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Transmission and Retention of *Salmonella enterica* by Phytophagous Hemipteran Insects

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Several pest insects of human and livestock habitations are known as vectors of *Salmonella enterica*; however, the role of plant-feeding insects as vectors of *S. enterica* to agricultural crops remains unexamined. Using a hemipteran insect pest-lettuce system, we investigated the potential for transmission and retention of *S. enterica*. Specifically, *Macrosteles quadrilineatus* and *Myzus persicae* insects were fed *S. enterica*-inoculated lettuce leaf discs or artificial liquid diets confined in Parafilm sachets to allow physical contact or exclusively oral ingestion of the pathogen, respectively. After a 24-h acquisition access period, insects were moved onto two consecutive noninoculated leaf discs or liquid diets and allowed a 24-h inoculation access period on each of the two discs or sachets. Similar proportions of individuals from both species ingested *S. enterica* after a 24-h acquisition access period from inoculated leaf discs, but a significantly higher proportion of *M. quadrilineatus* retained the pathogen internally after a 48-h inoculation access period. *S. enterica* was also recovered from the honeydew of both species. After a 48-h inoculation access period, bacteria were recovered from a significantly higher proportion of honeydew samples from *M. quadrilineatus* than from *M. persicae* insects. The recovery of *S. enterica* from leaf discs and liquid diets postfeeding demonstrated that both species of insects were capable of transmitting the bacteria in ways that are not limited to mechanical transmission. Overall, these results suggest that phytophagous insects may serve as potential vectors of *S. enterica* in association with plants.

In the United States, more than 9 million cases of food-borne illness are caused each year by consumption of contaminated food (1). According to the Centers for Disease Control and Prevention, almost half of those illnesses that occurred from 1998 to 2008 were attributable to contaminated fresh produce (1). Nontyphoidal *Salmonella* is the leading bacterial cause of food-borne illness in the United States, with an incidence of infection that has not significantly declined in more than a decade (2). The high frequency of outbreaks of produce-associated salmonellosis (3) suggests that humans are more likely to encounter *Salmonella enterica* from eating fresh produce than animal products (4).

Contamination of fresh produce most likely occurs prior to crop harvest due to the presence and long-term persistence of *S. enterica* in the environment and the recurrent introduction of the pathogen in agricultural production areas (5, 6). In the agricultural environment, soil, surface and irrigation water, animals, and contaminated seeds are considered candidate reservoirs and facilitators of initial contact between *S. enterica* and plants (4, 7). *S. enterica* populations tend to decline steadily over time on leaves of agricultural crops (2, 8, 9). However, higher survival rates have been observed on leaves cocolonized with plant pathogens (10, 11, 12). Similarly, infestation and feeding by some phytophagous insects enhanced the persistence of *S. enterica* on lettuce leaves (13). Persistence and growth of human bacterial pathogens on crop plants increase the chance that an infectious dose would survive until harvest, posing a public health threat (14, 15). Thus, the risk of a food-borne illness outbreak due to consumption of contaminated crop plants can be influenced by biotic factors, including phytophagous insects.

Insects can influence the dispersal and survival of bacterial pathogens in agricultural environments. Bacteria have evolved to establish specialized symbiotic or pathogenic associations with insects and to exploit them as vectors (16). Particularly, these intimate associations are highly developed within members of the *Enterobacteriaceae* (17). Phytophagous insects are largely recognized as vectors of enteric phytobacterial pathogens that cause important diseases on many crops (18, 19). The level of specificity and complexity of these symbiotic relationships vary depending on the bacteria-insect combination and the frequency of cooccurrence of both organisms within the same plant or ecological niche (19). A nonspecific association between the fireblight pathogen *Erwinia amylovora* and pollinating insects is now widely recognized, in which flower-visiting insects spread bacteria attached to their external surfaces to new infection sites (19). In other cases of specific interactions, bacteria are internalized and disseminated by insects that serve as both vectors and overwintering hosts. For example, *Pantoea stewartii* and *Erwinia tracheiphila* are transmitted by the corn flea beetle (*Chaetocnema pulicaria*) and the striped cucumber beetle (*Acalymma vittatum*), respectively, which deposit bacteria-contaminated frass into feeding wounds (19, 20). Although the mechanisms of internalization, survival, and/or transmission of these enteric phytobacterial pathogens by their insect vectors have been widely studied, the actual multiplication of these bacteria inside their insect vector remains unconfirmed. The fact that phytophagous insects feed frequently upon plant tissues and readily move from plant to plant, some of which are potentially contaminated with human pathogens, such as *S. enterica*, suggests that they could influence the dispersal of enteric pathogens in the field. Surprisingly, the potential for phytopha-

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gous insect pests of agricultural crops to be vectors of *S. enterica* has not previously been studied.

Aster leafhoppers (*Macrosteles quadrilineatus* Forbes [Hemiptera: Cicadellidae]) and green peach aphids (*Myzus persicae* Sulzer [Hemiptera: Aphididae]) are both common agricultural pests and vectors of phytobacterial pathogens of several agricultural crops, including lettuce (18, 19, 21, 22, 23), and have previously been demonstrated to become contaminated and harbor large *S. enterica* populations upon contact with contaminated plant material (13). Thus, the main objective of this study was to investigate the potential acquisition, retention, and transmission of *S. enterica* by these phytophagous hemipteran insects. We hypothesize that phytophagous insect pests could serve as potential vectors of human enteric bacterial pathogens on and among leaves.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Six *S. enterica* serovars, Cubana strain 98A9878 (24), Enteritidis 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 (25), 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. The six strains were mixed in a cocktail inoculum that was prepared as previously described (13). The *S. enterica* serovar cocktail was used to mitigate possible differences in the plant-microbe-insect interaction. Xylose lysine desoxycholate (XLD) agar (Difco), a *Salmonella* semiselective growth medium in which all chosen strains produce black colonies, was used to determine *S. enterica* populations from all samples. To verify that the black colonies recovered from XLD medium 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*. Samples were always direct plated and additionally enriched in kanamycin-amended medium in order to improve the sensitivity in detecting *S. enterica* in samples containing the pathogen even at very low concentrations.

Insect rearing. A colony of *M. quadrilineatus* was maintained on oat (*Avena sativa* L.) seedlings, and a colony of *M. persicae* was maintained on Chinese cabbage (*Brassica rapa*) in a controlled environment with a 16-h light (24°C) and 8-h dark (19°C) photoperiod on the campus of the University of Wisconsin-Madison. Insect colonies were maintained in separate rooms from *S. enterica* to prevent any potential contamination and to ensure that all insects used were free of the pathogen before each experiment. Even-aged cohorts of adult insects were used in all experiments, and only apterous adult *M. persicae* insects were included in experiments.

