CHARACTERIZING INSECT FEEDING BEHAVIORS WHICH INFLUENCE EPIPHYTIC *SALMONELLA ENTERICA* POPULATIONS

By

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Dissertation Abstract

The incidence of *Salmonella enterica* foodborne outbreaks linked to the consumption of raw produce has increased in the last decade. Although *S. enterica* populations naturally decline within the phyllosphere of healthy plants, high proportions of *S. enterica* outbreaks on produce suggest that co-existing biological and environmental factors – such as the presence of phytophagous insects – lead to enhanced bacterial survival or growth. While the complex pathosystem between plants, insects, and *S. enterica* had been identified, the entomological mechanisms leading to enhancement of epiphytic *S. enterica* populations had remained unknown. In this thesis, I review and characterize a series of insect feeding behaviors which manipulate the surface of the plant to transform a once inhospitable niche, to one that is conducive to *S. enterica*. I first identified that leaves damaged by Western Flower Thrips (*Frankliniella occidentalis,* Pergande, Thysanoptera: Thripidae) concurrently supported significantly higher *S. enterica* populations and faced greater rates of cellular damage than sites lacking feeding damage. Investigating piercing-sucking insects, I similarly identified elevated levels of cellular leaf damage on leaves damaged by Aster Leafhoppers (*Macrosteles quadrilineatus,* Forbes, Hemiptera: Cicadellidae*)*, but not by Green Peach Aphids (*M. persicae,* Sulzer, Hemiptera: Aphididae*).* Feeding preferences for leafhoppers shifted the distribution of *S. enterica* across tomato leaflets, and moreover, the presence of *S. enterica* altered the distribution of probing attempts. Our final study revealed that insect honeydew benefits *S. enterica* in a plant host-dependent manner. Together, these findings expand our understanding of insects as biomultipliers for *S. enterica* within the context of agroecosystems.

Chapter 1: Literature Review

SALMONELLA ENTERICA AS A FOODBORNE PATHOGEN

Salmonella is derived from the Enterobacteriaceae family, a family of Gram-negative bacteria that's further comprised of other disease-causing pathogens such as *Escherichia coli* and *Shigella*. Some members of the Enterobacteriaceae family, including *Salmonella,* are rod-shaped (bacilli) and mobile, using flagella to facilitate movement across or within substrates, and towards attractants and away from repellants (Berg, 2003). A flagellum is composed of three major structures: 1) the basal body, 2) the filament – acting as the primary motor, and 3) the transmembrane motor – connecting the basal body to the filament (Kerridge et al., 1962). Although *Salmonella* may survive without flagella, the absence of flagella strips the bacteria of protection, deters duplication abilities, and inhibits movement (Robertson et al., 2003; Weinstein et al., 1984). The genus *Salmonella* is only comprised of only two species. *Salmonella bongori* – the lesser studied and rarer occurring – is restricted to cold blooded hosts, such as lizards. Conversely, the species *Salmonella enterica* is highly prevalent within mammalian systems, and as such, is of significant global public health concern as it causes nearly 1.3 billion cases of illness annually.

S. enterica is comprised of six subspecies that are differentiated by their lipopolysaccharides (LPS; O – antigens), flagellar proteins (H – antigens), and capsular polysaccharides (Vi – antigens). Between these six subspecies, there are nearly 2,600 serovars that are either considered typhoidal or nontyphoidal. Briefly, the contraction of typhoidal serovars are restricted to humans and is manifested 1 to 3 weeks after inoculation in the form of typhoid fever, further accompanied by symptoms of weakness, constipation, and mild vomiting. Non-typhoid serovars, of which Enteritidis and Typhimurium are the most common, elicit intense gastrointestinal distress and abdominal cramps in humans and animal such as poultry and cattle. Non-typhoidal symptoms begin 12 to 72 hours after consuming contaminated products and may persist from 4 to 7 days afterwards. Although anyone is at risk of contracting

salmonellosis, the elderly, those with compromised immune systems, and infants are of greatest risk for severe, and potentially fatal, symptoms.

Salmonellosis has long been linked with the consumption of raw contaminated animal products, such as raw chicken or unpasteurized milk; however, *S. enterica*-contaminated fresh produce has recently emerged as the primary origin of salmonellosis (Hanning et al., 2009). Occupying plants as alternative hosts, *S. enterica* employs adaptation strategies to evade plant defense responses and may successfully internalize within plant tissues *(*Zarkani and Schikora*,* 2021; Jechalke et al., 2019; Golberg et al., 2011). But as a zoonotic pathogen, how does *S. enterica* arrive to plant niches? Thoroughly supported by a vast body of research, several abiotic components, either naturally occurring or introduced via human influence, are known to support and facilitate the dispersal of *S. enterica* populations (Barak and Schroeder, 2012). Contaminated irrigation water, for instance, exacerbates both the spread and magnitude of *S. enterica* contaminated produce, prompting bacterial populations to persist for weeks within the phyllosphere (Islam et al., 2004; Liu et al., 2018). Splash dispersal from raindrops has similarly been shown to spread *S. enterica* from its origin (Cevallos-Cevallos et al., 2012). With reference to animal agriculture, manure treated soils are commonly applied to maintain soil fertility, yet simultaneously compromise the safety of produce by enhancing enteric bacterial growth (Hruby et al., 2018; Jacobsen and Bech, 2012). Although poor agricultural practices and cross-contamination within distribution chains may well increase one's chance of foodborne illness, other discrete ecological occurrences – including the presence of insects or plant pathogens – may contribute to *S. enterica* persistence.

ENVIRONMENTAL SURVIVAL OF S. ENTERICA

While *S. enterica* may be acquired from the consumption of contaminated raw produce, hostile environmental conditions in the phyllosphere, such as direct UV radiation, desiccation, and a lack of nutrient availability, are a few of the limiting factors prompting bacterial populations

to decrease over time on leaves (Nyeleti et al., 2004). High proportion of *S. enterica* outbreaks on produce, however, indicate that these bacteria are quite adaptive, and able to exploit discrete plant niches to successfully persist.

Vulnerable to abiotic elements, *S. enterica's* populations congregate around ubiquitous leaf structures which typically provide protection or scarce nutritional resources. Acting as a doorway to the internal leaf lumen, *S. enterica* has been shown to conglomerate around stomatal openings (Kroupitski et al., 2009; Kroupitski et al., 2011). *S. enterica* leaf internalization via the stomata is highest when the plant host is induced by light and when plants are stored at higher temperatures highlighting the relevant impact of abiotic conditions upon bacterial proliferation success (Kroupitski et al., 2009). The arrival of other plant pathogenic bacteria such as *Pseudomonas siringae* however, result in stomatal closure and may therefore impact *S. enterica* internalization (Toum at al., 2016). Apart from stomata, Glandular type 1 trichomes, a specialized plant hair which secretes secondary metabolites, provides epiphytic bacteria with a beneficial and nutritional reservoir (Barak et al., 2011). Other factors such as leaf age, plant cultivar, and foliage type (i.e. leaves, roots, and fruits) furthermore impact colonization success (Barak et al., 2011; Jechalke et al., 2019). In addition to morphological characteristics of plants, *S. enterica* population dynamics are further influenced by other phyllosphere-ranging organisms. Enhanced colonization of *S. enterica* was observed upon plants co-inoculated with an additional plant pathogen such as *Xanthomonas gardeneri*, *X. perforans,* and downy mildew, as their infectious stages consequentially expose plant nutrients and provide direct access to leaf metabolites (Potnis et al., 2014; Potnis et al., 2015; Kwan at al., 2013; Simko et al., 2015). In conjunction with these plant pathogenic biological multipliers (biomultipliers), insects have similarly been identified as potential promoters of *S. enterica* survival.

INSECTS AS BIOLOGICAL MULTIPLIERS

The term ‘vector’ has been assigned to a plethora of insects across a range of ecological and epidemiological systems. Wilson et al., concluded that the definition of vector is highly context dependent. Within a medical framework, the term vector emphasizes hematophagous arthropods and their range of movement. Within an evolutionary framework, the fitness consequences between the pathogen and vector, and moreover the inner workings of transmission biology are typically emphasized (Wilson et al., 2017). Within a general context, however, the term vector is used to describe an invertebrate, typically an arthropod, which carries a disease agent from a reservoir to a susceptible host (Institute of Medicine, 2008). Nevertheless, previous studies have thoroughly demonstrated that insects can manipulate human enteric bacterial pathogens, such as *S. enterica*, directly and indirectly.

The concurrent possession of winged appendages and leg structures affords insects multiple avenues of movement, and as such, makes them effective mechanical vehicles for human enteric bacterial pathogens. Within rural settlement housing, cockroaches collected from pit latrines carried a higher mean of bacteria pathogens than insects derived from kitchens, reflecting the status of contamination between unique environments (Tatfeng et al., 2005). Within poultry dominated settings, several species of cockroaches (*Diploptera puncture)* have shown to mechanically transmit *Salmonella* by traversing from contaminated egg surfaces to neighboring uncontaminated eggs, consequently facilitating the movement of bacteria (Kopanic et al., 1994). Similarly, houseflies within proximity to humans and contamination sources (i.e. cow manure) are capable of contaminating water, human food, and even other living organisms following physical contact, with *Salmonella* populations persisting upon the exoskeleton of flies for up to 4 weeks (Ostrolenk et al., 1941). Aside from direct mechanical transmission, feeding events such as regurgitation and excretion, have further shown to directly introduce viable bacterial populations consequentially prompting elevated likelihood of foodborne outbreaks (Swinscoe et al., 2018, Nayduch et al., 2018). Given their intimate proximity to humans and

association with human enteric bacterial pathogens, the vector biology of synanthropic insects – such as flies and cockroaches – has been studied thoroughly. As such, expanding our understanding of insects outside of anthropogenic systems (such as those belonging to our agricultural crop systems) and their association with human enteric bacterial pathogens would develop our understanding of the role of insects as foodborne pathogen vectors.

Previously a subset of phytophagous insects had been identified as biomultipliers, enhancing *S. enterica* populations in association with produce (Soto-Arias et al., 2014). When exposed to tomato and lettuce plants, Aster leafhopper infestations significantly promoted *S. enterica* populations and persistence over time (Soto-Arias et al., 2013; Soto-Arias et al., 2014; Cowles et al., 2018). Although the presence of green peach aphids did not benefit *S. enterica* populations, excrement (honeydew) voided by these aphids still contained viable *S. enterica* populations. Viable bacterial populations were similarly identified in leafhopper honeydew. Although this complex relationship (between plants, phytophagous insects, and *S. enterica*) has previously been described, the feeding mechanisms by which these phytophagous insects manipulate the persistence of *S. enterica* populations, and inversely, the potential impact of *S. enterica* upon insect feeding behaviors, has not yet been investigated.

PLANT EXPLORATION AND PROBING BEHAVIORS BY INSECTS

Suitable for probing into plants, leafhoppers and aphids have highly modified mouthparts to access the plant vasculature. Generally, the mouth parts of leafhoppers and aphids comprise of a labrum, labium, and stylets. The labrum, a triangular shaped appendage with an indented groove, positions the insect stylets. Housed within the labium, a long tubular appendage otherwise referred to as the proboscis or stylet bundle, are the collection of stylets. Between accessing deep plant tissues to aiding in salivary sheath egestion, these piercing sucking mouthparts is what makes hemipteran insects successful worldwide agricultural pests, and plant

disease vectors (Valenzuela and Hoffmann, 2014; Stillson and Szendrei, 2020; Tipu et al., 2020).

Once a hemipteran arrives to a plant, the process of substrate exploration commences. Moving across the surface of a plant, the insect begins to orient their position and exhibit 'labial dabbing' by which their labium repeatedly taps the plant epithelium, and a drop of sheath saliva covers the tip of the stylet. Hemipteran insects produce two types of salivary secretions to facilitate vasculature feeding. Sheath saliva, the components that form salivary sheathes, solidifies immediately after exposure to the air, and covers the insect stylet entirely to create a protective shield against plant defenses. Although sheath saliva is composed primarily of proteins, it also contains traces of phospholipids and carbohydrates. Watery saliva, the second type of hemipteran salivary secretion, is made of digestive enzymes and facilitates the feeding process by liquefying plant material during stylet insertion. Subsequently after a suitable feeding location is selected, the stylets are thrust through the salivary droplet (forming the start of the salivary sheath) and moves into the plant. After puncturing the epidermis, an aphid's stylet transiently probes and injects adjacent cells with watery saliva, whereas the stylet of a leafhopper sieves intracellularly through leaves to reach the phloem (Hori K, 1976; Miles, 1999; Hunter and Backus, 1989). Despite the presence of primary defense compounds (i.e. trichomes, waxy cuticles, etc.) plants depend on phytohormones to further confer insect damage resistance. Depending on the intensity or sequence of attack, plants typically upregulate jasmonic acid (JA) or salicylic acid (SA) to mediate damage, but other lesser known phytohormones may concurrently be employed (Erb et al., 2009; Tooker and De Moraes, 2011; Mapes and Davies, 2001; Campos et al., 2009). Notably, infestation by green peach aphids results in upregulation of the SA pathway whereas Aster leafhoppers infestation brings upon elevated levels of JA (Mohase and van der Westhuizen, 2002; Kusnierczyk et al., 2008; Cowles et al., 2018). Together, the presence of insect infestation may trigger a series of phytohormonal responses, which in turn, might act as a deterrent to current or future insect feeders.

Prior to feeding, piercing-sucking insects, such as leafhoppers and aphids, undergo a series of behaviors assisting the selection of an appropriate host plant. Although these behaviors typically follow a sequence consisting of (1) plant exploration, (2) stylet probing, (3) fluid ingestion, and (4) feeding termination, environmental stimuli are concurrently interacting with the insect, and providing new sensory information about its food source (Nault and Rodriguez, 1985). Introduction to alternative environments, such as exposure to new host plants or encountering contaminated host plants, may elicit behaviors that violate these expected feeding sequences (Huang et al., 2018; Soldano et al., 2016).

HONEYDEW PRODUCTION

The vasculature of plants, comprising of the xylem and phloem, contains a slew of organic compounds that are sought by sap-feeders. Via the process of translocation, the phloem predominantly transports complex carbohydrates produced from the source organs (leaves) directly to sink organs (such as roots, developing seeds, or young plant shoots) under high osmotic pressure. In order to counterbalance the high-pressure environment of the vasculature, and moreover retain water and rare amino acids, vasculature feeding insects absorb essential nutrients, and void excess carbohydrates in the form of honeydew.

The chemical composition of insect honeydew depends on a variety of biological factors including the nutrient profile of the vasculature (Mittler, 1958), the duration of insect infestation (Hendrix et al., 1992), the climate (Seeburger et al., 2022) and interestingly, even the presence of ants (Yao and Akimoto, 2001; Yao and Akimoto, 2002). Although honeydew is commonly considered an excrement product, the supplementary ecological impact it holds should not be understated. In the face of nectar food shortages, moths have shown to exploit existing honeydew sources as a nutritional food buffer (Gardner-Gee et al., 2014; Moller, 1989). To supplement their diet, ants collect and ‘farm’ aphids for their honeydew in exchange for protection from aphid predators. Studies have even shown that the composition of aphid

honeydew shifts in the absence of ants, significantly reducing the quantity of melezitose – a trisaccharide synthesized within insect guts (Fischer and Shingleton et al., 2001). Although the presence of melezitose-rich honeydew is sought after by ant mutualists, this form of honeydew acts as a poor alternative food source for honeybees, and even leads to accelerated rates of crystallization within processed honey samples (Seeburger et al., 2022). When considering the phyllosphere and the plant pathogenic microbiota that may inhabit it, the presence of honeydew has been identified as a nutritional food source. When co-inhabiting broad bean plants, populations of *Pseudomonas syringae* – a plant pathogenic bacteria – were significantly higher in the presence of honeydew than in its absence (Smee and Hendry, 2022). Moreover, the carbohydrate rich reservoir which honeydew provides creates the foundation for fungal plant pathogens, such as sooty mold, to persist. Despite setting the foundation for numerous ecological interactions to occur, the impact that honeydew might hold on human enteric bacterial pathogen growth remains largely unknown.

Although viable populations of *S. enterica* were previously identified within green peach aphids and Aster leafhopper honeydew, the use of honeydew as a nutritional niche by *S. enterica* had not yet been established (Soto-Arias et al., 2014). Together, the rich concentrations of carbohydrates (i.e. glucose, fructose, and maltose) and the trace amounts of essential amino acids (i.e. glutamate, glutamine, and aspartate) within honeydew suggest potential for *S. enterica* bacterium to thrive (Shaaban et al., 2020).

INSECTS ENCOUNTERING BACTERIA

The mode by which insects navigate the world begins with an environmental stimulus, continues with a cascade of physiological responses, and culminates in a behavioral reaction. For instance, (1) a flee-flight response by a ladybird beetle (*Coccinellidae*) may partially be caused by a sudden movement of mechanoreceptors, thus indicating an unexpected disturbance of a leaf, or (2) a cohort of chemoreceptors which receive sex pheromone

attractants may likely prompt a moth to find a conspecific mate. Although a plethora of studies have assessed how and why insects move, the field of entomology has only begun to question the complexity of behavioral and molecular modalities when it comes to an insect's perception of a food source. Our vast improvement of scientific techniques and understanding within the last decade has prompted research groups to generate interest in the environmental triggers which elicit a behavioral response when insects encounter an adverse, pathogenic stimuli. Provided the high applicability of studying human-relevant illnesses within an entomological context, a majority of these studies focus upon Gram - negative bacterial pathogens, such as *S. enterica* and *E. coli*. Aside from generally expanding our knowledge of insect behavior, understanding this multi-dimensional line of study elucidates the mechanisms by which insects choose to avoid or approach contaminated foods. Taken together, this research is critical given that some phytophagous insects act as vehicles for pathogenic bacteria as previously mentioned. Western flower thrips, Aster leafhopper, and Green peach aphids, for instance, are considered biological multipliers of *S. enterica*, a Gram - negative human enteric bacterial pathogen which is frequently associated with large scale food borne outbreaks on both lettuce and tomatoes (Soto-Arias et al., 2014; Cowles et al., 2018). Characterizing how insects perceive *S. enterica* – potentially modifying their natural behaviors – we can further understand how other biological multipliers influence food borne outbreaks, and vice versa.

BACTERIAL-INDUCED BEHAVIORS AND AVOIDANCE

Anthropomorphically speaking, the human ability to taste develops our repertoire of foods we form preference and aversions to. While this ability may seem exclusive to complex mammalian systems, insects have shown to exhibit odor-evoked behaviors and discriminate between contaminated substrates. When searching for a source of food and concurrently encountering bacterially saturated substrates, insects undergo a series of responses. Simply put, however, an insect's immune system first responds, and a physical behavior is

subsequently elicited. This physical response is typically manifested by a movement away from the contaminant, a visceral reflexive movement (such as grooming), or a combination of both. When presented two agar substrates, *D. melanogaster* preferred the plain, non-contaminated agar over the side treated with LPS (Yanagawa et al., 2014). Within a general aversion-based bioassay, it was observationally discerned that some of these taste receptors likely reside upon the legs and mouthparts of insects, provided that insects exposed to LPS promptly moved away from the LPS contaminant and towards the non-contaminated or sugar enriched substrate. Furthermore, these clusters of gustatory neurons, when combined, functionally create taste receptors. Fascinatingly, it was additionally found that general exposure to LPS elicited an expression of taste receptors which are broadly associated with bitter foods (Soldano et al., 2017), suggesting the ability to form aversions associated with taste. This overt aversion to LPS was similarly observed when selecting a egg laying substrates of female *D. melanogaster* (Soldano et al., 2017). Regarding other LPS associated behaviors, Yanagawa et al., demonstrated that the application of *E. coli*, LPS derived from *E. coli*, and other abrasive chemicals to *D. melanogaster*'s wing-based taste receptors induced a grooming response (Yanagawa et al., 2014). This behavior is typically employed by insects to decrease the extent of microbial populations upon their exoskeleton, thereby reducing the potential for bacterial infections. One potentially problematic, yet intriguing, step of this investigation was the decapitation of insects in order to prevent ample movement. A complementary experiment, however, indicated that grooming behavior continued to occur 20 hours post decapitation, indicating a programed behavior to limit one's exposure to harmful bacterial pathogens. Furthermore, this lab also demonstrated that the application of Gram – positive bacteria such as *Lactobacterillus bulgaricus* and *Mycoplasma fermentans*, and fungal pathogens (*Beuveria bassiana*) did not elicit grooming induction independent of insect sex. Although this behavior, alongside grooming, exhibits severe aversion and avoidance strategies of LPS, and therefore Gram - negative bacteria, it remains unclear whether insects adversely respond to LPS all

together, or it's broken-down components which are comprised of a lipid anchor, carbohydrate chains, or the polysaccharide core. Together or individually, these components act as exotoxins which may immediately be detected by insect sensilla. Furthermore, it remains widely unknown where LPS is specifically recognized or broken down within the foregut of insects. Aside from adult fruit flies, the food preference and avoidance strategies have similarly been shown within *D. melanogaster* larval stages. Specifically, when exposed to foods contaminated with Gram - negative bacteria, and thus food which could contribute to a lethal infection, larvae began to adapt and avoid the bacterially saturated food (Surendran et al., 2017). This mediation is suspected to be initially recognized by the central nervous system and curtails the insect's food choice to prevent further bacterial infections. Despite the lack of an adaptive immune system these aversion and grooming behaviors across insect life stages serve as effective mechanisms to potentially thwart Gram - negative bacterial invasions, such as *E. coli* and potentially *S. enterica*. Further studies are essential to entirely understand how physiological signals mediate insect behaviors, and how these elicited behaviors in turn might impact the mechanical transmission of *S. enterica*. Moreover, investigations into how insects beyond Diptera, such as vasculature feeding hemipteran insects, respond to bacterial pathogens are highly relevant for food.

TASTE PERCEPTION IN HEMIPTERAN INSECTS

Most physiological taste-perception studies heavily utilize *D. melanogaster* as an ideal model organism given its high proportions of shared genes with humans, and to further build upon other studies which similarly utilized this insect. Although our knowledge regarding insect-taste recognition of pathogenic substrates has clearly been expanded, *D. melanogaster* possess a sponging -sucking mouthpart (a mode of feeding generally exclusive among flies) and thereby limits our knowledge on how insects employing other modes of feeding perceive the environment similarly, or vastly different. As previously found in *D. melanogaster*, the

potential exposure to LPS within the environment could potentially halt this leafhopper feeding sequence, by causing an immediate aversion; Given the lack of investigation, however, this speculation remains unexplored. Although the Aster leafhopper has not yet been considered for similar taste studies, a genome analysis identified both odorant and gustatory receptor genes in aphid and mosquito genomes, which both possess a piercing sucking mouthpart comparable to that of leafhoppers (Isono and Morita, 2010). In a series of bioassays, however, aphids had shown to not cultivate a taste aversion to lectin, a plant derived entomotoxic protein, unlike larval *D. melanogaster* in response to LPS. Although these are two vastly different compounds, it might imply that the feeding strategies of these two insects might impact food-aversion behaviors. Including behavioral studies within our pathosystem (plant, phytophagous insect, and *S. enterica*) would significantly expand our understanding of vector biology.

RELEVANCE TO GLOBAL HEALTH

Although the causal agent of food borne outbreaks is frequently placed on identifying the origin, research from the Barak and Groves laboratories highlights the necessity to additionally consider the biological agents enhancing the success of *S. enterica* survival. Comprising a majority of the worlds described animal species, insects have historically posed a ubiquitous threat to agriculture by devastating crop yields and concurrently jeopardizing the livelihood of farmers both small and corporate. While our study places emphasis upon *M. persicae*, *M. quadrilineatus,* and touches on *F. occidentalis*, these organisms represent phytophagous insects that feed on a variety of plant families including many crops under cultivation worldwide. Although the elimination of food-borne outbreaks altogether is implausible, understanding how biological multipliers benefit epiphytic *S. enterica* populations is the next rational step in pursing food safety intervention strategies, integrated pest management measures, and furthermore protecting the health of local and global communities at risk.

RESEARCH OBJECTIVES

Previously, the Groves and Barak lab had identified that the presence of select phytophagous insects enhance epiphytic survival of *S. enterica* upon lettuce and tomato hosts. While this novel pathosystem had been described, the behavioral mechanisms employed by insects that transforms the phyllosphere into a habitable niche for *S. enterica* had remained unexplored. Within this thesis, my aim is to elucidate these entomological mechanisms that contribute to produce-associated *S. enterica* foodborne outbreaks. My specific objectives were 1) to explore the extent of elicited cellular damage induced by phytophagous insects possessing rasping-sucking (Chapter 2), or piercing-sucking mouthparts (Chapter 3); 2) investigate Hemipteran feeding behaviors in response to *S. enterica* exposure (Chapter 3); and 3) distinguish the influence of honeydew upon epiphytic *S. enterica* populations in-vitro, and on novel host plants (Chapter 4). Practicing effective science communication, Chapter 5 was written in collaboration with the Wisconsin Initiative for Science Literacy (alternatively known as WISL) to translate my previously published work (Chapter 3) into an accessible format for non-scientific audiences. Previous studies composed by Dundore-Arias et al., and Cowles et al., had identified western flower thrips, Aster leafhoppers and green peach aphids as biological multipliers for epiphytic *S. enterica* populations. In conjunction with these findings – and moreover their robust nature as agricultural pests – I chose to further characterize feeding behaviors of these model organisms.

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Chapter 2: *Frankliniella occidentalis* facilitate *Salmonella enterica* survival in the phyllosphere.

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Author Contributions

VL, RG, JB conceptualized the study and had written and reviewed the final manuscript. VL and MM curated data and conducted formal analysis.

Abstract

The human enteric bacterial pathogen *Salmonella enterica* causes approximately 1.35 million cases of food borne illnesses annually in the United States. Of these salmonellosis cases, almost half are derived from the consumption of fresh, raw produce. Although epiphytic *S. enterica* populations naturally decline in the phyllosphere, a subset of phytophagous insects have recently been identified as biological multipliers, consequently facilitating the growth of bacterial populations. We investigated whether tomato leaves with macroscopic feeding damage, caused by infestation of adult Western flower thrips (Frankliniella occidentalis), support higher *S. enterica* populations. To explore this hypothesis, we assessed *S. enterica* populations in response to thrips feeding by varying insect density, plant age, and the gender of the insect. As a reference control, direct leaf damage analogous to thrips feeding was also evaluated using directed, hydraulic pressure. In a supplementary set series of experiments, groups of *F. occidentalis* infested tomato plants were later inoculated with *S. enterica* to determine how prior insect infestation might influence bacterial survival and persistence. Following an infestation period, leaves visibly damaged by adult *F. occidentalis* supported significantly higher *S. enterica* populations and resulted in greater amounts of electrolyte leakage (measured as electrical conductivity) than leaves lacking visible feeding damage. Plant age did not significantly influence *S. enterica* populations or estimates of electrolyte leakage, independent of initial infestation. Additionally, the gender of the insect did not uniquely influence *S. enterica* population dynamics. Finally, applications of aggressive water bombardment resulted in more electrolyte leakage than leaves damaged by *F. occidentalis*, yet supported comparable *S. enterica* populations. Together, this study indicates that *F. occidentalis* feeding is one of the many potential biological mechanisms creating a more habitable environment for *S. enterica*.

Introduction

In the United States alone, the human enteric bacterial pathogen *Salmonella enterica* is estimated to cause 1.35 million cases of food-borne illnesses annually (Centers for Disease Control and Prevention, 2020). While it is generally perceived that cases of salmonellosis are acquired from consumption of *S. enterica*-contaminated animal products, cross-contamination of fresh produce has become an overwhelming risk throughout the cultivation process, subsequently resulting in a growing rate of produce-associated salmonellosis over the past decade [1, 2]. According to the CDC, *S. enterica* is responsible for the majority of bacterial foodborne illness and, unlike other bacterial foodborne pathogens, the incidence of outbreaks has not diminished over the last decade. The switch in vehicles from contaminated animal products to fresh produce is posited as the leading reason for the ongoing salmonellosis outbreak occurrence. Identifying the biotic mechanisms that allow enteric human pathogens, such as *S. enterica*, to persist or grow on fresh produce pre-harvest is the first step in reducing the disease burden and increasing food safety.

Harsh environmental conditions, such as direct UV radiation or lack of available nutrients, make the phyllosphere of plants an adverse environment for *S. enterica* [3]. In fact, *S. enterica* populations have been observed to naturally decline on healthy leaves [4–6]. In spite of these challenges, *S. enterica* populations must persist or find advantageous niches or partnerships on plants in the field pre-harvest, because salmonellosis outbreaks from consumption of raw produce continue to occur regularly. In addition to agricultural practices acting as bacterial reservoirs [7], insects are also recognized as potential biological vectors of *S. enterica* given their unique behaviors as they facilitate the dispersal and enhance the persistence of the bacterium [8]. Multiple species of cockroaches and darkling beetles, for instance, phoretically transfer or mechanically vector *S. enterica*, thus promoting proliferation and dispersal of bacteria within poultry associated environments [9, 10]. To a similar extent, previous studies showed that infestations of the Aster leafhopper (Macrosteles quadrilineatus)

and the Green peach aphid (*Myzus persicae*) are associated with mechanical transmission of *S. enterica* to uncontaminated lettuce leaves [8]; interestingly, Aster leafhoppers were also found to ingest *S. enterica* and disperse the pathogen in honeydew [8]. In addition to assisting the movement of bacteria, phytophagous insect infestation on *S. enterica*-contaminated plants led to a prolonged persistence of the bacteria when compared to plants without insects [5, 11]. While the exact mechanisms causing prolonged persistence of the bacterium are unknown, unique insect feeding behaviors and associated damage to plants are suspected to provide a favorable environment for *S. enterica* persistence by providing a more direct route of entry to the apoplast of plant leaves, protected leaf interior, and/or to damaged cells leaking nutrients.

Western flower thrips, Frankliniella occidentalis Pergande (Thysanoptera: Thripidae) are considered a highly polyphagous insect species and employ a modified form of piercing-sucking feeding, whereby the stylets do not reach the vascular system of plants to withdraw vascular constituents [12]. Instead, adult, and immature *F. occidentalis* feed by initially puncturing surface mesophyll cells and then sucking out cellular contents. In this study, we tested the hypothesis that areas of leaves with macroscopic feeding damage caused by adult *F. occidentalis* support greater *S. enterica* populations than undamaged areas. Additionally, we investigated whether insect containment or open infestation of *F. occidentalis* would differentially affect the magnitude of leaf damage and *S. enterica* survival. We also postulated that the gender of *F. occidentalis* could influence the survivorship of *S. enterica*¸ similar to the results observed with transmission of Tomato spotted wilt tospovirus [TSWV; 13, 14], where adult male *F. occidentalis* possessed higher transmission efficiencies compared to females. The findings herein elucidate whether insect feeding behaviors, namely feeding damage, locally promotes the persistence of *S. enterica* populations.

Material & Methods

Bacterial strains and culture conditions

A kanamycin (Kan) resistant strain of *S. enterica* serovar Typhimurium 14028s used in this study was grown in lysogeny broth (LB; Difco, Dickinson and Company, Sparks, MD) at 37°C with shaking at 200 rpm.

Insect rearing

Colonies of *F. occidentalis* were maintained on green bean pods (Phaseolus vulgaris), where the insects utilize the pods as both an ovipositional substrate and a food source. A starter colony of *F. occidentalis* was provided by Dr. Thomas L. German, Professor Emeritus, University of Wisconsin, Madison, from a long-term colony maintained in their laboratory. Weekly, green bean pods were sterilized in a 5% Clorox solution for 15 min and rinsed twice with sterile water to remove potential pesticide residue prior to placement in rearing containers. Populations of *F. occidentalis* were maintained in 0.4 L plastic containers (Dart Container Corporation, Mason, MI) and held under a constant temperature (27°C) and a 16:8 (Light:Dark) photoperiod. Each container included a sheet of filter paper to prevent moisture from accumulating. Voucher specimens of adult female and male *F. occidentalis*, obtained from the original colony, have been deposited in the Wisconsin Insect Research Collection, University of Wisconsin-Madison (http://labs.russell.wisc.edu/wirc/).

