in sterile PBS in TissueLyser II (Qiagen). Bacterial homogenates were serially diluted in PBS and plated onto Xylose-Lysine Deoxycholate (XLD) agar (Beckton, Dickinson and Company) plates, followed by incubation at 37°C overnight for bacterial CFU counts. All animal care and procedures were in accordance with institutional policies for animal health and well-being and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

## Data Analysis

Synteny analysis was carried out using default parameters in the SyntTax web server (Oberto, 2013). Sequences were imported from GenBank. UV peak integration was conducted with proprietary Waters software, and analysis of mass spectral data was carried out using Thermo Scientific proprietary software. Means and standard error of the mean were calculated in Microsoft Excel v. 14.0.0.

# RESULTS

*ipdC is conserved in salmonellae*. *ipdC*, the gene encoding indole-3-pyruvate decarboxylase, a key enzyme in the IPyA pathway, is common in many members of *Enterobacteriaceae*; including organisms that are commonly associated with animals as well as with plants. Genomic analysis showed that all available *Salmonella* genomes harbor *ipdC,* but that it is absent in *E. coli*, *Shigella*, or *Erwinia* genomes and in some strains of *Enterobacter*. The presence or absence from the enterobacterial genomes does not seem to correlate with the source of the isolates, and *ipdC* was as likely to be found in the genomes of animal as of plant isolates of this genus. Synteny analysis revealed a distribution of orthologs in the genomes of many strains belonging to the *Enterobacteriaceae* where it is generally found within similar genomic context (**Figure 1**). In *Salmonella*, *ipdC* is located downstream of an ortholog of the glucokinase *glk*, and a putative ion channel protein known as YfeO. *glk* was conserved at this location in all genomes except in *Pectobacterium*, in which it is present at another site. Similarly, YfeO is present at different sites in *Citrobacter*, *Panteoa* and *Pectobacterium* genomes. Immediately downstream of *ipdC* in the *Salmonella* genome are an ortholog of the L-glyceraldehyde 3-phosphate reductase *gpr,* and an uncharacterized periplasmic protein YpeC. Although its function is currently unknown, *ypeC* was present at a similar location in all genomes. *gpr* (*yghZ*) was present between *ipdC* and *ypeC* except in *E. coli* and *Pectobacterium*. These results suggest that the *Salmonella ipdC* may encode an ortholog of a well-characterized indolepyruvate decarboxylase, responsible for IAA synthesis in other bacteria. Therefore, we tested whether *ipdC* is capable of directing IAA production in *S. enterica* sv Typhimurium.

## IAA Production by *Salmonella* via *ipdC*

The ability of *Salmonella* to produce IAA was first tested in culture in Minimal A medium using a colorimetric assay based on the Salkowski reagent. While there was no auxin production by either the wild type or the isogenic *ipdC* mutant grown in unammended Minimal A medium, production of IAA was detected in the medium supplemented with 1 mM L-tryptophan, the precursor for the IPyA pathway (**Figure 2A**). The intensity of absorbance at 540 nm after addition of the Salkowski reagent, which correlates with the presence of indole compounds, including IAA, was significantly lower in the culture filtrate extract of the *ipdC* mutant than that of the wild type strain (**Figure 2A**). When culture filtrate extracts were separated using HPLC, UV peaks corresponding to IAA and tryptophol were observed for the wild type, but were strongly reduced for the *ipdC* mutant (**Figure 2B**). Tryptophol is a product of a reduction of indole-3-acetaldehyde, the product of the reaction catalyzed by *ipdC*. The production of IAA by the wild type *S. enterica* sv. Typhimurium 14028 was confirmed by LC-MS/MS. Production of IAA was significantly reduced (but not completely eliminated) in the isogenic strain lacking *ipdC* (**Figure 2C**). In laboratory cultures, expression of the *ipdC* RIVET (recombinase-based *in vivo* expression technology) reporter was low (less than 10%) after 24 h of incubation, however, it increased by 72 h (**Figure 2D**). Consistent with the production of IAA in laboratory media, a recombinase *in vivo* expression technology (RIVET) reporter in *ipdC* was expressed in a laboratory medium at 45–55% (**Figure 2D**). Expression of the reporter in the *ipdC* background was considerably lower than in the wild type, suggesting a feedback regulation. However, supplementation of the medium with 50 μM IAA did not significantly affect expression of the *ipdC* RIVET reporter in the wild type or in the *ipdC* mutant. Despite clear differences in the amount of IAA produced in a minimal medium with and without tryptophan, the *ipdC* RIVET reporter was expressed at similar levels in the minimal medium with and without tryptophan (**Figure 2D**), suggesting that the availability of the substrate is critical for the function of the IpdC enzyme (as indirectly evidenced by the accumulation of IAA in culture) but not for transcription of the encoding gene.

## Plant Responses to IAA from *Salmonella*

To determine whether *Salmonella* produces auxin on plant surfaces and whether this bacterially-produced plant hormone

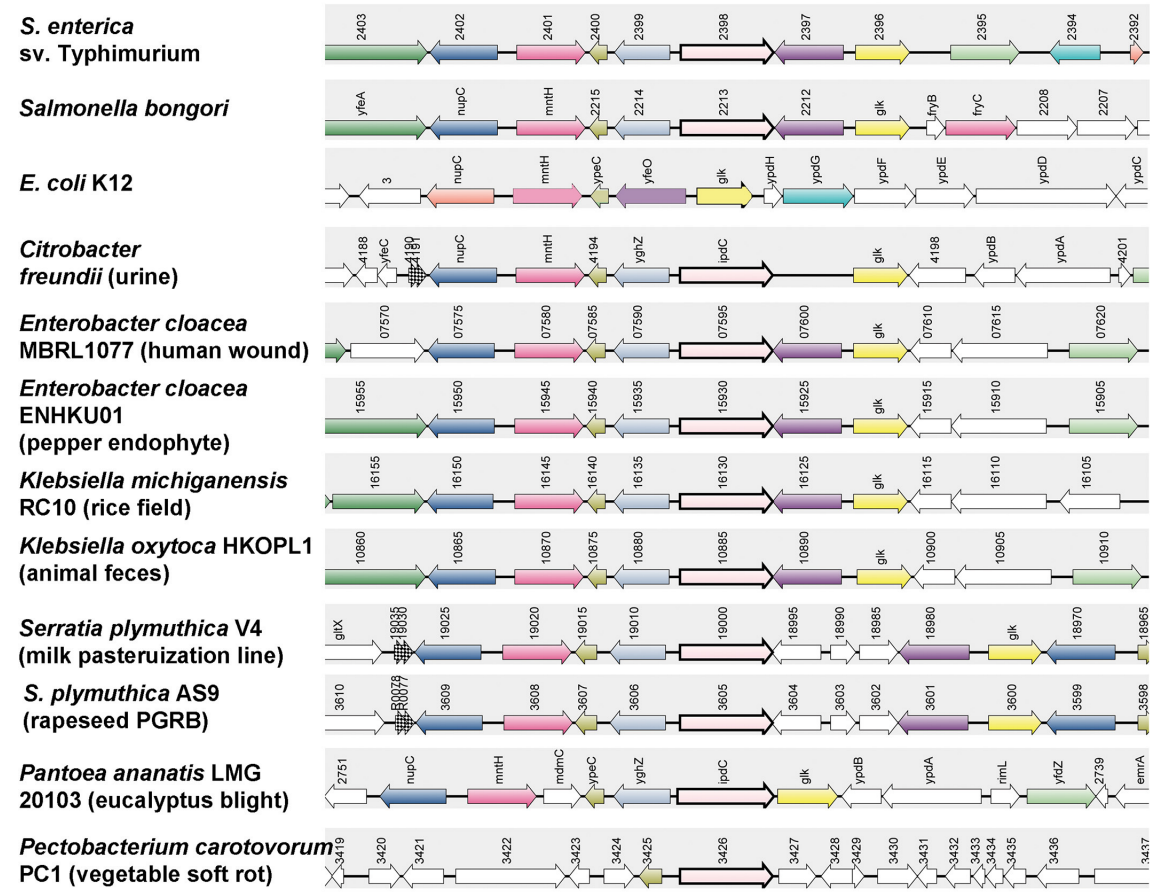


**FIGURE 1 |** Synteny analysis of *ipdC* in *Enterobacteriaceae*. Genes homologous to the *Salmonella ipdC* were retrieved from NCBI. Synteny analysis was carried out using SyntTax (Oberto, 2013). Orthologs of *ipdC* are shown as pink arrows with a thick black border.

has a function during colonization of plant hosts, *ipdC* expression was assessed in the *M. truncatula* rhizosphere. The *ipdC* RIVET reporter was resolved at ∼5–8% during the first 3 days of root colonization, reaching 22 and 37% at seven and 10 days post-inoculation, respectively (**Figure 3A**). When compared with the expression of the same reporter in Minimal A medium (**Figure 2D**), it does not appear that *ipdC* is expressed stronger in the rhizosphere, nevertheless, it is clear that *ipdC* is expressed in *S.* Typhimurium on roots during colonization. Next, we tested the consequences of the *ipdC* mutation using a *GH3::GUS* reporter in *M. truncatula*. Genes belonging to the *GH3* (Gretchen Hagen 3) family are among the regulators that control the dynamic process of endogenous auxin homeostasis (Yang et al., 2015). This *GH3::GUS* reporter is normally expressed in the stele of the root, while expression in the cortical cells results from exposure to exogenous IAA, including that produced by bacteria such as by the symbiotic *Sinorhizobium meliloti* (Mathesius et al., 1998, 2000; van Noorden et al., 2007). As shown in **Figure 3B**, the *GH3*::*GUS* reporter was broadly induced in the cortical tissue of *M. trucatula* roots after inoculation with the *S.* Typhimurium wild type strain, but only at discrete locations in the cortex after that with the *ipdC* mutant. Taken together, the activation of the plant *GH3::GUS* reporter by *Salmonella* and the expression of the *Salmonella ipdC* gene in the rhizosphere suggest that this human pathogen synthesizes auxin via IpdC during plant colonization and in quantities that are detectable by the plant cells and sufficient to cause changes in plant gene expression.

## Production of IAA Creates a Niche for *Salmonella*

To determine how *Salmonella* may benefit from the production of IAA, we first tested whether perception of the bacterially-produced IAA led to observable phenotypic changes in the inoculated plants. Secondary root initiation is a phenotype known to be regulated by IAA (Spaepen et al., 2014; Bensmihen, 2015). Therefore, we evaluated formation of secondary roots in the seedlings inoculated with the wild type *Salmonella* or the isogenic *ipdC* mutant. As shown in **Figure 3C**, few secondary roots had emerged in 2-week old *M. truncatula* seedlings that were not inoculated with *Salmonella* and treated with the nutrient solution only (control). Seedlings inoculated with the *S.* Typhimurium wild type strain showed 5–12 secondary roots, while seedlings inoculated with the *ipdC* mutant had 2–7 secondary roots per plant. When the wild type and the *ipdC*

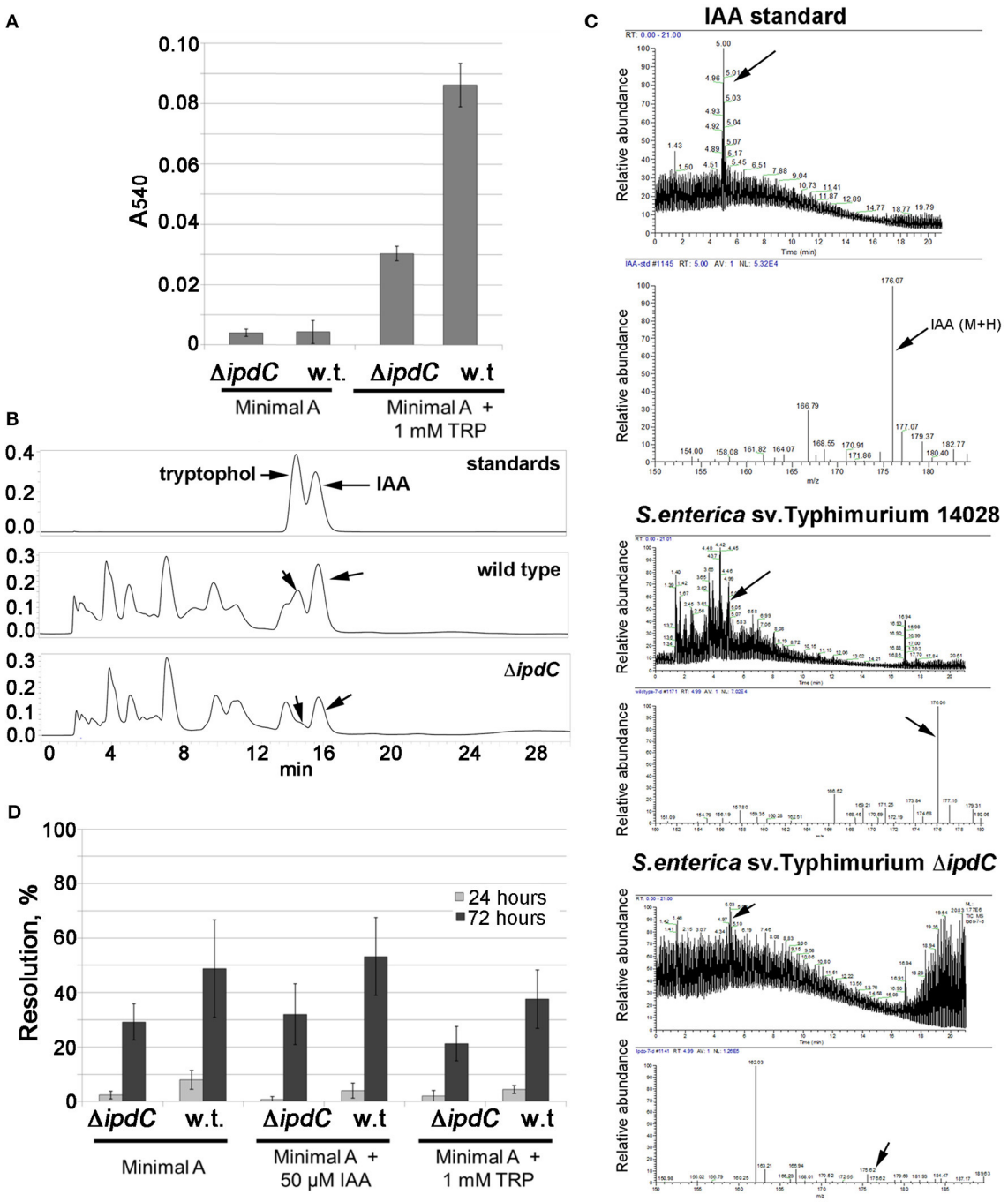


**FIGURE 2 |** Production of IAA in *ipdC*-dependent manner by *Salmonella enterica* sv. Typhimuirum 14028. **(A)** Detection of IAA by the Salkowski reagent. Results are from replicates from three independent experiments, averages are shown. Error bars are standard deviations. **(B)** HPLC detection of IAA and tryptophol in spent cultures of the wild type and the *ipdC* mutant. Hydrophobic fractions of *Salmonella* culture filtrates were subjected to reverse phase ($C_{18}$) liquid chromatography, eluted isocratically with acidified water/methanol and eluting substances were detected with a UV/VIS detector set to 230 and 280 nm. **(C)** Low Resolution Electrospray Ionization LC-MS of the hydrophobic fraction of *Salmonella* culture filtrates. LTQ LC-MS was carried out using a Grace Vydac 218TP C18 and acetonitrile/water gradient. **(D)** Expression of the *ipdC* RIVET reporter in the wild type and Δ*ipdC* backgrounds was measured in Minimal A medium with and without synthetic IAA or tryptophan in cultures incubated at 22°C for 72 h. Samples were streaked to xylose lysine deoxycholate (XLD) agar with kanamycin at 24 and 72 h. Plates were incubated at 37°C overnight and colonies were patched to LB agar with tetracycline to quantify resolution. Experiments were repeated five times (without technical replications), averages from the five experiments are shown. Error bars are standard deviations.

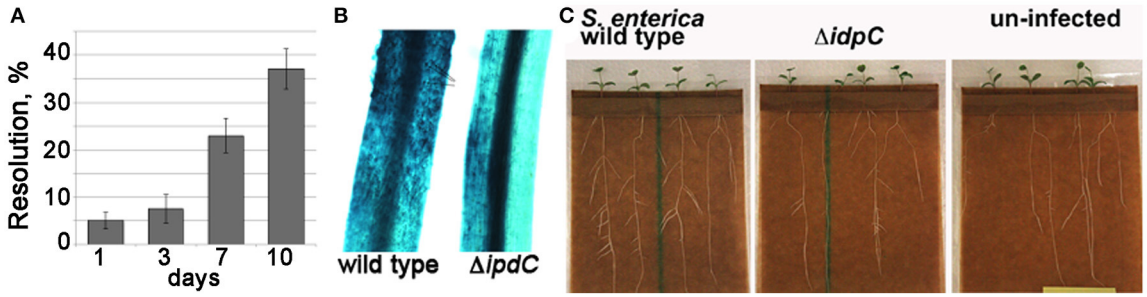


**FIGURE 3 |** *M. truncatula* responds to the activation of *ipdC* in the rhizosphere. **(A)** Resolution of the *Salmonella ipdC* RIVET reporter was measured on the seedlings grown in pouches from surface-sterilized *M. truncatula* Jemalong A17 seeds. **(B)** Activation of the *M. truncatula* auxin-responsive *GH3::GUS* reporter was measured as in Mathesius et al. (2003). Black arrows point to representative sites of *GH3::GUS* expression in the cortex, likely a result of the IAA production by the wild type *Salmonella*. Six seedlings were inoculated per treatment, and results of a representative experiment (6 days post- infection) are shown. Care was taken to excise root segments at the same developmental stage **(C)** Inoculation with the wild type *Salmonella* Typhimurium 14028 increases lateral root formation in *M. truncatula* Jemalong A17.

mutant were tagged with a plasmid constitutively expressing GFP, the *ipdC* mutant was found to be evenly distributed throughout the root surface and on root hairs, while the wild type tended to form large aggregates at the sites of the lateral root emergence (**Figure 4**). However, we did not observe differences in the total number of bacteria recovered from the seedlings (data not shown). In laboratory LB shake cultures, the wild type and the *ipdC* mutant grew with the same kinetics and to the same final optical densities at 22 and 37°C.

## Deletion of *ipdC* Reduces Virulence in the Mouse Model

We hypothesized that because IAA is a plant hormone, the function of *ipdC* may be confined to the interaction of *Salmonella* with its plant hosts. To test this hypothesis, the comparative ability of the *S.* Typhimurium wild type and *ipdC* mutant to colonize mice following an oral gavage was determined. The hypothesis that the mutation in *idpC* impacts virulence in mice was proven null (**Figure 5**). While the *ipdC* mutant did not differ from the wild type in its ability to establish within the intestine, it was deficient in the colonization of the liver and the spleen (**Figure 5**). However, due to the small number of animals infected per dose, robust statistical analyses of these data were not possible.

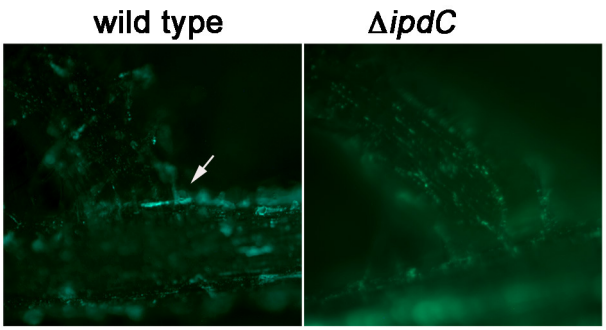


**FIGURE 4 |** Colonization of sites of lateral root emergence by *Salmonella* wild type and Δ*ipdC*. *M. truncatula* seedlings were inoculated with either CEC1000 or CEC1002 labeled with pGFP-ON. Fluorescence of the bacteria on roots of seedlings was documented 12 days after inoculation using Olympus BX41 epifluorescent microscope with 3.3 MPX Olympus camera. The white arrow points to the microcolony of the wild type *Salmonella* at the base of the lateral root.

## DISCUSSION

The presence of *ipdC* in *Salmonella* was first noted by Spaepen et al. (2007) while constructing a phylogenic analysis to support the differentiation of the *ipdC* product of *Azospirillum brasilense* as a phenylpyruvate decarboxylase in contrast to the classical indole-3-pyruvate decarboxylase product of *ipdC* in *Enterobacter cloacae* (Spaepen et al., 2007). Of the 33 species with genomes containing putative *ipdC* homologs considered in their analysis, *ipdC* of *S.* Typhimurium LT2 grouped independently but was most closely related to *E. cloacae* and *Pseudomonas putida*, the strains most distant from *A. brasilense*, supporting the hypothetical function of *Salmonella ipdC* in synthesis of auxin.

Our analysis of genomes in the currently available databases, revealed that *ipdC* is present in all *Salmonella* genomes, including that of the obligate human pathogen *Salmonella* Typhi. The synteny analysis presented here confirms also the common presence of *ipdC* in the genomes of both human and plant pathogenic *Enterobacteriaceae*.

The results of this work suggest that the *Salmonella ipdC* contributes to the production of IAA in laboratory cultures, that this gene is expressed in laboratory media and during root colonization, and that the product of the reaction involving IpdC activates plant auxin-responsive promoter. Inoculation of *M. truncatula* seedlings with the wild type *Salmonella,* but not that with the *ipdC* mutant resulted in increased production of lateral roots. Although the wild type and the *ipdC* mutant were recovered from the seedlings in similar numbers, the wild type cells tended to cluster at the sites of lateral root emergence, where high tryptophan availability to bacterial colonizers was reported with a whole-cell biosensor (Jaeger et al., 1999), whereas cells

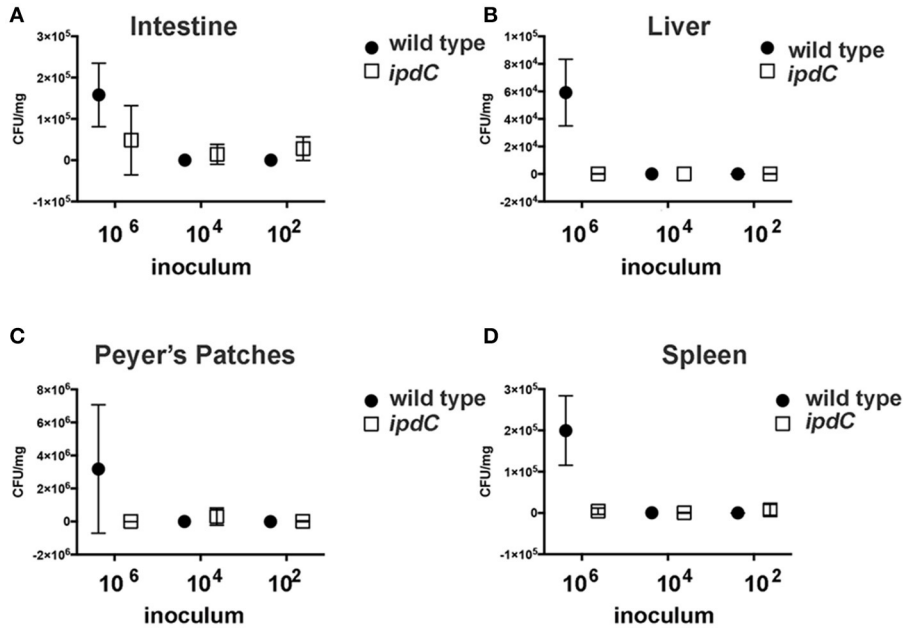


**FIGURE 5 |** Recovery of *Salmonella* from mouse organs. Three female BALB/c mice per three infectious doses were infected with the wild type *S.* Typhimurium 14028 or an isogenic *ipdC* mutant by oral gavage. After 7 days, surviving animals were euthanized, and organs harvested for the determination of bacterial load. Recovered CFU of the mutant (empty square) and the wild type (filled circle) are shown. Error bars are standard deviations. When error bars are not shown, this indicates that only a single animal was remaining upon completion of the experiment. *Salmonella* recovery from intestine **(A)**, liver **(B)**, Peyer's patches **(C)** and spleen **(D)** is shown.

lacking *ipdC* were evenly distributed in the rhizosphere. This indicates that *Salmonella* cells located at sites where tryptophan is likely abundant benefit from IAA production to achieve greater cell densities. (Brandl and Lindow, 1998) suggested that IAA production by bacterial plant colonists may increase their fitness by enhancing nutrient leakage from plant cells by cell wall remodeling (Brandl and Lindow, 1998). Collectively, these results point at the ability of *Salmonella* to manipulate their plant hosts through IAA production although the entire suite of plant functions subject to this manipulation is not yet known. Plant pathogens, epiphytes, symbionts and endophytes employ auxin to thwart plant defenses, create new organs (nodules and galls), alter plant tissue development, and induce leakage of nutrients (Lindow et al., 1998; Lindow and Brandl, 2003; Duca et al., 2014; Talboys et al., 2014; Ludwig-Müller, 2015). While we present evidence that *Salmonella* expresses *ipdC* in the plant rhizosphere and that plants sense and respond to the resulting presence of this exogenous auxin, the broader implications of our observations for the ecology of *Salmonella* on plants remain unknown. In interpreting the results of this study, it is important to recognize that they were conducted in growth pouches that are largely devoid of native phytomicrobiota, which can strongly influence the outcomes of interactions between human enteric pathogens and plants (Klerks et al., 2007a; Teplitski et al., 2011; Brandl et al., 2013; Gu et al., 2013b). It is not clear whether production of IAA by *Salmonella* is of consequence during interactions under the field conditions, in natural soils where *Salmonella* must compete with other rhizosphere microbes, many of which produce IAA as well. In natural environments, effects of IAA on plants are concentration-dependent: low concentrations could induce formation of lateral roots, suppress host defense responses, while at higher doses it can inhibit plant growth (Kazan and Manners, 2009; Duca et al., 2014; Ludwig-Müller, 2015). It is clear that the production of IAA is dependent on the availability of tryptophan. In the rhizosphere, bacteria that produce more tryptophan, rather than depend on exogenous sources, can produce more IAA and therefore tend to have an advantage (Duca et al., 2014). At least initially, in a gnotobiotic rhizosphere *Salmonella* appears to utilize tryptophan that is exuded from *M. truncatula* seedlings. It is not certain that *Salmonella* can scavenge sufficient tryptophan or synthesize it *in situ* when it competes with the native rhizosphere microorganisms. Therefore, the ecological role of *Salmonella*-synthesized IAA in natural interactions with plants remains to be elucidated.

The role for *ipdC* during *Salmonella* mouse infection was unexpected. We initially hypothesized that the production of IAA must represent a unique adaptation to the plant-associated lifestyle. Upon further reflection, it should come as no surprise that the same molecule may have beneficial functions in a number of environments, where successful opportunists (such as non-typhoidal *Salmonella*) have evolved to thrive. Studies suggest that in some bacteria, IAA aids in stress adaptation and protection against stressors such as UV, salt and acidity (Duca et al., 2014). It is possible that these functions of IAA explain the mouse phenotype of the mutant. It is also possible that the phenotype of the *ipdC* mutant in the mouse may be—at least partially—independent of IAA and rather dictated by other functions of IpdC. For example, while indolpyruvate decarboxylases are generally known to have high affinity for indolpyruvate, they can also use phenylpyruvate, pyruvate and

benzoylformate as substrates. It is, therefore, possible that IAA production is not the main, but rather incidental, role for these enzymes in some organisms (Duca et al., 2014). The conservation of *ipdC* in many bacterial animal pathogens that behave as opportunistic plant colonists and, reversely, in epiphytic bacteria that opportunistically colonize animal tissue, provides new incentives to gain insight into the function of this plant hormone in a larger biological context.

## ETHICS STATEMENT

Results of mouse experiments are described. Animal protocols were reviewed and approved by the University of Florida IACUC.

## AUTHOR CONTRIBUTIONS

CC, MB, and MT conceived the project and designed the experiments, CC and MT carried out experiments and data analyses in **Figures 1**, **2A,B,D**, **3A**, **4**. SG carried out chemical identification of IAA (**Figures 2B,C**). MdM designed and conducted mouse experiments.

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The other 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.

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# Long-Term Warming Shifts the Composition of Bacterial Communities in the Phyllosphere of *Galium album* in a Permanent Grassland Field-Experiment

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Global warming is currently a much discussed topic with as yet largely unexplored consequences for agro-ecosystems. Little is known about the warming effect on the bacterial microbiota inhabiting the plant surface (phyllosphere), which can have a strong impact on plant growth and health, as well as on plant diseases and colonization by human pathogens. The aim of this study was to investigate the effect of moderate surface warming on the diversity and composition of the bacterial leaf microbiota of the herbaceous plant *Galium album*. Leaves were collected from four control and four surface warmed (+2°C) plots located at the field site of the Environmental Monitoring and Climate Impact Research Station Linden in Germany over a 6-year period. Warming had no effect on the concentration of total number of cells attached to the leaf surface as counted by Sybr Green I staining after detachment, but changes in the diversity and phylogenetic composition of the bacterial leaf microbiota analyzed by bacterial 16S rRNA gene Illumina amplicon sequencing were observed. The bacterial phyllosphere microbiota were dominated by *Proteobacteria*, *Bacteroidetes,* and *Actinobacteria*. Warming caused a significant higher relative abundance of members of the *Gammaproteobacteria*, *Actinobacteria,* and *Firmicutes,* and a lower relative abundance of members of the *Alphaproteobacteria* and *Bacteroidetes*. Plant beneficial bacteria like *Sphingomonas* spp. and *Rhizobium* spp. occurred in significantly lower relative abundance in leaf samples of warmed plots. In contrast, several members of the *Enterobacteriaceae,* especially *Enterobacter* and *Erwinia,* and other potential plant or human pathogenic genera such as *Acinetobacter* and insect-associated *Buchnera* and *Wolbachia* spp. occurred in higher relative abundances in the phyllosphere samples from warmed plots. This study showed for the first time the long-term impact of moderate (+2°C) surface warming on the phyllosphere microbiota on plants. A reduction

of beneficial bacteria and an enhancement of potential pathogenic bacteria in the phyllosphere of plants may indicate that this aspect of the ecosystem which has been largely neglected up till now, can be a potential risk for pathogen transmission in agro-ecosystems in the near future.

**Keywords: global climate change, warming experiment, phyllosphere, *Sphingomonas*, *Enterobacteriaceae*, *Buchnera*, grassland**

## INTRODUCTION

Global warming is an ongoing climate change effect and will lead to an increase of the mean global air temperature by 2–3°C until 2050 as predicted by the Intergovernmental Panel on Climate Change (IPCC, 2013). Elevated surface temperature can affect plants and their symbiotic microbiota (the plant holobiont; Margulis, 1993; Vandenkoornhuyse et al., 2015) by influencing plant growth, health, and yield (Wu et al., 2011; Jansen-Willems et al., 2016). An important plant-associated habitat colonized by specific bacterial communities is the phyllosphere, the aerial part of plants dominated by leaves (Vorholt, 2012; Bringel and Couée, 2015). Estimated densities of leaf surface colonizing bacteria (epiphytic bacteria) range between $10^6$ and $10^7$ per $cm^2$ leaf surface (Vorholt, 2012). The composition of the phyllosphere microbiota can vary among plant species and is influenced by the geographical location, season, and different environmental conditions including temperature, rain, sunlight exposure, dryness (Whipps et al., 2008; Knief et al., 2010; Wellner et al., 2011; Rastogi et al., 2012; Copeland et al., 2015; Ding and Melchner, 2016). Phyllosphere inhabiting bacteria can be beneficial, pathogenic, or antagonistic (plant-protecting) for the host plant and can strongly contribute to plant health and yield by complex plant–microbe interactions (Vorholt, 2012; Bulgarelli et al., 2013; Brader et al., 2017). So far, only a few studies have investigated the effects of increasing temperature on the phyllosphere microbiota, beneficial microbes (Compant et al., 2010), richness of bacteria and fungi in the phyllosphere (Peñuelas et al., 2012), composition of the phyllosphere endophytic microbiota and the plant metabolism (Campisano et al., 2014). Furthermore, the combination of both elevated temperature and $CO_2$ led to an increase or decrease of specific phyllosphere inhabiting bacteria (Ren et al., 2015). Campisano et al. (2017) showed a shift of the endophytic community of *Vitis vinifera* induced by temperature changes and a stronger influence of the season, on bacterial taxa in stems (phyllosphere compartment) compared to roots. Respective seasonal shifts of the leaf microbiota were also shown in several other studies, e.g., by Redford and Fierer (2009), Ding and Melchner (2016). This demonstrates the higher sensitivity of the phyllosphere to temperature changes and illustrates the effects of climate changes. Agler et al. (2016) introduced the microbial "hub" – concept into the research on microbe–microbe interactions in the phyllosphere (van der Heijden and Hartmann, 2016). Based on this concept, specific highly interactive bacterial taxa play a key role in the phyllosphere microbiota by their interactions with several other community members. Changes in the relative abundance of those "hub" microbes induced by abiotic or host factors, like climate, distribution, or host resistance alleles, can strongly affect the total microbial community structure. Warming is one of the environmental factors, that can affect hub microbes and thus strongly impact the phyllosphere microbiota. This has not been studied in depth so far.

An increasing number of studies showed that the phyllosphere of plants can be efficiently colonized by human pathogens (Mootian et al., 2009; Oliveira et al., 2011, 2012; Holden et al., 2015). This is also critical due to the fact, that a high number of potentially pathogenic bacteria, including the possibility that some of them may be resistant to antibiotics, can be released into the environment from livestock husbandry and wastewater treatment plants (Heuer et al., 2011; Schauss et al., 2015, 2016).

Previous studies have shown, that moderate warming (+2°C) was more beneficial for plants whereas higher temperatures had adverse effects (Jin et al., 2011; Martinez et al., 2014). The effect of warming on different plant–insect interactions was investigated in several studies (Dong et al., 2013; Berthe et al., 2015; Kozlov et al., 2017). Studies on the effects of warming on plant–bacteria interactions were mainly focused on soil microbial communities (Sheik et al., 2011; DeAngelis et al., 2015; Romero-Olivares et al., 2017), thus little is known about the phyllosphere associated microbial communities. The impact of elevated temperature on the phyllosphere microbiota in long-term experiments to our knowledge is so far only studied by Ren et al. (2015) in the rice phyllosphere using a 16S rRNA gene amplicon pyrotaq sequencing approach.

Worldwide, grasslands cover approximately 26% of the world land area (Freibauer et al., 2004; Conant, 2010) and constitute an important ecosystem function including food for livestock and wild animals. However, long-term warming effects on the phyllosphere microbiota of a permanent grassland have not been studied so far.

In a 6.5-year experiment examining the effects of increasing the leaf surface temperature (by +2°C), an increase in the aboveground biomass was observed (Jansen-Willems et al., 2016). This experiment was used to get a first insight on the effect of a moderate, (+2°C) long-term warming on the composition of the phyllosphere inhabiting microbial community of the most abundant forb, *Galium album* Mill., within the permanent grassland. Total cell counts and bacterial 16S rRNA gene amplicon sequencing analyses using the Illumina sequencing technology were used to monitor these effects. This study was performed to test the hypothesis that long-term moderate (+2°C) surface warming affects the phyllosphere microbiota with respect to (1) the total abundance of phyllosphere bacteria and (2)

the diversity and phylogenetic composition of the bacterial communities in the phyllosphere.

## MATERIALS AND METHODS

### Field Site Description

The experiment was established on 24th January 2008 on the permanent temperate grassland of the Environmental Monitoring and Climate Impact Research Station at Linden in Germany and was run until 12th May 2014. The field site is located at 50°31.6′N and 8°41.7′E and sits 172 m above sea level. The mean annual temperature is 9.4°C and the mean cumulative annual precipitation is 558 mm (1998–2013). The mean winter (December–February) temperature over the period 2008–2014 was 1.4°C ± 5.1 and the mean summer (June–August) temperature over the corresponding time period was 17.6°C ± 5.2. The vegetation is characterized as a seminatural grassland dominated by *Arrhenatheretum elatioris* and *Filipendula ulmaria*. Among the forbs present in the grassland, *G. album* was the dominating species. The permanent grassland was treated like a meadow and was routinely cut twice a year from 1993 onward. *G. album* did then not grow over the autumn or winter months. Since 1995, 40 kg N $ha^{-1}$ per year was applied each spring. No plowing has been performed at the experimental site for more than 100 years. The soil on the field site was classified as a Fluvic Gleysol on loamy-sandy sediments over a clay layer.

### Experimental Setup

A 100 $m^2$ site area was divided into 16 equally sized plots, arranged in four rows of four plots for the experiment (Supplementary Figure 1). The plots were set up according to a Latin square (each treatment occurred once in each row and each column) in four randomly distributed plot replicates. The control and treated plots both appeared twice in each replicate. Over the "treated plots" an IR-lamp was set up to elevate the surface temperature of the plants and the soil resulting in a mean increase of surface temperature of +2°C (mean 1.9 standard error 0.03, measured 5 cm above soil). The temperature treatment was performed with Edison screw base ceramic infrared heaters of 230V and 250W with reflector and E27 ceramic lamp holder (Friedrich Freek GmbH, Menden, Germany). To avoid the displacement of the lamps (by external force) they were stabilized with three metal bars, and a metal plate above the lamps was used as rain protection. A metal plate for rain protection was also installed above the control plots for comparability to the elevated temperature treatments. The experiment ran for 6.5 years (2008–2014).

The area in the center of each plot (318 $cm^2$) directly underneath the IR-lamp was used for sampling. In order to prevent interference between the control and treated plots, the areas of sampling were 2.5 m apart from each other.

### Sample Collection

Leaf samples of the herbaceous plant *G. album* (dicot) were taken in the morning of 12th May 2014. All four replicates of the elevated temperature and control plots were sampled separately. Using a sterile pestle, wreaths of leaves were collected in a 540-mL sterile whirl-pack bag (Carl Roth GmbH, Karlsruhe, Germany) and directly stored in a dark precooled (−80°C) transport box. Samples for further molecular analyses were stored at −80°C immediately after they arrived at the laboratory.

### Total Cell Counts

The concentration of leaf-attached bacterial cells per gram of leaf fresh weight (FW) was determined by SybrGreen I (SG-I) staining using the method of Lunau et al. (2005). For this 4–6 g of leaf material of *G. album,* was placed in 50-mL falcon tubes and 30 mL autoclaved phosphate buffered saline (PBS; 130 mM NaCl, 7 mM $Na_2HPO_4$, 3 mM $NaH_3PO_4$ per liter; pH 7) was added to the samples. The tubes were shaken for 10 min to detach bacterial cells using a vertical shaker (level 8/Edmund Bühler, Tübingen). The detachment was repeated by a second washing step using an additional 30 mL PBS buffer. Both buffer fractions were combined for further analysis. Detached cells were fixed with 2% (v/v) glutaraldehyde (AppliChem) and the cells were stained with SYBR Green-I (SG-I; Sigma-Aldrich) solution. Fixed cells (filtered volume: 0.7 mL) were therefore collected on black polycarbonate filters (0.2 μm pore size, Millipore, Eschborn, Germany) using a SG-I moviol staining solution (Lunau et al., 2005). By epifluorescence microscopy at 1000× magnification with a Leica DFC 3000G microscope (Leica, Germany) filters were analyzed using the LAS X software for cell measurement. SG-I stained cells were counted from 10 digital images, which were generated with a Leica DFC 3000G (Leica, Germany) camera system. Mean values of the four plots were calculated from the mean values of the 10 picture counts. The respective standard deviation was considered by error propagation. The presence of statistically significant differences between treatments were measured with the Student's *t*-test in SigmaPLOT 11 (Systat Software Inc.).

### DNA-Extraction

Total DNA-extraction from whole frozen leaves was performed with the genomic DNA extraction kit for soil, the NucleoSpin® Soil Kit (Macherey Nagel, Germany). Approximately 170 mg of whole leaves were weighed and extracted according to manufactures instructions. Total leaves were used for DNA extraction in order to ensure that bacterial cells strongly attached to the leaf surface and partially embedded in a biofilm ("phylloplane") were include in the analysis. This was done in order to avoid the exclusion of the most active fraction of the phyllosphere microbiota. Endophytic bacteria were only partially co-extracted, because leaves remained intact after the first steps of DNA extraction. Extraction was started using lysis buffer SL1 and afterward steps 1–5 were repeated with lysis buffer SL2, thus the leaves were lysed twice. The supernatants were loaded on one NucleoSpin Soil Column for DNA binding. The silica membrane was centrifuged twice for washing and the DNA was eluted in two subsequent elution steps with 25 μL PCR water. The DNA containing

eluates were combined and DNA quality and quantity were investigated using a NanoDrop spectrophotometer (Thermo Scientific).

## 16S rRNA Gene Amplicon Illumina MiSeq Analysis

The diversity and phylogenetic composition of the bacterial communities were analyzed by 16S rRNA gene amplicon sequencing using universal bacterial 16S rRNA gene V5-V6 region targeting primers 799F (5′-AACMGGATT AGATACCCKG-3′) and 1115R (5′-AGGGTTGCGCTCG TTG-3′) which are recommended for the exclusion of chloroplast DNA amplification (Chelius and Triplett, 2001; Redford et al., 2010). The PCR amplification and Illumina 300 bp paired-end read sequencing was done by LGC Genomics (Berlin, Germany) with the Illumina MiSeq V3 system. Sequence libraries were demultiplexed with the Illumina bcl2fastq 1.8.4 software, reads were sorted by amplicon inline barcodes. Reads with missing barcodes, one-sided barcodes or conflicting barcode pairs were discarded. Barcodes, adaptors and primer sequences were clipped after sorting. Reads with a final length <100 bp after adaptor clipping were discarded. Sequences were oriented into the forward and reverse primer direction. Forward and reverse reads were combined using BBMerge 34.48[1]. Datasets of combined reads were analyzed in the SILVAngs analysis pipeline[2]. Using the SILVA Incremental Aligner [SINA version 1.2.10 for ARB SVN (revision 21008)] (Pruesse et al., 2012) all sequence reads were aligned against the SILVA SSU rRNA SEED database and quality controlled (Quast et al., 2013). Reads with more than 2% of ambiguities or 2% of homopolymers, respectively, were excluded from the analysis. Putative contaminations and artifacts, reads containing a low alignment quality (50 alignment identity, 40 alignment score reported by SINA) were detected and excluded from downstream analysis. After this initial quality control step, dereplication and clustering was performed with cd-hit-est (version 3.1.2[3]) (Li and Godzik, 2006) using *accurate mode*, disregarding overhangs, and using identity criteria of 1.00 and 0.98, respectively. The classification was done with the nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 123[4]) using standard settings (Camacho et al., 2009) of blastn (version 2.2.30+[5]). Each operational taxonomic unit (OTU) reference read classification was mapped onto all reads that were assigned to the respective OTU. This resulted in quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as, multiple rRNA operons. Reads missing any BLAST hits or reads with weak BLAST hits remained unclassified, where the function "(% sequence identity + % alignment coverage)/2" did not pass the value of 93. Then assignment of these reads to a meta group of "No Relative" in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011) were done. This method was first applied in the studies published by Ionescu et al. (2012) and Klindworth et al. (2013). Archaeal, chloroplast, mitochondria, and "No Relative" reads were excluded from the analysis. Only reads assigned to the bacterial phyla were used for further analysis. Amplicon sequence data were submitted to the sequence read archive (SRA) of the NCBI within the BioProject PRJNA400850 as BioSamples SAMN07562291 to SAMN07562298.

[1]http://bbmap.sourceforge.net/

[2]https://www.arb-silva.de/ngs/

[3]http://www.bioinformatics.org/cd-hit

[4]http://www.arb-silva.de

[5]http://blast.ncbi.nlm.nih.gov/Blast.cgi

## Statistical Analysis

Statistical analyses were performed by PAST software version 3.11 (Hammer et al., 2001). Non-metric multidimensional scaling (nMDS; Taguchi and Oono, 2005) and principal component analysis (PCA; Davis, 1986; Harper, 1999) were used to display the differences between the relative abundance pattern of leaf associated bacteria and the influence of the individual phyla or taxa to the differences between treatments. The differences between the control and treated plots were tested for statistical significance using a one-way ANOSIM (Clarke, 1993). nMDS and one-way ANOSIM analysis based on similarity matrices was calculated with the Bray–Curtis similarity index. SIMPER analyses (Clarke, 1993) were used to detect phyla and taxa of lower ranks, which contributed most to the differences between treatments using the same similarity index. Richness and diversity of the phyllosphere communities were calculated with Chao 1, Shannon, evenness and dominance indices (Harper, 1999) and rarefaction curve analyses (Krebs, 1989) considering the number of taxonomic paths and the number of individual reads per taxonomic path. Boxplots of cell counts, of the taxa contributing to the main differences and of the diversity indices were performed in SigmaPLOT 12.5 (Systat Software Inc.) and the significance of the differences between C and T plots were tested using Student's *t*-test with the same software.

# RESULTS

## Bacterial Abundance in the Phyllosphere of *G. album* of Control and Warmed Plots

The numbers of leaf-associated bacteria (stained by Sybr Green I after detachment from leaves) observed in leaves sampled randomly from *G. album* control plants (ambient temperature) (control plots; C 1–4) and the those on leaves exposed to elevated temperature (+2°C; elevated temperature plots; T 1–4) is shown in Supplementary Figure 1. The bacterial cell concentration was in the range of $10^5$ cells $g^{-1}$ leaf fresh weight (FW) for *G. album* leaves of C and T plots (**Figure 1** and Supplementary Table 1). Elevated temperature had no significant effect on the abundance of leaf-associated bacteria ($p > 0.05$, Student's *t*-test). Cell morphologies of bacterial cells detached from leaves collected from C and T plots were also similar. Most of the detached cells were rod-shaped with a mean size of 1.6 (±0.4) × 0.6 (±0.2) μm (C) and 1.7 (±1.1) × 0.6 (±0.2) μm (T), respectively.

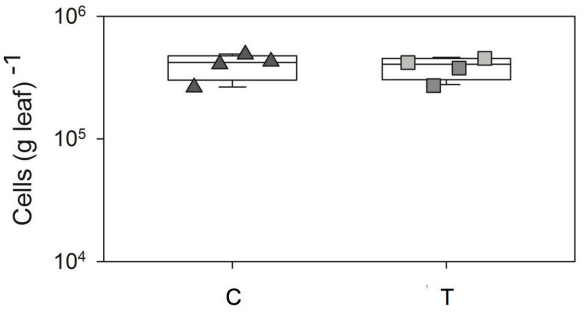


**FIGURE 1 |** Box-plots of total cell number counts per g *G. album* leaf of C and T plots. C = ambient temperature (C), T = elevated temperature (T). Squares = four C plots, triangles = four T plots, box-plots were performed in SigmaPLOT (Applied Maths).

## Analysis of the Phylogenetic Composition of the Bacterial Phyllosphere Microbiota

Bacterial 16S rRNA gene amplicon sequencing was performed from total DNA extracts obtained from *G. album* leaves, which were randomly collected from the four C and T plots. A total number of 638,626 paired end sequences with an average sequence length of 299 nucleotides (nt) were obtained from the eight analyzed leaf samples derived from eight plots. In summary, 204,704 sequences (32.1%) were rejected, because they failed the quality control of the SilvaNGS pipeline. Finally, 433,922 sequences (7,627–88,073 per sample) were subjected to analysis (Supplementary Table 2). A total number of 14,420 OTUs (2.3%) was obtained based on a 98% sequence similarity threshold from 59,031 clustered sequences (9.3%) including 360,471 replicates (56.4%). Respective OTUs were assigned to taxonomic paths (named as "phylogenetic groups") with a maximum resolution at the genus level. The relative abundance of chloroplast and mitochondrial 16S rRNA gene sequence was in the range of 1.9–34.4% and 0.5–12.3% for individual samples, respectively (Supplementary Figure 2). This was in the range reported also in other phyllosphere studies (Lopez-Velasco et al., 2011; Ferrando et al., 2012; Ren et al., 2015). All sequences which were identified as *Archaea* (304 sequences, 0.1%), chloroplasts (113,103 sequences; 19.2%), mitochondria (42,530 sequence, 7.2%) or which did not match any known taxa (sequence similarity < 93% to the next known taxa; summarized as "no relative"; 0.1%) were excluded from the analysis (Supplementary Figure 2). Only sequences assigned to the domain *Bacteria* were considered subsequently. Respective sequences were set to 100%.

## Elevated Temperature Effects on the Composition and Diversity of the Phyllosphere Microbiota

In summary, 28 different phyla or Candidatus phyla (18 from C and 26 from T plots) were detected in the phyllosphere of *G. album* leaves. Bacterial communities of both treatments were dominated by *Proteobacteria* including mainly *Alpha-*, *Gamma-*, *Beta-*, and *Deltaproteobacteria*, followed by *Bacteroidetes* and *Actinobacteria* (**Figure 2A**). Lower abundant phyla (contribution > 1%) detected in the phyllosphere of *G. album* leaves from C as well T plots, were *Firmicutes*, *Chloroflexi*, and *Acidobacteria* (**Figure 2A** and Supplementary Table 3).

Principal component analysis (**Figure 2B**) of the relative abundance patterns of bacterial phyla showed a distinct separation of the bacterial phyllosphere microbiota on *G. album* leaves of C and T plots ($p = 0.03$; one-way ANOSIM). According to those samples PCA biplots indicated, that changes in the relative abundance of *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* had the main impact on the differences between the bacterial phyllosphere microbiota of *G. album* leaves from C and T plots. *Alphaproteobacteria*, *Betaproteobacteria*, and *Bacteroidetes* were more abundant in control plots, while *Gammaproteobacteria, Deltaproteobacteria*, *Actinobacteria*, and *Firmicutes* were more abundant in warmed plots (**Figure 2B**).

Considering the highly diverse phylum *Proteobacteria* as one group, no significant differences were obtained with respect to relative abundance between control and warmed plots (**Figure 2C**). However, if most abundant classes within the *Proteobacteria* (representing >99% of all *Proteobacteria* sequences per sample) were considered separately (**Figure 2C**) significant differences were obtained for the relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria*, but no significant differences were seen for *Betaproteobacteria* and *Deltaproteobacteria* between C and T plots. The relative abundance of *Alphaproteobacteria* was significantly lower under warmed conditions (C: 60.1 ± 10.6%; T: 13.0 ± 10.6%), while the relative abundance of *Gammaproteobacteria* significantly increased under warmed conditions (C: 7.6 ± 3.2%; T: 57.9 ± 30.2%). The relative abundance of *Betaproteobacteria* was slightly but not significantly lower, while the relative abundance of *Deltaproteobacteria* was slightly but not significantly higher in warmed plots.

A significantly lower relative abundance of *Bacteroidetes* (C: 21.6 ± 13.1%; T: 4.1 ± 2.1%) and a significantly higher relative abundance of *Actinobacteria* (C: 1.0 ± 0.5%; T: 14.3 ± 12.6%) were detected on leaves from T compared to C plots (**Figure 2C**). A phylum with lower abundance that was affected by warming was *Firmicutes* (C: 0.6 ± 0.4%; T: 2.3 ± 0.6%), which was detected in significant higher relative abundance on leaves derived from T plots (**Figure 2C**).

For a more detailed insight into the effects of warming, the diversity and composition of the bacterial phyllosphere microbiota was analyzed at a higher phylogenetic resolution by comparing the relative abundance patterns of phylogenetic groups (mainly defined at the genus level). In total, 724 different phylogenetic groups (177–502 per sample) were detected (Supplementary Table 4). On average 9 (±1) and 12 (±6) phylogenetic groups had a relative abundance higher than 1% in leaf samples of C and T plots, respectively.

Non-metric multidimensional scaling of bacterial community patterns based on Bray–Curtis similarities revealed significant

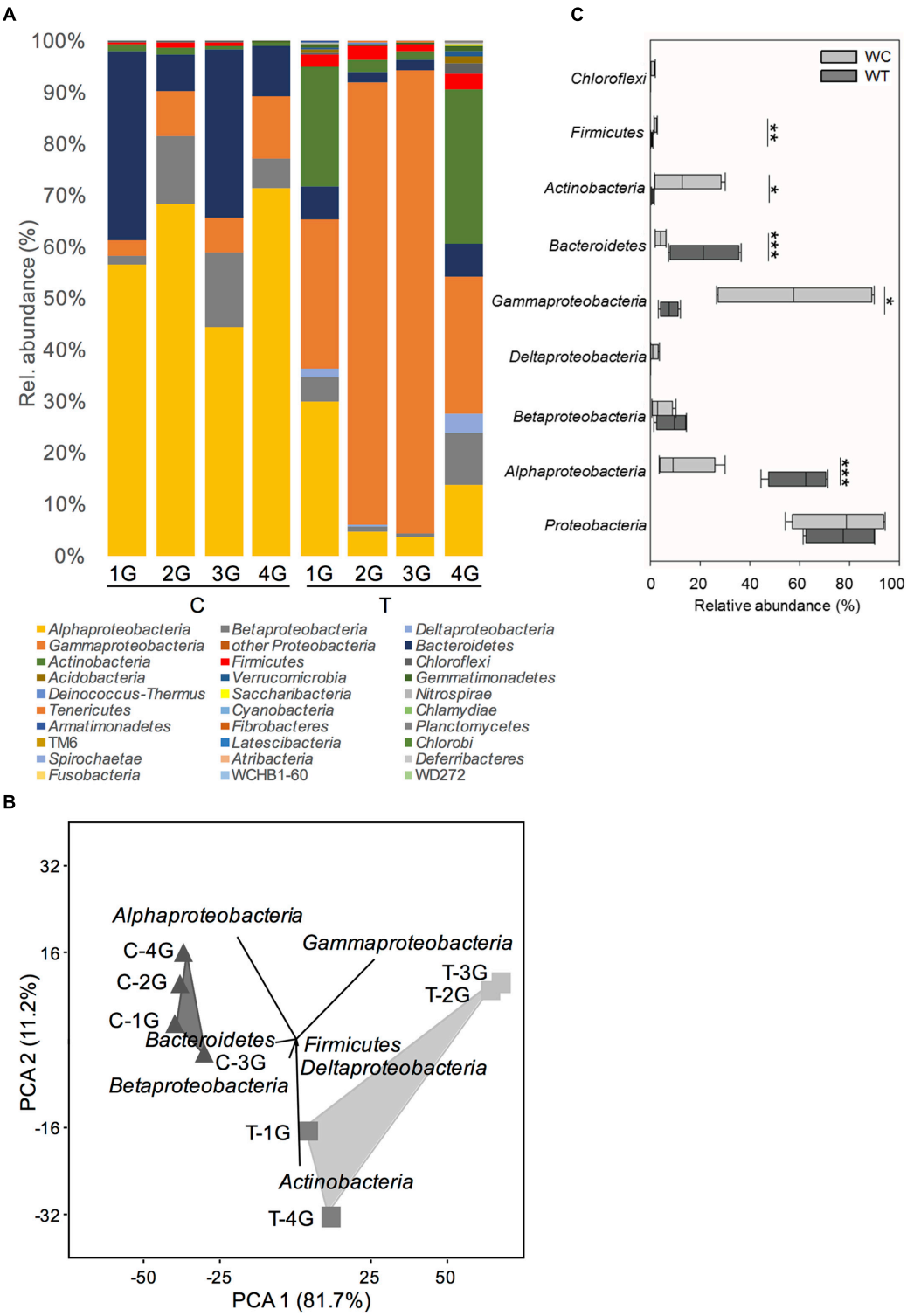


**FIGURE 2 |** Phylogenetic composition of phyllosphere inhabiting bacteria of *Galium album* leaves from plots with ambient temperature (C 1–4) and +2°C surface temperature elevated plots (T 1–4) resolved at the level of *Bacteria* phyla. **(A)** Relative abundance of different bacterial phyla on *G. album* leaves from C and T plots. **(B)** Principal component analysis (PCA) of relative abundance pattern of respective bacterial communities based on the phyla assignment. Eigenvalues for the compared principal components are given in brackets (%) at the respective axes of the graphs. The contribution of different taxonomic groups to the placement of the samples in the PCA plots are indicated as biplots in the graph, and **(C)** variation of the relative abundances of the most abundant phyla among *G. album* leaf samples from C and T plots. Asterisks are representing statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. G = *Galium album*; C = ambient temperature; T = elevated temperature; 1–4 = biological replicates.

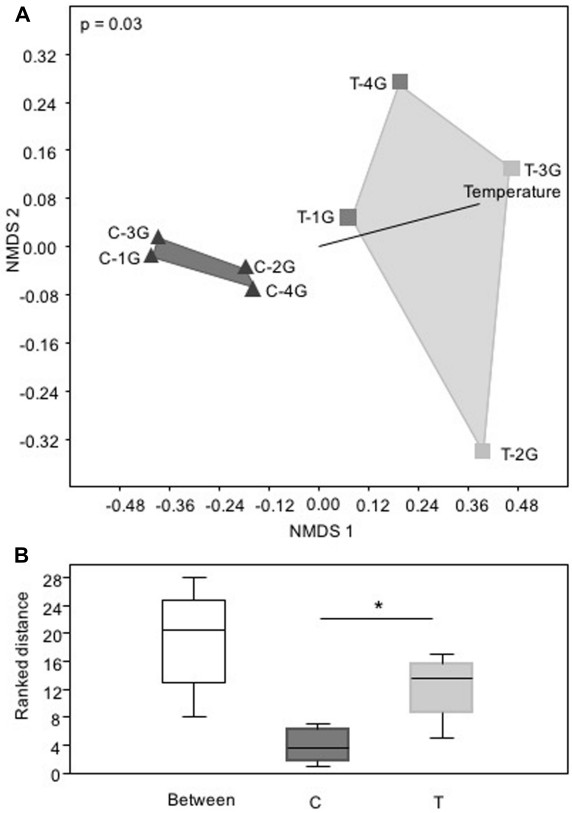


**FIGURE 3 |** Phylogenetic composition of phyllosphere inhabiting bacteria of *Galium album* leaves from plots with ambient temperature (C 1–4) and +2°C surface temperature elevated plots (T 1–4) with a resolution at the level of *Bacteria* taxa. **(A)** Non-metric multidimensional scaling (nMDS) analysis of relative abundance pattern of respective bacterial communities based on the taxa assignment, calculated with Bray–Curtis similarity matrix, and **(B)** Rank differences of the leaf microbiota from C and T plots analyzed at the level of bacterial taxa. Analysis was performed in PAST3 and based on a Bray–Curtis similarity matrix calculation. Box-plots were calculated with the interpolated quartile method. Outliers were not determined. Significant differences were determined by one-way ANOSIM. Asterisk is representing statistical significance: $^*p < 0.05$.

differences (corrected $p \leq 0.03$, one-way ANOSIM) between the bacterial community compositions of C compared to T leaves (**Figure 3A**). The inclusion of temperature as an environmental variable to the nMDS analysis revealed, that surface temperature correlated with the significant differences of the community patterns of the leaf microbiota derived from C and T plots (**Figure 3A**). The nMDS plot and the corresponding ranked distance analysis of the bacterial community patterns (**Figures 3A,B**) showed a higher variability (relative abundance patterns of different phylogenetic groups) obtained from leaf samples of the four T plots, but were very similar to the bacterial communities of the C plots. This indicated that leaf associated bacterial communities were much more stable under control than elevated temperature conditions.

This finding was supported by the comparison of bacterial communities using different diversity measurements (**Figures 4A–E**). Diversity indices were similar for the bacterial phyllosphere microbiota obtained from *G. album* leaves of the four independent C plots, but were much more variable for leaf samples derived from the four T plots. Based on the Chao 1 index (representing the total number of phylogenetic groups, **Figure 4A**) it could be shown, that the bacterial richness was more similar in *G. album* leaves grown in C (316–364) compared to T plots (223–646). The richness of phylogenetic groups of the bacterial communities in T plots was higher compared to C plots for two of the T plots (T-1G and T-4G) and lower for the other two T plots (T-2G and T-3G). The composition of the bacterial phyllosphere microbiota of leaves from C plots was less equally distributed (evenness index, **Figure 4B**) because of the high abundance of individual phylogenetic groups as indicated by high dominance values for C samples (**Figure 4C**). The dominance and evenness measurements of the microbial communities of the T plots again were highly variable among each other. Generally, the phyllosphere bacterial communities were much more equally distributed, without single abundant taxa and more diverse (Shannon index; **Figure 4D**) compared to C plots in two of the T plots (T-1 and T-4; **Figures 4B–D**). The opposite was obtained for the phyllosphere microbiota derived from the other two T plots (T-2 and T-3). The differences of the bacterial communities of the leaf samples from the four T plots among each other and in comparison to the C plots were also illustrated by the differences in the dynamics of the rarefaction curves (**Figure 4E**). Rarefaction curves of T samples had always a higher slope than the curves of the C samples indicating the presence of more complex bacterial communities under elevated temperature conditions.

## Phylogenetic Groups with Strong Impact on Differences in the Phyllosphere Microbiota under Control and Warmed Conditions

Principal component analysis (**Figure 5A**) of bacterial community patterns with a resolution at the level of phylogenetic groups and a respective similarity percentage (SIMPER, Supplementary Table 4) analysis were performed to determine the contribution of individual phylogenetic groups to the differences between bacterial communities obtained from control and warmed plots. In addition, *t*-tests were performed for each phylogenetic group to determine those, which were significantly impacted by warming. A total of 62 phylogenetic groups were found, which showed a significant change along with warming (**Figure 5B** and Supplementary Table 5).

Biplots in the PCA analysis (**Figure 5A**) and box plots of the ranking based on the contribution to the differences on the bacterial community patterns (SIMPER) (**Figures 5B, 6** and Supplementary Table 4) showed, that 10 phylogenetic groups contributed with 75% to the differences between the phyllosphere microbiota of *G. album* leaves derived from C and T plots.

Phylogenetic groups, which had the main contribution to the differences among the microbial communities on leaves of C and T plots were *Proteobacteria* and *Bacteroidetes*. Four of the 10 main contributing phylogenetic groups which occurred

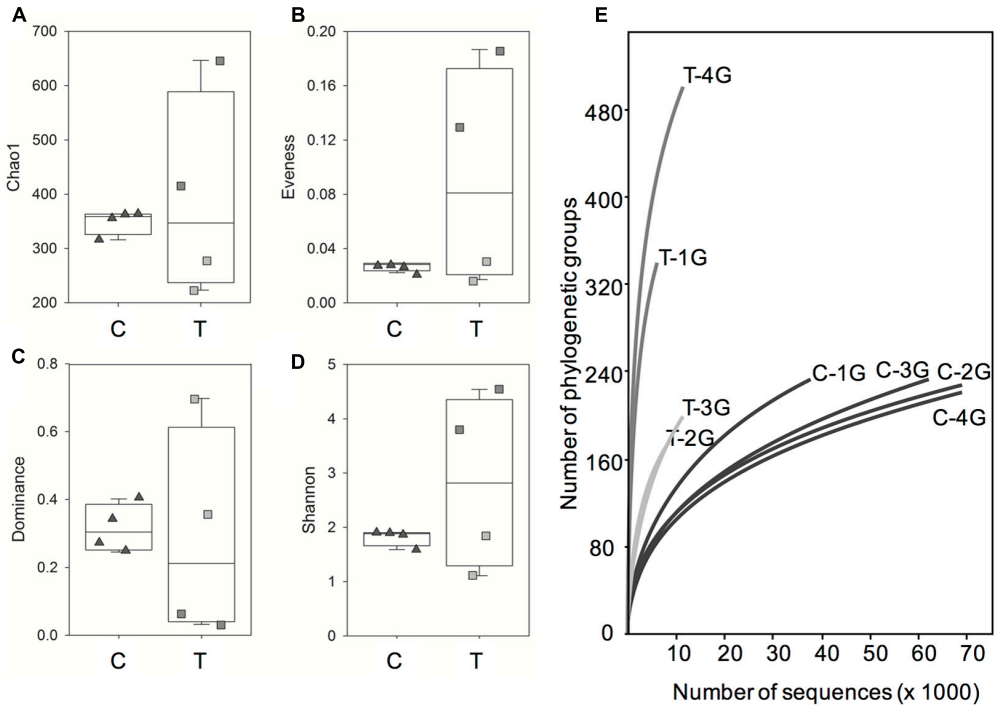


**FIGURE 4 |** Box-plots of alpha diversity indices Chao 1 **(A)**, evenness **(B)**, dominance **(C)** and Shannon index **(D)** and rarefaction curves **(E)** for the phyllosphere community of *Galium album* leaves from C and T plots. C = ambient temperature (C), T = elevated temperature (T). Triangles = four C plots, squares = four T plots. **(E)** Rarefaction curves (specimens versus taxa) based on Illumina 16S rRNA gene amplicon sequencing of microbial communities from all C (C 1–4) and T (T 1–4) plots.

with significant higher relative abundance under control conditions were *Sphingomonas* (tax ID T1; *Alphaproteobacteria*), *Hymenobacter* (T3, *Bacteroidetes*), *Aureimonas* (T7), and *Rhizobium* (T10) (both *Alphaproteobacteria*). Two phylogenetic groups also occurred in higher relative abundances under control conditions. They were uncultured *Comamonadaceae* (T6, *Betaproteobacteria*) and *Pedobacter* (T8, *Bacteroidetes*), but differences were not significant. The other four main contributing phylogenetic groups occurred in a higher relative abundance on leaves of T plots were *Gammaproteobacteria*: *Pseudomonas* (T2), *Erwinia* (T4), *Buchnera* (T5), and *Enterobacter* (T2). Differences in relative abundances were only significant for *Buchnera* and *Enterobacter,* because *Pseudomonas* and *Erwinia* occurred only in a high abundance in leaf samples derived from one of the four T plots, T-2G (83.5%) and T-3G (57.6%), respectively, and in lower relative abundance in leaf samples of the other three T plots (9.8 ± 1.2% and 0.8 ± 0.4%, respectively). The high relative abundance of those two phylogenetic groups in T-3G and T-2G was the main reason for the differences in diversity of two T plot samples compared to the other two T plots.