Plant inoculation. Lettuce (*Lactuca sativa* cv. Butterhead) plants were grown from certified, pathogen-free seeds in 10-cm pots containing Metro Mix 300 potting medium (Sun Gro Horticulture, Canada CM Ltd.) to standardize plant condition. Pots were kept in a growth chamber (28°C, 14 h of light and 10 h of dark, 60% relative humidity), where plants grew for 3 to 4 weeks prior to use in all experiments. Selected plants were dip inoculated with either sterile water, as a control, or with an *S. enterica* cocktail ($\sim 10^8$ CFU/ml) as previously described (13). *Salmonella enterica* inoculum was verified by serial dilution before and after plant dip inoculation by being plated on Luria-Bertani (LB) (Difco/Becton, Dickinson, Franklin Lakes, NJ) medium amended with kanamycin to ensure that the bacterial concentration was constant throughout the inoculation process. Plant inoculation with contaminated water was used to simulate overhead sprinkler irrigation.

***S. enterica* localization, excretion, and transmission assays.** The overall methodologies used corresponding to these assays are illustrated in Fig. 1. In these three assays, 343 *M. persicae* (203 and 140 exposed and nonexposed to *S. enterica*, respectively) and 493 *M. quadrilineatus* (273 and 220 exposed and nonexposed to *S. enterica*, respectively) insects were examined, as well as the corresponding honeydew samples and leaf discs or liquid diets. To investigate the potential for phytophagous insects to ingest *S. enterica* acquired from contaminated plants, adult *M. quadrilineatus* and *M. persicae* insects were allowed to feed on leaf discs (4-mm diameter) excised from *S. enterica*-inoculated lettuce ($\sim 10^4$ CFU/mm^2) for a 24-h acquisition access period (AAP). Insects were contained inside individual 1.5-ml microcentrifuge tubes, and leaf discs were carefully placed inside the cap of each tube (prior of closing the tube) to prevent direct contact with any other part of the tube. After the 24-h AAP, the population of *S. enterica* on the inoculated leaf discs averaged $\sim 10^3$CFU/mm^2, and at that time the insects were anesthetized by placing the tubes in an ice bath for 10 min, and adult insects were individually transferred to new, sterile microcentrifuge tubes where they were submersed in 100 μl of sterile water. Then, following previously published procedures with slight modifications (23), each sample insect was washed using agitation by placing the microcentrifuge tube on an 80-place rack attached to a Vortex mixer set at a slow speed for 2 min, to remove bacteria from the insect exterior, and a sample (20 μl) of the external wash was collected. After external wash, each insect was then homogenized in the remaining liquid with a MINIMITE cordless grinding tool (Dremel, Racine, WI), and both the wash and the homogenate were separately enriched and plated on LB-kan and XLD-kan, respectively. A homogenate sample was scored as positive for internal contamination only if the corresponding external wash was negative, and those samples were considered containing surviving *S. enterica* within the insect. These experiments were repeated 3 times, and 72 *M. persicae* and 71 *M. quadrilineatus* insects were examined. In a supplementary set of experiments, individual adult insects of both species were fed a synthetic liquid diet consisting of either *S. enterica* (10^6 CFU/ml) or sterile water, and both were mixed at a 1:1 ratio with a 10% glucose solution. These inoculum concentrations are equivalent to the bacterial concentration in contaminated manure (28) and lower than the inoculum used in similar studies (23, 29, 30). In our experiments, 200 μl of the liquid diet was initially pipetted into the inside of the cap of each sterile microcentrifuge tube and then tightly covered with 2 layers of stretched Parafilm to limit physical contact of the insect body with the diet but allowing oral ingestion. Previous studies have demonstrated that both leafhoppers and aphids can successfully feed through a Parafilm sachet and acquire and transmit bacterial pathogens through these artificial membranes (30, 31, 32, 33). After a 24-h AAP, insects were again anesthetized and individually transferred to two consecutive noninoculated leaf discs or two consecutive *S. enterica*-free liquid diets (only *M. quadrilineatus*) for a total of a 48-h inoculation access period (IAP). Specifically, adult insects were allowed access to each leaf disc or sachet for a 24-h IAP. In the case of *M. persicae*, high rates of mortality were observed in the experiment when aphids were fed only liquid diets (with and without *S. enterica*) for 72 h (24-h AAP and 48-h IAP); therefore, the experiment with this specific insect was halted. At each sampling point, insects were sampled and both the external wash and the homogenate were tested as described above. Additionally, excretion of *S. enterica* from adult insects was tested following similar procedures. Drops of honeydew (liquid excrement from hemipteran insects) accumulated on the sides and bottom of each microcentrifuge tube and were tested for *S. enterica* using selective enrichment (LB-kan) and plating (XLD-kan) after a 24-h AAP and a 24- and 48-h IAP. In the case of the honeydew collected at the 24- and 48-h IAP time points, a honeydew sample was scored as positive only if the corresponding noninoculated leaf or liquid diet tested negative for *S. enterica*. Our goal here was to ensure that the honeydew excreted by the respective insect was *S. enterica* positive as a consequence of the initial acquisition and not from subsequent reinoculation of leaf discs or liquid diets. These experiments were repeated 4 times, and 90 *M. persicae* and 169 *M. quadrilineatus* insects were examined.

To test if contaminated insects could transmit *S. enterica* to plants, adult *M. quadrilineatus* and *M. persicae* insects were allowed to feed on