Insect infestation and plant inoculation experiments

To investigate whether plant age together with *F. occidentalis* infestation density would alter *S. enterica* populations and electrolyte leakage, a no choice arena experiment was performed. Solanum lycopersicum (tomato, cv. Money Maker) seedlings were cultivated using Professional Growing Mix (Sunshine Redi-earth) in 6” pots held in a growth room maintained at a 16:8 (L:D) photoperiod and 24°C light and 19°C dark conditions for three and five weeks. In a

2X2 factorial design, replicate sets of adult *F. occidentalis* were transferred onto six separate three and five-week-old tomato plants, at densities of 5 (low density) or 20 (high density) individuals per plant. Each plant was contained in a 15.5 cm (ht) and 10 cm (diameter) Plexiglas tube, fashioned with thrips-proof screening (Green-tek Inc., Janesville, WI) at one end of the tube to prevent insects from escaping the experimental arena, and infested cages were held at 26°C temperature with a 16:8 (L:D) photoperiod. Seventy-two hours after the initial release of insects into cages, all adult *F. occidentalis* were removed, and the number of feeding lesion sites were visually assessed and counted. Subsequently, each plant was dip-inoculated in 450 ml of sterile water or a 108 CFU/ml suspension of *S. enterica* (each beaker containing 75 µL of Sil-Wet, a surfactant aiding solution adhesion) for one minute. For bacterial inoculum preparation, lysogeny broth was inoculated with bacteria from -80°C freezer stocks and incubated, shaking overnight at 37°C. Bacterial cultures were normalized to an optical density at 600 nm of 0.2 in sterile water. Inoculum populations were verified by enumerating populations following serial dilution, plating on LB-Kan agar (50 µg/ml), and growth overnight at 37°C. Dip-inoculated plants were then placed in a clear, uncovered plastic bin at 27°C temperature under a 16:8 (L:D) photoperiod.

To assess *S. enterica* populations, plants were sub-sampled 72 hours after dip-inoculation to determine whether bacterial populations were localized to damaged sites on leaves. We chose to examine *S. enterica* population dynamics 72 hours post inoculation, as bacteria begin to diverge in population size within the presence or absence of insect infestation upon tomato leaves at this point [5]. Specifically, one 10 mm diameter leaf disc with macroscopic feeding damage (silvering), and one leaf disc absent of visible (macroscopic) feeding damage were extracted from the same leaf on dip-inoculated plants 72 hours following inoculation. These samples derived from the 2X2 factorial design were individually homogenized in 500 µL of sterile water using a cordless Dremel tool, and further diluted 1:10 in sterile water. Homogenates were immediately plated on LB-Kan agar, incubated overnight at

37°C and enumerated after 24 hours. Experiments were performed with three biological replicates.

To further characterize the magnitude of cellular or leaf damage associated with 72-hour thrips infestation, measurements of electrical conductivity were obtained from a comparable set of three, 10 mm-diameter leaf discs with macroscopic feeding damage, and three visibly undamaged leaf discs from the same leaf on each of three plants. Each group of three leaf discs were individually placed in single wells of a 12-well tissue culture plate containing 4 ml of sterile water. Plates were positioned on a rotating table at 50 rpm for approximately 30 min, acting as a wash step to prevent remnant soil particles from influencing conductivity measurements [15]. Subsequently, water from each well was removed and replaced with fresh, sterile water, and electrical conductance was immediately measured. Electrical conductance was measured by pipetting 1 ml of water from sample wells onto a ECTestr11+ MultiRange electrical conductance probe to assess the extent of conductive solute leakage, here used as a proxy for cellular damage. After the initial assessment of electrical conductance, sample plates were left covered under light banks at an ambient temperature for 6 hours, after which a second and final conductivity measurement was taken. Measures of electrical conductance were calculated by subtracting between the two time points (initial and second) and were used to evaluate the extent of electrolyte leakage over a six-hour period. Differences in measured conductance were used for data analysis for each plant age and infestation density treatment group.

An additional, free choice experiment was performed to assess if *F. occidentalis* would similarly influence electrical conductivity estimates and *S. enterica* populations compared with results observed in the no choice experiments. Briefly, adult populations (females and males) of *F. occidentalis* were released onto tomato seedlings for infestation periods of either three, four, or five weeks, resulting in plants infested with various life stages of the insects and obvious sites with feeding damage (silvering). This release of *F. occidentalis* onto plants allowed insects to actively move and thereby feed wherever they chose, hence the name ‘free choice experiment’.

After the initial free-choice infestation period, 24 plants were randomly selected and removed from the experimental arena at one of the three time points post infestation and were dip inoculated into either a 450 ml aliquot of sterile water, or a 108 CFU/ml suspension of *S. enterica* (each beaker containing 75 µl of Sil-Wet) for one minute. Bacterial cultures were prepared as described above. Replicate sets of 10 mm-diameter leaf discs were extracted 3 days after dip inoculation from separate leaves on each of 24 biological replicates per time point following infestation. Electrical conductance and *S. enterica* populations were assessed as described previously.

To determine whether insect gender influences *S. enterica* population dynamics, adult populations of male or female *F. occidentalis* were provided access to separate plants. Six, 2 cm diameter, thrips-proof, Plexiglas clip cages were attached to the underside of three, middle S. lycopersicum leaflets on opposing leaves. Three clip cages were infested with three individual male or female *F. occidentalis*, whereas the remaining three clip cages remained empty. Three days post infestation, insects and clip cages were removed, and each plant was subsequently dip inoculated into either a 450 ml aliquot of sterile water, or a 108 CFU/ml suspension of *S. enterica* (each beaker containing 75 µl of Sil-Wet) for one minute. Bacterial cultures were prepared as described above. Replicate sets of 10 mm-diameter leaf discs were extracted 3 days after dip inoculation from separate leaves on each of the 4 individual plants following infestation by male or female thrips, and plants which remained absent of infestation. Electrical conductance and *S. enterica* populations were assessed as described previously. Experiments were performed with three biological replicates.

Biotic vs. abiotic damage

To better understand the interaction between biotic (*F. occidentalis*) and abiotic cellular damage and resulting *S. enterica* populations, water pressure was used to inflict direct, physical (abiotic) damage to S. lycopersicum leaves, and served as a reference control. Three leaflets,

from five-week-old plants randomly chosen for infestation, were faceted with three clip cages and infested with three adult *F. occidentalis*, whereas the remaining three clip cages on the opposite leaf remained empty on each plant. Additionally, six empty clip cages were applied to six leaflets between two opposing leaves for plants used as a control. At 72 hours after infestation, an inserted color cup was attached to a single action, siphon feed airbrush set (Paasche Airbrush Co., Kenosha, Wisconsin) and filled with sterile water, and a new set of five-week-old plants were randomly selected to receive short duration (5 sec) pulses of water at different pressures of 0.35, 1.41, or 3.78 kg cm-2 and directed to the underside of three middle leaflets. After inducing pressurized water damage, insects and clip cages were removed, and each plant was subsequently dip inoculated into either a 450 ml aliquot of sterile water, or a 108 CFU/ml suspension of *S. enterica* (each beaker containing 75 µl of Sil-Wet) for one minute. Bacterial cultures were prepared as described above. Replicate sets of 10 mm-diameter leaf discs were extracted three days after dip inoculation from separate leaves on each of the 4 plants per treatment group following infestation. Electrical conductance and *S. enterica* populations were assessed as described previously. Experiments were performed with three biological replicates. To examine cell membrane viability of areas damaged by *F. occidentalis* or water pressure application, whole leaves were extracted 72 hours after imposed damage (as previously described) and immediately submerged in 10 mL of 0.25% Evans blue dye and incubated on a rotating table at 80 rpm for 20 minutes [16]. Following incubation, whole leaves were rinsed with sterile water to remove residual dye and observed with a Leica MZFL3 stereoscopic microscope.

Statistical analysis

A one-way, analysis of variance (ANOVA) was used to assess if *S. enterica* populations or electrical conductance measurements varied between damaged and undamaged leaf discs derived from plants varying in age (3 or 5 weeks old), subjected to different *F. occidentalis*

infestation densities (5 or 20 adult thrips), subjected to male or female infestation, and when damaged by *F. occidentalis* or water pressure. Student's t-tests were performed to determine if feeding damage prompted a difference in electrical conductance of *S. enterica* populations when compared to undamaged plant tissues regardless of their treatment. To analyze the same response, student's t-tests were also applied to the free-choice experiment. Outliers within each experiment were kept.

Results

S. enterica populations and electrolyte leakage are greater in F. occidentalis damaged sites in no-choice experiments

To learn whether populations of *S. enterica* were directly influenced by localized macroscopic damage sites from insect feeding, bacterial population dynamics were assessed on damaged and macroscopically undamaged tomato leaves. In a controlled, no-choice environment, tomato plants from two age groups (3 or 5 weeks-old) were exposed to low (5 insects/cage) or high (20 insects/cage) densities of *F. occidentalis* to investigate if plant age and insect density influenced *S. enterica* populations. Within each treatment group, *S. enterica* populations were 1 log higher on macroscopically damaged leaf discs($P < 0.0001$; Fig 1A). To ascertain whether cellular damage co-occurred with *S. enterica* populations, electrical conductance was measured. Electrolyte leakage doubled on leaf discs in association with feeding damage ($P < 0.0001$; Fig 1B).

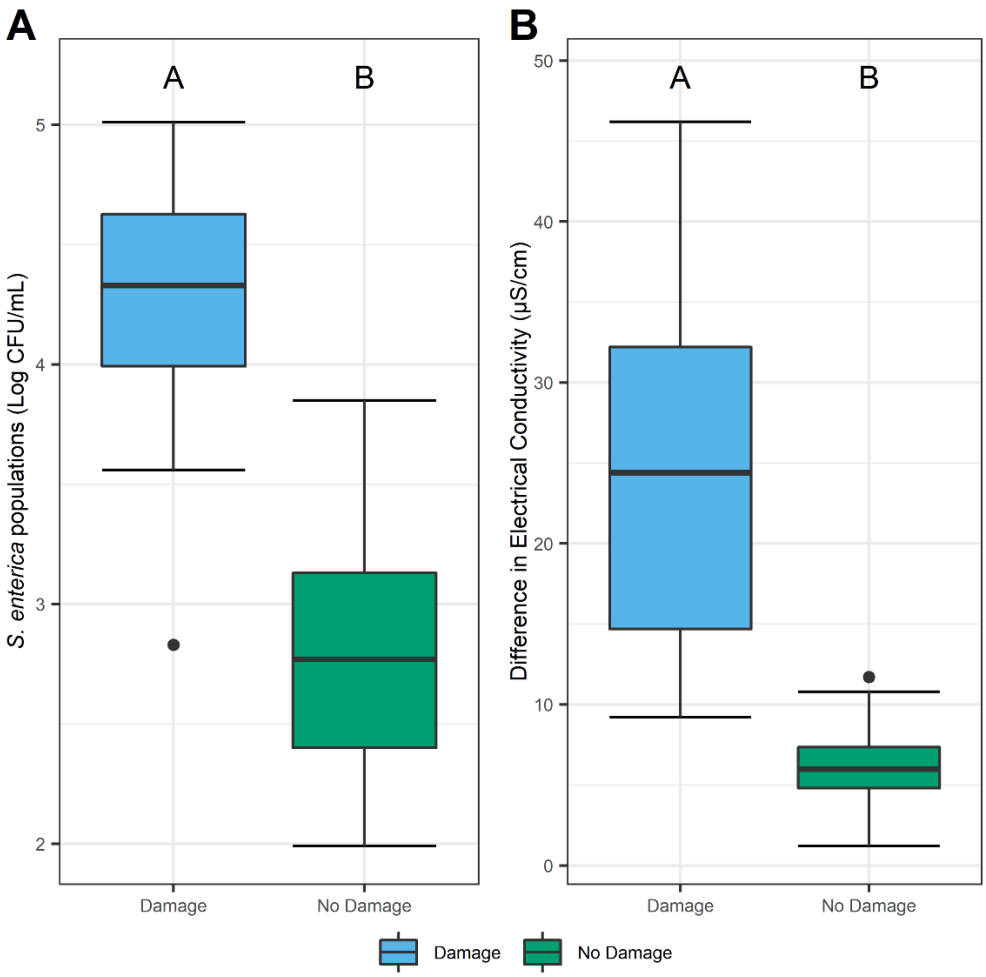

Fig 1. Impact of F. occidentalis damage on S. enterica populations and electrolyte leakage in a confined environment. In a no-choice experimental arena, tomato leaf areas with F. occidentalis damage (blue) had higher S. enterica populations (A) and greater differences in estimated electrolyte leakage (B) than undamaged sites (green). Means from each treatment group represent combined responses over plant age (3 or 5 weeks) and infestation density (5 or 20 insects per plant), as these main effects were non-significant. Measures of electrical conductance were calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over a six-hour period. Boxplots with different letters indicate a significant difference ($P < 0.05$), as determined by a student's t-test. Singular dots represent outlier points.

Next, we examined plant age and insect density. There was no significant differences when comparing treatment groups (variation in plant age and insect density) between macroscopically damaged leaves when assessing bacterial populations and electrolyte leakage ($P > 0.05$.; S1 Fig). Younger (3 weeks-old) tomato plants infested with higher, initial *F. occidentalis* populations densities (20 insects/cage) had twice as many macroscopic damaged areas when compared with plants exposed to lower infestation densities. ($P > 0.05$; Fig 2). However, older (5-week-old) tomato plants had similar numbers of feeding lesions regardless of initial *F. occidentalis* populations densities. Furthermore, the electrolyte leakage observed was similar to the results for the numbers of feeding lesions with more leakage on leaves exposed to more insects at 3 weeks but similar leakage on leaves at 5 weeks, independent of insect density ($P > 0.05$; S1 Fig).

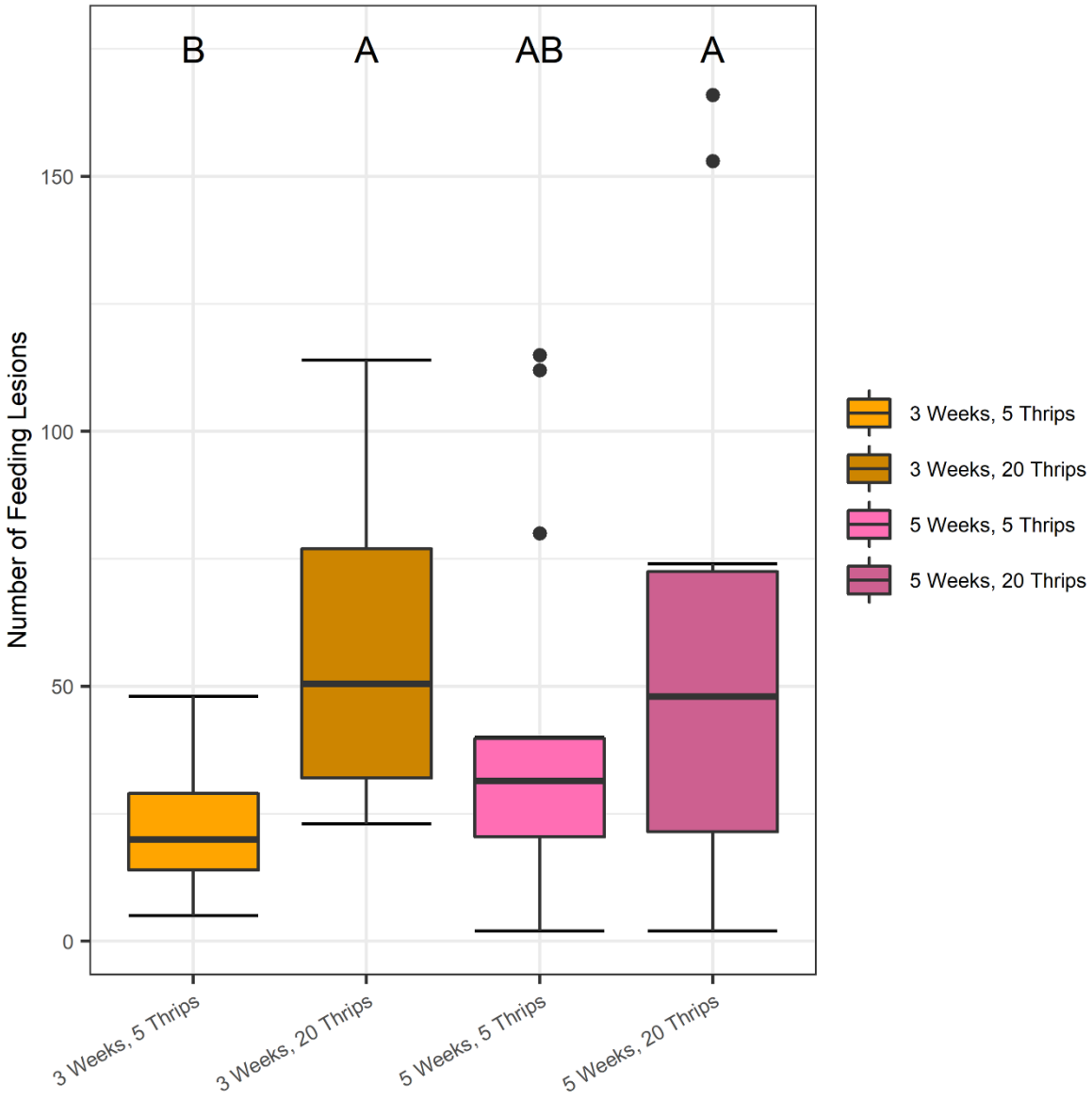

Fig 2. Quantity of *F. occidentalis* feeding lesions on tomato plants. In a no-choice experimental arena, younger (3 weeks-old) tomato plants with a greater, initial *F. occidentalis* infestation density (20 thrips/plant) exhibited significantly more individual feeding lesions than younger plants with lower, initial infestation densities (5 thrips/plant). Macroscopic feeding damage was visually assessed and counted after removing insects from each plant following a three-day infestation period. Data from each experimental replicate were combined and represented. Boxplots with different letters indicate a significant difference ($P < 0.05$), as determined by a one-way ANOVA test. Singular dots represent an outlier point.

We also examined insect gender. In an additional no-choice experiment, tomato leaves damaged by male or female *F. occidentalis* had significantly greater electrolyte leakage ($P < 0.0001$) and higher *S. enterica* populations ($P < 0.0001$), when compared with visibly undamaged tissues on infested plants, or plants entirely absent of insects (Fig 3A and 3B). *S. enterica* populations and electrolyte leakage were not significantly different between plants exposed to male or female insects ($P > 0.9801$, $P > 0.9628$; Fig 3A and 3B). Surprisingly, visibly undamaged leaf discs excised from infested plants had double the amount of electrolyte leakage than uninfested plants ($P < 0.0208$; Fig 3B).

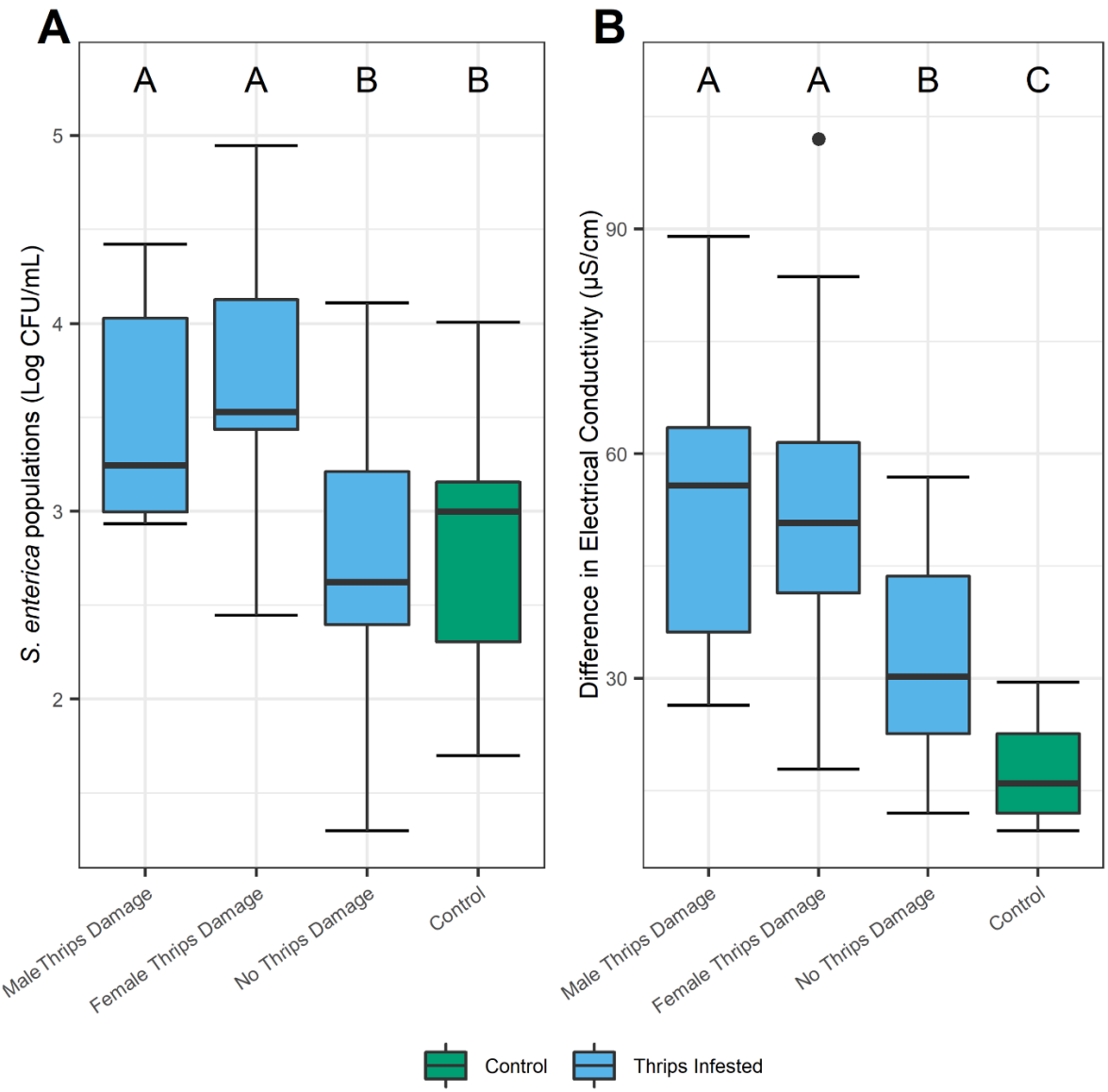

Fig 3. Male and female thrips damage enhances *S. enterica* populations and electrolyte leakage in a no-choice environment. Tomato leaf areas with *F. occidentalis* damage had significantly higher *S. enterica* populations (A) and significantly greater differences in electrolyte leakage (B) than undamaged sites. *S. enterica* population dynamics and electrolyte leakage were not significantly different between males and females. 'No Thrips Damage' represents undamaged leaf discs from *F. occidentalis* infested plants, whereas the 'Control' represents undamaged leaf discs from uninfested plants. Undamaged samples (No Thrips Damage) from plants previously infested by males or females were combined and means are represented. Measures of electrical conductance were calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were

used to evaluate the extent of electrolyte leakage over a six-hour period. Boxplots with different letters indicate a significant difference ($P < 0.05$), as determined by a one-way ANOVA test. Singular dots represent an outlier point.

To model the natural environment of insect infestation and subsequent *S. enterica* plant contamination, we constructed a free choice experimental arena with various life stages of *F. occidentalis* infested tomato plants for experimental durations of 3, 4, or 5 weeks. *S. enterica* populations were a log greater and electrolyte leakage tripled on leaves exhibiting *F. occidentalis* feeding damage when compared to undamaged sites ($P < 0.0001$; Fig 4A and 4B, respectively).

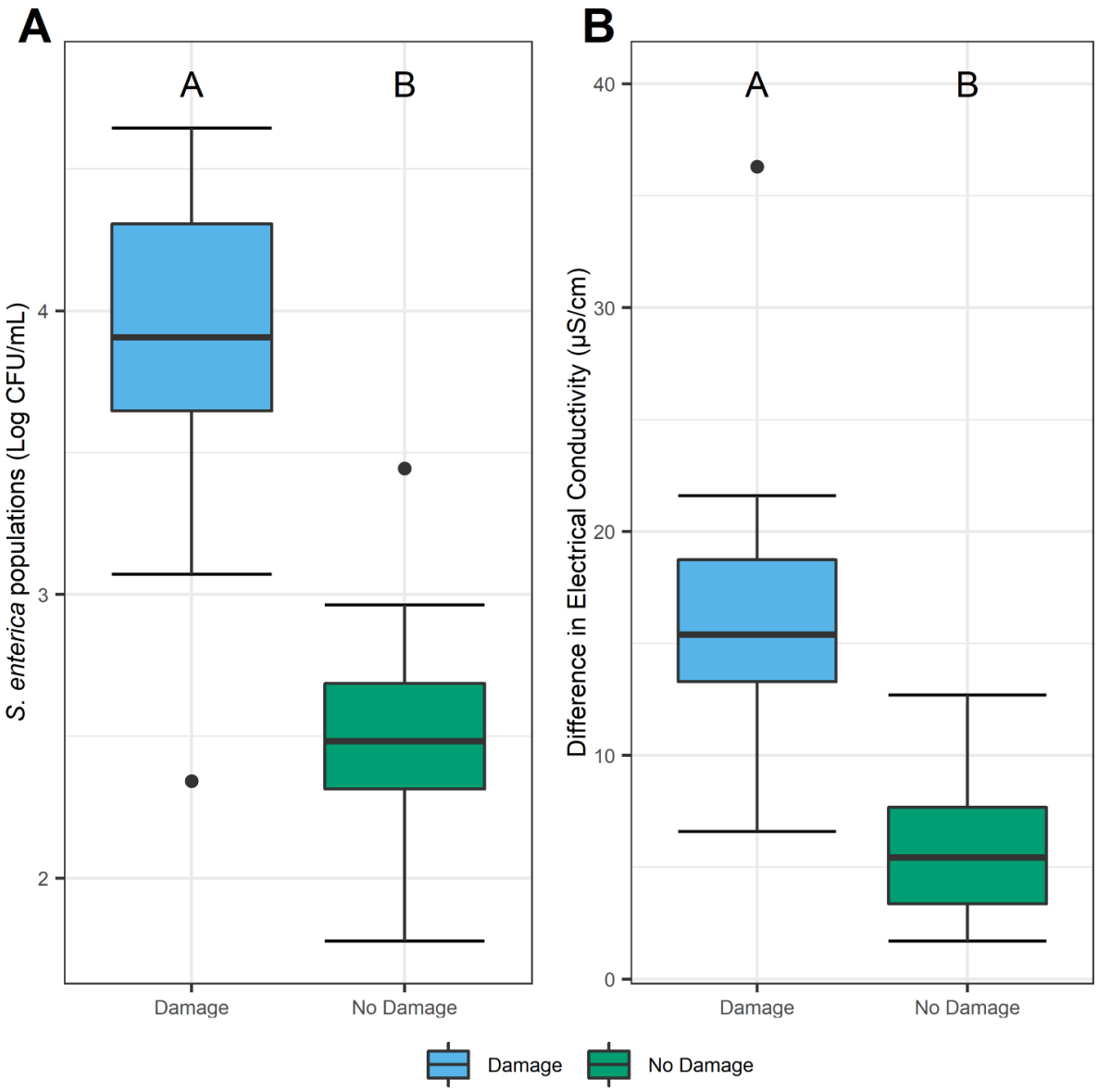

Fig 4. Thrips damage enhances S. enterica populations and electrolyte leakage in a free choice environment. In a free choice experimental arena, tomato leaf areas with F. occidentalis damage (blue) had higher S. enterica populations (A) and greater differences in estimated electrolyte leakage (B) than undamaged sites (green). Data from each infestation period of 3, 4 and 5 weeks were combined as S. enterica populations and electrolyte leakage estimates did not significantly vary over the experimental time periods. Measures of electrical conductance calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs were used to evaluate the extent of electrolyte leakage over a six-hour period. Boxplots with different letters indicate a significant difference ($P < 0.05$), as determined by a student's t-test. Singular dots represent an outlier point.

S. enterica populations are not exclusively dependent on cellular damage induced by F. occidentalis

Cellular damage induced by insect feeding was hypothesized to be a factor that could enhance *S. enterica* population persistence. In this study, pressurized water inoculations were used as a reference control to represent abiotic, or physical damage in comparison to the biotic damage imposed by thrips feeding. First, we observed that *S. enterica* populations decreased over 2 logs within seventy-two hours following inoculation without insect or abiotic damage ($P < 0.0001$; Fig 5). Interestingly, leaves physically damaged by medium (1.41 kg cm-2) or higher (3.78 kg cm-2) water pressure, together with leaves possessing *F. occidentalis* feeding sites, exhibited *S. enterica* populations more than a log greater than leaves damaged by low water pressure (0.35 kg cm-2) or those without any form of visible damage ($P < 0.0001$; Fig 5). Leaves mechanically damaged by these varied pressures (0.35, 1.41, or 3.78 kg cm-2) resulted in significantly greater electrolyte leakage than undamaged leaves ($P < 0.0106$; Fig 6). Leaf tissue with *F. occidentalis* feeding sites resulted in significantly lower cellular damage than leaves damaged by high water pressure sprays (3.78 kg cm-2), indicating that the aggressive bombardment of water resulted in a greater amount of cellular damage in comparison to thrips feeding (Fig 6). Surprisingly, there was not an association between cellular damage and *S. enterica* populations; and of further interest *F. occidentalis* feeding caused significantly less cellular damage than high pressure sprays (3.78 kg cm-2), but resulted in equivalent bacterial populations.

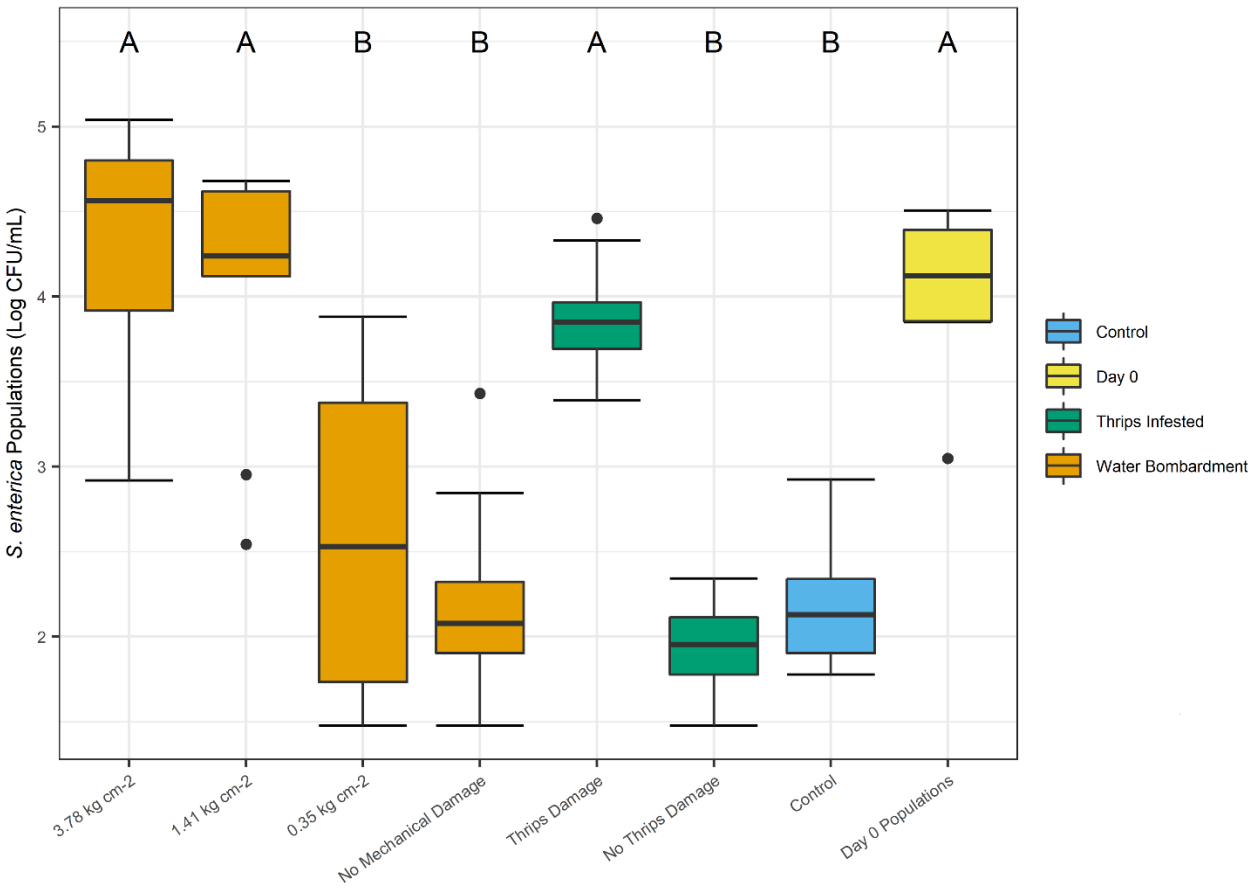

Fig 5. Impact of thrips damage and water bombardment on *S. enterica* populations.

