Interaction of Phytophagous Insects with *Salmonella enterica* on Plants and Enhanced Persistence of the Pathogen with *Macrosteles quadrilineatus* Infestation or *Frankliniella occidentalis* Feeding

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Abstract

Recently, most foodborne illness outbreaks of salmonellosis have been caused by consumption of contaminated fresh produce. Yet, the mechanisms that allow the human pathogen *Salmonella enterica* to contaminate and grow in plant environments remain poorly described. We examined the effect of feeding by phytophagous insects on survival of *S. enterica* on lettuce. Larger *S. enterica* populations were found on leaves infested with *Macrosteles quadrilineatus*. In contrast, pathogen populations among plants exposed to *Frankliniella occidentalis* or *Myzus persicae* were similar to those without insects. However, on plants infested with *F. occidentalis*, areas of the infested leaf with feeding damage sustained higher *S. enterica* populations than areas without damage. The spatial distribution of *S. enterica* cells on leaves infested with *F. occidentalis* may be altered resulting in higher populations in feeding lesions or survival may be different across a leaf dependent on local damage. Results suggest the possibility of some specificity with select insects and the persistence of *S. enterica*. Additionally, we demonstrated the potential for phytophagous insects to become contaminated with *S. enterica* from contaminated plant material. *S. enterica* was detected in approximately 50% of all *M. quadrilineatus*, *F. occidentalis*, and *M. persicae* after 24 h exposure to contaminated leaves. Particularly, 17% of *F. occidentalis*, the smallest of the insects tested, harbored more than 10^2 CFU/F. *occidentalis*. Our results show that phytophagous insects may influence the population dynamics of *S. enterica* in agricultural crops. This study provides evidence of a human bacterial pathogen interacting with phytophagous insect during plant infestation.

Introduction

The frequency and severity of produce-related foodborne illness outbreaks have increased in the last few decades [1,2]. Although consumption of fruits and vegetables has risen in recent years, these well-publicized foodborne outbreaks trigger consumer concerns about the safety of fresh produce, and impose a negative impact on the agricultural sector. In the US, *Salmonella enterica* is the number one cause of bacterial foodborne illness, and the incidence of infection has not declined over the past 15 years, and instead, has increased slightly since mid-2000s [3,4]. Recently, fresh produce has been linked to more salmonellosis outbreaks than any animal product; and now, plants are considered an important part of the life cycle of enteric human pathogens and as vectors to humans [5].

Human pathogens experience harsh conditions on the leaves of field-grown plants, and survival may depend on trophic interactions. Net growth of *S. enterica* on leaves is rare and populations tend to decline steadily overtime [6-8]. This suggests that multiplication factors are required to induce growth of bacterial populations or sustain infectious populations for extended periods, in what is normally described as a non-host environment. Liberation of plant nutrients by physical damage or plant pathogen infection has been shown to influence the survival of human pathogens [9-12]. The role of additional biological multipliers, such as phytophagous insects remains unexplored.
Bacteria have evolved to exploit insects as hosts and/or vectors. Several studies have found an intimate relationship between insects and members of the Enterobacteriaceae [13], the family to which Salmonella belongs. In fact, numerous insects, such as flies, beetles and cockroaches, are associated with human habitations and livestock facilities, and have been linked with the spread of S. enterica [14]. In all these studies, mechanical transfer of the bacterium on body surfaces after contact with contaminated materials has been suggested as the likely mechanism for movement [14-16]. Several phytophagous insects are considered as widespread pests of agricultural crops many of which are known to be competent vectors of plant pathogens, including members of the Enterobacteriaceae [13,17]. Insect feeding on plants raises the possibility of a biological interaction, in addition to simple physical contamination, between S. enterica and phytophagous insects [18].

Specifically, insect-feeding activity may influence foodborne pathogen populations on leaves. Feeding sites could represent a preferential niche that would allow bacterial multiplication due to access to nutrients liberated from surrounding damaged plant cells or protein/carbon-rich substances excreted during or after feeding by insects [18,19]. The effect of insect feeding on growth of human enteric bacteria on plant surfaces has been documented, however, only with Escherichia coli. Wasala and collaborators [19] reported that regurgitation spots of house flies (Musca domestica) represent a nutrient source that allows E. coli O157:H7 to multiply on spinach leaves. Additionally, Erickson et al. [18] observed higher E. coli O157:H7 populations on lettuce leaves that were inoculated soon after being fed upon by cabbage loopers (Trichoplusia ni). The effect of feeding by phytophagous insects on contaminated plants has not been studied, and the potential for insect activity to act as a ‘biomultiplier’ of S. enterica on agricultural crops remains unknown.