## Effects of Elevated Temperature on Phyla-Specific Bacterial Communities

Diversity measurements and community profiles were considered for the most abundant phyla including *Proteobacteria* (separated into *Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria*), *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (Supplementary Figures 3–10).

### *Bacteroidetes*

The total relative abundance of *Bacteroidetes* was negatively affected by warming (**Figure 2C** and Supplementary Figure 3a). The dominance index was significantly higher in C plots, because of the high relative abundance of two genera, *Hymenobacter* (T3) and *Pedobacter* (T8) under control conditions. The low relative abundance of those taxa in T plots led to a significantly higher diversity of *Bacteroidetes* on *G. album* leaves grown under elevated temperature (Supplementary Figure 3b). *Hymenobacter* and *Pedobacter*, contributed mostly to the differences between the *G. album* leaf microbiota from C and T plots (Supplementary Figure 3c). The boxplots of the ranked distance analysis demonstrated a high variability within the community composition in C and T plots (Supplementary Figure 11).

### *Alphaproteobacteria*

The total relative abundance of *Alphaproteobacteria* was also negatively affected by warming (**Figure 2C** and Supplementary Figure 4a). Significantly more single dominant phylogenetic groups, *Sphingomonas* (T1), *Aureimonas* (T7), and *Rhizobium* (T10), were detected in control compared to warmed plots (all $p < 0.05$; Supplementary Figures 4b,c). Due to the lower relative abundance of those genera, the diversity increased

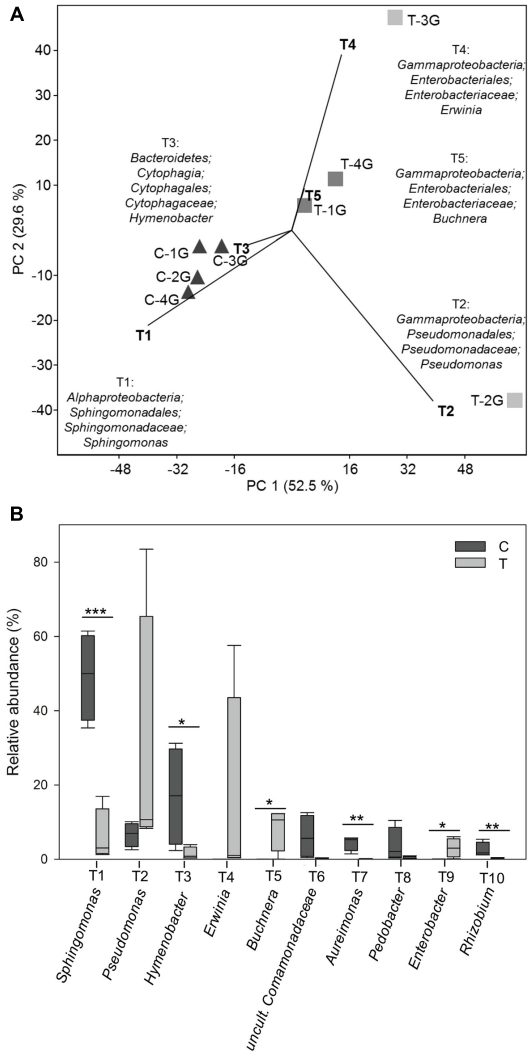


**FIGURE 5 | (A)** Principal component analysis (PCA) of relative abundance pattern of respective bacterial communities from *Galium album* leaves based on the taxa assignment. Eigenvalues for the compared principal components were given in brackets (%) at the respective axes of the graphs. The contribution of different taxonomic groups to the placement of the samples in the PCA plots are indicated as biplots in the graph. Different taxa were numbered with T1-T111 in accordance with their contribution to the differences between leaf microbiota from C and T plots; T1 represents the taxa with the highest contribution. **(B)** Relative abundance of the most abundant 10 taxa present on *Galium album* leaves from C and T plots. Asterisks are representing statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

significantly under warmed conditions (Supplementary Figure 4b). *Mesorhizobium* (T11) occurred in high abundance on leaves from T plots (especially high in one replicate: T-1G) and contributed also to the differences between *G. album* leaf microbiota from C and T plots (Supplementary Figure 4c). The *Alphaproteobacteria* community compositions showed significant differences with respect to the similarities within C and T plot replicates. The *Alphaproteobacteria* communities were markedly more similar within control plots compared to warmed plots as shown by ranked distance analysis (Supplementary Figure 11).

### *Gammaproteobacteria*

In contrast to the *Alphaproteobacteria*, the total relative abundance of *Gammaproteobacteria* was positively affected by warming (**Figure 2C** and Supplementary Figure 5a). In three of four warmed plots, *Gammaproteobacteria* were more equally distributed with less single dominant phylogenetic groups. Differences in the diversity indices were not significant here (Supplementary Figure 5b). Within this subclass the genera *Pseudomonas* (T2), *Erwinia* (T4), *Buchnera* (T5), and *Enterobacter* (T9) contributed most to the differences between the *G. album* leaf microbiota from control and warmed plots and each of them were abundant on leaves from T plots (Supplementary Figure 5c). Ranked distance analysis showed more similarity of the *Gammaproteobacteria* community within C plots and a significant higher variability of the community in T plots (Supplementary Figure 11).

### *Betaproteobacteria*

Warming had no significant effect on the total relative abundance of *Betaproteobacteria* communities (**Figure 2C** and Supplementary Figure 6a). Uncultured *Comamonadaceae* (T6) and *Massilia* (T13) were detected in high abundance in control plots. The dominance index was significantly higher under C compared to T (Supplementary Figure 6b). In consequence their absence led to a significantly more diverse community in the leaf phyllosphere of warmed plots (Supplementary Figure 6b). The phylogenetic groups assigned to SC-I-84 (T26) and *Duganella* (T37) were abundant in T plots and had together with T6 the highest contribution to the differences between leaf microbiota from C and T plots (Supplementary Figure 6c). The ranked distance analysis showed a high variability of *Betaproteobacteria* within C and T plots (Supplementary Figure 11).

### *Deltaproteobacteria*

The total relative abundance of *Deltaproteobacteria* was not influenced by warming (**Figure 2C** and Supplementary Figure 7a). In three of the four warmed plots, less single dominant phylogenetic groups, but a higher diverse community of *Deltaproteobacteria* was detected (Supplementary Figure 7b). The diversity indices were not significantly different. Within this subclass GR-WP33-30 (T29), *Haliangium* (T75), P3OB-42 (T138), uncultured *Sandaracinaceae* (T185) and group BIrii41 (T113) contributed mainly to the differences between the *G. album* leaf microbiota from C and T plots and were all abundant on leaves from T plots (Supplementary Figure 7c). The variability of the community was smaller within T plots compared to C plots (Supplementary Figure 11).

*Actinobacteria* and *Firmicutes* were the two main phyla, which occurred in significantly higher relative abundance in warmed plots (**Figure 2C**). Single dominant phylogenetic groups dominated *Actinobacteria* and *Firmicutes* communities of leaves from C plots, but not from T plots, which led to a higher diversity of *Actinobacteria* and *Firmicutes* under warmed conditions (Supplementary Figures 8a–j, 9a, and 10a, respectively).


**FIGURE 6 |** Relative abundance pattern of bacterial taxa present on *Galium album* leaves grown in control (C) and warming (T) plots. Analysis were performed at the level of taxonomic paths (resolved up to the genus level). Community pattern were compared by cluster analysis in PAST3 using UPGMA as clustering algorithm and Bray–Curtis similarity calculation. Taxa were sorted by their contribution to the differentiation between leaf microbiota from C and T plots by the SIMPER analysis using PAST. Data are shown for taxa with ≥0.1% contribution.

Within the *Actinobacteria,* the genus *Agreia* (T45) was dominant under control conditions (Supplementary Figure 9b). Uncultured members of the *Gaiellales* (T12), *Nocardioides* (T15), *Kineococcus* (T17), uncultured group 480-2 (T18), *Gaiella* (T19), uncultured group MB-A2-108 (T20), *Mycobacterium* (T23) and *Brevibacterium* (T30) were abundant on leaves from T plots (Supplementary Figure 9b). All nine phylogenetic groups contributed to the differences between the leaf microbiota from C and T plots. Ranked distance analysis showed, that the community patterns were more similar among C plots and had a high variability among T plots (Supplementary Figure 11).

Four main phylogenetic groups of *Firmicutes* had the strongest impact on the differences between control and warmed plots (Supplementary Figure 10b). *Streptococcus* spp. (T41) were abundant under C. *Bacillus* (T22), *Clostridium sensu stricto* 7 (T25) and *Paenibacillus* (T71) were detected with a high relative abundance in T plots. Ranked distance analysis showed a high variability of *Firmicutes* within control and warmed plots with a higher variability under elevated temperature (Supplementary Figure 11).

# DISCUSSION

Our study showed that surface warming did not strongly affect the abundance of bacteria in the phyllosphere but had several effects on the diversity and phylogenetic composition of the phyllosphere inhabiting bacterial communities. Bacterial richness and diversity increased with higher temperature. Ren et al. (2015) also reported an increase in bacterial richness and diversity in the rice leaf phyllosphere with the combined effects of elevated $CO_2$ and temperature ($eCO_2$+eT), but no effects were determined by analyzing each factor separately. Campisano et al. (2017) also showed, that higher growth temperatures (35°C compared to 15°C) led to a higher diversity of endophytic bacterial communities in above ground organs of *Vitis vinifera* plants grown under controlled conditions in greenhouse experiments.

The relative abundance and composition of the dominating bacterial phyla, *Proteobacteria, Bacteroidetes,* and *Actinobacteria,* were affected differently (**Figure 2A**). These three phyla were often described as phyllosphere-associated generalists occurring as most abundant phyla in the phyllosphere of different plant species (Bulgarelli et al., 2013; Bringel and Couée, 2015). The occurrence of these three phyla in the phyllosphere was also determined in several other studies (Kim et al., 2012; Rastogi et al., 2012; Horton et al., 2014; Bai et al., 2015; Dees et al., 2015). To understand the effects of surface warming on the bacterial microbiota in detail, required an in-depth study on the effects of the relative abundance of individual bacterial taxa within the abundant but also less abundant phyla, which were also significantly affected by warming, e.g., the *Firmicutes.* As also indicated by the genome sequence based study of leaf-derived bacterial isolates performed by Bai et al. (2015), different leaf associated members of the *Actinobacteria* for example had similar biological functions while the functions among different members of the *Firmicutes* were much more complex. Shifts of individual taxa can therefore strongly affect the functionality of the phyllosphere microbiota with respect to plant–microbe as well as microbe–microbe interactions. At present, the knowledge of functional traits of phyllosphere bacteria is limited and mainly focused on the culturable fraction of the leaf microbiota.

## Effects of Warming on Common Abundant Phyllosphere Inhabiting Bacteria

Several well-known phyllosphere inhabiting bacterial genera such as *Sphingomonas*, *Hymenobacter*, *Aureimonas, Pedobacter*, and *Rhizobium*, had the main impact on the differences of the community profiles of leaf samples from C and T plots. All were negatively influenced with respect to their relative abundance by warming. The potential functions of those phylogenetic groups and the reason and consequence for changes in their relative abundance will be discussed in more detail:

### *Sphingomonas*

Differences in the relative abundance of *Sphingomonas* (tax ID T1, **Figures 5**, **6**) made the highest contribution (26.4%; **Figure 6**) to the differences between the phyllosphere microbiota of *G. album* leaves from C and T plots. *Sphingomonas* occurred in a high relative abundance on leaves grown in C plots (49.2 ± 10.3%) and in a lower relative abundance on leaves grown in T plots (6.0 ± 6.3%; Supplementary Table 4). *Sphingomonas* are well-known as phyllosphere inhabiting bacteria of different plants and for their plant protective potential against pathogens (Delmotte et al., 2009; Innerebner et al., 2011; Vorholt, 2012). Ren et al. (2015) found by a 16S rRNA gene based 454 pyrosequencing approach, that *Sphingomonadaceae,* which includes *Sphingomonas* as type genus, showed a slight reduction under elevated temperature in the rice phyllosphere. Their lower relative abundance on plants grown under elevated temperature may cause a negative effect on plant protection and in consequence enhance the growth of plant pathogens. In a laboratory study of Innerebner et al. (2011) it was shown, that the population size of the plant pathogens *Pseudomonas syringae* pv. tomato DC3000 and *Xanthomonas campestris* pv. *campestris* LMG 568 was reduced on *Arabidopsis* leaves after leaf inoculation with a *Sphingomonas* sp. strain, that was isolated previously from the phyllosphere of grasses.

The lower relative abundance of *Sphingomonas* in the leaf phyllosphere of *G. album* grown in T plots may be explained by changes in the substrate availability on *G. album* plants grown under elevated temperature. Metaproteome studies of the phyllosphere microbiota showed a high abundance of sugar uptake transporters of *Sphingomonas* which indicated, that plant-released carbohydrates are the main carbon source of phyllosphere inhabiting *Sphingomonas* spp. (Delmotte et al., 2009). Jansen-Willems et al. (2016) showed, that the plant biomass increased in T plots, which indicated changes of the plant physiology and morphology. A first insight into metabolome profiles of *G. album* leaves of C and T plots indicated significant differences among leave metabolomes (unpublished data). The impact of changed substrate profiles of the plants in general will be an important aspect for the shift of the phyllosphere

microbiota. Bai et al. (2015) showed in a comparative genomic study of phyllosphere and root derived bacterial isolates, that phyllosphere bacteria contain a significantly higher fraction of annotated proteins linked to the carbohydrate and amino acid metabolism, indicating the importance of changes in substrate availability for phyllosphere inhabiting microbiota. If the changed substrate pattern available under elevated temperature did not match the optimal substrate utilization capacity of *Sphingomonas,* those may have been outcompeted by other phylogenetic groups, which could more efficiently use these substrates and occupy that novel ecological niche characterized by specific substrate profiles.

### *Hymenobacter*

The second most abundant phylogenetic group, the genus *Hymenobacter* was significantly less abundant in the phyllosphere of *G. album* in warmed plots (tax ID T3, **Figures 5**, **6**). It occurred with a high relative abundance in the *G. album* phyllosphere of C plots (C: 16.9 ± 11.7%) and with only 1.4 ± 1.5% relative abundance in the phyllosphere of *G. album* grown in T plots (Supplementary Table 4). The genus *Hymenobacter* contributed with 9.6% to the differences between the *G. album* leaf microbiota from C and T plots. Several studies showed that members of the genus *Hymenobacter* are common inhabitants of the phyllosphere (Ottesen et al., 2009; Leveau and Tech, 2011), but little is known about their function. Ding and Melchner (2016) described *Hymenobacter* as dominant endospheric bacteria in leaf samples from five plant species (*Ambrosia psilostachya* DC., *Asclepias viridis* Walt., *Panicum virgatum* L., *Sorghastrum nutans* (L.) Nash, and *Ruellia humilis* Nutt.).

Related to the microbial "hub" concept discussed by Agler et al. (2016), genera which occurred in a high relative abundance in the phyllosphere under control conditions, as *Sphingomonas* and *Hymenobacter,* may represent "hub" taxa within the phyllosphere microbiota of *G. album* plants. Their low relative abundance in the phyllosphere of *G. album* leaves grown under elevated temperature may be one reason for the high variability or high instability of the T microbiota, which was especially indicated by increased abundance of individual members of the *Gammaproteobacteria* including potential pathogenic genera, *Pseudomonas* or *Erwinia*. Copeland et al. (2015) described members of the genus *Sphingomonas* as leaf adapted taxa and *Pseudomonas* and *Erwinia* as taxa with moderate prevalence and variable abundance on leaves of different crop plants. Based on the microbial "hub" concept, the loss of central hub taxa may disturb the stability of the phyllosphere microbiota, if those strongly interacting taxa are functionally not replaced. Other taxa, which include potentially pathogenic bacteria may be able to grow in ecological niches which were previously occupied by the "hub" taxa without replacing their functionality, i.e., the high abundance of different *Gammaproteobacteria* in warmed plots. The localization of *Hymenobacter* in or at the surface of leaves of *G. album* is so far not known, but may be endospheric as indicated by Ding and Melchner (2016). Thereby *Hymenobacter* may be a "hub" taxon, which occupies another ecological niche than the mainly surface attached *Sphingomonas* (Delmotte et al., 2009; Vorholt, 2012).

### *Aureimonas*

Amplicon sequences assigned to the genus *Aureimonas* were also detected in significantly lower abundance on *G. album* leaves grown under elevated temperature with a relative abundance between 4.4 (±1.7)% in C and 0.08 (±0.03)% in T plots (**Figures 5B, 6** and Supplementary Table 4). The contribution of this genus to the differences between the phyllosphere microbiota from C and T plots was 2.6% (**Figure 6**). Little is known and reported about the genus *Aureimonas* and its occurrence in the phyllosphere. The genus *Aureimonas* belongs to the *Aurantimonadaceae,* which are very closely related to the genus *Rhizobium,* which contains many plant growth-promoting *bacteria*. Among the currently described ten species with validated names, four were isolated from the phyllosphere. *A. galii* and *A. pseudogalii* (Aydogan et al., 2016) were both isolated from *G. album* leaves, the type stain of *A. galii* even from leaves of the current experiment from plants grown under C. Strains of the same species were not cultured from leaves of plants grown under T, which supported the finding of the reduced abundance of this genus in warmed plots (data not shown). *A. phyllosphaerae* and *A. jatrophae* (Madhaiyan et al., 2013) were isolated from surface-sterilized leaf tissues of *Jatropha curcas* L. cultivars, which indicated an endophytic life style.

### *Pedobacter*

The second phylogenetic group of *Bacteroidetes,* which was among the ten phylogenetic groups that showed main contribution to the differences among the bacterial community composition under warmed conditions and occurred with a slightly lower but not significant relative abundance in warmed plots, was the genus *Pedobacter* (**Figures 5B**, **6**). *Pedobacter* spp. was detected also in other phyllosphere studies (Nissinen et al., 2012; Vorholt, 2012; Humphrey et al., 2014; Dees et al., 2015). *Pedobacter* infection was negatively correlated with herbivory and may affect plant–microbe–herbivore interactions in a similar manner as specific *Pseudomonas* spp. (*P. fluorescence*) (Humphrey et al., 2014).

### *Rhizobium*

Sequences assigned to the genus *Rhizobium* were also detected in significant lower relative abundance on *G. album* leaves grown in T (0.2 ± 0.2%) compared to C plots (2.5 ± 1.7%) (**Figure 5B** and Supplementary Table 4). They contributed with 1.4% to the differences between the leaf microbiota from C and T plots (**Figure 6**). Members of the genus *Rhizobium* were often reported as phyllosphere inhabiting bacteria (Wellner et al., 2011; Knief et al., 2012; Horton et al., 2014) with beneficial effects on the plant phyllosphere (Nandi et al., 1982). *Rhizobium* sp. are well known for their plant growth promotion and induction of systemic resistances, which positively affects plant fitness (Glaeser et al., 2016).

## Warming Effects on Potential Plant and/or Human Pathogens

### *Pseudomonas*

The genus *Pseudomonas* (tax ID T2, **Figures 5**, **6**) had the second strongest contribution (13.4%) to the differences among

the microbial community patterns of leaves after warming, due to an increased relative abundance of *Pseudomonas* on leaves derived from T plots (28.2 ± 31.9%) compared to C plots (6.6 ± 2.7%, Supplementary Table 4). The high abundance of sequences assigned to the genus were however, only detected on the leaf phyllosphere from one (T-2G: 83.5%) of four T plots with significantly higher abundance compared to C plots. The high abundance of *Pseudomonas* in one warmed plot sample was the main reason for the high dominance index value obtained for this T sample (**Figure 4C**), and had also a strong contribution to the high variability among the total bacterial community patterns of T plots (**Figure 3B**). Differences of the relative abundance of *Pseudomonas* were therefore not significant between the leaf microbiota from C and T plots. The genus *Pseudomonas* was often reported as a dominating phyllosphere inhabitant (Lopez-Velasco et al., 2011; Rastogi et al., 2012; Bodenhausen et al., 2013). Within the genus, some species are known as plant or human pathogens (Vorholt, 2012) while other species are well known for their plant protective function (Cronin et al., 1997; Mendes et al., 2011). Mendes et al. (2011) showed, that the presence of plant pathogenic fungi (*Rhizoctonia solani*) enhanced the abundance of the antagonistically acting *Pseudomonadaceae* haplotypes, and illustrated herewith the huge functional diversity even among strains of *Pseudomonas*. An increase in the abundance of distinct *Pseudomonas* spp. (especially *P. syringae*) was also positively correlated, whereas *P. fluorescens* infection was negatively correlated with herbivory (Humphrey et al., 2014). Positive or negative impacts of *Pseudomonas* in the phyllosphere were however, always strain dependent and could strongly vary among different *Pseudomonas* spp. isolated from plants (Mendes et al., 2011; Humphrey et al., 2014). The function of *Pseudomonas* present in the phyllosphere of *G. album* under control and warmed conditions needs to be further analyzed through an investigation of *Pseudomonas* spp. isolates obtained from the respective samples in parallel to the cultivation independent study (data not shown).

### *Enterobacteriaceae*

The phyllosphere microbiota of leaves derived from T plots contained a significantly higher abundance of the genus *Enterobacter* (**Figure 5B**). *Enterobacter* spp. contributed with 1.8% (**Figure 6**) to the differences between C (0.03 ± 0.02%) and T (3.0 ± 2.2%) derived phyllosphere bacterial communities, which was also shown with the respective biplot (tax ID T9, *Enterobacter,* **Figure 5A**). The genus *Erwinia* (biplot T4; **Figure 5A**) also had a strong contribution (9.6%) to the differences between leaf microbiota from C and T plots. *Erwinia* spp. occurred with a high relative abundance on *G. album* leaves obtained from one T plot (T-3G: 57.6%, Supplementary Table 4) of four, which led again to the strong variability of the bacterial community composition among leaf samples from elevated temperature plots (**Figure 3B**) and explained also the high dominance index value for the community composition on leaves from this plot (**Figure 4C**). Several species within the genus *Erwinia* are known to be plant pathogens (Starr and Chatterjee, 1972). Other low abundant members of the *Enterobacteriaceae* (*Pantoea, Proteus, Escherichia-Shigella, Serratia*) occurred in slightly higher abundances on *G. album* leaves from T plots, and other genera like *Buttiauxella, Citrobacter, Cronobacter, Klebsiella, Kluyvera, Pectobacterium, Providencia, Raoultella, Tatumella,* and uncultured *Enterobacteriaceae* were detected only on leaves grown in T plots (Supplementary Table 4) although the results were not statistically significant because of the variability within the bacterial community on leaves obtained from T plots. *Enterobacteriaceae* in general constituted 2.6% of all detected phylogenetic groups. In summary, the data suggests that an increase of the surface temperature by +2°C had a long-term effect on the microbial community of the *G. album* leaf phyllosphere with an increase of the relative abundance of *Enterobacteriaceae*. Rastogi et al. (2012) detected more *Enterobacteriaceae* and culturable coliforms in summer compared to winter samples on field grown lettuce leaves, and Ren et al. (2015) also detected slightly higher *Enterobacteriaceae* in the upper rice leaf phyllosphere with elevated temperature.

### Other Potential Pathogens

A significantly higher relative abundance of potential plant and/or human pathogens, as classified by the "Technische Regel für Biologische Arbeitsstoffe" (TRBA 466) for Germany as risk group 2 microbes, like members of *Curtobacterium, Clostridium, Peptoclostridium, Aeromonas, Rhodococcus, Acinetobacter, Burkholderia, Bacillus, Staphylococcus, Cellulomonas, Corynebacterium* and a slightly but not significantly higher relative abundance of the potential plant pathogen *Xanthomonas* were detected on leaves of *G. album* from T plots (Supplementary Tables 4, 5). In former studies it is already reported, that plants represent reservoirs for potential plant or human pathogens like *Pseudomonas, Staphylococcus, Enterococcus, Burkholderia,* and *Enterobacteriaceae* (Berg et al., 2014; Schikora and Schikora, 2014). Sydnor and Perl (2011) described genera like *Acinetobacter*, *Enterobacter*, *Klebsiella* (*K. pneumonia, K. oxytoca*), *Proteus*, *Pseudomonas*, and *Serratia* as antibiotic-resistant microorganisms, which play an important role in hospital-acquired infections. *Acinetobacter baumannii* is regarded as an emerging pathogen and one of most important multidrug-resistant bacteria in hospitals worldwide (Peleg et al., 2008; Antunes et al., 2014). These findings indicate a potential risk for an increase in potential plant and/or human pathogens on *G. album* leaves with elevated temperature due to global warming.

## Indication of Warming Effects on Arthropods and/or Nematodes

### *Buchnera*

*Galium album* leaf phyllosphere from elevated temperature plots harbored a significantly higher abundance of sequences assigned to *Buchnera* (C: 0.002 ± 0.002%; T: 8.3 ± 5.0%; **Figure 5** and Supplementary Table 4) with a contribution of 5.1% to the differences between the leaf microbiota from C and T plots (**Figure 6**). The strong impact of the high relative abundance of *Buchnera* on the microbial community differences among both treatments was also indicated by the respective biplot (**Figure 5A**; *Buchnera*, T5). *Buchnera* spp. are well known endosymbionts of aphids (Moran et al., 2008). The high relative abundance of

*Buchnera* in the warmed plots therefore indicated an enhanced occurrence of aphids on leaves of *G. album* in T plots, as shown in the Supplementary Figure 12 for a *G. album* plant from the same field side. Either aphids or *Buchnera* only were coextracted with plant material. Aphids constitute a globally distributed agricultural pest as sap-sucking herbivores, using large amounts of phloem as a nutritional source (Gray and Fraenkel, 1954). Our findings were in congruence with the results obtained by Barton and Ives (2014). They detected an increased aphid population growth rate on sweet corn grown under elevated temperature (elevated daily temperature of 4.87°C ± 0.14°C). Dong et al. (2013) also described, that experimental warming led to an increase in pest aphid abundance on wheat fields.

### *Wolbachia*

The increase of the relative abundance of insects or at least the enhanced relative abundance of insect symbionts was demonstrated for the genus *Wolbachia*. However, *Wolbachia* spp. occurred generally only in a low abundance. A significantly ($p \leq 0.01$, Student's *t*-test) higher relative abundance of *Wolbachia* was detected in the samples analyzed from T (0.2 ± 0.1%) compared to C (0.001 ± 0.001%) plots (Supplementary Tables 4, 5). *Wolbachia* spp. are intracellular bacteria found in ∼40% arthropods and filarial nematodes (Ferri et al., 2011; Zug and Hammerstein, 2012). Most of the filarial nematodes can cause dangerous filariasis (river blindness and elephantiasis) in humans, and arthropods can lead to dengue fever, yellow fever, malaria and Chikungunya virus (as reviewed by Slatko et al., 2014). Both of them, arthropods and nematodes, can be found in the phyllosphere (Erb et al., 2008; as reviewed by Lindow and Brandl, 2003; Müller et al., 2016).

As reviewed by Robinet and Roques (2010) there is an impact of global warming on several insects, which respond with earlier flight periods, enhanced winter survival and acceleration of development rates. In contrast, a long-term monitoring study of insects feeding on mountain birch (*Betula pubescens* ssp. *czerepanovii*) demonstrated, that climate change influenced insect herbivory in heavily stressed compared to pristine forests differently, and that herbivorous insects showed diverse responses to climate variations (Kozlov et al., 2017). Kozlov et al. (2017) highlighted the problems of predicting effects of global change on forest damaging insects, which requires long-term observations.

The higher relative abundance of *Buchnera* and *Wolbachia* on leaves of *G. album* were an indicator of increased numbers of arthropods and filarial nematodes with elevated temperature, which can represent a vector for potential human diseases (Tobias, 2016).

## Warming Effects on Plant Beneficial Methylotrophs Often Dominating the Phyllosphere

### *Methylobacterium*

Highly abundant and plant beneficial bacterial members of the phyllosphere microbiota are members of the genus *Methylobacterium* (Delmotte et al., 2009; Wellner et al., 2011; Knief et al., 2012; Vorholt, 2012). *Methylobacterium* spp. normally represent the most abundant methylotrophic bacteria. Sequences assigned to the genus *Methylobacterium* were only detected in low abundance (<0.1%, Supplementary Table 4) in the phyllosphere of *G. album* leaves. A non-significantly lower relative abundance of *Methylobacterium* sequences was observed with elevated temperature. One important reason for the low detection rate of *Methylobacterium* sequences may be due to the difficulties in the DNA extraction from this genus. Reisberg et al. (2012) mentioned the same problem by analyzing DNA extracts of the *Arabidopsis* phyllosphere. However, pure culture test experiments showed that DNA from *Methylobacterium* spp. could be efficiently extracted with the DNA extraction kit applied here for the leaf samples. Whether or not the relative abundance of *Methylobacterium* sp. in the *G. album* phyllosphere is common, needs to be investigated further.

## CONCLUSION

This is a first systematic study showing that global warming can have significant effects on the phyllosphere inhabiting bacterial communities caused by surface warming predicted within the next decades as consequence of global climate change. *G. album* was used as model plant and showed changes in the abundances of well-known members of plant phyllosphere bacteria in general and may therefore be representative for many land plants. Important plant protective and beneficial bacteria like members of the *Sphingomonas* and *Hymenobacter* were negatively affected with respect to their relative abundance. The loss of key or "hub" taxa may lead to a more imbalanced phyllosphere microbiota composition, which may then become more susceptible for a stronger colonization with plant and human pathogens. Significantly higher abundances of, e.g., *Enterobacteriaceae*, *Pseudomonas*, or *Acinetobacter* indicate that moderate surface warming may cause a increase of potentially pathogenic bacteria on leaves. The colonization of plants with those bacteria is also problematic due to the fact that antibiotic resistances, linked to respective taxa, may be distributed into the environment.

The increase of insect symbionts in the sequence datasets obtained from warming plots supports the findings of other studies, that surface warming increases the abundance of plant associated insects. Differences in carbon source patterns presented in leaves, but also changes in the leaf associated microbiota may contribute to the increase of insect abundance. However, the increased insect abundance acting as transmission vectors may fasten plant damage in agroecosystems and, thus, may also increase the risk for the spread of plant and human pathogens (Thomas, 2017). This can play a role along with global climate change and needs to be considered in further studies.

This study provided clear evidence that surface warming by +2°C will have a strong impact on the bacterial phyllosphere microbiota. Warming leads to an increase in ecosystem photosynthesis (see review by Wu et al., 2011) which effects the availability of carbon sources in the phyllosphere. Changes in the availability of carbon substrates will be in general one

of the key factors contributing to changes in the composition of the phyllosphere microbiota. Competition for substrates is often one of the main drivers in shaping microbial community compositions. The loss of abundant hub taxa may play an important role in the changes of the composition of phyllosphere microbiota. In addition, changes of environmental parameters may have also contributed to the microbiota shift. Jansen-Willems et al. (2016) determined a net loss in soil carbon in the T plots caused by an increased soil respiration with elevated temperature. As Luo (2007) discussed, changes in the soil and the increased aboveground biomass can also lead to plant stress caused by the higher water and nutrient demands of the plant, which may have impacted the leaf microbiota. However, this is not confirmed by other studies so far. Further studies are required to explain the effects in more detail and to estimate positive and negative effect of surface warming on the phyllosphere microbiota and in consequence on plants and also humans.

## AUTHOR CONTRIBUTIONS

SG and EA designed the project, analyzed and interpreted the data, and wrote the manuscript; GM, SG, and EA did the field sampling; EA performed the laboratory experiments; GM and CM were responsible for the long-term field experiment; PK got funding for the project; PK, GM, and CM made final approval of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00144/full#supplementary-material

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# Few Differences in Metabolic Network Use Found Between *Salmonella enterica* Colonization of Plants and Typhoidal Mice

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The human enteric pathogen *Salmonella enterica* leads a cross-kingdom lifestyle, actively colonizing and persisting on plants in between animal hosts. One of the questions that arises from this dual lifestyle is how *S. enterica* is able to adapt to such divergent hosts. Metabolic pathways required for *S. enterica* animal colonization and virulence have been previously identified, but the metabolism of this bacterium on plants is poorly understood. To determine the requirements for plant colonization by *S. enterica*, we first screened a library of metabolic mutants, previously examined in a systemic mouse typhoidal model, for competitive plant colonization fitness on alfalfa seedlings. By comparing our results to those reported in *S. enterica*-infected murine spleens, we found that the presence of individual nutrients differed between the two host niches. Yet, similar metabolic pathways contributed to *S. enterica* colonization of both plants and animals, such as the biosynthesis of amino acids, purines, and vitamins and the catabolism of glycerol and glucose. However, utilization of at least three metabolic networks differed during the bacterium's plant- and animal-associated lifestyles. Whereas both fatty acid biosynthesis and degradation contributed to *S. enterica* animal colonization, only fatty acid biosynthesis was required during plant colonization. Though serine biosynthesis was required in both hosts, *S. enterica* used different pathways within the serine metabolic network to achieve this outcome. Lastly, the metabolic network surrounding *manA* played different roles during colonization of each host. In animal models of infection, O-antigen production downstream of *manA* facilitates immune evasion. We discovered that *manA* contributed to *S. enterica* attachment, to seeds and germinated seedlings, and was essential for growth in early seedling exudates, when mannose is limited. However, only seedling attachment was linked to O-antigen production, indicating that *manA* played additional roles critical for plant colonization that were independent of surface polysaccharide production. The integrated view of *S. enterica* metabolism throughout its life cycle presented here provides insight on how metabolic versatility and adaption of known physiological pathways for alternate functions enable a zoonotic pathogen to thrive in niches spanning across multiple kingdoms of life.

**Keywords: *Salmonella* enterica serovar Typhimurium, plant-microbe interaction, food safety, mannose metabolism, fatty acid biosynthesis**

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## INTRODUCTION

The host ranges of most pathogens have boundaries that fall between the species and kingdom levels. However, a small group of microbes are able to transcend these boundaries to lead a cross-kingdom lifestyle, i.e., colonizing both animals and plants. For example, the nosocomial human pathogens *Enterobacter cloacae* and *Serratia marcescens* are also plant pathogens that cause bulb decay of onions and cucurbit yellow vine disease, respectively (Bishop and Davis, 1990; Hejazi and Falkiner, 1997; Sanders and Sanders, 1997; Bruton et al., 2003; Barak and Schroeder, 2012). *Burkholderia cepacia* and *Pseudomonas aeruginosa* are both responsible for pneumonia in cystic fibrosis patients as well as bacterial rots of onion (Barak and Schroeder, 2012). Dual pathogenicity, however, is not required for a cross-kingdom lifestyle. *Xylella fastidiosa* causes economically important diseases of grapes, citrus, and other plants but colonizes its insect vector without causing disease (Chatterjee et al., 2008). In order to succeed on such diverse hosts, cross-kingdom pathogens adopt strategies that range from generalist to specialist. Generalists use universal strategies to exploit animal and plant hosts, whereas specialists have dedicated mechanisms for interaction with each host (Kirzinger et al., 2011). Understanding the biology of cross-kingdom bacterial colonists on both animal and plant hosts leads to improved management of human, animal, and plant health.

This study focuses on the cross-kingdom enteric human pathogen *Salmonella enterica*, which colonizes agricultural plants as alternate hosts and uses them as a vector to humans (Barak and Schroeder, 2012). Numerous salmonellosis outbreaks linked to *S. enterica*-contaminated fresh produce have occurred in recent years; produce now ranks as the riskiest commodity for foodborne illness (Painter et al., 2013). Because the primary economic and pathological significance of *S. enterica* lies in the human-associated portion of its life cycle, the biology of this pathogen is much better understood in the context of its human and animal hosts compared to its plant hosts. However, in order to reduce foodborne illness, we need to understand the biology of *S. enterica* on plants, before the pathogen reaches humans and causes disease.

The cross-kingdom lifestyle of *S. enterica* requires that this bacterium attach to, colonize, and persist in divergent host environments. In animals, *S. enterica* colonizes the anaerobic intestinal lumen and causes gastroenteritis in humans (Wiedemann et al., 2014). *S. enterica* pathogenesis and metabolism have been best characterized in the murine host due to the tractability of mice as an experimental model. In a mouse typhoidal model, the pathogen migrates to lymphoid organs such as the spleen, invades epithelial and macrophage cells, and establishes a nutrient-limited intracellular niche called the *Salmonella*-containing vacuole (SCV) (Steeb et al., 2013; Wiedemann et al., 2014). Nutrients in the SCV are quantitatively limiting (i.e., low concentrations) rather than qualitatively limiting (i.e., few types of nutrients). In contrast to colonization of animal tissues, plant surfaces colonized by *S. enterica* are typically aerobic and sites like the rhizosphere can support high levels of bacterial replication (Kwan et al., 2015), though the nutritional drivers of this growth are still unclear. Amino acids were previously found to be limiting in alfalfa root exudates, similar to conditions encountered during colonization of the chicken intestinal lumen, but the ability of amino acid auxotrophs and transport mutants to grow in the root environment suggests that amino acid metabolism is not a determinant of *S. enterica* growth during plant association (Harvey et al., 2011; Kwan et al., 2015). A variety of sugars, organic acids, and other nutrients have also been identified on plant surfaces and in root exudates, but their contributions to plant colonization by *S. enterica* remains to be determined (El-Hamalawi and Erwin, 1986; Mercier and Lindow, 2000; Nelson, 2004; Roberts et al., 2009; Han and Micallef, 2016). The metabolic networks essential for *S. enterica* plant colonization are also largely unknown. Addressing this knowledge gap is critical to understanding how *S. enterica* succeeds on plants, a poorly-characterized part of the pathogen life cycle.

Differences in host physiologies and the host niches colonized by *S. enterica* led us to hypothesize that different metabolic networks are required for the distinct plant- and animal-associated lifestyles of *S. enterica*. In this study, we broadly examined the metabolic capabilities of *S. enterica* to identify a subset of metabolic networks that drives plant colonization by this bacterium. We used alfalfa seedlings as our model as they are the produce commodity most frequently contaminated with *S. enterica* (Callejón et al., 2015). By comparing the metabolic networks driving plant colonization by *S. enterica* to the known metabolic requirements for animal colonization, we provide a unique, integrated view of *S. enterica* metabolism throughout its life cycle.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. Strains were cultured on lysogeny broth agar (LB) medium at 37°C unless otherwise indicated. When necessary, antibiotics were added at the following concentrations: ampicillin (Amp), 100 μg/ml; chloramphenicol (Cm), 40 μg/ml; kanamycin (Kan), 50 μg/ml.

### Mutant Construction

Gene deletion mutants were generated in *S. enterica* KHLT2 with the λ-Red recombination method (Datsenko and Wanner, 2000) and transduced into *S. enterica* 14028S or *S. enterica* SL1344 by bacteriophage P22. A *S. enterica* 14028S Δ*rfbP*::Kan mutant was obtained from a deletion library generated by the same method (Santiviago et al., 2009). Due to lack of O-antigen production, this mutant could not be transduced into the SL1344 background. All mutants were confirmed by PCR, either with two flanking verification ("veri") primers or one flanking verification primer and an antibiotic-cassette specific primer (c1 or k2) (Datsenko and Wanner, 2000). Mutagenesis and verification primers are listed in **Table 2**. Double mutants were produced by transduction into a mutant background.

**TABLE 1 |** List of strains.

| Strain or plasmid | Genotype/Relevant markers | References |
|---|---|---|
| **STRAINS** | | |
| ***S. enterica* sv. Typhimurium, SL1344** | | |
| | WT; *hisG rpsL,* $St^R$ | Hoiseth and Stocker, 1981 |
| | Δ*fadD*::$Kan^R$ Δ*fadK*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*glpFK*::$Kan^R$ Δ*gldA*::$Cm^R$ Δ*glpT* Δ*ugpB*, $St^R$ | Steeb et al., 2013 |
| | Δ*rbsB*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*galP*::$Kan^R$ Δ*mglB*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*nanT*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*manX*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*hisP*::$Kan^R$ Δ*artP*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*malG*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*manX*::$Kan^R$ Δ*nagE*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*deoP*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*lldP*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*fucP*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*eutC*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*proP*::$Kan^R$ Δ*proW*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*ptsG*::$Kan^R$ Δ*manX* Δ*mglB* Δ*galP*, $St^R$ | Steeb et al., 2013 |
| | Δ*exuT*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*STM4432*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*idnT*::$Kan^R$ Δ*gntT*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*lysP*::$Kan^R$ Δ*hisP*, $St^R$ | Steeb et al., 2013 |
| | Δ*uhpT*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*yicE*::$Kan^R$ Δ*xapB* Δ*uraA* Δ*nupC* Δ*nupG*, $St^R$ | Steeb et al., 2013 |
| | Δ*glgP* Δ*malP*, $St^R$, $Cm^R$, $Kan^R$ | Steeb et al., 2013 |
| | Δ*mtlA* Δ*mtlD*, $St^R$, $Cm^R$, $Kan^R$ | Steeb et al., 2013 |
| | Δ*glpFK*::$Kan^R$ Δ*glpT* Δ*ugpB*, $St^R$ | Steeb et al., 2013 |
| | Δ*glpFK*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*lysA*::$Kan^R$ Δ*cadA*, $St^R$ | Steeb et al., 2013 |
| | Δ*sifB*::GFPweak::$Kan^R$ Δ*yabJ* Δ*thil*, $St^R$ | Steeb et al., 2013 |
| | Δ*manX*::$Kan^R$ Δ*manA*::$Cm^R$ *sifB*@GFPs, $St^R$ | Steeb et al., 2013 |
| | Δ*manA*::$Cm^R$ *sifB*@GFPs, $St^R$ | Steeb et al., 2013 |
| | Δ*pdxA* Δ*STM0163 sifB*@sYFP, $St^R$ | Steeb et al., 2013 |
| | Δ*lysA*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*ilvD*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*thrC*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*trpA*::$Kan^R$ Δ*pheA* Δ*tyrA*, $St^R$ | Steeb et al., 2013 |
| | Δ*proC*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*cysE*::$Kan^R$ Δ*metA*::$Cm^R$ $St^R$ | Steeb et al., 2013 |
| | Δ*leuB*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*purH*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*purA*::$Kan^R$, $St^R$ | Steeb et al., 2013 |
| | Δ*tsx*::$Cm^R$, $St^R$ | Steeb et al., 2013 |
| JDB 1332 | Δ*pdxA* Δ*STM0163 sifB*@sYFP +pKTKan, $St^R$ | This study |
| JDB 1339 | Δ*fabG*::CmR, $St^R$ | This study |
| JDB 1343 | Δ*fabG*::CmR + pEVS141($Kan^R$), $St^R$ | This study |
| JDB 1344 | Δ*fabG*::CmR + pEVS141Δ*gfp*::*fabHDG*($Kan^R$), $St^R$ | This study |
| JDB 1336 | Δ*gmd*::$Kan^R$, $St^R$ | This study |
| JDB 1337 | Δ*manA*::$Cm^R$, $St^R$ | This study |
| JDB 1340 | Δ*manA*::$Cm^R$ + pEVS141($Kan^R$), $St^R$ | This study |
| JDB 1341 | Δ*manA*::$Cm^R$ + pEVS141Δ*gfp*::*manA*($Kan^R$), $St^R$ | This study |
| JDB 1399 | Δ*serA*::$Cm^R$, $St^R$ | This study |
| JDB 1400 | Δ*serA*::$Cm^R$ + pEVS141Δ*gfp*::*serA*($Kan^R$), $St^R$ | This study |
| JDB 1342 | WT + pEVS141($Kan^R$) | This study |
| ***S. enterica* sv. Typhimurium, 14028S** | | |
| | WT | ATCC |
| | Δ*asnA*::Kan Δ*asnB*::Cm | Kwan et al., 2015 |
| | Δ*glyA*::*Tn*10d | Kwan et al., 2015 |
| | Δ*lysA*::Kan | Kwan et al., 2015 |
| | Δ*metC*::Kan | Kwan et al., 2015 |
| | Δ*pheA*::Kan | Kwan et al., 2015 |
| | *proC693*::*Mud*A | Kwan et al., 2015 |
| | Δ*trpB*::Kan | Hao et al., 2012 |
| | Δ*tyrA*::Kan | Kwan et al., 2015 |
| | Δ*rfbP*::$Kan^R$ | Santiviago et al., 2009 |
| JDB 1358 | Δ*gmd*::$Kan^R$ | This study |
| **PLASMIDS** | | |
| pKTKan | *gfp*, $Kan^R$ | Miller et al., 2000 |
| pEVS141 | *gfp*, $Kan^R$ | Dunn et al., 2006 |

## Complementation

The Δ*manA*::Cm, Δ*fabG*::Cm, and Δ*serA*::Cm strains were genetically complemented by cloning of the *manA, fabHDG,* or *serA* genes into pEVS141. The complementation plasmids were transformed into the respective mutant strains. Transformants were selected on LB+Kan and confirmed by PCR of the cloned gene(s) from extracted plasmid DNA. The same primers were used for cloning and PCR confirmation (**Table 2**). For chemical complementation, 0.2 mM mannose was provided to Δ*manA*::Cm; 0.1 mM cysteine was added as a supplement for Δ*cysE* Δ*metA*.

## Preparation of Inoculum

For all experiments except mutant analysis of *serA*, overnight bacterial cultures were suspended in sterile deionized water and normalized to $\sim 10^8$ CFU/ml by absorbance at $OD_{600}$ using a spectrophotometer. This suspension was diluted as needed. For experiments involving Δ*serA*::Cm, the WT, mutant, and complement bacterial suspensions were made from overnight cultures grow in liquid lysogeny broth rather than LB agar. Inoculum populations were verified by dilution plating on LB.

**TABLE 2 |** Primers used in this study.

| Primer name | Primer sequence (5′3′) |
|---|---|
| **λ-RED DELETION/VERIFICATION** | |
| fabG-RS-F | GGCGCTGTCTGCGGCACTTACGCAATAAAAGAGGAAAACCG |
| | TGTAGGCTGGAGCTGCTTC |
| fabG-RS-R | GGCCTTTCGCCCTAATAACGCAAATATTTTTCAATCGTGACA |
| | TATGAATATCCTCCTTAG |
| fabG-RS-veriF2 | GAAACCAGCGGCAGATAAGC |
| manA-RS-F | ATACCTCCCATTGATCTCCACATTGAAACAGGGCTTGATAGT |
| | GTAGGCTGGAGCTGCTTC |
| manA-RS-R | TATAAGCTTAGCAAGAGTTGTTAAAAAATTCAGTACGTTGCA |
| | TATGAATATCCTCCTTAG |
| manA-RS-veriF2 | CCTGTCCAGACGATGCCAAG |
| gmd-RS-F | AGAAAGTTACTCCCTAACGGGACTATTTGAGGAAATGAAAGT |
| | GTAGGCTGGAGCTGCTTC |
| gmd-RS-R | CCGCGATGGCCCGCCACAAAAATTCGTTGCTTATTCATTCCA |
| | TATGAATATCCTCCTTAG |
| gmd-veriR | CGTGACGATAGGTCATGGCA |
| STM2084-veriR2 | CGGCTTTTCATCGCCTGTTG |
| rfbP-veriF | CTTTCGATGTTGAGCGCGAG |
| rfbP-veriR | GCACCTGAGTTACGCTGCTA |
| serAredFor | AAAAGACAGGATCGGGGAAATGGCAAAGGTATCGCTGGAGGTGTAGGCTGGAGCTGCTTC |
| serAredRev | GCAGGTCATCTCCTGCCCATTTAGCGGAAATTAGTACAGCCATATGAATATC CTC CTTAG |
| serAconfirmFor | TTCCTTCACGACGCTGGC |
| serAconfirmRev | TGTTGAGGCAGGGAAACC |
| C1 | TTATACGCAAGGCGACAAGG |
| K2 | CGGTGCCCTGAATGAACTGC |
| **COMPLEMENTATION** | |
| fabH-AvrII-F | NNNCCTAGGTCGTGGTGGCTGCTGTTATT |
| fabG-BamHI-R | NNNGGATCCTCTTCCTGCTTAACGCCCAG |
| manA-AvrII-F | NNNCCTAGGTGTCCAGACGATGCCAAGTG |
| manA-BamHI-R | NNNGGATCCCGCGGGACGTAGCATAAAAG |
| serAForAvr | NNNCCTAGGAGGGTTGTTCGCCAACCG |
| serARevBam | NNNGGATCCACCAGAGAAAGGATGGGC |

## Plant Assays

Surface sanitized alfalfa seeds (International Specialty Supply) were prepared as described by Cowles et al. (2016).

For alfalfa attachment assays, 10 seeds or 10 3-day-old seedlings were placed in sterile 50 ml conical tubes and co-inoculated with 20 ml of a $10^3$ CFU/ml bacterial suspension of wild-type (WT) and mutant at a 1:1 (±0.1) ratio. For experiments using Δ*manA*::Cm, the WT, mutant, and complement bacterial suspensions were made from overnight cultures on M9 minimal agar media amended with 0.4% glucose and 0.2 mM histidine because LB likely contains low concentrations of mannose (our own unpublished results). Histidine was required because *S. enterica* SL1344 is a histidine auxotroph. For chemical complementation, mannose was added to this overnight culture because the surface polymers produced downstream of *manA* (see Discussion) that are known to mediate attachment must be pre-formed in culture; *S. enterica* does not grow during the first 4 h following inoculation into germinating seedling cultures–the time period used for this attachment assay (Becker et al., 2006; Kwan et al., 2015). Tubes were incubated for 4 h at room temperature with gentle shaking. Then, the water was decanted and seeds or seedlings were rinsed twice in 20 ml sterile water by vortexing on high for 30 s to remove loosely attached bacterial cells. Individual seeds or seedlings were homogenized in 300 ul sterile water and plated on LB and LB+Kan or LB+Cm. Plates were incubated at 37°C overnight, and populations were enumerated. The experiment was repeated at least three times.

For alfalfa colonization assays, 0.3 g surface sanitized alfalfa seeds were placed in plastic petri dishes and irrigated with 20 ml of a bacterial suspension containing $10^5$ CFU/ml. For co-inoculation (competition) assays, the bacterial suspension contained a 1:1 (±0.1) ratio of two bacterial strains. After a 1 h incubation at room temperature with constant gentle agitation, the irrigation water was replaced with fresh sterile water. Inoculated seeds were then incubated at 24°C on a rotating shaker set at ∼40 rpm and under constant light. Every 24 h, samples were removed, and the irrigation water was replaced. Seedlings with attached seed coats were sampled at 24 h

post-inoculation, and those without seed coats were selected at 48 and 72 h. At each time point, three seedlings were placed in a 50 ml conical tube containing 20 ml sterile water and vortexed on high for 30 s. Then, individual seedlings were homogenized in 500 µl sterile water and dilution plated on LB, LB+Kan, and/or LB+Cm. Plates were incubated at 37°C overnight, and then populations were enumerated. There were three replicates per treatment and the experiment was repeated at least three times. Because it was necessary to distinguish between WT and mutant cells in our competition assays (detailed below), pKT-Kan was transformed into the unmarked Δ*pdxA* ΔSTM0163 strain as a selection marker. No growth difference was observed between the marked and unmarked strains in LB (data not shown). For chemical complementation, the appropriate metabolite was added into the irrigation water at each water change.

For growth assays in the exudates of germinating plant seedlings, experiments were set up as described for the colonization assays above except that the inoculum concentration was $10^3$ CFU/ml and the irrigation water was never changed. Growth was examined in the exudates of three different plants: alfalfa, broccoli, and lettuce. Broccoli seeds (Johnny Seeds; Winslow, ME) were surface sanitized as described above for alfalfa seeds. Lettuce seeds (cultivar Green Towers; GeneFresh Technologies; Salinas, CA) were not sanitized because bleach sanitized seeds failed to germinate. Approximately 130 alfalfa (=0.3 g), lettuce (=0.125 g), or broccoli seeds (counted by hand) were placed in each petri plate. Planktonic bacterial cell populations were determined every 24 h, for 72 h, by removing 100 ul of the irrigation water and plating serial dilutions on LB. Plates were incubated at 37°C overnight and then populations were enumerated. No bacterial growth was observed for control samples that were not inoculated (data not shown). There were three replicates per treatment, and the experiment was repeated at least three times.

To determine Δ*manA*::Cm growth in more mature plant exudates, the experiment was conducted identically to the typical growth assay (above) except that alfalfa seeds were germinated in sterile water for 24 h prior to bacterial inoculation. This method contrasts with the normal experimental setup where bacteria were inoculated into sterile water containing yet ungerminated seeds.

## Seedling Toxicity Assays

Alfalfa seeds (0.3 g) were germinated in 20 ml of a 0.1 mM cysteine solution and incubated for 3 days at 24°C on a rotating shaking set at ∼40 rpm and under constant light. Seeds germinated in sterile water served as the control. The irrigation water was removed and replaced with sterile water or 0.1 mM cysteine daily. The length of five 3-day-old seedlings was determined using a ruler. The experiment was repeated three times.

## Metabolomics

Metabolites in alfalfa seedling exudates were identified by liquid chromatography-mass spectrometry (LC-MS) as described by Kwan et al. (2015). Briefly, 0.3 g surface sanitized alfalfa seeds were germinated in 20 ml of a *S. enterica* 14028S bacterial suspension ($10^3$ CFU/ml) or mock-inoculated with sterile water. At 0, 8, 24, 48, and 72 h, the irrigation water was removed, passed through a 0.22 µM PES membrane filter, and analyzed by LC-MS to identify metabolites present in alfalfa seedling exudates. At least two biological replicates, each produced by pooling three technical replicates collected concurrently, were analyzed for each treatment at each time point.

## Statistical Analyses

Statistical analyses were performed using R software (version 2.14.1; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org]). Bacterial population counts were $\log_{10}$-transformed prior to analysis except in seed and seedling attachment assays where counts remained untransformed. The mean population size (single inoculation) or percent total population (co-inoculation) were calculated at each time point for each experiment and then averaged across experiments. Biological replicates within an experiment were considered subsamples. For plant attachment assays, the percent of the total population comprised of the mutant at each time point was compared against the percentage of the mutant in the inoculum in paired, two-tailed *t*-tests assuming unequal variance. Differences among treatments at each time point in singly inoculated growth (alfalfa) and seedling toxicity were also evaluated by paired, two-tailed *t*-tests assuming unequal variance. Differences in growth between amino acid auxotrophs and WT in lettuce and broccoli seedling exudates were calculated. These differences were rank transformed (Conover, 2012), then compared to an expected value µ, 0, using one-sample *t*-tests. For plant co-colonization and co-growth assays, the average percent total population for each mutant was compared against the expected value of µ, 50, in one-sample *t*-tests. For plant complementation assays, differences among treatments at each time point were analyzed using Tukey's honestly significant difference (HSD) test (based on two-way analysis of variance [ANOVA] with strain and experiment as factors). Significance was set at a $p < 0.05$ for all aforementioned tests.

The ratios of nutrient concentrations in the presence and absence of *S. enterica* were compared against an expected µ value of 1 using one-sample *t*-tests ($p < 0.1$). A ratio of 1 indicates that *S. enterica* had no effect on the concentration of a particular nutrient in alfalfa exudates; a ratio less than 1 indicates nutrient depletion in the presence of *S. enterica*.

# RESULTS

## Metabolic Network Requirements for *S. enterica* Plant Colonization

To identify metabolic networks that contribute to plant colonization by *S. enterica*, we screened a library of *S. enterica* SL1344 mutants for colonization fitness on alfalfa seedlings (**Figure 1**). This set of mutants had previously been tested for spleen colonization in a mouse typhoid fever model (Steeb et al., 2013). Mutants defective in the biosynthesis of amino acids, vitamins, and purines were outcompeted by the isogenic WT, suggesting that these nutrients are limiting in or on seedling

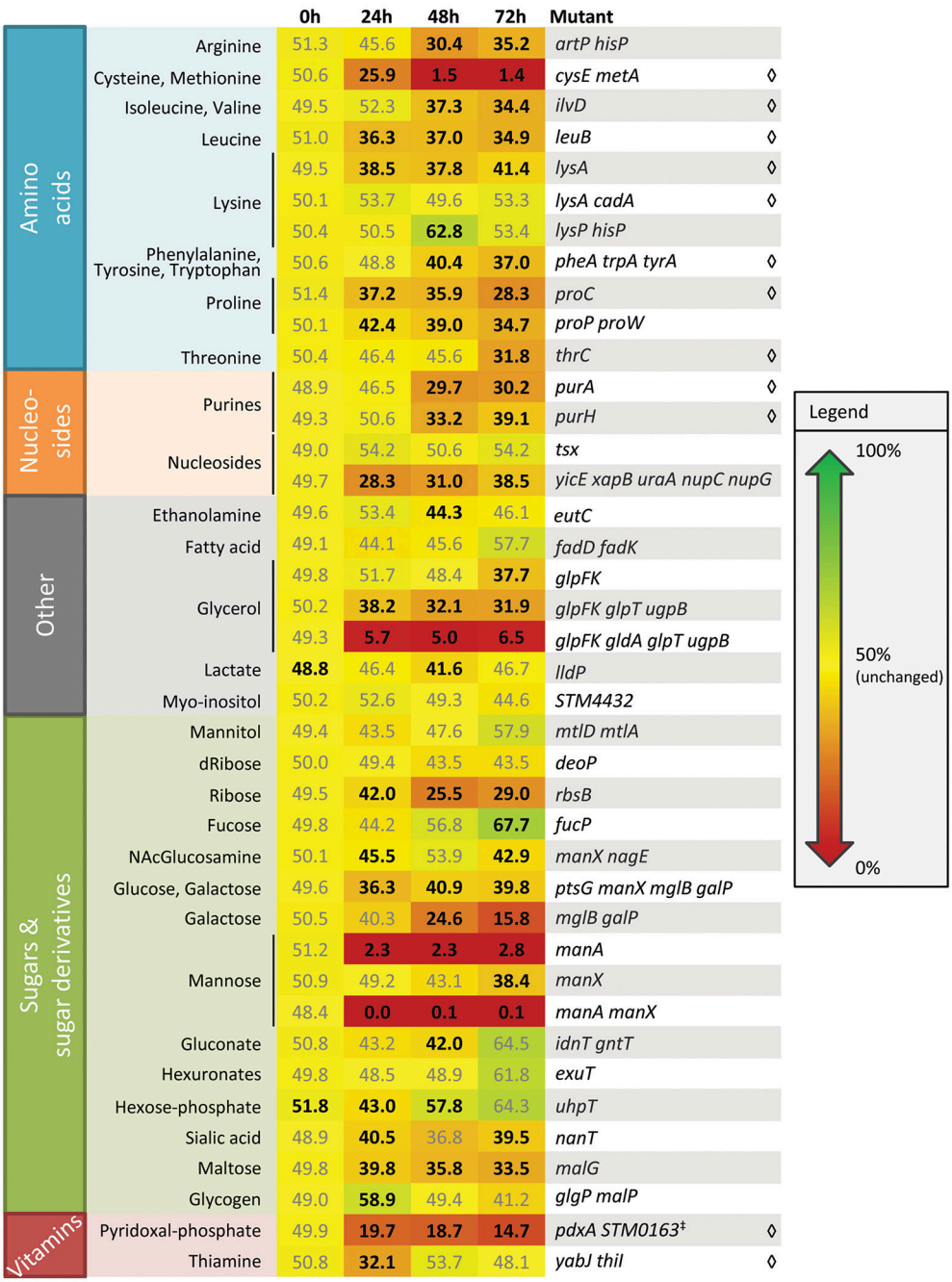


**FIGURE 1 |** Alfalfa seedling colonization fitness of *Salmonella* SL1344 metabolic mutants. Values indicate the percent of the total population comprised of mutant, following a 1:1 co-inoculation of WT and mutant. Cells containing these values are color coded as a heat map from 0 (red) to 100% (green) with yellow as 50%, i.e., equal to WT, for easy visualization of the data. Auxotrophic mutants are indicated by ◊; all other mutants lack genes for utilization of specific nutrients. The data shown are the means of three independent experiments with three replicates each. Bold values indicate a statistically significant difference from 50% (*t*-test, $p < 0.05$).

surfaces. Amino acid auxotrophs were also reduced in growth in alfalfa (Kwan et al., 2015), broccoli, and lettuce seedling exudates, indicating that amino acid limitation is common in the plant environment (Table S1). A *cysE metA* mutant, unable to biosynthesize the sulfur-containing amino acids cysteine and methionine, was severely compromised in its competitive colonization fitness vs. the WT, especially at 48 and 72 h. This fitness defect was partially complemented by addition of 0.1 mM cysteine but alfalfa seedlings were severely stunted (Figure S1). Seedlings germinated in the presence of cysteine were ∼50% shorter than their counterparts irrigated with water only, indicating that this amino acid is toxic to alfalfa development. Biosynthesis of many compounds is required for *S. enterica* colonization of both plant and animal hosts.

In addition to biosynthesis, nutrient transport also contributes to *S. enterica* plant colonization (**Figure 1**). A *glpFK gldA glpT ugpB* mutant, unable to transport or utilize glycerol and glycerol-3-phosphate, had a severe competitive disadvantage vs. the WT, suggesting that glycerol and glycerol-3-phosphate are major nutrient sources that drive *S. enterica* seedling colonization. Based on the mutant data, glucose, galactose, ribose, maltose, sialic acid, nucleosides, arginine, and proline are also likely present in the seedling environment and used to support bacterial colonization of seedlings. The temporal releases of nutrients in alfalfa exudates that can serve as sole carbon or nitrogen sources for *S. enterica*, as inferred from the mutant data and as directly measured by LC-MS (Tables S2–S4) are detailed in **Table 3**. Utilization of a variety of plant-derived nutrients, likely simultaneously, contributes to bacterial fitness during plant colonization.

## Fatty Acid Biosynthesis, but Not Catabolism, Is Required for Plant Colonization by *S. enterica*

Proteomic data indicate that the *S. enterica* fatty acid metabolic network is active during mouse spleen colonization and alfalfa seedling-associated growth (**Figure 2A**; Steeb et al., 2013; Kwan et al., 2015). Both degradation and biosynthesis of fatty acids are active in the animal model of infection and mutants defective in these pathways are less fit (Becker et al., 2006; Steeb et al., 2013). In contrast, fatty acid degradation appears to be inactive or dispensable for plant colonization. No proteins involved in β-oxidation of fatty acids were detected in a shotgun proteomic survey of the *S. enterica* proteome during growth in alfalfa seedling exudates (Kwan et al., 2015), and a *fadD fadK* mutant, unable to transport or activate fatty acids for β-oxidation, had no loss of fitness during competitive colonization of alfalfa seedlings (**Figure 1**). However, five proteins involved in fatty acid biosynthesis (AccA, AccB, AccC, AccD, and FabB) were identified in the *S. enterica* proteome during seedling-associated growth (Kwan et al., 2015), suggesting that this metabolic pathway was both active and required by *S. enterica* in this environment. To test this hypothesis, we examined a *fabG* mutant, defective in fatty acid biosynthesis, and found that it was non-competitive against the WT for seedling colonization (**Figure 2B**). The *fabG* mutant never exceeded 0.06% of the total bacterial population and frequently no *fabG* colonies were recovered. This mutant also exhibited a severe colonization defect even in the absence of competition from WT (**Figure 2C**). To better understand the nature of the defect, we examined the *fabG* mutant in each of the two distinct phases of plant colonization, attachment to plant surfaces followed by microbial growth. The *fabG* mutant was as competitive as WT for attachment to alfalfa seeds and 3-day-old seedlings (data not shown) but showed a growth defect in germinating alfalfa seedling exudates (**Figure 2D**). Both the growth and colonization defects of the *fabG* mutant were complemented by a plasmid-borne WT copy of *fabG* (**Figures 2C,D**). Taken together, fatty acid biosynthesis is not just a fitness factor but a determinant of growth during early seedling colonization by *S. enterica.*

**TABLE 3 |** Temporal utilization of nutrients by *S. enterica* in germinating alfalfa exudates that serve as sole C- or N-sources* or vitamins.

| Nutrient type | Nutrient | | | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|---|
| Amino acids | Aspartate | ‡ | | X | X | X |
| | Glutamine | ‡ | § | X | X | X |
| | Glycine | | § | X | X | X |
| | Proline | ‡ | § | X | X | X |
| | Serine | ‡ | § | X | X | X |
| | Alanine | ‡ | | - | X | X |
| | Arginine | ‡ | § | - | X | X |
| | Asparagine | ‡ | | - | X | X |
| Nucleosides | Adenosine | ‡ | | - | X | X |
| | Guanosine | ‡ | | - | X | X |
| | Uridine | ‡ | | - | X | X |
| | Inosine | ‡ | | - | X | X |
| Organic acids | Fumarate | ‡ | | - | X | X |
| | Glycerate | ‡ | | - | X | X |
| | Malate | ‡ | | - | X | X |
| | Succinate | ‡ | | - | X | X |
| Polyols | Glycerol-3-P | | § | X | X | X |
| | Glycerol | | § | – | - | X |
| Sugars | Maltose | | § | X | X | X |
| | Ribose | | § | X | X | X |
| | Glucose | | § | X | - | - |
| | Galactose | | § | - | X | X |
| Vitamins | Biotin | ‡ | | - | X | X |
| | Panthothenate | ‡ | | - | X | X |
| | Pyridoxal-P | | § | X | X | X |
| | Thiamine | ‡ | § | X | X | X |

**Compounds that can be used as sole carbon and nitrogen sources by S. enterica were identified by Gutnick et al. (1969). ‡, Nutrient utilization directly measured by LC-MS, as reported in Kwan et al. (2015) and this work. §, Nutrient utilization inferred from mutant data. Glutamine utilization inferred from Kwan et al. (2015). X, present and utilized by S. enterica as a nutrient. -, not present or no significant utilization by S. enterica as a nutrient. All nutrients measured by LC-MS were detected in plant exudates at all time points indicated by "-." Inferences made from mutant phenotypes were unable to distinguish between nutrient absence vs. lack of utilization by S. enterica.*

## Serine Biosynthesis Is Required for Plant Colonization by *S. enterica*

During alfalfa seedling-associated *S. enterica* growth and animal infection, serine metabolic network activity was detected (**Figure 3A**; Steeb et al., 2013; Kwan et al., 2015) and yet, serine biosynthesis is dispensable for mouse infection (Jelsbak et al., 2014). A *serA* mutant was poorly competitive for alfalfa seedling colonization at 48 and 72 hpi (**Figure 3B**) and grew poorly in root exudates (data not shown). The fitness of the *serA* mutant was fully restored by genetic complementation by a plasmid carrying a WT copy of *serA*. These data suggest that serine biosynthesis occurs during both plant and animal colonization but is only essential during seedling colonization.