Tomato leaf tissues macroscopically damaged by adult *F. occidentalis*, or mechanically damaged by 1.41 or 3.78 kg cm^{-2} of water pressure, harbor significantly greater *S. enterica* populations than low pressure (0.35 kg cm^{-2}) water treatments and undamaged leaves. Clip cages were fastened to each plant for three days containing three thrips or remained empty as a control. Three days after initial infestation, uninfested, or non-control, plants were subjected to mechanical damage induced by an airbrush paint atomizer, applying 0.35, 1.41 or 3.78 kg cm^{-2} of pressure for 5 seconds. After imposing the mechanical damage, each plant was dip-inoculated in a *S. enterica* solution for one minute and sampled for bacterial populations three days later. Three experimental replicates are represented, and different letters indicate significant differences between treatment groups ($P < 0.05$), as determined by a one-way ANOVA test.

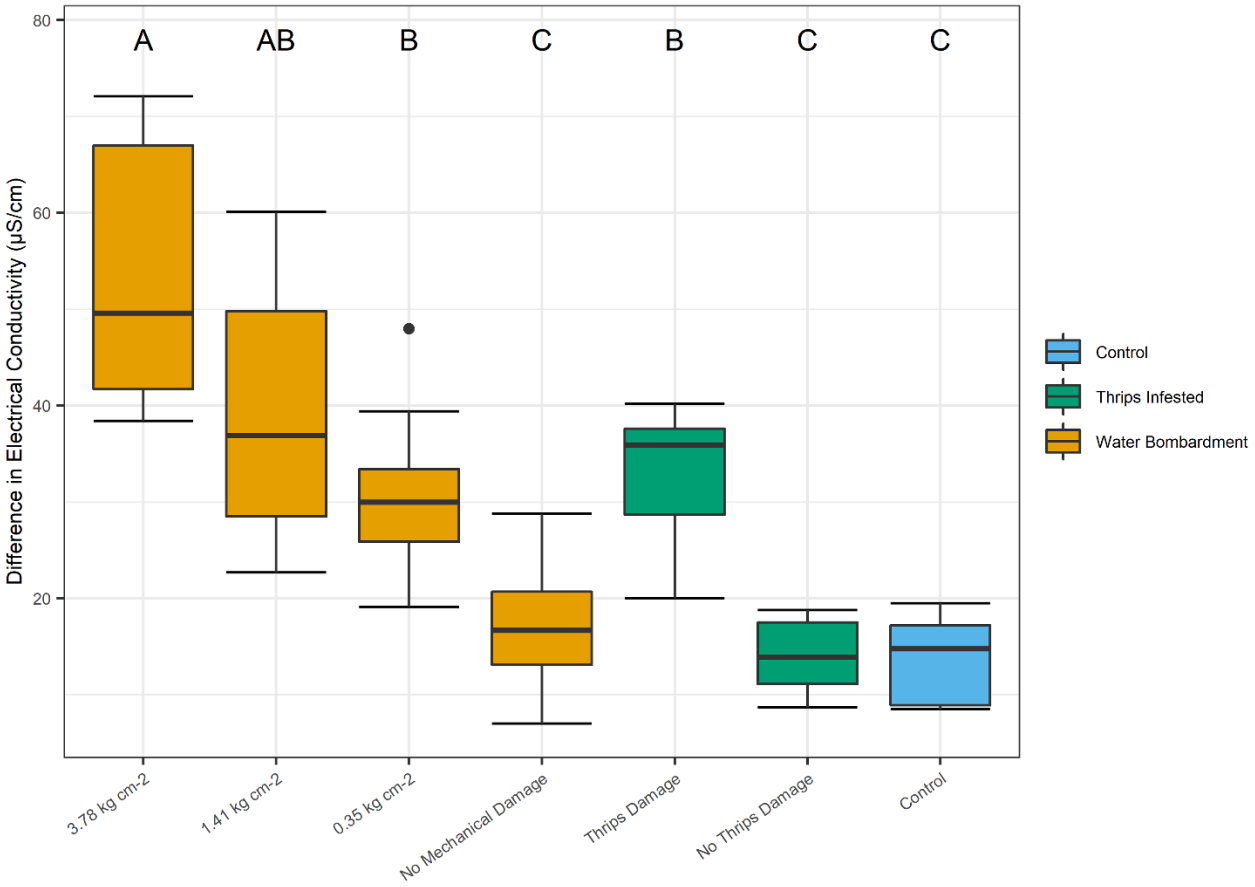

Fig 6. Impact of thrips damage and water bombardment on electrolyte leakage.

Tomato leaf tissues macroscopically damaged by adult F. occidentalis feeding or mechanically damaged by 0.35, 1.41 or 3.78 kg cm-2 of water pressure for 5 seconds elicit significantly greater electrical conductance than undamaged leaves. Leaves damaged by 3.78 kg cm-2 of water pressure resulted in significantly greater cellular damage than leaves damaged by F. occidentalis. Clip cages were fastened to each plant for three days containing three thrips or remained empty. Three days post infestation, uninfested plants were subjected to mechanical damage induced by a paint atomizer, applying 0.35, 1.41 or 3.78 kg cm-2 of water pressure for 5 seconds. Measures of electrical conductance for damaged and undamaged leaf discs were used to evaluate the extent of electrolyte leakage over a six-hour period. Each treatment group represents twelve plants. Three experimental replicates are represented, and different letters indicate significant differences between treatment groups ($P < 0.05$), as determined by a one-way ANOVA test.

Discussion

Although *S. enterica* is traditionally studied in the context of animal hosts, the number of salmonellosis cases attributed to consumption of fresh produce warrants an assessment of environmental factors and how they promote bacterial food-borne outbreaks. On healthy plants, *S. enterica* lacks the necessary mechanisms to maintain phyllosphere populations on its own [17], most likely due to its inability to degrade plant cell wall components and thereby liberate nutrients from plant cells. Maintaining bacterial populations or growing on the leaf surface requires necessary enzymes to breakdown plant cells or alter the cell's biochemistry. The prolific bacterial pathogen, Pseudomonas syringae for instance, produces biosurfactants that increase the rate of diffusion of water across plant cell cuticles subsequently prompting a release of foliar nutrients and making them successful, primary phyllosphere colonists [18]. Despite *S. enterica*'s inability to persist on its own, ubiquitous environmental stressors such as the presence of insects on plants, has previously been demonstrated to enhance *S. enterica* survival [5, 11], and thus increasing the risks for outbreaks of food borne illness from consumption of fresh produce. Although the fruits, of tomato plants are traditionally consumed and linked to food borne outbreaks, earlier investigations have previously isolated *S. enterica* from tomato fruit a month after initial *S. enterica* foliar inoculation. This affirms the relevance of assessing *S. enterica* populations upon tomato leaves in relation to food borne outbreak risks especially in the context of foliar feeding insects [19]. In the current study, we examined the interactions between *S. enterica*, *F. occidentalis*, and tomato plants, specifically investigating the effects of cellular damage induced by *F. occidentalis* on *S. enterica* populations, and further evaluating the influence of insect gender, plant age, and infestation density on bacterial populations.

In both choice and no choice experiments, we demonstrated that *S. enterica* population dynamics are influenced by insect feeding damage on tomato leaves (Figs 1 and 4). Prior research from the Barak and Groves laboratories determined that lettuce leaves previously

damaged by *F. occidentalis* harbor greater *S. enterica* populations compared to undamaged areas. Here, we expand our understanding of *F. occidentalis* as a biological multiplier for *S. enterica* on a new host plant L. solanaceae and further demonstrate how the timing of bacterial contamination can follow insect damage. While feeding, *F. occidentalis* ingest plant cell contents [12], including the cytoplasm [20] and chloroplasts [21]. Epidermal and mesophilic leaf cells impacted by *F. occidentalis*' piercing-sucking mouthparts are generally emptied during ingestion [12]. It is plausible to assume, however, that the remaining, and newly exposed, plant cell constituents could consequently benefit epiphytic bacteria as a nutrient source. One effective way to quantify the proportion of newly exposed foliar contents is to analyze the extent of electrolyte leakage. In the event of damage or death, plant cells lose membrane integrity causing electrolytes to leak into the surrounding and exposed environment [22]. Thus far, plant pathogen attacks and hostile environmental conditions such as drought have been implicated as biological factors inducing electrolyte leakage [23, 24]. Electrolyte leakage caused by Xanthomonas gardneri infection resulted in *S. enterica* growth on tomato plants [17]. Previous studies, however, have not yet investigated if insect feeding behaviors elicit a similar and measurable response. In this study, we found that leaves with feeding damage exhibit significantly higher levels of cellular damage when compared with areas absent of feeding lesions (Figs 1, 3 and 4). Furthermore, an assay testing cell viability indicated that areas damaged by *F. occidentalis* exhibited a combination of destabilized and dead cells, demonstrating that damaged cells continue to leak constituents after feeding has ceased (S2 Fig). The plant-derived solutes released from damaged cells might provide sufficient metabolic requirements for *S. enterica* growth, as seen with the rich composition of exudates released by roots or germinating seeds [25]. To expand our understanding on this subject, future studies characterizing the composition of cellular and chemical leakage could further reveal the direct effects of *F. occidentalis* feeding on *S. enterica* populations.

Thrips feeding is distinct between males and females. Female *F. occidentalis* are defined as penetrative feeders, causing extensive scarring to epidermal and mesophyll cells in large concentrated areas, emptying out the entirety of cellular contents [26]. Males, on the other hand, are considered shallow feeders, lightly puncturing numerous epidermal and mesophyll cells, ingesting relatively small amounts of cellular contents and producing microscopic (invisible) scarring [14]. The gender of thrips has been shown to influence the transmission of viral pathogens, including Tomato spotted wilt tospovirus [TSWV; 13, 27]. Comparatively, females feed by devastating plant cells, which limit viral infection and subsequently replication. Although the influence of insect gender on virus transmission has been broadly investigated [28–30], no study has considered *F. occidentalis* gender when investigating human enteric bacterial pathogens on plants. In the case of *S. enterica*, we found that the gender of the insect had no significant influence on bacterial population dynamics (Fig 3), leading us to question whether cellular damage alone is the mechanism promoting *S. enterica* survival on thrips infested plants.

Plant and insect interactions have co-evolved strategies to best minimize damage between one another. Plants, for one, have evolved resistance and tolerance strategies against herbivores, actively mitigating the extent of damage (through biochemical or morphological means) or minimizing the impact on plant fitness respectively [31–33]. Each of these innate defense mechanisms are found in plants but are inversely proportional between juvenile (vegetative) and reproductive developmental stages, and thus, pre-flowering plants could be considered the intermediate in terms of confronting herbivores with plant resistance or tolerance strategies [34, 35]. In our study, we found that five-week-old plants had similar lesion numbers regardless of densities of insects. This observation indicates that *F. occidentalis* feeding behavior may change as pre-reproductive plants age since the extent of damage as measured by conductivity followed a similar pattern as numbers of lesions. Fewer insects could cause extensive damage as pre-reproductive plants age altering the phyllosphere to a more

inhabitable environment for *S. enterica* as the plant begins production of the raw fruit commonly implicated in salmonellosis outbreaks.

We hypothesized that areas with greater damage, and thus a higher exposure to plant nutrients, would result in a higher overall *S. enterica* population. To better understand this interaction, we imposed varying levels of abiotic physical damage to emulate thrips feeding damage in isolation of other biotic interactions. Results from these experiments indicated that although an aggressive bombardment of water resulted in significantly greater cellular damage beyond that which we observed from *F. occidentalis* (Fig 6), *S. enterica* populations were similar across these levels (Fig 5). The data from this experiment suggests that *S. enterica* survival is not strictly correlated with the extent of cellular damage, as described and illustrated in our hypothetical model (Fig 7). Rather, there are likely other biological factors directly, or indirectly influenced by *F. occidentalis* feeding which may enhance epiphytic *S. enterica* population persistence. One possible explanation may be an upregulation of an immune response to insect feeding damage. Recent investigations have shown that the co-occurrence of phytophagous insects on *S. enterica* inoculated plants results in an active, up-regulation of both jasmonic and salicylic acid defense pathways, benefiting *S. enterica* epiphytic populations [5]. Similar to investigations with Auchenorrhyncha leafhoppers [36], thrips have been shown to elicit an upregulation of jasmonic acid defense pathways in response to feeding and ingestion [37, 38], and thus, may benefit *S. enterica* populations in this indirect way.

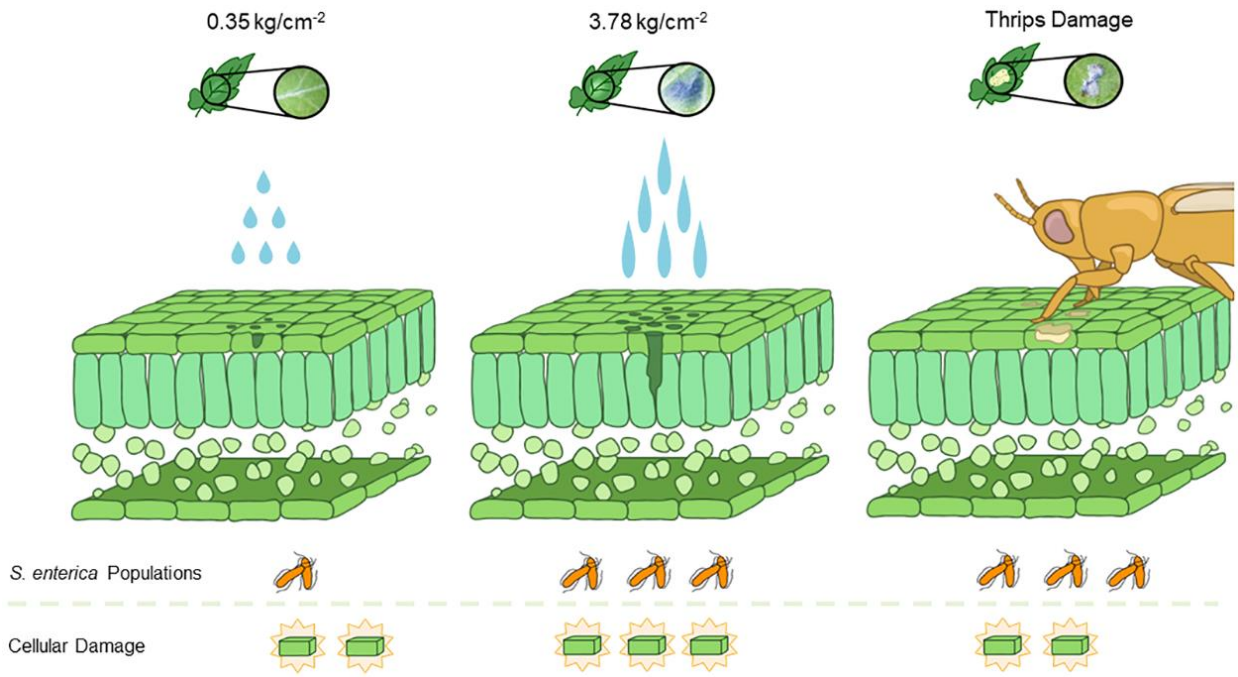

Fig 7. *Salmonella enterica* survival is not strictly correlated with the extent of cellular damage. The delivery of high-pressure water alone (middle) resulted in significantly greater amounts of cellular damage, when compared with areas possessing direct damage as a result of *F. occidentalis* feeding (right). Leaves damaged with lower water pressure (left) displayed significantly lower *S. enterica* populations in comparison to the two aforementioned groups. Regardless of the degree of cellular damage, *S. enterica* populations were similar between plants physically damaged by the highest water pressures and those compromised by *F. occidentalis* feeding, indicating that cellular damage is only one of the potential biological mechanisms which may enhance *S. enterica* populations in the plant phyllosphere.

In the context of agricultural ecosystems, we identified several important relationships between *F. occidentalis*, *S. enterica*, and the tomato phyllosphere. Our study indicated that growers may face a greater likelihood, and possibly a prolonged period of vulnerability to produce contamination with foliar feeding damage induced by thrips. More so, greater feeding damage likely indicates a greater proliferation, or protracted interval of risk of *S. enterica* suggesting appropriate pest management actions may be warranted where the risk of *S.*

enterica and *F. occidentalis* co-occurrence is greatest, regardless of the sex ratios observed in field populations.

Acknowledgments

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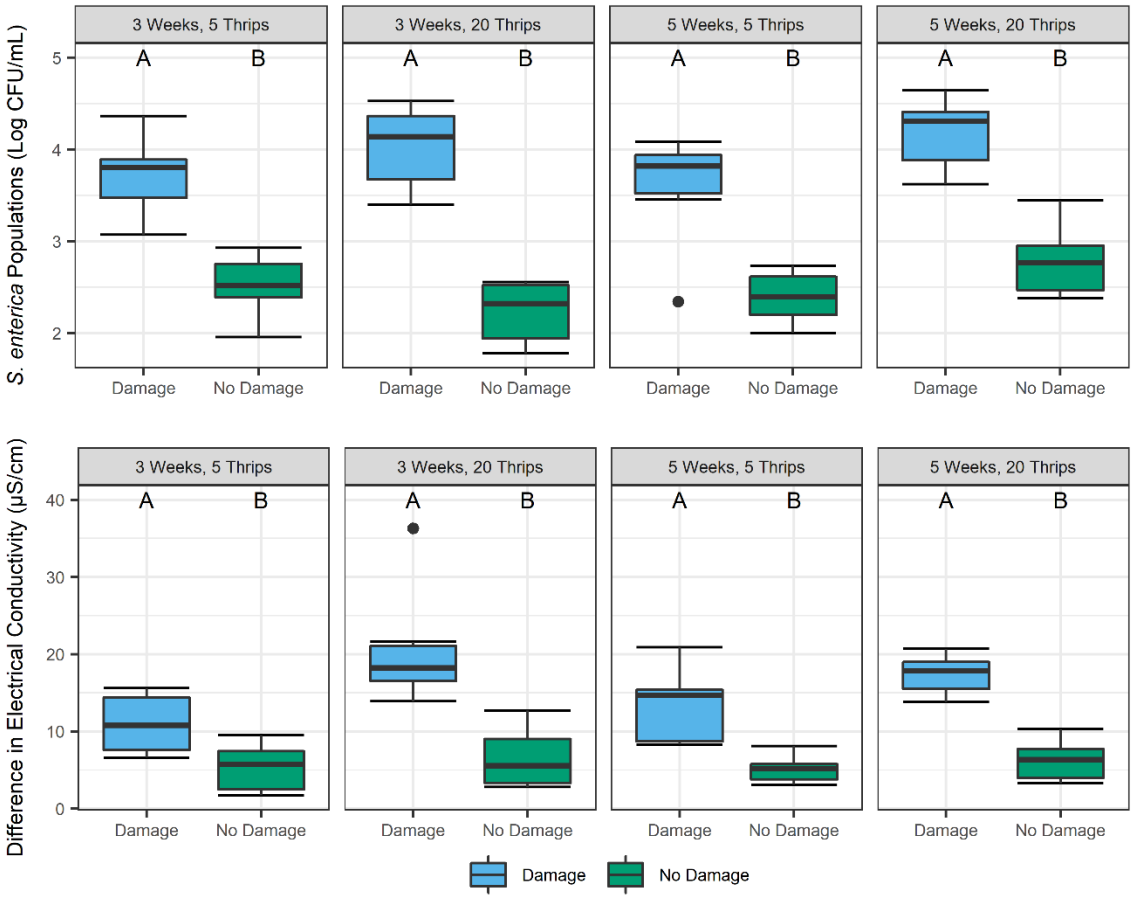
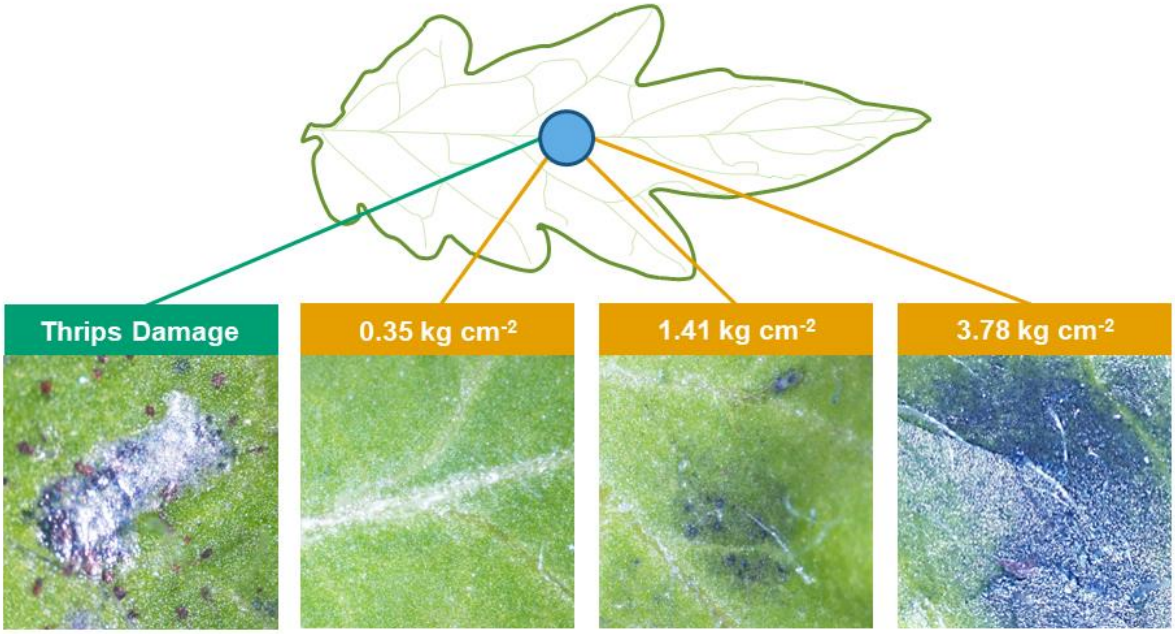
Supplementary Figures



S1 Fig. Thrips damage enhances S. enterica populations and electrolyte leakage in a no-choice environment. In a no-choice experimental arena, damaged leaf tissue exhibited higher S. enterica populations (top) and greater electrolyte leakage (bottom), regardless of plant age or initial F. occidentalis

infestation density. Damaged and undamaged leaf discs were extracted from each three or five-week-old plant with high (20 thrips/cage) or low (5 thrips/cage) infestation densities. Measures of electrical conductance for damaged and undamaged leaf discs were used to evaluate the extent of electrolyte leakage over a six-hour period. Boxplots with different letters within each treatment group indicate a significant difference ($P < 0.05$), as determined by a student's t-test. Singular dots represent outlier points.



S2 Fig. Evans blue staining of damaged tomato leaflets. Five-week-old tomato leaflets were subjected to F. occidentalis feeding for 72 hours, or an application of low (0.35 kg cm-2), medium (1.41 kg cm-2), or high (3.78 kg cm-2) water bombardment for five seconds. The blue dot on the drawn leaflet was the location where water pressure or contained thrips damage was applied. Whole leaves were extracted after imposed damage, and immediately dyed to visualize cell membrane viability.

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Chapter 3: Salmonella enterica changes *Macrosteles quadrilineatus* feeding behaviors resulting in altered *S. enterica* distribution on leaves and increased populations.

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Author Contributions

V.L, R.G, and J.B conceptualized the design of the study and provided revisions of the manuscript; V.L and E.G curated data and conducted formal analysis.

Abstract

Hemipteran insects are ubiquitous inhabitants of the phyllosphere. Changes in microbial phyllosphere communities have recently been demonstrated following infestation by *Macrosteles quadrilineatus* (Aster Leafhopper). Although epiphytic *Salmonella enterica* populations naturally decline in the phyllosphere of plants, *M. quadrilineatus* infestation facilitated the growth of the bacterial pathogen populations. Here, we demonstrate that cellular damage by insect stylet penetration results in a localized beneficial niche on the leaf surface, leading to enhanced *S. enterica* populations. We measured *S. enterica* populations and colonization patterns on plants infested with Hemipterans with distinct feeding behaviors. *M. quadrilineatus* infestation resulted in higher solute leakage and significantly greater bacterial populations than plants absent of insects. Following immigration via contaminated irrigation water, the highest populations of *S. enterica* are naturally found on the tips of tomato leaflets. We discovered *M. quadrilineatus* feeding preference altered the natural distribution of *S. enterica* populations, and that the presence of *S. enterica* altered the distribution of probing attempts. These findings elucidate how cellular damage resulting from insect feeding drives changes in bacterial colonization of the phyllosphere.

Introduction

Salmonella enterica, a human enteric bacterial pathogen, has recently been recognized as a member of the phyllosphere microbiome1,2. Unlike most members of this microbiome, the presence of *S. enterica* annually leads to food borne illness from the consumption of fresh, raw produce. In fact, cases of salmonellosis derived from the consumption of contaminated fresh produce has steadily increased over the last decade3. *S. enterica* colonization of the phyllosphere is reported to begin with contaminated irrigation water or direct application of raw (vs. composted) manure as a soil amendment4,5,6. Irrigation water is not only a conduit for *S. enterica* contamination directly to the phyllosphere, but can further spread the human pathogen

from plant to plant via splash dispersal7. Here again, application of raw manure has been implicated as an avenue for contamination of agricultural fields but also has been reported to stimulate enteric bacterial growth on subsequent crops1. These human-mediated practices, among many others, aid *S. enterica* in achieving access to preharvest produce, thereby generating a scenario that may lead to foodborne illness.

While pathogen introduction to leaves preharvest is the first step in a sequence culminating in human illness, *S. enterica* populations have been observed to decline in the phyllosphere of healthy plants8,9,10. Hostile environmental conditions, such as direct UV radiation, desiccation, and a lack of nutrient availability, are a few of the limiting factors prompting bacterial populations to decrease over time. The high proportion of *S. enterica* outbreaks associated with consumption of fresh, raw produce, however, indicate that these bacteria have evolved to exploit several biological niches to successfully persist. Following contamination of the phyllosphere via irrigation water, *S. enterica* populations concentrate around glandular trichomes and stomates, two ubiquitous leaf structures that exude scarce metabolites or provide leaf internalization access respectively, each resulting in a beneficial niche for epiphytic bacteria11. Furthermore, *S. enterica* successfully persists near leaf regions altered by phytobacterial pathogens, such as Xanthomonas species12. These authors hypothesize that *S. enterica* uses nutrients made available during the plant infection process.

Just as a subset of phytobacterial pathogens were discovered as potential biological multipliers, we previously identified phytophagous insects as additional promoters for *S. enterica* survival in the phyllosphere13. Specifically, our lab uncovered that *Macrosteles quadrilineatus* (Aster leafhopper) infestation significantly promoted *S. enterica* populations and persistence overtime on both lettuce and tomato leaves9,13. Although this relationship was previously discovered, the mechanisms by which the insect facilitates the persistence of these bacterial populations has not yet been established.

We hypothesize that insect feeding alters the phyllosphere from a *S. enterica* inhospitable habitat to an inhabitable niche. Members of the Hemipteran order of insects utilize a narrow and segmented piercing-sucking mouthpart, collectively composed of stylets, to feed on the phloem or xylem of plants. Although Hemipterans collectively share these mouthparts, different members of this important group employ unique probing and feeding strategies that elicit distinctive plant responses. For instance, the stylet of an aphid (Hemiptera, Stenorrhyncha, Aphidoidea) reaches the phloem via an intercellular pathway. After puncturing the epidermis, an aphid's stylet transiently probes and injects adjacent cells with watery saliva14,15 prompting upregulation of the salicylic acid pathway16,17. Contrastingly, leafhoppers (Hemiptera, Auchenorrhyncha, Cicadellidae) feed intracellularly by sieving through layers of cells to reach into the phloem, consequently upregulating the jasmonic acid pathway18. To date, the extent of cellular damage, elicited by feeding, has not been measured for Hemipterans. We hypothesize that these two modes of stylet penetration cause varying levels of cellular damage, and thus may uniquely alter the infested phyllosphere.

Here, we explore how differences between *M. quadrilineatus* and *M. persicae* feeding behaviors could influence the extent of cellular damage, and further how these differences may alter the phyllosphere for subsequent bacterial populations. We use *S. enterica* as a biological reporter of changes to the phyllosphere resulting from insect feeding, and lettuce and tomato plants were utilized as relevant plant systems for our experiments given their repeated association with domestic outbreaks of salmonellosis19,20. We hypothesize that preferred feeding locations of insects will experience higher levels of cellular damage, and in turn be associated with enhanced *S. enterica* populations21. To explore this hypothesis, we mapped preferential *S. enterica* colonization sites, preferred feeding locations of *M. quadrilineatus*, and examined whether earlier insect infestation influenced these distributions. In turn, we also investigated whether leaves previously contaminated with *S. enterica* would influence the feeding biology of *M. quadrilineatus* or *M. persicae* in the phyllosphere. Results from this study

illustrate how unique hemipteran feeding behaviors can alter the phyllosphere and subsequent microbial community, with special reference to *S. enterica*.

Materials and Methods

Bacterial strains, media, and culture conditions

A kanamycin (Kan) resistant strain of *S. enterica* serovar Typhimurium 14028 s, from -80 °C freezer stocks, were utilized and grown in a lysogeny broth (LB; Difco LB Broth) at 37 °C, shaking overnight at 200 rpm. *S. enterica* cultures were normalized to an optical density at 600 nm of 0.2 in sterile water. Inoculum preparations were verified by enumerating populations following serial dilution, plating on Kan amended plates, and incubated overnight at 37 °C.

Insect rearing

Colonies of *Macrosteles quadrilineatus* were maintained on oat seedlings (Avena sativa) under a constant temperature of 27 °C and a 16:8 (L:D) photoperiod. A colony of *Myzus persicae* was provided by Jason Timothy Ingram and Dr. Stewart Gray (Cornell University) and maintained on turnip plants (Brassica rapa) under the same controlled conditions of 27 °C and a 16:8 (L:D) photoperiod. Voucher specimens of adult female and male *M. quadrilineatus* and apterous *M. persicae* from our colony were deposited in the Wisconsin Insect Research Collection, University of Wisconsin (http://labs.russell.wisc.edu/wirc/).