In this study, we investigated the effect of feeding by phytophagous insects on survival of S. enterica in the phyllosphere. We chose lettuce as our model phyllosphere because it is a common host to the three phytophagous insects we chose as representative cell-content and phloem-sap feeders [20-22] and leafy greens are responsible for 23% of the foodborne illness outbreaks associated with contaminated produce [23]. Because the type of mouthparts will also determine the type of damage caused by the insect, and therefore, potentially influence bacterial populations, we examined the interaction of S. enterica with two types of insect feeding. Thrips are cell-content feeders that induce a condition described as ‘silvering’ on leaves, resulting from feeding damage using rasping-sucking mouthparts that damage surface epithelial cells. Hemipteran insects, such as aphids and leafhoppers, are phloem-sap feeders that ingest plant fluids without severe cellular damage to mesophyll cells. We found larger S. enterica populations on leaves co-infested with Macrosteles quadrilineatus. On plants infested with Frankliniella occidentalis, areas of silvering harbored higher S. enterica populations than areas without lesions. We also observed that insect feeding type did not influence insect contamination rates. However, S. enterica populations on individual insects varied by 2 logs.

Materials and Methods

Bacterial strains, media, and culture conditions

Six S. enterica serovars Cubana strain 98A9878 [24], Enteritiidis strain 99A-23 (California Health Department (CHD), July 2005 tomato outbreak), Newport strain 96E01152C-TX [25], Poona strain 00A3563 (CHD, cantaloupe outbreak), Schwarzengrund strain 96E01152C [21], Baildon strain 05x-02123 [26] and Mbandaka strain 99A1670 (CHD, alfalfa seed isolate) were used in this study. These strains were selected because they were responsible for salmonellosis outbreaks associated with contaminated fresh produce. Bacterial cultures were grown overnight on Luria-Bertani (Difco) agar (Difco) containing kanamycin (50 mg/liter) at 37°C. S. enterica strains were suspended from plates in sterile water to an optical density of 0.2 at 600 nm, which approximates 10^8 CFU/ml. S. enterica strains were always inoculated as a six-strain cocktail at 1:1:1:1:1:1 ratio per strain. A S. enterica strain cocktail was used to mitigate possible strain differences in the plant-microbe-insect interaction. Xylose Lysine Desoxycholate (XLD) agar (Difco), a Salmonella semi-selective growth medium in which all chosen strains produce black colonies, was used to determine S. enterica populations from both leaf and insect samples. To verify that the black colonies recovered with XLD were the inoculated strains, each strain was transformed with pKT-Kan that confers kanamycin resistance and constitutive green fluorescent protein expression [27] without affecting the survival and growth of S. enterica on roots [28].

Insect rearing

A Frankliniella occidentalis Pergande (Thysanoptera: Thripidae) colony was maintained on green bean pods (Phaseolus vulgaris L.) on the campus of the University of Wisconsin, Madison, Wisconsin. F. occidentalis colonies were maintained in plastic deli cups under ambient temperature and a 16:8 (L:D) photoperiod as previously described [29]. A colony of Macrosteles quadrilineatus Forbes (Hemiptera: Cicadellidae) was maintained on oat (Avena sativa L.) seedlings in a controlled environment with a 16:8 (L:D) photoperiod (24°C light; 19°C dark) [30]. A colony of Myzus persicae Sulzer (Hemiptera: Aphididae) was kindly provided by Dawn M. Smith (Cornell University), and established and maintained on Chinese cabbage (Brassica rapa) under similar controlled conditions as the M. quadrilineatus colony on the campus of the University of Wisconsin, Madison, Wisconsin.

Lettuce plant inoculation

Lettuce plants (Lactuca sativa cultivar butterhead), were cultivated in a growth chamber without insecticide treatments. Three-week-old plants were dip-inoculated with either sterile water, as a control, or a S. enterica cocktail suspension for 1 min. Plants were allowed to dry under a laminar flow hood and then kept in transparent plastic boxes at 25°C with covers on
samples were homogenized in 500 μl of sterile water, and sampled for cage and 3 cages per plant. Empty cages were placed on infestation exposed to the same conditions. All experiments were repeated at least three times.

Feeding experiments

Lettuce plants were dip-inoculated as described above. Twenty-four hours post inoculation, adult *F. occidentalis* were transferred to half of the lettuce plants at a density of 25 individuals per plant. *F. occidentalis*-infested and *F. occidentalis*-free plants were confined using a cage consisting of a 15 cm-diameter plexiglass tube covered with *F. occidentalis*-proof mesh that surrounded an individual plant and eliminated insect escape and plant exposure to unintended infestation. *S. enterica* leaf populations were enumerated prior to insect infestation, referred to as 0 day post infestation (dpi), and periodically at 4, 9, and 13 dpi. At each sampling time, two 5 mm-diameter leaf discs were excised from each plant. All samples were homogenized in 500 μl of sterile water, and dilution plated on XLD-kan agar, and incubated at 37°C overnight for bacterial-population enumeration. From *F. occidentalis*-infested plants, leaf discs were sampled for *S. enterica* from leaf areas with visible *F. occidentalis* feeding damage (silvering). Each treatment consisted of 8 plants individually potted, which were randomly arranged and exposed to the same conditions. All experiments were repeated at least three times.

The same protocol was followed, as described above, in a separate set of replicated experiments (silvering +/-), except adult *F. occidentalis* were added to all plants. Leaf discs were sampled for *S. enterica* from areas with and without *F. occidentalis* feeding damage within the same leaf. Eight, individually potted plants were used for each experiment and the experiment was repeated three times.