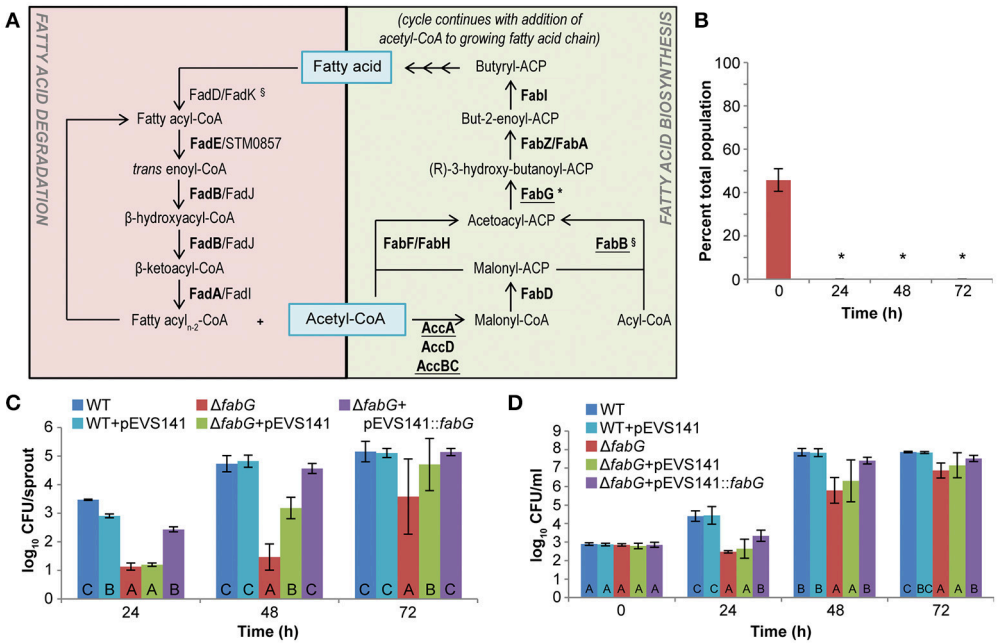


**FIGURE 2 |** The fatty acids metabolic network is required for *S. enterica* colonization of plants and animals. **(A)** Fatty acid degradation contributed to *S. enterica* colonization of animals (Steeb et al., 2013) whereas fatty acid biosynthesis was required for colonization of both animals and plants. Fatty acid network proteins were detected in proteomic screens of *S. enterica* during colonization of mice (bolded, Steeb et al., 2013) and growth in association with alfalfa seedlings (underlined, Kwan et al., 2015). §, significant colonization defect of mutant in mice (Barat et al., 2012; Steeb et al., 2013). *, significant colonization defect of mutant on alfalfa seedlings. **(B)** *S. enterica* SL1344 Δ*fabG* was non-competitive in alfalfa seedling colonization. Bars show the mean percent of the total population comprised of Δ*fabG* following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (Student's *t*-test, $n = 3$, $p < 0.05$). **(C,D)** Δ*fabG* was defective in alfalfa seedling colonization **(C)** and growth in seedling exudates **(D)**. These defects were complemented by addition of a plasmid-borne copy of *fabG* on pEVS141. Bars show the average population of *S. enterica* present on alfalfa sprouts **(C)** or the irrigation water containing seedling exudates **(D)**. Letters indicate statistically different groups within each time point (Tukey's HSD, $n = 45$, $p < 0.05$). For all experiments, data shown are the means of 3 independent experiments with 3 replicates each. Error bars indicate the standard deviation.

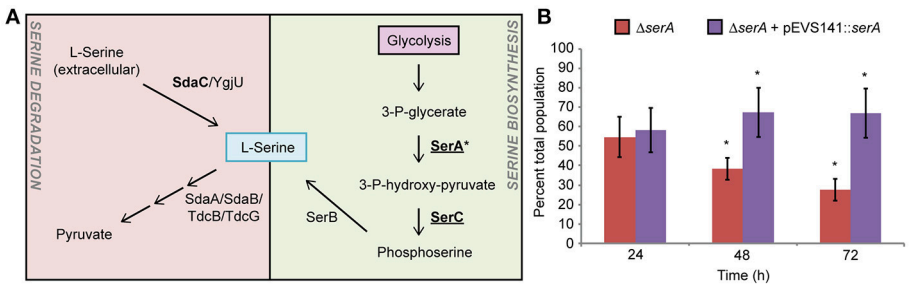


**FIGURE 3 |** Serine biosynthesis is required for *S. enterica* plant colonization. **(A)** Serine network proteins were detected in proteomic screens of *S. enterica* during colonization of mice (bolded, Steeb et al., 2013) and growth in association with alfalfa seedlings (underlined, Kwan et al., 2015). *S. enterica* SL1344 Δ*serA* was reduced in competitive fitness in alfalfa seedling colonization **(B)**. Bars show the mean percent of the total population comprised of Δ*serA* following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (Student's *t*-test, $n = 3$, $p < 0.05$).

## *manA* Is Required for *S. enterica* Plant Colonization

Proteomic data indicate that ManA is active during mouse spleen colonization and a *manA* mutant is less fit in infected mouse tissues (Steeb et al., 2013). A *manA* mutant was also non-competitive for alfalfa seedling colonization (**Figure 1**). ManA catalyzes the reversible isomerization of mannose-6-phosphate into fructose-6-phosphate. This defect was partially complemented by a plasmid carrying a WT copy of *manA* (**Figure 4A**). The fitness of the *manA* mutant was fully restored by addition of 0.2 mM mannose, but not 0.2 mM fructose, suggesting that the directionality of the reaction catalyzed by *manA in planta* is toward production of mannose-6-phosphate (data not shown). Mannose-6-phosphate is a precursor for the biosynthesis of the surface polymers colanic acid and O-antigen. The competitive colonization defect of the *manA*

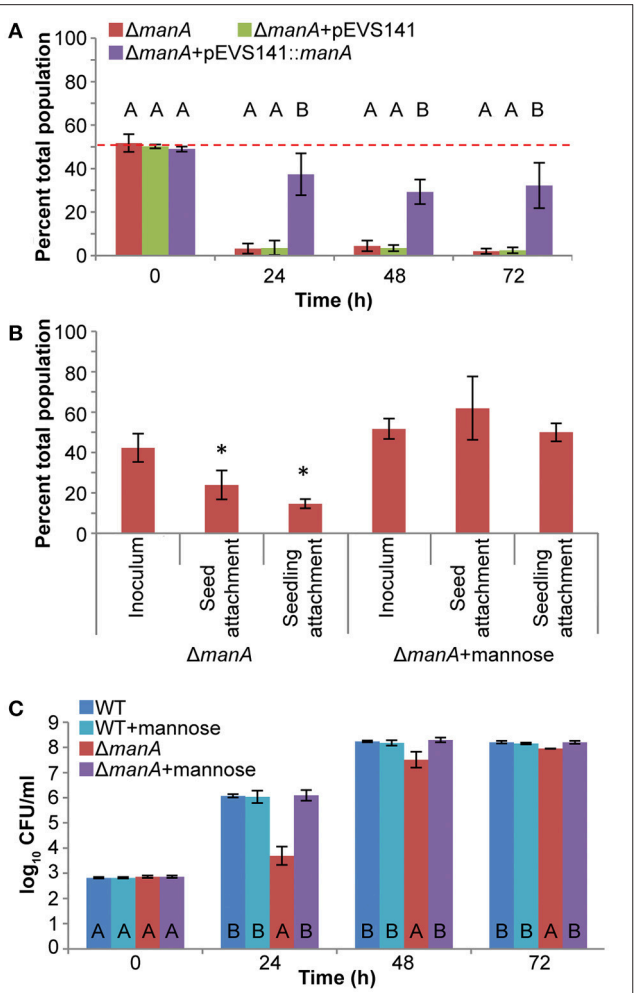


**FIGURE 4 |** Mannose is required for *S. enterica* plant colonization. **(A)** SL1344 Δ*manA*::Cm had a severe competitive colonization defect on alfalfa seedlings. Bars show the mean percent of the total population comprised of Δ*manA* following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (One-sample *t*-test, $n = 3$, $p < 0.05$). Letters above bars, statistically different groups within each time point (Tukey's HSD, $n = 9$, $p < 0.05$). **(B)** Mannose contributed to *S. enterica* attachment to alfalfa seeds and 3 day-old seedlings. The attachment defect of Δ*manA*::Cm was fully complemented by addition of 0.2 mM mannose. Bars show the mean percent of the total population comprised of Δ*manA* following a co-inoculation with the isogenic WT. *, statistically different from the inoculum (Paired *t*-test, $n = 6$, $p < 0.05$). **(C)** Mannose contributes to *S. enterica* growth in alfalfa seedling exudates *in vivo*. The growth defect of Δ*manA*::Cm was fully complemented by addition of 0.2 mM mannose. Letters at the bottom of the bars, statistically different groups within each time point (Tukey's HSD, $n = 36$, $p < 0.05$). For all experiments, data shown are the means of 3 independent experiments with 3 replicates each. Error bars indicate the standard deviation.

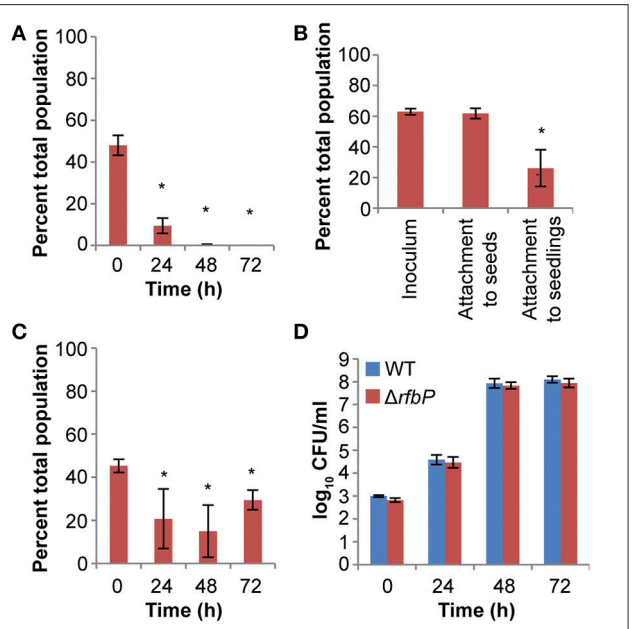


**FIGURE 5 |** O-antigen contributes to *S. enterica* plant colonization. **(A)** 14028S Δ*rfbP*, an O-antigen mutant, was less competitive than the isogenic WT in alfalfa seedling colonization. *, statistically different from 50% (Student's *t*-test, $n = 3$, $p < 0.05$). **(B)** O-antigen contributed to attachment to 3 d-old seedlings but not seeds. *, statistically different from inoculum (Paired *t*-test, $n = 6$, $p < 0.05$). **(C,D)** Δ*rfbP* had a subtle growth defect in alfalfa seedling exudates that is revealed only in competition with the isogenic WT. **(C)** *, statistically different from 50% (Student's *t*-test, $n = 3$, $p < 0.05$). **(D)** *, statistically different from WT (Paired *t*-test, $n = 6$, $p < 0.05$). WT and mutant were inoculated at a 1:1 ratio for all competition experiments **(A,B,C)**. All data shown represent the means of 3 independent experiments with 3 replicates each. Error bars indicate the standard deviation.

mutant stemmed from defects in both attachment, to alfalfa seeds and seedlings, and growth (**Figures 4B,C**). These defects were fully complemented by the addition of 0.2 mM mannose to the overnight culture (attachment) or the irrigation water (growth). The growth pattern of the *manA* mutant suggested that growth was delayed, but the growth rate was not impaired beyond the first 24 h. During the first 24 h, WT populations increased ~3 $\log_{10}$ (1000-fold) whereas the *manA* mutant growth was severely restricted to <1 $\log_{10}$ (<10-fold; **Figure 4C**). The *manA* mutant resumed growth between 24–48 h, increasing 3.8 $\log_{10}$, resembling WT replication during the 0–24 h period. Taken together, these data suggest that mannose may be a required metabolite that is absent from early seedling exudates. Alternately, *manA* may be required for plant host interactions.

## O-antigen Contributes to Distinct Steps in Plant Colonization

We hypothesized that the colonization defects of the *manA* mutant were due to defects in the production of extracellular polysaccharides from the mannose metabolic pathway, either O-antigen or colanic acid. We tested this hypothesis by examining the competitive colonization of *gmd* and *rfbP* mutants against the WT. A *gmd* mutant, unable to produce the GDP-fucose, is defective in colanic acid biosynthesis and a *rfbP* mutant fails to catalyze the first step in O-antigen assembly on the bacterial cell membrane. The *gmd* mutant was as competitive as the WT for seedling colonization and exhibited no attachment or growth defects (data not shown). The *rfbP* mutant was less fit than the WT in a competitive colonization assay (**Figure 5A**). This colonization defect was due to deficiencies in attachment and

competitive growth (**Figures 5B**,**C**, respectively). However, no growth defect was observed when the *rfbP* mutant was singly inoculated into plant cultures (**Figure 5D**). Our data indicate that O-antigen, but not colanic acid, contributed to distinct steps of *S. enterica* seedling colonization.

## DISCUSSION

It is widely accepted that *S. enterica* adopts a cross-kingdom lifestyle, actively colonizing and persisting on plants in between infection of animal or human hosts (Barak and Schroeder, 2012). We hypothesized that this human pathogen would employ distinct metabolic strategies for colonization of cross-kingdom hosts due to the inherent differences in plant and animal host physiology as well as the nature of the extracellular (plant) vs. intracellular (animal) niches. A recent mutant screen of metabolic mutant colonization of murine spleens gave us the opportunity to test our hypothesis. Direct comparison of the same set of *S. enterica* mutants in both environments is a powerful approach that avoids identification of artificial differences that may arise from comparisons based on disparate data sets. Whereas the SCV inside of animal cells contains high levels of reactive oxygen and nitrous oxide species and few sugars but sufficient amino acids, plant root exudates are aerobic and sugar-rich, but amino acid deficient. We were thus surprised to find that *S. enterica* generally depends on the same metabolic networks to exploit both plant and animal hosts.

Biosynthesis of essential cellular metabolites is a key metabolic function shared between the plant- and animal-associated lifestyles of *S. enterica*. However, the specific nutritional constraints on *S. enterica* growth differed between plant and animal hosts. Though amino acids were present in both host environments, as inferred from impaired colonization by mutants and measured depletion of these metabolites, amino acids were much more limiting during growth in plant seed exudates (alfalfa, lettuce, broccoli; Kwan et al., 2015 and this study) and tomato fruit (Han and Micallef, 2016) than in animal models of infection. Of the 13 amino acids which have been examined in murine models, only asparagine and proline biosynthesis were required for full fitness (Steeb et al., 2013; Jelsbak et al., 2014). In contrast, *de novo* biosynthesis of at least 8 different essential amino acids (out of 18 examined) were required in each plant environment for full *S. enterica* fitness (Kwan et al., 2015; de Moraes et al., 2017, and this study), though the exact amino acids varied by plant. Notably, methionine and tryptophan biosynthesis emerged as common requisites for the plant-associated lifestyle of *S. enterica* that are dispensable during animal infection. We recognize that levels of different amino acids will vary and depend on factors such as plant growth conditions, plant genotype, plant growth stage, as well as plant organ; yet, our study did examine root exudates from plants from three distinct orders while murine models are restricted to a single host (mouse) that carry a dysfunctional *Slc1 1a1* allele which makes them highly susceptible to systemic *Salmonella* infections. Collectively, the data presented herein and in the current literature indicate that the host-associated lifestyles of *S. enterica*, whether on plants or animals, involve exploitation of host nutrients as well as *de novo* biosynthesis of metabolites insufficiently available from the host.

The much greater variety of amino acids that must by produced endogenously during plant-associated growth compared to animal niches reflects the general low availability of these nutrients from plant hosts as well as the high bacterial titers ($\geq 10^7$ CFU/ml) in plant environments. For example, the average concentration of every amino acid except threonine in alfalfa seedling exudates was less than 70 uM, which is 3–185 times lower than reported in murine spleen tissue (Xiao et al., 2016). Plants may tightly control production and release of certain nutrients, e.g., cysteine, due to their toxicity (Figure S1). Thus, even dedicated plant colonists such as *P. fluorescens* and *P. tolaasii* must synthesize these metabolites to succeed in the plant environment (Simons et al., 1997; Chung et al., 2014). Because amino acid restriction is common in the plant environment, amino acid prototrophy appears as a universal plant colonization trait for a variety of plant-associated bacteria.

Utilization of multiple, diverse nutrients appears to be a general metabolic colonization strategy of *S. enterica*. During typhoid fever in mice, spleen colonization by *S. enterica* depended on the simultaneous exploitation of seven host nutrients - glycerol, fatty acids, N-acetylglucosamine, gluconate, glucose, lactate, and arginine (Steeb et al., 2013). During alfalfa seedling colonization, *S. enterica* was inferred to use glycerol, glucose, galactose, ribose, maltose, nucleosides, arginine, and proline to support its growth (this study), as mutants in these metabolic pathways were less fit. The exploitation of a greater range of sugars in the plant environment reflects the richness of sugars in plant exudates. In both plant and animal hosts, glycerol and its derivative glycerol-3-phosphate were metabolic drivers of *S. enterica* colonization. Mutants impaired in the transport and/or catabolism of these compounds were up to 10-fold less fit than the WT in competitive colonization of both hosts. Because these mutants were not non-competitive, we conclude that glycerol and glycerol-3-phosphate are not essential metabolic determinants of host colonization by *S. enterica*. Overall, most individual nutrients appeared to have small contributions to bacterial colonization. The availability or use of specific nutrients did not determine plant colonization ability; rather, high levels of *S. enterica* colonization is supported by the contributions of many plant-derived nutrients. This diversified metabolic strategy may enable *S. enterica* to colonize diverse hosts and better adapt to changes in the host environment, such as the alteration of plant exudate composition during plant development. However, the robust metabolism of *S. enterica* and the rich and dynamic nature of plant exudates present a complex challenge for designing strategies aimed at restricting or reducing enteric human pathogen growth on food crops. Identifying essential nutrients that *S. enterica* either cannot biosynthesize, e.g., minerals, or cannot import will be key to this endeavor.

While there are many similarities in *S. enterica* metabolism during plant and animal colonization, we highlight in this work three distinct differences in the metabolic networks used to support each bacterial lifestyle. For example, host differences and metabolic preferences lead to differential usage of the

metabolic network surrounding fatty acids. Proteomic data indicate that this metabolic network is active during mouse spleen colonization and alfalfa seedling-associated growth (Steeb et al., 2013; Kwan et al., 2015). Fatty acid biosynthesis by *S. enterica* is likely important for repair of cell membranes damaged during infection and production of new membrane components for replicating cells. Disruption of fatty acid biosynthesis in a *fabB* mutant resulted in a 100,000-fold loss in competitive fitness ($\log_2$ (competitive index) $< -16.7$) and rapid clearance of the bacterium from infected mice (Becker et al., 2006; Barat et al., 2012). The failure of a *fabG* fatty acid biosynthetic mutant to grow during the first 24 h in alfalfa seedling exudates and the restoration of its growth ability thereafter suggests that fatty acids were not available to *S. enterica* in early seedling exudates but became available as the seedlings matured. Even as this nutrient became available, it was not a preferred nutrient source for *S. enterica* based on the WT phenotype of the *fadD fadK* fatty acid utilization mutant. This result is consistent with the use of sugars as preferred carbon sources, which are abundant in plant exudates. This conclusion contrasts with *S. enterica* nutrition during systemic murine infection where fatty acids were a major nutrient source (Steeb et al., 2013). Though fatty acid degradation was not necessary for the phases of plant-associated growth (lag to late log phases) examined in this study, partly due to the lack of this nutrient in very early seedling exudates, this metabolic function may become more important as bacterial cells enter the less biosynthetically-active stationary phase. At this point, maintenance and repair of cell membranes will likely shift from use of phospholipids synthesized *de novo* to modification or degradation and repurposing of existing cellular lipids. A recent study of stationary phase *S. enterica* in tomato fruits appears to support this conclusion (de Moraes et al., 2017). Fatty acid degradation may be an example of divergent use of a single metabolic network, for cellular maintenance vs. nutrition, during plant and animal colonization.

Serine metabolism is another example of a distinct difference between *S. enterica* plant and animal colonization. SerA and SerC, two enzymes involved in serine biosynthesis via the glycolysis intermediate 3-phosphoglycerate, are produced both during growth in germinating alfalfa exudates (Kwan et al., 2015) and during animal colonization (Becker et al., 2006; Steeb et al., 2013). In contrast, the serine transporter SdaC and serine deaminases SdaA and SdaB were detected only during animal infection. Mutant analysis indicates that serine biosynthesis is required for alfalfa root colonization (this study) but dispensable during murine infection (Jelsbak et al., 2014). Serine biosynthesis occurs during both plant and animal colonization but is only essential for root colonization suggesting that sufficient serine may be available in animals or serine synthesis from glycine is energetically favorable during animal colonization. The latter is supported by the finding that the *gvc* operon, involved in this glycine-to-serine conversion, is active during animal infection (Eriksson et al., 2003). Taken together, the data suggests that serine is limited in both murine spleens and alfalfa root exudates but *S. enterica* uses different biosynthetic pathways to overcome that deficit during its plant- and animal-associated lifestyles.

We also examined the metabolic network surrounding mannose. A *manA* mutant was severely compromised in competitive colonization of both plants and animals. ManA, or phosphomannose isomerase (Pmi), catalyzes the reversible isomerization of mannose-6-phosphate into fructose-6-phosphate, a glycolytic intermediate. In both plants and animals, the reaction catalyzed by ManA is believed to operate in the reverse direction, diverting fructose-6-phosphate from glycolysis to yield mannose-6-phosphate (Steeb et al., 2013, this study). Mannose-6-phosphate is a precursor for the nucleotide sugars that form the backbones of the common enterobacterial extracellular polysaccharides colanic acid and O-antigen. A *pmi* mutant is avirulent in mice, and the loss of competitive colonization fitness in a *manA* mutant is attributed to loss of O-antigen (Collins et al., 1991; Steeb et al., 2013). O-antigen delays immune recognition and confers resistance to complement-mediated killing and antimicrobial peptides in animal models of infection (Duerr et al., 2009; Ilg et al., 2009). However, we found no role for *manA* in resistance to ROS, acidity, flavone, or the antimicrobial peptide polymyxin B in plant exudates (data not shown). In fact, O-antigen deficiency only partially explained the phenotype of the *manA* mutant *in planta.* O-antigen contributed to seedling but not seed attachment, and competitive growth fitness in the plant environment but not the inherent ability to grow in the absence of competition. We also found no role for colanic acid in *S. enterica* seed or seedling attachment, colonization, or plant-associated growth. These results are consistent with a previous report that identified a role for O-antigen, but not colanic acid, in alfalfa sprout attachment and colonization by *S. enterica* sv. Enteriditis (Barak et al., 2007). However, colanic acid has been reported to contribute to desiccation tolerance on leaf surfaces (Cowles et al., 2016). The results from this study suggest that physiological functions in addition to O-antigen and colanic acid are impaired in a *manA* mutant, which is also reduced in seed attachment and severely compromised in the ability to grow during the first 24 h following inoculation into a germinating seedling culture. We hypothesize that mannose-6-phosphate and/or a downstream metabolite essential for plant-associated growth is absent from early seed exudates and must be biosynthesized endogenously by *S. enterica* to permit bacterial proliferation.

Taken together, this study shows that there is high overlap in the metabolic networks that are required for both the plant- and animal-associated lifestyles of *S. enterica*. However, some of these networks can be used differently to enable *S. enterica* to colonize each host, reflecting the chemical environment provided by the host. Common, simple molecules such as sugars, organic acids, and amino acids comprise the diet of *S. enterica* in both plants and animals. This finding was initially surprising to us because plants and animals are physiologically distinct and we expected unique *S. enterica* metabolic profiles in these environments. However, plants and animals share the same basic cellular metabolites (i.e., glucose, glycerol, amino acids, etc.) and pathways; it is understandable that similar host nutrients support *S. enterica* colonization of plants and the SCV inside of animal cells. Thus, the success of *S. enterica* in both host environments is partly due to similar available metabolites from plant and animal

hosts. Additionally, the metabolic capacity of the bacterium—characterized by nutritional versatility, biosynthesis of limiting metabolites, and co-opting virulence pathways (e.g., the mannose metabolic network) for plant colonization—likely contribute to the ability of *S. enterica* to exploit cross-kingdom hosts.

## CONCLUSIONS

We have provided fundamental information about how a human enteric pathogen grows on and colonizes plants. This advancement is an important step to understand the biology of *S. enterica*, the leading bacterial pathogen of food-borne illness which is now most commonly linked to consumption of fresh produce. We examined *S. enterica* mutants defective in the metabolism of diverse nutrients for their ability to colonize plants in order to identify the traits that contribute to its success. A comparison with the metabolic requirements for splenic colonization revealed few differences in metabolic network use, e.g., amino acid and fatty acid metabolism and exopolysaccharide production. However, pathways within these networks served different functions facilitating S. *enterica* colonization of diverse niches. To thrive in distinct niches that span multiple kingdoms of life, *S. enterica* exhibits metabolic versatility and adaptation of physiological pathways for alternate functions. Having the capacity to colonize the food source (plants) of a preferred host (omnivore or herbivore) allows fluidity in the lifecycle of *S. enterica*.

## AUTHOR CONTRIBUTIONS

GK and JB conceived the study and wrote the manuscript. GK, JB, KC, TP, and DA-N analyzed the data. GK conducted the majority of the assays. TP conducted the metabolomics assays. BP and KC participated in mutant phenotyping. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00695/full#supplementary-material

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# Genome-Wide Comparative Functional Analyses Reveal Adaptations of *Salmonella* sv. Newport to a Plant Colonization Lifestyle

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Outbreaks of salmonellosis linked to the consumption of vegetables have been disproportionately associated with strains of serovar Newport. We tested the hypothesis that strains of sv. Newport have evolved unique adaptations to persistence in plants that are not shared by strains of other *Salmonella* serovars. We used a genome-wide mutant screen to compare growth in tomato fruit of a sv. Newport strain from an outbreak traced to tomatoes, and a sv. Typhimurium strain from animals. Most genes in the sv. Newport strain that were selected during persistence in tomatoes were shared with, and similarly selected in, the sv. Typhimurium strain. Many of their functions are linked to central metabolism, including amino acid biosynthetic pathways, iron acquisition, and maintenance of cell structure. One exception was a greater need for the core genes involved in purine metabolism in sv. Typhimurium than in sv. Newport. We discovered a gene, *papA*, that was unique to sv. Newport and contributed to the strain's fitness in tomatoes. The *papA* gene was present in about 25% of sv. Newport Group III genomes and generally absent from other *Salmonella* genomes. Homologs of *papA* were detected in the genomes of *Pantoea, Dickeya,* and *Pectobacterium,* members of the Enterobacteriacea family that can colonize both plants and animals.

**Keywords: tomato, plant-microbe interactions, comparative genomics, pan-genome, vegetable safety**

## INTRODUCTION

Salmonellosis outbreaks linked to the consumption of fruits (tomatoes, cucumbers, cantaloupes), leafy green vegetables and sprouts became an important public health issue over the last decade, defying the traditional notion that this pathogen is only associated with products of animal origin (Teplitski et al., 2009; Hernandez-Reyes and Schikora, 2013; Jackson et al., 2013; Wiedemann et al., 2014; Bennett et al., 2015). A CDC study identified *Salmonella* sv. Newport as the predominant serovar involved in outbreaks traced to vegetables. It was responsible for 57% of outbreaks associated with all fresh vegetables, and for 29% of outbreaks

associated with vine-stalk vegetables (such as tomatoes and cucumbers). Tomatoes were a major source of salmonellosis outbreaks, and were implicated in up to 90% of outbreaks linked to vine-stalk vegetables, with sv. Newport responsible for 32% of them (Jackson et al., 2013). An analysis of outbreak occurrence from 1990 to 2010 identified 15 outbreaks that were associated with fresh tomatoes, and sv. Newport was responsible for six of them (Bennett et al., 2015). The association of salmonellosis outbreaks with sv. Newport and fresh vegetables reported in independent geographical locations suggests that this serovar may have a close relationship with plants and/or has evolved to persist in the vegetable production environment. At least three hypotheses can explain this phenomenon.

One hypothesis for the overrepresentation of sv. Newport in produce-associated outbreaks is that it evolved functions that make it more fit in plants. This hypothesis is supported by the evidence that sv. Newport outcompetes other serovars during plant colonization. When tested for proliferation in tomatoes, *Salmonella* sv. Newport reached higher cell numbers in green and pink tomatoes than sv. Typhimurium, Braenderup, and Montevideo (Marvasi et al., 2013b). Cells of serovar Newport were also recovered at higher rates from tomato rhizosphere than those of the serovars Saintpaul, Typhimurium, and Montevideo (Zheng et al., 2013). At least in part, the ability of some strains of sv. Newport to be more competitive within tomatoes could be due to spontaneous non-*rdar* mutations, which increased *Salmonella* fitness inside tomatoes (Zaragoza et al., 2012).

Secondly, strains of serovar Newport may be more fit in plants than those of other serovars because they are better able to adapt their physiology to enter and proliferate in tomato tissues (Teplitski and de Moraes, 2018). Studies with regulatory mutants and mutants in metabolic pathways suggest that during tomato colonization, *Salmonella* utilizes carbohydrates and inorganic sources of nitrogen and then uses the acquired nutrients to synthesize amino acids, LPS, and capsule. For example, genes involved in amino acid biosynthesis and iron acquisition were important for *Salmonella* growth in tomato pericarps. Inside tomato fruits, at least 51 *Salmonella* genes were differently regulated, including *fadH*, involved in fatty acid degradation, and *cysB*, the regulator for cysteine biosynthesis and acquisition. Changes in surface structures are also part of the *Salmonella* strategy for proliferation within plant tissues. In unripe tomatoes, the *yihT* gene, involved in the synthesis of O-antigen, is required for successful colonization of pericarps (Noel et al., 2010; Marvasi et al., 2013a; Nugent et al., 2015; de Moraes et al., 2017). These observations suggest that the environment in plant hosts is nutritionally unbalanced, and *Salmonella* has to employ and coordinate a diverse set of functions to thrive and establish itself within a niche already occupied by the native microbiota. In many respects, these observations are in line with the requirements of other phytobacteria during their interactions with plants (Lindow and Leveau, 2002; Lindow and Brandl, 2003). However, an earlier analysis of the totality of functions used by *S.* Typhimurium to colonize tomatoes revealed that, despite some similarities with phytobacteria in its plant colonization strategies, it relies on a distinct set of functions to establish itself within tomatoes (de Moraes et al., 2017).

Lastly, the advantage of sv. Newport over other serovars in colonization of plants could be enhanced by the presence of intrinsic colonization factors in this *Salmonella* clade. Studies of *Salmonella* virulence in animals show how the serovars' genetic diversity can affect interactions with the hosts. For example, distribution of virulence plasmids and pathogenicity islands is serovar-specific, and the presence of these genetic determinants appears to impact host range (Barrow et al., 1987; Barrow and Lovell, 1988; Libby et al., 1997; Desai et al., 2013). Is it possible that sv. Newport has functions, not commonly present in other serovars of *Salmonella*, that allow it to colonize plants more efficiently?

In this study, we applied comparative genomics combined with transposon insertion screening analysis to identify unique features that distinguish sv. Newport from sv. Typhimurium in the *Salmonella*-tomato interaction model.

# MATERIALS AND METHODS

## Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *Salmonella enterica* and *Escherichia coli* strains were propagated in LB (Luria Bertani) broth (Fisher Scientific) at 37 or 30°C, as specified in the text. When necessary, bacteria were plated onto LB agar (Fisher Scientific) or XLD (Oxoid) agar plates. As appropriate, growth media were supplemented with 100 μg/ml ampicillin, 60 μg/ml kanamycin or 20 μg/ml chloramphenicol. Construction of mutants was done using λ Red mutagenesis (Datsenko and Wanner, 2000). Typically, entire ORFs (from the start to the stop codon) were excised and replaced with a frt-*kan*-frt or a frt-*cat*-frt cassette, and the kanamycin (or chloramphenicol) resistance gene was flipped out as in Datsenko and Wanner (2000).

## Transposon Insertion Library Screening in Tomato Pericarps

Tomato inoculation with a *S. enterica* Newport C4.2 transposon library was performed as previously done with *S. enterica* Typhimurium ATCC14028 (de Moraes et al., 2017). Tomatoes (cultivar Campari) were obtained from a local grocery store, where they are sold on the vine in a clam shell plastic container. The *S. enterica* sv. Newport transposon library was grown overnight in LB with the addition of kanamycin at 37°C, and the resulting cultures were resuspended in PBS. Approximately $10^8$ CFU/ml in 3 μL of PBS were inoculated into three shallow wounds in tomato pericarps, and six fruits were used. Tomato fruits were incubated at 22°C and a relative humidity of ∼60% for 7 days. Under these conditions, no gross changes in the appearance of the fruit were observed, although ripening clearly progressed. *Salmonella* was recovered by collecting 1 g samples of tomato tissue around ∼1 cm of the inoculation site; samples from the same fruit were combined and homogenized in a stomacher (Sevard). *Salmonella* cells were recovered by centrifugation and were then resuspended and cultured in 50 ml of LB broth for 6 h at 37°C and 250 rpm.

**TABLE 1 |** Strains used in this study.

| Strain name | Genotype | Source or construction |
|---|---|---|
| C4.2 | *Salmonella* sv. Newport C4.2 | *Salmonella* sv. Newport isolate from a tomato field in Virginia owned by DiMare |
| ISG9 | *Salmonella* sv. Newport C4.2 ppeg.4639::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG7 | *Salmonella* sv. Newport C4.2 *phoN*-FRT-*cm*-FRT | Strain carrying a FRT-*cm*-FRT insertion downstream of *phoN* |
| ISG10 | *Salmonella* sv. Newport C4.2 *glnA*::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG11 | *Salmonella* sv. Newport C4.2 peg.4132::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG12 | *Salmonella* sv. Newport C4.2 peg.4640::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG13 | *Salmonella* sv. Newport C4.2 *ilvD*::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG14 | *Salmonella* sv. Newport C4.2 *metA*::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG15 | *Salmonella* sv. Newport C4.2 peg.4638::FRT-cm-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG16 | *Salmonella* sv. Newport C4.2 *glnG*::FRT-*cm*-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG17 | *Salmonella* sv. Newport C4.2 *thrC*::FRT-cm-FRT | Constructed using Datsenko and Wanner mutagenesis |
| ISG19 | *Salmonella* sv. Newport C4.2 Δ*papA phoN*::*papA* | Constructed using Datsenko and Wanner mutagenesis |

## Transposon Insertion Library Construction and Analysis

The transposon insertion library construction and analysis in sv. Newport were performed as described before (de Moraes et al., 2017). Briefly, a library of *S. enterica* serovar Newport C4.2 Tn5 insertion mutants was constructed with a mini-Tn5 derivative into which we inserted an $N_{18}$ random barcode using PCR. This derivative was integrated into the genome using the EZ-Tn5 < T7/KAN-2 > promoter insertion kit (Epicentre Biotechnologies, Madison, WI, United States). The transposome complex was dialyzed against water before electroporation into fresh electrocompetent cells of *S. enterica* sv. Newport C4.2. Transformed cells were recovered on LB agar with kanamycin after overnight growth at 37°C.

Mapping of barcoded transposons to specific locations in the genome was performed as described before (de Moraes et al., 2017). Briefly, genomic DNA from the library was extracted using the GenElute bacterial genomic DNA kit (Sigma-Aldrich). DNA was fragmented by sonication and ligated to Illumina primers. This product was used to amplify the regions, including the $N_{18}$ barcode and the genomic DNA adjacent to the transposon insertion, using a stepwise nested PCR (de Moraes et al., 2017). Resulting amplicons were purified with the QIAquick PCR Product Purification kit (Qiagen), and 150-base reads were obtained at both ends. The resulting reads were mapped against the *de novo* assembled sv. Newport C4.2 genome (see below) using Bowtie2. The $N_{18}$ barcode tag for each mapped read was identified using custom Perl scripts. The same reads, trimmed to remove Tn5 sequences, were also employed to assemble the sv. Newport C4.2 genome, which was then annotated using the RAST package[1].

For experiments in tomatoes, transposons were quantified as before (de Moraes et al., 2017). In brief, bacteria were recovered from tomatoes and grown in LB+60 μg/ml kanamycin. Bacteria were pelleted, lysed and subjected to PCR using primers directly

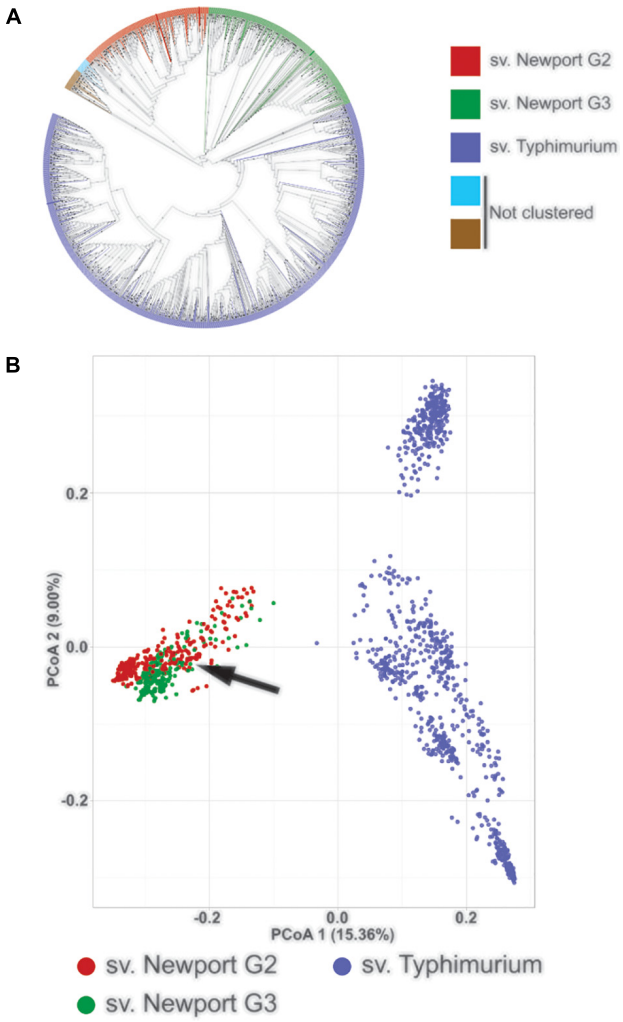


**FIGURE 1 |** Phylogenetic and comparative genomic analysis of the sv. Typhimurium and sv. Newport genomes. **(A)** Phylogenetic tree for 1,597 genomes of sv Typhimurium and sv Newport isolates, constructed using SNPs and minimal evolution over raw distance. Colors represent the major clades identified and dots represent branches with bootstrap values higher than 0.85. iTOL v4 (Letunic and Bork, 2016) was used to visualize the tree. **(B)** Principal Coordinate Analysis of the gene presence and absence profile of the same genomes. *Salmonella* sv. Typhimurium strains are represented in purple, sv. Newport Group II isolates in red and sv. Newport Group III strains in green. The arrow indicates strain C4.2 of sv. Newport, recovered from a tomato-linked outbreak of human salmonellosis and used in this study.

[1]http://rast.nmpdr.org/rast.cgi?page=JobDetails&job=211518

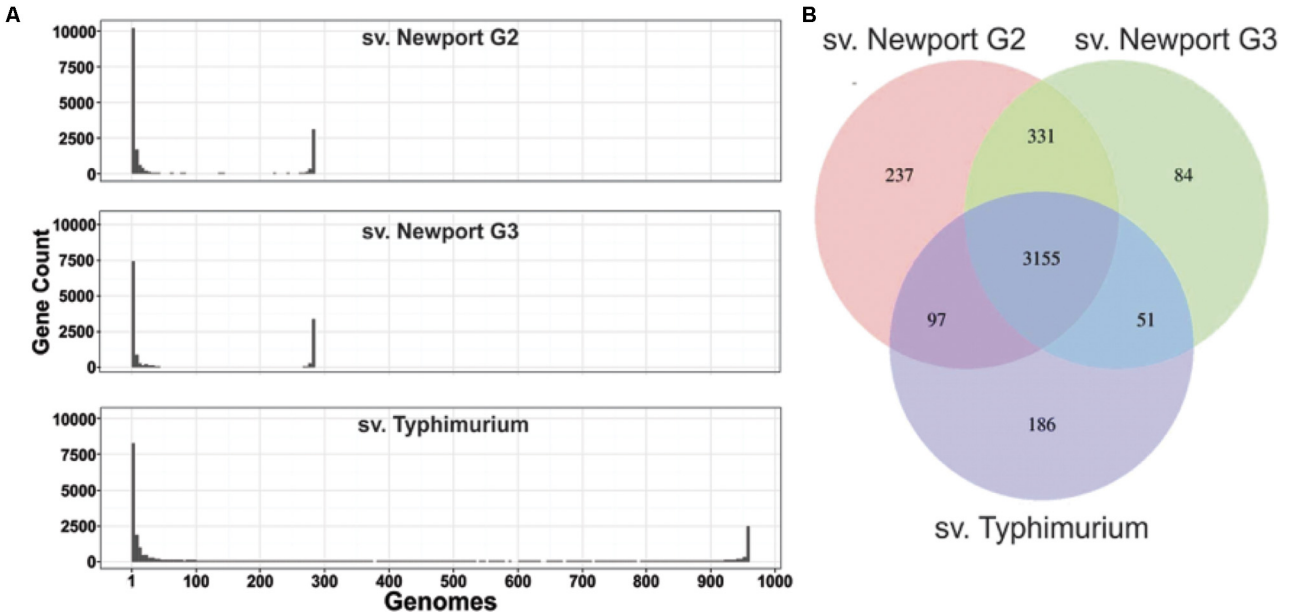


**FIGURE 2 |** *Salmonella* core, shell and accessory genomes. **(A)** Number of genes per number of *Salmonella* sv. Newport Group II, sv. Newport Group III and sv. Typhimurium genomes. Many genes were found in only one or a few genomes (peaks on the left of each plot). The core genes, present in almost all genomes of each group, are represented by the peak on the right of each plot. **(B)** Venn diagram representing the shared and unique elements of *Salmonella* core genes (shared by >95% of all members of each group) among the groups studied.

flanking the $N_{18}$ barcode. The frequency of each barcode was enumerated by Illumina sequencing of 20 bases. The aggregated abundances for the input and output libraries were statistically analyzed using edgeR, and the $\log_2$-fold changes and FDRs were reported.

## Genome Sequence Retrieval, Quality Control and Assembly

Raw reads from Illumina sequencing of *S. enterica* sv. Newport and sv. Typhimurium strains were recovered from the NCBI Sequence Read Archive (SRA). The SRA identifiers of the strains used are listed in **Supplementary Table S1**. We opted to assemble genomes *de novo* to remove biases associated with different assembly methods and to employ the same quality standards. The genomes of the type strains (GCA_000022165.1, GCA_000016045.1, and GCA_000171415.1) were recovered from the NCBI Genome databases. Read quality control and visualization were done using the package Trim Galore (Andrews et al., 2015). Genome assembly was done using SPAdes, using default parameters (Bankevich et al., 2012). Assessment of the genome assembly quality was done with CheckM (Parks et al., 2015). Genomes with more than 1% of contamination and less than 99% of completeness were excluded. Prokka was used to annotate genomes (Seemann, 2014). The resulting.gff files were fed into Roary to build the pan-genome matrix (Page et al., 2015). The analysis of the pan-genome matrix was performed using *ad hoc* R scripts. We used power-law regression to model the total size of the *Salmonella* pan-genome. To that end, analysis of random permutations of the addition of new genomes was performed and the number of new genes found per addition was recorded, and used in the regression to estimate the expansion of the pan-genome.

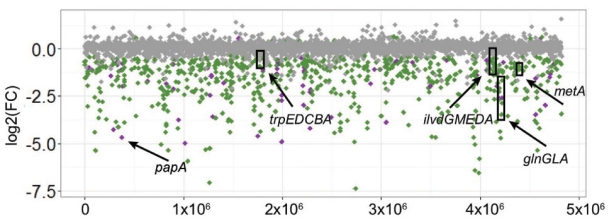


**FIGURE 3 |** Change in abundance of *Salmonella* C4.2 loci after screening in tomatoes. Relative abundance of transposon insertions in loci after the incubation was compared to the initial inoculum. The position of each locus on the *y*-axis represents $\log_2$(Fold Change) of relative abundance and on the *x*-axis represents a physical position on the *Salmonella* chromosome. Loci with a significant change in abundance (FDR < 0.1) are shown in green when shared between *S.* Newport C4.2 and *S.* Typhimurium ATCC 14028 and purple when unique to *S.* Newport C4.2. Loci with an FDR > 0.1 are shown in gray. Arrows point to loci and operons targeted for further experiments.

## Competition Assays

To confirm the results from the transposon insertion sequencing analysis, the fitness of individual isogenic mutants in relation to the wild-type was estimated. The bacterial population densities of overnight cultures of the wild type strain sv. Newport C4.2 and an isogenic mutant built in this background were set to

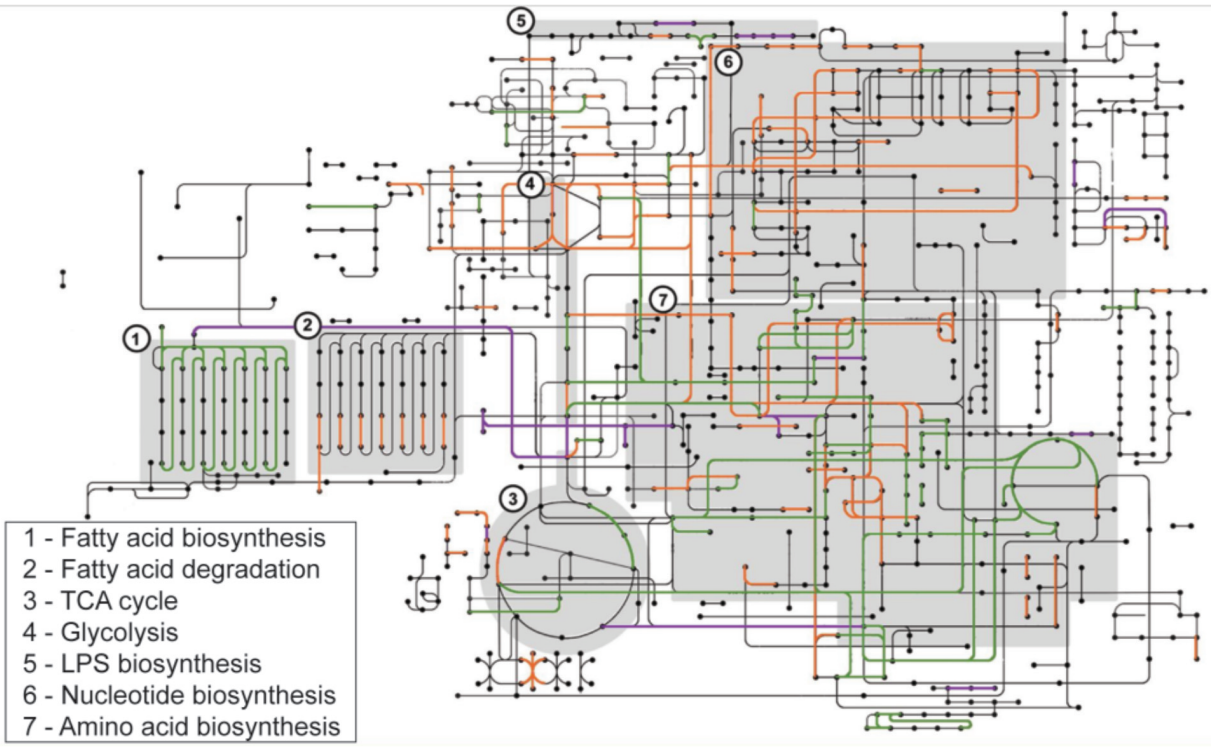


**FIGURE 4 |** Comparison of functions required for colonization of tomatoes between *S.* Newport C4.2 and *S.* Typhimurium ATCC 14028. *S.* Newport C4.2 metabolic pathways involved in tomato colonization are shown in purple, *S.* Typhimurium ATCC 14028 metabolic pathways involved in tomato colonization are shown in orange, and required pathways shared by both strains are shown in green. Pathways were identified as required for tomato colonization if the corresponding mutants were less represented in the output pool [$\log_2$(FC) < -0.5, FDR < 0.1]. Key metabolic steps are shown as shaded boxes. KEGG Orthology terms for the protein sequences corresponding to the underrepresented mutants were retrieved using BlastKOALA and mapped using KEGG Mapper. False color overlays were imposed in Adobe Photoshop 2014.

similar numbers by adjusting their $OD_{600}$ to the same level. Cells were then spun and resuspended in PBS to the original volume and mixed in a 1:1 ratio, followed by a 10,000-fold dilution in PBS. 3 μl of this mix were inoculated into the tomato pericarp in three separate wounds resulting in $\sim 10^3$ CFU per tomato fruit. To get an accurate count of the wild type and mutant cells in the inoculum, an aliquot was serially diluted and plated onto XLD agar and incubated overnight at 37°C; fifty randomly picked colonies were then patched onto LB agar with chloramphenicol and the initial mutant:wild-type ratio was determined.

The inoculated tomatoes were incubated at 22°C for 7 days. Wound sites were then sampled using a sterile loop and *Salmonella* cells were recovered from a streak on XLD plates. Fifty colonies were patched onto LB agar with chloramphenicol to determine the mutant:wild-type ratio in the recovered sample. The competition index (CI) was calculated using the formula $(MUT_{out}:WT_{out})/(MUT_{in}:WT_{in})$ (Noel et al., 2010). Statistical significance was determined using ANOVA against the CI of the neutral mutant ISG7. This neutral mutant was constructed with a FRT-*cm*-FRT insertion downstream of *phoN* known to not affect *Salmonella* fitness in tomatoes (Cox et al., 2013). The neutral phenotype of ISG7 was confirmed by competitions against the wild type. The software JMP version 12 was used for all CI analyses.

## Growth Curve and *rdar* Phenotype Characterization

Responses of the strains to oxidative stress were compared by diluting overnight cultures to $OD_{600}$ = 0.01 in 3 ml of LB with or without 0.5 mM paraquat. The cultures were incubated at 37°C with shaking at 200 rpm. 100 μl aliquots were collected hourly to estimate culture concentrations by serial dilution and plating onto LB agar. All experiments were replicated three times. The *rdar* phenotype was evaluated by spotting 5 μl of an overnight culture onto salt-less LB with Congo Red as described by Zaragoza et al. (2012).

# RESULTS AND DISCUSSION

## Clade Separation Using Phylogenetic Analysis

In this study, we compared the genes required for growth of an *S. enterica* sv. Newport strain in tomatoes to previous results (de Moraes et al., 2017) obtained for a Typhimurium

strain. We first used comparative genomics analysis to determine whether the strain of sv. Newport recovered from a tomato-linked outbreak and used in this study is an outlier within serovar Newport or whether it is a typical representative. A phylogenetic analysis, constructed with minimum-evolution using SNP distances obtained from 1526 *Salmonella* genomes (**Supplementary Table S1**), formed distinct clades for sv. Typhimurium and sv. Newport and displayed a small number of fast evolving genomes with no clear grouping (**Figure 1A** and **Supplementary Figure S1**). This result is consistent with previous *Salmonella* phylogenetic analyses, supporting the notion that sv. Typhimurium strains group as one clade and serovar Newport strains are divided into three different clades (Group I, Group II, Group III) (Sangal et al., 2010).

We identified the sv. Newport clades obtained in our analysis by placing the sv. Newport type strains SL257, known to be in Group II, and SL317, known to be in Group III, in the phylogeny tree. The tomato outbreak strain C4.2 used in this study was placed in sv. Newport Group III, consistent with a previous phylogenetic analysis (Cao et al., 2013). Based on its position in the phylogenetic tree, it appears to be a typical representative of the sv. Newport Group III.

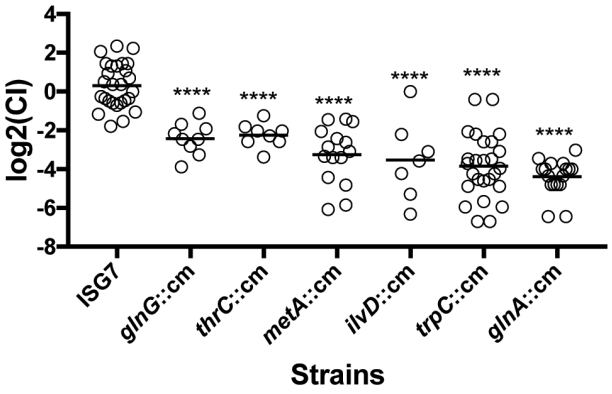


**FIGURE 5 |** Competitive fitness of isogenic mutants involved in amino acid biosynthesis. Approximately $10^3$ CFUs of a mix of the wild type and an isogenic mutant (1:1) were inoculated into each tomato, followed by 7-day incubation at 22°C. The Competitive Index CI was calculated using the formula (MUTout:WTout)/(MUTin:WTin). The replicates were obtained from six tomato fruits with three inoculation sites per fruit. Each dot represents a single replicate and the black bar represents the mean $\log_2$(CI) of all replicates. Statistical significance was established by comparing CI's using ANOVA and Tukey's *post hoc* test. Asterisks represent strains that are statistically different from the neutral mutant ISG7 ($P$-value $< 0.05$).

## Comparison of the *Salmonella* sv. Typhimurium and sv. Newport Pan-Genomes

The association of multiple strains of sv. Newport with recurrent produce-related outbreaks and the lack of such a strong association for sv. Typhimurium, coupled with the availability of parallel tools for the functional genomics characterization of the interactions of these organisms with diverse hosts, offers an opportunity to address the hypothesis that sv. Newport strains might have additional genes associated with success in tomatoes. Within 1526 *Salmonella* genomes that passed phylogeny quality controls, gene prediction using Prokka identified 31,675 gene orthologs. These data were used to build a pan-genome matrix (**Supplementary Table S1**) containing gene orthologs present in each genome. While sv. Typhimurium exhibited a small standard deviation in the number of genes per genome, the sv. Newport strains contained genomes that had up to 2,000 additional genes, primarily due to prophage and plasmids.

*Salmonella* sv. Typhimurium had an average of 4,628 genes per genome, while sv. Newport Group II had 4,554 genes per genome and sv. Newport Group III had 4,413 genes per genome (**Supplementary Figure S1**). The presence and absence gene profiles of each group were visualized by Principal Coordinate Analysis (**Figure 1B**). Principal Coordinate Analysis clustered the sv. Newport Groups II/III and sv. Typhimurium in two distinct groups separated by the first principal coordinate, showing that the difference among serovars corresponded to the presence and absence of genes, not only to serological or SNPs differences. The strain Newport C4.2, used in our model for tomato colonization, was within the cluster of sv. Newport Group III, confirming that its genetic profile is similar to most sv. Newport isolates.

Core and accessory genome analyses revealed similar genome structures for sv. Newport and Typhimurium. To further characterize the differences between *Salmonella* genomes, we assessed their core and accessory genome content. We used the definition of "core genome" as the genes shared by more than 95% of all the genomes per group, and "accessory genome" as the genes shared by less than 5% of the genomes per group. All the genes between these two classifications are defined as "shell genome." In sv. Newport Group II and sv. Newport Group III, 61–68% of all identified gene orthologs were part of the accessory genome and 11–12% of all identified gene orthologs were in the core genome (**Figure 2A**). The relative abundance of genes in the accessory genome in relation to the core genome was similar in the three *Salmonella* groups tested in this study. Moreover, these data showed that most of the *Salmonella* genes identified in the groups investigated here were in the accessory genome. The number of genes in the core genome was similar within all groups, ranging from 3,489 to 3,820 genes. All groups shared 3,155 genes (90%) of their core genomes. The sv. Newport groups shared consistently more of their core genomes than they shared with sv. Typhimurium (**Figure 2B**).

We also investigated whether the serovars' pan-genomes are open or closed according to the definition of (Medini et al., 2005; Tettelin et al., 2005). Using power-law regression, we found that all pan-genomes of *Salmonella* serovars analyzed in this study are closed, with an α parameter of 2.92, 2.94, and 2.39 (**Supplementary Figure S2**) for sv. Newport Group II, sv. Newport Group III and sv. Typhimurium, respectively. All regression curves exhibited similar slopes, although they reached saturation of new genes per genome at different points (around 20 for sv. Newport Group II and Group III, and 10 for sv. Typhimurium, **Supplementary Figure S2**). The genus

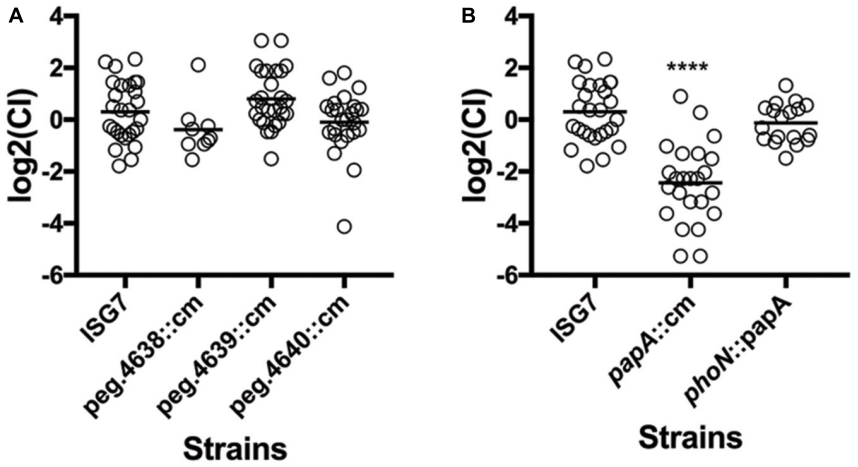


**FIGURE 6 |** Competitive fitness of isogenic mutants of genes unique to *S.* Newport C4.2. The Competitive Index CI was calculated using the formula (MUTout:WTout)/(MUTin:WTin) and inoculation and statistical analysis was done as described before. **(A)** CI of isogenic mutants for peg.4638, peg.4639, and peg.4640. **(B)** CI of isogenic mutants for *papA* and the complemented isogenic mutant Δ*papA phoN::papA*.

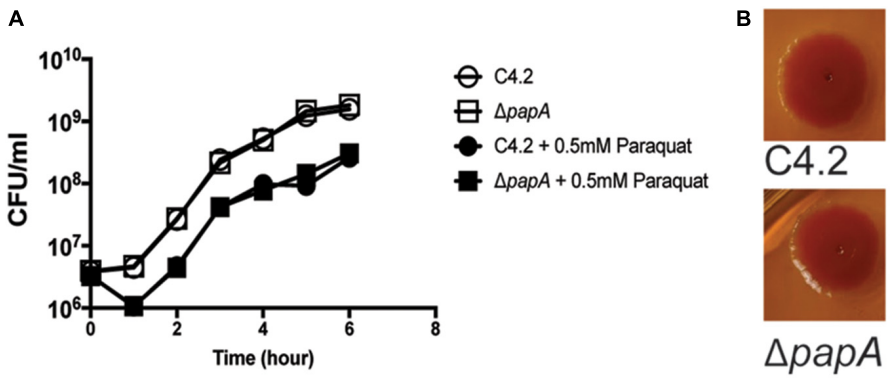


**FIGURE 7 |** Comparison of growth and colony morphology between *S.* Newport C4.2 and its *papA* mutant. **(A)** Growth of strains in LB medium at 250 rpm and 37°C in the presence and absence of 0.5 mM paraquat. Three independent replicates were used per condition. **(B)** Colony morphology of *S.* Newport C4.2 and its *papA* mutant on Congo Red indicator plates. The smooth surface and edges show the absence of the rough and dry phenotype.

*Salmonella* was previously identified as a closed genome species (Jacobsen et al., 2011).

The characterization of the presence/absence profile of genes in these genomes allowed the identification of 724 group-specific genes in the sv. Newport Group III shell genome and 84 group-specific genes unique to its core genome, compared with sv. Typhimurium. Further analysis was performed to determine if any of these genes were part of the genetic basis of the adaptation of the Group III Newport C4.2 strain to plant colonization.

## Transposon Insertion Sequencing Identifies Functions Required for Persistence in Tomatoes

To identify genome regions that encode functions important in colonization of tomato pericarps, we employed a library of transposon insertion mutants in sv. Newport C4.2, using the same screening methods applied before for sv. Typhimurium ATCC 14028 (de Moraes et al., 2017). The reads obtained from the transposon library screening were used to reconstruct the sv. Newport C4.2 genome (NCBI) using the RAST package. The fully annotated genome was deposited (see foot note text 1, username:guest, password:guest), and a comprehensive CSV file with locus tags, transposon insertion counts and orientation, and read counts is presented in **Supplementary Table S2**.

We mapped 4,811 coding sequences disrupted by the transposon, and mutants in 796 of these had a significant change in their abundance after growth in tomato wounds (FDR $< 0.1$). 781 of these mutants displayed reduced fitness and 15 had increased fitness (**Figure 3**). Most of the genes (689) whose disruption led to a reduction of fitness were shared by sv. Newport C4.2 and sv. Typhimurium ATCC 14028.

To understand the cellular functions employed by sv. Newport C4.2 to colonize tomatoes, we identified the metabolic functions involved in this interaction. We mapped the genes identified

as required for full fitness in pericarps and retrieved their COGs using the BlastKOALA web interface. Results were plotted against the sv. Newport C4.2 metabolic map. These results were compared with the outcomes of the sv. Typhimurium ATCC 14028 transposon insertion screening in a meta-analysis, with the goal of identifying potential serovar-specific factors. The comparison was done by overlapping the sv. Newport C4.2 and sv. Typhimurium ATCC 14028 metabolic maps. The main functions required for sv. Newport C4.2 colonization of tomato pericarps involved biosynthetic pathways such as amino acid and LPS biosynthesis, fatty acid catabolism and glycolysis. These same pathways were also required by sv. Typhimurium ATCC 14028 (**Figure 4**). The shared metabolic requirements by sv. Newport C4.2 and sv. Typhimurium ATCC 14028 corroborate the earlier conclusion that *Salmonella* relies on its robust and diverse metabolism to fully colonize tomatoes (de Moraes et al., 2017).