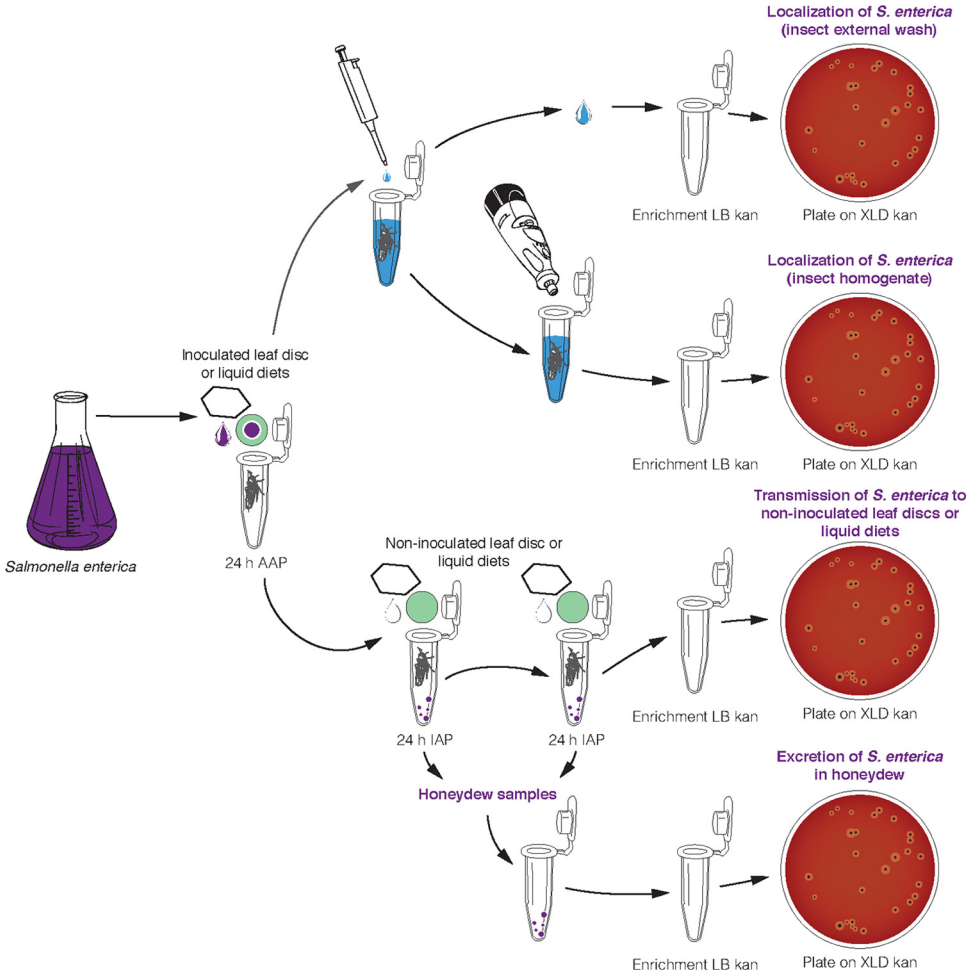

FIG 1 Schematic representation of the methodologies used to test the localization, excretion, and transmission of *Salmonella enterica* by phytophagous hemipterans in association with plants. *S. enterica* suspension (purple) was used to inoculate either lettuce leaf discs (green circles) or artificial liquid diets which were confined in Parafilm sachets (depicted as a pentagon with a liquid drop underneath). Leaf discs and liquid diets were placed and confined, respectively, inside the cap of sterile microcentrifuge tubes and were used to feed individual *Macrosteles quadrilineatus* and *Myzus persicae*. Red circles represent XLD agar plates, and small black circles within represent colonies of *S. enterica*.

inoculated leaf discs for a 12-h AAP and then transferred to new, sterile microcentrifuge tubes and allowed access to two consecutive noninoculated leaf discs for a total of a 24-h IAP. In this experiment, insects were moved through a series of leaf discs (inoculated and noninoculated); therefore, the AAP and each IAP were reduced to 12 h, considering that there was no need of an adjustment period to feed on liquid diets through Parafilm sachets. At the end of each 12-h period, the corresponding leaf discs were homogenized and subsequently enriched and plated on LB-kan and XLD-kan, respectively, and the percentage of *S. enterica*-contaminated leaf discs was determined. Leaf samples were homogenized and subsequently enriched and plated on LB-kan and XLD-kan, respectively. Results of these experiments were interpreted as the proportion of individual insects that successfully transmitted the bacteria from inoculated to healthy leaf discs, either by mechanical passage or contamination through excretion. In a similar set of experiments, insects were fed for a 24-h AAP on *S. enterica*-inoculated liquid diet and two consecutive noninoculated liquid diets, and at the end of each inoculation access period (24- and 48-h IAP), the corresponding liquid diets were sampled and the percentage of noninoculated liquid diets positive for *S. enterica* was determined. Here again, these results were interpreted as the proportion of individual insects capable of bacterial transmission from inoculated to noninoculated liquid diet. These experiments were repeated 4 times, and 131 *M. persicae* and 202 *M. quadrilineatus* insects were examined.

Statistical analysis. Consistently, *S. enterica* was not recovered from noninoculated control leaves, liquid diets, insects, or honeydew in any of the experiments; therefore, these data are not shown. No significant differences were found among replications of the overall experiments; thus, means from all the replicates of each experiment were combined. Proportions of *S. enterica*-contaminated leaves, liquid diets, insects, and honeydew were calculated, and significant differences among treatment main effects (diets and/or insect species) for individual experiments were tested with Pearson's chi-square test. Additionally, McNemar's test was used to test for marginal homogeneity in contamination rates over time, based on proportions of insects testing positive for *S. enterica*. This approach is commonly applied as a normal approximation on paired, nominal data expressed as a dichotomous trait, with matched pairs of subjects, and designed to determine whether marginal detection frequencies are equal. All statistical analyses were performed using R software (34).

RESULTS

***Salmonella enterica* is acquired and retained by phytophagous hemipterans.** Different variations on a common set of experiments were conducted in order to determine if phytophagous insects ingest *S. enterica*. Overall, after being fed either inoculated leaf discs or liquid diets, insects were washed and homogenized to

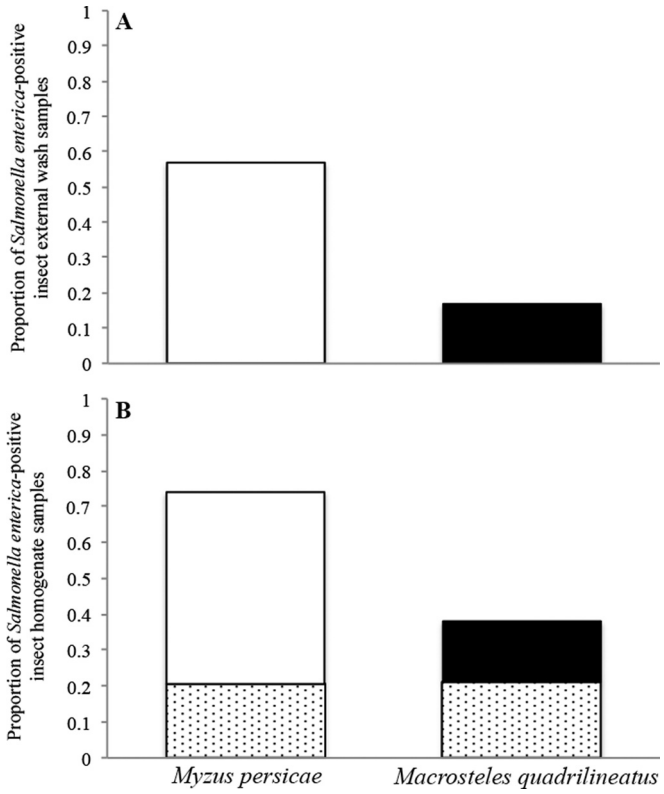

FIG 2 Localization of *Salmonella enterica* in or within phytophagous hemipterans. Proportion of *S. enterica*-positive samples resulting from insects that were fed inoculated leaf discs for a 24-h acquisition access period. Insects were scored as *S. enterica* positive after external wash (A) or homogenization of the whole sample (B). For homogenized samples, vertical bars represent the proportion of *S. enterica*-positive samples; dotted sections represent the proportion of samples that had a negative external wash but were positive after homogenization; and the solid sections represent the proportion of samples positive in both external wash and homogenization. Experiments were repeated 3 times, and *Myzus persicae* ($n = 72$) and *Macrosteles quadrilineatus* ($n = 71$) insects were examined. The proportion of *S. enterica*-positive *M. quadrilineatus* samples was smaller than that of *M. persicae* samples (chi-square analysis, $P < 0.01$).