In a separate set of replicated experiments, adult *M. quadrilineatus* and *M. persicae* were transferred to half of the *Salmonella*-inoculated plants and confined in individual clip-cages at a density of 4 *M. quadrilineatus* and 5 *M. persicae* per cage and 3 cages per plant. Empty cages were placed on insect-free plants. *S. enterica* populations were enumerated prior to insect introduction (0 dpi) and periodically at 2, 4 and 9 dpi as described above. Each treatment consisted of 8 plants planted in individual pots, which were randomly arranged and exposed to the same conditions. All experiments were repeated four times.

Microscopy

A subset of leaves from *S. enterica* inoculated plants were collected and examined microscopically with an Olympus BX-60 epifluorescence microscope (Opelco, Dulles, VA). In order to identify preferred colonization sites on leaves from plants exposed and non-exposed to insects, leaf tissue was mounted on microscopic slides and examined for green fluorescence from bacteria as previously described [31].

Insect contamination

Lettuce plants were dip-inoculated as described above. Ten leaves were carefully removed from control and inoculated plants 24 hours after inoculation with a sterile razor blade. Individual leaves were placed inside a sterile petri dish. Non-contaminated insects were collected from respective colonies, and placed in the bottom of each dish containing either the mock or a *S. enterica*-inoculated leaf at different densities due to different insect sizes (*F. occidentalis* = 10, *M. quadrilineatus* = 5, *M. persicae* = 7 per dish). Petri dishes were sealed with a strip of parafilm to prevent insect escape. Live insects were collected in sterile microcentrifuge tubes after a 24 h exposure to *S. enterica*-inoculated leaves, placed at -80°C for 30 min to kill them without affecting potential surface contamination, and enriched in LB overnight at 37°C. Insects in enrichment broth were homogenized and a sterile loop was used to streak the enriched sample onto XLD-kan to verify the presence of *S. enterica* in or on insect bodies. Appearance of black colonies 24 h post-streaking were scored as *S. enterica* positive. A subset of random *S. enterica* presumptive positive, black colonies was confirmed by PCR using primers that target the invA gene of *Salmonella* as previously described [32]. Additionally, leaf samples were collected and plated on XLD-kan agar before insects were added and after they were collected, to verify that inoculated leaves were contaminated with *S. enterica* and control leaves were not. Each treatment consisted of 10 petri dishes containing insects and the experiments were repeated four (*F. occidentalis*) or five (*M. persicae* and *M. quadrilineatus*) times, resulting in a minimum of 200 insects per treatment.

*S. enterica* population size per insect was determined following feeding on contaminated produce. Specifically, green bean pods were surface-sterilized by dipping in 10% bleach solution for 10 min, and placed in individual 50 ml conical tubes containing either 6 ml of sterile water or *S. enterica* suspension (prepared as described above). Conical tubes were placed horizontal in a shaking incubator at 37°C at 200 rpm overnight. Green beans were removed from the liquid and allowed to dry under a laminar flow hood and then placed in new sterile conical tubes. *F. occidentalis* were collected from the corresponding colony, and added to the conical tubes containing either the mock or the *S. enterica*-inoculated beans at a density of 15 *F. occidentalis* per tube. *F. occidentalis*-proof mesh was fixed to the tube cap to prevent insect escape or death from suffocation. Furthermore, green beans samples were collected, serially diluted, and plated on XLD-kan before insects were added and after they were collected, to verify that inoculated green beans were contaminated with *S. enterica* and control green beans were not. Live insects were collected in sterile microcentrifuge tubes after a 24 h contamination period, and placed at -80°C for 30 min to kill them without affecting potential surface contamination. Then, insects were homogenized in sterile water and *S. enterica* populations per insect were enumerated directly on XLD-kan. A subset of random *S. enterica* presumptive positive, black colonies was confirmed by PCR as described above. Each treatment consisted of 6 conical tubes and the experiment was repeated three times, resulting in a minimum of 100 insects per treatment.
Statistical analysis

To determine whether the average population or incidence of S. enterica differed between treatments or over time, analysis of covariance (ANCOVA) was used to test the potential effects of insect feeding on S. enterica populations on leaves, with treatment and time (dpi) as covariates. Bacterial counts were log transformed prior to analysis and repetitions of the experiment were considered as block factors. In this manuscript, the intercept parameter is described as the starting S. enterica population, and the slope parameter is described as a measure of S. enterica population persistence. In the special instance where both silvered and non-silvered leaf tissue was sampled, leaf samples from the same plants were randomly assigned to one of the two treatments prior to insect addition. Therefore, in the analysis of the silverying assay, the intercept was estimated in the same way, but the model was modified to disallow variation between treatments for the y-intercept. For the case of inoculated plants, the rate of decline of S. enterica populations was statistically higher than 50% of the total population of insects tested. All statistical analysis were performed using R software [33].