Plant assays

Solanum lycopersicum (tomato, cv. Money Maker), and Lactuca sativa (lettuce, cv. Butterhead) seedlings were cultivated using Professional Growing Mix (Sunshine Redi-earth) in 6″ pots held in a growth room maintained at a 16:8 (L:D) photoperiod and 24 °C light and 19 °C dark conditions. No plant material was collected. Seeds were bought commercially (Eden Brothers). Tomato plants were established and maintained for five weeks prior to all

experiments, whereas lettuce plants were grown and utilized after six weeks. Six sets of 4.5 cm diameter plexiglass clip cages, fashioned with insect-proof mesh at one end, were fastened with clips onto the abaxial (under) surface of two opposing leaves, three individually containing one adult *M. quadrilineatus* and the remaining three left empty as a control. Each clip cage was attached to the center of leaflets on each plant. Plants were held at a constant 24 °C temperature with a 16:8 (L:D) photoperiod and were randomly assigned treatment groups indicating the length of infestation. At each infestation period of 24, 48, or 72 h, individual *M. quadrilineatus* were removed, and leaf discs were excised from under each clip cage to assess for electrolyte leakage. Similar experiments were carried out with apterous *M. persicae* (single insect per cage) on tomato plants. An additional experiment evaluated whether electrical conductivity measurements differ on tomato leaflets infested with singular or multiple aphids (3 individuals). Before infesting plants, individual *M. quadrilineatus* were collected with a respirator whereas a wet brush was used to transfer *M. persicae*. After the initial collection, insects were placed into a container over ice to impede movement thereby facilitating the transfer into a clip cage. Insects were visually monitored for any movement immediately after plant application to ensure they were not injured during placement.

Cellular damage

To analyze the extent of cellular damage associated with insect probing and feeding, estimates of electrolyte leakage were obtained by measuring electrical conductivity as previously described22. Briefly, a set of three comparable 10 mm-diameter leaf discs from under clip cages with or without insects were placed in a single well of a 12-well tissue culture plate containing 4 ml of sterile water. Plates were positioned on a rotating table at 50 rpm for approximately 30 min, acting as a wash step. This wash step prevented any leaf contaminants, such as remnant soil, from affecting conductivity measurements. Water from each well was subsequently removed and replaced with fresh, sterile water, and electrical conductance was

immediately measured. Electrical conductance was measured by pipetting 1 ml of the aqueous solution from sample wells onto an ECTestr11 + MultiRange electrical conductance probe to assess the extent of conductive electrolyte leakage, here used as a proxy for cellular damage. After the initial assessment of electrical conductance, sample plates were left on a lit bench at ambient temperature (24 °C) for 6 h, after which a second and final conductivity measurement was taken. Differences in measured conductance between the two estimates were used for data analysis comparing each treatment group and used as a proxy for electrolyte leakage.

Distribution of Salmonella enterica on the leaf phyllosphere

To characterize the distribution of *S. enterica* on tomato and lettuce plants, attached leaflets and whole leaves, respectively were dip inoculated in a suspension of *S. enterica*. Replicate sets of tomato and lettuce plants were dip-inoculated for one minute in 450 ml of sterile water with the addition of 75 µL of Sil-Wet, or a 108 CFU/ml suspension of *S. enterica* prepared as described above with the addition of 75 µL of Sil-Wet. In each replicate, tip, middle and basal regions of whole leaves (lettuce) and leaflets (tomato) were randomized in a 2X2 factorial design, to receive either *S. enterica* suspensions or water controls. One-hour post-dip inoculation, clip cages were placed onto tip, middle and basal sections of leaflets or leaves for later assessments of electrolyte leakage and *S. enterica* population enumeration. Water and *S. enterica* dip-inoculated plants were then placed in clear, plastic bins held at 24 °C temperature under a 16:8 (L:D) photoperiod and sampled seventy-two hours post-inoculation. In a complementary experiment designed to evaluate the influence of leaf angle on *S. enterica* distribution and electrolyte leakage *S. enterica* dip-inoculated plants were placed into a modified container with plastic ramps positioning tomato leaves at a 65° vertical angle propping the tips of leaflets upwards and above the basal portions of leaves (e.g. petiole attachment). Prior to leaf excision for the two aforementioned experiments, each location (on the tip, middle and basal portions) was assigned a number and was entered into a random group generator, to prescribe

the areas which would be used to measure *S. enterica* populations, and associated electrolyte leakage (https://www.randomizer.org).

To assess *S. enterica* populations, plants were sampled seventy-two hours after dip-inoculation. Specifically, one 10 mm diameter leaf disc was excised from under clip cages on either lettuce or tomato. Samples were individually homogenized in 500 µl of sterile water using a cordless Dremel tool, and further diluted 1:10 in sterile water. Homogenates were immediately plated on LB-Kan, incubated overnight at 37 °C, and populations were enumerated after 24 h. Electrolyte leakage was assessed three days after dip-inoculation as previously described. A total of 3 experimental replicates were completed for each experiment.

S. enterica, plant, and insect interaction

An additional experiment was performed to determine if the presence of *M. quadrilineatus* or M. precise altered the natural distribution of *S. enterica* populations or the magnitude of electrolyte leakage on tomato leaves. Groups of tomato plants were randomly assigned to treatment groups (water, or *S. enterica*) and arranged as a randomized complete block. One-hour post dip-inoculation, one clear hinged lid container (8 × 5¾ × 3; Dart Container Corporation) was fastened onto a middle-aged leaflet. Clamshell containers were concurrently infested by five, adult M. quadrilineatus, or five apterous *M. persicae*, which were allowed to move freely around the entire leaflet, whereas a replicate set of clamshells remained empty for uninfested controls. Replicate sets of 10 mm diameter leaf discs were collected at the tip, middle and basal leaflet portions at 72 h post-infestation and were randomly selected for assessments of *S. enterica* populations or electrolyte leakage (e.g. cellular damage) utilizing a random group generator (https://randomizer.org). A total of 3 experimental replicates were completed.

To determine whether *S. enterica* could influence the feeding behavior of M. quadrilineatus, the distribution of salivary sheathes was observed on *S. enterica*-contaminated

tomato leaves. Groups of four tomato plants were randomly assigned to the following inoculation groups: whole leaf water inoculation, whole leaf *S. enterica* inoculation, or *S. enterica* inoculated onto basal, middle or tip portions of select leaflets, and organized as a randomized complete block design with 3 experimental replicates. Regions uncontaminated by *S. enterica* were inoculated with sterile water. One hour post dip-inoculation, sets of 5 adult *M. quadrilineatus* were released into experimental clamshells and allowed access to whole leaves with different inoculation treatments. Following 72 h of infestation, all insects were removed and whole leaflets were extracted, stained and cleared to enumerate salivary sheathes.

Salivary sheath staining and clearing procedure

To enumerate salivary sheaths associated with adult *M. quadrilineatus* feeding, experimental leaflets were extracted, and subsequently stained with 0.2% acid fuchsin in a 1:1 (vol/vol) solution of 85% glacial acetic acid and 95% ethanol, otherwise known as McBryde's acid fuchsin stain (23,24). Leaflets were fully submerged within the dye for 20 to 24 h at ambient temperature (24 °C). To remove chlorophyll and clear tissues, leaflets were soaked in 95% ethanol for 30 min, replacing the stained liquid with new ethanol every 10 min to ensure residual dye is washed off. Leaflets were then heated in a 1:1:1 (vol/vol/vol) solution in glycerol, 85% glacial acetic acid, and water, and individually boiled for 8 to 10 min to appear translucent. Salivary sheathes of individual leaflets were visually quantified under an Olympus SZ60 Stereoscope with a white background to better highlight embedded salivary sheathes.

To determine the response of adult M. quadrilineatus' to leaf surfaces contaminated with *S. enterica*, two observational experiments were performed. In a first set of experiments, one middle-aged leaflet was entirely inoculated with sterile water, *S. enterica*, or both treatments on separate ends (tip or basal end) of leaves. One-hour post-inoculation, a modified clam shell container was affixed to encase each experimental treatment. Each cage was placed on a container at a height that would mimic the natural position of the leaflet and adjusted to ensure

that the leaf did not touch the sides of the cage while still attached to the plant. Sets of five adult *M. quadrilineatus* per cage were released and allowed to move freely inside. Approximately 15 min post-infestation, a visual observation was made to assess the location (container, *S. enterica*- or water-inoculated regions) of individual leafhoppers while also noting the position of insects on either abaxial or adaxial leaf surfaces. A total of 8 different visual assessments over 2 h were conducted for each set of leafhoppers, leading to 16 observations per treatment group for one experimental replicate. A total of 5 experimental replicates were completed.

To further define whether *S. enterica* influenced *M. quadrilineatus* and *M. persicae* resting preferences, observations of adult insects were made in terms of their positions across leaflets or the experimental cage. Observations (basal, middle or tip) for sets of *M. quadrilineatus* (5 per plant) were recorded at 2-, 24-, and 48 h post infestation on tomato leaflets inoculated exclusively at the base, middle or tip, or entirely inoculated with *S. enterica* or water. Observations (*S. enterica* or water) for sets of *M. persicae* (1 per clip cage) were recorded at 24-, 48- and 72 h post infestation on *S. enterica* or water inoculated halves of leaflets (tip or basal end). The location of *S. enterica* inoculations were randomly assigned to leaf areas by utilizing a random group generator (https://randomizer.org). Differences in observed times and the alternative inoculation style between insect species was chosen to accommodate the smaller and lesser mobile apterous life stage of *M. persicae*, compared to the larger bodied and more mobile M. quadrilineatus. Groups of 4 plants were utilized for each treatment group with 3 experimental replicates. At the conclusion of these experiments, leaflets from *M. quadrilineatus* infested plants were removed and stained in an effort to count salivary sheathes 72 h following insect exposure, as mentioned above.

Statistical analysis

Student's t-tests were performed to compare estimates of electrical conductivity of leaflet samples that were uninfested or infested between the two experimental taxa (*M. quadrilineatus*

and *M. persicae*), for water inoculated treatments at 24, 48, or 72 h. A one-way, analysis of variance (ANOVA) was used to assess if *S. enterica* populations or electrical conductance measurements varied among regions on leaves/leaflets (notably the basal, middle, or tip regions) of uninfested tomato or lettuce plants in their natural position, tomato leaflets altered by a 65° upward-angled ramp, and tomato plants that were infested by either *M. quadrilineatus* or *M. persicae*. Furthermore, ANOVA was used to determine the distribution of *M. quadrilineatus* salivary sheathes across tomato leaflets uniquely inoculated at the base, middle or basal regions, or remained entirely inoculated with either sterile water or *S. enterica*. Interpolation was used to visualize estimated *S. enterica* populations outside of the pre-determined leaf excision points (tip, middle and basal regions) using the 'lattice' and 'akima' packages on R-Studio. An analysis of covariance (ANCOVA) was used to analyze the proportion of resting *M. quadrilineatus* and *M. persicae* across the experimental cage and tomato leaflets with half-inoculation of *S. enterica* and water on opposing leaflet ends. *M. quadrilineatus* resting preference for uniquely *S. enterica* inoculated surfaces (tip, middle and basal regions), or alternative surfaces (water, or experimental cage), was determined using a likelihood ratio chi-square test.

Results

M. quadrilineatus infestation (intracellular penetration) results in greater cellular damage than uninfested plants

To further investigate how different feeding styles alter the phyllosphere, we analyzed the extent of electrolyte leakage (using electrical conductivity as a proxy) on tomato plants in response to intracellular or intercellular penetration employed by leafhoppers and aphids, respectively. Tomato plants infested with leafhoppers had significantly higher levels of measured electrical conductivity at 24 h post-infestation (hpi) when compared to plants with no insects ($P < 0.0005$; Fig. 1a). After 24 hpi, measurements of electrical conductivity were not

significantly different on tomato plants infested by M. quadrilineatus, or without insects ($P > 0.05$; Fig. 1a). In the presence of aphids, measurements of electrical conductivity were not significantly different between plants with or without insects at the measurement timepoints of 24, 48, or 72 hpi ($P > 0.05$; Supplemental Fig. S1). A complimentary experiment determined that increasing aphid populations, from one individual to three, did not influence the extent of electrolyte leakage on tomato plants ($P = 0.398$; Supplemental Fig. S2). To determine if this pattern of electrolyte leakage following leafhopper infestation was independent of host, we tested lettuce plants and continued to observe higher electrical conductivity estimates at 24, 48, and 72 hpi ($P < 0.0001$; Fig. 1b). Electrical conductivity values were not measured in response to aphids on lettuce.

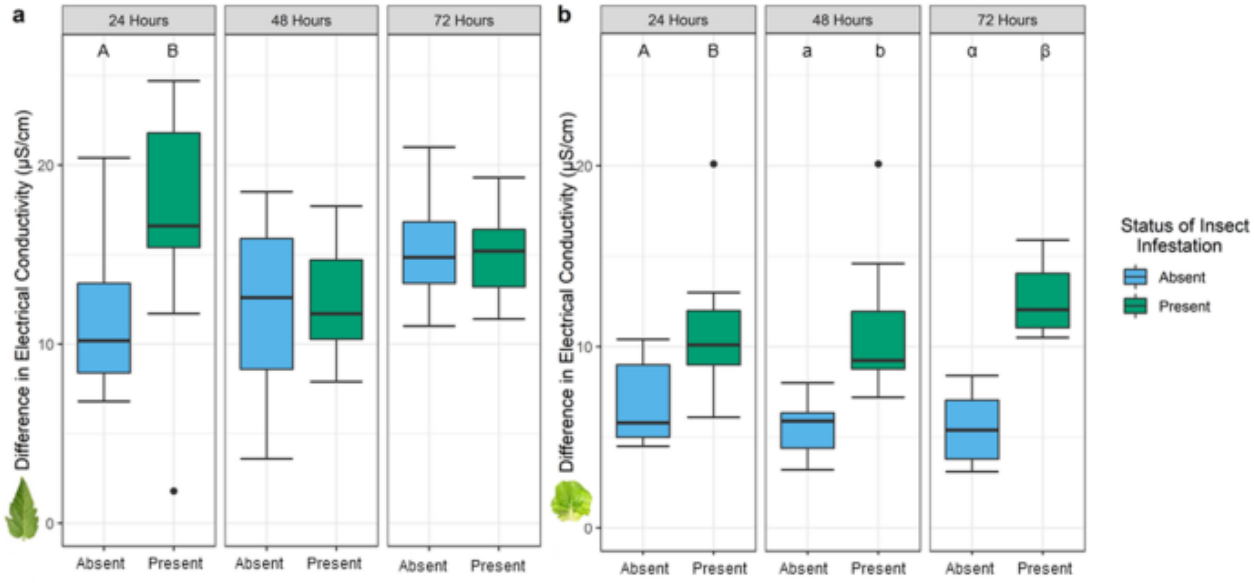

Figure 1. Infestation by *Macrosteles quadrilineatus* leads to an increase in measured cellular damage on tomato (a) and lettuce (b) leaves. When compared to uninfested areas, electrolyte leakage was significantly higher on tomato leaflets (a) infested for 24 h, and on lettuce leaves (b) infested for 24, 48, and 72 h ($P < 0.05$). Three clip cages were fastened onto a middle leaf each containing one leafhopper (green), and additional three clip cages remained empty (blue) on an opposing leaf. Electrical conductance was calculated by subtracting the final from the initial measurement for damaged and

undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. Letters above boxplot indicate significant differences between treatment groups within a single time point ($P < 0.05$). A student's t-test was used to assess significance between samples from infested or non-infested clip cages. Singular dots represent an outlier point.

S. enterica populations naturally accumulate at the tips of tomato leaflets

To better understand how leafhopper feeding alters the distribution of *S. enterica* in the phyllosphere, we examined how bacterial populations changed across the leaf surface. First, the natural distribution of *S. enterica* on lettuce and tomato plants was determined (Fig. 2). Tomato leaflets supported significantly higher *S. enterica* populations at the tip of leaflets, compared to samples measured from the basal regions ($P < 0.0007$; Fig. 2a). Measurements of electrical conductivity at the base of tomato leaflets were higher, but not significantly different than samples collected in the middle or tip regions of leaflets ($P = 0.0626$; Supplemental Fig. S3a). To test whether *S. enterica* population distribution across the tomato leaflet is influenced by gravity, a complementary experiment was designed to disrupt the natural tendency of leaflets to droop and consequentially result in liquid collecting on leaflet tips. When leaflets were placed in a more upright position (65° upward angle) after the dip-inoculation, the highest accumulation of bacterial populations shifted to the base ($P = 0.0068$; Supplemental Fig. S4), whereas measured electrical conductance remained higher, but not significantly different, at the basal region of the leaf ($P = 0.061$; Supplemental Fig. S5). On lettuce, *S. enterica* populations remained somewhat uniform across the leaf ($P = 0.87$; Fig. 2b) while the base of lettuce plants exhibited significantly higher electrical conductivity than the middle or tip regions ($P < 0.001$; Supplemental Fig. S3b). With improved knowledge of where *S. enterica* preferentially colonizes the phyllosphere, we could examine if leafhopper infestation alters bacterial distribution.

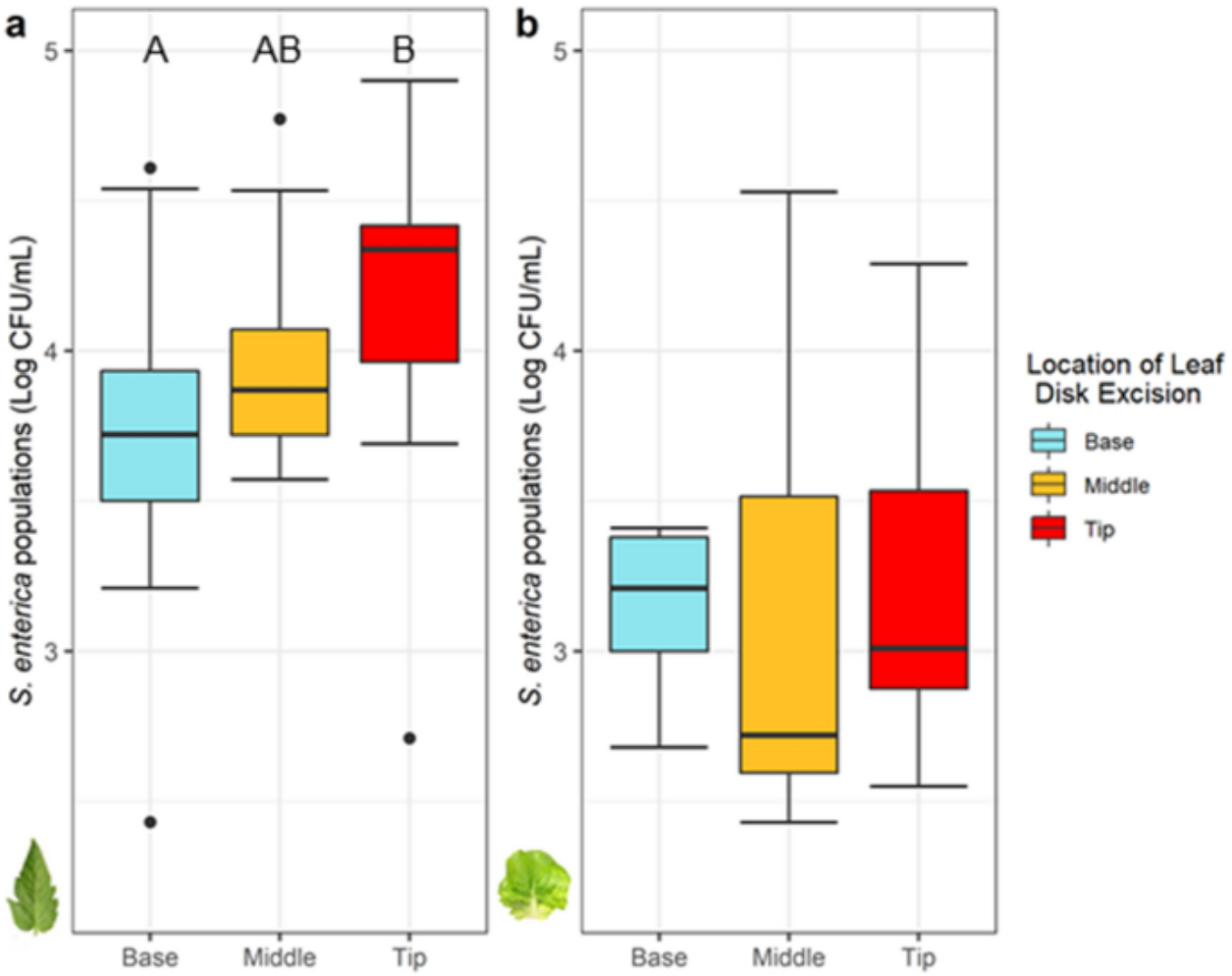

Figure 2. Tomato leaf architecture impacts the distribution of *S. enterica* populations, unlike lettuce. *Salmonella enterica* populations were significantly greater at the tip of tomato leaflets than the base (a) ($P < 0.05$), whereas lettuce leaves have a uniform bacterial distribution (b). Leaf discs were excised from pre-determined locations from the basal (blue), middle (orange), and tip (red) regions of leaves. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by a one-way ANOVA. Singular dots represent an outlier point.

M. quadrilineatus infested tomato leaflets had the greatest electrical conductivity and enhanced S. enterica populations

To test the hypothesis that cellular damage, prompted by leafhopper intracellular penetration, facilitates fundamental changes in the phyllosphere, we measured *S. enterica*

populations in response to *M. quadrilineatus* infestation (Fig. 3). After 72 hpi, *S. enterica* populations were approximately half a log higher on tomato plants infested with leafhoppers than *S. enterica* inoculated plants without insects, consistently indicating that leafhopper infestation enhances *S. enterica* populations ($P < 0.0001$; Fig. 3a). Similarly, plants infested by leafhoppers and contaminated with *S. enterica* exhibited significantly greater estimates of electrolyte leakage than plants without insects ($P = 0.0020$; Fig. 3b), and had higher, but not significantly different, electrical conductivity measurements than infested plants treated with water only. Infested plants had a roughly uniform distribution across the middle and tip regions of leaflets indicating a shift in expected natural bacterial populations. Although the *S. enterica* population was similar at the tip for either infested or leaves without insects, the base and middle locations of infested leaflets had significantly higher *S. enterica* than the same locations on uninfested leaflets ($P < 0.005$; Fig. 4a, b), suggesting that insect activity increased the local *S. enterica* populations in these regions of the leaflet. Electrical conductivity was not significantly different across a leaflet within any treatment group and was similar between the tip, middle, and basal regions of infested *S. enterica* tomato plants ($P > 0.05$; Supplemental Fig. S6). Greater cellular damage on infested, *S. enterica* inoculated leaflets prompted an additional set of experiments to determine whether *M. quadrilineatus* feeding and resting preference was influenced by *S. enterica* or water-inoculated leaves. Measurements of *S. enterica* populations and electrical conductivity within a randomized block design were similarly measured for *M. persicae*, yet no significant differences between infested and uninfested plants were observed ($P > 0.05$; Supplemental Fig. S7).

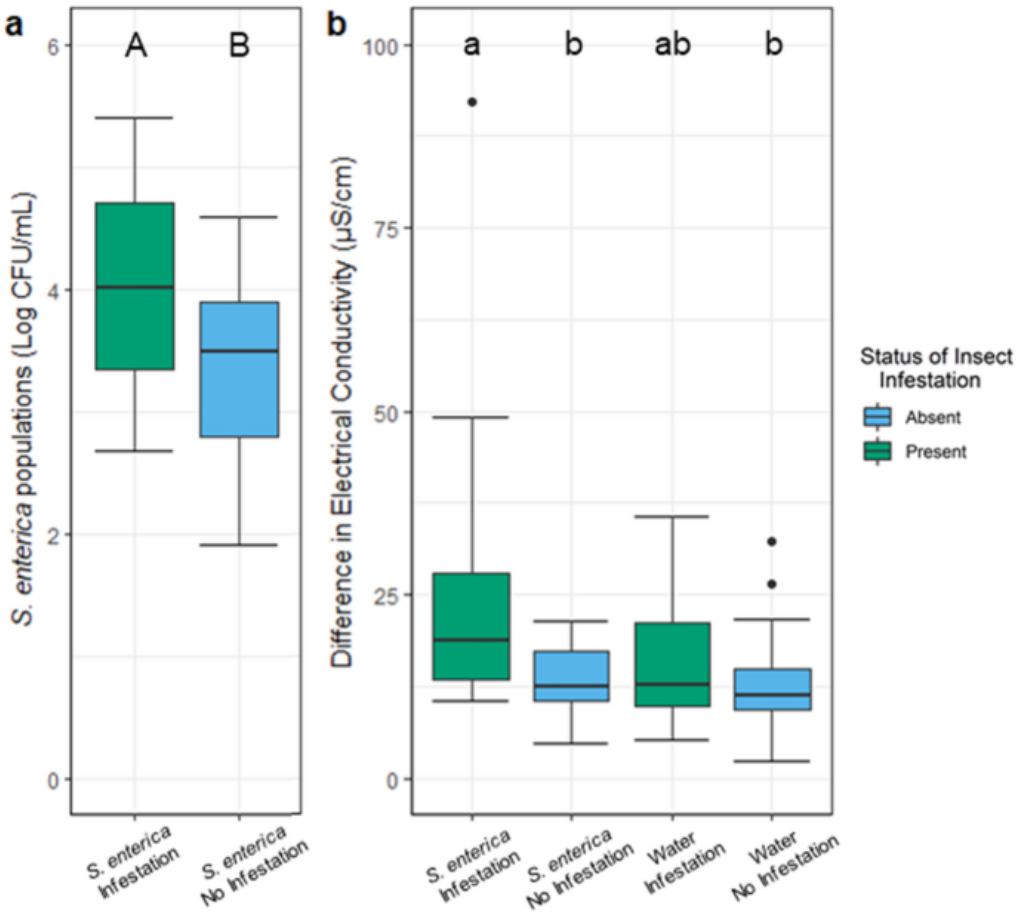

Figure 3. *Macrosteles quadrilineatus* infestation on *S. enterica* inoculated tomato plants led to heightened bacterial populations (a) and electrical conductivity (b) than plants absent insect infestation. Plants inoculated with either *S. enterica* or water (mock) were infested by adult *M. quadrilineatus* or remained absent of insects. Empty clip cages were applied for treatment groups with no infestation. Electrical conductance was calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. Each treatment group contains combined data from the tip, middle, and basal regions of leaves. *Salmonella enterica* populations were measured on water inoculated leaves but yielded 0 CFU and were thus excluded from the figure. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$) as detected by a one-way ANOVA. Singular dots represent an outlier point.

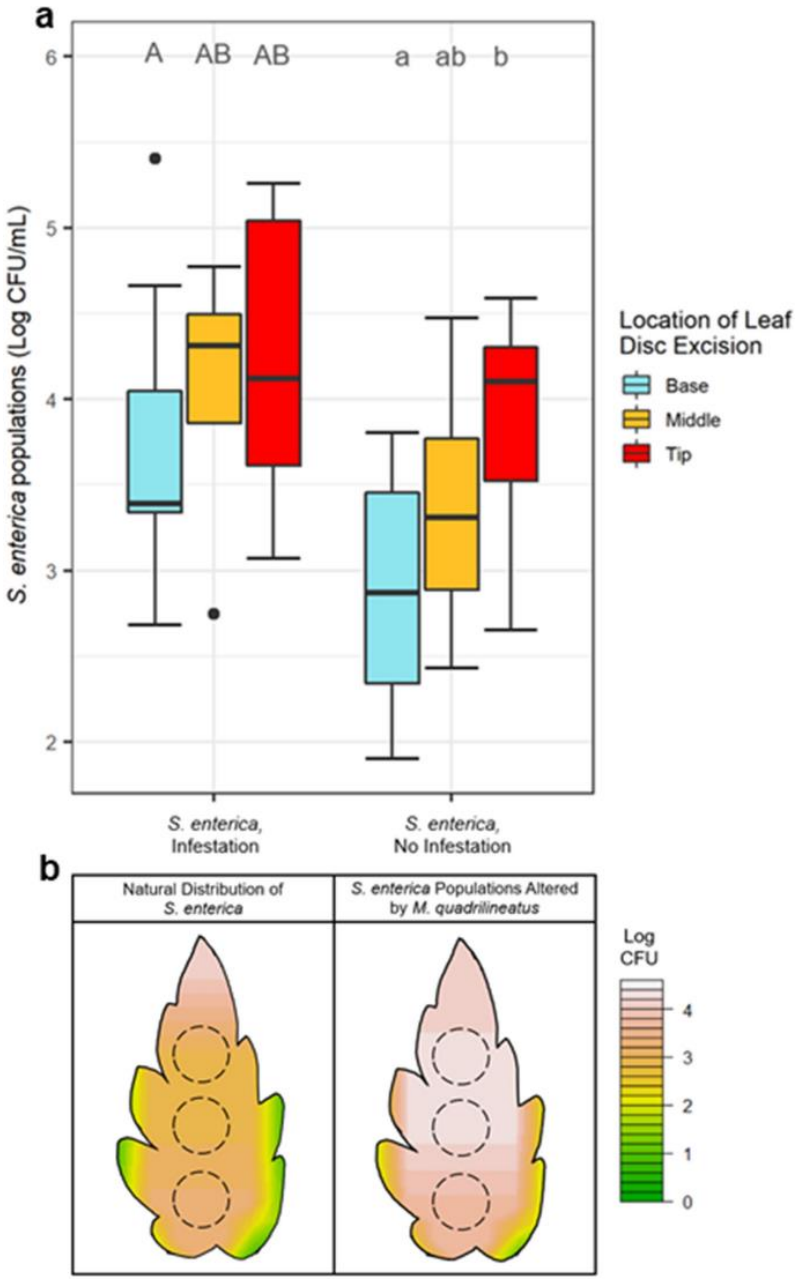

Figure 4. Adult *M. quadrilineatus* infestation on *S. enterica* inoculated tomato plants resulted in a redistribution of bacterial populations. In the absence of insects, *S. enterica* populations are significantly higher at the tips of leaflets ($P < 0.05$); however, after insect infestation, *S. enterica* populations are approximately uniform across the tip and middle (a, b) regions. The 2 X 2 factorial experiment included *S. enterica* or water inoculated plants that were either infested, or not infested by M. quadrilineatus. Empty clip cages were applied for treatment groups with no infestation. Leaf discs were excised from the tip, middle and basal regions of leaflets. *Salmonella enterica* populations were also

measured on water inoculated leaves but yielded 0 CFU, and were thus excluded from the figure. An interpolation graph was created to depict the shift in bacterial populations (Log CFU) over a 72 h post-infestation period. Singular dots represent an outlier point. Letters above boxplots indicate significant differences between leaf treatment groups within an insect infestation treatment ($P < 0.05$), as detected by a one-way ANOVA.

Adult M. quadrilineatus prefer water inoculated surfaces, over those inoculated with S. enterica

When provided a choice, adult *M. quadrilineatus* discriminated between non-plant and plant surfaces over a 2-h period, landing more frequently on water inoculated areas than on *S. enterica* inoculated areas ($P < 0.005$; Supplemental Fig. S8). When exposed to partially or entirely inoculated leaflets for a greater duration, *M. quadrilineatus* were observed to explore leaf surfaces at 2-h post infestation but migrated away from leaflets and onto the experimental cage after 48-h of exposure to *S. enterica* ($P < 0.001$; Fig. 5). Similar to M. quadrilineatus, sets of *M. persicae* were also exposed to inoculated tomato leaflets over a 72 h experimental interval. While a pattern of emigration from inoculated leaflets and towards the cage of the experimental arena emerged over the 72-h period of infestation, apterous *M. persicae* indicated no significant substrate preference ($P > 0.05$; Supplemental Fig. S9).