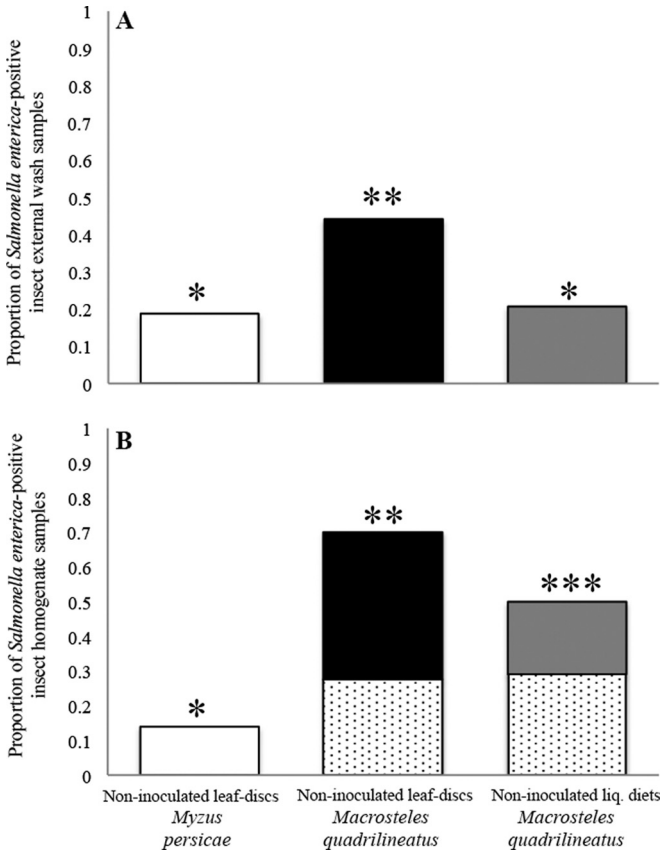

FIG 3 Localization and persistence of *Salmonella enterica* in or within phytophagous hemipterans. Proportion of *S. enterica*-positive samples resulting from insects that were fed an inoculated liquid diet confined in Parafilm sachets for a 24-h acquisition access period (AAP) and then moved to two consecutive noninoculated leaf discs (white and black bars) or noninoculated liquid diets (gray bars) for 48 h following AAP. Insects were scored as contaminated after external wash (A) or homogenization of the whole sample (B). For homogenized samples, vertical bars represent the proportion of *S. enterica*-positive samples; dotted sections represent the proportion of samples that had a negative external wash but were positive after homogenization; and the solid sections represent the proportion of samples positive in both external wash and homogenization. Experiments were repeated 4 times, and *Myzus persicae* ($n = 90$) and *Macrosteles quadrilineatus* ($n = 169$) insects were examined. Within each graph, bars sharing the same number of asterisks are not statistically different from each other (chi-square analysis, $P < 0.01$).

dislodge and recover *S. enterica* externally attached and internalized, respectively. When insects were fed inoculated leaf discs for a 24-h AAP, a significantly higher proportion of external wash and homogenized *M. persicae* than *M. quadrilineatus* insects was positive for *S. enterica* (Fig. 2A and B). However, the proportions of insects resulting in negative external contamination but positive for whole-insect homogenates were similar for both species (Fig. 2B). In subsequent experiments, both species were fed *S. enterica*-inoculated liquid diets through Parafilm sachets for a 24-h AAP and then moved to 2 noninoculated leaf discs or 2 noninoculated liquid diets for a 48-h IAP. Higher proportions of both external contamination and homogenate samples were observed when *M. quadrilineatus* insects were fed 2 consecutive noninoculated leaf discs than when *M. persicae* or *M. quadrilineatus* insects were fed noninoculated leaf discs or liquid diets, respectively (Fig. 3A and B). However, similar proportions of *M. quadrilineatus* insects that were solely positive for internal contamination (dotted section of the vertical bar) were observed independently of the diet (leaf disc or liquid). Relatively equal proportions of *M. persicae* insects that were internally contaminated were also externally contaminated, as signified by the lack of a dotted section of the vertical bar (Fig. 3B).

***Salmonella enterica* is excreted by phytophagous hemipterans.** Multiple assays were conducted in which the honeydew of *M. quadrilineatus* and *M. persicae* insects was collected at different time points following exposure to *S. enterica*-inoculated liquid diets. Approximately 50% of the honeydew samples from both species of insects were positive for *S. enterica* after insects were fed an inoculated liquid diet for a 24-h AAP (Fig. 4A). However, after a 24-h IAP, the proportion of *S. enterica*-positive honeydew samples was reduced in both *M. quadrilineatus* and *M. persicae* insects when insects were subsequently fed a noninoculated leaf disc, and those proportions were not significantly different between insects ($P > 0.01$). Contrastingly, at the same time point, a significantly higher proportion of *S. enterica*-positive honeydew samples was detected when *M. quadrilineatus* insects were fed a noninoculated