Results

Extended survival of S. enterica on lettuce leaves in areas damaged by cell-content feeders

Lettuce plants inoculated with S. enterica were exposed to F. occidentalis to investigate if insect infestation influenced S. enterica populations. Although plants were inoculated at a high concentration (10⁸ CFU/ml), bacterial populations that colonized leaves at the beginning of the insect infestation interval (0 dpi), averaged 10⁴ CFU/mm². S. enterica was not recovered from uninoculated control plants (data not shown). In the case of inoculated plants, S. enterica populations declined over time. Surprisingly, the final bacterial concentrations did not reach zero, even 14 days after inoculation. In addition to differences in bacterial populations over the sampling interval (dpi P<0.05); the slopes varied among experimental replications (exp: dpi P<0.05; Figure 1). However, no significant differences were observed in S. enterica populations (P>0.05) or population decline over time among plants exposed or non-exposed to F. occidentalis, evident in the interaction trt: dpi (P>0.05, Figure 1). F. occidentalis were freely released onto whole plants instead of being confined on individual leaves, or portions of leaves, allowing them to feed in an unrestricted manner over the entire plant. In turn, samples from the same plant were collected from different leaves, because of a lack of sufficient F. occidentalis feeding sites (silvering) on the same leaf potentially increasing the variability among samples.

However, using fluorescent microscopy, we consistently observed the presence of gfp-tagged S. enterica cells accumulating in areas that were fed upon by F. occidentalis (data not shown); therefore, the effect of F. occidentalis feeding damage was further investigated. Survival of S. enterica in feeding areas with obvious silvering was compared with areas without visible feeding damage in the same plant. Similar to our earlier findings, bacterial populations declined over the sampling interval (dpi P<0.05). But in contrast to the experiments with or without F. occidentalis, bacterial populations were similar among experimental replications (P>0.05) in these experiments. Overall, S. enterica population decline was delayed in areas with silverying (trt: dpi interaction, P<0.05), and sustained significantly higher bacterial populations even at 10 and 13 dpi when compared to undamaged areas (Figure 2). Consistently, S. enterica was not recovered from uninoculated control plants.

Enhanced survival of S. enterica on lettuce plants in the presence of certain phloem-sap feeding insects

To determine if S. enterica survival on lettuce was specific or unique to F. occidentalis feeding, S. enterica population survival was examined in plants exposed to phloem-feeding insects. In experiments where S. enterica-inoculated plants were exposed to M. persicae, significant variation in bacterial populations was observed along sampling days (dpi, P<0.05; Figure 3). However, there were no significant differences in S. enterica populations between treatments (P>0.05) or in population decline over time among plants exposed or non-exposed to M. persicae (trt: dpi, P>0.05). In contrast, exposure of plants to M. quadrilineatus enhanced S. enterica survival compared to plants that were not exposed to insects (P<0.05; Figure 4). Likewise, the rate of decline of S. enterica was significantly attenuated in the presence of M. quadrilineatus (trt: dpi, P<0.05), resulting in approximately half a log higher bacterial populations at 13 dpi in M. quadrilineatus-exposed plants.

Phytophagous insects become contaminated and harbor elevated S. enterica populations from contaminated produce

The potential for phytophagous insects to become contaminated with S. enterica from contaminated plant material was observed by detection of the bacteria in approximately 50% of all insects tested. A total of 241 F. occidentalis, 229 M. quadrilineatus, and 289 M. persicae were exposed to contaminated lettuce leaves for 24 h, and subsequently tested for the presence of S. enterica. Dead insects were not collected to assure that sampled insects were in contact with contaminated plant tissue. S. enterica was not isolated from untreated control treatments. From the exposed insects, 52% of F. occidentalis and M. persicae were positive for S. enterica, while in the case of M. quadrilineatus, the contamination rate was slightly lower (47%). Hypothesis testing with Z-scores (F. occidentalis Z=0.45, M. quadrilineatus Z=0.99, M. persicae Z=0.64) suggest that rates of contamination were not significantly different when compared to the remaining 50% of the corresponding insect population.

To further characterize the potential for insect contamination with S. enterica, S. enterica populations were enumerated from individual insects following feeding on contaminated plant tissue. F. occidentalis were used in this experiment because they are relatively tiny and slender (usually 1-2 mm long), and represent the smallest in size of the insects evaluated in this study. S. enterica was not isolated from untreated control treatments. Based on direct plating, 68% (98 out of 145) of the
F. occidentalis tested positive for contamination of S. enterica, while 32% (47 thrips) tested negative (Figure 5). Populations between 1-20 CFU’s were recovered from more than 30% (46 thrips) of the F. occidentalis; however, this value could be higher considering that insects that tested negative were not subjected to enrichment methods and could have had S. enterica counts below the detection limit. Interestingly, 17% (24 thrips) of the F. occidentalis harbored more than $10^2$ S. enterica CFU on their bodies (Figure 5). It is important to note that this method does not allow us to distinguish from S. enterica contamination of insect body versus cells ingested.