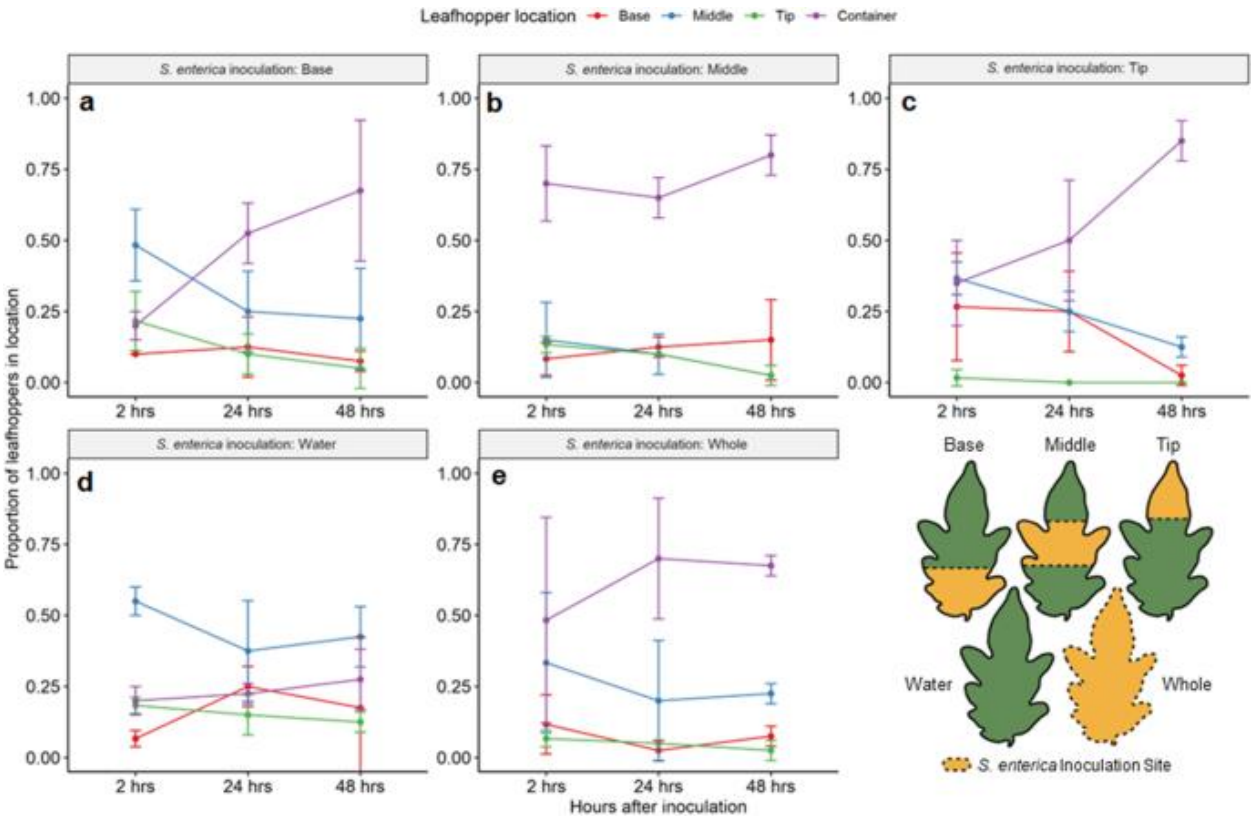

Figure 5. The proportion of *M. quadrilineatus* that emigrate away from leaflets inoculated with *S. enterica* increase over 48 h. Tomato leaflets were inoculated with *S. enterica* exclusively at the basal (a), middle (b) or tip (c) regions or were entirely submerged during inoculation (Whole; e). Similarly, one group of tomato leaflets were entirely inoculated by water (Water; d). One hour after *S. enterica* inoculation, five leafhoppers were placed in a container encasing one tomato leaflet, still attached to the plant. Observations were taken 2, 24, and 48 h after the initial infestation period. The proportion of insects within each treatment represent means from across three experimental replicates between four plants (N = 60 adult M. quadrilineatus).

Salivary sheath distribution is dependent upon S. enterica presence

To further define M. quadrilineatus' preferred feeding sites in relation to the presence of *S. enterica*, the presence of salivary sheathes across water and *S. enterica* inoculated leaflets were observed. Across all inoculation treatment groups, salivary sheathes were observed to be significantly less abundant on primary and secondary veins indicating a predominant preference

for tertiary, or lesser, veins ($P < 0.0001$; Supplemental Fig. S10). As a result, salivary sheathes located on primary and secondary veins were excluded from statistical comparisons of salivary sheath distribution across the basal, middle and tip regions of uniquely inoculated leaflets. Salivary sheathes were most regularly found on the middle of leaflets inoculated at the base or tip, and on leaflets inoculated entirely with *S. enterica* or water ($P < 0.0001$; Fig. 6). However, inoculation of *S. enterica* exclusively on the middle portions of leaflets, their preferred feeding location, resulted in a shift of salivary sheath distribution as significantly more salivary sheathes were found at the base of leaflets than the middle ($P = 0.0237$; Fig. 6). This finding demonstrates that even limited presence of *S. enterica* on a leaflet alters the preferred probing/feeding locations of adult *M. quadrilineatus*.

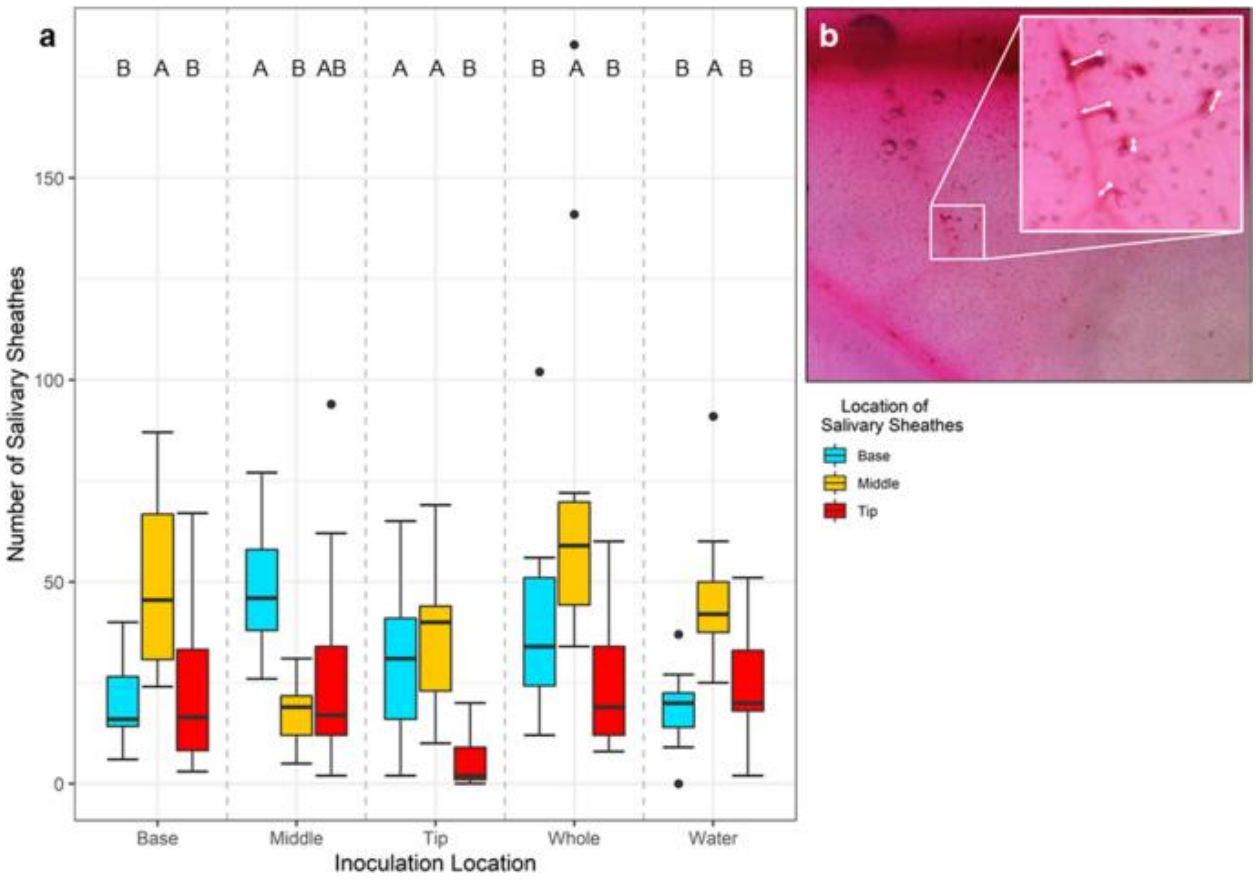

Figure 6. Salivary sheath distribution across tomato leaflets in response to *S. enterica* inoculations applied to different regions of plants, excluding those found on the primary and secondary veins. Tomato leaflets were inoculated with *S. enterica* exclusively at the basal, middle or tip regions, or were entirely submerged during inoculation (Whole). Similarly, one group of tomato leaflets were entirely inoculated by water (Water). One hour after *S. enterica* inoculation, five adult *M. quadrilineatus* were placed in a container encasing one tomato leaflet still attached to the plant. Leaflets were extracted 72 h post *S. enterica* inoculation and were subjected to staining and clearing procedures to count total salivary sheaths (a). Post clearing and staining procedures, salivary sheathes appeared as dark red in contrast to the pink leaflets (b). Salivary sheathes from three experimental replicates (n = 60 leaflets) are combined and represented above. Letters above boxplots indicate significant differences between leaf treatment groups within an insect infestation treatment ($P < 0.05$).

Discussion

The now frequent, reoccurrence of foodborne illness cases associated with consumption of fresh produce requires an in-depth assessment of environmental factors that increase the risks of continued outbreaks. In our current study, we examined the population dynamics of a foodborne pathogen through an entomological perspective, analyzing the interactions between *S. enterica*, plants, and phytophagous insects. Specifically, we investigated how changes in the phyllosphere resulting from unique insect feeding styles, impacted the longevity and persistence of *S. enterica* populations on the leaf surface.

Previous literature demonstrated that insects can manipulate human enteric bacterial pathogen populations directly, and indirectly. Within poultry dominated environments, cockroaches may mechanically transmit *S. enterica* by traversing from contaminated egg surfaces to uncompromised substrates, consequently facilitating the movement of bacteria25. Seaweed flies, intimately associated with decaying and pathogenic seaweed beds, excrete viable bacterial populations within intertidal zones, enhancing the potential transmission of E. coli (26). Despite *S. enterica* populations decreasing by 2 logs over a 13-day period upon tomato hosts, *M. quadrilineatus* enhances transmission of *S. enterica* from contaminated leaves to clean leaves or adjacent plants within an agriculturally-relevant context (8,13). Furthermore, excretion of viable *S. enterica* from *M. quadrilineatus* has also been documented (27). Yet, how phytophagous insects influence this increase of *S. enterica* persistence on leaves remains mostly unexamined.

In earlier studies, we observed that only *M. quadrilineatus* infestation led to an increase in *S. enterica* persistence, but no observed benefit occurred following *M. persicae* infestation8. The findings of this investigation point towards differences between the inter- and intracellular penetrative styles of feeding between these two taxa, and the resulting effects these styles may hold for *S. enterica* population dynamics within the phyllosphere. While both insects possess similar mouthpart structures, collectively referred to as stylets, their modes of reaching vascular

tissues are very distinct. Aphids, or intercellular feeders, begin probing at the junction of two epidermal cells and guide their stylet through intercellular spaces in the mesophyll and towards vascular bundles (28). Leafhoppers, considered as intracellular feeders, similarly begin feeding at a cell junction, but distinctly pierce through leaf mesophyll to reach the phloem29. Comparisons of the electrical conductivity response of leaflets infested by inter- and intracellular penetration revealed that *M. quadrilineatus* infestation elicits a greater magnitude of electrolyte leakage, and consequently greater cellular damage than *M. persicae* on tomato plants (Fig. 1; Supplemental Fig. S1). Furthermore, our current study demonstrated that plants contaminated by *S. enterica* and infested with *M. quadrilineatus* had the highest overall populations of bacteria and resulted in the greatest magnitude of electrolyte leakage (measured as electrical conductance) (Fig. 3a-b). In addition to their distinct feeding behaviors, leafhoppers possess a stylet bundle 5-times wider than those found on aphids (30,31). To compensate for the lesser stylet, we investigated the influence of higher aphid populations in a complementary experiment, yet found no measurable impact on enhanced electrolyte leakage, or cellular damage (Supplemental Fig. S2). Taken together, the wider stylet paired with intracellular lacerating types of feeding behavior by *M. quadrilineatus* may partially explain the enhanced magnitude of cellular damage on the phyllosphere of tomato plants (Fig. 1). These findings lead us to conclude that cellular damage induced by *M. persicae* probing behaviors does not manipulate the phyllosphere to the same extent as M. quadrilineatus.

As previously mentioned, *S. enterica* and *M. quadrilineatus* co-habitation on the same leaflet resulted in higher *S. enterica* populations and measured electrolyte leakage (aka cellular damage) compared to water inoculated leaflets with or without insects (Fig. 3a-b). These elevated levels of cellular damage are likely the result of greater probing frequencies and may indicate an unfavorable feeding environment for the insect, prompting them to more frequently probe and search for alternative food sources. Previous studies identified clusters of gustatory neurons, which when combined, functionally create taste receptors within insects (32). When

encountering food contaminated by lipopolysaccharides (LPS), a ubiquitous component found on gram-negative bacterial cells, Drosophila melanogaster not only avoids E. coli-contaminated foods but also commence a hygienic grooming regimen (33). This prompted behavior suggests that some insects can discriminate between LPS contaminated and non-contaminated food sources via gustatory cues. Although many of these studies focus on insects with sponging-sucking mouthparts, such as flies, a genome analysis identified both odorant and gustatory receptor genes in aphid and mosquito genomes, both of which possess piercing-sucking mouthparts comparable to that of M. quadrilineatus (34). In our experiments where we confined *M. quadrilineatus* and *S. enterica* together in more proximal environments, we propose that the adult leafhoppers could encounter higher traces of LPS and may modify their normal feeding behavior as a consequence. Due to the restricted movement in these instances, we surmise the heightened magnitude of electrolyte leakage is driven by a constant search for a non-contaminated substrate and thus, heightened occurrences of probing for a new food source on *S. enterica* inoculated plant (Fig. 3b). To further evaluate whether *S. enterica* presence alters M. quadrilineatus' movement, we provided *M. quadrilineatus* with contaminated (*S. enterica*) and non-contaminated (sterile water) tomato leaflet surfaces and monitored their resting or feeding locations every 15 min thereafter for over a two-hour period. Throughout the time course of these observational experiments, a pattern of substrate discrimination occurred (Supplemental Fig. S8). Most insects initially landed on the plastic container housing the experiment, but over time began to immigrate more often to water-inoculated surfaces than those with *S. enterica*. In a complementary experiment, adult *M. quadrilineatus* exposed to tomato leaflets inoculated at either tip or basal regions of leaves similarly preferred water inoculated regions at 2 h post exposure, but predominantly emigrated to the experimental container walls after 48 h (Fig. 5). Altogether, leaflets entirely or partially inoculated with *S. enterica* were less frequently visited at the last time point (48 h post infestation), whereas leaflets inoculated solely with water were occupied throughout the experiment. Contrasting this behavior, apterous *M. persicae* exhibited

no preference between *S. enterica* or alternative surfaces (Supplemental Fig. S9). This lack of substrate preference may result from the largely sessile lifestyle of aphids, in contrast to more mobile and alate leafhoppers. These avoidance behaviors by *M. quadrilineatus* in response to *S. enterica* inoculated leaflets suggest a capability of recognizing contaminated substrates similar to the responses described for *D. melanogaster*.

To evaluate the extent by which *M. quadrilineatus* might influence the distribution of bacterial populations across leaflets, we first defined the distribution of *S. enterica* and the magnitude of electrolyte leakage across tomato and lettuce leaves in the absence of any insects. Morphological features between pre-reproductive lettuce and tomato plants are vastly distinct and were hypothesized to impact the distribution of bacterial populations and electrolyte leakage. In our study, the leaf tips were the lowest positioned part of tomato leaflets and exhibited half a log higher *S. enterica* populations in comparison to basal regions (Fig. 2a). Here again, the nominal architecture of tomato leaves results in a natural 'drooping' of fully expanded leaf tips. In a complementary experiment, tomato leaflets were modified to reverse this normal positioning of leaf tips to basal regions, and we did observe a corresponding re-distribution of *S. enterica* where accumulations were enhanced on basal portions of leaves (Supplemental Fig S4). These findings suggest that during the application of an aqueous solution—such as contaminated irrigation water or even foliar-applied crop inputs—factors including gravitational force may influence aggregations of aqueous solutions on leaves (35). This suite of findings identified leaf positioning and morphology, in conjunction with gravitational forces, as dominant influences of *S. enterica* population distribution across tomato leaflets while unaffecting the degree of electrical conductivity estimates, or associated electrolyte leakage of leaf electrolytes (Supplemental Fig. S3b). Despite *S. enterica* populations being highest at the tips of unaffected leaflets, bacterial populations were comparable at the tip and middle portions of leaflets only after adult *M. quadrilineatus* infestation, suggesting an insect mediated influence (Fig. 4). To this finding, we hypothesized that leafhopper feeding is not uniform or homogeneous across whole

leaflets and that the distribution of leaf vascular bundles may influence where adult leafhoppers find preferential feeding sites. The diameter of primary and secondary angiosperm vascular bundles typically narrows from the base to the tip of leaves, presumably to maximize the efficiency of hydraulic conductivity using adhesive and cohesive forces (36,37). This natural tapering of vascular structures at the tips of leaves provides piercing-sucking insects with some limitations in the number of ideal feeding locations and we hypothesize that the variation in the dendritic nature of leaf venation may alter the distribution of *M. quadrilineatus* feeding sites, explaining the higher *S. enterica* populations in the middle of infested tomato leaflets38. In addition to frequently observing leafhoppers in middle portions of leaflet regions, salivary sheathes were also predominantly found in similar regions of water-inoculated leaflets indicating preferences for these vascular bundles across leaflets (Fig. 6). Despite being their preferred feeding site, *S. enterica* inoculation at the middle of leaflets appeared to influence adult *M. quadrilineatus* towards feeding at the non-contaminated basal and tip regions, away from the *S. enterica* middle regions (Fig. 5). Similarly, leaflets partially inoculated at the base and tip had the least amount of salivary sheathes at their base and tip, respectively. This consistent pattern of probing avoidance of contaminated regions suggests that *M. quadrilineatus* may exhibit discriminatory behaviors against leaflets where *S. enterica* was present, indicating that even limited exposure to *S. enterica* holds potential to alter natural feeding behaviors as seen on water inoculated leaflets.

Although *M. quadrilineatus* exhibited avoidance behaviors of partially inoculated leaflets, their mobile lifestyle illustrates their potential as a biological multiplier for *S. enterica*. During their exposure to partially inoculated leaflets, salivary sheathes were identified at the base, middle and tip, although nonuniformly, suggesting an exploratory behavior (Fig. 6). This movement across *S. enterica* contaminated leaflets and the subsequent aversion suggest a likelihood for emigrating to alternative food sources (Fig. 7). Logically, if *M. quadrilineatus* have previously encountered *S. enterica* contaminated leaves or plants, then mechanical

transmission of bacteria could further exacerbate the likelihood of *S. enterica* dissemination within contaminated agricultural crops and promote the possibility of food borne outbreaks.

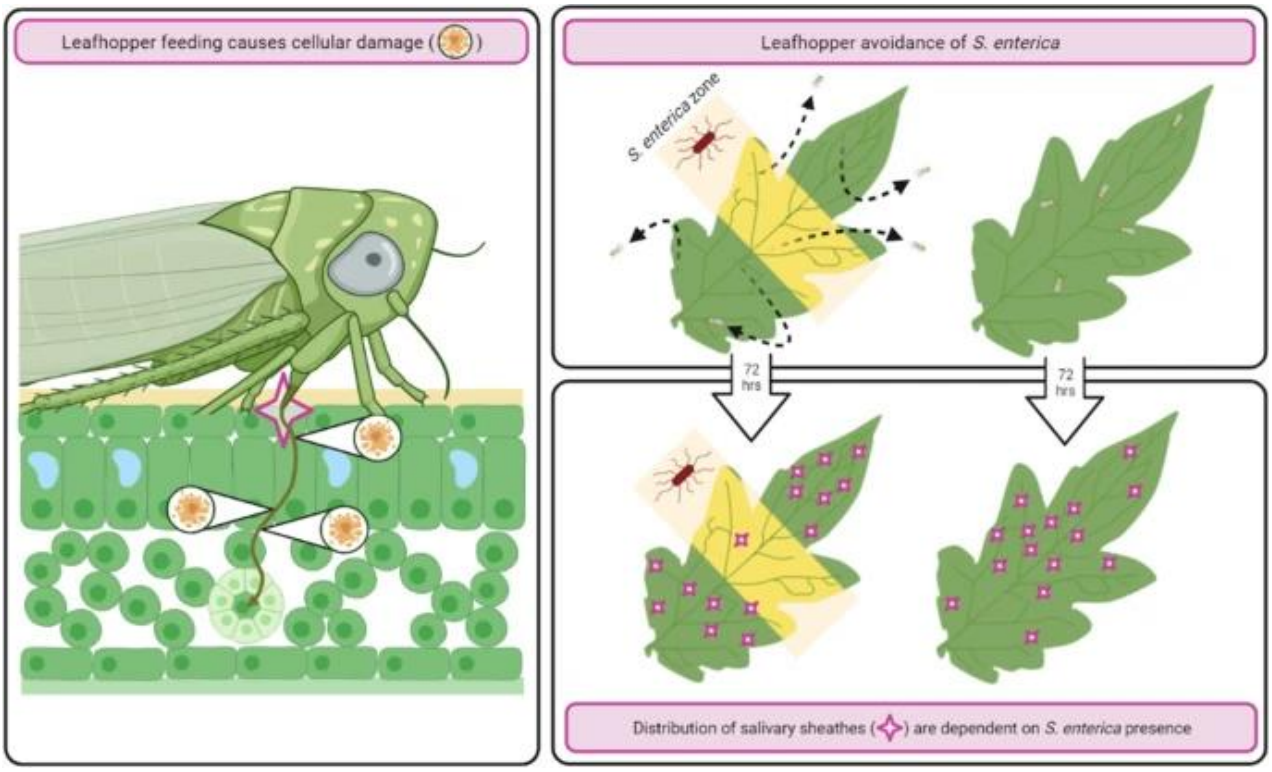

Figure 7. The presence of *S. enterica* alters *M. quadrilineatus* feeding behaviors. Infestation by *M. quadrilineatus* on *S. enterica* inoculated tomato plants resulted in significantly greater rates of localized cellular damage and bacterial populations than uninfested leaflets (left panel). Over a 48-h period of infestation, cohorts of *M. quadrilineatus* migrated away from tomato leaflets with partial, or entire, *S. enterica* inoculation (top). Cleaning and staining procedures 72-h afterwards demonstrated that zones of bacterial inoculation contained the least amount of salivary sheathes indicating an aversion to *S. enterica* within the phyllosphere (bottom). Image created through Biorender (biorender.com).

Within this study, we aimed to characterize insect feeding behaviors which could directly enhance *S. enterica* populations on tomato leaflets. Although we directly focused on cellular damage by stylet penetration, a suite of other phenomena (i.e. honeydew production and plant

immunity regulation) occurring in tandem necessitate further investigation. While these biological factors likely co-occurred, we identified prominent insect-mediated interactions involving cellular damage, unique insect feeding behaviors, and *S. enterica* populations, thereby demonstrating intracellular stylet penetration by *M. quadrilineatus* as a beneficial insect behavior for *S. enterica* persistence. Furthermore, we demonstrated that plant morphology directs the distribution of bacterial populations when dispersed aqueously yet may be manipulated in the presence of *M. quadrilineatus* due to increased stylet probing at preferred feeding sites. Although our results were collected under laboratory conditions, our findings elucidate how insects interact within the phyllosphere, and in turn, influence *S. enterica* population dynamics.

Acknowledgments

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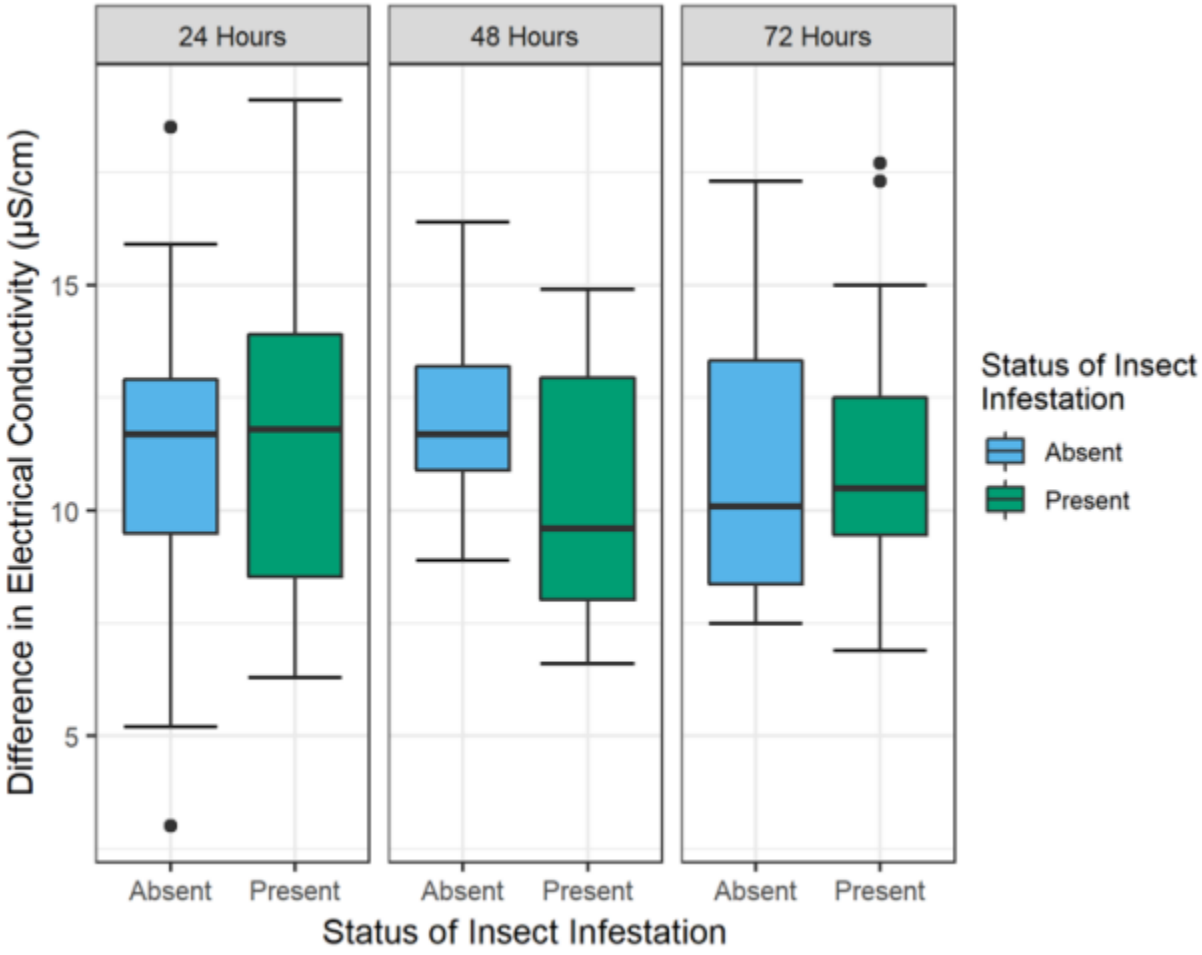
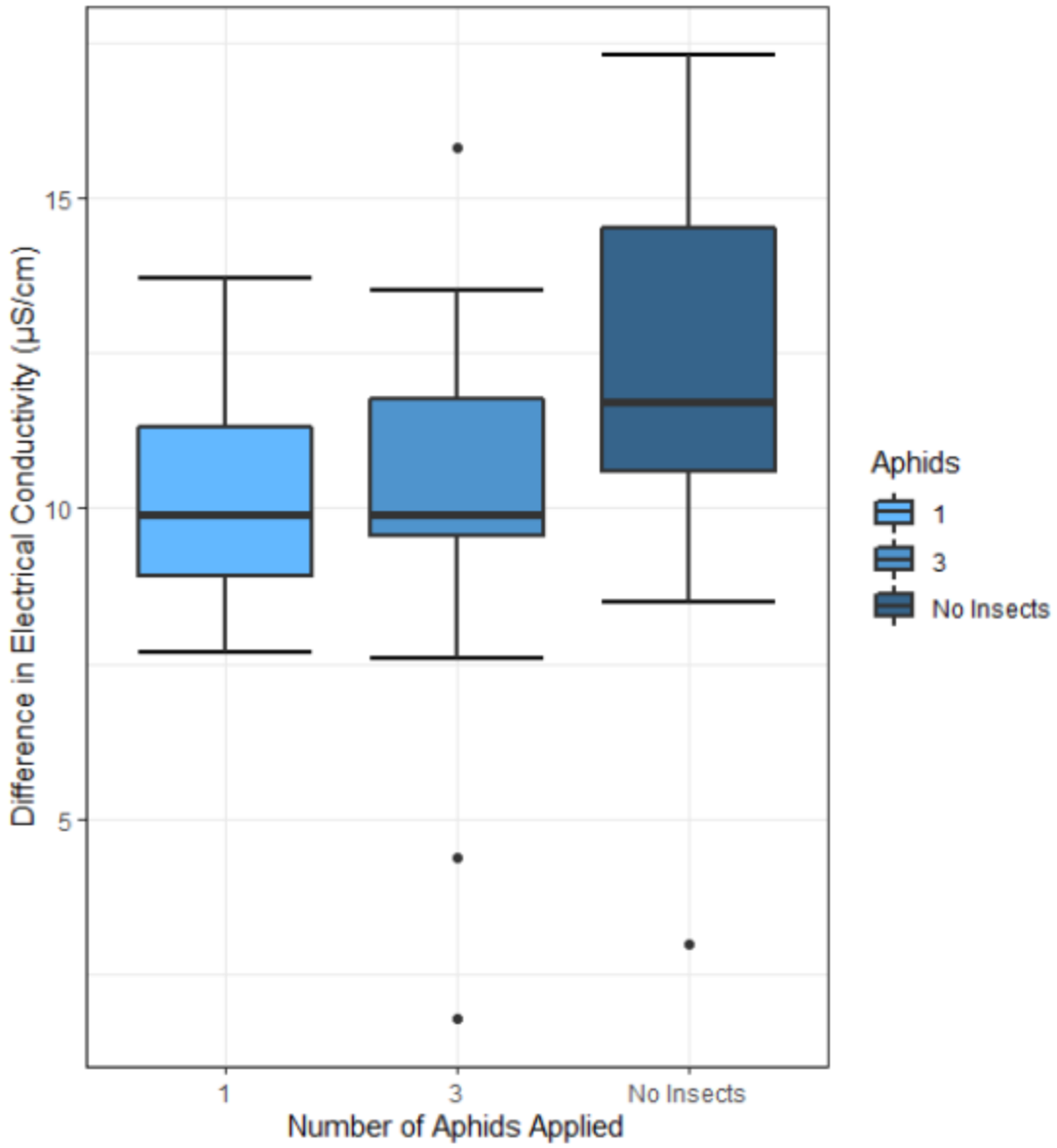
Competing Interests

The authors declare no competing interests.