Biosynthesis of amino acids by *Salmonella* during its interaction with tomatoes is of special interest for food safety. Different tomato cultivars are known to differ in the amounts of amino acids that accumulate within fruit during ripening (DiLeo et al., 2011; Osorio et al., 2011), and tomato genotypes with different amino acid profiles were shown to support different levels of *Salmonella* growth (Marvasi et al., 2014). To confirm the results obtained from the transposon insertion screening, we constructed isogenic mutants in the genes associated with amino acid biosynthesis and used these mutants to evaluate their fitness during tomato colonization in competition experiments against their parental strain. The genes selected were *thrC*, *metA*, *ilvD*, *trpC*, and *glnA*, involved in the biosynthesis of threonine, methionine, branched amino acids, tryptophan, and glutamine (respectively), and *glnG*, which codes for the master nitrogen regulator. Competition assays confirmed the results obtained from the transposon library screening. All isogenic mutants tested had a severe defect in fitness as estimated by their competitive indices (CI). The *glnA* isogenic mutant had the most severe defect in fitness [$\log_2$(CI) = −4.4], while the strain lacking the global nitrogen regulator had the smallest reduction of fitness [$\log_2$(CI) = −2.4] (**Figure 5**). The competition assays established that amino acid biosynthesis is a fundamental feature that confers an advantage for *Salmonella* to colonize tomato pericarps.

The main point of divergence between the sv. Newport C4.2 and sv. Typhimurium ATCC 14028 metabolic requirements for tomato colonization was the biosynthesis of nucleotides (**Figure 4**). Both purine and pyrimidine synthesis pathways identified as needed for efficient colonization by sv. Typhimurium ATCC 14028 at high titer were not under selection in the sv. Newport C4.2 transposon insertion screening at similar titers, indicating that *S.* Newport obtains sufficient purines and pyrimidines by scavenging in this environment. Notably, purine and pyrimidine biosynthesis was required by sv. Typhimurium ATCC 14028 even when inoculation was performed with a 10,000-fold lower titer (de Moraes et al., 2017). This result suggests that sv. Newport C4.2 could have a more efficient scavenging system for purines and pyrimidines, which could be advantageous for this strain during plant colonization.

## Phenotypic Analysis of the Genes Unique to sv. Newport Reveals Potential Adaptions to Persistence in Plants

The main objective of this work was to explore the hypothesis that the sv. Newport C4.2 isolate from a tomato outbreak has additional genetic features not present in Typhimurium that enable it to colonize plants. Many sv. Newport-specific genes under selection had functions that could not be identified by BlastKOALA or Pfam. A few of those genes had putative metabolic functions. The locus peg.4637-peg.4641 is composed of genes that are absent from other *Salmonella* genomes and code for predicted proteins with unknown functions and a D-glucuronate permease (peg.4640). Since D-glucuronate is present in tomato fruit, we hypothesized that the reduction of fitness resulting from transposon insertions in this region indicates that *Salmonella* may be using D-glucuronate permease to scavenge D-glucuronate to proliferate in tomato pericarps. We therefore explored the role of this region during tomato colonization using a competition assay with isogenic mutants for the genes peg.4638, peg.4639, and peg.4640. The competitive indices for these isogenic mutants were not reduced when compared to ISG7, a strain that carries a neutral mutation (**Figure 6A**). However, this competition assay was performed with an inoculation titer thousands of fold lower than when the phenotype was observed in the transposon screen. It remains possible that this nutrient is used by *Salmonella* when the bacterial population is high.

Another gene (peg.4132), coding for a small putative protein (44 amino acids), was present in the shell genome of *S. enterica* sv. Newport C4.2, and a corresponding mutant had a strong reduction of fitness [$\log_2$(FC) = −4.66] (**Figure 6B**). This gene was probably horizontally acquired: it is associated with a region containing mobile elements that includes remnants of phage genes, and it is located near a tRNA gene. The gene has a GC content of 40%, in contrast to the genome average of 52%. We further investigated the role of this gene during tomato colonization using competition assays with an isogenic mutant. The peg4132 gene was required for fitness of the strain *S. enterica* sv. Newport C4.2 in tomatoes [($\log_2$(CI) = −2.44] (**Figure 6**). The mutant did not have a growth defect, displayed normal resistance to oxidative stress and was indistinguishable from the wild type when tested for the *rdar* phenotype (**Figure 7**). Due to its potential requirement for the colonization of plants, we named peg4132 "*papA*" (**P**lant **A**ssociated **P**rotein **A**).

To exclude the possibility that the reduction of fitness in this mutant in tomatoes was a result of a polar mutation, *papA* was cloned with its native promoter region in the vector pKD3 and then reinserted in the *phoN* locus in the Δ*papA* strain, creating a complemented *papA* strain with a single copy of the gene, stably maintained on the chromosome. The *phoN* locus was previously shown to be neutral during tomato colonization (Cox et al., 2013). The colonization fitness measured by the competitive index (CI) was not significantly different between the wild type and complemented *papA* strains (**Figure 6B**), corroborating that *papA* is a factor required for full fitness in sv. Newport C4.2 colonization of tomato pericarps.

We analyzed the distribution of *papA* in the *Salmonella* genomes, and determined that this gene was exclusive to Newport Group III, where it was found in 71 genomes out of 306, most of them closely associated in one clade. Using BlastP over the NCBI microbial database, we found potential orthologs of *papA*. Interestingly, many of these orthologs were in other members of the Enterobacteriaceae family that have a lifestyle associated with soil and plants, including *Pectobacterium* ssp. and *Dickeya* ssp, suggesting that this gene may be involved in persistence in or on plants.

## AUTHOR CONTRIBUTIONS

MdM, MM, and MT conceived the study and designed the experiments. MdM, EBS, ISG, and PD conducted the computational analysis. MM conceived, designed and oversaw Tn-Seq experiments and the Tn-Seq data analyses. MdM and WC conducted experimental work. MdM, MT, SP, and MM wrote the paper.

## FUNDING

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## ACKNOWLEDGMENTS

We thank Julia McGuinness and Eric Solomon for assistance with competition experiments (**Figures 5**, **6**).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00877/full#supplementary-material

**FIGURE S1 |** Number of genes per genome. The total number of genes per genome was plotted for the groups sv. Newport GII, sv. Newport Group TIT and sv. Typhimurium. The upper whisker extends to the highest value within 1.5 times the interquartile range. The lower whisker extend to the lowest value within 1.5 times the interquartile range. Outliers were defined as values higher than 1.5× interquartile range ± third quartile, or lower than 1.5 ± first quartile, and were plotted as dots. The thick black line within the boxes represents the mean.

**FIGURE S2 |** Total number of genes and new genes per genome obtained using random permutations. **(A,C,E)** Total number of genes per genome added. **(B,D,F)** New genes per genome and $\alpha$ and $\theta$ values from power law regression. **(A,B)** sv. Newport Group II. **(C,D)** sv. Newport Group III. **(E,F)** sv. Typhimurium.

**TABLE S1 |** Pangenome matrix containing genomes ID, metadata, and presence/absence profile of genes.

**TABLE S2 |** Genome Annotation of Newport C4.2 plus Tn5 fitness screening data compared to Typhimurium.

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# South Indian Isolates of the *Fusarium solani* Species Complex From Clinical and Environmental Samples: Identification, Antifungal Susceptibilities, and Virulence

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Members of the *Fusarium solani* species complex (FSSC) are the most frequently isolated fusaria from soil. Moreover, this complex solely affects more than 100 plant genera, and is also one of the major opportunistic human pathogenic filamentous fungi, being responsible for approximately two-third of fusariosis cases. Mycotic keratitis due to *Fusarium* species is among the leading causes of visual impairment and blindness in South India, but its management is still challenging due to the poor susceptibility of the isolates to conventional antifungal drugs. Aims of the present study were to isolate South Indian clinical and environmental FSSC strains and identify them to species level, to determine the actual trends in their susceptibilities to antifungal therapeutic drugs and to compare the virulence of clinical and environmental FSSC members. Based on the partial sequences of the translation elongation factor 1α gene, the majority of the isolates—both from keratomycosis and environment—were confirmed as *F. falciforme*, followed by *F. keratoplasticum* and *F. solani sensu stricto*. *In vitro* antifungal susceptibilities to commonly used azole, allylamine and polyene antifungals were determined by the CLSI M38-A2 broth microdilution method. The first generation triazoles, fluconazole and itraconazole proved to be ineffective against all isolates tested. This phenomenon has already been described before, as fusaria are intrinsically resistant to them. However, our results indicated that despite the intensive agricultural use of azole compounds, fusaria have not developed resistance against the imidazole class of antifungals. In order to compare the virulence of different FSSC species from clinical and environmental sources, a *Drosophila melanogaster* model was used. MyD88 mutant flies having impaired immune responses were highly susceptible to all the examined fusaria. In wild-type flies, one *F. falciforme* and two *F. keratoplasticum* strains also reduced the survival significantly. Pathogenicity seemed to be independent from the origin of the isolates.

**Keywords: keratomycosis, *Fusarium solani* species complex, *F. falciforme*, molecular identification, antifungal susceptibility, *Drosophila melanogaster*, virulence**

## INTRODUCTION

The genus *Fusarium* is a large group of hyaline filamentous fungi firstly described by Link (1809). According to the recent literature, it comprises approximately 200–300 species belonging to 20–22 species complexes (O'Donnell et al., 2013, 2015; Al-Hatmi et al., 2016). Fusaria are common soil saprophytes; however, they are also known as phytopathogens (Coleman, 2016). Two *Fusarium* species were recently included in the list of the top ten plant pathogenic fungi with both economic and scientific importance (Dean et al., 2012). The members of this genus may also interact with plants as endophytic root colonizers (Bacon and Yates, 2006); furthermore, they may be responsible for a wide range of human infections in either immunocompetent or immunocompromised patients (Garnica and Nucci, 2013). In accordance with the current species complex descriptions, at least ten of them have been reported to have human pathogenic representatives (Al-Hatmi et al., 2016). Last but not least, plumbing systems are also proven environmental reservoirs of human-pathogenic *Fusarium* species (Short et al., 2011).

Taxonomy of the genus *Fusarium* is changing intensely since 2011, when the era of the dual nomenclature ended (Hawksworth et al., 2011) and a comprehensive phylogenetic study of the genus discovered that the traditionally known *Fusarium* is not monophyletic (Gräfenhan et al., 2011). Based on these results, Gräfenhan et al. (2011) proposed to restrict the name *Fusarium* to the *Gibberella* clade and at the same time to reallocate the medically important *Fusarium solani* species complex (FSSC) and *Fusarium dimerum* species complex (FDSC) to other genera. After the release of this study, Lombard et al. (2015) were the first who suggested to use *Neocosmospora solani* instead of *F. solani*, and *Neocosmospora falciformis* instead of *F. falciforme*. However, in this study we would like to follow a previously published proposal of Geiser et al. (2013) by keeping the historical concept of *Fusarium* and use the names well-known in medical mycology.

In South India, a frequent scenario of fungal keratitis (keratomycosis) is that agricultural workers are infected after a corneal injury caused by plant or soil materials during their regular activities (Dóczi et al., 2004; Homa et al., 2013). Based on the recent reports, *Fusarium* species—and among them the members of the FSSC—are the most frequently isolated causative agents of fungal keratitis in this region (Chakrabarti and Singh, 2011; Homa et al., 2013; Hassan et al., 2016). The FSSC comprises at least 60 haplotypes, out of which 22 have been reported to have clinical associations (van Diepeningen et al., 2014; Al-Hatmi et al., 2016) with poor susceptibility to commonly used antifungal drugs (Azor et al., 2007). As consequence of the narrow range of therapeutic options, the treatment of *Fusarium* keratitis is extremely challenging and the lack of a prompt and effective therapy often results in corneal opacification or complete blindness (Shukla et al., 2008). Therefore, the rapid identification of the causative agent and the determination of its antifungal susceptibility are essential to choose the best therapeutic option. Presumably, the intensive agricultural and clinical (mis)use of antifungal compounds have also influenced the current susceptibility profile of the genus (Al-Hatmi et al., 2016). Thus, besides clinical studies, it is also crucial to evaluate the development of antifungal resistance among environmental strains to follow up the impact of fungicides used in the field.

Among FSSC species, *F. falciforme* was the most prevalent species isolated from human mycotic keratitis in South India (Homa et al., 2013; Hassan et al., 2016; Tupaki-Sreepurna et al., 2017a,b). However, it is unclear what lies in the background of its dominance: its environmental frequency or its high virulence. FSSC is reported to be more virulent than other species complexes of the genus (Mayayo et al., 1999); however, the virulence of different FSSC species has not been compared before. To answer the questions above, virulence studies are inevitable.

The objectives of the present study were (I) to isolate FSSC strains from keratomycosis patients, agricultural source and natural environments in South India; (II) to identify the strains at the species-level using molecular methods; (III) to determine their *in vitro* susceptibilities to commonly used antifungal agents; (IV) to compare the species diversity and the antifungal susceptibility profiles of the clinical and environmental isolates; (V) to compare the virulence of different clinical and environmental FSSC members; and (VI) to present and discuss the clinical details of the investigated keratomycosis cases.

## MATERIALS AND METHODS

### Patients Specimens and *Fusarium* Isolates

A total of 22 *Fusarium* isolates derived from patients with keratomycoses attending the Aravind Eye Hospital and Postgraduate Institute of Ophthalmology (Coimbatore, Tamilnadu, India) along with 20 environmental FSSC isolates from the same region were investigated (**Table 1**). Corneal scrapings were performed by an ophthalmologist under strict aseptic conditions, on each base of the corneal ulcer using a Kimura's spatula after instillation of 4% preservative-free lidocaine. Materials obtained from scraping the leading edge and the base of the ulcers were inoculated directly onto 5% sheep blood agar, chocolate agar, potato dextrose agar (PDA) and into brain heart infusion broth without gentamicin sulfate (Himedia Laboratories, India). Sheep blood agar and chocolate agar plates were incubated at 37°C, while PDA plates and brain heart infusion bottles were incubated at 27°C for 3 weeks.

To isolate fusaria from environmental sources, soil and plant parts (i.e., root and stem) were collected from gardens, parks, yards and agricultural fields in the surrounding regions of Coimbatore in November 2012. One gram of each collected soil sample was suspended in 10 ml sterile distilled water. The stock solutions were diluted 10 and 100 times and spread over Rose Bengal-Chloramphenicol agar (Himedia Laboratories, India) plates. The collected plant parts were pre-washed in sterile distilled water, surface-sterilized in 75% ethanol for 5 min and in 95% ethanol for 5 min, then rinsed in sterile distilled water for three times to remove ethanol residues. The sterilized parts were cut into small pieces, placed on Rose Bengal-Chloramphenicol agar plates and incubated at 25°C for 72 h. All fungal colonies from Rose Bengal-Chloramphenicol agar were subcultured into PDA plates using the cross-streak method. Then *Fusarium*-like

**TABLE 1 |** Molecular identification and antifungal susceptibilities of *Fusarium* strains isolated from keratitis cases and environmental samples in South India.

| SZMC No. | Year of isolation | Origin | State | Species based on *TEF1* | Accession No. of *TEF1* | MIC (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | AMB | CLT | ECN | FLC | ITC | KTC | NTM | TRB |
| **CLINICAL ISOLATES** | | | | | | | | | | | | | |
| SZMC 11438 | 2005 | Gobichettypalayam | Tamilnadu | *F. falciforme* | HE647902 | 2 | 8 | 2 | >64 | >32 | 32 | 16 | 64 |
| SZMC 11439 | 2005 | Erode | Tamilnadu | *F. falciforme* | HE647903 | 4 | 16 | 2 | >64 | >32 | 16 | 8 | 64 |
| SZMC 11441 | 2005 | Gobichettypalayam | Tamilnadu | *F. falciforme* | HE647906 | 8 | 16 | 2 | >64 | >32 | 32 | 8 | 4 |
| SZMC 11407 | 2005 | Coimbatore | Tamilnadu | *F. falciforme* | HE647909 | 0.25 | 8 | 1 | >64 | >32 | 8 | 8 | 4 |
| SZMC 11408 | 2005 | Palladam | Tamilnadu | *F. falciforme* | HE647910 | 0.5 | 8 | 1 | >64 | >32 | 16 | 8 | 16 |
| SZMC 11442 | 2005 | Coimbatore | Tamilnadu | *F. falciforme* | HE647911 | 4 | 16 | 2 | >64 | >32 | 16 | 8 | 32 |
| SZMC 11443 | 2004 | Palladam | Tamilnadu | *F. falciforme* | HE647914 | 4 | 16 | 1 | >64 | >32 | 8 | 8 | 16 |
| SZMC 11411 | 2005 | Kangeyam | Tamilnadu | *F. falciforme* | HE647915 | 4 | 8 | 2 | >64 | >32 | 32 | 8 | 32 |
| SZMC 11412 | 2005 | Kangeyam | Tamilnadu | *F. falciforme* | HE647916 | 2 | 16 | 2 | >64 | >32 | 32 | 8 | 32 |
| SZMC 11414 | 2004 | Palghat | Kerala | *F. keratoplasticum* | HE647919 | 2 | 8 | 1 | >64 | >32 | 8 | 8 | 16 |
| SZMC 11447 | 2005 | Harur | Tamilnadu | *F. falciforme* | HE647924 | 1 | 4 | 1 | >64 | >32 | 32 | 8 | 0.5 |
| SZMC 11448 | 2005 | Coimbatore | Tamilnadu | *F. falciforme* | HE647927 | 4 | 8 | 4 | >64 | >32 | 32 | 8 | 32 |
| SZMC 11449 | 2004 | Palghat | Kerala | *F. falciforme* | HE647928 | 8 | 16 | 2 | >64 | >32 | 16 | 16 | 32 |
| SZMC 11419 | 2005 | Coimbatore | Tamilnadu | *F. falciforme* | HE647929 | 4 | 8 | 8 | >64 | >32 | 64 | 8 | 32 |
| SZMC 11425 | 2005 | Krishnagiri | Tamilnadu | *F. falciforme* | HE647937 | 4 | 16 | 1 | >64 | >32 | 16 | 8 | 16 |
| SZMC 11454 | 2005 | Tirupur | Tamilnadu | *F. falciforme* | HE647944 | 4 | 2 | 1 | >64 | >32 | 8 | 8 | 0.5 |
| SZMC 11455 | 2005 | Tiruchengodu | Tamilnadu | *F. falciforme* | HE647945 | 4 | 8 | 4 | >64 | >32 | 64 | 16 | 16 |
| SZMC 11456 | 2005 | Kangeyam | Tamilnadu | *F. falciforme* | HE647946 | 0.5 | 8 | 0.5 | 32 | >32 | 2 | 8 | 4 |
| SZMC 11457 | 2005 | Tirupur | Tamilnadu | *F. falciforme* | HE647948 | 4 | 8 | 4 | >64 | >32 | 32 | 8 | 64 |
| SZMC 11431 | 2005 | Erode | Tamilnadu | *F. falciforme* | HE647949 | 4 | 8 | 4 | >64 | >32 | 64 | 8 | 32 |
| SZMC 11432 | 2005 | Tirupur | Tamilnadu | *F. falciforme* | HE647950 | 1 | 8 | 2 | >64 | >32 | 8 | 8 | 16 |
| SZMC 11458 | 2005 | Ottanchatram | Tamilnadu | *F. falciforme* | HE647951 | 2 | 4 | 8 | >64 | >32 | 8 | 4 | 4 |
| | | MIC range (μg/ml) | | | | 0.25–8 | 2–16 | 0.5–8 | 32–>64 | >32 | 2–64 | 4–16 | 0.5–64 |
| | | $MIC_{50}$ (μg/ml) | | | | 4 | 8 | 2 | >64 | >32 | 16 | 8 | 32 |
| | | GM MIC (μg/ml) | | | | 3.2 | 8.8 | 2.1 | >64 | >32 | 18.1 | 8.5 | 19.0 |
| **ENVIRONMENTAL ISOLATES** | | | | | | | | | | | | | |
| SZMC 21329 | 2012 | Soil, flower garden | Tamilnadu | *F. falciforme* | MG272421 | 4 | 16 | 8 | >64 | >32 | 16 | 16 | 64 |
| SZMC 21330 | 2012 | Soil, flower garden | Tamilnadu | *F. keratoplasticum* | MG272422 | 4 | 16 | 8 | >64 | >32 | 64 | 8 | 32 |
| SZMC 21331 | 2012 | Soil, outdoor flowerpot | Tamilnadu | *F. falciforme* | MG272423 | 2 | 16 | 2 | >64 | >32 | 64 | 8 | 4 |
| SZMC 21332 | 2012 | Soil, outdoor flowerpot | Tamilnadu | *F. falciforme* | MG272424 | 4 | 8 | 8 | >64 | >32 | 64 | 16 | 32 |
| SZMC 21333 | 2012 | Soil, outdoor flowerpot | Tamilnadu | *F. falciforme* | MG272425 | 4 | 8 | 8 | >64 | >32 | 16 | 16 | 32 |
| SZMC 21334 | 2012 | Soil, outdoor flowerpot | Tamilnadu | *F. falciforme* | MG272426 | 4 | 8 | 8 | >64 | >32 | 64 | 8 | 64 |

*(Continued)*

**TABLE 1** | Continued

| SZMC No. | Year of isolation | Origin | State | Species based on *TEF1* | Accession No. of *TEF1* | MIC (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | AMB | CLT | ECN | FLC | ITC | KTC | NTM | TRB |
| SZMC 21335 | 2012 | Soil, banana tree | Tamilnadu | *F. falciforme* | MG272427 | 4 | 16 | 4 | >64 | >32 | 32 | 8 | 32 |
| SZMC 21336 | 2012 | Soil, banana tree | Tamilnadu | *F. falciforme* | MG272428 | 4 | 16 | 4 | >64 | >32 | 32 | 8 | 32 |
| SZMC 21337 | 2012 | Soil, banana tree | Tamilnadu | *F. falciforme* | MG272429 | 2 | 16 | 2 | >64 | >32 | 64 | 8 | 4 |
| SZMC 21338 | 2012 | Soil, garden | Tamilnadu | *F. falciforme* | MG272430 | 1 | 8 | 16 | >64 | >32 | 64 | 8 | 16 |
| SZMC 21339 | 2012 | Soil, yard | Tamilnadu | *F. falciforme* | MG272431 | 4 | 8 | 4 | >64 | >32 | 64 | 16 | 32 |
| SZMC 21340 | 2012 | Soil, yard | Tamilnadu | *F. falciforme* | MG272432 | 4 | 8 | 4 | >64 | >32 | 64 | 16 | 32 |
| SZMC 21342 | 2012 | Soil, yard | Tamilnadu | *F. falciforme* | MG272433 | 4 | 8 | 1 | >64 | >32 | 16 | 8 | 8 |
| SZMC 21343 | 2012 | Soil, park | Tamilnadu | *F. falciforme* | MG272434 | 4 | 8 | 2 | >64 | >32 | 16 | 8 | 16 |
| SZMC 21344 | 2012 | Soil, park | Tamilnadu | *F. falciforme* | MG272435 | 2 | 16 | 8 | >64 | >32 | 32 | 8 | 16 |
| SZMC 21345 | 2012 | Soil, maize field | Tamilnadu | *F. falciforme* | MG272436 | 4 | 8 | 2 | >64 | >32 | 8 | 8 | 16 |
| SZMC 21346 | 2012 | Soil, sorghum field | Tamilnadu | *F. falciforme* | MG272437 | 2 | 8 | 2 | >64 | >32 | 16 | 8 | 8 |
| SZMC 21348 | 2012 | Soil, unknown cultivated field | Tamilnadu | *F. solani s. str.* | MG272438 | 2 | 8 | 16 | >64 | >32 | 8 | 8 | 4 |
| SZMC 21350 | 2012 | Sorghum, root | Tamilnadu | *F. falciforme* | MG272439 | 2 | 8 | 2 | >64 | >32 | 4 | 8 | 16 |
| SZMC 21351 | 2012 | Tomato, root | Tamilnadu | *F. falciforme* | MG272440 | 4 | 16 | 1 | >64 | >32 | 2 | 8 | 16 |
| MIC range (μg/ml) | | | | | | 1–4 | 8–16 | 1–16 | >64 | >32 | 2–64 | 8–16 | 4–64 |
| $MIC_{50}$ (μg/ml) | | | | | | 4 | 8 | 4 | >64 | >32 | 32 | 8 | 16 |
| GM MIC (μg/ml) | | | | | | 3.0 | 10.6 | 4.0 | >64 | >32 | 24.3 | 9.5 | 17.8 |

*AMB, amphotericin B; CLT, clotrimazole; ECN, econazole; FLC, fluconazole; GM, geometric mean; ITC, itraconazole; KTC, ketoconazole; MIC, minimum inhibitory concentration; $MIC_{50}$, MIC for 50% of the tested isolates; NTM, natamycin; SZMC, Szeged Microbiological Collection, University of Szeged, Szeged, Hungary; TEF1, translation elongation factor 1α; TRB, terbinafine.*

colonies were purified and identified by macro- and microscopic characteristics. From both clinical and environmental samples, the purified fungal colonies were sub-cultured and stored on PDA plates at 4°C until further investigations.

## Molecular Identification

Isolates suspected to be *Fusarium* sp. based on their macromorphological characteristics and microscopic features were further subjected to molecular identification. All isolates were grown in Potato Dextrose Broth (Sigma-Aldrich, USA) at 25°C in a shaker (New Brunswick Scientific Co., Inc., USA) at 220 rpm for 5 days, and subsequently genomic DNA was extracted with the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, USA) in accordance with manufacturer's instructions. FSSC isolates were selected using the FSSC-specific PCR as described by He et al. (2011) and confirmed with an *Eco*RI digestion-based PCR-RFLP method (Homa et al., 2013). For the species-level identification of FSSC-positive isolates, the 5′ portions of translation elongation factor 1α (*TEF1*) coding region and introns were amplified (O'Donnell et al., 1998). After Sanger sequencing (LGC Genomics GmbH, Germany) the *TEF1* sequences were deposited in the GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) under the accession numbers listed in **Table 1** and used as BLAST (Altschul et al., 1990) queries against the *Fusarium* MLST database (http://www.westerdijkinstitute.nl/fusarium/) (O'Donnell et al., 2010).

All the confirmed isolates were deposited in the Szeged Microbiological Collection (SZMC; http://szmc.hu/; http://www.wfcc.info/ccinfo/collection/by_id/987) under the strain numbers listed in **Table 1.**

## Phylogenetic Analysis

Besides the FSSC strains isolated from clinical and environmental sources, two clinical members of the *F. dimerum* species complex (SZMC 11496 and SZMC 11540) were involved in the analysis as an outgroup. The sequences were aligned by Muscle v3.8.31 (Edgar, 2004) and manually refined in BioEdit v7.1.3.0 (Hall, 1999). Substitution models for the final alignment were selected by the $AIC_c$ function in jModelTest 2.1.10 (Posada, 2008). Trees were inferred by Maximum Likelihood (ML) and Bayesian MCMC approaches. ML bootstrapping was performed in PhyML 3.0 under the TrN+G model of sequence evolution, using the nearest-neighbor interchange branch swapping algorithm and 1000 replicates of non-parametric bootstrap analysis (Guindon and Gascuel, 2003). ML bootstrap values >69% were considered as significant support (Soltis and Soltis, 2003). Bayesian MCMC analyses were run in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). One cold and three incrementally heated chains were run in two replicates sampling every 100th generation. Chain length was set to 10,000,000 generations and a burn-in value of 100 000 generations was chosen using the Tracer 1.4 software (Rambaut and Drummond, 2007). Post-burn-in trees were summarized in a 50% majority rule consensus tree in MrBayes. Posterior probabilities >0.94 were considered as significant.

## Antifungal Susceptibility Tests

Antifungal susceptibility tests were performed as described in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution method (Clinical Laboratory Standards Institute, 2008). Pharma grade powders of amphotericin B (AMB), clotrimazole (CLT), econazole (ECN), fluconazole (FLC), itraconazole (ITC), ketoconazole (KTC), terbinafine (TRB) (Sigma-Aldrich, USA), and commercially available natamycin (NTM) eye drops (Lalitha et al., 2008a) (Natamet, 5% suspension, Sun Pharmaceutical Ind. Ltd., India) were included in the tests. Conidial suspensions were prepared in 0.85% saline solution from 5-day-old cultures grown on PDA plates and diluted in RPMI-1640 medium (Sigma-Aldrich, USA) adjusting the final inoculum density to $10^4$ CFU/ml. Fungal growth was evaluated after incubation for 48 h at 35°C without shaking. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of an antifungal agent that inhibited completely the growth of the tested isolates compared to the drug-free control medium. For FLC and KTC, the MICs were defined as the lowest concentrations of the drugs that cause approximately 50% reduction in growth. $MIC_{50}$ was determined as the MIC inhibiting the growth of 50% of all the tested isolates. *Aspergillus flavus* ATCC 204304 and *Candida krusei* ATCC 6258 were included as quality control strains. Each experiment was performed in triplicates.

## Survival Experiments in *Drosophila melanogaster*

To examine the background of *F. falciforme* dominance in South Indian human keratomycosis cases, the virulence of six FSSC isolates, i.e., *F. falciforme* SZMC 11407, SZMC 11408, and SZMC 21332, *F. keratoplasticum* SZMC 11414 and SZMC 21330, and *F. solani s. str.* SZMC 21348 was examined in *D. melanogaster*.

Conidial suspensions were prepared with sterile phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) from 5-day-old cultures grown on PDA plates at 35°C. The final inoculum densities were adjusted to $1 \times 10^7$ conidia/ml with PBS.

*Drosophila* stocks were raised and kept following the infection on standard cornmeal agar medium at 25°C. The Oregon R strain, originally obtained from the Bloomington stock center, was used as the wild type throughout the experiments. MyD88c03881 flies having impaired immune responses were described previously (Tauszig-Delamasure et al., 2002). Infection was performed by dipping a thin needle in a suspension of fungal conidia ($10^7$ conidia/ml) or PBS for the uninfected control, and subsequently the thorax of the anesthetized fly was pricked. Flies were counted at different points of time to monitor survival. Flies were moved into fresh vials every other day. Each experiment was performed with approximately 60 flies for each genotype. The results shown in **Figure 1** are representative of at least three independent experiments.

## Statistical Analyses

All statistical analyses were performed in SigmaPlot (version 14.0). Two sample *t*-test was used to reveal significant differences between the antifungal susceptibility profiles of clinical and

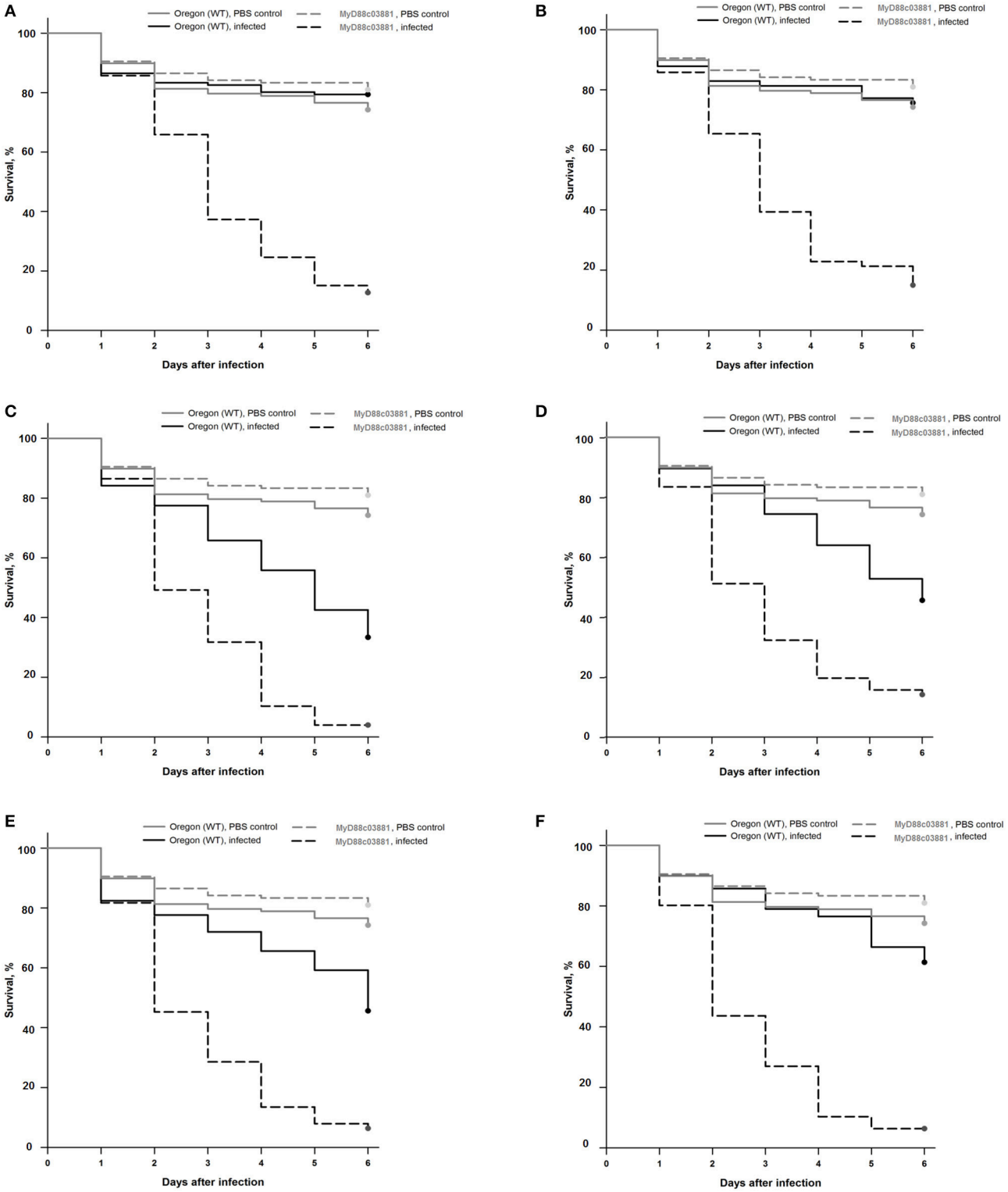


**FIGURE 1 |** Survival rates of wild-type (Oregon) and MyD88c03881 mutant flies infected with clinical *F. falciforme* SZMC 11407 and SZMC 11408 **(A,B)**, clinical *F. keratoplasticum* SZMC 11414 **(C)**, environmental *F. keratoplasticum* SZMC 21330 **(D)**, environmental *F. falciforme* SZMC 21332 **(E)**, and environmental *F. solani s. str.* SZMC 21348 **(F)** strains. The control groups were injected with sterile PBS.

environmental isolates. Fisher's exact test was applied to compare the species composition of clinical and environmental isolates. Kaplan-Meier survival curves were generated in order to present the results of the survival experiments in *D. melanogaster*. The Log-Rank statistic was used to decide whether there is a statistically significant difference between the curves. To identify the group—or groups—of flies that differ from the others, the Holm-Sidak multiple comparison procedure was applied (Glantz, 2012). Significance level was set at $p < 0.05$.

## Ethics Statement

Due to its observational nature, no formal ethics approval was required for this study. In order to protect the patients' anonymity, identifying information were not included in the manuscript.

# RESULTS

## Clinical Characteristics of Patients With *Fusarium* Keratitis

Clinical data are available for all cases but one (Strain No. SZMC 11425) (**Table 2**). Out of the 22 keratitis isolates, 20 were isolated from patients residing in Tamilnadu, while the rest were from Kerala. Majority of the infections ($n = 13$) were registered between June and August. None of the patients had any underlying conditions. Half of the patients reported trauma as a predisposing factor, while 10 patients could not recall any injury prior to the infection. The severity of the ulcer was recorded as mild in six, moderate in seven and severe in eight cases. The most common therapeutic approach was the combined topical application of NTM, ITC, and ECN eye drops ($n = 16$), which were supplemented with systemic KTC in 12 cases. Surgical intervention (therapeutic penetrating keratoplasty, TPK) was needed in four severe cases, where the topical and systemic drugs could not improve the patients' condition. The registered final outcomes were as follows: 16 patients were healed completely; the therapy failed in five cases and one patient was lost to follow-up.

## Identification of the *Fusarium* Isolates Based on Molecular Markers

BLAST searches with the partial *TEF1* sequences revealed that most of the isolates derived from both human keratomycoses and the environment belong to *F. falciforme* ($n = 41$; FSSC 3 + 4, O'Donnell et al., 2008). A clinical (SZMC 11414) and an environmental (SZMC 21330) isolate were identified as *F. keratoplasticum* (FSSC 2, Short et al., 2013), while another isolate from soil (SZMC 21348) was confirmed as *F. solani s. str.* (FSSC 5, Schroers et al., 2016; **Table 1**). Statistically significant association between the investigated FSSC species and their source was not detected. In order to examine the phylogenetic distribution of the isolates, a phylogenetic reconstruction was also performed using the above-mentioned *TEF1* locus, which confirmed the BLAST-based identifications (**Figure 2**).

## Antifungal Susceptibilities

**Table 1** summarizes the MIC values of the eight investigated antifungal agents. Clinical and environmental strains showed similar susceptibilities. However, environmental isolates proved to be significantly ($p = 0.01$) less susceptible to ECN than the clinical FSSC isolates. In all other cases, statistically significant differences were not detected between these two populations. The lowest MICs were recorded for AMB and ECN (0.25–16 μg/ml). MIC values of CLT and NTM were between 2 and 16 μg/ml, while the activities of TRB and KTC varied in the MIC ranges of 0.5–64 and 2–64 μg/ml, respectively. ITC and FLC proved to be ineffective in the tested concentration ranges.

## Virulence

In the case of wild type Oregon flies, the clinical *F. falciforme* strains SZMC 11407 and SZMC 11408 and the environmental FSSC 5 strain SZMC 21348 proved to be avirulent; the survival rates 6 days post infection (dpi) were 79 ± 21%, 76 ± 6%, and 61 ± 15% (**Figures 1A,B,F**). At the same time, infection with the *F. keratoplasticum* strains SZMC 11414 and SZMC 21330 and the environmental *F. falciforme* strain SZMC 21332 resulted in a significant reduction in the survival rate compared to the control group, the 6 dpi survival rates were 33 ± 18%, 46 ± 12%, and 46 ± 23% (**Figures 1C–E**), respectively. All six tested strains reduced the 6 dpi survival rates of the MyD88-mutant MyD88c03881 flies to 4–15% (**Figures 1A–F**).

# DISCUSSION

The population of tropical/subtropical countries such as India is more prone to eye infections, especially to fungal keratitis caused by *Fusarium* spp. generally due to the climatic conditions. Regular monitoring of the disease is essential for its effective management (Lalitha et al., 2008b; Kredics et al., 2015).

As it is shown in **Table 2**, we found the highest incidence of FSSC keratitis cases in July. Previously, Lin et al. (2012) also observed an uneven distribution of *Fusarium* keratitis cases throughout the year in South India with a major peak of registered cases in July. This peak was associated with the windy season in June-July, when dust particles are presumed to be the main causes of ocular trauma (Lin et al., 2012). This theory is reinforced by the clinical records summarized in **Table 2**, where dust was mentioned as a predisposing factor for the infection only in June and July. A minor peak of keratitis cases in January—which was detected by Lin et al. (2012) and was attributed to the intensive agricultural activities of the harvest season resulting in elevated concentrations of conidia in the air and frequent ocular injuries due to soil or plant debris particles—was not observed in our study. Interestingly, Lin et al. (2012) also observed that environmental humidity (dry and wet season) was not a significant factor in the seasonal patterns of fungal keratitis.

We observed some major variations especially in the risk factors and treatment of *Fusarium* keratitis cases when compared our data with the study of Walther et al. (2017) from Germany. Based on the clinical details, trauma was the most frequently recorded predisposing factor for *Fusarium* keratitis in India (Bharathi et al., 2007; Tupaki-Sreepurna et al., 2017a), whereas keratomycoses were rare in temperate climates and more commonly associated with the use of soft contact lenses (Keay et al., 2011; Walther et al., 2017). As shown in **Table 2**, most

**TABLE 2 |** Clinical details of *Fusarium* keratitis cases from South India.

| Strain No. | Month of presentation | History of trauma | VA at presentaion | Ulcer severity | Hypopyon | Antifungal treatment | | Surgery | Subconjunctival injection | Perforation | FinalVA | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Topical | Systemic | | | | | |
| SZMC 11438 | March | Mud | 6/60 | Moderate | No | NTM, ITC, ECN | No | No | No | No | 6/12 | Recovered |
| SZMC 11439 | June | No | 6/9 | Moderate | No | NTM, ITC | No | No | No | No | 6/9 | Recovered |
| SZMC 11441 | June | FB | HM | Severe | Yes | NTM, ITC, ECN | KTC | TKP | Yes | No | 1/60 | Failure |
| SZMC 11407 | July | Dust | 6/6 | Moderate | No | NTM | No | No | No | No | 6/6 | Recovered |
| SZMC 11408 | August | No | 6/6 | Mild | No | NTM, ITC, ECN | No | No | No | No | 6/6 | Recovered |
| SZMC 11442 | July | Paddy husk | PL | Severe | Yes | NTM, ITC, ECN | KTC | TKP | Yes | No | 1/60 | Failure |
| SZMC 11443 | December | Stick | 1/60 | Moderate | Yes | NTM, ITC, ECN | KTC | No | No | No | 6/60 | Recovered |
| SZMC 11411 | July | No | 6/12 | Mild | CF | NTM, ITC, CLT | No | No | No | No | 6/6 | Recovered |
| SZMC 11412 | July | No | 6/60 | Moderate | Yes | NTM, ITC, ECN | KTC | No | Yes | No | 6/24 | Recovered |
| SZMC 11414 | December | No | FCF | Severe | Yes | NTM, ITC, ECN | KTC | TKP | No | No | 6/24 | Failure |
| SZMC 11447 | March | No | 2/60 | Moderate | No | NTM, ITC, ECN | KTC | No | No | No | NA | Failure |
| SZMC 11448 | June | Dust | 6/24 | Mild | No | NTM, ITC | No | No | No | No | 6/24 | Recovered |
| SZMC 11449 | December | No | 6/12 | Mild | No | NTM, ITC, ECN | KTC | No | No | No | 6/18 | Recovered |
| SZMC 11419 | August | FB | 6/12 | Moderate | No | NTM, ITC, ECN | No | No | No | No | 6/24 | Recovered |
| SZMC 11425 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| SZMC 11454 | March | No | PL | Severe | Yes | NTM, ITC, ECN | KTC | No | No | No | PL | Recovered |
| SZMC 11455 | March | No | 1/60 | Mild | No | NTM, ITC, ECN | No | No | No | No | 6/9 | Recovered |
| SZMC 11456 | January | No | PL | Severe | Yes | NTM, ITC, ECN | KTC | TKP | No | Yes | NA | Failure |
| SZMC 11457 | June | FB | 6/9 | Mild | No | NTM, ITC | No | No | No | No | 6/6 | Recovered |
| SZMC 11431 | June | FB | PL | Severe | Yes | NTM, ITC, ECN | KTC | No | Yes | No | 6/36 | Recovered |
| SZMC 11432 | June | Cow's tail | FCF | Severe | Yes | NTM, ITC | KTC | No | No | No | 6/36 | Recovered |
| SZMC 11458 | June | Dust | 3/60 | Severe | Yes | NTM, ITC, ECN | KTC | No | Yes | No | 6/24 | Recovered |

*ECN, econazole; FB, foreign body; FCF, counting fingers close to face; HM, hand motion; ITC, itraconazole; KTC, ketoconazole; n.a., not available; NTM, natamycin; PL, perceive light; SZMC, Szeged Microbiological Collection, University of Szeged, Szeged, Hungary; TKP, therapeutic keratoplasty; TN, Tamilnadu, VA, visual acuity.*

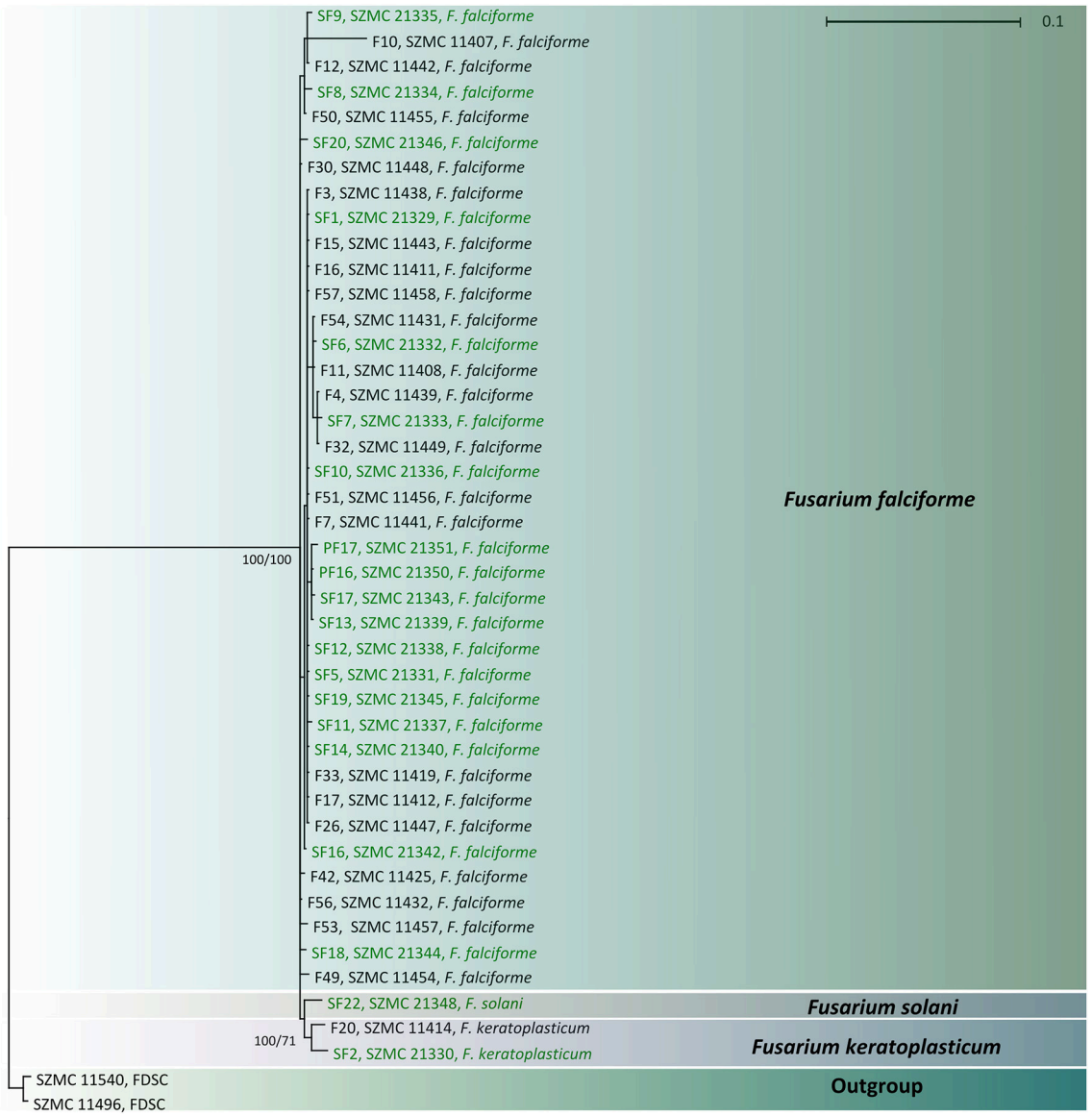


**FIGURE 2 |** Combined phylogenetic tree based on the partial sequences of translation elongation factor 1α genes of clinical (indicated with black letters) and environmental (indicated with green letters) FSSC isolates. Only Bayesian posterior probability and ML bootstrap support values >0.95 and 60% are shown at the nodes.

of the patients at the Aravind Eye Hospital were treated with the topical applications of NTM, ECN and ITC along with systemic KTC, while in Germany, AMB and VRC were the most frequently used antifungals not just in topical, but also in invasive and systemic therapeutic approaches. Surgical intervention was performed in four out of the 21 cases in the present study, while Walther et al. (2017) reported that nine out of 15 cases required TPK. Despite the differing therapeutic approaches, comparing the outcomes we could not find any differences between the two investigations; therapeutic failures were reported 3/15 times (20.0%) by Walther et al. (2017) and 5/21 times (23.8%) in the present study.

Several papers reported that *Fusarium* species—particularly members of the FSSC—are the predominant etiological agents of keratomycosis (**Table 3**). However, based on the English language literature available in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Google Scholar (https://scholar.google.hu/) databases, species level identification is still not a common practice with *Fusarium* keratitis cases. In South India, *F. falciforme* proved to be the most common FSSC species followed by *F. keratoplasticum* (Homa et al., 2013; Hassan et al., 2016; Tupaki-Sreepurna et al., 2017a,b). These results were similar to those observed in the present study (**Table 1**). In the work of Tupaki-Sreepurna et al. (2017a), seven out of

**TABLE 3 |** Literature overview of *Fusarium* keratitis studies with species-level data.

| Reference | Sampling period | Region | Species complex distribution (n) | Species distribution in the FSSC (n) | Gene(s) used for molecular analysis | Specific comments |
|---|---|---|---|---|---|---|
| **ASIA** | | | | | | |
| Sun et al., 2015 | 2002–2011 | Central China | FSSC (386)<br>FFSC (254)<br>FOSC (11) | *F. solani s. str.* (132)<br>*F. falciforme* (126) | *TEF1* | Species-level data are not available in English for the remaining 128 FSSC isolates |
| Homa et al., 2013 | 2010–2011 | South India | FSSC (53)<br>FDSC (6)<br>FFSC (6)<br>FOSC (3)<br>FIESC (2) | *F. falciforme* (45)<br>*F. keratoplasticum* (3)<br>*F. solani s. str.* (1)<br>FSSC 6 (1)<br>FSSC 33 (2) | *TEF1* | – |
| Hassan et al., 2016 | 2012–2013 | South India | FSSC (54)<br>FDSC (7)<br>FFSC (3)<br>FOSC (1) | *F. falciforme* (45)<br>*F. keratoplasticum* (8)<br>*F. lichenicola* (1) | *TEF1*, *RPB2* | – |
| Tupaki-Sreepurna et al., 2017a | 2012–2014 | South India | FSSC (9)<br>FSAMSC (1) | *F. keratoplasticum* (7)<br>*F. falciforme* (2) | *TEF1*, *RPB2* | – |
| Tupaki-Sreepurna et al., 2017b | n.a. | South India | FSSC (43)<br>FFSC (9)<br>FDSC (1) | *F. falciforme* (36)<br>*F. keratoplasticum* (6)<br>Unnamed FSSC sp. (1) | *TEF1*, *RPB2*, *TUB2*, ITS | – |
| Chang et al., 2006 | 2005–2006 | Hong Kong, Singapore | FSSC (20) | *F. keratoplasticum* (20) | *TEF1*, *RPB2*, ITS | Contact lens-associated cases |
| **EUROPE** | | | | | | |
| Walther et al., 2017 | 2014–2015 | Germany | FSSC (13)<br>FOSC (6)<br>FFSC (3) | *F. petroliphilum* (6)<br>*F. keratoplasticum* (3)<br>*F. falciforme* (1)<br>*F. solani s. str.* (1)<br>FSSC 9 (1)<br>FSSC 25 (1) | *TEF1* | Mostly contact lens-associated cases |
| Dalyan et al., 2015 | 1995–2015 | Turkey | FSSC (2)<br>FFSC (1) | *F. solani s. str.* (2) | *TEF1*, ITS | |
| **NORTH-AMERICA** | | | | | | |
| Chang et al., 2006 | 2005–2006 | USA | FSSC (30)<br>FOSC (7) | *F. petroliphilum* (13)<br>*F. keratoplasticum* (12)<br>*F. falciforme* (n.a.)<br>FSSC 6 (n.a.)<br>FSSC 7 (n.a.) | *TEF1*, *RPB2*, ITS | Contact lens-associated cases |

*FDSC, Fusarium dimerum species complex; FFSC, Fusarium fujikuroi species complex; FIESC, Fusarium incarnatum-equiseti species complex; FOSC, Fusarium oxysporum species complex; FSAMSC, Fusarium sambucinum species complex; FSSC, Fusarium solani species complex; FSSC 6, FSSC 7, FSSC 9, FSSC 25 and FSSC 33, unnamed FSSC species; ITS, internal transcribed spacer region; n.a., not available; RPB2, the second largest subunit of RNA polymerase II gene; TEF1, translation elongation factor 1α; TUB2, beta-tubulin.*

nine isolates were *F. keratoplasticum*, and the remaining two were identified as *F. falciforme*. This difference in the frequency of the two species could be explained by the small sample size. In contrast to these data from India, in Hong Kong and Singapore *F. keratoplasticum*, while in the USA, *F. petroliphilum*, *F. keratoplasticum* and *F. falciforme* were isolated from *Fusarium* keratitis cases. All of these species were identified in 2005 and 2006 during the multistate *Fusarium* keratitis outbreak associated with the use of Bausch and Lomb ReNu contact lens solution (Chang et al., 2006). Finally, in Germany *F. petroliphilum* and *F. keratoplasticum*, while in Turkey *F. solani s. str.* dominated among *Fusarium* keratitis isolates (Dalyan et al., 2015; Walther et al., 2017). *Fusarium lichenicola* was not isolated either from the environment or from clinical specimens in our study. According to our literature overview of *Fusarium* keratitis studies in **Table 3**, it is obvious that this species is an extremely rare causative agent of this disease. Previously, only Hassan et al. (2016) reported a single *F. lichenicola* isolate from keratomycosis from the Aravind Eye Hospital in Coimbatore, Tamilnadu.

Although our phylogeny (**Figure 2**) has been inferred using only the partial *TEF1* gene, it could be used to identify the isolates tested. *TEF1* is widely used to investigate the phylogenetic relationships of fusaria at the interspecific level (Debourgogne et al., 2012), but this locus alone is not appropriate to examine intraspecies relationships. According to O'Donnell et al. (2015), *TEF1* and the largest (*RPB1*) and the second largest subunit (*RPB2*) of the DNA-directed RNA polymerase II are the three most informative loci for phylogenetic species recognition in the genus *Fusarium*. Multilocus sequence typing (MLST) schemes including additional loci, e.g., the beta-tubulin gene, the internal transcribed spacer (ITS) region and the large ribosomal subunit gene (LSU) have also been proposed for fusaria (van Diepeningen

**TABLE 4 |** Literature overview of antifungal susceptibility data available for FSSC strains isolated from human keratomycoses.

| Reference | Sampling period | Tested isolates (n) | MIC ranges (μg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | AMB | CLT | CSP* | ECN | FLC | ISV | ITC | KTC | MCN | NTM | NYS | PSC | TRB | VRC |
| **INDIA** | | | | | | | | | | | | | | | | |
| Homa et al., 2013 | 2010–2011 | 53 | 0.125–>64 | 4–>64 | n.a. | 8–>64 | n.a. | n.a. | ≥64 | n.a. | n.a. | 2–>64 | n.a. | n.a. | 1–>64 | 0.125–>64 |
| Hassan et al., 2016 | 2012–2013 | 54 | 0.5–8 | 1–16 | n.a. | 2–8 | 8–16 | n.a. | 2–16 | 1–16 | 1–8 | 4–16 | 8–16 | n.a. | n.a. | 0.5–8 |
| Tupaki-Sreepurna et al., 2017a | 2012–2014 | 9 | 2–>32 | n.a. | ≥16 | n.a. | n.a. | n.a. | 16–>32 | n.a. | n.a. | 2-4 | n.a. | n.a. | n.a. | 8->32 |
| **EUROPE** | | | | | | | | | | | | | | | | |
| Walther et al., 2017 | 2014–2015 | 13 | 0.5–4 | n.a. | 16 | n.a. | n.a. | 16 | 16 | n.a. | n.a. | 4-16 | n.a. | 16 | 64 | 1-16 |
| Dalyan et al., 2015 | 1995–2015 | 2 | 0.25–1 | n.a. | >16 | n.a. | >64 | n.a. | >64 | n.a. | n.a. | n.a. | n.a. | >16 | n.a. | 8-16 |

**MECs, minimal effective concentration values.*

*AMB, amphotericin B; CLT, clotrimazole; CSP, caspofungin; ECN, econazole; FLC, fluconazole; ISV, isavuconazole; ITC, itraconazole; KTC, ketoconazole; MCN, micafungin; n.a., not available; NTM, natamycin; NYS, nystatin; PSC, posaconazole; TRB, terbinafine; VRC, voriconazole.*

et al., 2015). As it was previously described by Zhang et al. (2006) based on the phylogenetic analysis of the FSSC, we also found that clinical and environmental members of this species complex share a common evolutionary origin.

Susceptibility data on fusaria were not congruent in the literature (**Table 4**). According to Walther et al. (2017), FSSC and non-FSSC isolates may be easily separated based on their *in vitro* susceptibility to TRB. In their study, FSSC showed MICs higher than 32 μg/ml, while FOSC (*F. oxysporum* species complex) and FFSC (*F. fujikuroi* species complex) strains had MICs lower than 8 μg/ml (Walther et al., 2017). Our MIC data for the FSSC isolates did not confirm this suggestion; we observed a wide range of MICs for TRB (0.5–64 μg/ml). However, there are two fundamental differences between these reports: the geographical location and the species diversity. In contrast to the study of Walther et al. (2017), where *F. petroliphilum* and *F. keratoplasticum* were the most prevalent among the FSSC isolates, most of the strains in the present study were identified as *F. falciforme*.

In contrast to the currently reported susceptibility results, one of our previous studies revealed higher MIC values for all the tested antifungal drugs (Homa et al., 2013). However, in another South Indian keratitis study, Shobana et al. (2015) reported lower azole MICs (especially for ITC) for fusaria. According to Al-Hatmi et al. (2016), *Fusarium* spp. were intrinsically resistant to azoles. In agreement with this finding, we also found that ITC and FLC did not inhibit the growth of the investigated strains.

Triazole fungicides (i.e., hexaconazole, propiconazole, triadimefon, and tricyclazole) are commonly used for crop protection in India, especially in the Southern parts of the country (Chowdhary et al., 2012). The high exposure of environmental fungi to these compounds persisting in soil for a long time, may increase the risk of resistance development. For instance, the azole resistance of the common opportunistic human pathogen *Aspergillus fumigatus* was attributed to the non-medical use of these antifungals in the past few years (Van der Linden et al., 2011; Chowdhary et al., 2013; Azevedo et al., 2015; Berger et al., 2017). Although fusaria have not yet been investigated in this respect, we presume that resistance might emerge among them as a result of the permanent presence of fungicides in the environment.

When comparing our antifungal susceptibility data with previous studies, we also found similarities (**Table 4**). AMB and ECN were the most effective antifungal drugs against the majority of our isolates. Similarly, AMB showed the lowest MICs in other studies (Dalyan et al., 2015; Hassan et al., 2016; Walther et al., 2017). In accordance with the literature, all the MICs of NTM in our study were ≤16 μg/ml (Hassan et al., 2016; Tupaki-Sreepurna et al., 2017a; Walther et al., 2017), which is probably within the clinically achievable levels of this drug in eye tissue (Lalitha et al., 2008a).

Although species-specific clinical breakpoints are still not available for the genus *Fusarium*, CLSI epidemiological cutoff values (ECVs) were reported by Espinel-Ingroff et al. (2016) for AMB, posaconazole, voriconazole and ITC. These values may not able to help in predicting the clinical response to therapy but could possibly help to identify the so-called "non-wild-type

isolates" or isolates that are less susceptible to the antifungal drugs. The authors defined "non-wild-type" as the population of strains in a species-drug combination with a detectable acquired resistance mechanism (Espinel-Ingroff et al., 2016). The ECVs for AMB and ITC are 8 μg/ml and 32 μg/ml, respectively. Based on these values, our isolates proved to be less susceptible to ITC (MIC > 32 μg/ml) than wild-type FSSC strains. At the same time, the MICs of AMB were below the ECV (**Table 1**).

*Drosophila melanogaster* has been previously described as a suitable invertebrate host model to study the *in vivo* virulence and pathogenesis of clinically important filamentous fungi (i.e., *Aspergillus* spp., Mucorales spp., *Scedosporium* spp. and *Fusarium* spp.) (Hamilos et al., 2012). This fly has a genetically tractable and well-characterized innate immune system, which is regulated by two distinct signaling pathways: the immune deficiency and the Toll pathways. While the first one is important in the defense against Gram-negative bacteria, the latter one has a key role in the immunity against Gram-positive bacteria and fungi (Lemaitre and Hoffmann, 2007). MyD88 is an adapter in the Toll pathway and its overexpression induces the expression of the antifungal peptide drosomycin (Tauszig-Delamasure et al., 2002).

Previously, Lamaris et al. (2007) infected wild-type Oregon and Toll-deficient flies with a clinical *F. verticillioides* (formerly *F. moniliforme*, a member of the FFSC) strain. Both wild-type and mutant flies were susceptible to this fungus, but in Toll-deficient flies a more acute infection and higher mortality rates were observed (Lamaris et al., 2007). As it was expected, we also found lower survival rates in case of the MyD88-mutants than the wild-type flies. Our results reconfirmed that MyD88 was essential for *D. melanogaster* to recognize and eliminate fusaria.

Although *D. melanogaster* proved to be highly susceptible to *F. keratoplasticum* (**Figure 1**) in our study, intraspecies differences in the virulence of *F. falciforme* isolates suggest that virulence is more like a strain-specific, than a species-specific feature. These results were in agreement with the hypothesis of Zhang et al. (2006), namely, that susceptible patients are infected with the most prevalent fungi in their environment.

In conclusion, our results confirmed that *F. falciforme* was the most prevalent species of the FSSC in South India isolated from both *Fusarium* keratitis patients and environmental sources. Antifungal susceptibility and virulence of clinical and environmental isolates were similar. However, we found major differences in the most common etiological agents, compared to North-American, European and other Asian countries. In the consequences of the high incidence of *Fusarium* keratitis and the significant rate of treatment failure, regular clinical studies are still necessary to develop an effective management of this disease in South India.

## AUTHOR CONTRIBUTIONS

TP: contributed to the design and implementation of the research, participated in drafting the manuscript; LK, VN, and CV: contributed to analyze the results and helped in drafting the manuscript; LG: participated in molecular identification and the antifungal susceptibility tests; PM: collected all the clinical data reported in the manuscript and participated in the morphological identification process; RS and ÁC: performed the virulence studies; MH: performed the antifungal susceptibility tests, drafted the manuscript and designed the figures and the tables. All authors read and approved the final manuscript.

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# Leafhopper-Induced Activation of the Jasmonic Acid Response Benefits *Salmonella enterica* in a Flagellum-Dependent Manner

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Enteric human pathogens such as *Salmonella enterica* are typically studied in the context of their animal hosts, but it has become apparent that these bacteria spend a significant portion of their life cycle on plants. *S. enterica* survives the numerous stresses common to a plant niche, including defense responses, water and nutrient limitation, and exposure to UV irradiation leading to an increased potential for human disease. In fact, *S. enterica* is estimated to cause over one million cases of foodborne illness each year in the United States with 20% of those cases resulting from consumption of contaminated produce. Although *S. enterica* successfully persists in the plant environment, phytobacterial infection by *Pectobacterium carotovorum* or *Xanthomonas* spp. increases *S. enterica* survival and infrequently leads to growth on infected plants. The co-association of phytophagous insects, such as the Aster leafhopper, *Macrosteles quadrilineatus*, results in *S. enterica* populations that persist at higher levels for longer periods of time when compared to plants treated with *S. enterica* alone. We hypothesized that leafhoppers increase *S. enterica* persistence by altering the plant defense response to the benefit of the bacteria. Leafhopper infestation activated the jasmonic acid (JA) defense response while *S. enterica* colonization triggered the salicylic acid (SA) response. In tomato plants co-treated with *S. enterica* and leafhoppers, both JA- and SA-inducible genes were activated, suggesting that the presence of leafhoppers may affect the crosstalk that occurs between the two immune response pathways. To rule out the possibility that leafhoppers provide additional benefits to *S. enterica*, plants were treated with a chemical JA analog to activate the immune response in the absence of leafhoppers. Although bacterial populations continue to decline over time, analog treatment significantly increased bacterial persistence on the leaf surface. Bacterial mutant analysis determined that the bacterial flagellum, whether functional or not, was required for increased *S. enterica* survival after analog treatment. By investigating the interaction between this human pathogen, a common phytophagous insect, and their plant host, we hope to elucidate the mechanisms promoting *S. enterica* survival on plants and provide information to be used in the development of new food safety intervention strategies.

**Keywords: biomultiplier, jasmonic acid, bacterial flagellum, plant immunity, pathogen-triggered immunity**

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## INTRODUCTION

According to the Centers for Disease Control and Prevention (CDC), the human enteric bacterial pathogen *Salmonella enterica* is estimated to cause disease in more than 1.2 million people annually in the United States. Salmonellosis is most often characterized by gastroenteritis that may result in diarrhea, fever, and abdominal cramping. Of the *S. enterica*-related illnesses that are acquired in the United States, more than 90% are estimated to have come from a food source with 46% involving fresh produce. *S. enterica* is ubiquitous in the plant environment, is commonly isolated from water sources used for irrigation or pesticide application, and persists for months on the roots and leaves of agricultural crops (Islam et al., 2004a,b; Haley et al., 2009). The phyllosphere (the above-ground parts of the plant) is a hostile environment with fluctuations in temperature, water and nutrient availability, and UV irradiation (O'Brien and Lindow, 1989; Islam et al., 2004b; Barak and Liang, 2008; Poza-Carrion et al., 2013). The ability of *S. enterica* to overcome these environmental stresses contributes to the incidence of human disease due to the consumption of contaminated fresh produce (Gorski et al., 2011; Painter et al., 2013).