Figure 1. *Salmonella enterica* population dynamics on plants exposed to *Frankliniella occidentalis*. Lettuce plants were exposed (open circles) or non-exposed (close circles) to *F. occidentalis*. Shown is the mean log population of *S. enterica* on lettuce leaf samples (CFU/mm²) at 0, 4, 9 and 13 days post infestation. The data represent the means of three independent experiments. Lines (black, exposed; dotted, non-exposed) correspond to a linear regression model, and shaded areas to their associated 95% confidence interval.

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In this study, *F. occidentalis*, *M. quadrilineatus*, and *M. persicae* were used as model systems to study the role of insects as potential biomultipliers of *S. enterica* on plants. Thrips, leafhoppers, and aphids were selected because they are both common agricultural pests and vectors of phytobacterial pathogens of several agricultural crops, including lettuce [20-22,34-36]. These insects have two distinct types of mouthparts and unique feeding strategies that enabled comparison of feeding behaviors and their respective influence on the colonization of plants by *S. enterica*.

![Figure 2](image.png)

**Figure 2.** Extended survival of *Salmonella enterica* on lettuce leaves in areas damaged by *Frankliniella occidentalis*. Open circles represent areas with feeding damage and close circles areas without feeding damage caused by *F. occidentalis*. Shown is the mean log population of *S. enterica* on lettuce leaf samples (CFU/mm²) at 0, 4, 9 and 13 days post infestation. The data represent the means of three independent experiments. Lines (black, exposed; dotted, non-exposed) correspond to a linear regression model, and shaded areas to their associated 95% confidence interval.

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**Discussion**

In this study, *F. occidentalis*, *M. quadrilineatus*, and *M. persicae* were used as model systems to study the role of insects as potential biomultipliers of *S. enterica* on plants.
It was observed that feeding damage caused by *F. occidentalis* enhanced survival of *S. enterica* in comparison with areas of the same plant where feeding lesions were not visible. *F. occidentalis* have rasping-sucking mouthparts and feed by rasping the surface of the leaves and ingesting fluids of the mesophyll and epidermal cells of leaf tissues [37,38]. Specifically, the thrips mandible pierces a hole in the leaf and cell contents are ingested via a cibarial pump which extracts cellular contents through maxillary stylets [39]. However, the feeding process combines periods of probing or stylet penetration, and non-probing [38], and the cell damage is correlated to the frequency of probing and the duration of each

Figure 3. *Salmonella enterica* population dynamics on plants exposed to *Myzus persicae*. Lettuce plants were exposed (open circles) or non-exposed (close circles) to *M. persicae*. Shown is the mean log population of *S. enterica* on lettuce leaf samples (CFU/mm²) at 0, 2, 4 and 9 days post infestation. The data represent the means of four independent experiments. Lines (black, exposed; dotted, non-exposed) correspond to a linear regression model, and shaded areas to their associated 95% confidence interval.

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probe, from the time of insertion to removal of the maxillary styles [38]. We hypothesized that *F. occidentalis* feeding damage allowed *S. enterica* survival by providing direct access to cellular cytoplasm for successful colonization. However, it is also recognized that this feeding behavior can result in an extreme plasmolysis leaving completely empty cells, which after a short period of time, cannot provide appropriate nutrients to sustain growth of the pathogen. This may explain why similar declines in *S. enterica* population rates were observed among plants exposed to both infested and uninfested treatments. It is possible that the sampled tissue from *F. occidentalis*-infested plants was too damaged or

Figure 4. Increased survival of *Salmonella enterica* on plants exposed to *Macrosteles quadrilineatus*. Lettuce plants were exposed (open circles) or non-exposed (close circles) to *M. quadrilineatus*. Shown is the mean log population of *S. enterica* on lettuce leaf samples (CFU/mm²) at 0, 2, 4 and 9 days post infestation. The data represent the means of four independent experiments. Lines (black, exposed; dotted, non-exposed) correspond to a linear regression model, and shaded areas to their associated 95% confidence interval.

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damaged leaves over fresh leaves has been previously reported as a mechanism that enables the uptake of symbiotic gut bacteria [41]. In our study, significant \textit{S. enterica} populations were recovered from \textit{F. occidentalis}-damaged areas of lettuce leaves. Although \textit{F. occidentalis} lesions may appear inconsequential to consumers, our results suggest that thrips feeding scars not only reduce aesthetic quality, but can also serve as potential reservoirs of human pathogenic bacteria on plants and may increase the food safety risk.

Results from the current study suggest that human bacterial pathogen survival can be influenced by the presence of specific phytophagous insect taxa, with unique feeding strategies. We initially hypothesized that feeding of both \textit{M. persicae} and \textit{M. quadrilineatus} may fail to induce \textit{S. enterica} persistence or growth in the phyllosphere, on the premise that they do not facilitate direct access to cellular cytoplasm for pathogen use. Most hemipterans depend exclusively on phloem sap as their primary source of nutrients, and they possess highly modified piercing-sucking mouthparts that allow them to ingest fluids from plant vascular, epidermal, and/or mesophyll cells [17]. Mouthparts consist of a needle-like stylet bundle and a salivary canal that are used to ingest plant fluids and also deliver saliva into the feeding site. However, substantial differences among hemipteran feeding mechanisms have been described [17,42]. Interestingly, we found different effects on \textit{S. enterica} persistence on leaves infested with \textit{M. persicae} or \textit{M. quadrilineatus}. It is possible that differences in stylet penetration behaviors could influence \textit{S. enterica} survival in this study. Unlike the intercellular penetration style of sternorrhynchan stylets, the intracellular stylet of auchenorrhynchan stylets, such as those of leafhoppers [42], could have benefited \textit{S. enterica} through leaking of phloem sap, similar to that which can occur with feeding by \textit{F. occidentalis}.