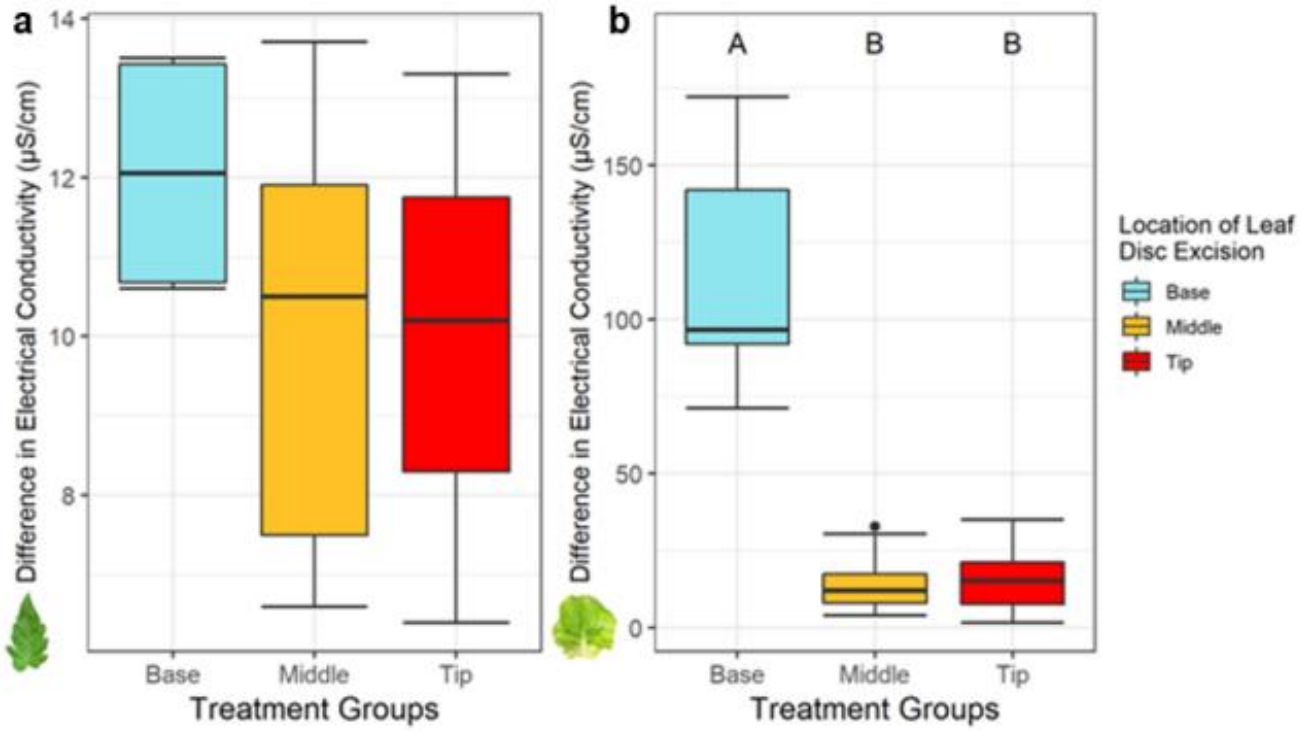
Supplementary Information



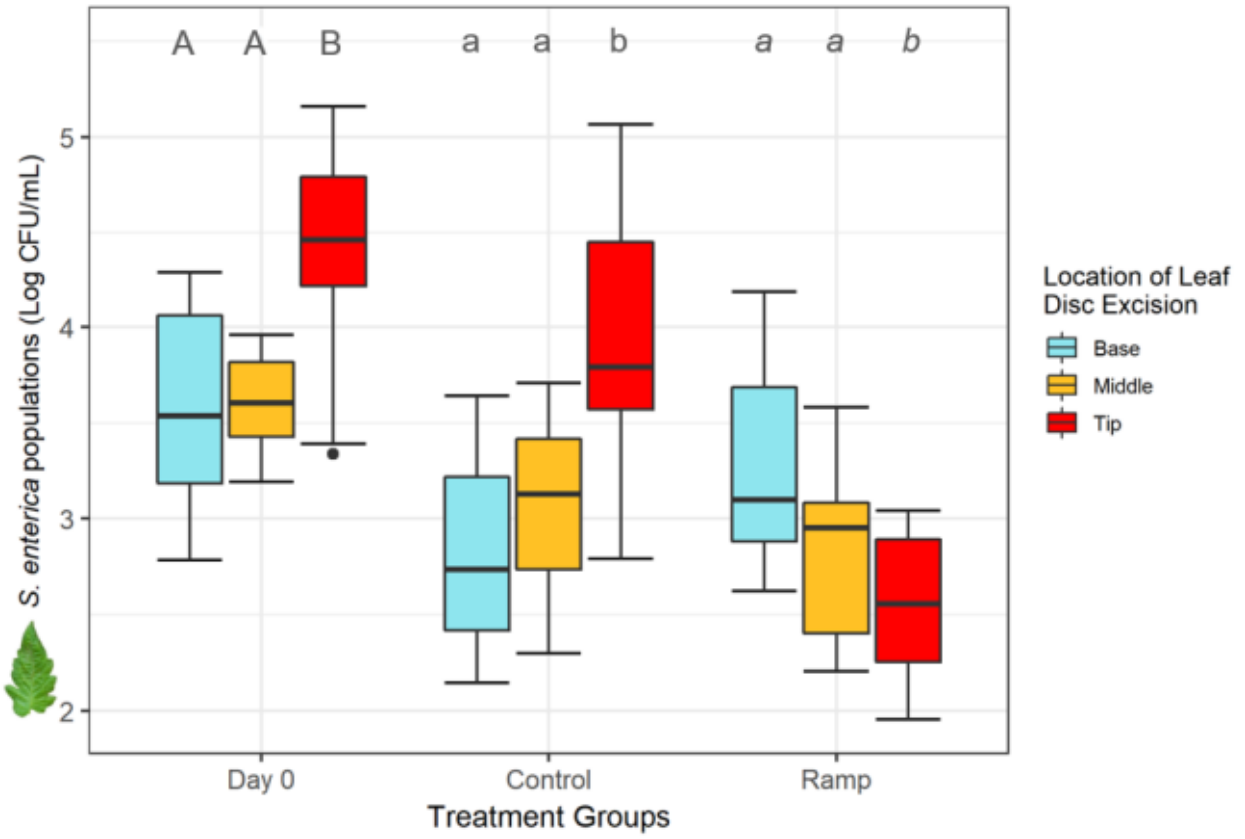
Supplemental Figure S1. Influence of Myzus persicae infestation upon electrical conductance measured at 24-, 48- or 72-hours post-infestation. Three clip cages were fastened onto a middle leaf, each containing one aphid (green), and three additional clip cages remained empty (blue) on the opposite tomato leaf. Measures of electrical conductance were calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. A student's t-test was used to assess significance between samples from infested, or non-infested clip cages. Singular dots represent an outlier point.



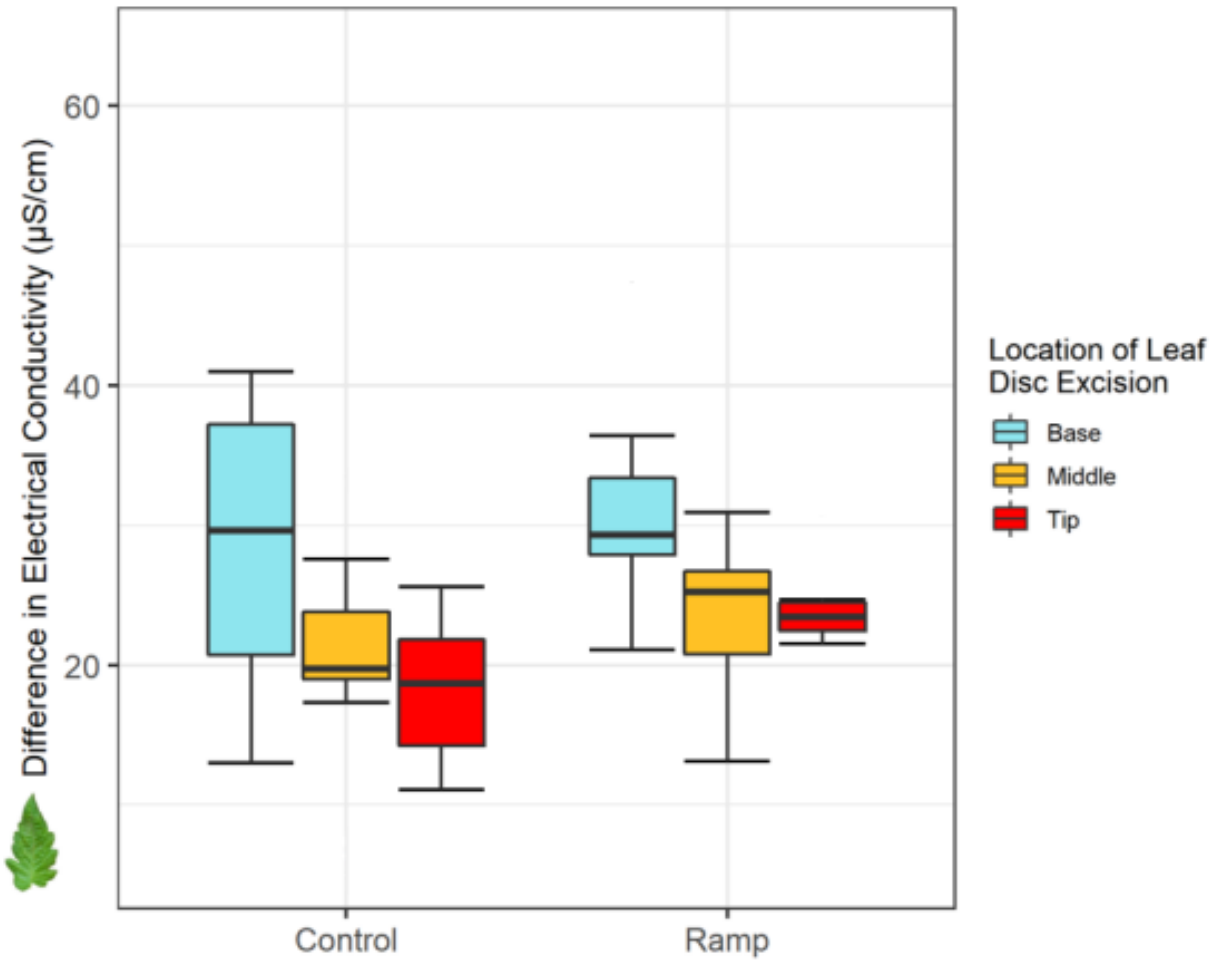
Supplemental Figure S2. Regardless of initial infestation density, populations of *Myzus persicae* allowed to feed for 72 hours did not elicit an increase in electrical conductance. Clip cages were fastened onto the middle of a leaflet containing one (light blue) or three (blue) aphids or remained empty (dark blue). Measures of electrical conductance were calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. A one-way ANOVA was used to assess significance between samples from infested, or non-infested clip cages. Singular dots represent an outlier point.



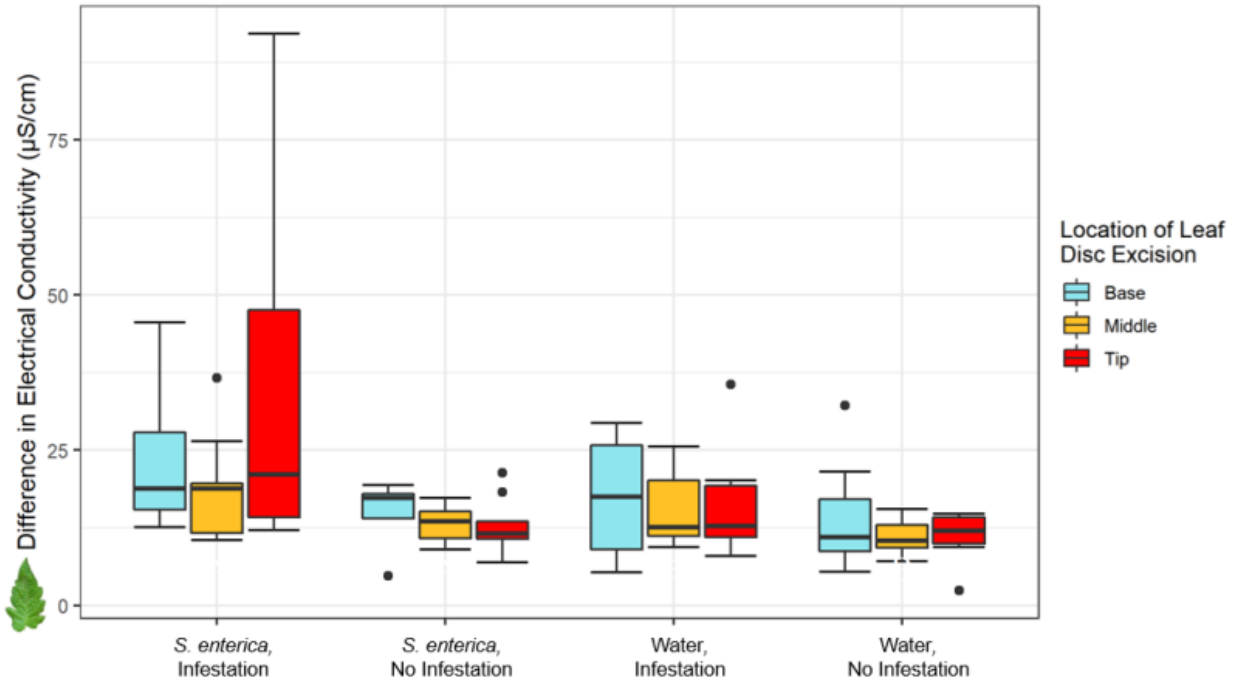
Supplemental Figure S3. Estimates of electrical conductance varied among regions of leaves in both lettuce and tomato. Tomato leaflets had a higher, albeit non-significant estimate of mean electrical conductance at its basal region (Supplemental Fig. S3a), whereas lettuce leaves had a significantly greater electrical conductance at its base (Supplemental Fig. S3b). Leaf discs were excised from pre-determined locations from the base (blue), middle (orange), and tips (red) of leaves (Fig. 3a). Measures of electrical conductance were calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by a one-way ANOVA. Singular dots represent an outlier point.



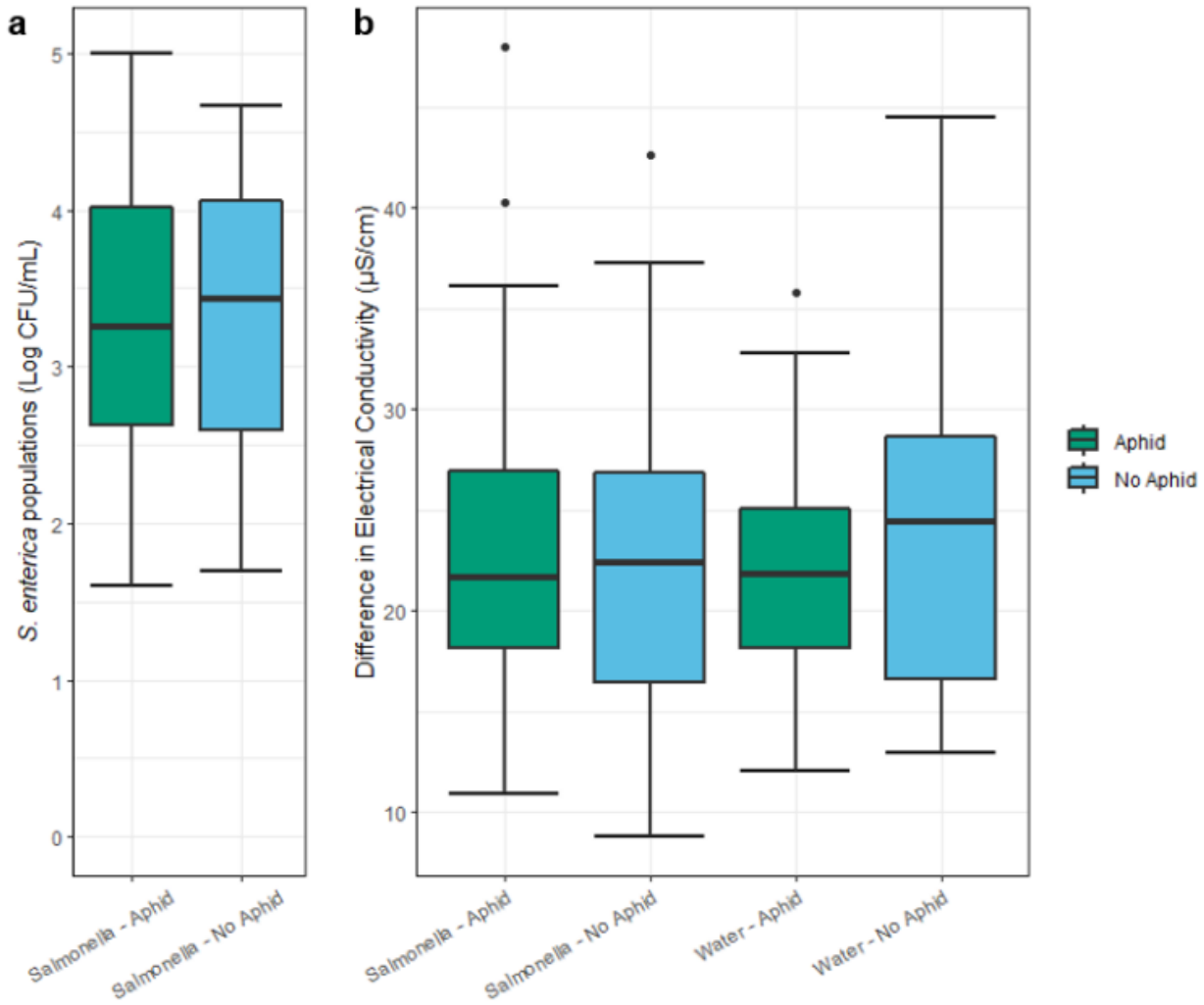
Supplemental Figure S4. The position of tomato leaflets impacts the resulting distribution of S. enterica in the phyllosphere. The tips (red) of unmanipulated tomato leaflets (Control & Day 0) support significantly higher S. enterica populations than the basal (blue) regions, whereas tomato leaflets facing upward (65o upward angle) had the greatest bacterial populations at its base (blue). Tomato plants were dip inoculated in an S. enterica or water solution and immediately placed into position. Control plants were directly placed into empty bins, whereas ramp treated plants were placed into bins with Plexiglass ramps at an approximately 65° upward angle for leaflets to vertically rest upon. After 72 hours, each plant was removed from its bin and measured for S. enterica populations across the base, middle or tip regions from among randomly chosen leaflets. S. enterica populations were measured on water inoculated leaves but yielded 0 CFU and were thus excluded from the figure. Each treatment group represents 16 biological replicates. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by a oneway ANOVA. Singular dots represent an outlier point.



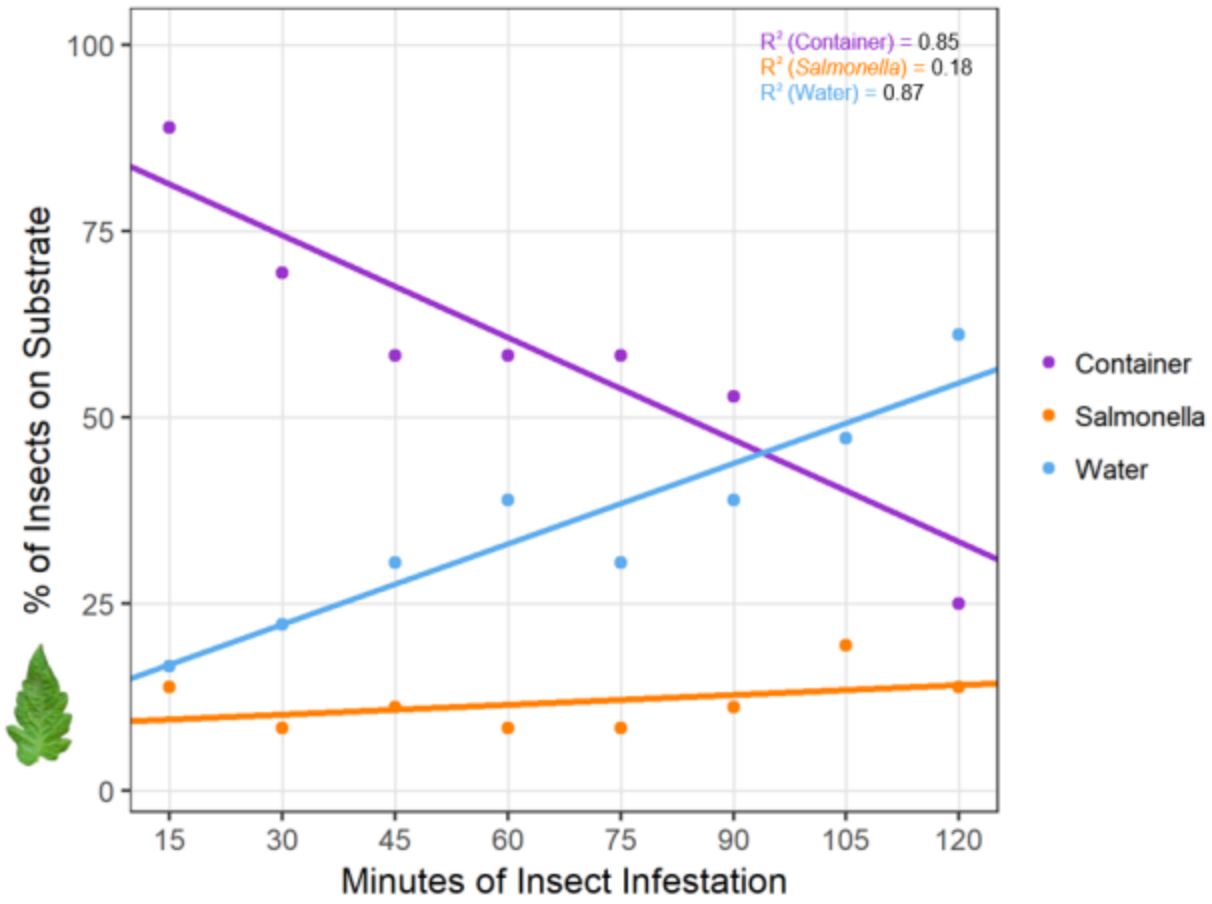
Supplemental Figure S5. Estimates of electrical conductivity did not change across a tomato leaflet in response to altered leaf positions. The base (blue) of tomato leaflets had a higher, but not significantly different extent of measured electrical conductance when compared to estimates at leaf tips (red; $P > 0.05$). Tomato plants were dip inoculated in an S. enterica or water solution and immediately placed into position. Control plants were directly placed into empty bins, whereas ramp treated plants were placed into bins with Plexiglass ramps an approximately 65° upward angle for leaflets to vertically rest upon. After 72 hours, each plant was removed from its bin and assessed for electrolyte leakage across the base, middle or tip regions from among randomly chosen leaflets. Data from S. enterica and water inoculated plants are both combined and represented in the figure above. Measures of electrical conductance were calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. Each treatment group represents 16 biological replicates.



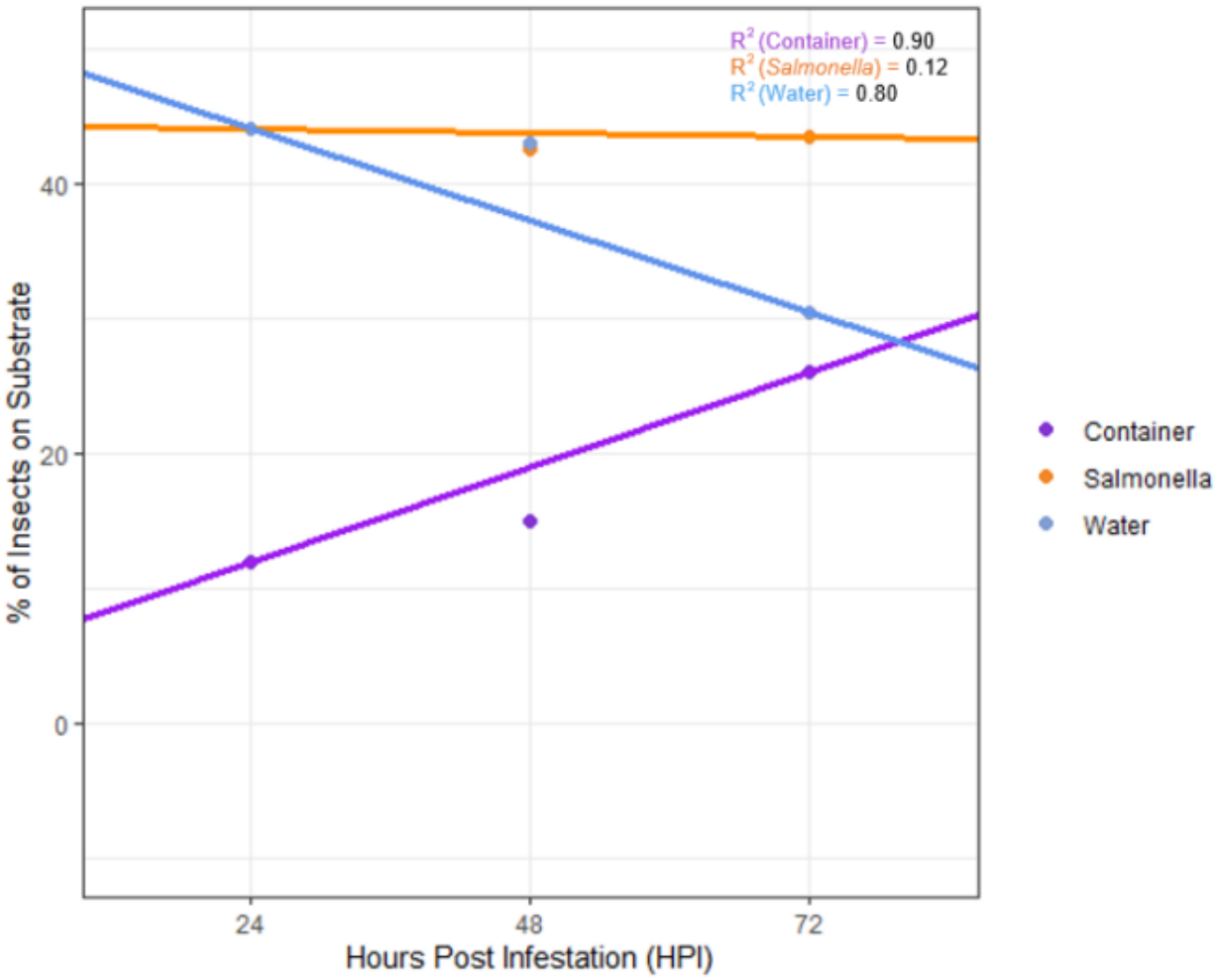
Supplemental Figure S6. Adult M. quadrilineatus infestation did not shift the natural magnitude of electrical conductivity on tomato plants. Among treatment groups, estimates of electrolyte leakage (measured as changes in electrical conductivity) was uniform across the basal (blue), middle (orange) and tip (red) regions of tomato leaflets. Salmonella enterica or water inoculated plants were either infested or remained un-infested using adult M. quadrilineatus. Leaf discs were excised from the tip, middle and basal regions of leaflets. Singular dots represent an outlier point.



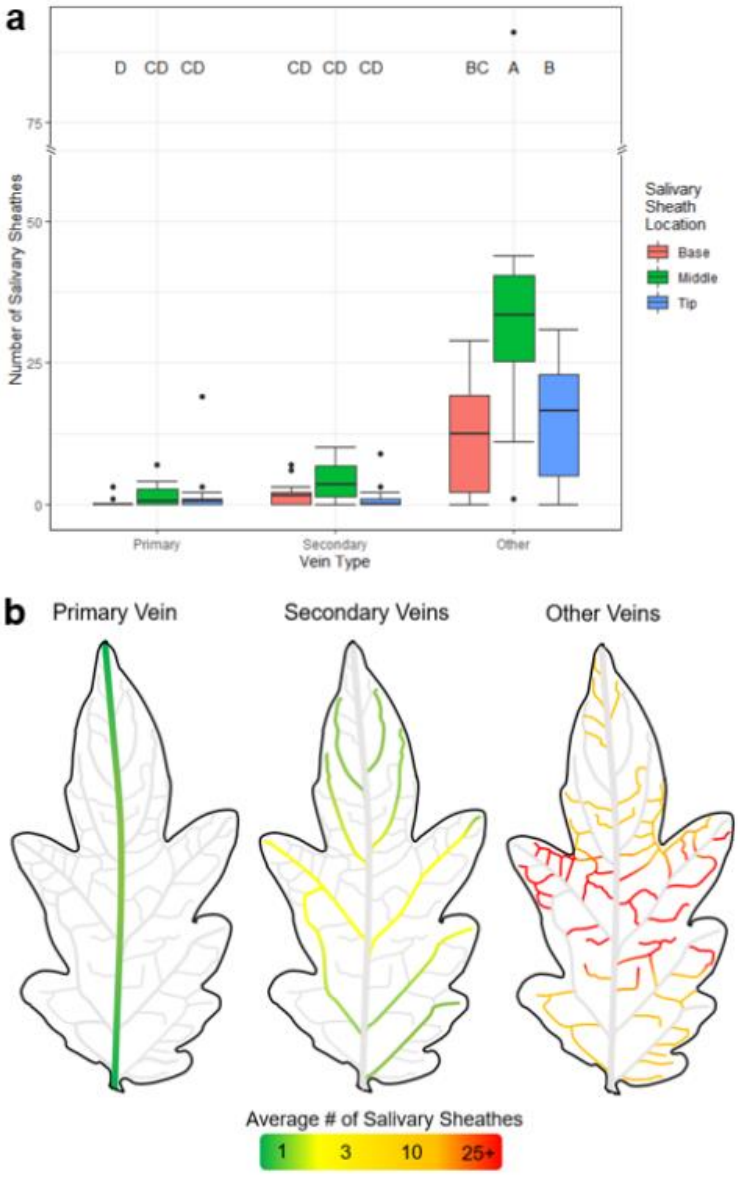
Supplemental Figure S7. *Myzus persicae* infestation on *S. enterica* inoculated tomato plants did not lead to heightened bacterial populations and electrical conductivity. Plants inoculated with either S. enterica or water were infested by apterous M. persicae or remained absent of insects. Empty clip cages were applied for treatment groups with no infestation. Electrical conductance was calculated by subtracting the final from the initial measurement for damaged and undamaged leaf discs and were used to evaluate the extent of electrolyte leakage over six hours. Each treatment group contains combined data from the tip, middle, and basal regions of leaves. Salmonella enterica populations were measured on water inoculated leaves but yielded 0 CFU and were thus excluded from the figure. Singular dots represent an outlier point.



Supplemental Figure S8. The percentage of adult M. quadrilineatus resting on water inoculated tomato leaves increased over a two-hour duration. Over a two-hour period, replicate sets of adult M. quadrilineatus were placed in containers encasing a tomato leaflet still attached to the plant. One leaf had the distal (tip) half inoculated with S. enterica, and the basal portion remained inoculated with sterile water. Remaining leaves had inoculation positions switched. Observations were taken every 15 minutes, starting 15 minutes after the initial insectplant exposure. The percent of insects on either substrate from two styles of inoculation (S. enterica and sterile water on the same leaf on either location) represent means of six experimental replicates and are represented as single points per 15 minute interval.



Supplemental Figure S9. The proportion of M. persicae that remain on leaflets inoculated with S. enterica over 72 hours. Tomato leaflets were partially inoculated with S. enterica on the right or left side of leaflets. One hour after S. enterica inoculation, one aphid was placed in a clip cage located on the middle of an inoculated tomato leaflet. Locations (Cage, S. enterica inoculated, or water inoculated regions) of individual insects were taken 24-, 48-, and 72 hours after the initial infestation period.