To survive on the leaf surface, epiphytic bacteria actively migrate to preferential niches such as trichomes, stomates, and cell junctions (Haefele and Lindow, 1987; Kinkel, 1997; Monier and Lindow, 2003; Remus-Emsermann et al., 2012). In these sites, bacteria find increased nutrient availability and protection against fluctuations in temperature, humidity, and UV radiation (Leveau and Lindow, 2001). In general, bacteria utilize many different types of motility, including swimming, swarming, twitching, gliding, and sliding (Henrichsen, 1972; Macnab, 1996; Mattick, 2002; Harshey, 2003; Mignot et al., 2007; Kearns, 2010). Perhaps the most well studied, swimming motility utilizes the flagellum to propel a bacterium through a semi-solid or liquid medium. The flagellum is a complex organelle in terms of both structure and regulation and is important for bacterial survival in many niches, including the surfaces of leaves (for recent review, see Rossez et al., 2015). For example, the plant pathogen *Pseudomonas syringae* requires flagellum-dependent swimming motility for full fitness on the surface of bean leaves. Motility mutants are less resistant to UV irradiation and desiccation, and mutant populations are reduced compared to the wildtype parent in colonization assays (Haefele and Lindow, 1987; Lindow et al., 1993). Similarly, *S. enterica* utilizes flagella for swimming and swarming motility, and flagella contribute to bacterial attachment to basil leaves (Berger et al., 2009).

Although the bacteria successfully locate preferred sites on the leaf surface, *S. enterica* populations slowly decline over time (Islam et al., 2004a,b; Barak et al., 2011). Unlike phytobacterial pathogens, *S. enterica* cannot liberate nutrients from the plant, leaving the human pathogen in an environment where resources are limiting. However, biotic factors, such as infection with phytobacterial pathogens, can release nutrients and allow for proliferation or enhanced survival of human bacterial pathogens. Depending on the species, *Xanthomonas*-induced necrosis and water-soaking leads to increased persistence of *S. enterica* serovar Typhimurium (*S.* Typhimurium), or even replication in the case of *Xanthomonas euvesicatoria* and *Xanthomonas gardneri*, on tomato leaves (Potnis et al., 2014, 2015). Similarly, tissue damage caused by the soft-rotting bacterium *Pectobacterium carotovorum* subsp. *carotovorum* enhances *S.* Typhimurium persistence on romaine lettuce leaves (Kwan et al., 2013). Recently, our lab expanded the list of biological factors that enhance *S. enterica* survival (biomultipliers) to include phytophagous insects and demonstrated that insect infestation increases *S. enterica* persistence on plants (Soto-Arias et al., 2013, 2014). Specifically, the presence of Aster leafhopper (*Macrosteles quadrilineatus*), a common phloem-feeding pest of agricultural crops, significantly improved *S. enterica* persistence on lettuce leaves (Soto-Arias et al., 2013). Furthermore, phytophagous insects that encounter *S. enterica*-contaminated plants represent a risk for transmitting disease as they frequently move within and between plants while feeding and can be contaminated both externally and internally with *S. enterica* (Soto-Arias et al., 2014). Thus, phytophagous insects represent a risk factor for the dispersal of *S. enterica* amongst plants in an agricultural setting.

The mechanism(s) by which leafhopper infestation promotes *S. enterica* persistence on plants are unknown. Reviewing the list of environmental stresses present in this environment, we hypothesize that leafhoppers alter the plant immune response in a way that benefits bacterial survival on leaves. Plant immunity is characterized by a multi-layer defense response that is reminiscent of animal innate immunity. Pattern recognition receptors (PRRs) detect conserved pathogen associated molecular patterns (PAMPs) from invading microorganisms which trigger a cascade of responses (for reviews, see Garcia and Hirt, 2014; Melotto et al., 2014). Initially, the plant responds within seconds to minutes with changes in ion fluxes and extracellular alkalinization and increased production of reactive oxygen species (ROS). These changes are followed in the next minutes to hours with increases in ethylene production, stomatal closure, mitogen-activated protein kinase (MAPK) signaling, and transcriptional reprogramming. Within hours to days, accumulations in callose [β-(1,3)-glucan polymer] deposition, salicylic acid (SA) production, and defense gene transcription are observed. The defense hormones SA and jasmonic acid (JA) are well characterized signaling hormones critical to effective plant immunity. Generally, the SA pathway is induced by the plant in response to biotrophic pathogens while the JA pathway is activated in the presence of necrotrophic pathogens or chewing herbivores. Although not a strict rule, the SA and JA pathways are typically antagonistic; activation of one pathway leads to inhibition of the other (Pena-Cortes et al., 1993; van Wees et al., 2000; Spoel, 2003; Van der Does et al., 2013; Wei et al., 2014). Crosstalk between the two pathways is thought to fine tune the plant immune response to pathogens (Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Pieterse et al., 2012). Disruption or alteration of the SA and JA pathways could have downstream effects on the plant immune response and impact microbial survival.

Aster leafhoppers feed by inserting their stylets through plant cells to reach the phloem. Although this feeding style causes little cellular damage compared to chewing herbivores such as

caterpillars, leafhoppers induce the JA response in *Arabidopsis thaliana* and tobacco (*Nicotiana attenuata*) plants (Sugio et al., 2011; Kallenbach et al., 2012). In contrast, in *A. thaliana*, *Medicago truncatula*, and lettuce (*Lactuca sativa*), inoculation with *S. enterica* leads to induction of the SA pathway (Iniguez et al., 2005; Klerks et al., 2007; Schikora et al., 2008; Jayaraman et al., 2014). Phytophagous insects have developed strategies to manipulate plant immunity to the benefit of the insect. As one example, the Colorado potato beetle (*Leptinotarsa decemlineata*) utilizes bacteria found in the beetle's oral secretions to reduce the JA response to the beetle by preemptively activating the SA response using the oral bacteria (Chung et al., 2013a,b). In this way, the beetle manipulates the host into responding to the bacteria, taking advantage of the antagonistic nature of the JA and SA pathways, and giving the herbivore, and their offspring, easier access to plant nutrients. The interaction between *S. enterica* and leafhoppers may not be as directly symbiotic as the Colorado potato beetle and its oral microflora, but we hypothesize that leafhopper-induced activation of the JA response may lead to antagonistic suppression of the SA response and indirectly create a more permissive environment for *S. enterica*.

In this study, we tested the hypothesis that infestation with leafhoppers alters the plant immune response and increases *S. enterica* persistence. Several multistate and international outbreaks have been linked to *Salmonella*-contaminated tomatoes (Cummings et al., 2001; Centers for Disease Control and Prevention, 2005, 2007; Gupta et al., 2007; Greene et al., 2008), making tomato a relevant plant host for studying *S. enterica* persistence. We chose to examine tomato plants as a way to compare the effects of leafhoppers on *S. enterica* populations in a second plant host. Additionally, we used the JA-inducible proteinase inhibitor gene *pin1* and the SA-inducible pathogenesis related protein gene *pr1a1* as established markers (Tornero et al., 1994; Fowler et al., 2009) to monitor the two plant defense responses with quantitative PCR. Further, we examined the effect of a chemical JA analog on *S. enterica* persistence and investigated the importance of the bacterial flagellum in this system.

# MATERIALS AND METHODS

## Bacterial Strains, Media, and Culture Conditions

The nalidixic acid-resistant strain of *S. enterica* serovar Typhimurium 14028s (*S.* Typhimurium; Cowles et al., 2016) was used as wildtype in this study (**Table 1**). The *fliF*::Kan mutant was generated using primers listed in **Table 2** with the $\lambda$Red recombinase method (Datsenko and Wanner, 2000), and the *motA*::Kan mutant was obtained from a mutant library collection (Santiviago et al., 2009). These deletion–insertion mutations were transduced into a fresh *S.* Typhimurium background using P22 (Gemski and Stocker, 1967) and confirmed by PCR (see **Table 2** for confirmation primers). Bacterial cultures were grown in lysogeny broth (LB) at 37°C with shaking at 200 rpm. The antibiotics nalidixic acid (Nal) and kanamycin (Kan) were used at concentrations of 20 and 50 μg/ml, respectively. Strains used in this study are shown in **Table 1**, and primers used to confirm bacterial mutants are shown in **Table 2**.

**TABLE 1 |** List of strains.

| Strain designation | Genotype | Reference or source |
|---|---|---|
| ***Salmonella enterica* strains** | | |
| JDB682 | *S. enterica* serovar Typhimurium 14028s | ATCC |
| JDB1034 | *S. enterica* serovar Typhimurium 14028s; $Nal^R$ | This study |
| JDB903 | Δ*fliF*::Kan | This study |
| JDB902 | Δ*motA*::Kan | This study |

## Insect Rearing

A colony of Aster leafhoppers (*M. quadrilineatus*) was maintained on oat plants (*Avena sativa* L.) in a controlled environment with a 16 h photoperiod with 24°C light and 19°C dark. Even-aged, 3- to 5-day-old adult insects were used for all experiments.

## Plant Inoculation and Infestation

*Solanum lycopersicum* (tomato) cultivar MoneyMaker seedlings were cultivated in Professional Growing Mix (Sunshine Redi-earth) with a 16 h photoperiod at 24°C for 5 weeks. For colonization assays, bacterial cultures were grown overnight in LB and normalized to an optical density at 600 nm ($OD_{600}$) of 0.2 in sterile water. An $OD_{600}$ of 0.2 corresponds to a bacterial population level of $\sim 10^8$ CFU/ml, and this inoculum level was used for all experiments. Prior to inoculation, 0.025% Hi-Wett (Loveland Products, Inc.) was added to water or the bacterial inoculum. Pots containing tomato plants were dip-inoculated by inverting plants in either sterile water or the bacterial inoculum for 1 min with agitation to prevent bacterial cell settlement (**Figure 1**). Dip-inoculated plants were incubated under the blower in a SterilGARD Class II Biosafety Cabinet (The Baker Company) for 1 h to dry the leaves. After the 1-h drying period, half of the water-dipped and half of the bacterial-dipped plants were fitted with empty clip cages on two middle leaflets (as defined in Potnis et al., 2014; **Figure 1**). Clip cages are two cm diameter plexiglass cylinders topped with plastic screen that were attached to the upper leaf surface to trap insects on the leaflet (**Figure 1**). The remaining dip-inoculated plants were fitted with clip cages containing four adult leafhoppers each. Plants were incubated at high humidity in lidded, plastic bins under grow lights with a 16 h photoperiod at room temperature (∼26°C). At multiple time points post-inoculation and/or post-infestation, leaf samples were taken from within clip cages using destructive sampling to determine bacterial populations and collect samples for RNA extraction.

## Chemical Analog Treatment

For exogenous induction of the JA response, 5-week-old MoneyMaker tomato seedlings were sprayed with a fine mist of sterile water or methyl jasmonate (MeJA; 0.000001%; Sigma Aldrich, St. Louis, MO, United States). The MeJA concentration was chosen to most closely mimic the plant response to a

**TABLE 2 |** List of primers.

| Primer | Primer sequence (5′–3′) | | % Efficiency |
|---|---|---|---|
| | Forward | Reverse | |
| **Quantitative PCR analysis**[a] | | | |
| *act41* | GCTCTTGACTATGAACAGGAAC | AAGGACCTCAGGACACCG | 104 |
| *ubi3* | GCCGACTACAACATCCAGAAGG | TGCAACACAGCGAGCTTAACC | 105 |
| *pin1* | GCTAAGGAAATAATTGAGAAGGA | TAAGTCACCACAGGCATT | 102 |
| *pr1a1* | TCAAAGAGCTGATGACTGTG | GTACCATTGCTTCTCATCGT | 102 |
| **Mutant construction and confirmation**[b] | | | |
| *fliF*::Kan construction | ATGAGTGCGACTGCATCGACTGC AACCCAACCTAAACCTCGTGTA GGCTGGAGCTCCTTC | GATCGTTACTCATCCACTGGCG AATGACCAGCGCCACC ACCATATGAATATCCTCCTTAG | |
| *fliF*::Kan confirm | CGTTGCGATGGTGCTGTGGG | GCGCGTCAACTGCGGACGTA | |
| *motA*::Kan confirm | GCGGACACCTTGGGGCACTC | TGGACGCTCACTGGAATAAAGCG | |

[a]*Quantitative PCR primers were used to examine transcription of plant genes (as described in the Section "Materials and Methods").* [b]*Mutant construction primers were used for the λRed recombinase method, and mutant confirmation primers were used to amplify across potential deletion–insertion sites and compare the resulting product sizes to that observed when using wildtype genomic DNA as a template.*

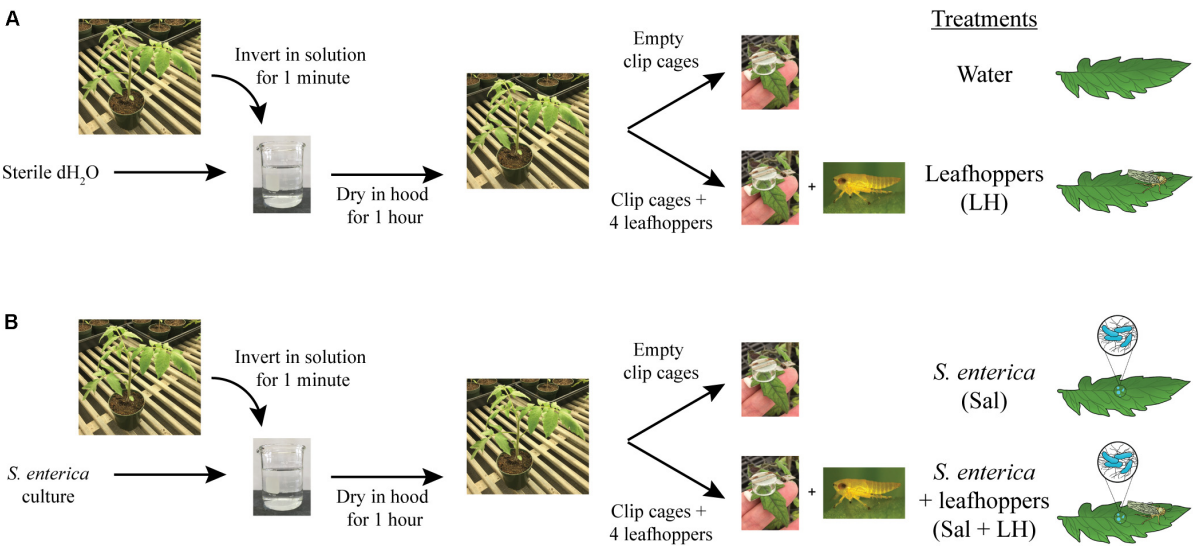


**FIGURE 1 |** Inoculation and infestation assay. Tomato plants were dip-inoculated by inverting plants in either sterile water **(A)** or bacterial inoculum **(B)**. Dip-inoculated plants were dried for 1 h. Then, half of the water-dipped **(A)** and half of the bacterial-dipped plants **(B)** were fitted with clip cages, to trap insects on the leaflet, containing four adult leafhoppers each. The remaining dip-inoculated plants were fitted with empty clip cages.

leafhopper infestation; optimization experiments determined that there were no observable changes in plant morphology, and the level of JA induction was similar to that seen in response to the insects (data not shown). Plants were incubated for 24 h at high humidity and then dip-inoculated in water or bacterial suspension as described above. Dipped plants were incubated at high humidity as described above. At days 0, 3, 6, 10, and 14 (post-dip inoculation), leaf samples were taken from middle leaflets using destructive sampling to determine bacterial populations and collect samples for RNA extraction.

## Bacterial Population Sampling

At indicated time points, one 79 $mm^2$ leaf disc was taken from each of two leaflets on middle leaves (Potnis et al., 2014). Samples from four plants per treatment per time point were transferred to microfuge tubes and homogenized in 500 μl of sterile water using a 4.8 V rotary tool (Dremel, Mount Prospect, IL, United States) with microcentrifuge tube sample pestle attachment (Thermo Fisher Scientific). Homogenates were diluted 1:10 in sterile water and spiral plated (Autoplate 4000, Spiral Biotech, Norwood, MA, United States) on LB Nal plates. Resulting colonies were counted after overnight incubation at 37°C to determine bacterial populations. Experiments were performed with three biological replicates.

## RNA Isolation

At indicated time points, two 79 $mm^2$ leaf discs were taken from each of two leaflets on middle leaves (Potnis et al.,

2014) and combined for a total of four leaf discs per plant. Samples from four plants per treatment per time point were collected and frozen at −80°C for further processing. RNA was extracted using the PureLink RNA Purification kit (Invitrogen) with some modifications. Briefly, four leaf discs were homogenized with a mortar and pestle in the presence of liquid nitrogen. Ground tissue was transferred to a small weigh boat containing 1 ml Trizol, mixed with a pipette tip, and transferred to a microcentrifuge tube. Samples were then processed according to manufacturer's instructions and eluted in 100 µl volume of RNase-free water. RNA was treated with Turbo DNA-free (Ambion) for 30 min using the manufacturer's protocol and quantified by NanoDrop (Thermo Fisher Scientific). RNA was isolated from three biological replicates for each experiment.

## cDNA Synthesis and Real-Time PCR

cDNA synthesis was performed using the iScript cDNA synthesis kit according to manufacturer's instructions (Bio-Rad) with 1.5 µg total RNA as input. Real-time PCR primers were designed with Beacon Designer software (Premier Biosoft International) avoiding template secondary structure (**Table 2**). Primer efficiencies (**Table 2**) were calculated using serial dilutions of MoneyMaker genomic DNA and CFX Manager 3.0 software (Bio-Rad). Reference transcripts were chosen based on published works: *act41* and *ubi3* (Rotenberg et al., 2006). Stable expression between treatments was validated using the Best Keeper program and four independent RNA samples from each treatment. Real-time PCR experiments were performed as described (Jahn et al., 2008; Cowles et al., 2016). Experiments were performed using the CFX96 Real-Time System and analyzed with the CFX Manager 3.0 software (Bio-Rad). The mean *Cq* of each target transcript was normalized by the mean *Cq* of each reference gene using the formula: $2^{(-(Cq\ \text{target} - \text{Cq reference}))}$. As previously described (Rotenberg et al., 2006), we determined the relative expression ratio (RER) of the target gene by dividing the normalized target RNA by a calibrator consisting of the average of the normalized values of the control samples (expression after water treatment in these experiments).

## Statistical Analysis

All statistical analyses were performed using R software (version 2.14.1; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria[1]) as described (Kwan et al., 2015). Briefly, three biological replicates were performed for each experiment, and samples taken from one replicate were considered as subsamples. To determine whether bacterial population results differed between treatments in the leafhopper and chemical analog experiments, analysis of covariance (ANCOVA) was used as previously described (Soto-Arias et al., 2013), with treatment and time as covariates. For real-time PCR analysis, four samples were compared for each treatment at each time point using Tukey's HSD test. Results were considered statistically significant at $P < 0.05$.

[1]http://www.R-project.org

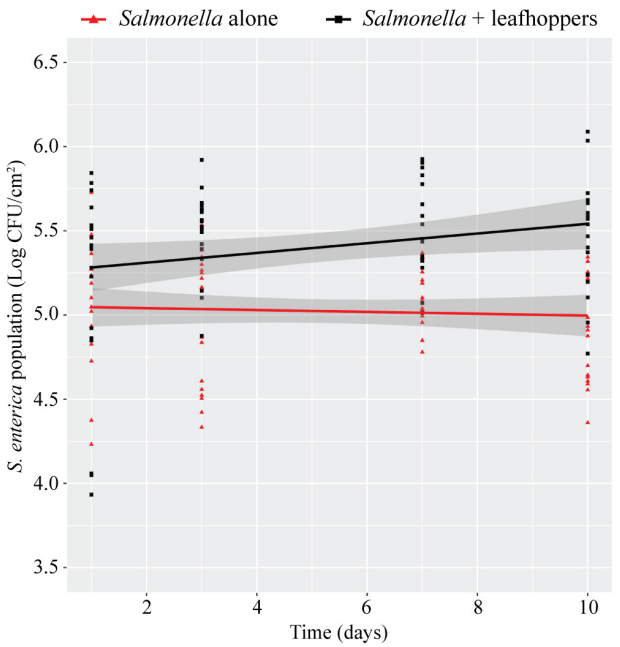


**FIGURE 2 |** Leafhopper infestation leads to increased persistence of *S.* Typhimurium populations. Bacterial populations on leaves were monitored over time either in the presence (black squares) or absence (red triangles) of leafhoppers. All data points from three independent experiments were combined and presented as *S.* Typhimurium populations (log CFU/cm$^2$) over a 10-day period. Lines (black, *S.* Typhimurium with leafhoppers; red, *S.* Typhimurium alone) correspond to a linear regression model, and shaded areas correspond to the respective 95% confidence interval.

# RESULTS

## The Presence of Leafhoppers Enhances *S.* Typhimurium Persistence on Tomato Leaves

Previously, we had shown that infestation with phytophagous insects promotes *S. enterica* survival on the leaves of lettuce plants (Soto-Arias et al., 2013). To determine if this effect occurs in additional plant species, bacterial populations were monitored on tomato plants (*S. lycopersicum*) infested with leafhoppers (**Figure 2**). The presence of leafhoppers significantly enhanced the persistence of *S.* Typhimurium populations over time compared to plants that were inoculated with bacteria alone (**Figure 2**). After 10 days, *S.* Typhimurium populations were approximately ½ log higher in the presence of leafhoppers than in plants that were not infested with insects (**Figure 2**).

## Co-inoculation of *S.* Typhimurium and Leafhoppers Alters the Plant Immune Response

To test the hypothesis that insect infestation changes the plant immune response to the benefit of *S.* Typhimurium, plant defense gene expression was monitored over time in tomatoes inoculated with leafhoppers, *S.* Typhimurium, or a combination

of leafhoppers and *S.* Typhimurium. The JA-inducible proteinase inhibitor gene *pin1* and the SA-inducible pathogenesis related protein gene *pr1a1* were used as established markers (Tornero et al., 1994; Fowler et al., 2009) to monitor the two plant defense responses with quantitative PCR. Day 3 post-inoculation was chosen as the initial time point to monitor plant immune gene expression as it was the time when bacterial populations began to diverge when comparing treatments with or without leafhopper infestation (**Figure 2**). Compared to the negative control (plants treated with water), tomatoes that were infested with leafhoppers showed a significant induction of *pin1* expression and displayed an intermediate level of *pr1a1* expression on day 3 post-inoculation (**Figures 3A,B**). Contrastingly, tomato plants treated with *S.* Typhimurium had no change in *pin1* expression compared to the water control, but had a significant induction of *pr1a1* expression on day 3 (**Figures 3A,B**). Plants that were simultaneously treated with both leafhoppers and *S.* Typhimurium responded with an increase in *pin1* expression but no significant change in *pr1a1* gene expression on day 3 (**Figures 3A,B**). To examine the impacts on later stages of colonization, plant defense gene expression was also measured at day 6 post-inoculation. By day 6, plants that were treated with both leafhoppers and *S.* Typhimurium induced both *pin1* and *pr1a1* while treatment with *S.* Typhimurium alone led to an intermediate induction of *pin1* expression compared to the water control (**Figures 3C,D**). By day 6, *pin1* and *pr1a1* levels were not significantly different from the negative control following leafhopper infestation (**Figures 3C,D**).

## Exogenous Treatment With Methyl Jasmonate Is Sufficient to Increase Bacterial Persistence

To differentiate the effects of leafhoppers on the plant immune response from other potentially beneficial impacts of insect infestation on *S.* Typhimurium, we artificially induced the JA defense response with exogenous application of a chemical JA analog, MeJA. Due to biosafety limitations when studying *S.* Typhimurium, MeJA treatment was performed 24 h prior to bacterial dip-inoculation instead of concurrently as done in experiments with leafhoppers (**Figure 1**). Leaves sprayed with MeJA displayed an induction of *pin1* expression that was comparable to the leafhopper-induced JA response (**Figure 3**) and lasted through day 6 post-bacterial inoculation (**Figures 4A**, **5A**). MeJA treatment led to a significant increase in *S.* Typhimurium population persistence compared to negative control plants that were sprayed with sterile water (**Figure 4B**).

## The Bacterial Flagellum Is Necessary for Enhanced *S.* Typhimurium Persistence After MeJA Treatment

To identify bacterial factors that impact the connection between *S.* Typhimurium persistence and the immune response, we examined bacterial populations in two motility mutants: *fliF*::Kan and *motA*::Kan. As motility has previously been linked to bacterial persistence on plant leaves (Haefele and Lindow, 1987; Lindow et al., 1993) and the *S. enterica* flagellum is a known stimulus of the plant immune response (Garcia et al., 2014), we hypothesized that the flagellum may play a role in bacterial persistence in our experimental system. The *fliF* gene encodes the inner membrane ring required for construction of the bacterial flagellum, and *motA* encodes one of the membrane proteins that acts as a stator in the flagellar motor (Block and Berg, 1984; Homma et al., 1987a,b; Blair and Berg, 1990). Although both mutants are non-motile, the *fliF*::Kan mutant does not make a flagellum while the *motA*::Kan mutant makes a non-functional flagellum (Block and Berg, 1984; Homma et al., 1987a,b; Blair and Berg, 1990). Plants were treated with MeJA (as described above) and then dip-inoculated with either wildtype, the *fliF*::Kan mutant, or the *motA*::Kan mutant. While MeJA treatment resulted in a significant increase in the persistence of wildtype *S.* Typhimurium, populations of the *fliF*::Kan mutant were not significantly different on plants treated with MeJA or water (**Figure 4B**). Examination of *pin1* expression revealed that JA induction did not persist as long in plants inoculated with the *fliF*::Kan mutant compared to wildtype (**Figures 4A,C**). Unlike the *fliF*::Kan mutant, MeJA enhanced persistence of the *motA*::Kan mutant compared to the water control (**Figure 5B**), and JA induction continued through day 6 after inoculation for the *motA*::Kan mutant (**Figure 5C**). To further characterize the effects of these treatments on plant immunity, we also monitored transcription of the SA response using *pr1a1* primers (**Figure 6**). Expression of *pr1a1* was significantly induced after MeJA treatment and bacterial dip-inoculation in all tested strains at variable time points (**Figure 6**).

# DISCUSSION

Although best characterized for its role as a human enteric pathogen, *S. enterica* colonizes plants as part of its lifecycle and as a means for returning to an animal host (for review, see Barak and Schroeder, 2012). Although *S. enterica* cannot disrupt plant cells such as plant pathogens to release nutrients from the leaf surface, the bacteria successfully survive in the plant environment and persist for months with a slow decline in populations over time (Islam et al., 2004a,b). Biomultipliers, such as phytobacterial pathogens or phytophagous insects, enhance *S. enterica* survival by altering the plant environment to the benefit of the enteric pathogen (Kwan et al., 2013; Soto-Arias et al., 2013; Potnis et al., 2014, 2015). Understanding the mechanisms for the interaction between *S. enterica* and these biomultipliers is important in order to reduce the risk of human disease from contaminated produce. In this study, we describe how phytophagous insects modulate plant immunity, and further, we provide a mechanism linking the bacterial flagellum and the plant JA response to *S. enterica* persistence.

Here, we examined the impact of one common phytophagous insect, the Aster leafhopper, and further characterized its role as a biomultiplier of *S.* Typhimurium populations. Using a similar methodology as described in this study (**Figure 1**), we had previously shown that leafhoppers enhance *S. enterica* persistence on lettuce plants (Soto-Arias et al., 2013). Those experiments demonstrated that, regardless of treatment,

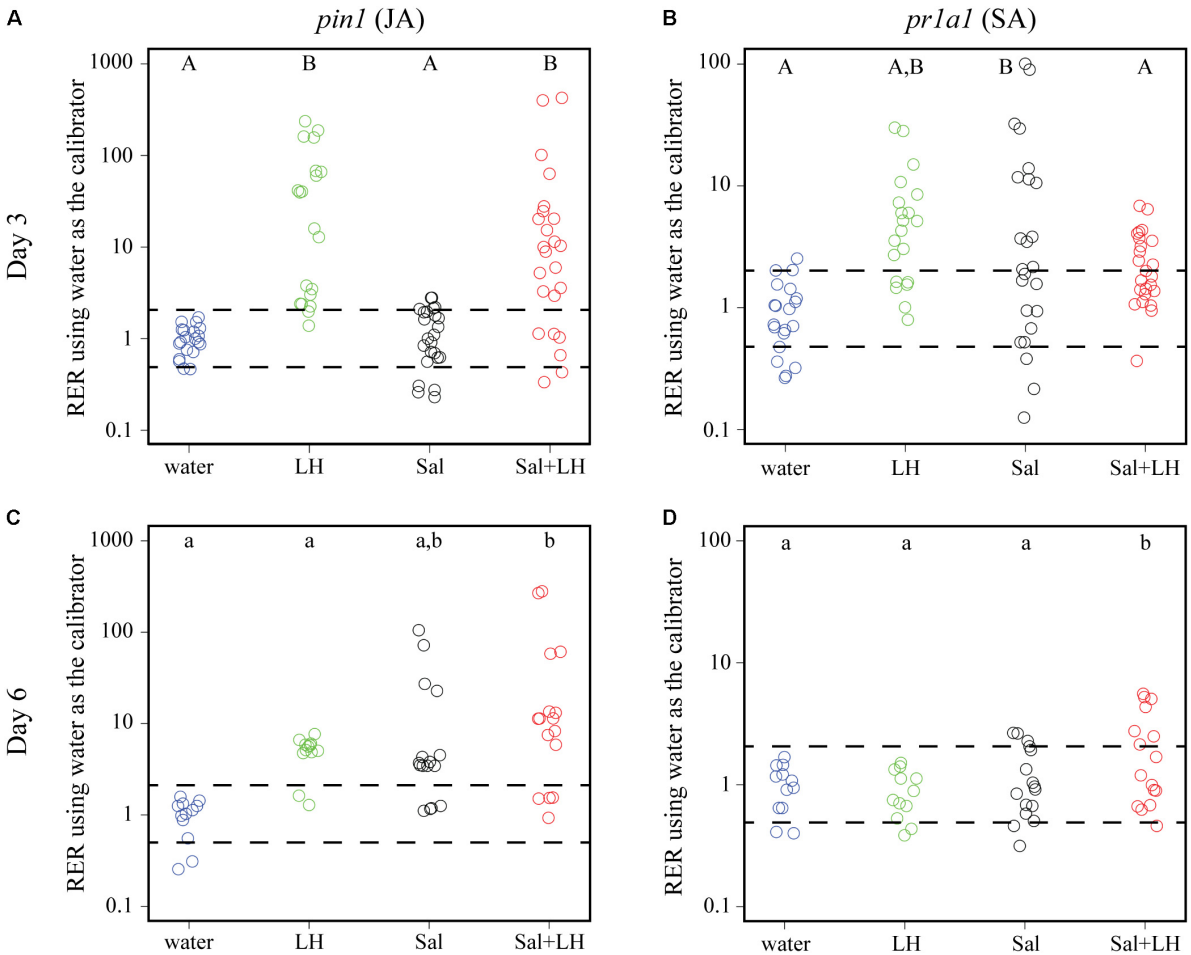


**FIGURE 3 |** Transcription of plant defense genes changes in response to leafhoppers (LH; green circles), *S.* Typhimurium bacteria (Sal; black circles), or co-inoculation of bacteria with leafhoppers (Sal+LH; red circles). Plant gene expression was quantified at days 3 **(A,B)** and 6 **(C,D)** post-inoculation. Data for the JA-inducible *pin1* **(A,C)** and SA-inducible *pr1a1* **(B,D)** are displayed as relative expression ratios (RER) using transcription in plants treated with water (blue circles) as the negative control and calibrator. The dashed line indicates a twofold change in transcription, and each circle represents data collected from one tomato plant. Transcript levels were quantified from three biological replicates with samples taken from at least four plants per treatment per time point. Letters denote significant differences between treatments within a single time point for that specific plant defense gene ($P < 0.05$).

*S. enterica* populations slowly decline over time. Infestation with leafhoppers increased the longevity of a cocktail of *S. enterica* strains (Cubana, Enteritidis, Newport, Poona, Schwarzengrund, Baildon, and Mbandaka) on lettuce leaves compared to plants without insect infestation (Soto-Arias et al., 2013). In this study, we demonstrate that, in the absence of leafhoppers, *S.* Typhimurium populations on tomato leaves also show a slow decline (**Figure 2**). However, two noticeable differences can be observed when comparing the data from lettuce to those collected from tomato. First, in the absence of leafhoppers, the decline in *S.* Typhimurium populations on tomato leaves appears to be slower (**Figure 2**) than the decline that was observed on lettuce (Soto-Arias et al., 2013). Second, in contrast to the experiments performed with lettuce (Soto-Arias et al., 2013), leafhopper infestation on tomato plants led to a constant *S.* Typhimurium population with no appreciable loss of overall numbers over the course of the experiment (**Figure 2**). The maintenance of a steady bacterial population level indicates that either the bacteria remain viable with no significant growth, or bacterial replication rates are equivalent to bacterial death, leading to no observable changes in overall bacterial numbers. The differences observed between bacterial populations on these two plants could also reflect differences in leafhopper feeding behavior on the two plant hosts. Aster leafhoppers overwinter on grains but will infest vegetable crops such as carrot, celery, potato, radish, and lettuce during the growing season (Hagel et al., 1973; Beanland et al., 2005). Although the leafhoppers utilize many different crops as sources of food or refuge, leafhopper reproduction consistently occurs only on lettuce, suggesting lettuce is a more preferred plant host. Thus, on lettuce, the leafhoppers may locate feeding sites quickly and create fewer damage sites (e.g. salivary sheaths) (Miles, 1972; Backus, 1988). Contrastingly, tomato plants, a Solanaceae plant like potato, could be considered a non-preferred host plant for the insects, and the leafhoppers may spend more time probing

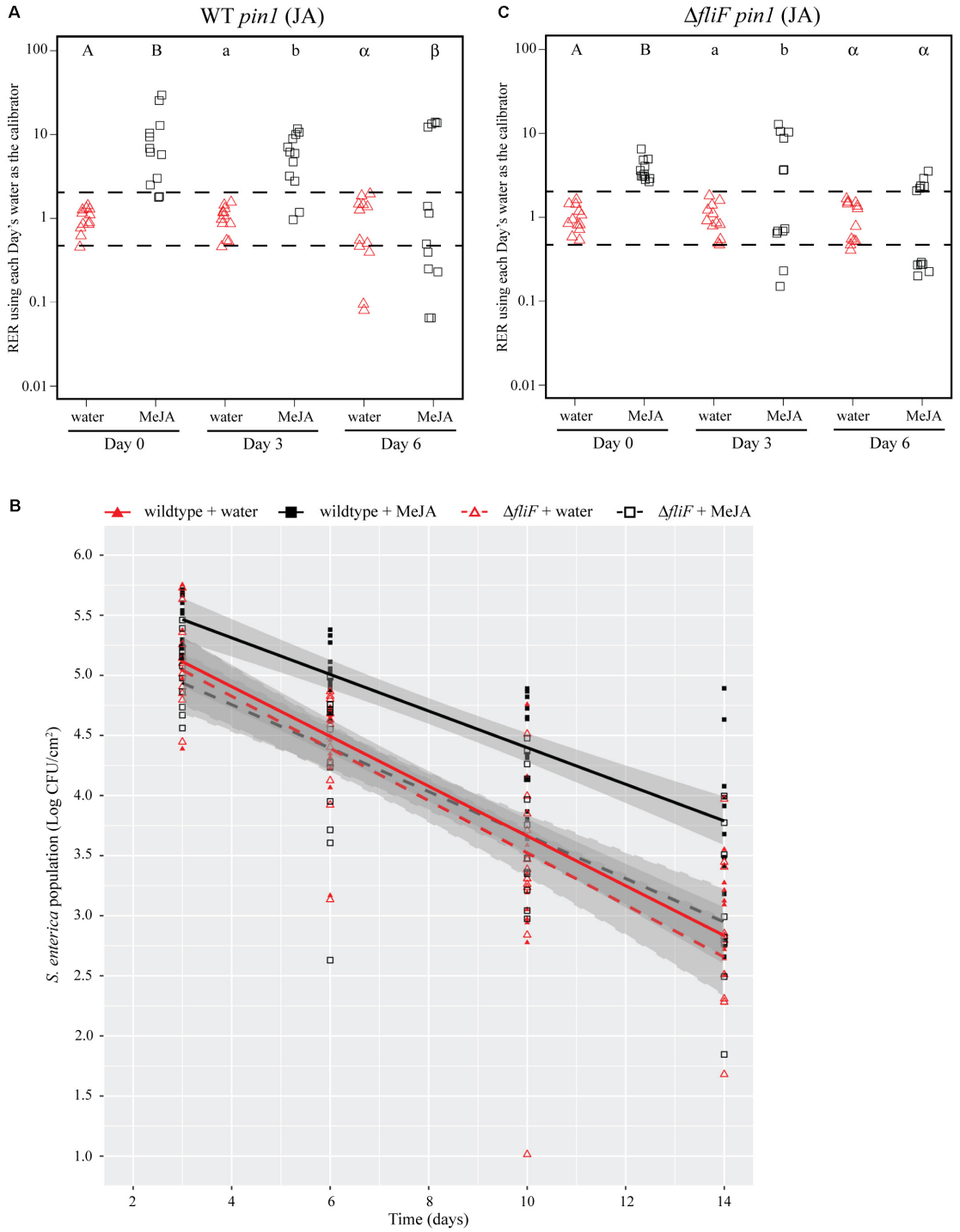


**FIGURE 4 |** Induction of the JA response with a chemical analog increases *S.* Typhimurium survival over time. **(A,C)** Plant *pin1* gene expression was quantified at days 0, 3, and 6 post-inoculation with wildtype *S.* Typhimurium **(A)** or the *fliF*::Kan mutant **(C)** for plants sprayed with water (red triangles) or methyl jasmonate (MeJA; black squares) and displayed as relative expression ratios (RER) using transcription on plants treated with water as the calibrator. The dashed line indicates a twofold change in transcription, and each symbol represents data collected from one tomato plant. Transcript levels were quantified from three biological replicates with samples taken from four plants per treatment per time point. Letters denote significant differences between treatments within a single time point ($P < 0.05$). **(B)** Bacterial populations on leaves were monitored over time following either treatment with water (red triangles) or MeJA (black squares) 24 h prior to bacterial dip inoculation. All data points from three independent experiments were combined and presented as *S.* Typhimurium populations (log CFU/cm$^2$) over a 14-day period. Lines (red, solid: wildtype *S. enterica* after water treatment; black, solid: wildtype *S.* Typhimurium after MeJA treatment; red, dashed: *fliF*::Kan after water treatment; black, dashed: *fliF*::Kan after MeJA treatment) correspond to a linear regression model, and shaded areas correspond to the respective 95% confidence interval.

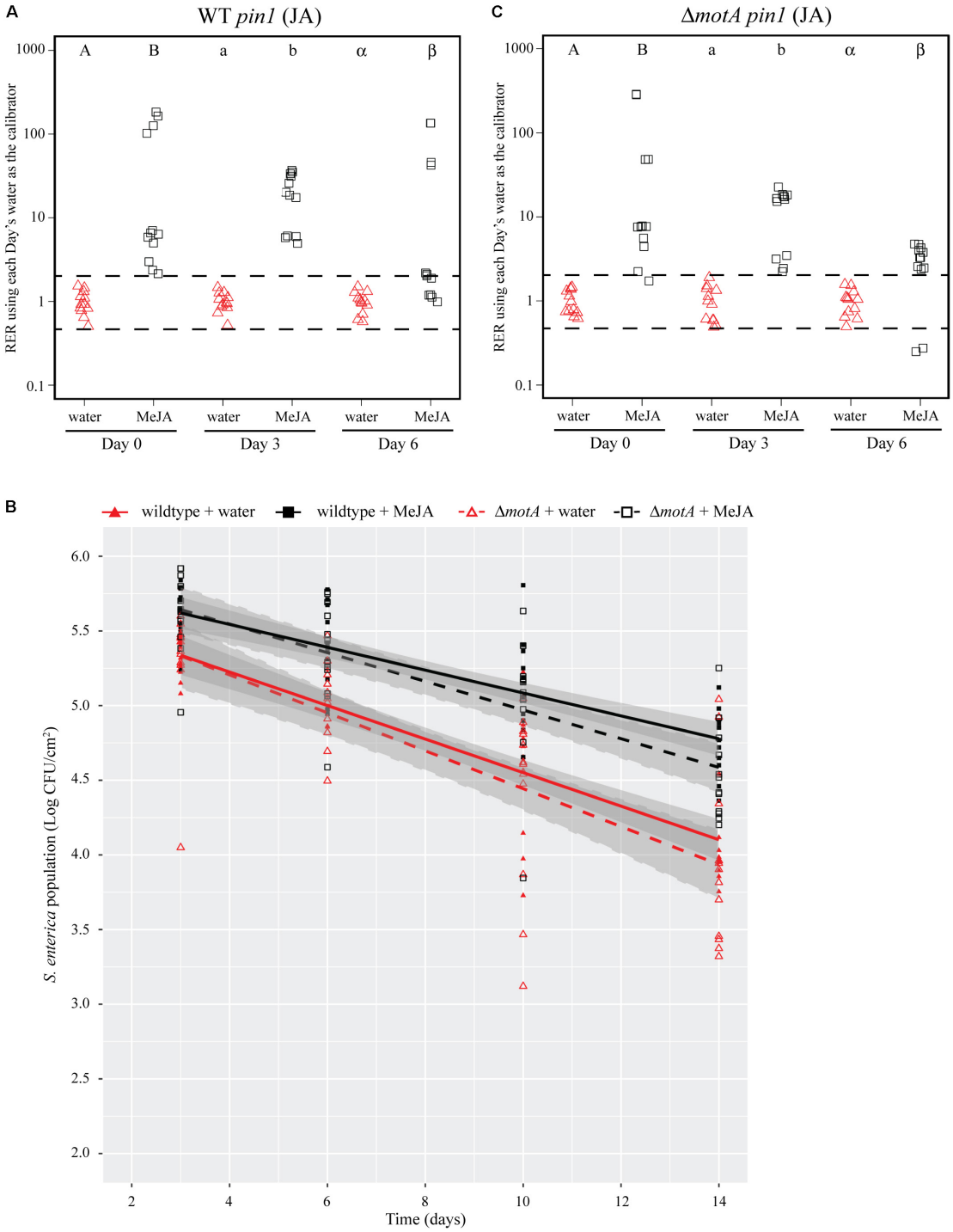


**FIGURE 5 |** Induction of the JA response with a chemical analog increases *S.* Typhimurium survival over time. **(A,C)** Plant *pin1* gene expression was quantified at days 0, 3, and 6 post-inoculation with wildtype *S.* Typhimurium **(A)** or the *motA*::Kan mutant **(C)** for plants sprayed with water (red triangles) or MeJA (black squares) and displayed as relative expression ratios (RER) using transcription on plants treated with water as the calibrator. The dashed line indicates a twofold change in transcription, and each symbol represents data collected from one tomato plant. Transcript levels were quantified from three biological replicates with samples taken from four plants per treatment per time point. Letters denote significant differences between treatments within a single time point ($P < 0.05$). **(B)** Bacterial populations on leaves were monitored over time following either treatment with water (red triangles) or MeJA (black squares) 24 h prior to bacterial dip inoculation. All data points from three independent experiments were combined and presented as *S.* Typhimurium populations (log CFU/cm$^2$) over a 14-day period. Lines (red, solid: wildtype *S.* Typhimurium after water treatment; black, solid: wildtype *S.* Typhimurium after MeJA treatment; red, dashed: *motA*::Kan after water treatment; black, dashed: *motA*::Kan after MeJA treatment) correspond to a linear regression model, and shaded areas correspond to the respective 95% confidence interval.

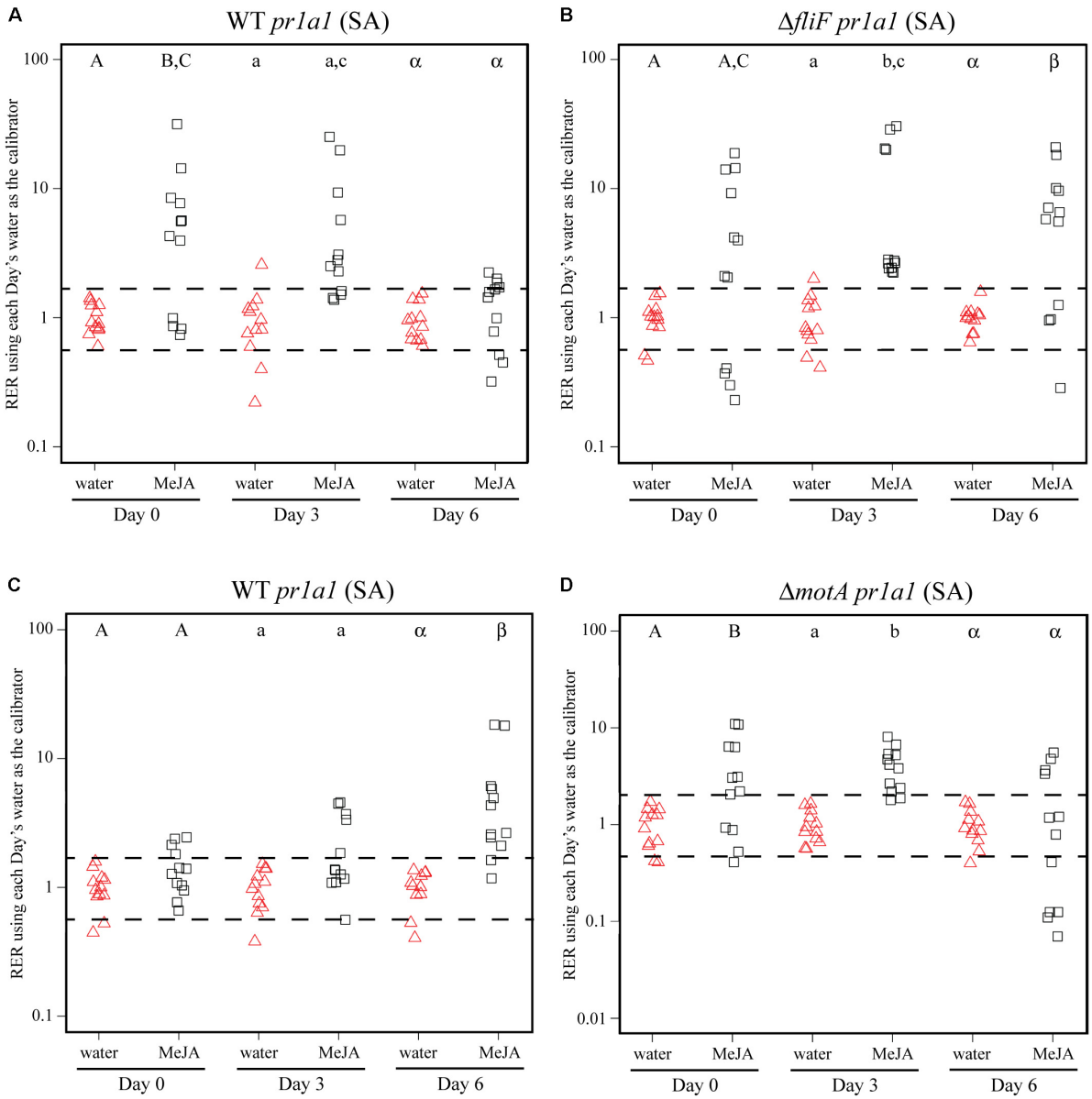


**FIGURE 6 |** MeJA treatment and *S.* Typhimurium inoculation lead to activation of the SA response. Plant *pr1a1* gene expression was quantified at days 0, 3, and 6 post-inoculation with wildtype *S.* Typhimurium (WT; **A,C**), the *fliF*::Kan mutant **(B)** or the *motA*::Kan mutant **(D)** for plants sprayed with water (red triangles) or MeJA (black squares) and displayed as relative expression ratios (RER) using transcription on plants treated with water as the calibrator. The *fliF*::Kan and *motA*::Kan mutants were tested in separate experiments, and the corresponding wildtype data are displayed for each experiment. The dashed line indicates a twofold change in transcription, and each symbol represents data collected from one tomato plant. Transcript levels were quantified from three biological replicates with samples taken from four plants per treatment per time point. Letters denote significant differences between treatments within a single time point in each experiment ($P < 0.05$).

the plant in search of nutrients. The increased probing has the potential to increase the defense response. Although we do not have data from lettuce as a comparison, we observed a robust induction of the JA response (12- to 236-fold) after 3 days of leafhopper infestation (**Figure 3A**). Interestingly, by 6 days post-infestation, *pin1* levels were not significantly different between water-treated plants and leafhopper-treated plants (**Figure 3C**). This reduction in the JA response over time may suggest that leafhoppers stop feeding at later points in the experiment and may be further evidence that tomato are not a preferred food source. Future experiments could address these questions by examining the lettuce defense responses in comparison to the tomato response presented here and monitoring leafhopper feeding patterns using these plant systems.

We hypothesize that leafhopper-induced alterations to plant immunity provide a benefit to *S.* Typhimurium and extend bacterial survival on the leaf surface. By day 3 post-inoculation, co-inoculation of leafhoppers with *S.* Typhimurium led to a

significant induction of the JA response while there was no change in the SA response until day 6 (**Figure 3**). Due to the strong, sustained induction of *pin1* expression, we chose to focus on the role of the JA response in enhancing *S.* Typhimurium persistence. To determine if changes in the JA response are sufficient for the effects on bacterial persistence, plants were treated with the analog MeJA to activate the immune response in the absence of leafhoppers. If MeJA treatment provided the same advantage as leafhopper infestation for *S.* Typhimurium populations, then we could rule out other mechanisms that explain the effect that leafhoppers have on bacterial populations, such as providing increased nutrients during insect feeding. Our results demonstrate that MeJA treatment enhanced bacterial persistence (**Figure 4B**), indicating that induction of the JA response is involved in the effect on bacterial populations. Contrasting to the leafhopper experiments (**Figure 2**), *S.* Typhimurium populations continued to decline over time after MeJA treatment (**Figure 4B**). As discussed above, leafhopper infestation resulted in a flat slope for the linear regression line describing bacterial populations while mock-treated plants had slowly declining levels of *S.* Typhimurium (**Figure 2**). In the MeJA experiments, we observed that both the water-treated and the MeJA-treated plants had a sharper decline in *S.* Typhimurium populations compared to the leafhopper experiment (**Figures 4**, **5** compared to **Figure 2**). Statistical analyses indicate that we cannot attribute the differences in slopes to the different experimental procedures used for these experiments. In the MeJA experiments, plants were sprayed with water or MeJA 24 h prior to bacterial inoculation while this treatment was not used in the leafhopper experiment. We found that bacterial populations on water-treated plants from the leafhopper experiment (**Figure 2**) were not significantly different from populations on water-treated plants in the MeJA *motA*::Kan experiment (**Figure 5**) at days 3, 6, and 7 but both populations were statistically different than those seen on water-treated plants in the MeJA *fliF*::Kan experiment (**Figure 4**) on those days ($P < 0.05$). The leafhopper experiment data (**Figure 2**) were statistically different from both MeJA experiments (**Figures 4**, **5**) by day 10 ($P < 0.05$). Biological variability does not explain these results as biological replicates within each experiment (each figure represents three independent experiments for those treatments) were not significantly different from one another ($P > 0.05$). This statistical analysis does preclude any conclusions that can be made about the sufficiency of JA induction on bacterial population enhancement. However, whole genome transcriptome analyses examining the plant response to insect herbivory demonstrate that plants alter transcription in multiple pathways, including primary metabolism, signaling, cell wall modification, and oxidative stress (Schwachtje and Baldwin, 2008; Kerchev et al., 2012). In the face of such a widespread response, we would have been surprised to find that JA induction alone was sufficient to effect bacterial persistence. Thus, although induction of the JA response (by leafhopper infestation or MeJA treatment) provides a benefit to *S.* Typhimurium, we hypothesize that additional mechanisms are likely present that also affect bacterial survival. When leafhoppers feed on leaves, they must excrete excess carbohydrates to counterbalance the difference in osmolarity that they encounter in the phloem (Wilkinson et al., 1997). These excretions are termed "honeydew," and we have data showing that *S. enterica* can use honeydew as a nutrient source (J. Dundore-Arias, personal communication). Release of nutrients from the leaf during leafhopper feeding or excretion of honeydew by the insects could provide a nutrient source for *S. enterica* in the otherwise nutrient poor environment on the leaf surface. Utilization of insect byproducts has been described for other human enteric pathogens as regurgitation by house flies provides nutrients for pathogenic *Escherichia coli* and leads to bacterial replication on spinach leaves (Wasala et al., 2013). Future analysis of honeydew as a nutrient source for *S. enterica* and quantification of leafhopper excretions on the leaf surface will elucidate the impact of nutrient availability on *S. enterica* survival.

The data presented in this study demonstrate that the presence of leafhoppers alter hormone production (**Figure 3**). Alone, *S.* Typhimurium induces the SA response; tomato plants that are inoculated with *S.* Typhimurium and infested with leafhoppers activate both the JA and SA pathways. Similarly, MeJA induction of the JA response in conjunction with *S.* Typhimurium inoculation leads to activation of both the JA and SA pathways (**Figures 4–6**). In many cases, activation of one pathway suppressors activation of the other pathway (Pena-Cortes et al., 1993; van Wees et al., 2000; Spoel, 2003; Van der Does et al., 2013; Wei et al., 2014). In nature, plants are often invaded by multiple pests at the same time. The antagonism between the SA and JA pathways allows the plant to prioritize one pathway over the other to provide the most effective response to its attackers (Kunkel and Brooks, 2002; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008). Despite much work describing the antagonistic nature of the SA and JA pathways, it is now accepted that the story is more complicated than the simple model where one pathway is activated while the other pathway is inhibited. For example, evidence from *A. thaliana* shows that treatment with low concentrations of the two hormones transcriptionally activates both defense pathways while higher concentrations produce the more typical antagonistic effect (Mur et al., 2006). Thus, the relationship between the SA and JA defense responses is dependent on the relative concentration of each hormone and also may be specific to the organism that initially stimulates the response. The data from this study suggest that simultaneous introduction of a phytophagous insect and a bacterium leads to a synergy between the SA and JA pathways. One limitation of this work is the localized nature of the sampling technique. In the leafhopper experiment (**Figures 2**, **3**), leaf samples for bacterial population and RNA analyses were taken from the localized insect infestation site within clip cages. Although the plant immune response initially responds in a localized manner, the plant prepares for further pathogen invasion in a systemic fashion (for review, see Henry et al., 2013). Immune activation leads to induction of the systemic acquired resistance in distal leaves and often includes accumulation of plant hormones like SA. To expand on our understanding, future studies examining the systemic response to these experimental conditions could reveal more information regarding the potential synergistic effects of the SA and JA response in this system.

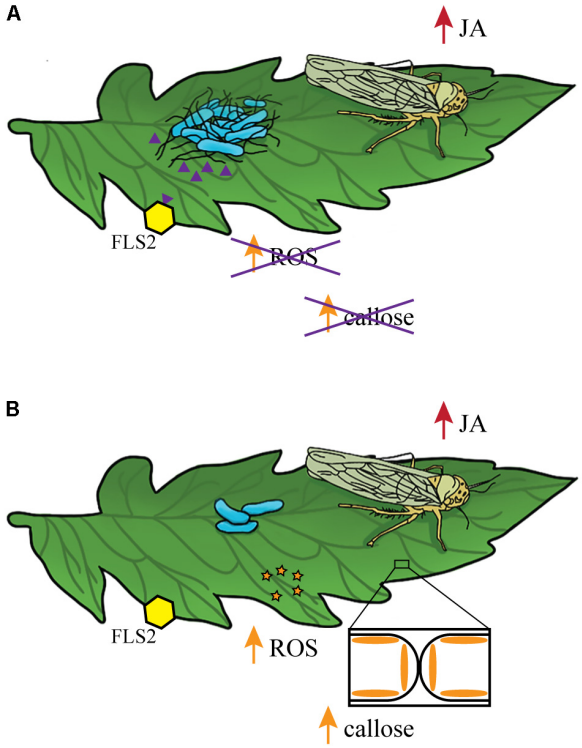


**FIGURE 7 |** Model describing the link between the bacterial flagellum and the positive effects of leafhopper infestation on *S.* Typhimurium persistence. **(A)** Leafhopper infestation leads to increased populations of flagellated *S.* Typhimurium (blue) on the leaf surface. Leafhopper-induction of the JA response (red) in combination with recognition of extracellular *S.* Typhimurium flg22 peptides (purple triangles) by FLS2 (yellow hexagon) inhibit ROS activation and callose deposition. **(B)** Although leafhoppers induce the JA response (red), non-flagellated *S.* Typhimurium populations (blue) decline due to activation of ROS activation (orange stars) and callose deposition.

To further define the mechanisms that enhance bacterial survival, we examined the importance of the flagellum, with known roles in motility and immune recognition, in bacterial persistence on tomato leaves. For these experiments, we characterized two mutants with defects in structural or functional aspects of flagellar-based motility. The non-flagellated *fliF*::Kan mutant is not motile and does not secrete the immunostimulatory flagellin subunit while the *motA*::Kan mutant produces an intact flagellum but is not motile due to a non-functional flagellar motor (Block and Berg, 1984; Homma et al., 1987a,b; Blair and Berg, 1990). Although non-motile, the *motA*::Kan mutant could use the non-functional flagellum for attachment, or the presence of extracellular flagellin monomers in the mutant strain could stimulate an immune response. The difference in the two mutant strains provides a distinction between a requirement for motility (*fliF*::Kan) and a requirement for the presence of the flagellum for attachment or stimulation of an immune response (*motA*::Kan). After pretreatment with MeJA to induce the JA response, the *motA*::Kan mutant displayed an increase in bacterial persistence (**Figure 5B**) while the *fliF*::Kan mutant populations were not significantly different on plants treated with MeJA or water (**Figure 4B**). The *motA*::Kan results indicate that motility is not required for *S.* Typhimurium to benefit from the MeJA-induced JA response as both the wildtype and non-motile *motA*::Kan strain have enhanced persistence after MeJA treatment. The *fliF*::Kan data suggest that the presence of the flagellum is needed to enhance bacterial persistence, perhaps through a mechanism involving attachment or recognition by the immune system. We predict that a connection to the immune response is more likely due to evidence that *S.* Typhimurium can attach to plant leaves in a flagellum-independent manner (Berger et al., 2009). Previous work demonstrated that, although a *S. enterica* serovar Senftenberg flagellum mutant has reduced attachment to basil leaves, *S.* Typhimurium (the serovar used in this study) can attach to leaves even in the absence of the flagellum (Berger et al., 2009). Thus, we hypothesize that the effects on immune recognition are a more likely mechanism to explain the role of flagella in this system.

The best characterized bacterial PAMP is the flagellin peptide flg22. This conserved N-terminal region of the flagellin protein is present in both *S.* Typhimurium flagellin proteins FliC and FljB (Garcia et al., 2014) and is recognized by the PRR flagellin-sensing protein 2 (FLS2). FLS2 recognition activates a cascade of responses categorized as pathogen-triggered immunity (PTI) (for review, see Melotto et al., 2014). A second PRR FLS3 was recently identified in tomato that recognizes a distinct region of flagellin called flgII-28 (Hind et al., 2016). Previous work in *A. thaliana* demonstrated that activation of the JA and SA pathways can influence PTI (Yi et al., 2014). Treatment with the flg22 peptide led to SA accumulation, increased ROS production, and increased callose deposition (Meng et al., 2013; Garcia et al., 2014). However, pretreatment with hormone analogs such as the JA mimics coronatine (COR) and MeJA led to a reduction in flg22-induced activation of ROS and callose accumulation (Yi et al., 2014). These results indicate that when the JA response is activated, flg22-induced responses are reduced. However, pretreatment with hormone analogs in the absence of flg22 has no effect on ROS production or callose deposition (Yi et al., 2014). The interaction between the JA response and flg22-induced changes in immunity could explain why wildtype *S.* Typhimurium and the *motA*::Kan mutant benefit from MeJA induction of the JA pathway while the *fliF*::Kan mutant does not (for model, see **Figure 7**). We chose not to include the SA response in our model because, although it is also activated under these conditions, there were no significant differences in *pr1a1* activation between wildtype and the *fliF*::Kan mutant (**Figure 6**), indicating that the SA pathway is not likely to be responsible for the population differences observed for these strains. When inoculated alone, *S. enterica* flg22 stimulates the immune response which rapidly produces increased levels of ROS and callose deposition (Garcia et al., 2014). Pretreatment with MeJA or infestation with leafhoppers in conjunction with *S.* Typhimurium inoculation activates the JA response (**Figures 3–5**). Thus, we predict that inoculation with wildtype or the *motA* mutant, which both produce the flg22 peptide, would lead to a reduction in ROS levels and callose deposition (**Figure 7**). Inoculation with the *fliF* mutant, which does not

make extracellular flagellin, would result in normal levels of ROS activation and callose deposition in response to immune activation (**Figure 7**). These differential immune responses could explain the differences in bacterial persistence that were observed in the MeJA experiments between the wildtype, *motA*::Kan mutant, and *fliF*::Kan mutant (**Figures 4**, **5**). Without flagella, the *fliF*::Kan mutant must still contend with ROS activation and callose deposition, and induction of the JA pathway is irrelevant to bacterial persistence in the absence of the flagellin protein. Future experiments looking at these downstream outputs of JA- and flg22-induced activation of the immune response could further elucidate the mechanism that leads to enhancement of *S. enterica* persistence.

In summary, this work provides several important contributions to the study of human enteric pathogens in plant hosts. We present another example of a synergistic response between the SA and JA immune pathways that adds to a growing body of work that challenges the dogma of a strictly antagonistic interaction between these two pathways. Further, we define a potential mechanism by which phytophagous insects enhance human enteric pathogen survival on leaves (**Figure 7**). Our data suggest that leafhopper-induced activation of the JA response in conjunction with plant responses to flg22 benefit *S. enterica* survival while also providing evidence that suggests additional mechanisms may remain to be discovered. We have advanced the fundamental knowledge describing how the phyllosphere community is influenced by insect herbivory and filled in gaps in understanding the biology of tri-tropic interactions between an enteric human pathogen, a phytophagous insect, and a plant host.

## AUTHOR CONTRIBUTIONS

KC, RG, and JB conceived the study, analyzed the data, and wrote the manuscript. KC conducted the assays. All authors have read and approved the final manuscript.

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REVIEW
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# The Hurdle Approach–A Holistic Concept for Controlling Food Safety Risks Associated With Pathogenic Bacterial Contamination of Leafy Green Vegetables. A Review

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Consumers appreciate leafy green vegetables such as baby leaves for their convenience and wholesomeness and for adding a variety of tastes and colors to their plate. In Western cuisine, leafy green vegetables are usually eaten fresh and raw, with no step in the long chain from seed to consumption where potentially harmful microorganisms could be completely eliminated, e.g., through heating. A concerning trend in recent years is disease outbreaks caused by various leafy vegetable crops and one of the most important foodborne pathogens in this context is Shiga toxin-producing *Escherichia coli* (STEC). Other pathogens such as *Salmonella, Shigella, Yersinia enterocolitica* and *Listeria monocytogenes* should also be considered in disease risk analysis, as they have been implicated in outbreaks associated with leafy greens. These pathogens may enter the horticultural value network during primary production in field or greenhouse via irrigation, at harvest, during processing and distribution or in the home kitchen/restaurant. The hurdle approach involves combining several mitigating approaches, each of which is insufficient on its own, to control or even eliminate pathogens in food products. Since the food chain system for leafy green vegetables contains no absolute kill step for pathogens, use of hurdles at critical points could enable control of pathogens that pose a human health risk. Hurdles should be combined so as to decrease the risk due to pathogenic microbes and also to improve microbial stability, shelf-life, nutritional properties and sensory quality of leafy vegetables. The hurdle toolbox includes different options, such as physical, physiochemical and microbial hurdles. The goal for leafy green vegetables is multi-target preservation through intelligently applied hurdles. This review describes hurdles that could be used for leafy green vegetables and their biological basis, and identifies prospective hurdles that need attention in future research.

**Keywords: *Escherichia coli*, foodborne disease, food system, *listeria*, primary production processing, *Salmonella*, spoilage**

## INTRODUCTION

Vegetables are an essential part of the human diet and an important source of minerals and vitamins. They are often eaten raw or only minimally processed (Goodburn and Wallace, 2013), particularly leafy green vegetables (LGV) such as baby leaf spinach and Swiss chard, rocket and different types of lettuce. Consumption of fresh produce in general has increased over the past two decades (Olaimat and Holley, 2012). At the same time, the number of foodborne disease outbreaks associated with consumption of LGV has increased (Castro-Ibáñez et al., 2017). The chief bacterial pathogens associated with these outbreaks are Shigatoxin-producing *Escherichia coli* (STEC), *Salmonella* spp., *Yersinia* spp. and *Listeria monocytogenes* (**Tables 1**, **2**). In addition to being vehicles of human pathogens, ready-to-eat (RTE) salads may also be vehicles for bacteria with genes coding for resistance to specific antibiotics (Campos et al., 2013).