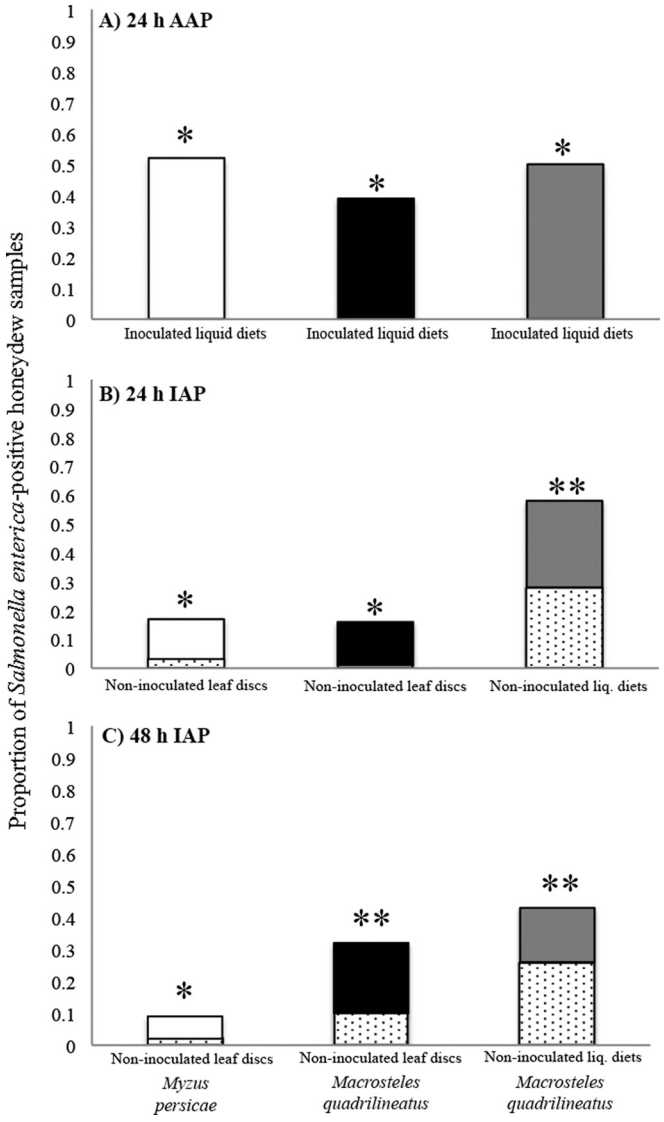

FIG 4 Excretion of *Salmonella enterica* in honeydew of phytophagous hemipterans. Proportion of *S. enterica*-positive samples resulting from insects that were fed inoculated liquid diets confined in Parafilm sachets for a 24-h acquisition access period (AAP) (A) and then on two consecutive noninoculated leaf discs (white and black bars) or noninoculated liquid diets confined in Parafilm sachets (gray bars) for a 48-h inoculation access period (IAP) (B and C). Vertical bars represent the proportion of *S. enterica*-positive samples; dotted sections represent the proportion of honeydew samples that were positive, but the corresponding noninoculated leaf discs or liquid diet was negative for *S. enterica* at the time when the sample was collected; and the solid sections represent the proportion of samples positive in both the honeydew and the corresponding noninoculated leaf discs or noninoculated liquid diet. Experiments were repeated 4 times, and honeydew samples from *Myzus persicae* ($n = 90$) and *Macrosteles quadrilineatus* ($n = 169$) insects were examined. Within each graph, bars sharing the same number of asterisks are not statistically different from each other (chi-square analysis, $P < 0.01$).

liquid diet confined in Parafilm sachets (Fig. 4B). Interestingly, at the 48-h IAP time point, proportions of *S. enterica*-positive honeydew samples from *M. quadrilineatus* insects were significantly higher than those of *M. persicae* insects, independent of the diet (leaf or liquid). Moreover, in the case of *M. quadrilineatus* insects fed noninoculated leaf discs, there was a higher proportion of samples that were positive for *S. enterica* at the 48-h IAP time point than at the 24-h IAP time point. In fact, unlike at the 24-h IAP time, at the 48-h IAP time point there was a substantial amount of *M. quadrilineatus* samples in which solely the honeydew (and not the leaf) was *S. enterica* positive, 0.01 at the 24-h IAP time point compared to 0.1 at the 48-h IAP time point (Fig. 4C). In *M. quadrilineatus* insects fed noninoculated liquid diets, the proportion of positive honeydew samples was slightly lower at the 48-h IAP time point than at the 24-h IAP time point. In this case, although the proportion of exclusively honeydew-positive samples was maintained, the proportion of double-positive samples (honeydew and liquid diet) was lower at the 48-h IAP time point than those observed at the 24-h IAP time point, 0.17 compared to 0.30, respectively. These results suggest that *S. enterica* acquired by *M. quadrilineatus* insects is retained and excreted following exposure to the inoculated source, and *S. enterica*-positive honeydew could contribute to inoculation of hemipteran feeding sites.

***Salmonella enterica* is transmitted by phytophagous hemipterans.** Transmission experiments in which insects acquired the pathogen from either inoculated leaves or synthetic liquid diets showed that both *M. quadrilineatus* and *M. persicae* insects could acquire and transmit the pathogen for up to 48 h following acquisition (Fig. 5 and 6). In the case of *M. quadrilineatus*, the highest transmission rate was observed when insects acquired *S. enterica* from inoculated leaves and transmitted it to noninoculated leaves after either 12- or 24-h IAPs (Fig. 5). The transmission rate decreased significantly when the acquisition source was changed to a liquid diet confined in Parafilm sachets, which presumably limited mechanical contamination of the insects. However, even at the 48-h IAP time point, the transmission rate was approximately 20% when insects acquired from and transmitted the pathogen to liquid diets (Fig. 5). Similarly, the highest transmission rate of *S. enterica* by *M. persicae* insects was observed when aphids acquired the pathogen from inoculated leaves and transmitted it to noninoculated leaves at the 12-h IAP time point. However, the transmission rates were reduced at both the 24- and 48-h IAP time points, independent of the source of inoculum (Fig. 6).

***Salmonella enterica* transmission and excretion by *M. quadrilineatus* insects correlated with recovery from within the insect.** Matched-pair comparisons of individual *M. quadrilineatus* insects for *S. enterica* presence over time were performed (Table 1). The comparison of excretion and transmission of *S. enterica* demonstrated that when insects were fed noninoculated leaf discs during the IAP, there was a high number of samples in which only the leaf disc or both the leaf disc and the corresponding honeydew tested positive, compared to only the honeydew being positive for *S. enterica*. The opposite was observed when insects were fed noninoculated liquid diets following acquisition, where in most samples only the honeydew or both the honeydew and the liquid diet were *S. enterica* positive. This result demonstrated that acquired *S. enterica* is excreted and transmitted, but honeydew is unlikely the sole source of contamination to plants. The comparison of *S. enterica* excretion and localization showed that when *M. quadrilineatus* insects were fed noninoculated leaf discs following acquisition, the majority of the insects were externally or internally positive for *S. enterica*, independent of whether the honeydew was *S. enterica* positive. In contrast, when *M. quadrilineatus* insects were fed noninoculated liquid diets following acquisition, the proportions of positive honeydew samples at the 24-h AAP and