Supplemental Figure 10. Macrosteles quadrilineatus salivary sheathes are less frequently found on primary or secondary veins across water inoculated tomato leaflets. Five adult M. quadrilineatus were confined to a water inoculated tomato leaflet for 72 hours and were allowed to actively move and feed. Infested leaflets were removed and subjected to staining and clearing procedures. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by a one-way ANOVA (a). Salivary sheathes were assesed upon primary, secondary, and other lesser veins (b). Salivary sheathes from three experimental replicates (n = 15 leaflets) were combined and represented above.

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Chapter 4: Honeydew impacts *Salmonella enterica* populations in a host-dependent manner.

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V.L, R.G, and J.B conceptualized the design of the study and provided revisions of the manuscript; V.L and E.G conducted experiments, curated data, and conducted formal analysis.

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Abstract

Feeding behaviors, such as mechanical transmission and foliar feeding damage, have implicated *Myzus persicae* and *Macrosteles quadrilineatus* as biological multipliers for epiphytic *S. enterica* populations. Little attention, however, has been placed on the potential impact of honeydew – a carbohydrate dominated exudate produced by sap-feeding insects – upon *S. enterica* population dynamics. Using honeydew from *M. persicae* and *M. quadrilineatus* that had fed on unique plant hosts that were fresh (uninfested) or previously infested, we investigated the benefit of honeydew arrival for *in vitro* and epiphytic *S. enterica* populations. Regardless of the initial host plant taxa, honeydew significantly benefits populations of *in vitro S. enterica*. Honeydew from previously infested celery plants supported significantly higher *in vitro* bacterial populations than honeydew from fresh plants. Contrary to tomato hosts, the presence of honeydew upon *S. enterica*-contaminated celery leaflets did not significantly benefit epiphytic bacterial populations further indicating that the effectiveness of honeydew is partially dependent on the bacterial colonized plant taxa. Honeydew joins the growing list of factors commonly occurring in agricultural cropping systems that act as biological multiplier for *S. enterica* populations further elucidates our understanding of the role which insects play in increasing the risk of salmonellosis foodborne outbreaks from consumption of raw produce.

Key Words: Salmonellosis, Foodborne, Leafhopper, Aphid, Plant

Introduction

Predominantly composed of a wide assortment of carbohydrates, honeydew holds immense ecological impact. The mutualistic relationship between ants and aphids, for instance, are an intensely studied honeydew-based interaction (for a review, refer to Nelson and Mooney, 2022). Briefly, ants provide *Myzus persicae* (Green Peach Aphid) protection from epiphytic ranging predators in exchange for aphid honeydew. Studies have even shown that ants cater to

particular *M. persicae* species, preferring those that produce honeydew containing melezitose – a sugar unavailable in plants yet physiologically synthesized by aphids (Fischer and Shingleton, 2001). Staying within the lens of insect-insect interactions, pollinators have been observed to glean previously deposited honeydew as a complementary nutritional source, whereas other insects, such as *Episyrphys balteatus*, utilize volatile compounds within honeydew to target *M. persicae* prey (Cameron et al., 2019; Leroy et al., 2011). Considering plant-insect interactions, plants heavily visited by hemipteran pests, and thus are consequentially drenched in honeydew, act as carbohydrate-rich reservoirs for fungal plant pathogens such as sooty mold (Dhami et al., 2013, Kamikawa et al., 2018). Whether honeydew provides a nutritional niche for phyllosphere-colonizing *S. enterica* has not yet been investigated.

M. persicae and *Macrosteles quadrilineatus* (Aster Leafhoppers), have been classified as biological multipliers of epiphytic *Salmonella enterica,* a human enteric bacterial pathogen, populations across a variety of plant taxa (Soto-Arias et al., 2014, Cowles et al., 2018, Harrod et al., 2022). When infested by *M. quadrilineatus*, previously contaminated tomato leaves faced significantly higher levels of damage (measured by solute leakage) and supported significantly higher *S. enterica* populations than uninfested leaflets (Harrod et al., 2022). Leaflets infested by *M. persicae*, however, did not significantly impact bacterial population or elicit greater levels of solute leakage. Nevertheless, transmission experiments indicated that both insects ingested and retained *S. enterica* from liquid diets and lettuce leaf substrates, and successfully transmitted bacterial populations 48 hours after acquisition (Soto-Arias et al., 2014). Moreover, viable *S. enterica* populations were detected within both *M. quadrilineatus* and *M. persicae* honeydew – a carbohydrate saturated aqueous exudate produced by vasculature feeders – after exposure to contaminated food sources (Soto-Arias et al., 2014). The fate of *S. enterica* populations in the presence of insect honeydew, however, remains unexplored.

Salmonella enterica is the leading causal agent of food borne illness in the United States leading to nearly 1.35 million cases of salmonellosis and 26,500 hospitalizations every year

(Center for Disease Control and Prevention, 2022). Although the acquisition of salmonellosis is traditionally associated with the consumption of raw animal products, nearly half of all *S. enterica* foodborne outbreaks are associated with the consumption of contaminated fresh produce. Moreover, cases of produce-associated salmonellosis have risen over the last decade, highlighting the necessity of investigating contamination events which contribute to re-occurring foodborne outbreaks (Lynch et al., 2009; Walsh et al., 2014, Callejon et al., 2015).

Despite the numerous food quality and preventative measures put into place, there are several routes for *S. enterica* contamination to occur pre-harvest. Applications of contaminated irrigation water or manure-treated soils, for instance, are two common agricultural events linked to widespread dissemination of *S. enterica* upon plants, prompting bacterial populations to persist for weeks within the plant phyllosphere or soil (Islam et al., 2004; Ganyu et al., 2018; Hruby et al., 2018, Liu et al., 2018). Despite the prevalence of produce-associated salmonellosis outbreaks, adverse environmental conditions (such as UV radiation from sunlight, or nutrient limitation) lead to poor fitness of *S. enterica* populations within a healthy-plant phyllosphere. This natural deterioration of *S. enterica* populations paired with the re-occurrence of salmonellosis foodborne outbreaks, however, suggests other agricultural factors – such as insects – likely enhance epiphytic bacterial populations thereby acting as biological multipliers.

Within this study, we tested the hypothesis that the presence of insect honeydew enhances *S. enterica* population dynamics. Honeydew from *M. quadrilineatus* and *M. persicae* were each collected from 2 distinct plant hosts (oat and celery for *M. quadrilineatus*, and turnip and celery for *M. persicae*) that were either fresh, or previously infested. Celery and tomato plants were selected and contaminated with *S. enterica* to further determine whether the success of *S. enterica* is influenced by honeydew type, or the contaminated host plant. To determine differences between honeydew samples, we analyzed the remnant levels of salicylic acid – a phytohormone mediating defense against plant pathogens and stylet-feeding insects – and estimated levels of sugars within honeydew. Altogether, we expanded our understanding on

the insidious impact that phytophagous insects can play on food safety and add honeydew to a growing list of biological multipliers that influencing epiphytic *S. enterica* populations, and ultimately increase the risk of foodborne outbreaks.

Materials and Methods

Insect Rearing and Honeydew Collection

Colonies of *Macrosteles quadrilineatus* were maintained on oat seedlings (*Avena sativa*) and celery plants (*Apium* graveolens) under a temperature of 27 ˚C and 19 ˚C and a 16:8 (L:D) photoperiod. A colony of *Myzus persicae* was provided by Jason Timothy Ingram and Dr. Stewart Gray (Cornell University) and maintained on turnip plants (*Brassica rapa*) and celery plants under the same controlled conditions of 27 ˚C and 19 ˚C and a 16:8 (L:D) photoperiod. Voucher specimens of adult and female *M. quadrilineatus* and apterous *M. persicae* from our colony were deposited in the Wisconsin Insect Research Collection of University of Wisconsin (http://labs.russell.wisc.edu/wirc/).

Honeydew was collected from each plant host species and plant host damage (fresh plant or previously infested plant) of *M. persicae* (celery, and turnip) and *M. quadrilineatus* (celery and oat). To determine whether plant status is reflected within insect honeydew – measuring salicylic acid concentrations, estimating solute sugars, and using *S. enterica* population density as reporters – insects were either provided fresh, or plants previously infested plants. ‘Fresh’ plants faced infestation by 30 insects (either *M. quadrilineatus* or *M. persicae* on their respective plant hosts) for 72 hours. During this infestation period, a plastic sheet covered in Parafilm (Laboratory Sealing Film; Type M) was inserted into each colony for 72 hours, after which droplets of honeydew were collected as composite samples into an Eppendorf tube and stored in a -80˚C until used for assays. Plants ‘previously infested’ were exposed to approximately 100 insects for 1 week, after which all insects were removed. This previously infested plant was then re-infested by 30 insects for 72 hours. During this second

infestation period, honeydew was collected as a composite sample. To collect honeydew samples from *M. quadrilineatus* and *M. persicae* feeding upon an artificial diet, 200 µL of 20% glucose were pipetted into the inside of Eppendorf tubes and tightly wrapped with a layer of parafilm (otherwise known as Parafilm sachets). To purge insects of plant-derived honeydew for our controls, insects were placed into a sachet for 24 hours, and subsequently moved to a new sachet for 72 hours where accumulated honeydew was subsequently collected and stored in a -80˚C until used for later assays

Bacterial Strains, Media, and Culture Conditions

A kanamycin (Kan) resistant strain of *S. enterica* serovar Typhimurium 14028s from -80˚C freezer stocks, were utilized and grown in a lysogeny broth (LB; Difco LB Broth) at 37˚C, shaking overnight at 200 rpm. *S. enterica* cultures were normalized to an optical density at 600 nm of 0.2 in sterile water. Inoculum preparations were verified by enumerating populations after serial dilution, plating on Kan amended plates, and incubated overnight at 37˚C.

Plant Assays

Apium gravolens (Celery), *Solanum lycopersicum* (Tomato), *Avena sativa* (Oat), and *Brassica rapa* (Turnip) seedlings were cultivated using Jolly Gardener (Pro-Line, C/GP Germinating Mix) in 6” pots held in a growth room maintained at a 16:8 (L:D) photoperiod and 24˚C light and 19˚C dark conditions. Seeds were bought commercially. Tomato, oat, and turnip plants were established and maintained for five weeks prior to all experiments, whereas celery plants were grown and utilized after eight weeks.

We first identified the impact of insect honeydew upon *S. enterica* population dynamics in the absence of a plant host (*in vitro*). *S. enterica* populations were normalized to 10^8 and subsequently diluted to 10^4. Honeydew from *M. persicae* (Celery: Fresh, Previous Damage and Turnip: Fresh, Previous Damage) or *M. quadrilineatus* (Celery: Fresh, Previous Damage and

Oat: Fresh, Previous Damage) was combined with *S. enterica* in a 0.01:1 (v/v) solution. To assess the success of *S. enterica* in the presence of honeydew, we also amended *S. enterica* with M9 (minimal growth medium amended with 20% glucose) to a 0.01:1 (v/v) and another sample with only *S. enterica*. Replicate sets of 3 tubes per treatment were incubated at 28˚C (200 rpm) for 24 hours. Samples were then plated onto Kan plates, incubated at 37˚C and enumerated after 24 hours. A total of 3 experimental replicates were completed.

To characterize *S. enterica* populations dynamics on tomato and celery plants, whole plants were dip inoculated in a suspension of *S. enterica*. Replicate sets of 4 tomato or celery plants per honeydew treatment group were dip-inoculated for one minute in 450 ml of a 10^8 CFU/ml suspension of *S. enterica* prepared as described above with the addition of 75 µL of Sil-Wet. One-hour post-dip inoculation, 5 µL of honeydew was applied to the middle of middle-aged terminal tomato and celery leaflets. To locate the exact location of honeydew deposition, sets of 4.5 cm diameter plexiglass clip cages were fastened with clips surrounding the droplet. *S. enterica* dip-inoculated plants were then placed in clear, plastic bins held at 24°C under a 16:8 (L:D) photoperiod. To enumerate epiphytic *S. enterica* populations, honeydew-treated leaflets were sampled 24 hours after dip-inoculation. One 10-mm diameter leaf disc was excised from under clip cages on either tomato or celery leaflets. Samples were individually homogenized in 500 µL of sterile water using a cordless Dremel tool, and further diluted 1:10 (v/v) in sterile water. Homogenates were immediately plated on LB-Kan plates, incubated overnight at 37 ˚C, and *S. enterica* populations were enumerated 24 hours later. A total of 3 experimental replicates were completed.

Mass Spectrometry and Soluble Sugar Analysis

The soluble sugars (Brix˚) of insect honeydew, and vasculature samples of fresh celery, oat, and turnip plants were measured using a digital refractometer (Model HI 96801, Hanna Instruments). Plant vasculature was collected by excising small fragments of plant stems from

middle-aged leaflets and laying them flat on one 1x3” sheet of Parafilm. The stems were then rolled from one end to form a tight bundle and placed within an Eppendorf tube. Each sample was then centrifuged for 10 minutes at 10000 rpm, and vasculature samples accumulated on the bottom were transferred to new tubes and kept in the -80˚C until further use.

The methods used for measurement of salicylic acid were modified from Balcke et. al. Multiple samples for each sample type were collected. Samples of the same type were first pooled together to yield enough material to generate 3 replicate pooled samples for each sample type. Prior to LC/MS/MS, samples were processed by solid-phase extraction (SPE). SPE was performed using a vacuum manifold in two sequential batches. For all SPE replicates, 10uL sample volumes were diluted with 5uL deuterated salicylic acid (d4-SA) at 10pmol/uL in methanol followed by 485uL 1% formic acid in water. SPE was performed using Sep-Pak tC18 (Waters) cartridges containing 100mg sorbent in a 1mL cartridge format. After each step, any liquid remaining in the outlet tubing was drained to waste prior to the next step. Cartridges were first wet by addition of 1mL methanol and equilibrated with 1mL 1% formic acid in water. Samples were then applied to the sorbent and the flow-through collected. Bound material was washed with 1mL 1% formic acid in water and eluted with 1mL 5% concentrated ammonium hydroxide (v/v) in methanol. Eluates were collected into 2mL centrifuge tubes and immediately placed on dry ice. With each SPE batch, an additional 2 SPE were performed on the d4-SA alone spiked into 495uL 1% formic acid. SPE eluates were dried by centrifugal vacuum concentrator (Speed-Vac). 5uL methanol was added to each dried eluate followed by 95uL 0.3mM ammonium formate, pH 3.5, 0.1% formic acid in water.

Data were collected on a Sciex 5500 QTRAP triple-quadrupole mass spectrometer coupled to an Agilent 1100 HPLC stack. This stack consisted of a Nanopump operating in normal mode, a uWPS autosampler with a 40uL loop, and a thermostatted column compartment. The LC column was a GL Sciences Insertsustain AQ-C18, 2.1mm x 150mm with 3uM particles. LC solvents consisted of A: 0.3mM ammonium acetate pH 3.5, 0.1% formic acid

in water; and B: 0.3mM ammonium acetate, pH 3.5, 0.1% formic acid, 90% acetonitrile. A 15-minute gradient was used with initial conditions of 5%B for 1 minute, ramping to 98%B at 9 minutes. Hold for 0.5 minutes, ramp down to 5%B at 10 minutes and equilibrate for 5 additional minutes. The flow rate was 300uL/min and the column was held at 35C. The mass spectrometer was operated in negative ion MRM mode. Native salicylic acid transitions were 136.8/93.0 (quantifier) and 136.8/65.0 (qualifier). Labeled salicylic acid (d4-SA) transitions were 140.8/97.0 (quantifier) and 140.8/69.0 (qualifier). Quantifier transitions used the settings CE -22V and CXP -5V. Qualifier transitions used CE -37V and CXP -6V. Additional instrument parameters were as follows: CUR 40, CAD medium, IS -3000, TEM 550, GA1 50, GS2 50, DP -40 EP -9.8. Q1 and Q3 were operated at unit resolution. Injection volumes for all analyses were 5uL. Data were collected using Analyst 1.6.3 and quantitation was performed by measuring the are ratio of native salicylic acid to d4-SA and multiplying by the concentration of d4-SA, which had been spiked into samples at 5pmol/uL.

Processing of d4-SA through the solid-phase extraction step resulted in a small but reproducible signal in the MRM channels for native salicylic acid. Therefore, this was subtracted from the salicylic acid measured in samples. This was done by averaging the two d4-SA blanks processed along with each batch and subtracting from the samples in that batch. The average salicylic acid concentration measured in the blanks was 36.3ng/mL for batch 1 and 32.8ng/mL for batch 2.

Statistical Analysis

A one-way, analysis of variance (ANOVA) was used to assess the impact of *M. persicae* or *M. quadrilineatus* honeydew treatments upon *in vitro S. enterica* cultures, epiphytic *S. enterica* populations on tomato and celery host plants, and concentrations of SA between insect types. Bacterial counts were log transformed prior to analysis. Results were considered

statistically significant at $P < 0.05$. All statistical analysis was preformed using R software (version 4.2.2).

Results

Honeydew benefits *S. enterica* populations, *in vitro*.

To determine whether insect honeydew benefit *S. enterica* populations in the absence of a plant host, *in vitro* experiments were performed. In addition, we hypothesized that honeydew may have different qualities from insects feeding on plants that had previously hosted the same insect compared to plants without previous insect feeding which we called “fresh.” Feeding on fresh or previously infested plants, *M. persicae* honeydew was collected from celery or turnip plants, whereas *M. quadrilineatus* honeydew was collected from celery or oat plants. Following collection, cultures of *S. enterica* were amended with the aforementioned honeydew treatments. *S. enterica* grew almost 1 log in 24 h following *M. quadrilineatus* honeydew amendments (Figure 1). *S. enterica* cultures treated with *M. quadrilineatus* honeydew from previously infested celery plants resulted in significantly higher bacterial populations than *M. quadrilineatus* honeydew from fresh celery plants (Figure 1; $P < 0.05$), *S. enterica* amended with M9 - 20% glucose (Figure 1; $P < 0.05$), and samples of *S. enterica* without honeydew amendments (Figure 1; $P < 0.001$). *S. enterica* also grew in *M. persicae* honeydew amended cultures, but to a lower final population compared to cultures amended with *M. quadrilineatus* honeydew. The same differential pattern of *S. enterica* growth was observed with *M. persicae* honeydew-amended cultures where honeydew from heavily infested celery plants resulted in significantly higher *S. enterica* populations than honeydew from fresh celery plants (Figure 2; $P < 0.05$), M9 - 20% glucose (Figure 2; $P < 0.05$), and samples where *S. enterica* had no amendments (Figure 2; $P < 0.001$). Surprisingly, no differential growth was observed when *S. enterica* was amended with honeydew from oat-fed *M. quadrilineatus* or turnip-fed *M. persicae* with regard to the history of infestation of the host. *S. enterica* cultures amended with honeydew, however, were

significantly higher than cultures without honeydew (Supplemental Figure 2; $P < 0.001$, Supplemental Figure 3; $P < 0.001$).

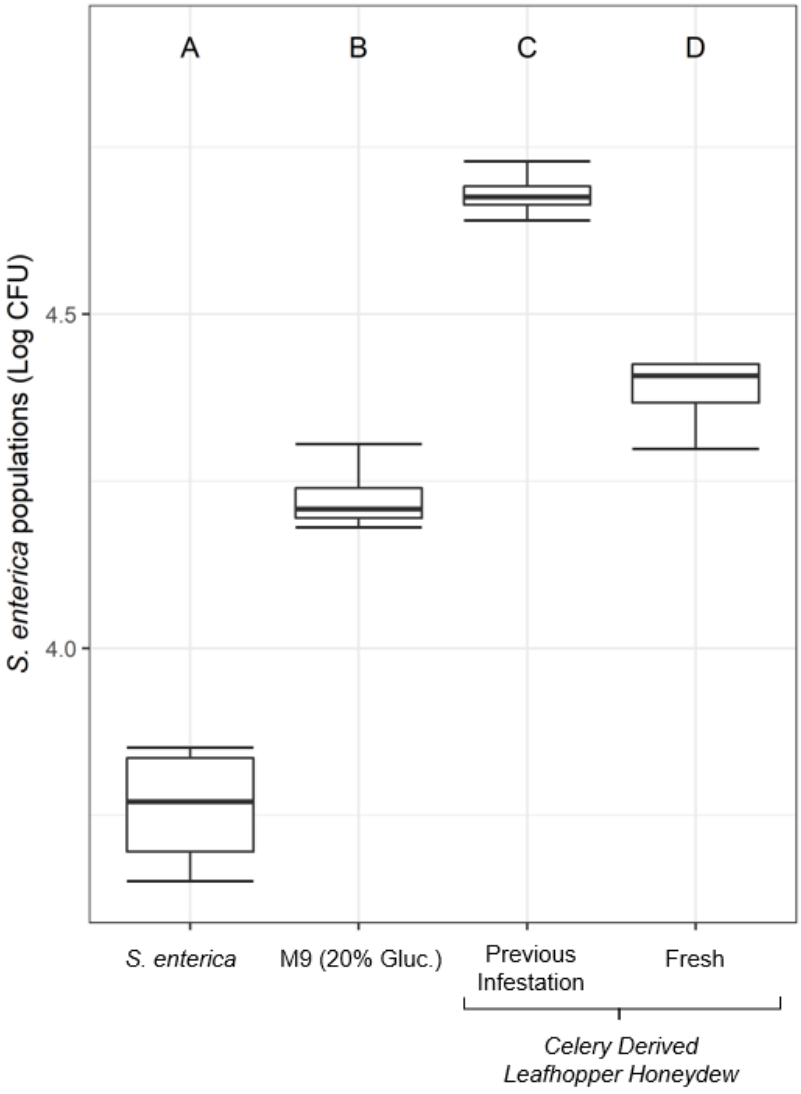


Figure 1. Cultures of *S. enterica* amended with celery-derived *M. quadrilineatus* honeydew result in significantly higher bacterial populations than untreated samples. Honeydew derived from previously infested celery plants supported significantly higher bacterial populations than honeydew derived from fresh celery plants, or samples treated with M9 (20% glucose). Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.

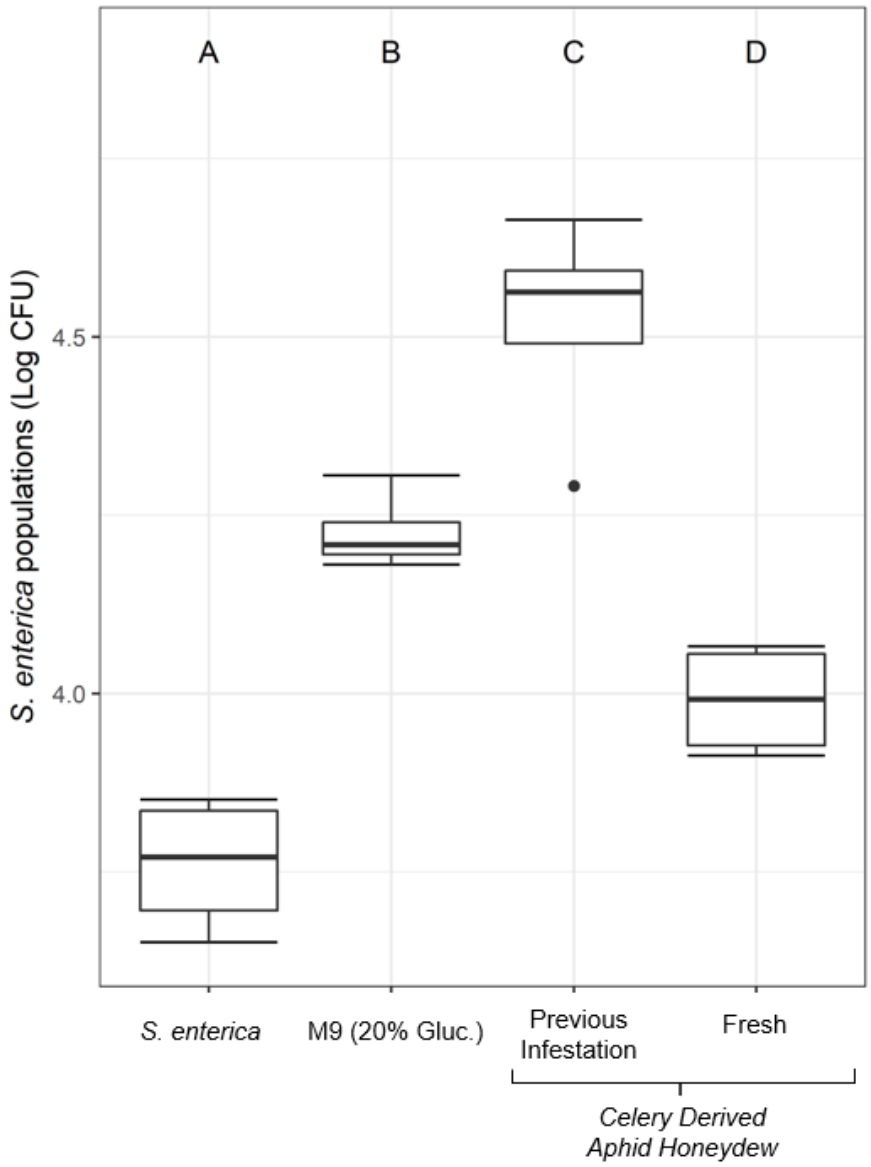


Figure 2. Cultures of S. enterica amended with celery-derived *M. persicae* honeydew result in significantly higher bacterial populations than untreated samples. Honeydew derived from previously infested celery plants supported significantly higher bacterial populations than honeydew derived from fresh celery plants, or samples treated with M9 (20% glucose). Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.

Status of plant damage significantly impacts SA concentrations within honeydew.

To determine whether insect infestation host history impacts the composition of honeydew, we analyzed SA concentrations using LC/MS/MS (Table 1). SA was found in all honeydew samples. Hosts that previously had been infested had the highest SA concentrations. Comparing within insect types, *M. quadrilineatus* and *M. persicae* honeydew derived from previously infested celery plants contained significantly higher concentrations of SA than honeydew from fresh celery plants ($P < 0.05$), and honeydew from glucose-fed insects ($P < 0.0001$). Not surprisingly, honeydew from fresh celery plants fed upon by both *M. quadrilineatus* and *M. persicae*, had significantly higher concentrations of SA than honeydew from glucose-fed insects ($P < 0.0001$).

	Treatment	***SA Concentrations (ng/mL)***
A	Aphid – Glucose	36.26
B	Aphid – Fresh	880.64
C	Aphid – Previous Infestation	1110.43
a	Leafhopper – Glucose	32.82
b	Leafhopper – Fresh	556.68
c	Leafhopper – Previous Infestation	870.66

Low SA° High SA°

Table 1. Concentrations (ng/mL) of salicylic acid within composite honeydew samples processed by solid-phase extraction and LC/MS/MS. Honeydew was collected from insects that had fed on previously infested celery plants ('Previous Infestation'), fresh celery plants ('Fresh'), or on a 20% glucose solution ('Glucose').

The presence of honeydew enhances *S. enterica* populations on tomato leaves, but not on celery leaves.

To further characterize honeydew as a biological multiplier for epiphytic *S. enterica* populations, we treated *S. enterica*-colonized tomato and celery plants with droplets of insect honeydew. Honeydew treatments resulted in *S. enterica* populations growing up to 1.5 logs on tomato leaves compared to *S. enterica* populations that did not receive honeydew, independent of insect taxa and infestation history (Figure 3). Applications of honeydew derived from *M. persicae* or *M. quadrilineatus* fresh or previously infested celery plants resulted in significantly higher *S. enterica* populations than untreated tomato leaflets (Figure 3; $P < 0.05$). Tomato leaflets amended with oat-derived *M. quadrilineatus* honeydew or turnip-derived *M. persicae* honeydew supported significantly higher populations than non-treated tomato leaflets (Figure 4-5, respectively; $P < 0.05$). In contrast to tomato, addition of honeydew to celery leaves colonized by *S. enterica* did not result in significant population changes (Supplemental Figure 4). Neither insect taxa nor host infestation history influenced *S. enterica* populations colonizing celery leaflets ($P < 0.05$). *S. enterica* populations on celery leaves treated with oat-derived *M. quadrilineatus* honeydew or turnip-derived *M. persicae* honeydew contained similar bacterial populations than untreated tomato or celery leaves (Figure 4-5, respectively; $P > 0.05$).

Supplemental Figures and Tables

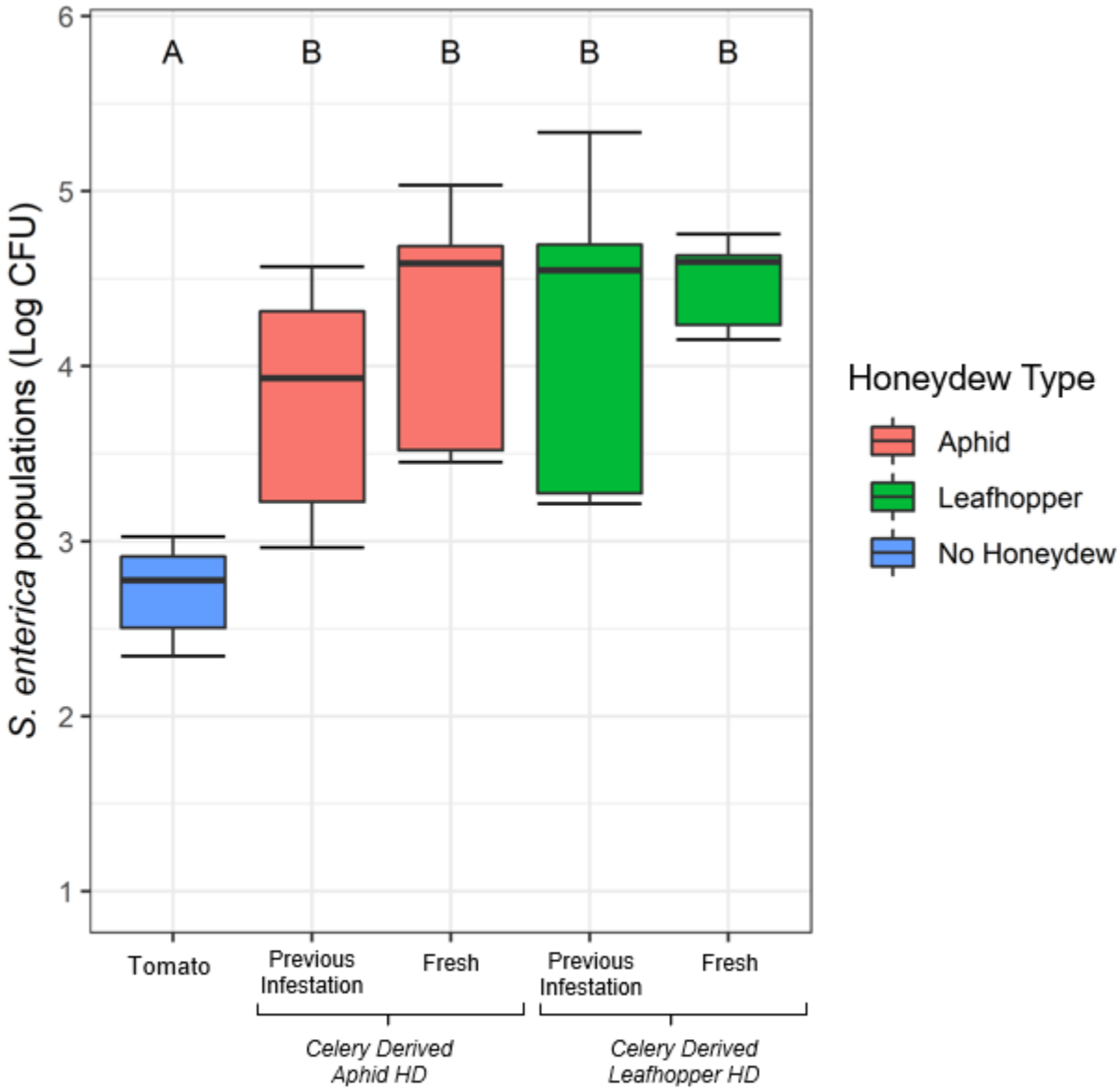


Figure 3. The presence of celery-derived *M. quadrilineatus* (green) or *M. persicae* (red) honeydew on tomato leaflets significantly benefits epiphytic *S. enterica* populations. Honeydew from fresh or previously infested celery plants was collected and deposited onto dip-inoculated celery host plants. Leaf discs were processed, and bacterial populations were enumerated 24 hours after dip-inoculation. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.