Ready-to-eat salads are a convenient way to ensure intake of vegetables, but consumers need to be confident that the products are safe to eat. However, analysis of RTE salads in Finland has shown that the bacterial quality and safety of packaged fresh LGV is often poor (Nousiainen et al., 2016). It is unclear whether production, processing or distribution practices (or all of these) are responsible for RTE salads of substandard quality, but there is clearly a need for improvements regarding suppression of pathogenic microbes within the entire horticultural value network for LGV, from farm to fork.

Ensuring the microbial safety of fresh LGV presents a unique challenge because the products are consumed raw, with no kill step of pathogens (e.g., heating) at any point in the chain to prevent transmission (Gil et al., 2015). Attempts has been done with aqueous ozone, that could potentially lead to a log 2 reduction of *E. coli,* without detrimental effects on the chemical characteristics of the vegetables (Karaca and Velioglu, 2014). In some countries disinfectant wash, e.g., a chlorine solution, is an option, but it is unclear if this is sufficient to guarantee the food safety due to the risk of internalized bacteria that cannot be reached. There are also indications that the bacteria can adapt to the sanitizer stress with increased resistance to hydrogen peroxide and calcium hypochlorite (Kyle et al., 2010) in combination with increased expression of virulence determinants—potentially increasing the risks. Furthermore, standard commercial washing and distribution conditions may be insufficient to reliably control human pathogens on fresh produce (Hutchison et al., 2017). Pathogens can be adept at adhering to leaf surfaces and potentially penetrating into internal leaf structures, which limits the usefulness of chemical sanitation methods in preventing pathogen transmission via contaminated produce (Lynch et al., 2009).

Critical steps in the value network for LGV are: (1) Primary production, (2) harvest, (3) washing and processing, (4) packaging, and (5) handling, distribution, display, and retail. It is vital to take account of all these steps when considering microbial safety. For example, in an *E. coli* outbreak in Sweden in 2005 with 135 cases, production conditions were implicated in the safety of the final produce because cattle shedding STEC at a farm upstream from the irrigation point resulted in STEC-contaminated lettuce (Söderström et al., 2008). Irrespective of contamination source, there are numerous abiotic and biotic variables that influence conditions on the surface of leafy greens (**Figure 1**). This in turn modulates continued growth and survival of introduced pathogenic microbes (Tomás-Callejas et al., 2011). For example, *E. coli* and *Salmonella* achieve high numbers on young leaves, suggesting that leaf age affects pre-harvest and post-harvest colonization by pathogens (Brandl and Amundson, 2008).

Overall, microbial hazards are significant for fresh produce. Therefore, ways to reduce sources of contamination and a deeper understanding of pathogen survival and growth on fresh produce are required to reduce the risk to human health and the associated economic consequences (Alegbeleye et al., 2018). As there is no single solution for ensuring safety of LGV, the hurdle approach appears to be an appropriate treatment system for this market section of produce. In the next sections of this review, we provide essential background by describing disease outbreaks and then define the hurdle concept. In terms of scope, our review assesses abiotic and biotic parameters relevant for bacterial pathogens on leafy greens, in order to understand the biological underpinnings of hurdles, and summarizes existing hurdles. We also consider potential hurdles and the research needed to develop these.

## NATURE OF RECORDED DISEASE OUTBREAKS

EFSA (2013) ranked combinations of pathogens and foodstuffs of non-animal origin including fruit, vegetables including leafy green, salads, seeds, nuts, cereals, herbs, spices, fungi, and algae (EFSA, 2013). The food safety concerns are a consequence of that many foods of non-animal origin are consumed as ready-to-eat foods in which the constituents are raw or minimally processed (e.g., fresh-cut and prepacked).

EFSA noted that food of non-animal origin such as vegetables were implicated in 10% of the reported outbreaks during 2007–2011. However, those outbreaks were larger and more serious as they involved 26% of the cases, 35% of the hospitalizations, and 46% of the deaths. The huge VTEC O104 outbreak linked to consumption of contaminated fenugreek sprouted seeds in Germany 2011 as well as other European countries influenced these numbers (Beutin and Martin, 2012). However, if excluding this outbreak, still 18% of the cases were linked to consumption of foods of non-food origin.

The risk ranking was done based on following criteria: (a) Strength of association between consumption of the foodstuff and disease associated with the specific pathogen; (b) Incidence of illness and burden of the disease; (c) The dose response curve—high rank if the curve indicated that low doses could cause infection and disease; (d) Amount consumed of the vegetable; (e) Prevalence of contamination found on the vegetable, and (f) Growth potential of the pathogen during the shelf life.

Subsequently, the risk ranking identified *Salmonella* in leafy greens eaten raw, as the main concern. Moreover, the combinations of *Salmonella* spp. and bulb or stem vegetables;

**TABLE 1 |** Examples of foodborne disease outbreaks linked to leafy vegetables from 2000 onwards, starting with the most recent.

| Year | Country | Product/crop | Pathogen | Isolation from implicated food | Cases (deaths) | References |
|---|---|---|---|---|---|---|
| 2016 | U.K. | Salad mix | *E. coli* O157 | No | 161 (2) | Public Health England (PHE), 2016 |
| 2015/2016 | U.S. | Ready-to-eat salad mix | *L. monocytogenes* | Yes | 19 (1) | CDC, 2016; Self et al., 2016 |
| 2015 | U.K. | Pre-packed salad | *E. coli* O157 | No | 38 | Public Health England (PHE), 2015 |
| 2014 | Norway | Salad mix (probably radicchio rosso as it has a longer shelf-life than other ingredients in salad mix) | *Y. enterocolitica* O:9 | No | 133 | MacDonald et al., 2016 |
| 2013/2014 | Norway | RTE salad mix (imported rocket, baby spinach and red rhubarb, washed and bagged in Norway) | *Salmonella* Coeln (*Salmonella enterica* spp. *enterica*) | No | 26 | Vestrheim et al., 2016 |
| 2013 | U.S. | Romaine lettuce (from Mexico) | *Cyclospora* | No | 631 | Buss et al., 2016a,b |
| 2013 | Sweden | Mixed green salad served at restaurant | *E. coli* O157 | No | 19 | Edelstein et al., 2014 |
| 2012/2013 | Canada | Lettuce served at fast food chains | *E. coli* O157 | No | 31 | Tataryn et al., 2014 |
| 2012 | Finland | Frisée salad from Netherlands | *Cryptosporidium parvum* | No | >250 | Åberg et al., 2015 |
| 2012 | U.K. | Pre-cut mixed salad (including leaves from growers in the UK, Spain, Italy and France) | *Cryptosporidium parvum* | No | >300 | McKerr et al., 2015 |
| 2012 | U.S. | Bagged salad (romaine, iceberg lettuce, cabbage, carrots) | *E. coli* O157 | No | 17 (2) | Marder et al., 2014 |
| 2012 | U.S. | Pre-packed organic spinach and salad mix | *E. coli* O157 | Yes | 33 | CDC, 2012b |
| 2011 | Norway | Salad mix containing radicchio rosso | *Y. enterocolitica* O:9 | No | 21 | MacDonald et al., 2011 |
| 2011 | U.S. | Romaine lettuce | *E. coli* O157 | No | 58 | CDC, 2012a; Slayton et al., 2013 |
| 2010 | U.S. | Romaine lettuce (shredded) | *E. coli* O145 | Yes | 27 | CDC, 2010; Taylor et al., 2013 |
| 2010 | Denmark | Lollo bionda lettuce (from France) | Entero-toxigenic *Escherichia coli* | Yes | 260 | Ethelberg et al., 2010 |
| 2008 | Finland | Ready-to-eat iceberg lettuce imported from Central Europe | *Salmonella* Newport/Reading | No | 107 (2) | Lienemann et al., 2011 |
| 2007 | Sweden | Baby spinach (imported) | *Salmonella.* Paratyphi B variant Java (*Salmonella* Java) | No | 172[a] | Denny et al., 2007 |
| 2006 | U.S. | Spinach | *E. coli* O157 | Yes | 191 (5) | CDC, 2006; Grant et al., 2008 |
| 2005 | Sweden | Iceberg lettuce | *E. coli* O157 | No | 135 | Söderström et al., 2008 |
| 2005 | Finland | Iceberg lettuce (from Spain) | *Salmonella Typhimurium* DT 104B | Yes | 60 | Takkinen et al., 2005 |
| 2005 | U.K. | Iceberg lettuce | *Salmonella* Typhimurium DT104 | No | 96 | HPA, 2005a,b |
| 2004 | U.K. | Lettuce | *Salmonella* Newport | No | >360 | Gillespie, 2004; HPA, 2004; Irvine et al., 2009 |
| 2004 | Denmark, Norway, Sweden | Rocket (from Italy) | *Salmonella* Thompson | Yes | 100 | Nygård et al., 2008 |
| 2003 | U.K. | Iceberg lettuce (from Spain) | *Salmonella* Braenderup | Yes | 29 | Gajraj et al., 2012 |
| 2000 | U.K. | Lettuce | *Salmonella* Typhimurium DT 104 | No | 361 | Horby et al., 2003 |
| 2000 | Iceland, U.K., Netherlands, Germany | Lettuce | *Salmonella* Typhimurium DT204b | No | 392 | Crook et al., 2003 |

[a]*Cases also in UK and Denmark.*

tomatoes; and melons was also a concern. The combination of pathogenic *Escherichia coli* and fresh legumes or grains were also suggested posing a risk.

A total of 29 outbreaks that occurred world-wide between 2000 and 2016 are listed in **Table 1**. Bacteria were responsible for most of these outbreaks, with STEC and *Salmonella* being

**TABLE 2 |** Summary of characteristics of microorganisms of most concern on leafy greens from a food safety perspective.

| Microorganism | Size (μm) | | Motility | pH range | | | Temperature range (°C) | | | Metabolism | Energy source | Environment | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diameter | Length | | Min. | Optimum | Max. | Min. | Optimum | Max. | | | | |
| STEC | 1.1–1.5 | 2.0–6.0 | Peritrichous flagella or nonmotile | 3.5 | | 9.0 | 15 | 21–37 | 45 | Facultative anaerobic | Glucose and other carbohydrates | Primary reservoir is the bovine intestinal tract | Park et al., 1999; Kaper et al., 2004; Brenner, 2005 |
| *Salmonella* | 0.7–1.5 | 2.0–5.0 | Peritrichous flagella | 4.0 | 6.6–8.2 | 9.0 | 5.3 | 37 | 45 | Facultative anaerobic | Amino acids, nitrate, nitrite, ammonia | Warm- and cold-blooded animals, humans, eggs, milk and dairy products | Matches and Liston, 1968; Page and Solberg, 1980; Brenner, 2005; Jay et al., 2005 |
| *Yersinia* | 0.5–0.8 | 1.0–3.0 | Peritrichous flagella; motile at temperatures below 30°C. *Y. pestis* always nonmotile | 4.0 | 7.0–8.0 | 10.0 | −2 | 28–29 | 45 | Facultative anaerobic | Sucrose (cannot utilize rhamnose) | Domestic and wild mammals and birds feces, water, vacuum-packed meats, seafood, vegetables, milk, pigs (most prominent reservoir) | Bottone and Mollaret, 1977; Hanna et al., 1977; Stern et al., 1980; Brenner, 2005; Jay et al., 2005 |
| *Listeria* | 0.4–0.5 | 1.0–2.0 | Peritrichous flagella, tumbling motility | 4.3 | 6.0–8.0 | 9.4 | >0 | 30–37 | 45 | Facultative anaerobic | Require carbohydrate as primary energy source, glucose is preferred source | Soil, vegetation, meat (fresh and frozen), water, poultry, and cattle | Petran and Zottola, 1989; Pine et al., 1989; George et al., 1996; te Giffel and Zwietering, 1999; Jay et al., 2005; De Vos et al., 2009 |
| *Shigella* | 1.0–3.0 | 0.7–1.0 | Nonmotile | 5.0 | 6.0–8.0 | 9.0 | 10 | 37 | 48 | Facultative anaerobic | Glucose and other carbohydrates | Intestinal tract of humans and primates | Small et al., 1994; Brenner, 2005; Jay et al., 2005 |
| *Cryptosporidium* | 4.0–6.0 | | Gliding motility | 2.0 | | 10.0 | 4 | | 15 (they can survive at higher temperatures, but oocyst infectivity is inactivated at higher temperatures) | Obligate intracellular coccidian parasite | Amylopectin is the energy reserve needed for excystation and invasion of host cells | Surface waters. Sporulated oocysts are shed in the feces of infected hosts. By contamination of the environment, food or water, oocysts can be ingested by other suitable hosts | Vetterling and Doran, 1969; Fayer and Ungar, 1986; Rose et al., 1997; Brush et al., 1998; Smith and Rose, 1998; Jay et al., 2005; King et al., 2005; Wetzel et al., 2005 |

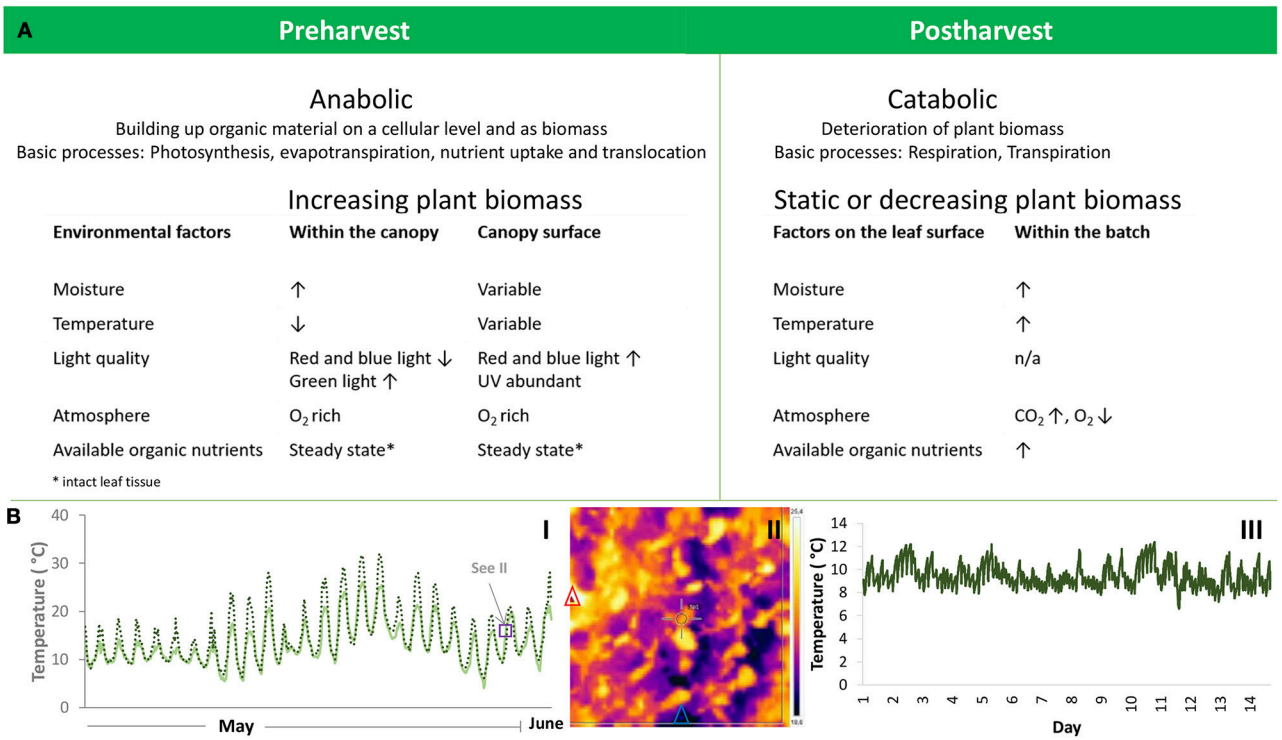


**FIGURE 1 |** Environmental conditions prevailing during pre- and post-harvest of leafy vegetables and affecting the fate of phyllosphere organisms. **(A)** Change of the plant matrix from pre- to post-harvest and related alterations in the physio-chemical phyllosphere environment. **(B)** Temperature as an example of fluctuations in the phyllosphere environment during pre-harvest (I: field environment; II canopy surface) and post-harvest (III: household refrigerator). I: Diurnal changes in atmospheric temperature (solid line) and crop stand temperature near the soil surface (broken line) monitored in southern Sweden during 3 weeks in May and June 2014 in a baby leaf spinach crop. Atmospheric temperature was recorded once per hour and crop stand temperature every 30 min. The purple box indicates the time window for assessment of leaf temperature displayed in II. II: Leaf surface temperature of field-grown baby leaf spinach. The image was taken with an infrared camera. The red and blue triangles indicate the sites with the highest and lowest temperatures in the section. III: Temperature fluctuations in a household refrigerator where temperature was recorded every 5 min during a 14-day period. (Pictures and original data in the figures have previously not been published. Photo by Beatrix Alsanius).

the most commonly implicated infectious agents. The STEC outbreaks were more commonly reported in the U.S. and Canada, while the majority of the *Salmonella* outbreaks were reported in European countries (**Table 1**). Almost half of all cases were associated with consuming LGV outside the home, e.g., in restaurants, fast food outlets or school cafeterias. In general, vegetables are more frequently consumed by women (Takkinen et al., 2005; CDC, 2006, 2012b; Söderström et al., 2008; MacDonald et al., 2011; Marder et al., 2014) and this is reflected in the majority of cases reported being female (**Table 1**). Between 2000 and 2008, the crops associated with listed outbreaks were unspecified "lettuce," iceberg lettuce, rocket and (baby) spinach, while in more recent epidemics salad mixes ("RTE salad mix," "pre-cut mixed salad," "bagged salad," "pre-packed salad") and romaine lettuce were implicated (**Table 1**).

Considering the ephemeral nature of leafy vegetables, which means that they need to be eaten quickly or disposed of, in conjunction with the fact that the incubation period of enteric pathogens can be several days, it is not surprising that the causative pathogen is only detected in suspected produce in less than one-third of cases (**Table 1**). This inability to conclusively link a causal agent to a disease outbreak is a hallmark of many foodborne illnesses. Therefore, most outbreak investigations rely on epidemiological evidence to obtain an indication of the possible contamination source and transmission path. In Finland, restaurants are advised to keep frozen samples of served foods for at least 2 weeks, to enable microbiological investigations after possible outbreaks (Åberg et al., 2015). This system for internal quality control should be encouraged in other countries and the storage period required should be extended to at least 4 weeks, since it typically takes more than 2 weeks from point of infection to confirmation (CDC, 2017). To further complicate source tracking, many LGV products implicated in outbreaks are mixes of varieties with different origins (MacDonald et al., 2011; McKerr et al., 2015). For example, in an outbreak of yersiniosis in Norway in 2011, the salad mix implicated contained four varieties of leafy vegetables originating from 12 suppliers in two countries, while the vegetables were mixed, washed, and packaged in Norway (MacDonald et al., 2011).

Identification of the offending produce enables subsequent trace-back investigations, which can identify potential suspects responsible for the original contamination further upstream within the value network (Horby et al., 2003; Slayton et al., 2013; Public Health England (PHE), 2015; Buss et al., 2016a,b).

However, there have only been two outbreaks for which bacteria matching the outbreak strain have been isolated from environmental samples on, or in close proximity to, suspected farms (CDC, 2006; Grant et al., 2008; Söderström et al., 2008). Sweden and the U.S. experienced EHEC outbreaks in 2005 and 2006, respectively, and in both cases the outbreak strain was isolated from cattle (both countries) and feral swine (U.S. only) at or adjacent to the specific primary production site (CDC, 2006; Grant et al., 2008). Irrigation water contaminated with feces is often suggested to be the culprit for LGV contamination with pathogens (Ethelberg et al., 2010; Åberg et al., 2015). Furthermore, in a Finnish *Cryptosporidium* outbreak in 2012, it was speculated that contaminated water splash from heavy rain events during the growing period caused infection of growing produce in the field (Åberg et al., 2015).

The rapid distribution of foodstuffs via complex distribution networks has the capacity to distribute foodborne infection swiftly and widely. When only a few cases are infected by each pathogen in a country, outbreaks can be very difficult to detect locally or nationally. Therefore, international communication networks such as the Rapid Alert System for Food and Feed (RASFF) and genotyping approaches such as PulseNet are very important in outbreak investigations focusing on leafy vegetables distributed to different countries (Nygård et al., 2008).

## THE HURDLE CONCEPT

Food of animal origin can undergo a thermal process in order to inactivate human pathogens. Leafy green vegetables (LGVs) are eaten raw and cannot be treated the same way due to induced physiological damages and deterioration of the organoleptic properties. The limitation of disinfectants to completely eradicate food borne pathogens on fresh produce could partly be explained by the localization of bacteria in protected niches, cracks and crevices, or that they are even internalized and protected from direct contact (Sela and Manulis-Sasson, 2015). Secondly, biofilm formation on the plant's surface protects the pathogens from antimicrobial agents. The challenge is thus to design a network for safe LGV from field to fork that preserves freshness, prolongs shelf-life and maintains quality. However, there is no single risk mitigation measure that can achieve acceptable food safety for LGV. This lack of efficient kill-step means that the fresh produce industry must rely on preventive measures such as good agricultural practice (GAP) and hazard analysis of critical control points (HACCP) (Sela and Manulis-Sasson, 2015). This approach requires decisions based on evidence and a framework for describing the decision process within the supply chain (Monaghan et al., 2017).

Hurdles, or "the hurdle approach," is a strategy employed in the food chain for meat and animal-based products (Leistner, 2000), but can be adopted as a novel approach for LGV. The hurdle approach has been proposed to some extent before. Some studies have suggested the use of natural agents as hurdles, other the use of bacteriocins, modified athmosphere and a strict management of temperature and storage times (Wadamori et al., 2017). But, regardless of the type of hurdles, the basis of the hurdle concept is a combined approach incorporating two or more practices (or hurdles), each of which may not be sufficient to control foodborne pathogens by itself, but together they can reduce or eliminate microbial hazards (Leistner, 2000). The main aim of the hurdle approach is to achieve microbial safety, product stability, organoleptic and nutritional quality, and economic viability of food production. "Fit for purpose" is key with the approach, as each aim must be achieved without detriment to the other aims.

The magnitude of microbial reduction achieved through use of hurdles will vary depending on circumstances in primary production, *e.g.* whether a large microbial burden (both pathogens and spoilage organisms) is assumed or not. For milk, an example of a foodstuff with high microbial burden, the key hurdles are pasteurization, cool storage and protection against cross-contamination. pasteurization of milk (72°C for 15 s) can reduce the bacterial load by around 7 $\log_{10}$ units for *L. monocytogenes*, *Salmonella Typhimurium*, and pathogenic *E. coli* (Pearce et al., 2012). However, pasteurization is not an option for fresh leafy greens. Instead, the equivalent key element of safe LGV production must be to minimize the pathogen and spoilage burden pre-harvest and to maintain a low burden throughout the whole food value network.

## PATHOGENIC ORGANISMS MOST LIKELY TO BE FOUND ON LGV

Shigatoxigenic *E. coli*, *Salmonella*, *L. monocytogenes*, *Yersinia* spp., *Shigella,* and *Cryptosporidium* are the pathogens of greatest concern as regards LGV and food safety (**Table 2**). Apart from the protozoan *Cryptosporidium*, these are all facultative anaerobic bacteria and the majority are motile. They can grow across a wide pH range, from strongly acidic (pH 2 and 3.5 for *Cryptosporidium* and STEC, respectively) to alkaline (pH 9–10). They have an optimum temperature for growth of between 21 and 37°C, although some are capable of growth at much lower temperatures, close to 0°C (e.g., *Y. enterocolitica* and *L. monocytogenes*) (**Table 2**). Some are widespread in the environment (i.e., *L. monocytogenes*) and most are found in the gut and feces of warm-blooded animals.

## ABIOTIC FACTORS TO BE CONSIDERED IN THE HURDLE APPROACH

### Temperature

Environmental conditions prevailing during pre- and post-harvest of leafy vegetables and affecting the fate of phyllosphere organisms are summarized in **Figure 1**. One of the most influential drivers of plant and microbial activities and growth is temperature. Within the path taken by LGV through the food system, there are three marked phases that can be delineated, namely pre-harvest, harvest, and post-harvest. Each stage displays distinct temperature kinetics due to the physiological status of the plant material and the surrounding ambient temperature conditions.

For a human pathogenic bacterium residing in the phyllosphere, the most critical factor is the temperature on the leaf surface, which is a function of wind speed, air temperature, transpiration rate, and diffusion resistance in plant tissues (Gates, 1968; Larcher, 2003).

There are complex interactions between the parameters influencing leaf temperature. For example, cushion, and rosette plants (e.g., baby spinach, Swiss chard, rocket) form a closed canopy close to the soil surface and are less prone to convection energy losses (cooling by wind), which in turn allows temperatures to rise above air temperature (**Figure 1**). However, this effect only applies to the interior of the canopy, while leaves in the exterior canopy border interfacing with the atmosphere are more exposed to convection losses (Larcher, 2003). Temperature relationships relating to plant architecture have not been studied specifically for head-forming leafy vegetables, such as different types of lettuce.

Horticultural production systems incorporate many management practices, including mulching, row covering and low tunnels, which significantly alter the thermal environment of cropped plants (Krug et al., 2002). For example, temperature fluctuations (amplitude) are greater under black polyethylene film and lower under white polyvinylchloride film (Takakura and Feng, 2002). Apart from the thermal impact, mulch using polymeric films shields the crop from the soil surface and thereby reduces contamination of the foliage through soil splash.

Harvesting is a decisive intervention in the physiological and thermal conditions of leafy vegetables. From a physiological perspective, harvesting involves detachment of the leaf and hence interruption of the water flow continuum. Harvested leaves are subject to respiration, leading to a temperature increase, and to dehydration, especially if the relative humidity of the surrounding air is low. To maintain organoleptic properties, leaf integrity and the bioactive compound content, harvested leaf biomass is generally stored under cool conditions during transport, processing and packaging, distribution, retail and before preparation and consumption. Leafy green vegetables are highly perishable and any signs of decay strongly diminish their appeal to consumers. Moreover, during decay they also lose organic nutrients from the leaf matrix, favoring microbial growth. Harvested leafy vegetables should thus be cooled as soon as possible to prevent water loss and respiration and to deter microbial proliferation (Paull, 1999). Different cooling methods are available, e.g., room cooling, forced air cooling, hydro cooling, vacuum cooling, water-spray vacuum cooling etc. (Thompson et al., 2002). These methods differ in terms of their cooling efficiency, and thus in their effect on the final produce quality, so the method employed should be chosen with respect to target produce, infrastructure and economic considerations at the processing facility. In addition, positioning of harvested trays affects the rate of cooling, which progresses more rapidly in trays placed in the middle of a stack than in trays at the top or bottom of the stack (Rediers et al., 2009).

There is of course a time lapse between harvesting and the onset of adequate cooling temperatures. In this context, seasonality is important. Onset of target cooling temperatures is delayed when harvest takes place at elevated outdoor temperatures, which can ultimately reduce produce quality and increase microbial proliferation (Rediers et al., 2009; Garrido et al., 2015). High outdoor temperatures during harvesting also promote respiration, so pre-cooling using a hydro or vacuum method may be preferable as this increases leaf water content (Garrido et al., 2015). In summary, important hurdles to consider are preferentially harvesting LGV at lower outdoor temperatures and using a pre-cooling method by which target temperature can be reached faster or respiratory water loss can be mitigated.

Post-harvest temperature management is the most critical hurdle for LGV. According to the University of Florida's Institute of Food and Agricultural Sciences (UF-IFAS) recommendations for processing, post-harvest temperature for LGV should not exceed 5°C (Garrido et al., 2004). In reality, however, there are large variations in the ability to maintain this target temperature throughout storage, processing, distribution, and retail of LGV.

Post-harvest temperature management can change in different phases. A comprehensive study by Rediers et al. (2009) found that temperature was well managed during the post-harvest path of endive until distribution to restaurants, whereupon breakdowns in temperature maintenance occurred, with temperature increases of up to 4°C. Considerable temperature fluctuations were also noted during up to 4 days of storage at restaurants (Rediers et al., 2009). In contrast, Zeng et al. (2014) found that low temperatures are often not maintained during transport, retail storage, and even display (Zeng et al., 2014). Transfer points within post-harvest paths seem especially vulnerable to failures to maintain low target temperatures (Koseki and Isobe, 2005).

There is strong consumer awareness that LGV need to be refrigerated (Jacxsens et al., 2015), but measurements of the actual temperature achieved during domestic storage (refrigeration) show that home refrigerators may be unable to maintain isothermal conditions consistently below the target 5°C (**Figure 1**). The type and design of the refrigerator, its placement within the kitchen (close to a heat source), operating conditions, the temperature setting and the number of door openings affect the temperature distribution within refrigerators (Laguerre et al., 2002). Despite the fact that LGV are highly perishable, appropriate domestic storage is not always achieved. A study by Marklinder et al. (2004) found that almost one-third of respondents stored RTE salads in the warmest locations of their refrigerator, where mean storage temperature was 6.2°C and the highest refrigeration temperatures recorded were 11.3–18.2°C (Marklinder et al., 2004). From a food safety perspective, such high refrigeration temperatures should be considered an abuse temperature regime under which many human pathogenic bacteria are capable of growth. Similar data have been reported previously (Carrasco et al., 2007), and in a recent study the risk of waste of fresh-cut iceberg salad (partly due to microbial aspects) increased manifold with higher fridge temperatures (Manzocco et al., 2017).

Few studies have examined the temperature dynamics of perishable produce at the point of display. Thomas et al. (2014) monitored temperature dynamics during 3 days at 24 schools using cooling wells, cooling plates or healthy carts. They found that the temperature of the LGV displayed varied substantially

and that it remained below 5°C only in a "holding cooler." The mean time above 5°C for the other devices they tested followed the order: loose cooling pan>cooling plate>cooling well>display cooler (Thomas et al., 2014).

The fate of foodborne pathogens inoculated onto different horticultural commodities eaten raw and exposed to different temperature regimes has been well analyzed. The majority of these studies have analyzed *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* but the temperature regimes employed differ, although most have been conducted under isothermal conditions. It is important to note that the term "abuse" temperature' used in the literature does not consider one specific threshold, but is arbitrarily assigned in different studies. In general, the literature shows that proliferation of *E. coli* O157:H7 is slowed down at lower temperatures (8°C compared with 12°C), but that this decline can be negated by extended storage time (Luo et al., 2009). This pathogen does not grow, but survives, at 5°C (Luo et al., 2010). Similar results have been reported for *Salmonella* Hadar inoculated on washed and shredded white cabbage, with survival but no growth at 4°C, but growth observed at 12°C and 20 °C (Piagentini et al., 1997; Delbeke et al., 2015).

In a study where *E. coli* O157:H7 gfp+, *L. monocytogenes*, and *Y. enterocolitica* were inoculated onto baby spinach and stored at 8 and 15°C for 7 days, only populations of *Y. enterocolitica* increased significantly and exclusively under temperature abuse (15°C) (Söderqvist et al., 2017). Intactness of LGV has an impact on nutrient availability, and hence proliferation of contaminants. Khalil and Frank (2010) found that the occurrence of *E. coli* O157:H7 was particularly promoted on bruised parsley and bruised spinach at 12°C during 4 days of storage and that growth of *E. coli* O157:H7 on bruised spinach, but not on bruised coriander cilantro, romaine lettuce and parsley, was selectively promoted after 3 days of storage at 8°C (Khalil and Frank, 2010). Interestingly, processing treatment affects the fate of *E. coli* O157:H7 during storage at different temperature regimes (Lopez-Velasco et al., 2010). At low temperature (4°C), *E. coli* O157:H7 is internalized into cut iceberg lettuce tissue more efficiently than at higher temperatures (7, 25, and 37°C) (Takeuchi and Frank, 2000).

In a study that simulated temperature conditions during transport and retail, *E. coli* O157:H7 counts were found to be approximately 3 log colony-forming units (CFU)/g in transport and the population increased by 0.1 to 3.0 log CFU/g during retail storage, but did not increase further during retail display (Zeng et al., 2014). In similar temperature simulations, *L. monocytogenes* substantially increased under retail storage temperatures (by 3.0 log CFU/g) and retail display temperatures (by 1.1 log CFU/g) (Zeng et al., 2014). Considering the interaction between human pathogens and temperature conditions, a hurdle approach for LGV must include strict temperature management to avoid temperatures that can support growth of pathogens. Access to data on the temperature history of LGV batches can be an important complement, so that storage times can be adjusted according to previous temperatures experienced by the produce.

## Irradiation

Light is a critical factor in crop production, due to its impact on plant biology. Light quality (spectral distribution) and quantity (day light integral, light intensity) and diurnal changes (light period/day length, sunfleck and diurnal artificial light modulation) are all important in governing plant physiological and metabolic processes. By extension, parameters that influence vital plant processes will also have an indirect impact on phyllosphere microbiota residing on the leaf surface. This indirect effect is primarily mediated by nutrients leached from the leaf onto the surface, but temperature and humidity are secondary light effects to be considered, as they have a direct impact on bacteria (Alsanius et al., 2017). Interestingly, recent findings demonstrate that light can have a direct influence on the metabolism of phyllosphere bacteria, even those that are nonphototrophic (Gharaie et al., 2017).

Only a few studies have considered the impact of light source, quantity and quality on the epiphytic phyllosphere microbiota. Of these, most consider light quality interactions, while light source and quantity receive less attention. It has been shown that variations in cumulative photosynthetically active radiation (PAR) in a lettuce crop under field conditions cause a shift in the abundance of bacterial families, with e.g., Betaproteobacteria decreasing and Gammaproteobacteria increasing between the highest and lowest light regimes (Truchado et al., 2017). However, it is not clear whether this effect is due to variations in PAR, light quality or other abiotic factors associated with shielding. Light quality studies have predominantly focused on the impact of different UV-bands on epiphytic bacteria. An impact of light source and light quality has been demonstrated in greenhouse experiments, where UV-B irradiation changed the microbial phyllosphere community structure of various crops (Newsham et al., 1997; Jacobs and Sundin, 2001; Kadivar and Stapelton, 2003). However, pigmented phyllosphere bacteria are equipped with UV-B protection strategies, to mitigate DNA damage (Jacobs et al., 2003). Other protection strategies for preventing detrimental UV-A effects are based on quenching reactive oxygen species, e.g., using an avobenzone-like compound that absorbs UV-A found in *Methylobacterium*, a phyllosphere resident (Yoshida et al., 2017). Furthermore, blue light receptor domains have been found in several phyllosphere bacteria and human pathogens, which indicates that the ability to detect blue light may be more common than previously thought (van der Horst et al., 2007; Ondrusch and Kreft, 2011; Wu et al., 2013; Río-Álvarez et al., 2014; Alsanius et al., 2017; Gharaie et al., 2017). Recent results indicate that both light quality and nutritional factors are critical for bacterial nutrient utilization and propagation of phyllosphere bacteria and may also dictate colonization success (Gharaie et al., 2017).

In the context of light microbe interactions in crop stands, it is worth mentioning that the ambient light quality conditions only reflect those to which the top of the crop is exposed. When a covering canopy has developed, this results in light quality stratification as basal leaves are shaded (Alsanius et al., 2017). While managing irradiation to mitigate enteric pathogens may not be feasible in a field setting, it may have some

potential in a greenhouse setting where light conditions are more controlled. However, much work still needs to be done in order to determine whether light manipulation is an achievable hurdle and irradiation currently does not make any practical contribution as a hurdle.

## Water Availability and Moisture/Humidity

Leaf moisture is a function of the dynamics of air and leaf temperature and relative air humidity, and thus also of crop transpiration. Leaf wetness, by contrast, is very difficult to define, because various portions of leaves and canopies are wet and dry at different times (Huber and Gillespie, 1992). Leaf moisture is also dependent on plant morphological properties, such as leaf topography, cuticle morphology and composition, and the density of trichomes. The fate of an bacterium on the leaf surface is dependent on the leaf surface structure in combination with the amount of nutrients and water available (Monier and Lindow, 2005). If bacterial cells arrive on the leaf surface within droplets, such as rain splash or irrigation water droplets, they can move with these droplets across the leaf surface and aggregate at sites where water remains for the longest time during subsequent drying (Monier and Lindow, 2005). The crop surface is exposed to sunlight and desiccation, which leads to shorter survival times of pathogenic microorganisms on crops compared with in water and soil. If pathogenic microorganisms remain viable in the soil, they are able to re-contaminate plants during irrigation and rainfall (Steele and Odumeru, 2004). Although there is evidence that both *S. enterica* and *E. coli* O157 have the ability to colonize some plant species, they usually fail to grow on leaves under dry conditions (Lindow and Brandl, 2003).

One prominent cultural management measure affecting leaf moisture and relative humidity in the crop stand is irrigation. Moreover, irrigation water of inferior hygienic quality (surface water, reclaimed water, non-treated and treated sewage water) is listed as one of the most decisive vehicles for human pathogen transmission to LGV. As leafy vegetables have a shallow root system, they need to be irrigated regularly (e.g., every 3 days under sunny and warm Scandinavian conditions, with an irrigation water volume of 5 mm/event). Bacterial pathogens contained in surface water used for irrigation are generally re-isolated from the surface of the irrigated crop (Islam et al., 2004; Ijabadeniyi et al., 2011). Irrigation water may also mediate internalization of human pathogens into the leaf tissue, using the stomata as points of entry (Kroupitski et al., 2009a). Several measures have been suggested to prevent transmission of human pathogens through irrigation water (Allende and Monaghan, 2015). First, it is important to monitor pathogen and indicator species in water sources. Second, available physical and chemical water treatment methods to remove human pathogens from the irrigation water source should be applied. Third, the irrigation method used should be considered (Allende and Monaghan, 2015). Methods such as subsurface drip irrigation avoid direct contact with the edible parts of the crop, but more knowledge is required on the risks posed by root contamination and internalization of bacteria. Soil splash created by "rain-sized" water droplets can transfer enteric bacteria from soil to leaves (Monaghan and Hutchison, 2012), but drip and overhead sprinkler irrigation do not influence the survival of *E. coli* O157:H7 on the lettuce leaf surface (Moyne et al., 2011). This means that there is a complex web of conditions affecting the microbial quality of irrigation water (Pachepsky et al., 2011; Liu et al., 2013).

## Nutrients

Phyllosphere bacteria, including introduced pathogenic bacteria, must have access to nutrients in order to either sustain or grow populations. There is a lack of published data regarding nutrients encountered on the surface of LGV, but well over 180 plants have been assessed to establish the kinds of nutrients that may be present in the phyllosphere (Tukey, 1970). Therefore, predictions concerning the nutritional environment of LGV surfaces can be made based on generalities established for the wide variety of plants already examined (Morgan and Tukey, 1964; Tukey, 1966, 1970). The types of nutrients that are commonly present on the leaf surface can be categorized into inorganic and several classes of organic molecules (Morgan and Tukey, 1964). Inorganic elements or ions include boron, calcium, copper, iron, sodium, magnesium, nitrogen, phosphorus, silica, strontium, sulfur, and zinc (Tukey and Mecklenburg, 1964; Mecklenburg et al., 1966; Tukey, 1966, 1970).

Organic molecules include most of the known amino acids (Tukey, 1966, 1970; Rodger and Blakeman, 1984), as well as many organic acids, with a preponderance of tricarboxylic acid (TCA) cycle intermediates such as citric, fumaric and succinic acid (Morgan and Tukey, 1964; Tukey, 1966, 1970). Carbohydrates are of especial interest, due to their ability to readily support growth of enteric bacteria such as *E. coli*, and dominant phyllosphere sugars are fructose, glucose and sucrose (Morgan and Tukey, 1964; Tukey, 1966, 1970; Fiala et al., 1990; Dik et al., 1992; Mercier and Lindow, 2000; Leveau and Lindow, 2001; Aruscavage et al., 2010). Phylloplane nutrients may be of endogenous origin (Brown, 1922; Morgan and Tukey, 1964; Tukey and Mecklenburg, 1964; Mecklenburg et al., 1966; Leveau and Lindow, 2001), through passive leaching from within leaves themselves, or of exogenous origin, via deposits such as pollen, insect frass, and aphid honeydew (Rodger and Blakeman, 1984; Dik et al., 1991, 1992; Stadler and Müller, 2000). Two distinct hallmarks of the phyllosphere with respect to nutrients are nutrient scarcity and heterogeneous distribution, which represents a challenge for phyllosphere bacteria (Frossard et al., 1983; Haller and Stolp, 1985; Fiala et al., 1990; Leveau and Lindow, 2001). The heterogeneous distribution of nutrients indicates that nutrients leach in a non-random fashion across the leaf surface, with cracks and natural openings representing sites where greater amounts of nutrients are present (Leveau and Lindow, 2001). There is direct evidence that these phyllosphere nutrients are taken up and utilized by microbial residents and also that they represent an important source supporting microbial activities (Frossard et al., 1983; Rodger and Blakeman, 1984; Leveau and Lindow, 2001). Activity of fungi and bacteria are often restricted on the leaf surface due to nutrient limitations and there is a positive correlation between the amount of available nutrients and microbial growth (Bashi and Fokkema, 1977; Fokkema et al., 1979; Rodger and Blakeman, 1984;

Mercier and Lindow, 2000). Leaching is enhanced by high water content and a moist or wet leaf releases more nutrients into the phyllosphere than a drier counterpart (Tukey, 1966). Wounds and injuries that breach the leaf surface, whether by biological or mechanical means, increase nutrient availability, with a concomitant increase in growth of microbes on the plant surface, including *E. coli* (Tukey and Morgan, 1963; Dingman, 2000; Jablasone et al., 2005; Aruscavage et al., 2008, 2010). Consequently, processing practices that inflict wounds or injuries, such as minimal processing of leafy greens in the form of chopping and shredding, cause a nutritional pulse that increases growth of *E. coli* and other resident microbes (Brandl, 2008). In conclusion, approaches to mitigating the risk of foodborne illnesses associated with produce and produce spoilage should include practices that aim to decrease leaching of nutrients. Considering that leaching is greatest from leaves that are injured, older and/or wet (Morgan and Tukey, 1964; Tukey, 1970; Aruscavage et al., 2010), then appropriate hurdles for enteric bacteria would be to avoid extended post-harvest storage periods and to avoid post- and processing approaches where leaves are either wetted for extended periods or subjected to practices that cause wounding or injuries.

## Oxygen/Package Atmosphere

Once harvested, leaves will continue to respire and if placed in sealed packages they will modify their own atmosphere due to consumption of ambient oxygen ($O_2$), while releasing carbon dioxide ($CO_2$). The aim of modified atmosphere packaging (MAP) is to extend the shelf-life of produce by slowing down respiration of the leaves. This is achieved by altering the gaseous environment, which can be accomplished either by harnessing innate produce respiration (passive MAP) or by adding or removing gases to manipulate the levels of $O_2$ and $CO_2$ (Oliveira et al., 2010). In general, a lower level of $O_2$ (3–6%) and a higher level of $CO_2$ (2–10%) induce a lower respiration rate in produce. Nitrogen may be used as a filler gas, to prevent collapse of the package (Sandhya, 2010). Of the gases used in MAP, $CO_2$ is the only one with demonstrated antimicrobial activity olive (Oliveira et al., 2015). However, this inhibitory effect is not general and depends on many factors related to the microorganism, storage conditions, and product characteristics. Produce for which MAP is used may be vulnerable from a food safety perspective, as the associated extension of shelf-life may allow growth of pathogenic bacteria, while also inhibiting growth of organisms that usually make the consumer aware of spoilage by off-odors (Farber et al., 2003). The $O_2$ and $CO_2$ levels that develop inside the package due to different types of packaging films do not appear to affect survival and growth of E. coli O157:H7 on different types of fresh produce at 5°C, because growth of E. coli O157:H7 is predominantly dependent on temperature and to a lesser extent on atmospheric conditions (Abdul-Raouf et al., 1993; Oliveira et al., 2010; Abadias et al., 2012). Similar findings have been reported for *S. Typhimurium* and *S. choleraesuis* (Oliveira et al., 2010; Lee et al., 2011). The above also holds true for the psychrotrophic pathogen *L. monocytogenes*, which is of particular interest as it can grow at refrigerator temperatures. There is convincing evidence that *L. monocytogenes* populations grow unaffected, or persist, in MAP with high levels of $CO_2$ at between 3 and 14°C (Kallander et al., 1991; Carlin et al., 1996; Jacxsens et al., 1999; Scifo et al., 2009; O'Beirne et al., 2015). Furthermore, high $CO_2$ has been shown to promote growth of *L. innocua*, a bacterium which is often used as a valid model organism instead of the pathogenic *L. monocytogenes* (Geysen et al., 2006; Escalona et al., 2007). Thus, there is a risk that the level of *L. monocytogenes* will exceed the EC limit (100 CFU/g) if it is present even at low initial levels when stored in MAP. However, there are conflicting results, e.g., in one study an *E. coli* O157:H7 population was noticeably reduced in a high-oxygen (70 kPa $O_2$) modified atmosphere (MA) compared with a low-oxygen (5 kPa $O_2$) MA (Lee et al., 2011). It has also been shown that near-ambient air conditions support lower populations of *E. coli* O157:H7 when stored at 4°C than when stored in active MAP conditions with high $CO_2$ (Sharma et al., 2011).

Apart from the proven importance of proper storage temperatures for fresh produce stored in MAP, other biological characteristics are also important. It has been shown that MAP increases the ability of different isolates of *E. coli* to survive gastric acid challenge on lettuce stored at abuse temperatures (≥15°C) (Chua et al., 2008). Storage under near-ambient air atmospheric conditions can potentially promote higher expression levels of *E. coli* O157:H7 virulence factors on lettuce, which could therefore affect the severity of *E. coli* O157:H7 infections associated with leafy greens (Sharma et al., 2011).

To summarize the role of atmosphere, it is likely that pathogens are more influenced by type of vegetable than by type of atmosphere (Jacxsens et al., 1999). If MAP is used to increase shelf-life, it should be applied with other preservation techniques to ensure inhibition of pathogens, as the MAP technique itself does not appear to represent an impediment to enteric bacteria (Scifo et al., 2009).

## pH

Human pathogenic bacteria have a narrow span of pH conditions in which they can survive on the leaf surface (**Table 2**). A pH value below 4.6 will inhibit growth and toxin production of many pathogens, however, many microorganisms, e.g., *Cryptosporidium* and STEC, are capable of growth or survival at pH values below this limit (**Table 2**). Lowering the pH of LGV to prevent growth of pathogenic bacteria may be a problem if it impairs taste due to fermentation (Leistner, 2000). Another issue is that some bacteria can develop resistance against acidity or can increase their virulence when exposed to environmental stress factors, such as osmotic stress, starvation, and suboptimal pH (Kroll and Patchet, 1992; Leistner, 2000). *Listeria monocytogenes* can develop resistance against acidity (pH 3.5) depending on other environmental conditions (Koutsoumanis et al., 2003). It has also been shown that *E. coli* O157:H7 can survive acidic conditions, with survival rates influenced by temperature (Conner and Kotrola, 1995). However, Menz et al. (2011) found that growth and survival of *E. coli* O157:H7 and *S. Typhimurium* in beer was prevented when the pH was lowered to 4.0 (Menz et al., 2011). In a study where *S. enterica* was spot-contaminated on lettuce leaves and lettuce pieces were then submerged in acid solution (pH 3.0), increased acid tolerance of attached bacteria

compared with planktonic cells was observed (Kroupitski et al., 2009b). This means that altering the pH is probably not a viable hurdle in practical use to prevent growth of pathogenic bacteria on leafy vegetables at any step of the food chain.

## Biotic Factors Critical for the Hurdle Approach

In addition to abiotic factors such as temperature, nutrients, water and pH, biotic factors must be taken into consideration when trying to combine different hurdle approaches. In this review, we focus solely on the presence of other bacteria on the leaf surface.

## Microbiome Associated With Leafy Greens

The native microbiota of leaves consists of millions of phylloepiphytes (bacteria of the phyllosphere; Lopez-Velasco et al., 2013). This diverse bacterial community varies due to morphological and chemical differences between plant genera. For example, Hunter et al. (2010) found that on all lettuce (*Lactuca sativa*) accessions they analyzed, the dominant species were from the Pseudomonadaceae and Enterobacteriaceae families, but that *Erwinia* and *Enterobacter* genera differed significantly between the accessions. Jackson et al. (2013) found that the amount of total culturable bacteria on salad vegetables ranged from $8 \times 10^3$ to $5.5 \times$ 108 CFU/g, with the cultured isolates belonging mainly to *Pseudomonas*, *Chryseobacterium*, *Pantoea*, and *Flavobacterium*. Culture-independent analysis of the same samples revealed that Gammaproteobacteria, Betaproteobacteria, and Bacteroidetes were the dominant lineages, including genera hosting plant pathogens such as *Pseudomonas*, *Ralstonia*, *Stenotrophomonas*, *Erwinia* and *Xanthomonas*, and genera hosting human pathogens such as *Serratia*, *Enterobacter*, *Bacillus* and *Staphylococcus*. There was no difference in microbial composition between organically and conventionally grown produce (Jackson et al., 2013).

Leafy green vegetables are significant vectors of human pathogens (**Table 1**). Pathogens that exist as endophytes are often involved in food-borne disease outbreaks of bagged lettuce and spinach, as they are more protected against external procedures such as washing (Uhlig et al., 2017). Although human pathogens are not adapted for growth in the phyllosphere, it has been shown that *E. coli* O157:H7 and *Salmonella* can survive at low levels over extended periods of time on plants in the field (Williams and Marco, 2014). Williams and Marco (2014) characterized bacteria on romaine lettuce grown in the laboratory and under field conditions and found that field-grown plants contained 10- to 100-fold higher bacterial load than laboratory-grown plants. Furthermore, field-grown plants contained significantly higher proportions of Gammaproteobacteria, represented primarily by Enterobacteriaceae and Moraxellaceae, among which the family Enterobacteriaceae in particular includes several well-known pathogens such as *E. coli* and *Salmonella*. In contrast, laboratory-grown plants were enriched with Betaproteobacteria, represented by the Comamonadaceae and Burkholderiaceae families (Williams and Marco, 2014). The relative quantities of *Erwinia*, *Acinetobacter* and *Alkanindiges* bacteria were significantly higher on field grown plants, whereas laboratory-grown plants carried significantly more representatives of *Comamonas*, *Limnobacter* and *Pelomonas* (Williams and Marco, 2014). It has been found that the uptake of *E. coli* O157:H7 can be mediated by the microbe itself. In a study by Solomon and Matthews (2005) in which lettuce plants were irrigated with *E. coli* O157:H7 or FluoSpheres, particles similar in size to bacterial cells but devoid of bacterial surface moieties, appendages or adaptive responses, it was found that both *E. coli* O157:H7 and FluoSpheres were internalized into growing plants and were present within root and leaf stem tissue. These findings suggest transport of spheres from the root up into edible tissue and, because the level of uptake of FluoSpheres and *E. coli* O157:H7 was similar, they indicate that entry of *E. coli* O157:H7 into lettuce plants may be a passive event (Solomon and Matthews, 2005).

## Antagonism and Pathogens on Leafy Greens

Co-existence in bacterial communities is controlled by access to space, nutrient use and availability, production of antimicrobial compounds and other strategies to acquire resources. An invading microorganism, including a human pathogen, must successfully co-exist and compete with an already adapted and established bacterial community in order to establish (Lopez-Velasco et al., 2013). Biodiversity seems to affect this delicate equilibrium and a more diverse community is correlated with reduced levels of *Salmonella* colonization, presumably due to increased presence of antagonistic bacteria (Jackson et al., 2013). Plant microbiome transplantations using freshly transferred and cryopreserved field microbiota have been found to increase bacterial diversity and to result in a bacterial composition similar to field plant microbiota that seems to be stable over time (Williams and Marco, 2014). To examine the effect of an exogenous potential pathogenic organism on the indigenous bacterial communities in the phyllosphere, Williams and Marco (2014) inoculated *E. coli* O157:H7 onto laboratory-grown romaine lettuce plants containing or lacking the field plant microbiota and found that it resulted in significant shifts in the abundance of certain taxa in both groups. Specifically, bacterial species of the genus *Microbacterium* were significantly enriched on *E. coli* O157:H7-containing plants with transplanted phyllosphere microbiota. This genus has previously only been found on field-grown lettuce and has been shown to exhibit antagonistic activity against *E. coli* O157:H7, so it has been investigated as a biocontrol agent against fungal plant pathogens (Barnett et al., 2006; Pereira et al., 2009; Lopez-Velasco et al., 2012).

Lopez-Velasco et al. (2013) found that 15 genera found on spinach could reduce the growth rate of *E. coli* O157:H7 *in vitro*. The majority (83%) of these antagonistic bacteria belonged to the same taxonomic class as *E. coli* (Gammaproteobacteria), and the remainder belonged to Firmicutes (7%), Bacterioidetes (5%), Actinobacteria (2%), and a small proportion of Alphaproteobacteria and Betaproteobacteria. The mechanisms responsible for the antagonistic effect were reported to be acid production or nutrient competition, mainly of carbon sources.

However, the reducing effect was significantly smaller when the antagonists were co-cultured with the pathogen on detached spinach leaves than when grown *in vitro* (Lopez-Velasco et al., 2013). *Escherichia coli* growth-stimulating bacteria belonging to Actinobacteria (33%), Bacterioidetes (33%), Alphaproteobacteria (26%), and Betaproteobacteria (6%) were also found. Research on microbe-human pathogen interactions in the phyllosphere is still in the fledgling stages, but insights gained from microbial control of plant pathogenic organisms indicate that such interactions are extremely intricate and complex. A holistic approach, including plant physiological factors and phenology, is therefore of great importance for successful hurdle development based on antagonistic mechanisms.

## Bacteriophages in Food Safety of Leafy Greens

During the last 10 years the possible use of bacteriophages has received more attention from the European Union food safety authorities as a practical food safety risk mitigation tool. One important question for regulatory agencies is whether the use of bacteriophages are acting as processing aids or food additives. Processing aids would be the case if reducing numbers of pathogens, but not preventing reinfection in the next steps of the food chain, while if considering use of bacteriophages as a food additive the foods treated with bacteriophages should be safe even in the case of recontamination. The European Food Safety Authority (Anonymous, 2009) issued an opinion on the use of bacteriophages and their modes of action. It appears to be not sufficient to conclude whether bacteriophages in general should be seen as a processing aid or food additive. It was recommended whether the use of bacteriophages should be considered a processing aid or food additive ought to be decided on a case by case basis for each food-pathogen-bacteriophage matrix.

Bacteriophages have two principal mechanisms for reducing number of food borne pathogens either by being temperate by causing "lysis of bacteria from within" where the lysis is caused by multiplication of phage within the bacteria i.e., having a temperate cycle or virulent where the phages are causing lysis without replication i.e., "lysis from without." One attractive feature given the host specificity of bacteriophages is the ability to eliminate the targeted pathogens specifically while leaving the microflora unharmed (Moye et al., 2018). This in contrast to decontamination methods such as heat treatment or treatment with chlorine or organic acids that kill microorganisms indiscriminately and consequently with an impact on the quality and shelf life of the foodstuff. Kazi and Annapure (2016) and references therein noted that bacteriophages intended for use in food should be strongly lytic, their host range should cover all epidemiologically important strains of the target microorganism and be stable in the intended environment of use (Kazi and Annapure, 2016). If needed a mixture of phages should be used (phage cocktails) to ensure reduction, or preferably elimination, of relevant pathogens. Ensuring a longer shelf life biopreservation and biosanitation by removal of biofilms are further possible areas where bacteriophages could be useful.

Bacteriophages could be used both at the pre-harvest and harvest stages. For example, cattle might be treated with bacteriophages to reduce the shedding of *E. coli* O157: H7 (Sheng et al., 2006), according to this study the numbers were reduced but not eliminated. Hence, one could use the possibility of phage therapy for cattle grazing upstream in irrigation water catchment areas or grazing nearby–i.e., an indirect hurdle.

For vegetables, Sharma et al. (2009) found that bacteriophages reduced the number of *E coli* on lettuce and cantaloupe after 2 days of spraying with phage cocktail (ECP-100) (Sharma et al., 2009). It has been concluded that bacteriophages could reduce the number of pathogens by at least one log or 90% (Zaczek et al., 2015). So, at least for Enterohaemorhagic *E. coli*, *Salmonella* Sp, and *Listeria monocytogenes*, the use of bacteriophages could be useful as one amongst several hurdles to ensure food safety. The metabolic activity of the bacteria is very important for the efficacy of bacteriophages. For example, one should not expect a high effect if the temperature is low.

# POTENTIAL HURDLES

Minimally processed foods are in increasing consumer demand possibly amplifying the consumer risks (Ohlsson and Bengtsson, 2002). In particular, risk mitigation and preservation options are needed to prolong shelf lives and to improve food safety for the following food groups; (i) minimally processed, convenient foods, (ii) chilled foods with "invisible technology", (iii) healthful foods with less salt and/or fat, (iv) less packaged foods. To achieve these aims Singh and Shalini (2016) suggested combining hurdle technologies with predictive microbiology models) and HACCP to achieve food safety and longer shelf lives (Singh and Shalini, 2016).

Vegetable foods such as leafy greens is one example of such a minimally processed food group. The leafy green food chain does not include a pathogen kill step such as boiling, before consumption. This presents an opportunity for pathogens and spoilage bacteria to multiply resulting in food safety risks if consumed, or shortened shelf lives with consequent increasing volumes of food waste. It has been suggested that hurdles or combinations of different preservation methods and Leistner (2000) suggested there were more than 60 potential hurdles such as temperature, acidity (pH), organic acids, or and competitive microorganisms including bacteriophages that could be considered (Leistner, 2000). The preservation methods may reduce bacterial numbers (bacteriocidic) or inhibit growth (bacteriostatic). For example an uninterrupted cold chain could be an helpful hurdle as bacterial growth are limited, while the bacteria contaminating the food before subject to the cold chain would usually remain throughout, and represent a constant risk.

We believe that the leafy green food chain would benefit by combining the HACCP with predictive microbiology and hurdles (combinations of preservation techniques) as a contextual model for improving food safety. However, any combinations of hurdles and the foreseen impact on pathogens should be validated before implemented on an industrial scale. One reason for this need for validation is that the estimates for pathogen reductions

on leafy green are very context specific where e.g., species of pathogens might influence the impacts of hurdles. For example *E. coli* O157:H7 survives in acidic environments, but better at lower temperatures (Hsin-Yi and Chou, 2001). Consequently, we agree with (McMeekin et al., 2000) that understanding of physiological processes occurring near the growth/no growth interface is crucial quantification of the impact of hurdles and their intelligent application (McMeekin et al., 2000).

As mentioned previously, the value network of LGV does not start with the processing of leaves but already with the seeds. Use of the hurdle approach in LGV production to date has focused mainly on post-harvest measures, even though most of the contamination risks occur pre-harvest, in primary production (**Table 3**). It is worth noting that LGV are exposed during the entire value chain to unpredictable environments and that boundary layers are not removed when eaten raw. Thus LGV and their environmental footprint are ingested, which makes the hurdle approach more challenging than that used for other products, e.g., dairy and meat.

In some cases, more controlled production conditions (soilless production systems) can result in lower initial microbial load. For example, it has been shown that at the end of lettuce shelf-life, soil-grown lettuce can have 1.5 log higher total coliforms than lettuce grown in a soilless system (Selma et al., 2012). In reality, most LGVs are produced in open fields, which means that insights regarding the biology of microbe-microbe and plant-microbe interactions are essential when seeking to determine the route of foodborne pathogen contamination (Critzer and Doyle, 2010) and to develop novel approaches to inhibit or inactivate microbes on LGV.

Possible hurdles for inclusion in the LGV chain could be low temperatures at harvest and along the food chain until consumption (processing, packing, storage, distribution, and pre-consumption), low pH, biopreservation with competitive microflora, aseptic packaging, and washing. The water used for rinsing could be UV-treated and electrolyzed (Rahman et al., 2016) or e.g., chlorine dioxide could be added (Van Haute et al., 2017). By judiciously introducing hurdles such as rinsing and low temperatures (e.g., 0–4 C) at harvest and post-harvest and by using aseptic packaging, a low pathogen and spoilage burden can be maintained and possibly even reduced up to the point of consumption.

Cross-contamination with human enteric pathogens is one of the major issues related to processing of LGV (Allende et al., 2008; Holvoet et al., 2012). Several hurdle options have been explored regarding decontamination of leaves during the washing procedure, with the pathogens commonly considered being *E. coli*, *Salmonella*, and *L. monocytogenes*. These treatments have led to reductions in pathogens, but none of the methods has proven sufficient to eliminate the pathogenic bacterial load completely. A number of studies have investigated the effect on foodborne pathogens of addition of chlorine to the wash water (Behrsing et al., 2000; Beuchat et al., 2004; Gragg and Brashears, 2010; Davidson et al., 2013; Omac et al., 2015; Pezzuto et al., 2016; Guzel et al., 2017). The efficacy of hypochlorite treatment is dependent on the microbial load of the inoculated pathogen and the target organism (Pezzuto et al. (2016). *Listeria monocytogenes* and *L. innocua* (used as a surrogate for *L. monocytogenes*) have been shown to resist treatment even when hypochlorite is administered in high concentrations, but this treatment is still

**TABLE 3 |** Summary of risk steps in the production of leafy green vegetables where contamination of human pathogens could occur and suggested hurdle options for these.

| | Field/production | Harvest | Process/wash | Pack./storage | Distribution | Consumer |
|---|---|---|---|---|---|---|
| Risk steps | Irrigation<br>Soil amendments<br>Plant protection actions<br>Contamination via farm equipment<br>Flooding incidents<br>Heavy rainfall<br>Production location<br>Proximity to animal rearing operation<br>Wild animals<br>Plant damage | Weather conditions at harvest<br>Temperature<br>Machine hygiene<br>Hand harvest hygiene<br>Field containers<br>Irrigation of harvested product to avoid dehydration<br>Cooling of harvested product<br>Plant damage | Wash line hygiene<br>Wash water<br>Employee hygiene<br>Clean containers<br>Storage time before wash<br>Temperature<br>Plant damage<br>Mode of drying the product | Packing line hygiene<br>Storage time before packing<br>Employee hygiene<br>Labeling<br>Type of packaging<br>Temperature<br>Plant damage | Sanitation of vehicles in transport of unpacked product<br>Temperature | Labeling of product (e.g., RTE or not)<br>Food handling<br>Clean utensils<br>Appropriate storage temperature<br>Clean wash water |
| Organisms | *EHEC, *E.coli, Salmonella enterica, Campylobacter, Shigella* spp., *Cyclospora cayatenensis, Chryptosporidium, Yersinia pseudotuberculosis, Listeria monocytogenes* | | | | | |
| Hurdle options | Disinfection of irrigation water<br>Fences for wild animals<br>No organic fertilizers | Cooling of produce | Unbroken temperature chain<br>Disinfection of wash water with sanitizing agents<br>Disinfection of wash lines | Unbroken temperature chain<br>Modified atmosphere<br>Relative humidity<br>Disinfection of pack lines | Unbroken temperature chain | |

*Blue: Risk steps identified by Gorny et al. (2006).*
*Green: List of multiple outbreaks on leafy green vegetables reported in at least three regions of the world, including illness and deaths (FAO WHO, 2008).*

more successful than washing without an additive (Beuchat et al., 2004; Omac et al., 2015; Guzel et al., 2017). Due to their mode of action (they produce inhibitory compounds at low temperature conditions, but do not grow), lactic acid bacteria are considered plausible candidates in LGV processing. Gragg and Brashears (2010) examined the possibility of using lactic acid bacteria alone, and in combination with a chlorine wash, in order to reduce the total bacterial load of spinach leaves. They found that when inoculated at a density of log 6 CFU/g, the chlorine wash reduced *E. coli* O157:H7 by approximately log 1, the lactic acid bacteria reduced *E. coli* by log 1.4 CFU/g and the multi-hurdle treatment reduced the load by log 1.9 CFU/g.

Although chlorinated water has been proven to be the most efficient sanitizer of LGV, several other have been tested, with varying degrees of efficiency being reported. For example, peroxyacetic acid has been used against *E. coli* O157:H7 and *L. monocytogenes* (Baert et al., 2009) and peracetic acid, percitric acid, sodium bicarbonate and vinegar have been used against *Salmonella* and *Listeria* (Pezzuto et al., 2016). Edible plant extract, such as the pulp and juice of lime fruits and oregano, has been used against *Salmonella*, *E. coli* O157:H7 and *Shigella sonnei* (Orue et al., 2013).

Vurma et al. (2009) examined the possibility of implementing a sanitizing step already in the cooling process by combining vacuum cooling with gaseous ozone. This multi-hurdle approach resulted in a reduction of log 2.4 CFU/g (initial density log 7CFU/g), but the process damaged the leaves. With an optimized procedure where no leaf damage occurred, the reduction in *E. coli* O157:H7 was log 1.8 CFU/g, which is comparable to that achieved with a chlorine wash (Vurma et al. (2009).

Irradiation with UV-C has antimicrobial effects and is sometimes suggested for use as a surface sterilizer of vegetables and other foodstuff, but also for disinfection of water. Hagele et al. (2016) examined the possibility of using low-dose UV-C irradiation (1.2 $kJ/m^2$) on leaf surfaces of endives and for decontamination of the processing water. They found that the reduction in total aerobic bacteria was log 0.9 CFU/g using only UV-C treatment and log 2.1 CFU/g if the UV-C treatment was preceded by washing the endive leaves in warm water (45°C). Treatment of the wash water reduced the total aerobic count in the water by log 1.3 CFU/mL (Hagele et al. (2016).