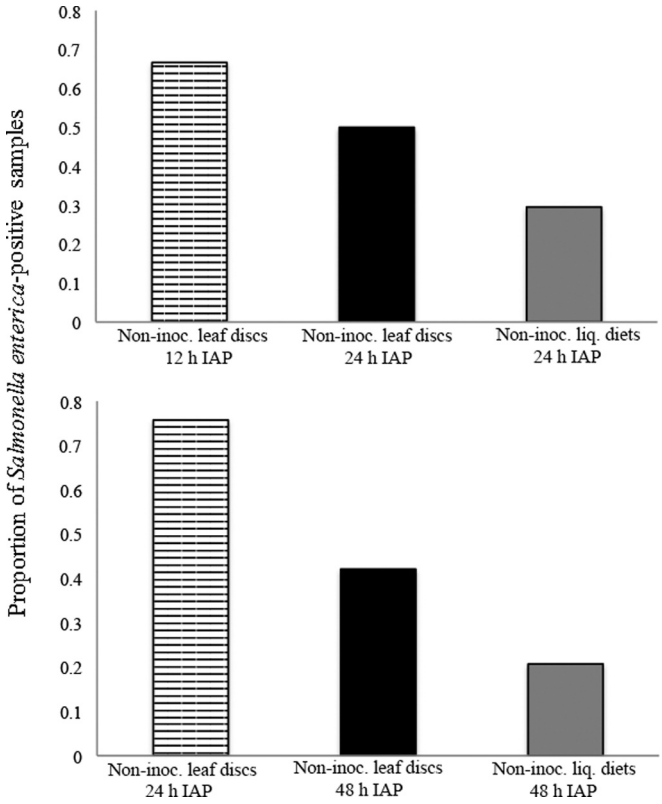

FIG 5 Transmission of *Salmonella enterica* by *Macrosteles quadrilineatus*. Proportion of *S. enterica*-positive samples resulting from transmission from inoculated leaf discs to noninoculated leaf discs (horizontal-stripe bars) or from inoculated liquid diets confined in Parafilm sachets to noninoculated leaf discs (black bars) or to noninoculated liquid diets confined in Parafilm sachets (gray bars) at different inoculation access periods (IAP). Experiments were repeated 4 times, and the corresponding samples from *M. quadrilineatus* ($n = 202$) insects were examined. Within each graph, the proportions of *S. enterica*-positive samples between treatments were significantly different from each other (chi-square analysis, $P < 0.01$).

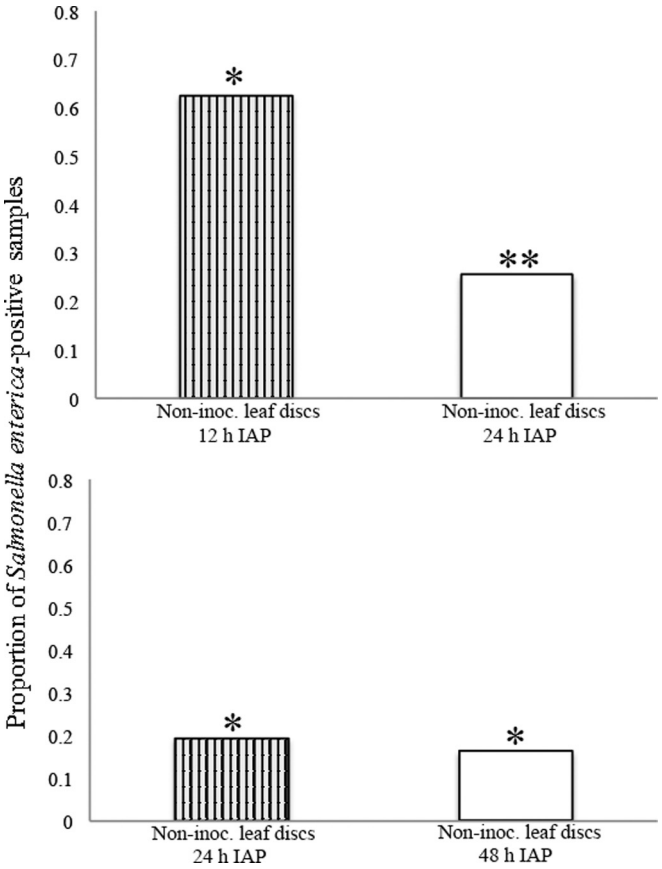

FIG 6 Transmission of *Salmonella enterica* by *Myzus persicae*. Proportion of *S. enterica*-positive samples resulting from transmission from inoculated leaf discs (vertical-stripe bars) or inoculated liquid diets confined in Parafilm sachets (white bars) to noninoculated leaf discs at different inoculation access periods (IAP). Experiments were repeated 4 times, and the corresponding samples from *M. persicae* insects ($n = 131$) were examined. Within each graph, bars sharing the same number of asterisks are not statistically different from each other (chi-square analysis, $P < 0.01$).

the 24- or 48-h IAP time points were significantly higher than the proportions of samples positive for external wash (0.36, 0.40, and 0.29 compared to 0.06, 0.02, and 0.08, respectively). However, there were no significant differences between the proportions of samples that were only positive for either honeydew (24-h AAP or 24- and 48-h IAP) or insect homogenate. Moreover, the proportion of double-positive samples (honeydew and homogenate) was more than twice (0.42, 0.34) the proportion when only one parameter was positive at the 24 (0.17, 0.08)- and 48 (0.08, 0.15)-h IAP time points. In summary, it appears that diet interacts with the inoculation interval following acquisition to influence localization and excretion. Similarly, the analysis of proportions for transmission and localization of *S. enterica* showed that internally contaminated insects transmitted *S. enterica* to both noninoculated leaf discs and liquid diets. This result was reflected by the high proportion of double-positive samples (homogenate and diets) and the low proportion of samples in which only the leaf disc or liquid diet was positive for *S. enterica* but the corresponding insect homogenate was negative. However, there was also a large proportion of insect samples whose homogenized sample was *S. enterica* positive at the 48-h IAP time point, for which the corresponding leaf discs or liquid diets were negative. This result suggests that transmission of the ingested *S. enterica* was far less than 100% efficient. Moreover, there were no significant differences between proportions of positive, noninoculated leaf discs or liquid diets and the externally washed *S. enterica*-positive samples (Table 1).

To determine if *S. enterica* transmissions, excretions, and localizations are similar among phytophagous hemipterans, we conducted the same matched-pair analysis for *M. persicae* insects fed inoculated liquid diets for 24 h and subsequently transferred to 2 consecutive noninoculated leaf discs (see Table S1 in the supplemental material). In this case, the proportion of samples in which both of the compared parameters were negative was particularly high. In fact, only the proportions of honeydew samples at the 24-h AAP time point were significantly higher than the corresponding noninoculated leaf disc at the 24-h IAP time point and the external wash sample and homogenized *S. enterica*-positive samples at the 48-h IAP time point. These results suggest that with *M. persicae* specifically, the ingestion of *S. enterica* through Parafilm sachets and its excretion through the honeydew had no direct effect on transmission or localization of the pathogen.