Feeding behavior, instead of mouthpart type, may correlate with human bacterial pathogen survival in infested leaves. Miles [43] described two different feeding strategies used by all hemipterans: “sheath feeding” in which insects protect their stylet tips with a sheath made of solidifying saliva, and “lacerate-and-flush” feeding in which stylets puncture plant tissues and rupture cellular matter, while releasing watery saliva, and then ingest the resulting fluid. Later, Backus et al. [42] renamed the second strategy as cell rupture feeding. Although, it has long been thought that the sheath feeding is the primary strategy used by most auchenorrhynchan species, various studies have more recently reported \textit{Empoasca} spp. leafhoppers (Hemiptera: Cicadellidae), as cell rupture feeders, not salivary sheath feeders [42,44]. This feeding strategy involves ingestion of mesophyll cell contents, and comprises two sub-strategies that vary in duration and intensity of cell laceration, which can be alternated on different tissues or host plants [42]. Although it has not yet been described, it is possible that in our study \textit{M. quadrilineatus} used an intermediate feeding strategy that allowed the enhanced persistence of \textit{S. enterica} by causing less drastic mesophyll cell damage and release of phloem-sap contents. Additionally, it is possible that the larger stylets of \textit{M. quadrilineatus}, compared to \textit{M. persicae}, caused more physical damage to

### Figure 5.

**Frequency distribution of \textit{Salmonella enterica} population size per \textit{Frankliniella occidentalis}**. Shown is the number of \textit{F. occidentalis} (N=145) from which specific \textit{S. enterica} populations were recovered after 24 h acquisition access period. Populations represent the number of \textit{S. enterica} CFU counted per individual \textit{F. occidentalis} homogenized, except for 100+ that also include populations that were too high to count. The zero column are those \textit{F. occidentalis} which were not carrying \textit{S. enterica} or whose populations were below the level of detection without enrichment. Data from three independent experiments were combined.

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- Damaged leaves over fresh leaves have been previously reported as a mechanism that enables the uptake of symbiotic gut bacteria [41]. In our study, significant \textit{S. enterica} populations were recovered from \textit{F. occidentalis}-damaged areas of lettuce leaves. Although \textit{F. occidentalis} lesions may appear inconsequential to consumers, our results suggest that thrips feeding scars not only reduce aesthetic quality, but can also serve as potential reservoirs of human pathogenic bacteria on plants and may increase the food safety risk.

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**Phytophagous Insects-Salmonella Interactions**
Plant defense response to herbivores may influence human pathogen populations. Feeding strategies that cause more aggressive damage to plant tissue, such as chewing, rasping-sucking, or repeated perforation of multiple plant cells stimulate the plant’s jasmonic acid dependent and independent wound-responses [17,45]. Several types of Lepidopteran caterpillars, Coleoptera, Tetranychid mites, Thysanoptera, and certain other Hemipteran leafhoppers are known to cause these types of injuries when feeding on plant material [17,46,47]. On the other hand, other Hemipteran insects such as whiteflies and some aphids follow intercellular pathways in the leaf as they probe for suitable feeding sites, causing minimal to unnoticeable cellular damage [17]. This type of feeding behavior mimics infection processes of biotrophic phytopathogens and usually plants respond with salicylic acid (SA) dependent pathways [17,46,48]. It is possible that plant-wounding responses induced by M. quadrilineatus feeding could have indirectly benefited S. enterica colonization of lettuce plants by antagonizing defense responses associated with pathogen establishment and infection, such as SA-dependent and – independent defenses and pathogenesis related proteins. Plant response to thrips and aphid feeding involves several signaling pathways associated with both pathogen infection and wounding [45,46]. Moreover, Mouttet et al. [47] reported that production of secondary metabolites by rose (Rosa hybrida cv. Sonia) plants previously infected with a plant pathogen could have had an adverse effect on aphids and thrips feeding. However, Erickson and collaborators observed lower E. coli O157:H7 populations internalized within leaves previously exposed to insects, including aphids and thrips [18]. In our study, S. enterica populations, among plants exposed to F. occidentalis or M. persicae, were similar to those without insects, suggesting that defense responses induced by aphids or thrips do not have a relevant effect on S. enterica populations. Moreover, whether S. enterica contamination of plants positively or negatively affects the behavior of phytophagous insects remains unknown.