There are multiple risks of contamination of produce by human enteric pathogens pre-harvest. One of the most common sources of such contamination is irrigation water (Brandl, 2006; Jongman and Korsten, 2017). There are ways of treating the irrigation water prior to use that reduce the bacterial load in the water, such as mechanical filtration using a 0.7 μm filter. This filter size is not sufficient to remove microbial cells, however, although it removes larger particles present in the water that could harbor microorganisms. Another possibility is to use advanced oxidation technology (AOT), where UV-light in combination with a titanium dioxide membrane ($TiO_2$) has an oxidative effect on microorganisms, for example *E. coli* and intestinal enterococci. López-Gálvez et al. (2018) treated water used for sprinkler irrigation of baby leaf spinach with chlorine dioxide ($ClO_2$; 1–3 mg/L) in a field setting and found that the treatment reduced the load of culturable *E. coli* by log 0.2–0.3 CFU/100 mL water. However, the chlorine dioxide was suspected to have a bacteriostatic effect on the *E. coli*, which caused cells to change into a viable but not culturable state (VBNC). Other factors that are considered to pose a risk of microbial contamination in the pre-harvest phase include contamination via farm equipment, heavy rainfall and flooding incidents, proximity to animal rearing facilities, poor hygiene of workers in the field and poor hygiene of crates and containers used in harvest (Gorny et al., 2006). Presence of wild animals and instances of plant damage by equipment used in the field or by weather events should also be considered. Plant damage can provide potential entry points for internalization of human enteric pathogens (Brandl, 2008; Hartmann et al., 2017). In summary, plant damage can occur at all stages of the production chain, both in the field and in processing and distribution, and it is important to minimize such damage in order to maintain high quality throughout the food chain.

## CONCLUSIONS

In order to improve food safety in general, supporting data need to be more easily available to assist primary producers in their decision-making process. There is also a need to develop hygiene criteria to aid validation of proposed interventions. No single risk mitigation measure can achieve food safety for leafy green vegetables. Instead, a combination of mitigation measures, or hurdles, aimed at controlling and/or eliminating pathogens could ensure food safety and quality and freshness. Based on a review of existing literature, it can be concluded that one key element of safe leafy green vegetable production is a low pathogen and spoilage burden pre-harvest. It can also be concluded that the food chain for leafy green vegetables must be a managed in a holistic way for efficient maintenance of food safety and quality. All stakeholders in the food system need to acknowledge their involvement in food safety. Among measures to mitigate the risk of foodborne illnesses being transmitted by produce, avoiding processing practices that involve significant injury to leaves is paramount.

Choice of hurdle must depend on the expected pathogen burden of harvested leafy green vegetables and the food safety objectives at the point of consumption. For example, under EU Regulation 2075/2005, the food safety objective, and consequently the end-product food safety microbiological criterion, for *Salmonella* is absence of the microbe in a 25-g sample of ready-to-eat leafy greens at the point of consumption. Therefore in addition to hurdles applied in the harvest and post-harvest stages of the leafy green vegetable value network, hurdles or risk mitigation measures must be adopted in primary production at all growth stages.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# *Salmonella enterica* Growth Conditions Influence Lettuce Leaf Internalization

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Human pathogens on plants (HPOP) have evolved complex interactions with their plant host. Stomatal internalization is one such mode of interaction, where bacteria are attracted to stomata and penetrate into the substomatal cavity by a process mediated by chemotaxis. Internalization enables HPOP to evade the hostile environment of the leaf surface and find a protected, nutrient-rich niche within the leaf. Numerous studies have documented attachment and entry of the foodborne pathogens, *Salmonella enterica* and *Escherichia coli* into stomata. Internalization, however, varies considerably among different pathogens and in different plants, and both bacterial and plant's factors were reported to influence HPOP attachment and internalization. Here we have studied the effect of laboratory growth conditions, on the internalization of *Salmonella enterica* serovar Typhimurium (STm) into lettuce leaf. We have further tested the potential involvement of universal stress-proteins in leaf internalization. We found that STm grown in Luria Bertani broth devoid of NaCl (LBNS), or in diluted LB (0.5×LB) internalized lettuce leaf better (62 ± 5% and 59 ± 7%, respectively) compared to bacteria grown in LB (15 ± 7%). Growth under non-aerated conditions also enhanced STm internalization compared to growth under aerated conditions. Growth temperature of 25 and 37°C did not affect STm internalization, however, growth at 42°C, significantly augmented leaf internalization. Since, the tested growth conditions represent moderate stresses, we further investigated the involvement of five universal-stress genes in STm leaf internalization following growth in LBNS medium. Knockout mutations in *ydaA, yecG, ybdQ*, and *uspAB*, but not in *ynaF*, significantly reduced STm internalization compared to the wild-type (wt) strain, without affecting bacterial attachment and motility. Transduction of the mutations back to the parent strain confirmed the linkage between the mutations and the internalization phenotype. These findings support a specific role of the universal-stress genes in leaf internalization. The present study highlights the complexity of bacterial internalization process and may provide partial explanation for the variable, sometimes-contrasting results reported in the literature regarding stomatal internalization by HPOP. Characterization of the regulatory networks that mediate the involvement of *usp* genes and the tested growth factors in STm internalization should contribute to our understanding of human pathogens-plant interactions.

**Keywords: *Salmonella*, internalization, stomata, attachment, leaf, lettuce, stress, *E. coli***

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## INTRODUCTION

Consumption of fresh vegetables has consistently increased over the last decades, since they are considered an important part of a healthy and low-calorie diet (Tirpanalan et al., 2011). Concomitantly, there is an increase in outbreaks of food-borne illness associated with the consumption of fresh produce (Brandl and Sundin, 2013; Mritunjay and Kumar, 2015). Lettuce is one of the vegetables, which often linked to outbreaks of foodborne diseases. For example, from 1994 to 2011, more than 20 alerts associated with contaminated lettuce were reported through the RASFF portal of the EU (Tirpanalan et al., 2011). Although efforts to develop chemical and technological means to disinfect vegetables are ongoing (Meireles et al., 2016), there is currently no treatment that can effectively kill foodborne pathogens on fresh produce (Goodburn and Wallace, 2013).

Numerous studies have documented the ability of foodborne pathogens, such as *Escherichia coli* and *Salmonella enterica,* to colonize plants and interact with the plant immune system (Schikora et al., 2012; Garcia and Hirt, 2014) and are considered human pathogens on plants (HPOP). Several reports have demonstrated that HPOP may enter the plant tissues through roots or via natural openings on the plant surface, such as stomata, lenticels or sites of biological or physical injury (Itoh et al., 1998; Seo and Frank, 1999; Takeuchi and Frank, 2001; Solomon et al., 2002; Kroupitski et al., 2009; Deering et al., 2012; Martínez-Vaz et al., 2014). Internalization enables HPOP to evade the hostile environment of the soil and the plant surfaces and find a protected, water- and nutrient-rich niche within the plant. Internalization may also protect internalized HPOP against surface sanitation used by the fresh produce industry (Gil et al., 2009; Niemira and Cooke, 2010). Consequently, understanding the factors that affect plant internalization by HPOP is needed toward developing new approaches to enhance the safety of fresh produce.

Attachment and internalization of HPOP are complex processes depending on both bacterial and plant factors. Plant's factors include, plant growing conditions (Ailes et al., 2008; Ge et al., 2012; Lopez-Galvez et al., 2018), development stage (Brandl and Amundson, 2008; Kroupitski et al., 2009; Pu et al., 2009), plant's cultivar (Jablasone et al., 2005; Mitra et al., 2009; Barak et al., 2011; Quilliam et al., 2012), as well as contamination site (Hirneisen et al., 2012). Bacterial factors that affect attachment and internalization include, cell concentration (Pu et al., 2009; Erickson et al., 2010; Ge et al., 2012), bacteria strain (Klerks et al., 2007; Wright et al., 2013; Kljujev et al., 2018) and biofilm formation (Yaron and Römling, 2014).

It has been demonstrated that preadaptation of human pathogens, including *Salmonella enterica*, to mild stresses enhanced their survival under more extreme stresses, as well as under other stress, in a process termed, cross protection (Jenkins et al., 1998; Bunning et al., 1990; Chung et al., 2006; Yuk and Schneider, 2006; Alvarez-Ordóñez et al., 2015). It has been reported that exposure of bacteria to the vegetable environment stimulates a cellular response required to colonize the animal host intestine (Goudeau et al., 2013). More recently, Fornefeld et al. (2017) have demonstrated that pregrowth of *Salmonella* Typhimurium LT2 in lettuce medium, rather than in rich laboratory broth (Luria Bertani, LB) resulted in better survival of the pathogen in soil microcosms. It has been suggested that the medium used to pregrow *Salmonella* can influence its fate in the environment (Fornefeld et al., 2017). Likewise, we hypothesize that pregrowth conditions of laboratory-grown *S.* Typhimurium may also affect bacterial response required for interaction with the plant host, and specifically, affect stomatal internalization using lettuce leaf model system.

## MATERIALS AND METHODS

### Bacterial Strains, Inoculum Preparation, and Fluorescence Labeling

*Salmonella enterica* serovar Typhimurium (STm) SL1344 strains and *E. coli* O157:H7 EDL933 strain were labeled with mCherry-fluorescence protein (mCherry) by electroporating plasmid pKB2690, containing the mCherry gene and ampicillin resistance gene (Sason et al., 2009). Bacterial cultures were kept in Luria-Bertani (LB; 10 g Bacto-peptone, 5 g Yeast Extract, 10 g NaCl) broth containing 25% glycerol at −80°C. For each experiment, bacteria were streaked on LB agar for overnight and fresh colony were re-suspended in 10 ml LB broth and grown with shaking (150 rpm) for 18–20 h at 37°C to generate the inoculum for the internalization assay. In some cases, as indicated, bacteria were grown at 25 and 42°C. Other growth media that were used, included LB broth without NaCl (LBNS), and water-diluted LB broth (0.5×LB). Where indicate, bacteria were grown in LB broth without shaking or on LB agar plates. Overnight liquid cultures were washed twice with sterile saline (0.85% NaCl) by centrifugation (2700 g, 10 min) and re-suspended in saline to a final concentration of about $10^8$ colony-forming units (CFU) per ml.

### Generation of Knockout Mutants in Universal Stress Genes

Site-directed mutagenesis was performed as described by Datsenko and Wanner (2000) using primers specific to each of the mutated genes (**Table 1**). The absence of the intact gene in the mutants and the authenticity of the nearby DNA sequences were confirmed by PCR and sequence analyses using upstream- and downstream-chromosomal derived primers in combination with the respective Km-cassette derived primer. A list of primers used to generate the mutants and to confirm their sequence is present in **Table 1**.

To confirm the linkage between the leaf internalization phenotype and the *usp* mutations, each of the mutation was further transferred to the STm wt strain by P22 HT int-105 transduction, as described (Kroupitski et al., 2013). Transductants were isolated on Km plates and the presence of the specific mutation was confirmed by PCR using primers presented in **Table 1**.

### Reverse Transcription Real Time PCR

STm SL1344 strain was grown in various media and growth conditions, as described above. Three milliliters of an overnight

**TABLE 1 |** Primers used in this study.

| Primer | Forward sequence | Reverse sequence | References/ source |
|---|---|---|---|
| uspAB | TCATGAGCGCAATCAAGCTCACCACCACCAAACCG CATAACGCGCTGGTCTGTAGGCTGGAGCTGCTTCG | GCAGCGGCACAATCAGCATGTCAACGTGAACGGTG TTGATCAGCTGGCGCCATATGAATATCCTCCTTAG | This study |
| uspA-RT | ACGTCAATCTGGGCGATATG | GCTCAGGGTTTCAGTGATAGG | This study |
| yecG | TCTATCGCCCGCCCTGTTCAGGCGAAAGTAAGCCT GATTACTCTCGCTTCTGTAGGCTGGAGCTGCTTCG | TGCCGAGCAGGACGCGCGCGAAAAGAAACTGTGG TTATGGTTGCCGCAAACATATGAATATCCTCCTTAG | This study |
| yecG-RT | AGGATTTACGCGCGGTTATG | ACTCACCTGATGCGATGAAAG | This study |
| ybdQ | GCGCAACAGGATGGCGTCATTCATCTGTTGCATGTA CTGCCTGGGTCCGCGCATATGAATATCCTCCTTAG | ACCACGCTTGAGGCGTTAGACCCCAACAGGTGTGT CGTGATGGACGGATTTGTAGGCTGGAGCTGCTTCG | This study |
| ybdQ-RT | AAACGATGGTGGGACACTTC | GACATCCGCATCCAGTTCTT | This study |
| ynaF | CCCATCGATATTTCAGATTCAGAATTAACTCAACGC GTGATTTCGCATGTTGTAGGCTGGAGCTGCTTCG | GAGCATTCCGCATGACGCACAACGGCTGCGGCGT TGGAACCCAACAGATACATATGAATATCCTCCTTAG | This study |
| ynaF-RT | CTTCACTGGGACTGGCTTATT | GCGGGAAGGTTGAATTTCTTG | This study |
| ydaA | CTGCGATGACAGTTGTAAGGAGACCCTGTATGGCT ATGTATCAAAATATGTGTAGGCTGGAGCTGCTTCG | CAGTTCAACCGGTGTTTGATACTCATCAGGCTTAA TGACTAACAGGTCGCCATATGAATATCCTCCTTAG | This study |
| ydaA-RT | CCGTGTGGATGGTCAAAGAT | TCGTTGAGAGCATTGTGATAGG | This study |
| rpoD-RT | GGCTCGTTTGTCCGATCTTAT | CTTCGTCATCATCCAGGTCTTC | This study |
| k2 | CGGTGCCCTGAATGAACTGC | | Datsenko and Wanner, 2000 |
| kt | | CGGCCACAGTCGATGAATCC | Datsenko and Wanner, 2000 |
| *uspAB*test | GAAACTGGCCCGCTTTTT | TATAGACCAGACGCGGTCTTAGC | This study |
| *yecG*test | GCACAATCTCATATTCTTGCAATC | GTGGCGACGTTCCCTAAG | This study |
| ybdQtest | CGGTTGATGTTTTTGAAATGG | GGCAGGGCGTGTAAGTTTT | This study |
| ynaFtest | AGTATCGTGGAGCAGCACCT | GGCGATGATGATTGATTTGA | This study |
| | | | This study |
| ydaAtest | GCGCTTCCTCTGTTTCATTC | AATAGGGTATTGGCCGGATG | This study |

cultures (about $10^9$ cells) were harvested by centrifugation (2700 g, 10 min) and total RNA was isolated by the RNAqueous® kit (Ca. No. AM1912), according to the manufacturer instructions (Ambion). DNase treatment and cDNA synthesis were performed using Maxima first strand cDNA synthesis kit with dsDNase (Thermo Scientific; Ca No. K16171). Primers for real time PCR of *uspA, yecG, ybdQ, ydaA, ynaF,* and *rpoD* genes were selected from STm SL1344 genomic sequence using the Syntezza-Israel IDT web portal (Integrated DNA Technologies; Leuven, Belgium), through the primer quest tool and are listed in **Table 1**. Fast SYBR® Green Master mix (AB#4385612), 96-wells plates (AB#4346906) and adhesive covers (AB#4311971) were purchased from Applied Biosystems. Real time PCR reactions (10 μl), included 1.72 ng cDNA, 0.2 μM each forward and reverse primers and 50% volume of Fast SYBR® Green Master mix (Applied Biosystems). Transcription of *uspA, yecG, ybdQ, ydaA,* and *ynaF* genes was evaluated by Real time PCR using Applied Biosystems StepOnePlus™ PCR system (Applied Biosystems, Foster City, CA, United States). The PCR conditions comprised 20 s at 95°C followed by 40 cycles at 95°C 3 s and 60°C for 30 s. To verify the specificity of each primer, a melting-curve analysis was included (60–95°C with fluorescence measured every 0.3°C). The threshold cycle ($C_T$) value and relative quantification (RQ) level were determined by StepOne™ Software 2.1 (Applied Biosystem). The house-keeping gene, *rpoD* (Kjeldgaard et al., 2011) served as a reference gene for normalization. Each run included a negative control and a cDNA reaction without reverse transcriptase to rule out DNA contamination. All experiments were performed in triplicates.

## Lettuce Preparation

Fresh whole iceberg lettuce (*Lactuca sativa*) was purchased at a local retail store, and used immediately or kept in a refrigerator for 1 day. Before each experiment, the lettuce temperature was equilibrated at room temperature. The outermost leaves of the lettuce head were aseptically removed and the inner 2–3 leaves were taken for the experiments. The leaves were aseptically cut into ca. 3 × 3 cm pieces using a sterile scalpel, as described previously (Kroupitski et al., 2009).

## Leaf Attachment and Internalization Assays

Interaction of bacteria with leaf pieces were tested, as described before (Kroupitski et al., 2009). Briefly, lettuce pieces were submerged in 30 ml saline in a 50 ml sterile polypropylene tube (Labcon, Petaluma, CA, United States), at one piece per tube. The leaves were pre-conditioned for 20 min under high intensity light (100 μE $m^{-2}$ $s^{-1}$) at 30°C. The saline was then replaced with 10 ml of mCherry-labeled bacterial suspension (in saline) containing about $10^8$ CFU per ml. The tubes were incubated for 2 h under the same conditions. Leaf pieces were rinsed twice for 1 min in excess of saline to remove unattached

bacteria and an internal small piece (10 × 5 mm) was excised and viewed immediately under confocal laser-scanning microscopy (CLSM). Bacterial localization was determined on the leaf surface (attachment) and in deeper layers (internalization) in 30 randomly chosen microscopic fields (magnification ×40). Internalization was quantified as the incidence of internalized bacteria, i.e., percentage of fields (×40) containing ≥1 internal mCherry-tagged bacteria in 30 microscopic fields of the same leaf tissue. STm attachment was scored as the number of microscopic fields containing: 0, 1–10, 10–50, 50–100, and >100 fluorescent cells per 90 fields (×40). Each experiment included three lettuce pieces from different leaves (in total 3 × 30 microscopic fields were examined per experiment), and was repeated independently at least three times at different days, with different lettuce heads.

## Confocal Laser-Scanning Microscopy

mCherry-fluorescent bacteria were visualized by CLSM (Olympus IX81, Tokyo, Japan), using 40 × 0.7 objective. Fluorescence bacteria were visualized using excitation wavelength of 543 nm and a BA560-600 nm emission filter. Chlorophyll autofluorescence was detected using 488 nm excitation wavelength and emission filter BA 660 IF. Transmitted light images were obtained using Nomarski differential interference contrast (DIC).

## Motility Tests

Swarming and Swimming motility assays were performed, as described previously with minor changes (Kjeldgaard et al., 2011). Briefly, freshly grown bacterial cultures were suspended in saline to a final concentration of ca. $10^8$ CFU/ml. For swarming motility assay, bacterial suspensions (1 μl) were inoculated on the center of a swarm plate containing nutrient broth (NB) supplemented with 0.5% glucose and 0.6% agar. For testing swimming motility, a similar inoculum was inoculated with a sterile needle in the center of a well, in 12-wells plates, containing NB-glucose with 0.3% agar. The plates were incubated at 30°C for 18 h in a humid chamber. Swarming motility was assessed by measuring the radius of the swarm from the point of inoculation. Swimming motility was determined visually by the presence of turbidity in the entire volume of the inoculated well. The experiments were repeated independently five times. Nonmotile STm mutants, *fliGHI,* and motA (Kroupitski et al., 2009), served as an internal negative control for both motility assays. Swarming motility was presented as the average radius of the colony from the point of inoculation and standard errors of the means.

## Statistical Methods

Comparison of the incidence of bacterial attachment and internalization was performed by ANOVA using the program Instat, version 3.0.6 (GraphPad Software, Inc., La Jolla, CA, United States). The comparisons of the relative quantity of the amplified mRNA in the reverse transcription real-time PCR experiments were done by unpaired Student's *t*-test using GraphPad software. Statistical significance was set at a one tailed $P \leq 0.05$.

# RESULTS

## Effect of Growth Conditions on *Salmonella* Internalization

Growth of STm in LB broth resulted in the lowest incidence of internalization (15% ± 7), while growth in LBNS, or diluted LB (0.5×LB) resulted in a fourfold higher incidence of internalization, 62% ± 5 and 59% ± 7, respectively (**Figure 1**).

Growth temperature of 25 or 37C yielded similar incidence of *Salmonella* internalization (19% ± 6 and 16% ± 3, respectively), however, growth at 42°C significantly increased bacterial internalization (60% ± 6; **Figure 2A**).

Growth of STm in LB broth at 37°C, without shaking, also enhanced internalization (61% ± 11) compared to growth in shaking culture (18 ± 6%) (**Figure 2B**). On the other hand, bacteria that were grown on LB agar were poorly internalized (6 ± 1%) (**Figure 2B**).

## Effect of LBNS on *E. coli* Internalization

Previous studies in our laboratory have failed to show substantial leaf internalization in the case of *E. coli* O157:H7 EDL933 pregrown in LB broth (data not shown). Given our findings regarding the effect of growth medium composition on STm internalization, it was of interest to examine, whether this is a more general phenomenon. Therefore, leaf internalization of *E. coli* O157:H7 pregrown in either LB broth or in LBNS was also tested. Similar to *Salmonella*, pregrowth of *E. coli* strains in LBNS broth resulted in higher leaf internalization compared to growth in LB broth (28 ± 3% and 1.8 ± 1.2%, respectively) (**Figure 3**).

In order to document the localization of LB- and LBNS-grown *E. coli* cells within the leaf tissue, mCherry-labeled bacteria

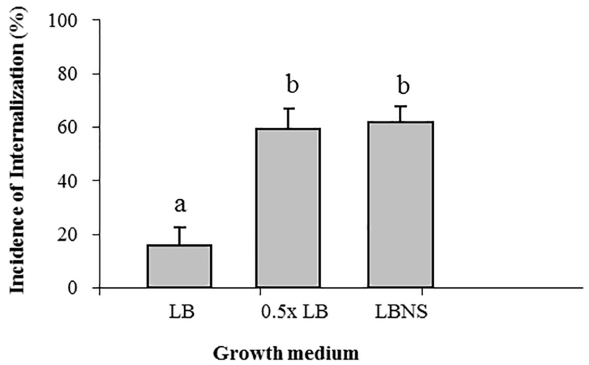


**FIGURE 1 |** Effect of growth medium on the internalization of *Salmonella* in lettuce leaf. *Salmonella* was grown overnight in the following media: LB, LBNS, water-diluted LB (1:1) with shaking at 37°C, washed and tested for leaf internalization. Incidence of bacteria in internal leaf tissues was calculated as the percentage of microscopic fields (×40) harboring ≥1 mCherry labeled bacteria in 30 randomly chosen microscopic fields of leaf tissue. The data denote the average and standard error (SE) of four independent experiments each performed in triplicates (3 × 30 fields per experiment). Different letters indicate significant difference ($P < 0.05$) between means of internal fields harboring bacteria, according to ANOVA Tukey–Kramer Multiple Comparisons Test.

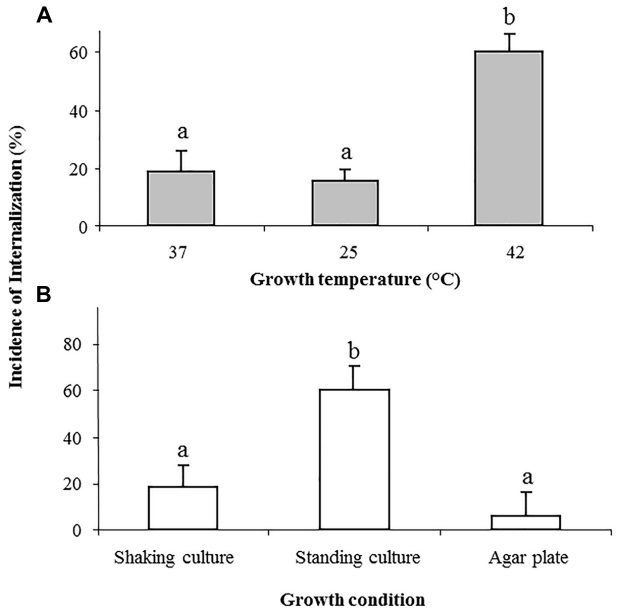


**FIGURE 2 |** Effect of growth temperature and growth conditions on the internalization of *Salmonella* in lettuce leaf. *Salmonella* was grown overnight in LB broth at the following temperatures: 37, 25, and 42°C with shaking **(A)**, or in LB without shaking, as well as in LB plates, both at 37°C **(B)**. Cells were washed and tested for internalization. The incidence of internalization was calculated and is presented as described in the legend of **Figure 1**. The data denote the average and SE of four independent experiments each performed in triplicates. Different letters indicate significant difference ($P < 0.05$) between means of internal fields harboring bacteria, according to ANOVA Tukey–Kramer Multiple Comparisons Test.

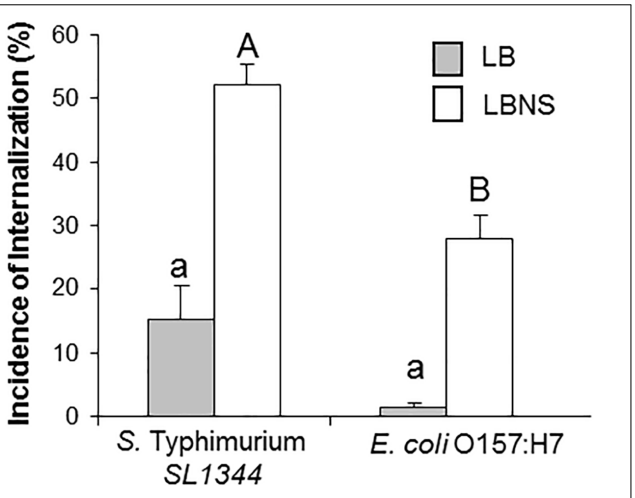


**FIGURE 3 |** Internalization of *E. coli* in lettuce leaf. *Salmonella* SL1344, and *E. coli* O157:H7 were grown overnight in LB or LBNS medium, washed and tested for internalization. The incidence of internalization was calculated and is presented as described in the legend of **Figure 1**. The data are the average of 4 independent experiments each performed in triplicates. Different letters indicate significant difference ($P < 0.05$) according to ANOVA Tukey–Kramer Multiple Comparisons Test.

were visualized by confocal microscopy. LBNS-grown bacteria were observed attached to the leaf surface with a distinct clustering pattern near and within stomata (**Figures 4A,B**). Images taken at various depths underneath the leaf surface demonstrated the presence of tagged bacteria within stomata and in the intercellular space (apoplast) of the spongy parenchyma in up to 55 μm depth (**Figure 4C**). A three-dimensional reconstruction model of fluorescent images taken at the same leaf region shows the presence of *E. coli* cells (pink) within a stomate (shown by the white rectangle) as well as in deeper layers of the leaf (**Figure 4D**). Similar z-section images and 3D reconstruction model performed on fluorescent images taken from leaves interacted with LB-grown *E. coli*, show no bacterial cells underneath the leaf surface (**Figures 4E,F**).

## Involvement of *Salmonella* Universal Stress Proteins in Leaf Internalization

The extrinsic factors that enhanced STm internalization, included mild stresses, such as growth in low NaCl broth (LBNS), nutrients deficiency (diluted LB broth), high-temperature (42°C), and low-oxygen content (standing culture without shaking). *Salmonella* possess at least five genes (*ydaA, yecG, ybdQ, ynaF,* and *uspAB*), encoding universal stress proteins, whose function in *Salmonella*-plant interaction is not known. To study possible involvement of these genes in *Salmonella* internalization, mutations in *ydaA, yecG, ybdQ, ynaF*, and *uspAB* were generated in STm SL1344. The wild type (wt) strain and the five isogenic mutants were grown in LBNS broth, and tested for leaf internalization. Mutants *uspAB, ydaA, yecG, ybdQ*, but not *ynaF*, demonstrated significant reduction in internalization efficiency compared to the wt strain (**Figure 5A**). Representative confocal microscopy images illustrating the localization of wt STm and two mutant strains on the leaf surface and underneath the surface are presented in **Figure 5B**). To confirm that the phenotype of the mutants was linked to the presence of the specific knockout mutations, each of the mutations was transferred back to the wt strain by transduction. The transductants harboring mutations in *uspAB, ydaA, yecG,* and *ybdQ*, but not in *ynaF,* were also impaired in leaf internalization phenotype, similar to the original knockout mutants (**Figure 5C**). These findings provide further evidence regarding the role these genes have in STm leaf internalization.

The wt and *ynaF* mutant strains display substantial attraction to stomata and were located in high numbers within the leaf tissue. In contrast, only few cells of the *yecG* mutant strain were observed on the leaf surface, including stomata, while no cells were observed underneath the surface **Figure 5B**).

It is possible that the low internalization phenotype displayed by the mutants merely reflect impaired attachment to the lettuce leaf, or lack of motility, which is required for leaf internalization (Kroupitski et al., 2009). Confocal microscopy visualization demonstrated that both the wt and its isogenic mutants displayed comparable attachment to the leaf surface (**Table 2**). Furthermore, both swarming motility (**Table 3**) and swimming motility (data not shown) of all the mutants were similar to that of the wt strain, suggesting that *uspAB, ydaA, yecG,* and *ybdQ* genes have a specific role in leaf internalization, which does not involve motility or attachment.

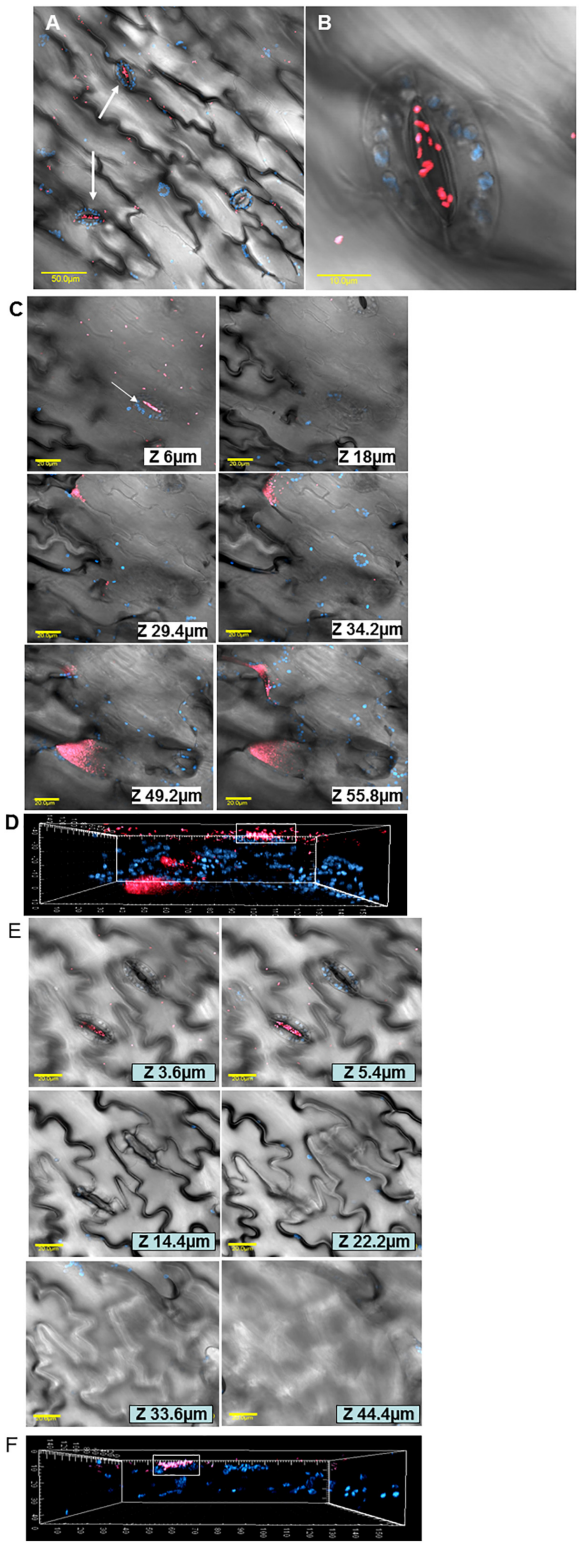


**FIGURE 4 |** Microscopic analysis of *E. coli* O157:H7 interactions with lettuce leaf following growth in LB or in LBNS. Confocal microscopy images of mCherry-tagged *E. coli* (pink) grown in LB broth show both diffuse attachment to the leaf surface and stomata-clustering **(A)**. A higher magnification of a *(Continued)*

**FIGURE 4 |** Continued
single stoma harboring *E. coli* cells is also presented **(B)**. Representative photomicrographs images showing LB-grown bacteria in various depth underneath the leaf surface **(C)** and a three-dimensional reconstruction of fluorescent images taken at the same leaf section, shown above, demonstrates the existence of bacteria (pink) in deeper leaf tissues **(D)**. In contrast, to LB-grown bacteria, following growth in LBNS, *E. coli* cells are detected in stomata but apparently no cell is observed within the leaf tissue **(E)**. Indeed, a three-dimensional reconstruction of confocal microscopy images taken at the same leaf section shown in **(E)**, shows no bacterial cells in the inner leaf tissues **(F)**. Blue fluorescence indicates auto-fluorescence of chlorophyll within chloroplasts of guard-cells and parenchymal cells. The fluorescent images in **(A–C,E)** were overlaid with the transmitted light image obtained using Nomarski differential interference.

## Transcriptional Induction of Universal Stress Protein Genes Under Growth in LBNS

The data presented suggest that growth of STm under suboptimal conditions may induce the expression of the *usp*- and other genes that may facilitate leaf internalization. Since, the phenotypic analysis of the 5 *usp* genes was performed following growth of STm in LBNS, expression of the 5 *usp* genes was assessed following growth in LBNS compared to growth in LB at 37°C. Transcription of all the tested genes was induced following growth in LBNS compared to LB (**Figure 6**).

# DISCUSSION

We have previously, demonstrated that *Salmonella enterica* cells cluster near lettuce stomata and penetrate through the stomatal opening into the inner leaf tissue in a process that involved chemotaxis (Kroupitski et al., 2009). The ability of HPOP to internalize leafy greens has been documented in many studies (Itoh et al., 1998; Seo and Frank, 1999; Takeuchi and Frank, 2001; Kroupitski et al., 2009; Golberg et al., 2011; Deering et al., 2012), however, it seems that internalization varies greatly in different studies and is influenced by genetic factors and environmental of both bacteria and plants (Mitra et al., 2009; Zhang et al., 2009; Deering et al., 2012; Wright et al., 2013).

In most studies, HPOP internalization was examined following growth in rich laboratory media (Itoh et al., 1998; Seo and Frank, 1999; Takeuchi and Frank, 2001; Solomon et al., 2002; Kroupitski et al., 2009; Niemira and Cooke, 2010; Golberg et al., 2011). In the present study, we have examined the idea that modulation of the growth conditions may affect STm internalization. We have demonstrated that leaf internalization was enhanced following pregrowth of STm under suboptimal growth conditions, representing bacterial adaptation to mild stresses, such as low salt (LBNS medium), $O_2$-limitation, low nutrients (0.5×LB) and temperature (42°C). The obtained effect was not limited to STm, at least in regard to pregrowth in LBNS, as growth of *E. coli* O157:H7 in this medium has also improved leaf internalization compared to growth in LB broth. Internalization of *E. coli* O157:H7 in leafy vegetables is a controversial issue. Some researchers

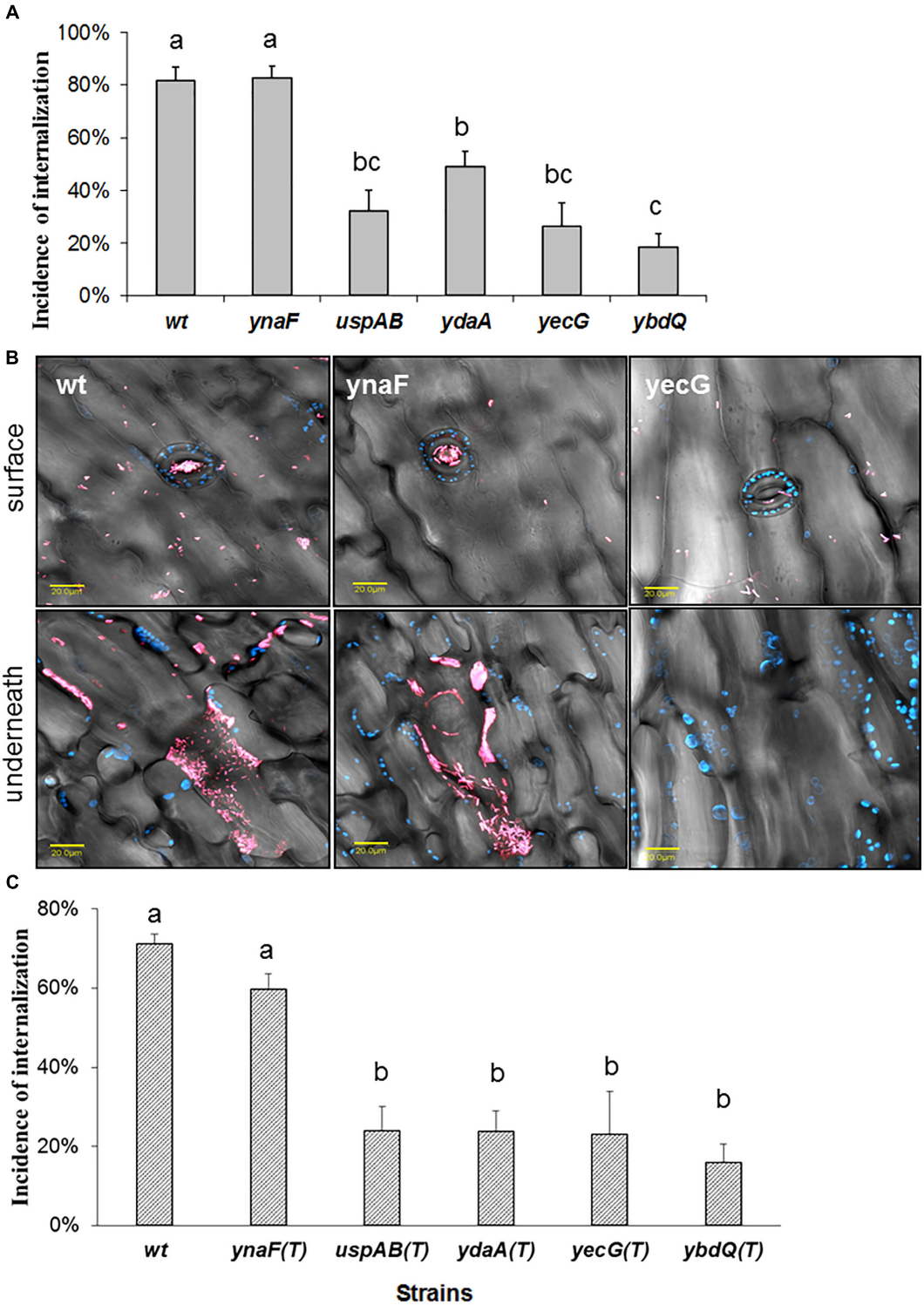


**FIGURE 5 |** Effect of mutations in genes encoding universal stress proteins on *Salmonella* internalization. *Salmonella* wt and mutant strains were grown in LBNS and tested for internalization as described in **Figure 1**. The incidence internalization in lettuce leaf is presented **(A)**. Representative confocal microscopy image taken on the leaf surface, or stacks of images taken along a z-section underneath (inside), show *Salmonella* distribution on the surface and within the leaf tissue **(B)**. Blue color indicates auto-fluorescence of chloroplasts and pink color represent mCherry-tagged cells. The fluorescent images were overlaid with the transmitted light image obtained using Nomarski differential interference. The incidence of internalization of transductants harboring mutations in usp genes is presented in **(C)**. The data presented in **(A,C)** are the average and SE of 6 independent experiments each performed in triplicates (3 × 30 fields per experiment). Different letters indicate significant difference ($P < 0.05$) according to ANOVA Tukey–Kramer Multiple Comparisons Test.

**TABLE 2 |** Attachment of STm wild-type and mutant strains to lettuce leaf.

| | Number of microscope fields out of 90 (means ± SD) harboring mCherry-tagged STm cells | | | | |
|---|---|---|---|---|---|
| **Strains** | **0** | **1–10** | **10–50** | **50–100** | **100 ≤** |
| wt | 0 | 4 ± 3 | 53 ± 11 | 23 ± 6 | 8 ± 8 |
| Δ*ynaF* | 0 | 1 ± 0.5 | 47 ± 14 | 28 ± 11 | 13 ± 8 |
| Δ*uspAB* | 0 | 16 ± 7 | 47 ± 10 | 16 ± 6 | 6 ± 4 |
| Δ*ydaA* | 0 | 5 ± 3 | 57 ± 11 | 17 ± 6 | 10 ± 10 |
| Δ*yecG* | 0 | 21 ± 7 | 47 ± 12 | 10 ± 3 | 10 ± 10 |
| Δ*ybdQ* | 0 | 11 ± 7 | 48 ± 9 | 27 ± 8 | 4 ± 4 |

**TABLE 3 |** Swarming motility assay.

| **Strains** | **Swarm distance (mm ± standard error)** |
|---|---|
| wt | 36.4 ± 3.0 |
| Δ*ynaF* | 36.4 ± 3.5 |
| Δ*uspAB* | 40.6 ± 1.2 |
| Δ*ydaA* | 36.2 ± 1.9 |
| Δ*yecG* | 36.8 ± 0.9 |
| Δ*ybdQ* | 39.8 ± 1.4 |
| Δ*fliGHI* | 44 ± 3.0 |
| Δ*motA* | 33 ± 3.0 |

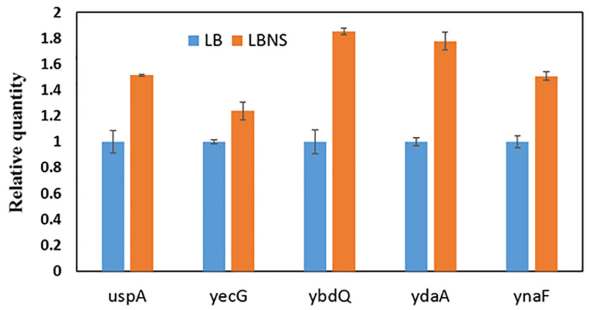


**FIGURE 6 |** Relative quantification of gene expression in STm cells grown in LBNS (orange columns) compared to growth in LB broth (Blue columns) at 37°C. Gene expression was normalized to the expression of *rpoD* under the same growth conditions. Bars represent the standard deviation of three repeats. Each of the tested genes was significantly ($P < 0.05$) induced upon growth in LBNS compared to LB, according to unpaired Student's *t*-test.

have reported that *E. coli* O157:H7 may reside within leaf tissue (Seo and Frank, 1999; Takeuchi and Frank, 2000, 2001; Erickson et al., 2010), while others failed to demonstrate *E. coli* O157:H7 internalization into lettuce leaves, regardless the type of lettuce, age of plant, or strain (Zhang et al., 2009; Deering et al., 2012). Similarly, Mitra et al. (2009) also reported lack of internalization of *E. coli* O157:H7 in intact spinach leaves. Previous studies in our laboratory with *E. coli* O157:H7 also failed to demonstrate substantial internalization using our lettuce leaf model, describe in this study (unpublished data). While, other plant and bacterial factors may account for these contradictory results, it is possible that difference in bacterial growth conditions, or exposure to other extrinsic factors, prior to the internalization assay, may have account for this variability in the internalization efficiency.

The detailed mechanisms through which growth conditions alter the capacity of bacteria to internalize leaves are not known. It is anticipated that pregrowth conditions may adapt bacteria to cope with the plant host. In the case of pregrowth in low NaCl medium, it was previously reported that the expression of curli, also known as thin aggregative fimbriae (tafi) is induced during growth of *Salmonella* and *E. coli* in low osmolarity medium, such as LBNS (Zogaj et al., 2001). Curli are bacterial adhesin that mediate attachment to various surfaces and contribute to biofilm formation on abiotic surfaces (Jain and Chen, 2007). Several studies have documented the involvement of curli also in the attachment of *Salmonella* and *E. coli* to plants (Barak et al., 2005; Jeter and Matthysse, 2005; Boyer et al., 2007; Macarisin et al., 2012; Yaron and Römling, 2014). Furthermore, curli were shown to enhance the transfer of *S.* Typhimurium from contaminated irrigation water to parsley and contributed to *Salmonella* plant internalization (Lapidot and Yaron, 2009). Thus, it is possible that curli may also influence STm internalization into lettuce leaves. Testing internalization of STm curli mutants should provide a better understanding regarding their potential role in lettuce leaf internalization.

Leaf surface is considered a hostile environment, where both phytopathogens and HPOP encounter multiple stresses, such as limited nutrients, UV irradiation, temperature fluctuations and desiccation (Brandl, 2006; Delaquis et al., 2007; Underwood et al., 2007; Heaton and Jones, 2008). It has been suggested that leaf internalization is a stress evasion strategy adapted by some phytopathogens and HPOP (Heaton and Jones, 2008). It might be hypothesized that pregrowing of *Salmonella* under non-optimal conditions may have induced a general stress response to overcome anticipated stresses, which among other features, induces mechanisms that contributes to leaf internalization. This idea is supported by the recent study of Fornefeld et al. (2017), who demonstrated that STm cells grown in lettuce-medium persist longer in soil microcosm compared to cells grown in LB broth (Fornefeld et al., 2017).

Adaptation of *Salmonella* to environmental conditions is mediated largely by overlapping regulatory systems that control the expression of numerous genes (Alvarez-Ordóñez et al., 2015). Universal stress proteins (USPs) are prevalent in all three domains of life. In bacteria, USP have a role in adaptation to several stresses, including oxidative stress, high temperature, low pH and hypoxia and are likely to contribute to the adaption of bacterial pathogens to the human host environment (Kvint et al., 2003; Liu et al., 2007; Persson et al., 2007; O'Connor and McClean, 2017). Knowledge regarding the role of USPs in phytopathogen-plant interaction is very limited (Ramachandran et al., 2014, transcriptomic base), and no data were reported regarding their potential role in human pathogen-plant interactions. Knockout mutants of STm in *uspAB, yecG, ydaA, ynaF, and ydaA,* encoding for UspAB, UspC, UspE, UspF, and UspG, respectively, were tested for their capacity to adhere to and internalize lettuce leaves. Mutations in *uspAB, ydaA, yecG,* and *ybdQ* significantly reduced STm internalization compared

to the wt strain, while mutation in the *ynaF* gene showed no phenotype. Similarly, transduction of the mutations in *uspAB, ydaA, yecG,* and *ybdQ* back to the wt strain, resulted in a similar phenotype. It seems that expression of both *uspAB, ydaA, yecG,* and *ybdQ* is needed for leaf internalization.

To further examine if these genes are indeed induced during growth of STm in LBNS, RT-RT PCR analysis demonstrated that each of the studied USP genes was induced under growth in LBNS compared to growth in LB at 37°C. The finding that *ynaF* expression was also induced, although the phenotype of the *ynaF* deletion mutant was not affected, implies that not all genes induced under growth in LBNS are necessarily critical for leaf internalization.

Remarkably, the attachment of all the mutants to the leaf surface was comparable to that of the wt strain (data not shown), indicating that *uspAB, ydaA, yecG,* and *ybdQ* were not merely required for bacterial attachment.

It has been reported that in *S.* Typhimurium the *uspA* gene is induced by metabolic, oxidative, and temperature stresses, and that mutation in *uspA* gene leads to reduced stress tolerance (Liu et al., 2007). UspA contributed to the *in vivo* virulence of *S.* Typhimurium in mice and to survival within the host (Liu et al., 2007). Additionally, *Salmonella* Enteritidis *uspAB* mutant had a decreased ability to contaminate eggs and to persist in harmful environments, such as in the oviduct and eggs shell (Raspoet et al., 2011). It has been reported that *Salmonella* faces similar stress in both mammalian and plant hosts (Schikora et al., 2011; Barak and Schroeder, 2012; Goudeau et al., 2013; Wiedemann et al., 2015; Cox et al., 2018). Our findings, regarding the involvement of specific USPs in STm colonization of internal leaf tissue, further support the idea that bacterial adaptation to stresses may be advantageous to confront comparable stresses in both mammalian- and plant-hosts.

## CONCLUSION

Internalization of STm in lettuce leaf was affected by bacterial growth conditions. Exposure of the pathogen to mild stresses enhanced leaf internalization, possibly due to bacterial preadaptation, which also contributes to lettuce leaf internalization. The universal stress genes *uspAB, ydaA, yecG,* and *ybdQ*, but not *ynaF* gene, are required for lettuce leaf internalization. Further characterization of their role in STm internalization is needed in order to better understand how *Salmonella* and possibly other HPOP adapt to the hostile plant environment.

## AUTHOR CONTRIBUTIONS

SS and YK designed the study and analyzed the results. YK, RP, and EB performed the experiments, except for RT-RT PCR. RG planned and performed the RT-RT PCR experiments, analyzed, and summarized the results. SS and YK wrote the manuscript.

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# *Salmonella* Establishment in Agricultural Soil and Colonization of Crop Plants Depend on Soil Type and Plant Species

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Human pathogenic bacteria, such as *Salmonella enterica*, are able to colonize crop plants. So far, not much is known about biotic and abiotic factors influencing this colonization in field soil. This understanding, however, is imperative for the provision of safe fresh produce to the consumer. In this study, we investigated the effects of soil type, organic fertilization, plant species and the way of *Salmonella* entry into the plant production system, on the survival of *S. enterica* in soil as well as the colonization of plants. The selected *S. enterica* serovar Typhimurium strain 14028s, *S.* Typhimurium strain LT2 and *S.* Senftenberg were able to persist in soil for several weeks. *Salmonella's* persistence in soil was prolonged in loamy, if compared to sandy soil, and when applied together with organic fertilizer. The leaves of lettuce and corn salad were colonized by *S. enterica* providing evidence for internalization from the soil *via* the root. Colonization rates were affected by soil type, plant species and *S. enterica* strain. Overall, *S. enterica* was detected in leaves of 0.5–0.9% of the plants, while lettuce was more frequently colonized than corn salad. Plants grown in sandy soil were more often colonized than plants grown in loamy soil. After spray inoculation, *S. enterica* could be detected on and in leaves for several weeks by cultivation-depending methods, confirmed by confocal microscopy using GFP-labeled *S.* Typhimurium 14028s. On the one hand, transcriptome data from *S.* Typhimurium 14028s assessed in response to lettuce medium or lettuce root exudates showed an upregulation of genes associated with biofilm formation and virulence. On the other hand, lettuce inoculated with *S.* Typhimurium 14028s showed a strong upregulation of genes associated with plant immune response and genes related to stress response. In summary, these results showed that organic fertilizers can increase the persistence of *Salmonella* in soil and that soil type and plant species play a crucial role in the interactions between human pathogens and crop plants. This understanding is therefore a starting point for new strategies to provide safe food for the consumer.

**Keywords: internalization, plant defense, *Salmonella*, persistence, soil, crop plants**

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## INTRODUCTION

The number of disease outbreaks, associated with fresh food of non-animal origin contaminated with human pathogens, increased in the EU from 2008 to 2011. Among them, the top ranked pathogen-food associations were raw eaten leafy greens contaminated with *Salmonella* ssp. (EFSA, 2013). In 2016, 0.21% of the 2,429 vegetable samples were positively tested for different *Salmonella* serovars, most of them at the retail stage (EFSA, 2017b). More recently, the *S. enterica* serovar Agona outbreak in several EU countries in 2018 was linked to ready-to-eat products containing cucumbers (EFSA, 2018). One potential source of contamination is the soil on which the plant is growing. Although the risk of plant colonization by human pathogens from soil is lower than, e.g., in hydroponic solution (Hirneisen et al., 2012), internalized *Salmonella* would be protected against post-harvest processing treatments and might therefore pose a risk for human health. Today, the pre-harvest colonization of leafy greens by *Salmonella* and factors influencing the extent of this contamination are not fully understood.

Potential sources of soil contamination with human pathogens are manure, used as organic fertilizer, and contaminated irrigation water (Beuchat, 2002). Pigs are typical hosts of *Salmonella*. Between 2006 and 2007, about 10.3% of the slaughter pigs in the EU were *Salmonella*-positive (EFSA, 2008). *S. enterica* serovar Typhiumurium (*S.* Typhimurium) was the most commonly reported serovar in pig herds and the second top-ranked serovar isolated from pork (EFSA, 2017b). Poultry are other typical hosts of *Salmonella*. For example, in 2017 a multi-country outbreak of *S.* Enteritidis was linked to eggs from Poland (EFSA, 2017a). Therefore, *Salmonella* can reach agricultural soils *via* fertilization with pig or poultry manure. Previous reports showed that *Salmonella* persist in soil for at least 21 days when originating from pig manure (Pornsukarom and Thakur, 2016) and up to one year when originating from poultry manure (Hruby et al., 2018), indicating that soils can act as a long-term *Salmonella* reservoir. Factors influencing the survival of *S. enterica* in manure and manure-amended soil were reviewed in Ongeng et al. (2015) and include the physical and chemical characteristics of both manure and soil, and also weather and atmospheric conditions, biological interactions, agricultural and livestock management practices, strain variation and physiological age of the cells.

Once *Salmonella* is in the soil, the most probable routes of contamination of crop plants are *via* the rhizosphere and root and *via* soil splashing on leaves, flowers or fruits during overhead irrigation or rainfall. All might result in internalization of *Salmonella* into the edible plant parts, as reviewed by Hirneisen et al. (2012). For example, even though the numbers of *S. enterica* internalized in lettuce were reported to decrease over time of cultivation, bacteria persisted in plant tissues (Jablasone et al., 2005). The observed negative correlation between internal colonization and plant mass (Franz et al., 2007), could indicate an infection rather than a contamination or colonization and point to a potential disadvantage for the host plant.

Nonetheless, not much is known about factors influencing the colonization of plants by *S. enterica* directly or indirectly such as soil type, organic fertilizer application, plant species, or *Salmonella* strain. Furthermore, reports on the internalization of *Salmonella* into lettuce plants were not always consistent, or reported only low numbers of cases (Honjoh et al., 2014). This supports the notion that internalization rates are rather low and/or depend on certain experimental conditions. One possible explanation for the low internalization rate of human pathogens into plant tissues might be the fact that *Salmonella* triggers a plant immune response, which might, to a certain level, prevent the colonization.

In this study, we investigated the colonization of leafy greens *via* soil inoculated with *S.* Typhimurium strains 14028s and LT2 as well as *S.* Senftenberg. Furthermore, in microcosm experiments we assessed the potential of different factors to influence the persistence of *Salmonella* in soil. These factors included soil texture, application of organic fertilizers (pig manure, chicken litter), *Salmonella* strain and manner of *Salmonella* application. We hypothesized that those factors could influence the persistence of *Salmonella* in soil and could also directly or indirectly influence the colonization rate of crop plants. In parallel, adaptations of *S. enterica* to the plant environment as well as the plant immune response to *Salmonella* inoculation were investigated on transcriptome level and the potential internalization routes were explored using confocal laser scanning microscopy (CLSM) and GFP-labeled *S.* Typhimurium 14028s. Our results show that the persistence of *Salmonella* in soil is enhanced in loamy soil, if compared to sandy soil and by an application of organic fertilizer, however, only when *S. enterica* was introduced into the plant production system together with the fertilizer. The colonization rates of lettuce and corn salad were relatively low and varied substantially between the soil types, plant species and *Salmonella* strains. CLSM confirmed secondary root emerging zones, root hairs, stomata and hydathodes as potential internalization sites of *Salmonella*. The transcriptome analyses showed that *S.* Typhimurium 14028s responded differently to lettuce medium and lettuce root exudates, indicating an active adaptation process. Furthermore, the lettuce transcriptome revealed that *S.* Typhimurium 14028s triggered a plant immune response, supporting the hypothesis that both organisms, *Salmonella* and the colonized plant, play an active role during the colonization process.

## MATERIALS AND METHODS

### *Salmonella* Strains Used in This Study

*Salmonella enterica* serovar Senftenberg (*S.* Senftenberg), obtained from Nicola Holden, The James Hutton Institute, Dundee, and John Coia, SSSCDRL, Glasgow (Scotland, United Kingdom) (Elviss et al., 2009), *S. enterica* serovar Typhimurium 14028s (*S.* Typhimurium 14028s) ATCC 14028, obtained from Dr. Isabelle Virlogeux-Payant, INRA Tours (France) and *S. enterica* serovar Typhimurium strain LT2 (*S.* Typhimurium LT2) DSM 18522 (Jarvik et al., 2010) were used in this study. Rifampicin resistant mutants were obtained by overnight culture on plates with LB agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) containing rifampicin

(50 mg/L, SERVA, Heidelberg, Germany) as described previously (Fornefeld et al., 2017).

To visualize the colonization patterns in lettuce (*Lactuca sativa* L. cultivar Tizian) and corn salad (*Valerianella locusta* L. cultivar Verte à coeur plein 2), *S.* Typhimurium 14028s was GFP-labeled with the plasmid pSM1890 (Haagensen et al., 2002). To this end, a triparental mating was performed between *S.* Typhimurium 14028s, *Escherichia coli* carrying the *gfp*-tagged IncQ plasmid pSM1890 GFP derived from the IncQ plasmid pIE723 (Richter and Smalla, 2007), and *E. coli* carrying the helper plasmid R751 (Thorsted et al., 1998). The strains were streaked on LB plates containing the respective antibiotics, incubated overnight at 37°C. Cells were harvested with a loop and resuspended in 1 mL of 10 mM $MgCl_2$. Cell solutions were combined and mixed, the suspension was centrifuged (4000 × *g*, 3 min) and the supernatant was removed. Cells were resuspended in the residual liquid and placed on a filter disc (0.22 μm, Durapore® membrane filters, Merck, Darmstadt, Germany) on LB and incubated overnight at 28°C. The following day, cells were resuspended from the filter by vortexing in 5 mL of 10 mM $MgCl_2$. Transconjugants were selected by plating of serial dilutions on LB plates containing rifampicin 50 mg/L, gentamicin 10 mg/L (Carl Roth GmbH + Co. KG) and streptomycin 50 mg/L (Carl Roth GmbH + Co. KG) and incubation at 28°C. After 24 h, green fluorescent colonies were picked and plated on XLD agar (Carl Roth GmbH + Co. KG), subsequently incubated for 24 h at 37°C to confirm the presence of *Salmonella,* indicated by the formation of black colonies.

## Origin and Characteristics of Agricultural Soils, Manure, and Chicken Litter

Two types of agricultural soil were chosen for the experiments, a sandy and a loamy soil. The sandy soil was characterized as Arenic-Luvisol with less silty sand and 5.5% clay, pH 6.1 and organic carbon content of 0.9% (diluvial sand, DS) and the loamy soil as Gleyic-Fluvisol with heavy sandy loam and 27.5% clay, pH 6.7 and organic carbon content of 1.8% (alluvial loam, AL) (Rühlmann and Ruppel, 2005; Schreiter et al., 2014).

Pig manure used in this study was obtained from an experimental pig husbandry farm at the Friedrich-Loeffler-Institute in Braunschweig, Germany. Chicken litter was obtained from a free-range chicken stable in Lower Saxony, Germany. Pig manure and chicken litter were stored at 4°C until the start of the experiment. Both fertilizers were tested negatively for the presence of *Salmonella* before the experiments. The matrix characterization of the organic fertilizers was performed by LUFA Nord-West (Hameln, Germany) and results are given in **Supplementary Table S1**.

## Design of the Experiment

A greenhouse microcosm experiment was performed to investigate the influence of organic fertilizer application (pig manure and chicken litter), *Salmonella* strain and manner of *Salmonella* application on the persistence of *Salmonella* in agricultural soils (DS and AL soil) as well as on the colonization of plants (lettuce and corn salad).

For the fertilizer treatments, soils were mixed with organic fertilizers to obtain a maximum N application of approximately 170 kg N/ha. Therefore, the N content of the organic fertilizers was determined (see above). The amount of fertilizer was calculated considering a depth of incorporation of 5 cm into the soils and equals 0.23 and 0.26 g N/kg DS and AL soil, respectively, due to different densities of the two soils. The amount of 170 kg N/ha and year is the maximum amount allowed to be applied on agricultural fields in Germany (Bundesministerium der Justiz und für Verbraucherschutz, 2017). Water was added to the fertilizer treatment and the control soil without fertilizer to obtain a maximum water holding capacity ($WHC_{max}$) of 50%.

About 28–44 days after the amendment with organic fertilizers (mixing day), lettuce and corn salad were transferred at a 2–3 leaf stage to the soil treatments in 100 mL polystyrene flower pots. In the following, this day is referred to as day zero. Special care was taken during the transfer to avoid contamination of the leaves with soil particles. The plants were pre-grown in planting trays containing a mixture of 1:1 (vol/vol) DS or AL soil mixed with bedding substrate (Substrat 1, Klasmann-Deilmann GmbH, Geeste, Germany) to support the germination and growth of the seedlings.

*Salmonella* strains were mixed into the soil either together with the organic fertilizers (on mixing day) to simulate a contamination by fertilization ("stored" treatment) or directly before the transfer of the plants (on day 0), simulating the contamination by irrigation water ("fresh" treatment). Therefore, *Salmonella* strains were first streaked on lettuce medium (LM) agar plates which were freshly prepared as described previously (Fornefeld et al., 2017) containing 50 mg/L rifampicin. After overnight incubation at 28°C, *Salmonella* strains were harvested from agar plates, resuspended in 10 mM $MgCl_2$ solution and the number of bacterial cells was estimated by the measurement of the optical density (OD) at 600 nm. All strains were inoculated at final cell number of $10^6$ colony forming units (CFU) per g dry soil.

The pots were watered from below, as needed, and incubated in the greenhouse at 20°C, 16 h light.

## Quantification of *Salmonella* in Soil by Plate Counts

On mixing day, as well as during the incubation period (on days 4, 7, 21, 35, or 49 after planting), soils of 4 replicate pots per treatment were mixed thoroughly and from each pot 1 g of soil was transferred to 50 mL screw cap centrifugation tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany), 9 mL of 10 mM $MgCl_2$ solution was added and the soil slurry was mixed by vortexing for 1 min. $MgCl_2$ was used in order to allow comparisons to previous experiments. Duplicates of serial dilutions were dropped (10 μL) in two technical replicates on XLD agar containing rifampicin 50 mg/L. CFU were enumerated after an overnight incubation at 37°C. Additionally, 100 μL and 1000 μL were spread on XLD agar (Carl Roth GmbH + Co. KG) when low cell counts of *Salmonella* were expected.

## Quantification of Phyllosphere Colonization

In order to investigate the relation between persistence of *Salmonella* in soil and colonization of crop plants, lettuce and corn salad leaves were sampled during the growth period, in parallel to the soil sampling (on days 4, 7, 21, 35, or 49 after planting). Only leaves that were not in contact with the soil were sampled. Leaves were sampled using sterilized scissors and forceps and two plants were pooled into one single 50 mL screw cap centrifugation tube. Afterwards, the leaves were shredded with sterilized scissors and 10 mL Buffered Peptone Water (BPW, Carl Roth GmbH + Co. KG) was added. Following the overnight incubation at 37°C under shaking conditions (140 rpm), 10 μL of the suspension was transferred to 190 μL Rappaport Vassiliadis Broth (RVS, Carl Roth GmbH + Co. KG) in 96-well plates and incubated overnight at 42°C. Ten microliter of both enrichments (BPW and RVS) were dropped on XLD plates containing rifampicin (50 mg/L) and incubated overnight at 37°C to confirm the presence of *Salmonella*.

## Statistical Analysis

For *Salmonella* CFU counts in soil, slopes of linear regressions were analyzed by a generalized linear model using the mixed procedure (SAS 9.4; SAS institute Inc., Cary, NC, United States). Tukey tests were performed with the procedure glimmix of SAS. Plant colonization in dependency of time after the transfer of the plants was analyzed by a linear model using the lm function (R; version 3.2.1). Differences in numbers of colonized plants between treatments were analyzed by Pearson's Chi-squared test for count data using the chisq.test function. Results were considered significant when $p < 0.05$.

## *Salmonella* Persistence in the Phyllosphere

To investigate the ability of *Salmonella* to persist on and in lettuce and corn salad leaves, the plants were spray-inoculated with different cell counts of *S.* Typhimurium 14028s and over time, the presence and quantity of *Salmonella* was determined by enrichment and direct CFU count approaches. The rifampicin resistant *S.* Typhimurium 14028s strain was pre-grown on LB plates containing rifampicin (50 mg/L) overnight at 37°C. Bacterial cells were harvested and resuspended in 10 mM $MgCL_2$ containing Tween 20 (0.02%, SERVA). Lettuce and corn salad plants at 5–10 leaf stage, which were pre-grown in potting soil, were sprayed with *S.* Typhimurium 14028s suspensions in cell counts between $10^7$ and $10^2$ CFU/mL. Spray-inoculated leaves were sampled on days 0 (about 1 h after spray inoculation), 7, 21, and 35. To avoid the sampling of leaves that were not inoculated, on day 0 leaves were labeled. The samples were weighed and homogenized in 10 mM $MgCl_2$ solution by an electric mortar on ice. *S.* Typhimurium 14028s CFUs were quantified by plating a dilution series on XLD agar as described for the quantification of *Salmonella* in soil samples. In parallel, residual undiluted plant leaf homogenates were used for enrichment in BPW and RVS as described above to test for the presence of *Salmonella*. The cell counts of *Salmonella* in the leaves were displayed as log (CFU per g leaf fresh weight). The plants were grown in the greenhouse at 20°C, 16 h light.