TABLE 1 Comparison of correlated proportions of the presence of *Salmonella enterica* between matched pairs of samples based on individual *Macrosteles quadrilineatus*

Matched pair of samples	Proportion[a]			
	−−	+−	−+	++
Excretion and transmission of *S. enterica*				
A) Honeydew 24-h IAP and leaf disc 24-h IAP	0.49	**0.01**	**0.35**	0.15
A) Honeydew 48-h IAP and leaf disc 48-h IAP	0.47	**0.10**	**0.21**	0.22
B) Honeydew 24-h IAP and liquid diet 24-h IAP	0.42	**0.28**	**0**	0.30
B) Honeydew 48-h IAP and liquid diet 48-h IAP	0.52	**0.27**	**0.05**	0.16
Excretion and localization of *S. enterica*				
A) Honeydew 24-h AAP and insect external wash 48-h IAP	0.36	0.19	0.26	0.19
A) Honeydew 24-h AAP and insect homogenate 48-h IAP	0.22	**0.08**	**0.39**	0.31
A) Honeydew 24-h IAP and insect external wash 48-h IAP	0.49	**0.04**	**0.35**	0.12
A) Honeydew 24-h IAP and insect homogenate 48-h IAP	0.27	**0.03**	**0.57**	0.13
A) Honeydew 48-h IAP and insect external wash 48-h IAP	0.43	**0.13**	**0.25**	0.19
A) Honeydew 48-h IAP and insect homogenate 48-h IAP	0.25	**0.04**	**0.42**	0.29
B) Honeydew 24-h AAP and insect external wash 48-h IAP	0.42	**0.36**	**0.06**	0.16
B) Honeydew 24-h AAP and insect homogenate 48-h IAP	0.27	0.23	0.22	0.28
B) Honeydew 24-h IAP and insect external wash 48-h IAP	0.40	**0.40**	**0.02**	0.18
B) Honeydew 24-h IAP and insect homogenate 48-h IAP	0.33	0.17	0.08	0.42
B) Honeydew 48-h IAP and insect external wash 48-h IAP	0.50	**0.29**	**0.08**	0.13
B) Honeydew 48-h IAP and insect homogenate 48-h IAP	0.43	0.08	0.15	0.34
Transmission and localization of *S. enterica*				
A) Leaf disc 24-h IAP and insect external wash 48-h IAP	0.33	0.23	0.18	0.26
A) Leaf disc 24-h IAP and insect homogenate 48-h IAP	0.18	**0.12**	**0.32**	0.38
A) Leaf disc 48-h IAP and insect external wash 48-h IAP	0.35	0.21	0.22	0.22
A) Leaf disc 48-h IAP and insect homogenate 48-h IAP	0.22	**0.08**	**0.35**	0.35
B) Liquid diet 24-h IAP and insect external wash 48-h IAP	0.57	0.21	0.13	0.09
B) Liquid diet 24-h IAP and insect homogenate 48-h IAP	0.38	**0.12**	**0.33**	0.17
B) Liquid diet 48-h IAP and insect external wash 48-h IAP	0.68	0.11	0.12	0.09
B) Liquid diet 48-h IAP and insect homogenate 48-h IAP	0.44	**0.06**	**0.36**	0.14

[a] The number listed is the proportion of the total samples observed with the given contamination pattern. If the +− proportion was significantly different than the −+ proportion based on McNemar's test ($P < 0.01$), the values are shown in bold. The −− and ++ proportions are given for reference purposes. Insects were fed an inoculated liquid diet for a 24-h acquisition access period (AAP) and then fed 2 consecutive noninoculated leaf discs (A; $n = 77$ insects) or noninoculated liquid diets (B; $n = 88$ insects) for a total of a 48-h inoculation access period (IAP).

DISCUSSION

We tested the hypothesis that phytophagous insect pests could serve as potential vectors of human enteric bacterial pathogens on and among leaves. Our results demonstrated that two commonly occurring hemipteran insect pests, *M. quadrilineatus* and *M. persicae*, acquired *S. enterica* upon feeding on an inoculated diet. The proposed mechanisms of acquisition include external adhesion of the bacterium to the insect body or ingestion during feeding. Additionally, both species excreted *S. enterica* through honeydew and transmitted the pathogen to noninoculated leaf surfaces and, in the case of *M. quadrilineatus*, to noninoculated liquid diets as well. In view of the current lack of epidemiological data from similar studies conducted under field conditions, results from this research point to a set of potential mechanisms by which phytophagous insects may interact with *S. enterica* in agricultural environments to influence the spread and persistence of the pathogen (Fig. 7).

Phytophagous hemipterans may contribute to *S. enterica* dispersal and colonization of the plant phyllosphere. Hemipteran insects could most certainly encounter a human enteric pathogen in the field, principally as a result of feeding or wandering on contaminated plant surfaces. In the field, plant surfaces can become contaminated with *S. enterica* in multiple ways (4), increasing the chances that phytophagous insects could infest and establish on contaminated plants. Such insects that encounter *S. enterica* on the leaf surface for brief periods of time could further distribute the pathogen along the same or adjacent leaves, simply by adhesion to the exoskeleton. It is certainly plausible to assume, however, that this redistribution of the pathogen could assist *S. enterica* to reach and exploit preferential colonization niches around the phyllosphere, even facilitating internalization. For example, phytophagous insects can move bacterial cells to the abaxial side of the leaf, containing higher densities of stomata, which are entry points used by bacteria to gain access and penetrate lettuce leaves (35, 36). Phytophagous insects can also increase the probability that *S. enterica* is placed on or near sources of nutrients such as wounds, trichomes, cuticle cracks, or insect feeding sites, contributing to the persistence of the bacteria on the leaf surface (4, 13). Hence, insect movement on infested plants may directly influence the spatial distribution and persistence of *S. enterica* on leaves.

Excretion of ingested *S. enterica* onto leaves could further represent an important mechanism of transmission. Insects can ingest *S. enterica* while feeding on parts of the plant previously colonized by the bacterium. In the current study, the fact that *S. enterica* was excreted in honeydew by both *M. quadrilineatus* and