It seems likely that only insect feeding behaviors that cause direct damage to plant cells tended to enhance the longevity of S. enterica on lettuce. However, the ability of phytophagous insects to become contaminated with the human pathogen seems to be independent of feeding strategy used. It was demonstrated that F. occidentalis, M. quadrilineatus, and M. persicae could become contaminated with S. enterica from contaminated plant tissues including lettuce leaf and green bean pods. Particularly, F. occidentalis, the smallest of the insects tested, harbored large S. enterica populations after a 24 h access period to contaminated plant material. It is well known that adult thrips are not strong flyers; however, they are quite active and can move quickly on the surface of leaves. In fact, the wandering behavior, which involves roaming, scraping of their heads, and search for new-feeding sites, is characteristic of thrips when they are not probing [37]. This, in addition to their thigmotactic behavior that brings them in close contact with their host plant [49], suggests their potential to influence the persistence and potentially the spread of S. enterica-adhered to their bodies over leaf surfaces and flowers. It is acknowledged that the use of high inoculum concentrations that are unlikely to occur in natural environments could have increased the probability of movement of bacteria by insects. Nevertheless, it is important to emphasize that bacterial populations at the time of infestations were similar to concentrations of Salmonella recovered from drainage water [50] and wound-inoculated tomatoes after treatment with chlorine water [40]. Taken together, these results highlight the potential role of insect pests of agricultural crops to influence the population dynamics of the human pathogen, S. enterica. Although in this study it was not determined whether S. enterica could adhere to the outside of phytophagous insects or be ingested, the potential for these insects to be biological vectors of S. enterica remains to be determined. Furthermore, since S. enterica was recovered from insect bodies and insect damaged plant material, insects or insect-damaged plant tissue could be exploited as a novel sentinel strategy for S. enterica-contaminated crop monitoring.

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Author Contributions

Conceived and designed the experiments: JPSA RG JDB. Performed the experiments: JPSA. Analyzed the data: JPSA RG JDB. Contributed reagents/materials/analysis tools: RG JDB. Wrote the manuscript: JPSA RG JDB.

References

1. Fletcher J, Leach JE, Eversole K, Tauxe R (2013) Human pathogens on plants: designing a multidisciplinary strategy for research. Phytopathology 103: 306–315. doi:10.1094/PHYTO-09-12-0236-IA. PubMed: 23406434.
2. Lynch MF, Tauxe RV, Hedberg CW (2009) The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. Epidemiol Infect 137: 307–315. doi:10.1017/ S0950268808001969. PubMed: 19200406.
3. Batz MB, Hoffmann S, Morris JG (2011) Ranking the Risks: The 10 Pathogen-Food Combinations with the Greatest Burden on Public Health. University of Florida. Gainsville, FL: Emerging Pathogens Institute.
4. CDC (2011) Vital signs: incidence and trends of infection with pathogens transmitted commonly through food --- foodborne diseases active surveillance network, 10 U.S. sites, 1996—2010. MMWR Morb Mortal Wkly Rep 60: 749–755. PubMed: 21659984.
5. Barak JD, Schroeder BK (2012) Interrelationships of food safety and plant pathology: the life cycle of human pathogens on plants. Annu Rev Phytopathol 50: 241–266. doi:10.1146/annurev-phyto-081211-172936. PubMed: 22656644.
6. Islam M, Morgan J, Doyle MP, Phatak SC, Millner P et al. (2004) Fate of Salmonella enterica serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. Appl Environ Microbiol 70: 2497–2502. doi:10.1128/AEM. 70.4.2497-2502.2004. PubMed: 15066849.
7. Islam M, Morgan J, Doyle MP, Phatak SC, Millner P et al. (2004) Persistence of Salmonella enterica serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with...
contaminated manure compost or irrigation water. Foodborne Pathog Dis 1: 27–35. doi: 10.1089/1535-2490-5-4.2008.0147. PubMed: 18532219.

8. Zheng J, Allard S, Reynolds S, Millner P, Arce G et al. (2013) Colonization and internalization of salmonella enterica in tomato plants. Appl Environ Microbiol 79: 2494–2502. doi: 10.1128/AEM.03704-12. PubMed: 23702609.

9. Arucavage D, Miller SA, Ivey ML, Lee K, Lejeune JT (2008) Survival and dissemination of Escherichia coli O157:H7 on physically and biologically damaged lettuce plants. J Food Protect 71: 2384–2388. PubMed: 19244686.

10. Yamazaki A, Holmes J, Hutchins WC, Wang L, Ma J et al. (2011) Commensal effect of pectate lyases secreted from Dickaea dandantii on proliferation of Escherichia coli O157:H7 EDL933 on lettuce leaves. Appl Environ Microbiol 77: 156–162. doi: 10.1128/AEM.01079-10. PubMed: 21075884.

11. Barak JD, Lian AS (2008) Role of soil, crop debris, and a plant pathogen in Salmonella enterica colonization of tomato plants. PLOS ONE 3: e1657. doi: 10.1371/journal.pone.0001657. PubMed: 18301739.