## Lettuce Transcriptome Profile

To investigate the response to colonization by *S.* Typhimurium 14028s, lettuce plants were exposed to *Salmonella* under sterile conditions. To this end, lettuce seeds were surface-sterilized by washing 5 times for 1 min with sterile tab water, followed by a sterilization step with 3% NaClO (Carl Roth GmbH + Co. KG) for 4 min and subsequently 4 washing steps with sterile tab water for 1 min. The sterilized seeds were placed on ¼ Murashige and Skoog agar medium (MS, Duchefa Biochemie, Haarlem, The Netherlands) at pH 5.6, including vitamins and 5 g/L sucrose. The seeds were incubated overnight in the dark and subsequently for additional 4 days in a climate chamber at 21°C, 16 h light. At the one-leaf stage, plants were transferred to 5 mL ¼ MS medium in 6-well plates (3 plants per well, in 3 replicates) and further incubated for 24 h. After this pre-adaption, MS medium was inoculated with *S.* Typhimurium 14028s resuspended in 10 mM $MgCl_2$ to obtain a final cell count of $10^8$ CFU/mL, 10 mM $MgCl_2$ solution was used as negative control. After incubation for 24 h, plants were harvested, immediately frozen in liquid nitrogen, ground with sterile mortar and pestle and stored at −80°C until the RNA extraction.

## *Salmonella* Transcriptome Profile

To determine the transcriptional response of the rifampicin-resistant *S.* Typhimurium 14028s to different plant-related environments, we designed a new experimental approach. *Salmonella* was grown at 37°C for 18–20 h in LB broth with rifampicin (50 mg/L). The stationary phase cells were pelleted at low speed (1,500 × *g*, 10 min), washed twice in 10 mM $MgCl_2$ and adjusted to $OD_{600\ nm} = 1$. Two milliliter of this suspension were pipetted into cellulose ester dialysis tubes with a pore size of 100 kDa (Spectrum Europe B.V., Breda, The Netherlands). Closed dialysis tubes were placed in 50 mL screw cap centrifugation tubes containing: (i) 30 mL of a minimal medium (MM); (ii) 30 mL of a lettuce medium (LM); or (iii) 30 mL of lettuce root exudates resuspended in $MgCl_2$ (LE). Lettuce root exudates were collected as described previously (Witzel et al., 2017) with some modifications. Briefly, roots of plants were immersed in sterile distilled water for 1 h, and then transferred to fresh bi-distilled water for another 4 h. The exudates from approximately 25 plants were pooled into a single sample. The medium was filtered and concentrated 10-fold by freeze-drying. The lyophilized root exudates were re-suspended in 10 mM $MgCl_2$ prior to use. All treatments were performed in triplicates. The MM consisted of 20% M9 salts (Sigma-Aldrich Chemie GmbH, München, Germany; 5× concentrated), 2 mM $MgSO_4$ and 1.23 mM glucose in sterile deionized water. The screw cap centrifugation tubes were incubated at 28°C for 24 h while shaking at 180 rpm. Thereafter, 2 × 0.5 mL from each dialysis tube were mixed with RNAprotect (QIAGEN, Hilden,

Germany), incubated for 5 min and centrifugated at high speed (4,000 × *g*, 10 min).

## RNA Extraction, Quantitative PCR, Library Preparation, and Sequencing

RNA was extracted from 0.1 g of lettuce leaves using the RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. DNA was digested using the PerfeCTa DNase I (Quanta BioSciences, Gaithersburg, MD, United States). For qPCR analysis, RNA was first transcribed to cDNA using the qScript cDNA Synthesis Kit (Quanta BioSciences) following the manufacturer's instructions. The subsequent library preparation and Illumina HiSeq sequencing were performed at the IIT GmbH, Bielefeld, Germany.

From *S.* Typhimurium 14028s cell pellets obtained as described above, total RNA was extracted using the RNeasy Mini Kit (QIAGEN). The rRNA was removed using the Ribominus Kit (Thermo Fisher Scientific, Darmstadt, Germany) with some modifications concerning the specificity of *Salmonella* 23S rRNA (Mühlig et al., 2014). For fragmentation of the RNA a Covaris S220 Focused-ultrasonicator (Covaris, Woburn, MA, United States) was used (180 s, 175 W peak power, 10% puty, 200 cycles). The fragments were dephosphorylated and phosphorylated using antarctic phosphatase (NEB, Frankfurt, Germany), SUPERase• In and T4PNK (Thermo Fisher Scientific) to gain a common phosphorylation state for all fragments. The library preparation was done with the TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA, United States) according to the manufacturer's instructions and the SuperScript IV cDNA synthesis Kit (Thermo Fisher Scientific). Illumina HiSeq sequencing was performed at the ZIEL – Institute for Food & Health, Core Facility Microbiome/NGS, Technische Universität München, Freising, Germany.

## Sequence Processing and Statistical Analyses of Lettuce Transcriptome

Fastq files were trimmed using trimmomatic (Bolger et al., 2014) and then mapped to the *L. sativa* L. cultivar Tizian genome (Verwaaijen et al., 2018) using top hat (Kim D. et al., 2013). The resulting BAM files were read into read explorer (Hilker et al., 2016) and read counts were obtained. Kyoto Encyclopedia of Genes and Genomes (KEGG) mappings for transcripts were obtained by aligning translated transcript sequences to the *Arabidopsis thaliana* protein set (TAIR10, November 2010, arabidopsis.org) using a local implementation of BLAST (Altschul et al., 1997). For each sequence the best hit was used for KEGG pathway analysis and only hits with an $E < 1^{-10}$ were used. Statistical analysis was carried out in R (R Core Team, 2018). Differentially abundant genes were determined using DESeq2 (Love et al., 2014). Enrichment analysis of Gene Ontology (GO) terms and KEGG pathways was done with R library gage (Luo et al., 2009) and the KEGG pathway gene set for *A. thaliana* was generated using function kegg.gsets. KEGG pathway maps were plotted with R package pathview (Luo and Brouwer, 2013). The raw sequences were deposited in the Sequence Read Archive database (NCBI) with the Bioproject ID PRJNA507508.

## Sequence Processing and Statistical Analyses of *S.* Typhimurium 14028s Transcriptome

The sequence analyses were performed using the Bowtie2 (Version 0.6), cufflinks (Version 2.2.1.0), cuffmerge (Version 2.2.1.0), cuffquant (Version 2.2.1.1) and cuffdiff (Version 2.2.1.5) (Langmead et al., 2009; Trapnell et al., 2010) where the FPKM (Fragments Per Kilobase Million) and the significant differences between the treatments were calculated as false discovery rate (FDR)-adjusted *p*-values of the test statistic (*q*-values). Values differing by more than twofold and *q*-values below 0.05 were considered significantly increased or decreased in gene expression. Not all genes detected in the *S.* Typhimurium 14028s from the lettuce treatments were detected in those from minimal medium and vice versa. These genes were also considered significantly up- and downregulated, respectively. With the web-based tool PANTHER[1] significant enriched GO terms among the significant upregulated genes were identified. The Euler diagrams were created using R package eulerr (Larsson, 2018). The raw sequences were deposited in the Gene Expression Omnibus database (NCBI) with the accession number GSE123152 (GSM3497440-GSM3497442 for MM, GSM3497446-GSM3497448 for LE, GSM3497452-GSM3497454 for LM).

## Visualization of Plant Colonization

To visualize the colonization patterns of *Salmonella* on lettuce and corn salad, the plants were incubated with GFP-labeled *S.* Typhimurium 14028s and subsequently investigated using the SP8 confocal laser scanning microscope (CLSM, Leica Microsystems, Wetzlar, Germany). Lettuce and corn salad plants were grown under sterile conditions in 6-well plates as described above. The ¼ MS medium in the wells was inoculated with *S.* Typhimurium 14028s GFP cells to obtain a final cell count of $10^8$ CFU/mL. After 1, 3, or 5 days, the leaves and roots were stained with propidium iodide solution (1 μg/mL) for 5 min and subsequently mounted on microscope slides in 4',6-Diamidin-2-phenylindol (DAPI) solution (10 μg/mL).

To visualize the colonization of leaf surfaces, a contamination *via* irrigation water was simulated. Lettuce and corn salad leaves were spray-inoculated with a 10 mM $MgCl_2$ with 0.05% Tween 20 containing *S.* Typhimurium 14028s GFP ($10^8$ CFU/mL). After incubation for 6 days at 20°C in the greenhouse, small squares of the leaves were cut with a sterile scalpel and stained as described above with propidium iodide and DAPI.

All observations were performed using: excitation 405 nm, emission 430–480 nm (presented as blue), followed by a second sequential scan of excitation 488, emission 500–550 nm (presented as green) and excitation 561 nm, emission 600–680 nm (presented as red), including autofluorescence of chloroplasts.

[1] http://geneontology.org

## RESULTS

### *Salmonella* Persisted in Agricultural Soils Throughout the Vegetation Period

Contamination with human pathogenic bacteria may occur at all stages of plant production, including the growing period. Since the establishment and persistence of *Salmonella* in agricultural soil(s) are prerequisites of successful plant colonization, we investigated for how long after introduction *Salmonella* would persist in such an environment. Therefore, three different *S. enterica* strains: *S. enterica* serovar Typhimurium strain 14028s (*S.* Typhimurium 14028s), *S.* Typhimurium strain LT2 (*S.* Typhimurium LT2), and *S. enterica* serovar Senftenberg (*S.* Senftenberg) were inoculated into two agricultural soils: diluvial sand (DS) and alluvial loam (AL). Bacteria were inoculated at a cell count of $10^6$ CFU/g of soil. We simulated two different entry routes: (i) *via* organic fertilizer, in this case *Salmonella* was introduced together with chicken litter or pig manure on mixing day, which were selected because both are often contaminated with *Salmonella* and could be potential contamination sources. (ii) *via* irrigation, in this case *Salmonella* was introduced with water on the day of planting, simulating the use of contaminated irrigation water. The CFU counts were monitored during the entire growth period in the different soil conditions. Regardless of the soil type, fertilizer, crop plant or the *Salmonella* strain, *Salmonella* persisted in soil throughout the monitored period, which encompassed the average growth duration for lettuce or corn salad (**Figure 1**). Even though *Salmonella* was detected in the soil during the entire growth phase, the CFU numbers decreased steadily under all conditions. The results suggest that these human pathogenic bacteria can persist in agricultural soils.

### Soil Type and Fertilizer Application Influenced the Persistence of *Salmonella*

The observed long-term persistence of *S. enterica* in soil motivated us to assess factors with potential impact on the persistence such as: plant species, soil type, fertilizer application and manner of *Salmonella* introduction. In order to compare the changes in abundance of *Salmonella* strains in the different experimental variants, the decrease in CFU was plotted as a function of log CFU/g dry soil and days after inoculation. In the next step, the decrease rates of all experimental variants were calculated and presented as slopes of linear regressions, exemplarily shown in **Figure 1** for *S.* Typhimurium 14028s applied to manure-treated or control DS (**Figure 1A**) and AL (**Figure 1B**) soils. Based on the slopes of linear regressions, factors influencing the decrease in *Salmonella* CFU were analyzed using a generalized linear model (**Table 1**). Soil type, fertilizer application and the manner of *Salmonella* introduction significantly influenced the decrease rates ($p < 0.05$), while the plant species did not ($p = 0.18$). Therefore, to improve the readability, we averaged the slopes from lettuce and corn salad samples for each experimental variant (**Table 2**). The data representing the single plant samples can be found in **Supplementary Table S2**. The decrease rates of *Salmonella* CFU counts ranged from −0.008 to −0.110 log (CFU/day). Interestingly, decrease rates in DS soil were higher than in AL soil with $-0.076 \pm 0.014$ and $-0.034 \pm 0.015$ log (CFU/day), respectively (Tukey test, $p < 0.05$). A significant interaction was observed in the linear model between fertilizer application and the manner of *Salmonella* introduction. In case of introducing *Salmonella* together with fertilizer, the decrease rates of *Salmonella* were lower compared to the *Salmonella* introduction at transplanting. This indicates a better survival of the strains when introduced to plant production systems *via* organic fertilizers 1 month prior to planting (**Table 2**). For the tested *Salmonella* strains, the means between the decrease rates were not significantly different (Tukey test, $p > 0.05$) and ranged from $-0.048 \pm 0.029$ (*S.* Typhimurium 14028s) to $-0.052 \pm 0.020$ (*S.* Senftenberg) and $-0.064 \pm 0.028$ (*S.* Typhimurium LT2). In summary, these results revealed that soil type, organic fertilizer and the *Salmonella* inoculation route were major factors influencing the persistence of *S. enterica* in soil.

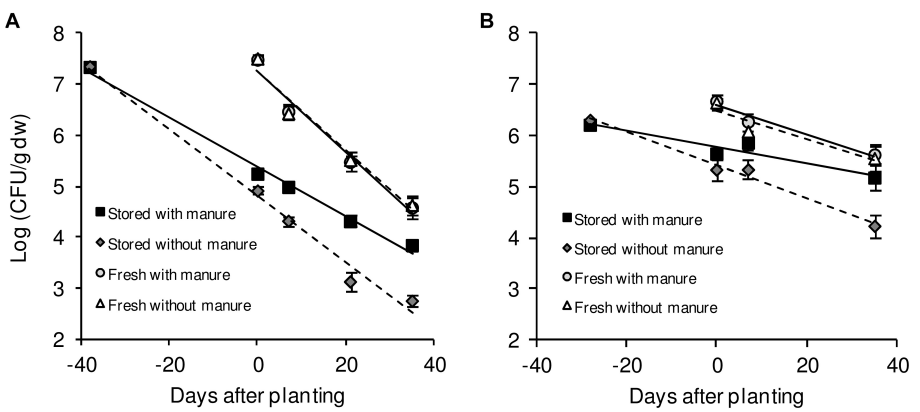


**FIGURE 1 |** CFU counts of *S.* Typhimurium 14028s in DS and AL soil. Exemplarily shown is the decrease of *S.* Typhimurium 14028s colony forming unit (CFU) counts in diluvial sand (DS) **(A)** and alluvial loam (AL) **(B)** soil treated with or without pig manure. The *Salmonella* strain was introduced together with manure application (stored) or on day 0 directly before planting of lettuce (fresh). Shown are the mean values for treatments and respective standard deviations ($n = 8$). Samples with lettuce and corn salad as crop plants were averaged. Solid and dashed lines indicate linear regressions for soil treated with manure or without manure, respectively.

**TABLE 1 |** Factors influencing the persistence of *Salmonella* in agricultural soils.

| Factor | *p*-value |
|---|---|
| Plant (lettuce, corn salad) | 0.1751 |
| Soil type (DS, AL) | < 0.0001 |
| Fertilizer (without, pig manure, chicken litter) | 0.0016 |
| *Salmonella* inoculation (day 0 or mixing day) | 0.0002 |
| Fertilizer application × *Salmonella* inoculation | 0.0085 |

*The decrease in Salmonella CFU counts was analyzed with a generalized linear model based on the slopes of linear regressions. p-values below 0.05 were considered significant.*

**TABLE 2 |** Decline of *Salmonella* in different experimental variants.

| | | | *Salmonella* strains | | |
|---|---|---|---|---|---|
| **Soil type** | **Inoculation time** | **Fertilizer** | **14028s** | **Senftenberg** | **LT2** |
| AL | Mixing day (stored) | Without | −0.030 | −0.034 | −0.052 |
| | | Pig manure | −0.016 | −0.021 | −0.022 |
| | | Chicken litter | −0.008 | −0.024 | −0.018 |
| | Day 0 (fresh) | Without | −0.030 | −0.041 | −0.063 |
| | | Pig manure | −0.028 | −0.040 | −0.053 |
| | | Chicken litter | −0.026 | −0.048 | −0.052 |
| DS | Mixing day (stored) | Without | −0.088 | −0.076 | −0.110 |
| | | Pig manure | −0.049 | −0.053 | −0.059 |
| | | Chicken litter | −0.074 | −0.067 | −0.082 |
| | Day 0 (fresh) | Without | −0.073 | −0.068 | −0.083 |
| | | Pig manure | −0.078 | −0.073 | −0.086 |
| | | Chicken litter | −0.080 | −0.079 | −0.093 |

*Decrease in log (CFU/g dry weight) counts (n = 4 per time point and experimental variant) of Salmonella strains in different treatments and soil types shown as slopes of linear regressions. Values represent means of slopes from lettuce and corn salad samples for the different treatments in log (CFU/day). For a better readability, the color code indicates a range between high and low decrease in CFU (red and yellow color, respectively).*

## Plant Species and *Salmonella* Strain Determined the Colonization Rate of the Phyllosphere

We then asked whether the differences in persistence of *Salmonella* in agricultural soils would influence the colonization rate of crop plants. To answer this question, lettuce and corn salad plants grown under the different conditions described above were harvested during the growth period and analyzed for the presence of *Salmonella* in the phyllosphere. A combination of non-specific enrichment and selective media was used to decide if leaf samples were *Salmonella*-positive. To reduce the number of samples, leaves of two individual plants were pooled into one sample. This resulted in the presented percentage range of plants colonized by *Salmonella* (**Figure 2**). For a better readability, the data were gathered into groups representing the analyzed factors: soil type, plant species, *Salmonella* strain, manner of *Salmonella* inoculation and the type of organic fertilizer (pig manure, chicken litter, without). In total, the fraction of colonized plants in *Salmonella*-contaminated soil (total sampled plants = 3,024) ranged from 0.5 to 0.9%. In general, a higher percentage of plants was colonized in the *Salmonella*-contaminated DS soil (0.9–1.5%) compared to the AL soil (0.3–0.5%) (**Figure 2**, Pearson's Chi-squared test, $p < 0.05$). On average, a higher proportion of lettuce compared to corn salad plants was colonized (0.7–1.4% vs. 0.3–0.4%, respectively, $p < 0.05$ for the maximum values, $p = 0.12$ for the minimum values). *S.* Typhimurium 14028s colonized the highest fraction of lettuce plants (1.6–3%), followed by *S.* Senftenberg (0.4–0.8%) and *S.* Typhimurium LT2 (0.2–0.4%), which displayed the lowest ability to colonize lettuce ($p < 0.05$). For corn salad, no differences between the *Salmonella* strains were observed regarding the number of colonized plants ($p > 0.05$). Furthermore, similar numbers of colonized plants were obtained in the organic fertilizer and control soil treatments ($p > 0.05$). The manner of *Salmonella* introduction (applied with organic fertilizer on day of mixing or on transplanting day zero, about one month after fertilization) did not affect the number of colonized plants in the two soils (**Figure 2**, $p > 0.05$). Interestingly, the overall colonization rate seemed to decrease over the time of plant growth period. However, this decrease was not significant due to the high variability (**Figure 3**, $p > 0.05$).

## *S.* Typhimurium 14028s Persisted Better on Lettuce Than on Corn Salad

One of the biggest differences in the comparisons presented above was the difference in colonization rates between lettuce and corn salad. In order to gain more insights into the ability of *Salmonella* to persist on and in those two crop plants, lettuce and corn salad leaves were spray-inoculated with different cell counts of *S.* Typhimurium 14028s. The presence of *Salmonella* was subsequently determined by CFU counts and in parallel by enrichment and selective plating, during the following 35 days. Generally, directly after spraying (day 0) the CFU of *Salmonella* per g leaf were not different between lettuce and corn salad leaves (**Figure 4**, *t*-test, $p > 0.05$). Over time, however, the CFU on corn salad leaves decreased much faster. *Salmonella* CFU were still quantifiable on day 35 on lettuce leaves spray-inoculated with the highest two cell counts ($10^7$ and $10^6$ CFU/mL) while for corn salad, CFU were quantifiable only until 7 days after spray inoculation. However, after a non-specific enrichment, *Salmonella* was detectable in both plants 35 days post-inoculation. While for corn salad, *Salmonella* was detectable on day 35 only on leaves inoculated with the highest cell count of $10^7$ CFU/mL, *Salmonella* was still detectable on lettuce leaves down to the applied cell count of $10^5$ CFU/mL.

## Colonization Patterns of Lettuce and Corn Salad Plants by *S.* Typhimurium 14028s

The very diverse results obtained for the persistence on lettuce and corn salad implied that the colonization patterns may differ and that *Salmonella* behaves differently depending on the host plant. To verify this assumption, we assessed the colonization patterns on lettuce and corn salad plants. We used *S.* Typhimurium 14028s expressing the green fluorescent protein (GFP) and a CLSM approach. Two different inoculation methods were used: either plants grown under sterile conditions were

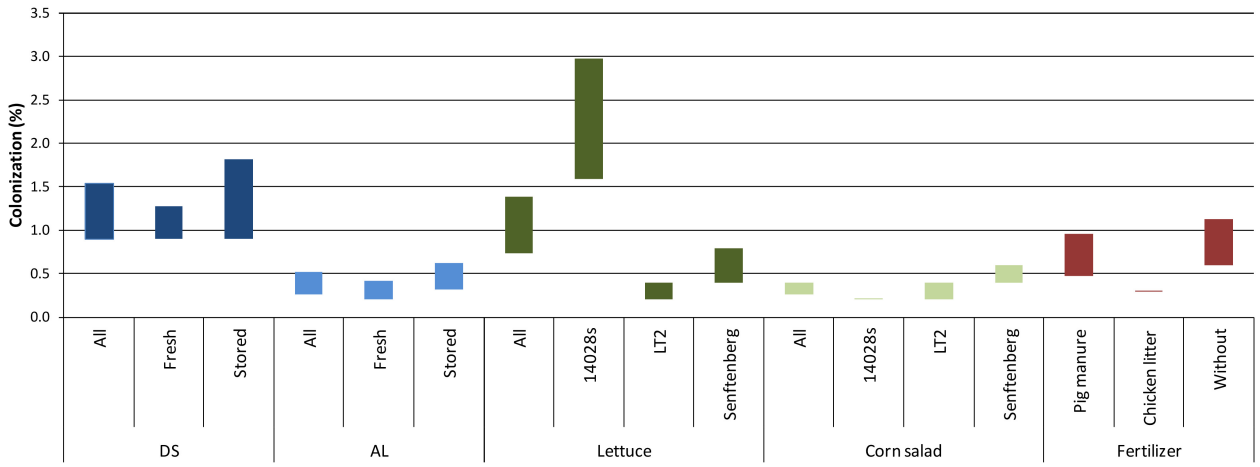


**FIGURE 2 |** Colonization rates of lettuce and corn salad plants by *Salmonella*. Two plants were pooled into one single 50 mL screw cap centrifugation tube. Only leaves that were not in contact with the soil were sampled. The range of colonized plants (*Salmonella* detected in leaf samples after enrichment) is indicated by colored bars showing the range between the minimal and maximal possible colonization rate. The data are grouped, representing the assessed factors: soil type [diluvial sand (DS)/alluvial loam (AL)], plant (lettuce/corn salad), *Salmonella* strain (14028s/LT2/Senftenberg), time of *Salmonella* application [mixing day (stored)/day zero (fresh)] and fertilizer application (pig manure/chicken litter/without). In total, 3,024 plants were sampled excluding the control plants without *Salmonella* inoculation.

inoculated with *Salmonella* in 6-well plates, or plants grown in soil were sprayed with *Salmonella*. In the first approach, the colonization patterns on roots were assessed. Roots of both plants were colonized in a similar manner (**Figure 5**). Very striking was the augmented colonization in the cavities between primary and the emerging secondary root (**Figures 5A,C**). *Salmonella* was also detected in the rhizoplane. However, this technique did not allow the detection of *Salmonella* in the deeper root tissues. Regarding lettuce leaves, the hydathodes showed a high abundance of *Salmonella,* also the space in the cavity below the stomata-like opening was frequently colonized (**Figure 6**). Interestingly, not all hydathode openings were equally colonized, indicating differences in their physiological activity. Leaves of corn salad plants were colonized in a more uniform pattern. *Salmonella* cells were detected attached to the cuticula and also inside the leaf tissue (spongy parenchyma) as well as in the stomatal cavities (**Figure 7**).

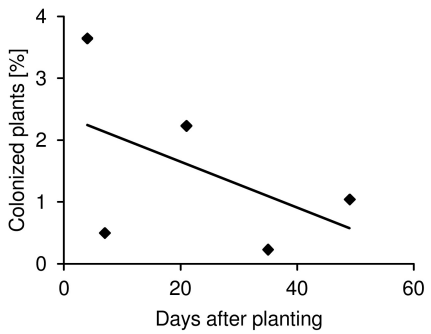


**FIGURE 3 |** Time-dependent colonization of plants by *Salmonella*. The maximum proportion (maximum range) of lettuce and corn salad plants colonized by *S. enterica* over the experimental period is shown. The plant and soil types as well as the *Salmonella* strains are averaged. The solid line shows the linear regression, which in tendency is decreasing but not significantly (linear model analysis, $p > 0.05$, $R^2 = 0.53$).

## S. Typhimurium 14028s Induced the Defense Response in Lettuce

The very close association between lettuce and *Salmonella* made us wonder how the plant perceives the bacteria. In order to assess how lettuce responses to colonization with *Salmonella,* the transcriptome of whole lettuce plants inoculated with *S.* Typhimurium 14028s was compared to plants exposed to $MgCl_2$ (control). Data were obtained from Illumina HiSeq-sequencing of three independent control samples and three independent inoculated plant samples, each comprising three individual plants 24 h after inoculation with *Salmonella*. We obtained approx. $1.23 \times 10^8$ raw reads per sample, of which 93.85% passed quality control. Of the quality-filtered reads, 64.27% were uniquely mapped to the genome of lettuce (*Lactuca sativa* L. cv. Tizian), of which 49.05% could be assigned to a feature. The expression of 1,722 gene orthologs was significantly upregulated in the *Salmonella*-exposed plants, if compared to the $MgCl_2$ control, while the expression of 1,103 gene orthologs was significantly downregulated ($p < 0.05$, **Supplementary Table S3**).

Among the genes with the highest increase in their expression levels after exposure to *Salmonella,* we detected several genes related to defense mechanisms, those genes code for Metalloendoproteinase 1, Pathogenesis-Related Protein PR-1, Glucan Endo-1,3-Beta-Glucosidase and Ethylene-Responsive Transcription Factor 1B. Functional analysis of the differentially expressed genes confirmed that defense-related GO terms were enriched in the group of upregulated genes. Regulation of salicylic acid (SA) biosynthesis as well as the synthesis of lignan

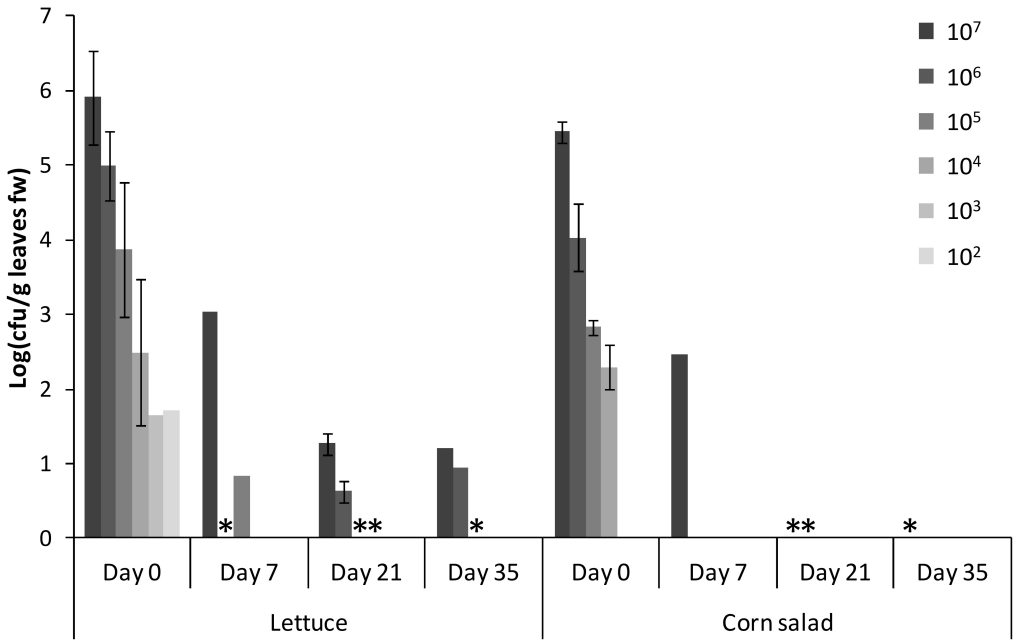


**FIGURE 4 |** Persistence of *S.* Typhimurium 14028s on lettuce and corn salad leaves. Bars display *Salmonella* CFU counts per g leaf fresh weight (fw) and corresponding standard deviations ($n$ = 4) during the days after spray inoculation of the leaves. Asterisks indicate a detection of *Salmonella* only after enrichment. Different cell counts of *Salmonella* (CFU per mL) sprayed on day 0 are indicated by different shades of gray.

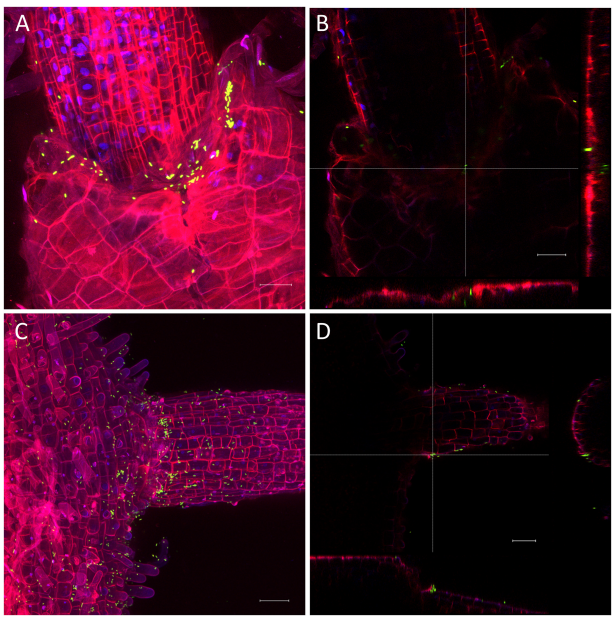


**FIGURE 5 |** Colonization pattern of *Salmonella* on roots. *S.* Typhimurium 14028s GFP on root of lettuce **(A,B)** and corn salad **(C,D)** grown under sterile conditions. Plants were inoculated with GFP-expressing *Salmonella* for 3 days. Maximum projection images **(A,C)** and orthogonal scalings **(B,D)** indicate nucleus in blue, cell walls in red and GFP-labeled *Salmonella* cells in green. Orthogonal scalings show *S.* Typhimurium 14028s GFP cells in the cavity between primary and secondary root **(B,D)**. The scale bars indicate 30 μm **(A,B)** and 40 μm **(C,D)**.

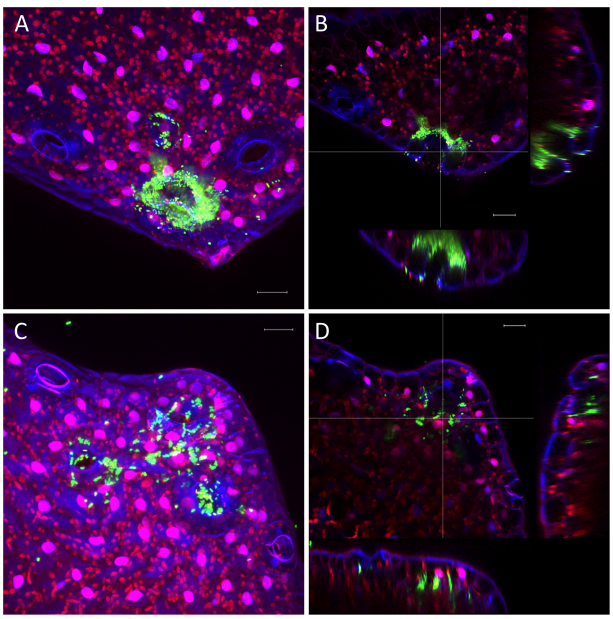


**FIGURE 6 |** Colonization pattern of *Salmonella* on lettuce leaves. Hydathode regions of lettuce leaves inoculated with *S.* Typhimurium 14028s GFP. Leaves were inoculated with *S.* Typhimurium 14028s GFP for 24 h. Maximum projection images **(A,C)** and orthogonal scalings **(B,D)** show hydathodes, nucleus and cuticula in blue, autofluorescence of chloroplasts in red and GFP-labeled *Salmonella* cells in green. Orthogonal scalings show *S.* Typhimurium 14028s GFP cells inside the hydathode openings **(B,D)**. The scale bars indicate 20 μm.

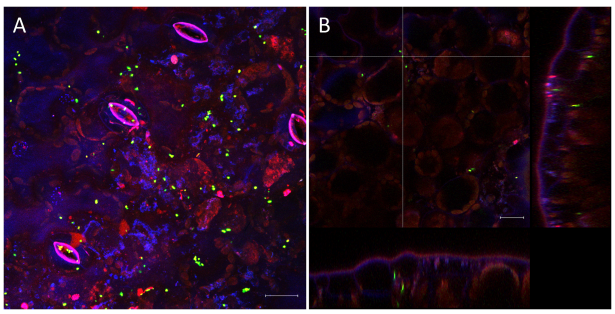


**FIGURE 7 |** Colonization pattern of *S.* Typhimurium 14028s GFP on corn salad leaves. Representative images were taken 6 days after spray inoculation with *S.* Typhimurium 14028s GFP. Maximum projection image **(A)** and orthogonal scaling **(B)** show stomatal openings and cuticula in blue, autofluorescence of chloroplasts in red and GFP-labeled *Salmonella* cells in green. Orthogonal scaling shows *S.* Typhimurium 14028s GFP cells attached to the cuticula but also inside of spongy parenchyma and stomatal cavities **(B)**. The scale bars indicate 20 μm.

and lignin, oxylipins was enriched more than 10 times. Other functional groups included response to stress-related hormones [SA, jasmonic acid (JA), ethylene], defense response or signaling pathways (**Figure 8**). In-depth analysis of the upregulated genes revealed the presence of multiple very prominent defense-related genes. Several receptors (*FLS2, SOBI1, CERK1, CRK2* and *19, RLK1, RPK2, PK1, RLP2,* and *FER* (*feronia*)), numerous transcription factors (*WRKY19, 23, 30, 33, 40, 41, 50, 55, 58, 70, 71, 75*) together with *MYB4, 44* and *108* and *BHLH61* were among them. Additionally, included were multiple kinases: *MPK3, MKK5, YDA, WAK2, 3, WNK 11* and *WAKL 2, 3, 9, 14* and *15*. Furthermore, several ethylene-related genes (*ERF1A, 1B, 2, 4, 5, 9, 37, 98,* and *110* as well as *ERS1* and *EBF2*) and ubiquitin-related (*PUB17, 22, 23,* and *PP2B13*) genes were upregulated in response to *Salmonella*, all demonstrating that lettuce responded to the colonization with *Salmonella* with a very complex immune response. Water homeostasis- and ion transport-related genes were downregulated in our system. Among them were such prominent genes as the phosphate transporter *PHO1* and *IRT2*. A selection of defense-related GO categories is shown in **Figure 9**. A full list of enriched GO terms can be found in **Supplementary Table S4**.

## *Salmonella* Adapted to the Plant Host Nutrients and Adjusted Its Physiology

In a complementary step, we assessed the *S.* Typhimurium 14028s response to lettuce-based medium (LM) or lettuce root exudates (LE). Minimal medium (MM) was used as control. In total, expression of 129 or 264 genes was upregulated in *Salmonella* exposed to LM or LE, while 43 and 62 genes were significantly downregulated, respectively (**Figure 10**). The complete list of differentially expressed genes including the respective fold-changes and *p*-values is provided in **Supplementary Tables S5, S6**. GO term enrichment analysis[2] of all differentially expressed genes revealed six GO terms including, glyoxylate cycle, translation, peptide biosynthetic process, peptide metabolic process, cellular amide metabolic process and amide biosynthesis enriched among genes upregulated in response to LE (**Figure 10**). Surprisingly, no overrepresentation of functional categories was found in response to LM or in the downregulated genes.

In *Salmonella* exposed to LE, genes associated with amino acid biosynthesis showed the highest upregulation, if compared to MM. Among the genes with the highest increase in expression were *amtB, asnA*, and *asnB* as well as *gltI*, associated with ammonium transport, L-asparagine biosynthesis and glutamate/aspartate transport, respectively. Expression of *cysK* coding for a cysteine synthase A was increased in response to LE, if compared to response to LM. Expression of genes involved in isoleucine synthesis was upregulated in bacteria exposed to the root exudates (*ilvE*, *ilvA*, *ilvD*, *ilvL*, *ilvJ*) or in both LE and LM (*ilvG*), if compared to MM. The expression of *proP* was higher in LE, compared to LM. The *proP* gene is involved in proline and/or betaine transport and osmoregulation (Culham et al., 1993) and supported the colonization of alfalfa seedlings by *Salmonella* (Kwan et al., 2018). Gene expression of *argR*, which likely plays a role in the control of arginine biosynthesis and aerobic catabolism (Park et al., 1997), was increased in response to LE. Also expression of *ilvG* and *ilvE*, which are part of the ilvGEDAYC cluster encoding enzymes responsible for isoleucine and valine biosynthesis (Blazey and Burns, 1982) was upregulated. Interestingly, expression of *metW*, *metY,* and *metZ*, associated with methionine biosynthesis (Alaminos and Ramos, 2001; Hwang et al., 2002) was decreased in *Salmonella* exposed to root exudates. Furthermore, expression of several genes associated with *Salmonella* virulence was upregulated, including sulfurtransferase encoding gene *glpE* (compared to LM) and the cytoplasmatic membrane protein encoding gene *dsbB* (in LE compared to MM). Also, expression of *fdnI* and *ogt* was upregulated in response to LE (compared to LM), both were associated with the ability of *Salmonella* to invade host epithelial cells (Lee et al., 2018). Biofilm and curli-associated genes *luxS* and *csgB* were highly expressed in response to LE (compared to MM and LM, respectively).

In contrast to the clear and specific response to LE, *Salmonella* exposed to LM regulates genes associated with a rather general stress response. Among genes with the highest upregulation were *ibpA, clpB, dnaK,* and *dnaJ*. The first encodes a small heat shock protein, reported to stabilize and protect proteins from denaturation and proteolysis during heat and oxidative stresses. Proteins bound to the IbpAB complex were efficiently refolded and reactivated by ClpB, DnaK, and DnaJ, representing ATP-dependent chaperone systems (Melkina et al., 2011). Besides the stress response genes, expression of amino acid biosynthesis genes was increased, e.g., *asnA,* associated with L-asparagine biosynthesis. Furthermore, expression of *feoA* and *feoB*, which code for ferrous iron uptake transporter proteins A and B, was upregulated. Additionally, expression of genes associated with infection and host-pathogen interaction was increased (*feoB*, STM1808, STM0082), as well as genes associated with biofilm formation (*yaiC, luxS*). Interestingly, *sulA,* which is associated with filamentation and downregulation of SPI-1 as

[2]http://www.geneontology.org

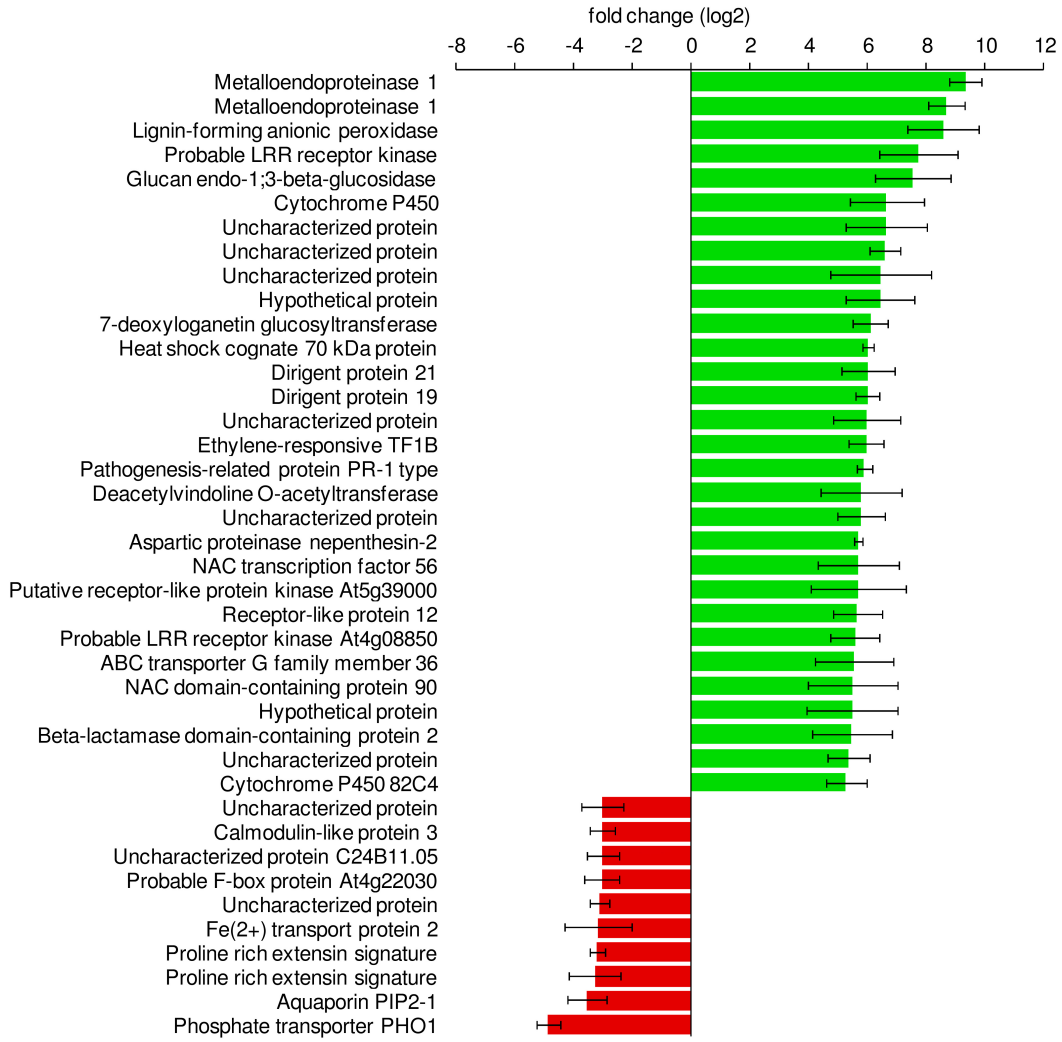


**FIGURE 8 |** Differentially expressed lettuce genes in response to *S.* Typhimurium 14028s. Sterile lettuce plants were exposed to *S.* Typhimurium 14028s or 10 mM $MgCl_2$ for 24 h. The 30 genes with the highest increase in fold change and the 10 with the highest decrease in fold change are shown. Standard errors are indicated by error bars ($p < 0.05$, $n = 3$).

well as *celC*, encoding a protein able to degrade cellulose-type substrates, were highly expressed in response to both LM and LE.

## DISCUSSION

### Persistence of *Salmonella* in Soil Is Influenced by the Soil Type and Application of Organic Fertilizer

Untreated livestock waste used as soil fertilizer has been already postulated as a potential route of contamination with *Salmonella* and other human pathogens (Beuchat, 2002). Pre-harvest contamination is of particular concern, since *Salmonella* can internalize into plant tissue and therefore limit the efficacy of post-harvest sanitizers (Solomon and Sharma, 2009). One of the factors influencing the extent of internalization might be the actual cell count of *Salmonella* in soil (Cooley et al., 2003; Hirneisen et al., 2012). Therefore, the identification and understanding of factors that reduce the persistence of *S. enterica* in soil would help to lower the risk of leafy green contamination. Consequently, the fate of *S. enterica* in the manure-amended soil-plant ecosystem and influencing factors are subject to intensive research (reviewed in Ongeng et al., 2015). The persistence of *Salmonella* was better in AL (loamy) than in DS (sandy) soil, which might be connected to the higher nitrogen and organic carbon contents in AL soil and the higher content of clay (Rühlmann and Ruppel, 2005), which is known for its positive effect on retention of organic and anorganic compounds (Basak et al., 2012). In contrast, a previous study indicated that the number of cultivable *S.* Typhimurium LT2 decreased faster in soil treated with sewage sludge (Fornefeld et al., 2018). The authors suggested that stress imposed by the sewage sludge

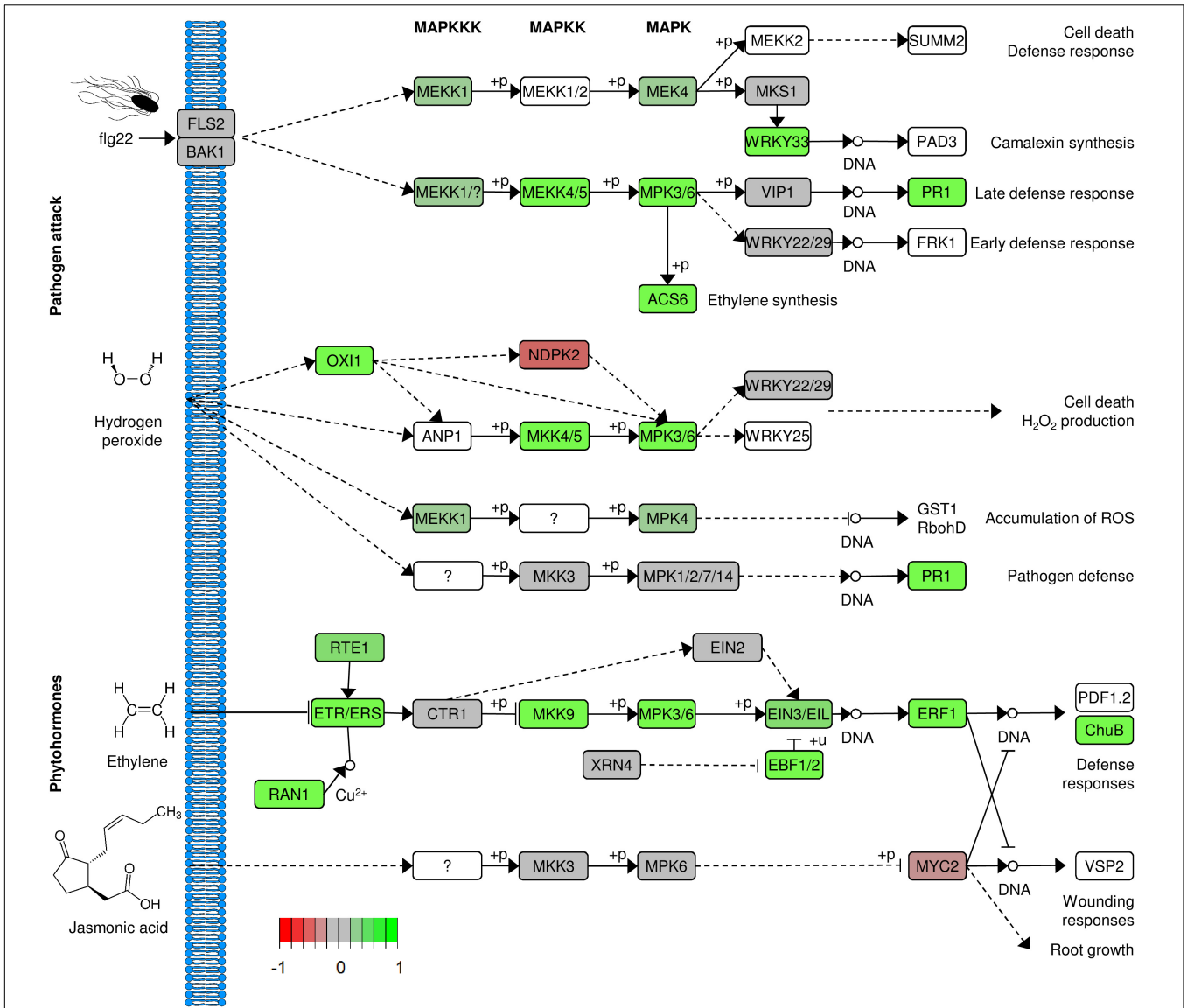


**FIGURE 9 |** Lettuce response to *S.* Typhimurium 14028s. Changes in signaling pathways related to plant defense response after 24 h exposure to *S.* Typhimurium 14028s, compared to exposure to 10 mM $MgCl_2$. Genes were translated to amino acid sequences and those were assigned to Arabidopsis protein orthologs. Up- and down regulated genes in the particular pathways are indicated in green and red, respectively. White background indicates genes not found in the analysis. The figure was based on KEGG and Pathview.

forced *Salmonella* to enter the viable but non-culturable (VBNC) state. Our results support therefore the hypothesis that one of the main factors driving the persistence of *Salmonella* in soil might be the availability and composition of nutrients. The usage of high quality manure (e.g., high in organic matter, low in easily available nutrients) might reduce the persistence of *Salmonella* in soil, as previously suggested by Franz and van Bruggen (2008) for *E. coli* O157:H7. Another cause for the better survival of *S. enterica* in AL compared to DS soil might be the different soil bacterial community structure (Schreiter et al., 2014). Thus, the survival of *S.* Dublin in soils with different soil type and management regime was reported to be correlated with differences in microbial communities rather than with differences in physicochemical properties (Moynihan et al., 2015).

## Root Internalization and Colonization of Plant Leaves

Internalization of *S.* Typhimurium LT2, *S.* Typhimurium ATCC14028, and *S.* Typhimurium S1 into the root of different plants, including lettuce, was demonstrated recently using a sterile system and subsequent FISH-CLSM analysis (Kljujev et al., 2018). In the case of basil, internalization of *S.* Thompson strain FMFP 899 *via* roots was demonstrated using *Salmonella*-soaked germination discs using high cell counts of *Salmonella*

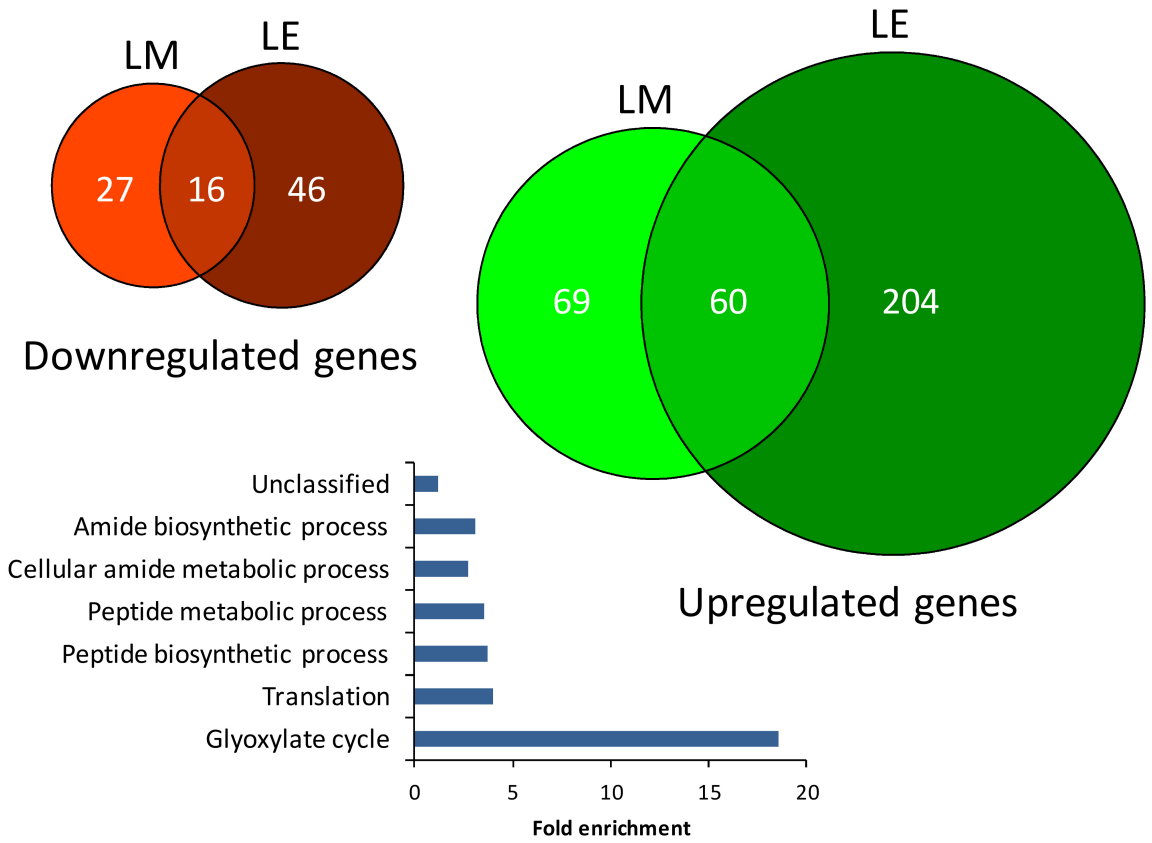


**FIGURE 10 |** Euler diagram of differentially expressed *Salmonella* genes in lettuce medium and lettuce exudates. The up- and downregulated genes from *S.* Typhimurium 14028s exposed to lettuce-based medium (LM) and lettuce exudates (LE) compared to the minimal medium (MM) as control are shown in green and red, respectively. Indicated are genes with significantly higher or lower expression ($p < 0.05$) as well as genes for which the expression was not detected in the treatment or the control. The bar plot indicates significantly enriched GO-terms of *Salmonella* from genes that were differentially regulated in expression after exposure to LE compared to MM as control.

for inoculation (Li and Uyttendaele, 2018). In the present study, we could observe all tested *Salmonella* strains in lettuce and corn salad leaves, indicating that the bacteria were able to internalize into the roots and spread within the plant, probably through the vascular system. Previous studies suggested that *Salmonella* preferentially colonizes emerging zones of secondary roots, rather than root hairs or the root surface. This was confirmed in this study by CLSM. Lateral root cracks were suggested to be sources of nutrients and potential entry sites of *Salmonella* and other bacteria into the plant (Dong et al., 2003). However, these internalization events were rather rare, with an average of 0.7–1.4% of positive plants for lettuce and 0.3–0.4% of positive plants for corn salad. The low rate of internalization might be the reason for the scarcity of internalization-related quantitative data in the literature, suggesting that experiments designed for small numbers of investigated plants likely resulted in (false) negative observations. A comparable range of 2.9% *Salmonella*-colonized plants (1 out of 35) was observed for lettuce after surface sterilization (Honjoh et al., 2014).

Interestingly, although we have shown that organic fertilizers can increase the survival of *S. enterica* in soil, the application of pig manure and chicken litter did not affect the colonization rate of the lettuce or corn salad. This is in line with a previous study, showing that sewage sludge amendment does not promote the internalization of *S.* Typhimurium LT2 into lettuce plants (Fornefeld et al., 2018). Nonetheless, we cannot exclude that the increase in *Salmonella* persistence after fertilizer application will ultimately result in a slightly higher colonization rate. Further experiments are required to precisely answer this question.

Intriguingly, a higher proportion of plants were colonized if the plants were grown in DS soil. Although this seems to be in contrast to the better survival of *Salmonella* in AL, a possible explanation might be different bacterial motility, affected by soil physicochemical properties. In this study, the high clay content in AL soil provided a large surface for microbial attachment and might have led to a decreased mobility of *Salmonella,* which eventually led to a reduced exposure of plant roots and finally resulted in lower colonization rates. Additionally, clay minerals are small, generating a large surface area with combined hydrophilic and hydrophobic properties that can provide adsorbed nutrients to other microorganisms (Cuadros, 2017). Differences in the colonization rate between DS and AL soils might be related to differences in the microbial

communities. For example, Klerks et al. (2007) reported that internalization was more frequent in axenically grown plants than in soil-grown plants. This was associated with reduced competition between the bacterial community and *Salmonella* and the resulting fitness advantage.

Strikingly, our results from the spray inoculation experiment showed that *S.* Typhimurium 14028s was more persistent on lettuce than on corn salad leaves. This might be caused by the differences in surface properties, leaf structures, microclimates and nutrient availability on the leaves. For example, in the case of tomato, plant surface compounds and exudates had a significant effect on *S. enterica* growth and colonization efficiency (Han and Micallef, 2016). Also the hydrophobic properties of the epicuticular wax layer appeared to be important for the colonization of lettuce leaves by *S.* Senftenberg (Hunter et al., 2015). Additionally, the better persistence on lettuce than on corn salad plants might have been associated with differences in the plant defense system. For example, the number of infiltrated *E. coli* O157:H7 isolate Sakai in lettuce and spinach leaves did not increase over time, indicating an arrested/restricted bacterial growth (Wright et al., 2017). Nonetheless, in the same study the authors observed an increase in bacterial number of >400-fold after infiltration of *Nicotiana benthamiana* leaves, suggesting that this difference was due to differences in the plant defense system.

## Induction of Defense Response in Response to *S.* Typhimurium 14028s

Lettuce response to *Salmonella* colonization seems very clear. The upregulation of multiple defense-related genes indicated that as in case of e.g., *A. thaliana* (Schikora et al., 2011), lettuce perceives *Salmonella* and induces its immune system. Numerous upregulated genes point to rather typical Pattern-Triggered Immunity (PTI) response, among them were receptors which recognize such Pathogen-Associated Molecular Patterns (PAMPs) (*FLS2* and *CERK1*), also the induced expression of several MAP kinases and WRKY transcription factors indicated the ability to perceive the bacteria and to react. Very interesting was the fact that in addition to the upregulated expression of many genes involved in the early response to bacterial invader and signaling cascades, also genes associated with the remodeling of the cell wall were upregulated. Several lignin synthases and enzymes involved in lignin synthesis were among them. In summary, the observed response is very similar to an immune response to typical plant pathogens. Interestingly, a recent report suggested that growth of *S.* Typhimurium 14028s is not affected by the *FLS2*-mediated plant defense in *Medicago truncatula.* At the same time, the growth of *S.* Typhimurium strain LT2 was reduced on the wild-type plant but not on the *FLS2*-deficient mutant (Wahlig et al., 2019). The authors hypothesized that these differences were associated with differences in sensitivity toward reactive oxygen species caused by the defect of *rpoS* in strain LT2. This difference might be one possible explanation for the observed lower colonization rate of lettuce by *S.* Typhimurium LT2 compared to *S.* Typhimurium 14028s.

## Transcriptional Changes of *S.* Typhimurium 14028s in Response to Plant Extracts and Root Exudates

From the three tested *S. enterica* strains, *S.* Typhimurium 14028s was the most successful in colonizing lettuce plants, indicating its ability to adapt to the conditions of lettuce rhizosphere and plant tissues. To assess this potential adaptation, we exposed *S.* Typhimurium 14028s to lettuce root exudates (LE) or lettuce-based medium (LM). Analysis of the transcriptome data showed enrichments in GO terms associated with glyoxylate cycle as well as other metabolic and biosynthetic processes in response to root exudates. The glyoxylate cycle was reported to be upregulated under conditions of oxidative stress but plays also an important role in pathogenesis (Ahn et al., 2016). Furthermore, genes associated with *Salmonella* virulence and biofilm formation were upregulated. This is in line with previous observations, showing that *S.* Typhimurium reacted to root exudates with the induction of pathogenicity-related genes (Klerks et al., 2007). Our study revealed the upregulation of *glpE,* which codes for a sulfurtransferase that was shown to contribute to virulence of *S.* Typhimurium in the murine model (Wallrodt et al., 2013). The expression of *dsbB* was also induced in response to LE. This cytoplasmic membrane protein plays a role in SPI-1 regulation and functions by reoxidizing the DsbA protein. DsbA is required for full virulence in a number of pathogenic organisms, such as *Vibrio cholera*, *E. coli* and *Yersinia pseudotuberculosis*, for the proper function of their type III secretion system (T3SS) (Lin et al., 2008). Furthermore, DsbA is required for the proper folding of SpiA of the SPI-2-encoded T3SS (Miki et al., 2004). Interestingly, the quorum sensing-related *luxS* gene was induced in response to both LE and LM. LuxS was previously shown to play an indirect regulatory role in biofilm formation (Ju et al., 2018). Additionally, the expression of the minor curli subunit gene *csgB* was upregulated in response to LE, it mediates the formation of curli by the conversion of CsgA from a soluble protein to an insoluble fiber (Hammer et al., 2007) and was reported to be regulated by a sigma subunit of RNA polymerase, $\sigma^S$ (RpoS) encoded by the *rpoS* gene (Robbe-Saule et al., 2006), which was also upregulated in response to LE. Curli assembly is important for attachment, the first step in biofilm formation. Interestingly, in the LT2 strain the *rpoS* gene has a defect, which might explain the observed lower colonization rate of lettuce. Another GO term complex of upregulated genes in response to LE, was associated with amino acid biosynthesis. Since root exudates are sugar-enriched but amino acid-limited, this finding was less surprising (Kwan et al., 2018). Among them, *cysE*, involved in cysteine synthesis, was shown to be required for colonization of alfalfa seedlings (Kwan et al., 2018). The observed induced expression in response to LE indicates that this gene might be also important for the colonization of lettuce roots.

The lettuce-based medium (LM) used in this study simulates rather the conditions within the plant or in damaged plant tissues. Here, a high proportion of genes associated with stress response followed by amino acid biosynthesis, was upregulated. Furthermore, the expression of *feoA* and *feoB* was significantly increased, both code for ferrous iron uptake transporter

proteins A and B and play an important role in *Salmonella* pathogenesis (Kim H. et al., 2013). Further genes upregulated in response to LM, were: STM1808, which was reported to support the growth of *Salmonella* during systemic infection of mice (Karlinsey et al., 2012) and STM0082, also referred to as gene *srfN,* which was reported for *S. enterica* to be important for intra-host fitness even though, the exact function of this gene is not known so far (Osborne et al., 2009). Strikingly, in *Salmonella* exposed to root exudates the expression of this latter gene was below detection limit. Expression of *sulA,* known to induce the filamentation in *S.* Typhimurium, preventing the invasion of epithelial cells and also associated with a downregulation of SPI-1-related gene expression (Humphrey et al., 2011), was induced upon the exposure to lettuce-based medium. Similar to the response to root exudates, genes associated with biofilm formation were upregulated also in response to LM. Among them was *yaiC*, also referred to as *adrA,* encoding a GGDEF protein, involved in stimulation of cellulose production (Brombacher et al., 2006), one of the major constituents of the *Salmonella* biofilm matrix (Da Re and Ghigo, 2006). The induced expression of the quorum sensing-related *luxS*, which may (indirectly) play a regulatory role in biofilm formation (Ju et al., 2018) was already mentioned above. Very important seems the upregulated expression of *celC*. Its expression was induced in response to both LM and LE. The gene *celC* encodes a protein able to degrade cellulose-type substrates (Yoo et al., 2004) and therefore might play a role in biofilm formation and even the degradation of plant cell walls (Yaronand and Romling, 2014).

In summary, the transcriptome analysis revealed that *S.* Typhimurium 14028s responded to plant compounds by adapting its metabolism. Furthermore, genes associated with virulence, pathogen-host-interactions and biofilm formation were induced, providing evidence that plants are recognized and used by *Salmonella* as alternative hosts. Interestingly, the adaptations to LE and LM on transcriptome level were quite distinct, suggesting that *Salmonella* distinguishes between the conditions that prevail in the rhizosphere and inside plant tissues. This might be crucial for the colonization efficiency and persistence.

## CONCLUSION

The *S. enterica* strains used in this study were able to survive in soils for several weeks, longer in loamy than in sandy soil and when applied together with the organic fertilizer. Both, lettuce and corn salad were colonized by *S. enterica*, providing evidence for the internalization from the soil *via* the root system. Colonization rates were affected by soil type, plant species and *S. enterica* strain, while lettuce was more frequently colonized than corn salad and plants grown in the sandy soil were more often colonized than plants grown in the loamy soil. *S.* Typhimurium 14028s responded to lettuce medium and lettuce root exudate medium by upregulation of genes associated with metabolism, stress response as well as biofilm formation and virulence, indicating that plants are recognized and used by *Salmonella* as alternative hosts. Also, lettuce reacted to *S.* Typhimurium 14028s with strong upregulation of genes associated with plant immune response, indicating that as in case of Arabidopsis, plants perceive *Salmonella* very similar to typical plant pathogens. Taken together, our results reveal that successful strategies for prevention of food-associated disease outbreaks will need to regard the plant production environment as a whole system, including the soil type, the fertilization management practice and maybe most importantly the crop plant.

## AUTHOR CONTRIBUTIONS

SJ, JS, RG, KS, and AS were responsible for research design and concept and wrote the manuscript. SJ, JS, MB, and AS performed the experiments and laboratory work. BF processed the plant transcriptome sequences. SJ and JS processed the *Salmonella* transcriptome sequences. SJ, JS, and AS analyzed the data.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00967/full#supplementary-material

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