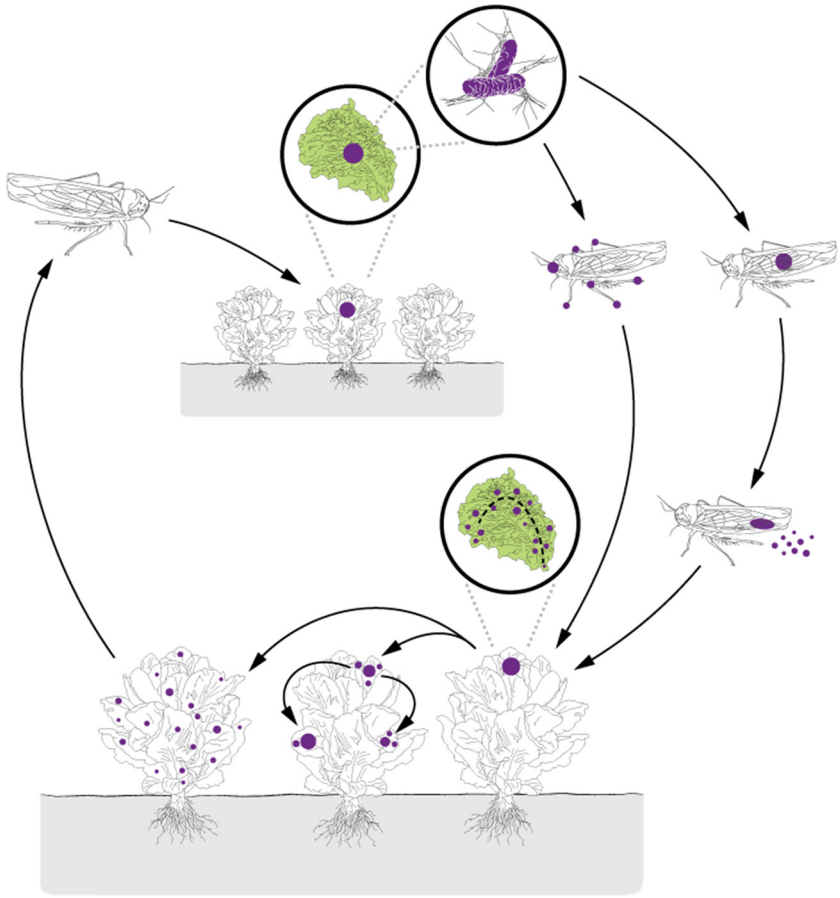

FIG 7 Proposed model of interactions between *Salmonella enterica* and phytophagous hemipterans in agricultural environments. Insects can become contaminated upon contact with *S. enterica* surface-contaminated plants (purple dots). *S. enterica* can adhere to various structures of the exoskeleton and mouthparts of the insects but can also be ingested. Ingested bacteria can be excreted in droplets of honeydew that are released onto leaves. *S. enterica* distribution on the leaf surface is altered by insect feeding, excretion, and movement (dashed line) behavior. Both internally and externally contaminated insects may increase the risk of dispersal of the pathogen within or among plants.

M. persicae insects demonstrates that ingested bacteria survived the passage through the alimentary canal and could subsequently be dispersed on plants by the carrier insect. Furthermore, all leafhoppers, and some aphids, forcibly direct droplets of their excrement (honeydew) away from themselves, in order to prevent self-contamination (37) and possibly to disorient predators. However, at high population densities, insects can also come into contact with honeydew or droplets released on the plant surface (37). Thus, *S. enterica*-contaminated honeydew may represent a mechanism of dispersal that allows contamination of both plant and insects.

In general, vectors are normally defined as organisms, usually arthropods, or fomites that carry a disease agent from a reservoir to a susceptible host (38). Lasky (39) defined a vector, with respect to food safety, as "a living carrier that serves as a vehicle of transmission of an infectious agent, but not necessarily as the reservoir, and facilitates exposure of a host to the pathogen." In the case of plant pathogens, vectors are loosely defined as organisms that can introduce a pathogen into a plant to cause infection, by carrying the pathogen internally or externally (40). In the case of phytobacterial pathogens, transmission can occur by physical contact of plant tissues with contaminated mouthparts, legs, and bodies or deposition of contaminated saliva or feces on leaf surfaces (18, 40). Previous studies demonstrated that 50% of *Frankliniella occidentalis*, *M. quadrilineatus*, and *M. persicae* insects became contaminated with *S. enterica* from feeding on contaminated plant material (13). Furthermore, *S. enterica* was isolated from both tomato and lettuce plants in areas damaged by contaminated *F. occidentalis*, which had previously fed on *S. enterica*-inoculated green beans (41). Also, the presence of *M. quadrilineatus* and feeding damage of *F. occidentalis* enhanced the longevity of *S. enterica* on lettuce (13). In this study, both internally and externally contaminated *M. quadrilineatus* and *M. persicae* insects were able to transmit *S. enterica* to noninoculated leaves or liquid diets. The mechanisms and associated pathways of transmission of the pathogen may have included one or a combination of transport routes on body parts and deposition of contaminated oral secretions or honeydew. Therefore, based on the working definitions of vectors, and our results, we propose that phytophagous insects should be considered potential vectors of *S. enterica* in and among plants.

Synanthropic and coprophagic insects are recognized as vectors of human enteric pathogens, and many have been implicated in not just the dispersal but also in the survival and multiplication of *S. enterica* (42). In addition to their association with unsanitary conditions, some of these insects are known for their ability to

indiscriminately change their habitats from urban to rural, or from livestock to produce fields, as well as their dietary flexibility, alternating between fecal material and plants, fruits, and vegetables (28, 29, 42, 43). In a field survey, leafhoppers were some of the most abundant and consistently captured insects in both rangeland and leafy green production areas, in which *Escherichia coli* O157:H7-positive flies were also found (28). Similarly, the presence of *S. enterica* in flies captured from the surroundings of animal farms has been demonstrated (42). It is known that the high protein content in honeydew attracts many other insects, such as flies, ants, and other predators, which require a substantial amount of protein to develop mature eggs (29, 44). In fact, Talley et al. (28) suggested that the honeydew in aphid-infested lettuce plants could have attracted filth flies (Muscidae and Calliphoridae) from rangelands that contained fresh cattle manure or composting operations to leafy green fields. Thus, the in-field epidemiology of *S. enterica* in association with plants may be influenced by multiple species of insects occupying different ecological niches.

Hemipteran insects can acquire phyllobacteria during probing and feeding on host plants (18, 45), and those bacteria are able to pass through the stylet and adhere to gut epithelial cells and then are excreted in the honeydew (46). Additionally, multiple studies have shown that *Salmonella* spp. can attach, survive, and even replicate in or on confinement insects (42). Our study unifies these observations and demonstrates that phytophagous hemipterans can be sources of *S. enterica*, and these insects are regarded as key pest species of crops implicated in food-borne illness outbreaks, such as lettuce and tomatoes (1, 3, 26). Internalization and movement of *S. enterica* within plant tissues, including the vascular system (2, 36, 47), suggest that phloem feeding insects could potentially acquire the pathogen while feeding on the vascular tissues of *S. enterica*-colonized plants. However, this hypothesis remains untested.

Insect honeydew is an aqueous solution consisting of various sugars and amino acids and constitutes a significant nutrient source and growth medium that could ultimately increase the survival of bacteria on the plant surface (23, 46, 48). In an ongoing set of replicated experiments, an approximate 2-log increase in concentrations of *S. enterica* was observed when the bacterial suspension was amended with honeydew collected from *M. quadrilineatus* and cultured overnight (J. P. Soto-Arias, unpublished data). The nutritional value of honeydew, and the copious amount that is excreted by phytophagous hemipterans, suggests that populations of *S. enterica* on the leaf surface might benefit from an infestation of those insects and their associated excreta. However, the availability and usage of honeydew by *S. enterica* may be influenced by the presence of other phyllobacteria; thus, further studies to evaluate the effects of honeydew on natural phyllosphere populations are necessary.

Overall, our findings strengthen the need for further investigations to evaluate the potential acquisition and transmission of *S. enterica* by phytophagous insects under field conditions and to better determine their contribution as a risk factor for dispersal of human pathogens in a field prior to harvest.

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