12. Kwan G, Charkowski AO, Barak JD (2013) Salmonella enterica suppresses Pectobacterium carotovorum subsp. carotovorum population and soft rot progression by acidifying the microaerophilic environment. mBio 4(1): e00057-12.

13. Nadarasa A, Slavrinides J (2011) Insects as alternative hosts for plant-pathogenic bacteria. FEMS Microbiol Rev 35: 555–575. doi: 10.1111/j.1574-6976.2011.02264.x. PubMed: 21251027.

14. Blazir JM, Lienau EK, Allard MW (2011) Insects as vectors of foodborne pathogenic bacteria. Terr Arthropods Rev 4: 5–16. doi: 10.1163/18748311X543896.

15. Kopanic RJ, Sheldon BW, Wright CG (1994) Cockroaches as vectors of phytopathogenic bacteria. FEMS Microbiol Rev 35: 555–575. doi: 10.1111/j.1574-6976.2011.02264.x. PubMed: 21251027.

16. Blazar JM, Lienau EK, Allard MW (2011) Insects as vectors of foodborne pathogenic bacteria. Terr Arthropods Rev 4: 5–16. doi: 10.1163/18748311X543896.

17. Mian LS, Maag H, Tacal JV (2002) Isolation of Commensal effect of pectate lyases secreted from Dickaea dandantii on proliferation of Escherichia coli O157:H7 EDL933 on lettuce leaves. Appl Environ Microbiol 77: 156–162. doi: 10.1128/AEM.01079-10. PubMed: 21075884.

18. Mian LS, Maag H, Tacal JV (2002) Isolation of Commensal effect of pectate lyases secreted from Dickaea dandantii on proliferation of Escherichia coli O157:H7 EDL933 on lettuce leaves. Appl Environ Microbiol 77: 156–162. doi: 10.1128/AEM.01079-10. PubMed: 21075884.

19. Parker WE, Collier RH, Ellis PR, Mead A, Chandler D (2002) Matching transmission by females. Entomol Exp Appl 88: 9–15. doi: 10.1046/j.1570-7458.1998.00340.x.

20. Stafford CA, Walker GP, Ullman DE (2011) Infection with a plant virus modifies vector feeding behavior. Proc Natl Acad Sci U_S_A 108: 2320–2325. doi: 10.1073/pnas.1005731108. PubMed: 21663672.

21. Chisholm IF, Lewis T (1994) A new look at thrips (Thysanoptera) mouthparts, their action and effects of feeding on plant tissue. Bull Entomol Res 74: 663–675. doi: 10.1016/S00704853(99)01408-4.

22. Selway AJ, Archer DL, Goodrich RM, Barta JT, Schneider KR (2006) Chlorine disinfection of tomato surface wounds contaminated with Salmonella spp. Hort Technol 16: 253–256.

23. De Vries EJ, Bos RA, Jacobs G, Breeurev HAJ (2006) Western flower thrips (Thysanoptera: Thripidae) preference for thrips-damaged leaves over fresh leaves enables uptake of symbiotic gut bacteria. Eur J Entomol 103: 779–786.

24. Backus EA, Serrano MS, Ranger CM (2005) Mechanisms of hopperburn: an overview of insect taxonomy, behavior, and physiology. Annu Rev Entomol 50: 125–151. doi: 10.1146/annurev.ento.50.052304.151355.

25. Miles PW (1972) The saliva of Hemiptera. Adv Insect Physiol 6: 75–148. doi: 10.1016/S0065-2806(08)60277-5.

26. Jin S, Chen ZM, Backus EA, Sun XL, Xiao B (2012) Characterization of EPG waveforms for the tea green leafhopper, Empoasca vitis Góthe (Hemiptera: Cicadellidae), on tea plants and their correlation with stylist activities. J Insect Physiol 58: 1234–1244. PubMed: 22750027.

27. Abe H, Ohnishi J, Narusaka M, Seo S, Narusaka Y et al. (2005) Function of jasmonate in response and tolerance of Arabidopsis to thrips feeding. Plant Cell Physiol 46: 68–80. doi: 10.1038/sj.pcp.4501686. PubMed: 15048512.

28. Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. Plant Physiol 125: 1074–1085. doi: 10.1104/pp.125.2.1074. PubMed: 11511062.

29. Frankliniella occidentalis (Pergande) and a pathogen sharing a host plant: evidence for indirect plant-mediated interactions. PLOS ONE 6: e18840. doi: 10.1371/journal.pone.0018840. PubMed: 21611161.

30. Inoue HD, Ahmer BM, Stone JM et al. (2005) Regulation of enteric endophytic bacterial colonization by plant defenses. Mol Plant Microbe Interact 18: 169–178. doi: 10.1094/MPMI-18-0169. PubMed: 15720086.

31. Lloyd CR (2009) Western flower thrips (Frankliniella occidentalis) management on ornamental crops grown in greenhouses: have we reached an impasse? Pests Technol 3: 1–9.

32. Jacobsen CS, Bech TB (2012) Soil survival of Salmonella and transfer to freshwater and fresh produce. Food Res Int 45: 557–566. doi: 10.1016/j.foodres.2011.07.026.