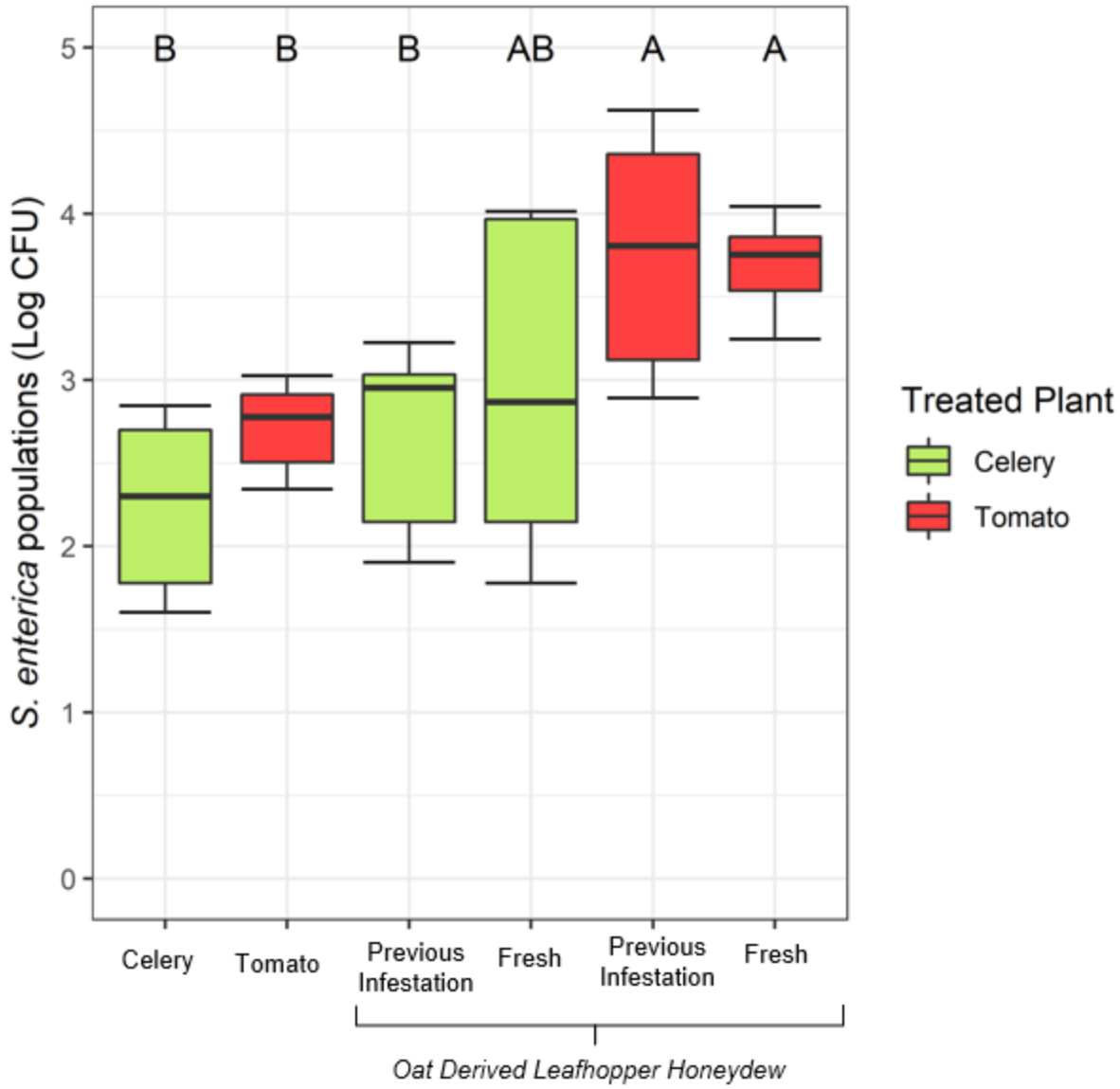


Figure 4. The presence of oat-derived *M. quadrilineatus* honeydew on tomato leaflets significantly benefits epiphytic *S. enterica* populations. Honeydew from fresh or previously infested oat plants was collected and deposited onto dip-inoculated celery (green) or tomato (red) host plants. Leaf discs were processed, and bacterial populations were enumerated 24 hours after dip-inoculation. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.

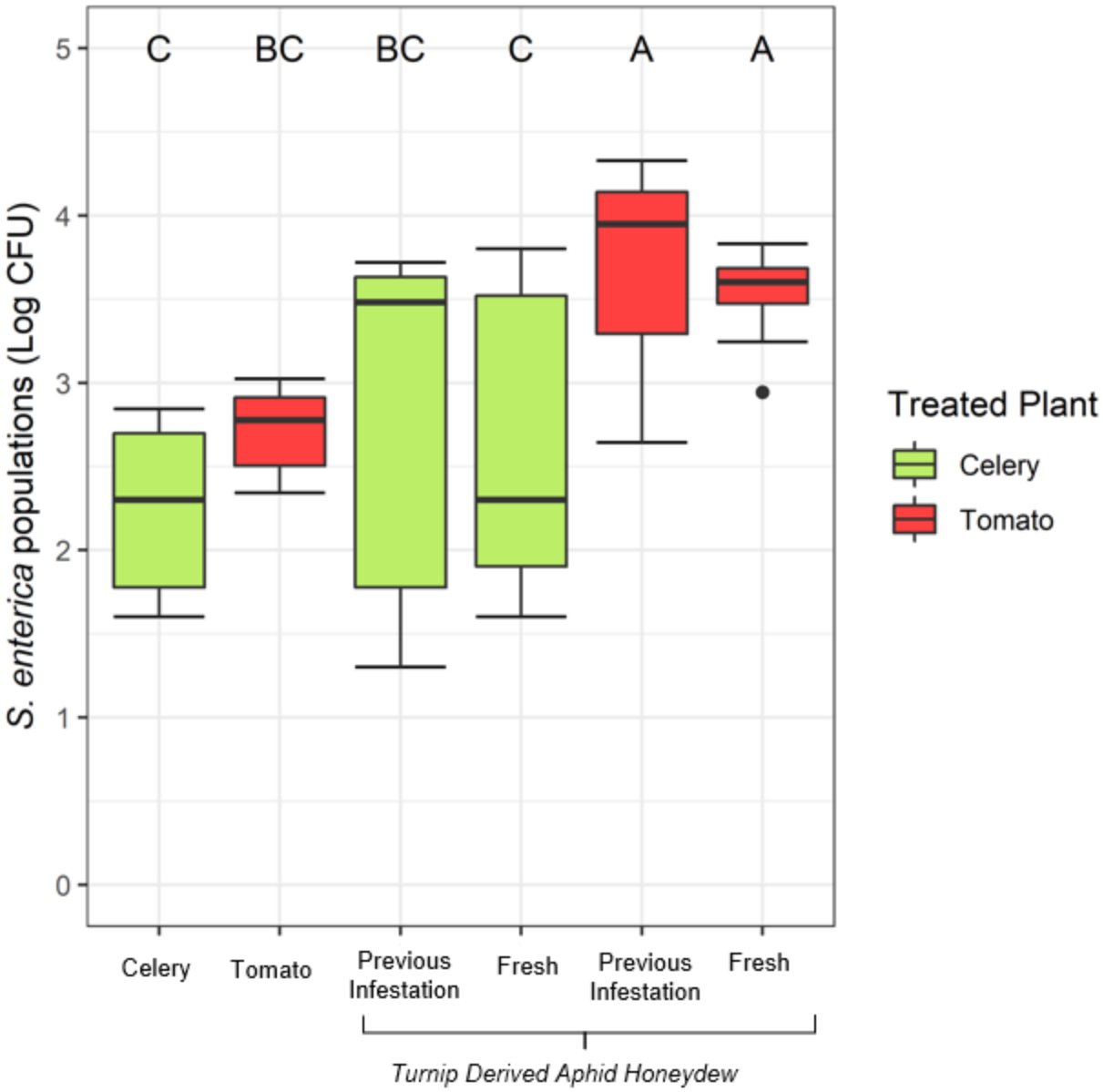


Figure 5. The presence of turnip-derived *M. persicae* honeydew on tomato leaflets significantly benefits epiphytic *S. enterica* populations. Honeydew from fresh or previously infested oat plants was collected and deposited onto dip-inoculated celery (green) or tomato (red) host plants. Leaf discs were processed, and bacterial populations were enumerated 24 hours after dip-inoculation. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by a one-way ANOVA. Singular dots represent an outlier point.

Discussion

Facing hostile environmental conditions within the phyllosphere of a healthy plant, *S. enterica* depends upon the intervention of biological multipliers to successfully persist. As solely vasculature feeders, *M. quadrilineatus and M. persicae* provide *S. enterica* a growth medium in the form of honeydew – a carbohydrate rich exudate – consequentially enhancing epiphytic bacterial populations and exacerbating the risk for produce-associated foodborne outbreaks. Using *S. enterica* as a biological reporter, our current study examines bacterial population dynamics in response to an assortment of insect honeydew. Specifically, we investigated whether the initial host plant taxa, infestation history status of the plant which honeydew was produced from (fresh or previously infested), or the bacterial colonized host plant taxa impacted *S. enterica* populations uniquely. Examining honeydew as a nutritional reservoir for *S. enterica*, this study further characterizes the role of *M. quadrilineatus* and *M. persicae* as common members of the agricultural environment that may impact the safety of raw produce production.

Although *M. quadrilineatus* and *M. persicae* possess narrow and segmented stylets used to access an assortment of phloem or xylem constituents, they employ unique piercing strategies which may impact their honeydew composition. *M. persicae*, for instance, stealthily reaches the vasculature via intercellular penetration, whereas *M. quadrilineatus* probes voraciously through cells (intracellularly; Escudero-Martinez et al., 2020). When assessing feeding damage, leaves infested by *M. quadrilineatus* face significantly higher levels of solute leakage than uninfested leaves and has also been reported to elicit upregulation of jasmonic acid (JA) and salicylic acid (SA) (Harrod et al., 2022, Cowles et al., 2018). Despite not resulting in higher levels of solute leakage, previous studies have demonstrated that elevated levels of *M. persicae* infestation elicit a greater competition of resources, and as such, induce a greater level of foliar SA expression than uninfested leaflets (Cao et al., 2016). To better understand whether status of the host plant (i.e. taxa or insect infestation history) impacts honeydew composition,

we analyzed the concentration of SA and observed the concentrations of soluble sugars (measured by Brix˚). Although identifying SA within *M. quadrilineatus* and *M. persicae* honeydew is novel within our study, previously groups had similarly detected remnant SA within other vasculature-feeding insects highlighting the potential for plant chemical components to be reflected within honeydew (Schwartzberg and Tumlinson, 2013, VanDoorn et al., 2015). Notably, we found the highest levels of SA within *M. persicae* honeydew from previously infested celery plants, and the lowest in leafhopper honeydew from plants not previously fed upon *(*Figure 3). Comparing SA concentrations within insect types, honeydew derived from previously infested plants contained significantly higher concentrations of SA, than their fresh counterparts. We were surprised that SA was detected in honeydew from both *M. quadrilineatus* and *M. persicae* that fed upon glucose, suggesting that SA continued to be excreted from the insect long after it stopped feeding from plant hosts. We also observed that honeydew from previously infested plants contained higher sugar concentrations, with the exception of *M. persicae*-derived honeydew from previously infested celery plants (Supplemental Figure 1). Furthermore, *M. persicae* honeydew contained higher concentrations of sugars than the vasculature of their host plant, whereas *M. quadrilineatus* honeydew contained comparable concentrations (Supplemental Figure 1). Corresponding to our findings, other studies had identified elevated levels of glucoisate and significantly enhanced amino acid to sugar concentrations within plant phloem following infestation by *M. persicae* upon *Brassiva pekinesis* (Chinese Cabbage) indicating a vascular phytochemical shift in response to infestation (Cao et al., 2016). Having established the range of SA and sugar concentrations across honeydew types, we subsequently explored whether *in vitro* and epiphytic *S. enterica* would utilize honeydew as a nutritional substrate.

Widely considered polyphagous pests, *M. quadrilineatus* and *M. persicae* feed upon a variety of common agricultural crops, and thus, participate in plant-switching (Hoy et al., 1992;

Blackman and Eastop, 2000). In conjunction with previous studies, we've similarly identified remnant phytohormones and sugars within insect honeydew, and as such, gained interest in investigating whether honeydew derived from one host plant might differentially impact *S. enterica* population when deposited on the same or different host plant type (Wäckers, 2011; Fischer et al., 2005; Yao and Akimoto, 2001). As before, we utilized *M. quadrilineatus* honeydew from celery or oat plants, and *M. persicae* honeydew from celery or turnip plants that were fresh or previously infested. Despite benefiting *in vitro* populations of *S. enterica* (Figures 1-2), the presence of celery-derived *M. persicae* or *M. quadrilineatus* honeydew upon previously colonized celery leaves did not significantly change *S. enterica* populations compared to untreated leaflets (Figure 3). Similarly, the presence of oat-derived or turnip-derived honeydew from *M. quadrilineatus* or *M. persicae*, respectively, on celery leaves did not significantly promote bacterial growth when compared to untreated celery plants (Figure 4-5). Interestingly, turnip-derived *M. persicae* honeydew had the highest estimated levels of sugars yet failed to induce epiphytic bacterial growth on celery (Supplemental Figure 1). All forms of honeydew, however, did significantly enhance *S. enterica* populations *in vitro* and upon previously colonized tomato host plants compared to honeydew-absent cultures or plants without honeydew (Figure 1 – 2, ; 3 – 5; Supplemental Figure 2 - 3). Applied globally as a natural medicine, the deterred success of *S. enterica* on celery leaflets may be explained by the plant's unique chemical characteristics. Rich in flavonoids and phenolic compounds, applications of celery leaf extract significantly restrain Gram-positive and Gram-negative bacterial colonization and deter reactive oxygen species (ROS) during inflammation (Aboody, 2021; Shin et al., 2019; Zhou et al., 2009). Within agricultural settings, amending chicken feed with celery extract results in inhibited *S. enterica* growth (Nuningtyas et al., 2020). Among other factors, these findings indicate that plant cultivars are a primary component that determines the success of epiphytic *S. enterica* colonization, further highlighting the importance of characterizing anti-microbial qualities employed by plants (Barak et al., 2011).

Within this study, we've identified *M. quadrilineatus* and *M. persicae* honeydew as a biological multiplier epiphyte populations of *S. enterica* in the phyllosphere. Although our study focused upon honeydew, future studies should consider and explore deeper into the interactions between plants, insects, and phyllosphere bacteria. Future studies would be informative in identifying the phytohormonal cascades in response to *S. enterica* and honeydew epiphytic arrival. While the elimination of foodborne outbreaks altogether is implausible, understanding how insect biological multipliers benefit *S. enterica* populations is the next step in pursing integrated pest management measures, and furthermore protecting the health of our local and global communities at risk.

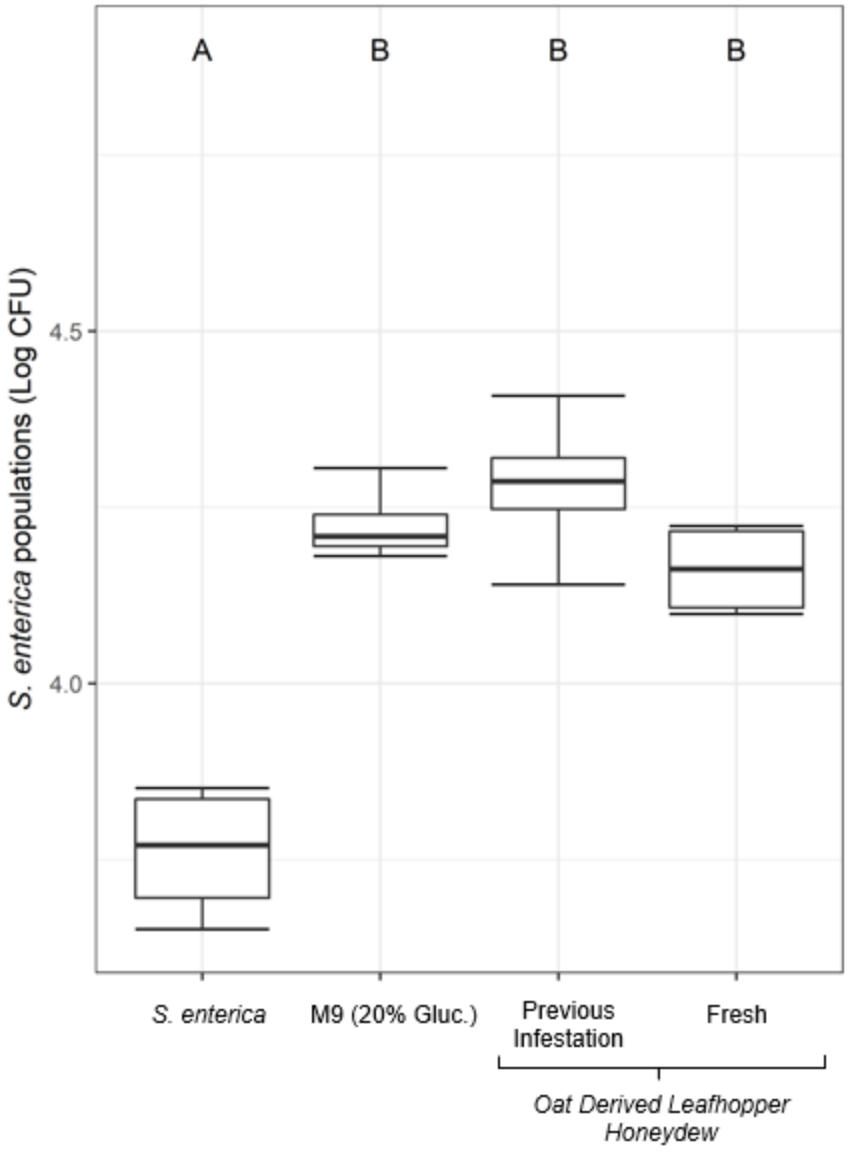
Acknowledgments

Funding was provided by USDA-NIFA 2016-67017-24422 and the Food Research Institute at the University of Wisconsin – Madison.

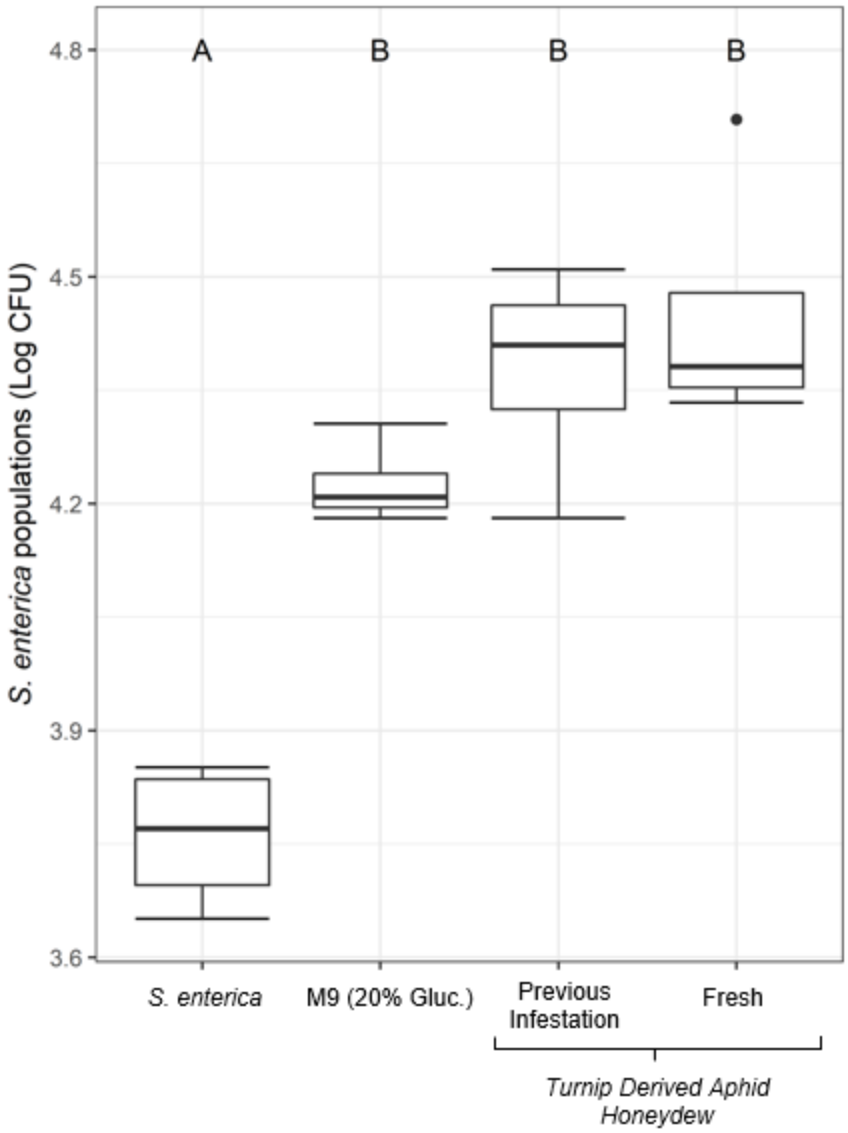
Supplemental Materials

Treatment Groups	***Brix°***
HD: Leafhopper – Celery (Previous Infestation)	6.5
HD: Leafhopper – Celery (Fresh)	5.0
HD: Aphid HD – Celery (Previous Infestation)	10.5
HD: Aphid HD – Celery (Fresh)	15.5
HD: Leafhopper – Oat (Previous Infestation)	1.1
HD: Leafhopper – Oat (Fresh)	0.3
HD: Aphid – Turnip (Previous Infestation)	27.2
HD: Aphid – Turnip (Fresh)	20.7
Vasculature: Celery	6.4
Vasculature: Oat	0.3
Vasculature: Turnip	17.1

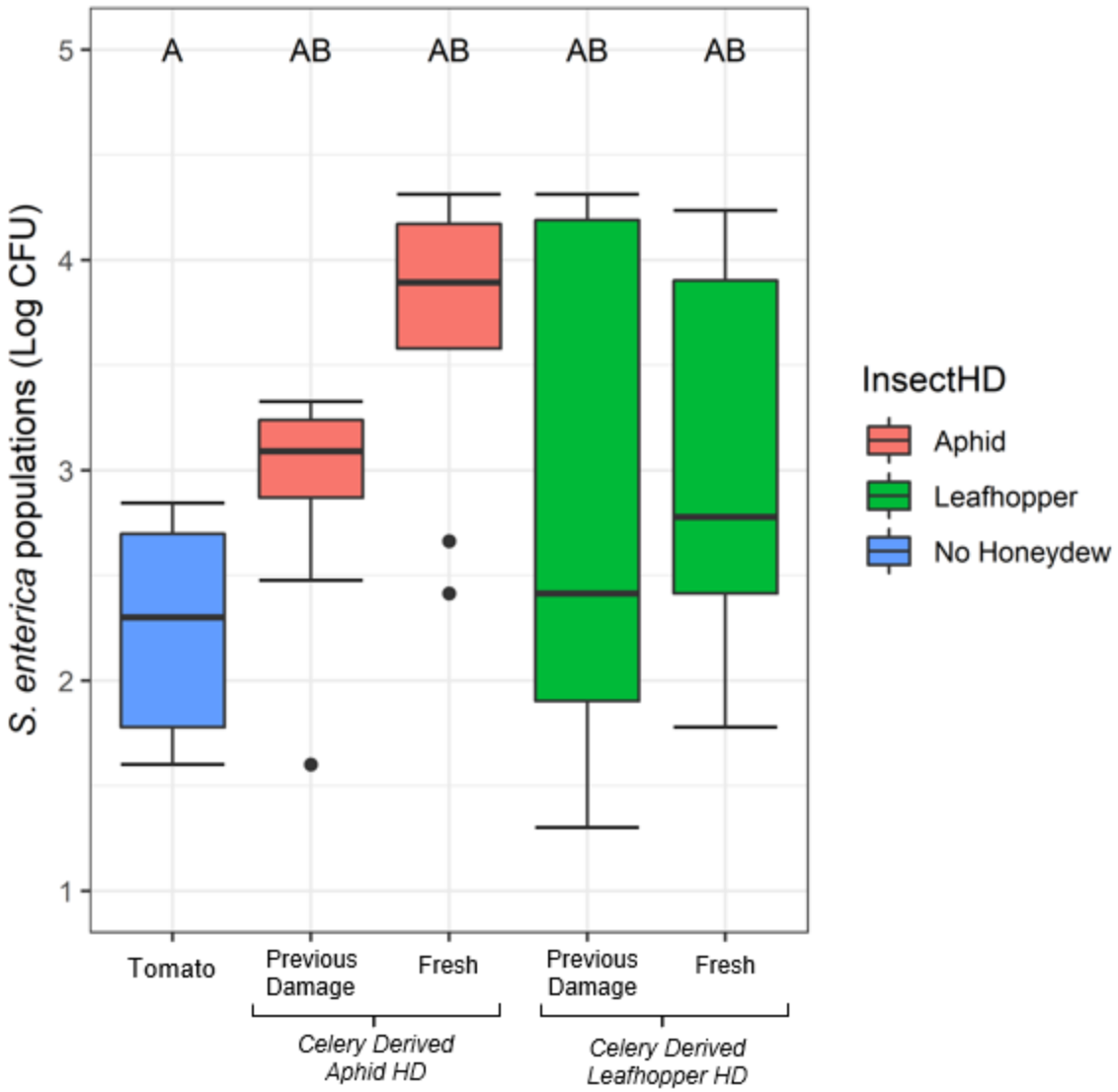
Supplemental Table 1. Observations of sugar concentrations of composite *M. quadrilineatus* or *M. persicae* honeydew. Honeydew was collected from each plant host species and plant host damage (fresh plant or previously infested plant) of *M. persicae* (celery, and turnip) and *M. quadrilineatus* (celery and oat). The soluble sugars (Brix˚) of insect honeydew, and vasculature samples of fresh celery, oat, and turnip plants were measured using a digital refractometer (Model HI 96801, Hanna Instruments).



Supplemental Figure 2. Cultures of *S. enterica* amended with oat-derived *M. quadrilineatus* honeydew result in significantly higher bacterial populations than untreated samples. Honeydew derived from previously infested celery plants supported similar levels of bacterial populations as honeydew derived from fresh celery plants, or samples treated with M9 (20% glucose). Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.



Supplemental Figure 3. Cultures of *S. enterica* amended with turnip-derived *M. persicae* honeydew result in significantly higher bacterial populations than untreated samples. Honeydew derived from previously infested celery plants supported similar levels of bacterial populations as honeydew derived from fresh celery plants, or samples treated with M9 (20% glucose). Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.



Supplemental Figure 4. The presence of celery-derived *M. quadrilineatus* (green) or *M. persicae* (red) honeydew on celery leaflets do not significantly benefits epiphytic *S. enterica* populations. Honeydew from fresh or previously infested celery plants was collected and deposited onto dip-inoculated celery host plants. Leaf discs were processed, and bacterial populations were enumerated 24 hours after dip-inoculation. Letters above boxplots indicate significant differences between treatment groups within each experiment ($P < 0.05$), as detected by an ANOVA. Singular dots represent an outlier point.

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Chapter 5: WISCONSIN INITIATIVE for SCIENCE LITERACY (WISL)

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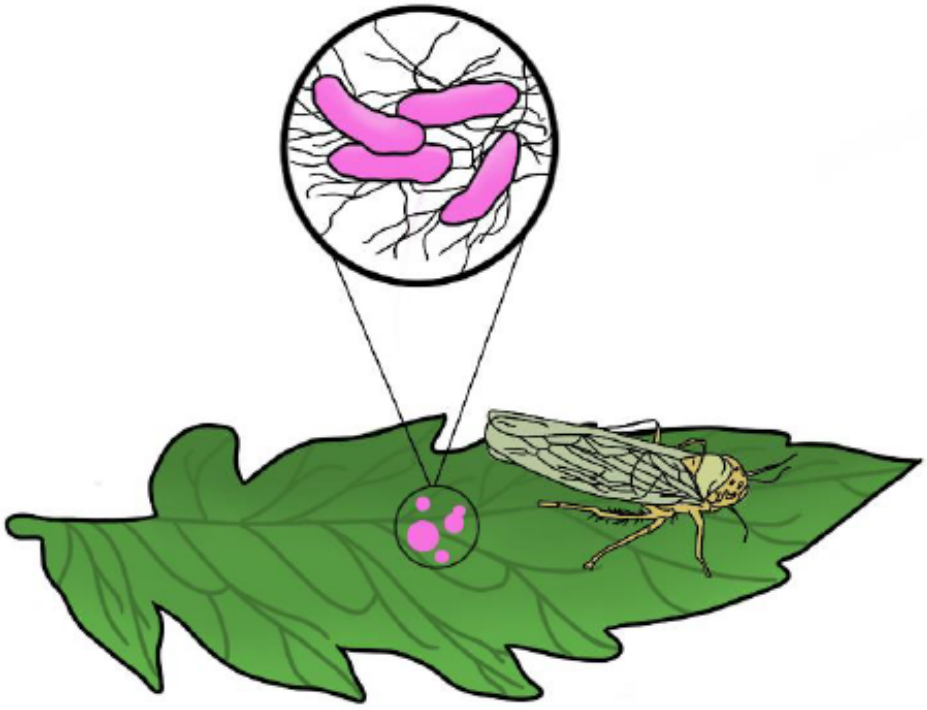
Author Contributions

V.L.H wrote, and conceptualized the outline of the document, and revisions were provided and made by the staff at the WISL office. This work will be published by the WISL on their website (http://scifun.org/Thesis_Awards/thesis_awards.html)

Notes: The format of Chapter 5 is outside of the traditional formatting requirements for a doctoral dissertation trhough the UW-Madison Graduate School. This format, however, has been approved by the WISL committee, and will be adjusted if the UW-Madison Graduate school requests for formating adjustments.

PRELUDE



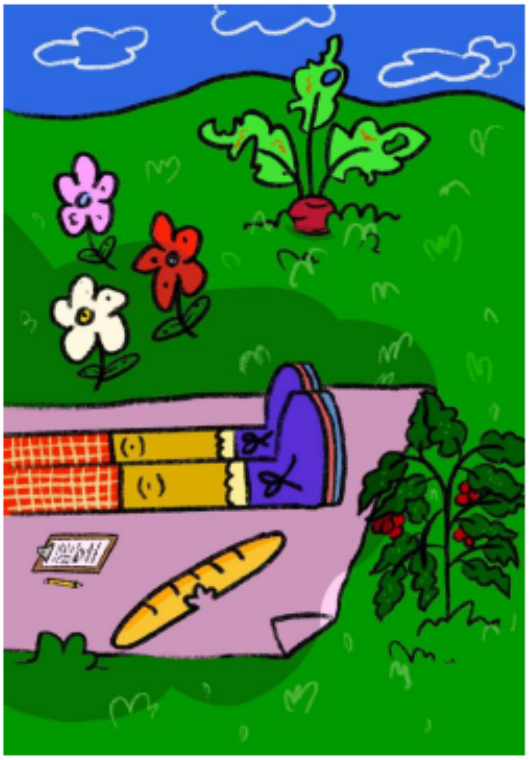
Regardless of the number of high-impact publications or the novelty of their work, a scientist is only as good as their ability to translate the importance of their findings – Especially so, to non-scientists. In typical Ph.D. fashion, the end of my time as a graduate student will be marked by a public oral presentation followed by a private defense of my thesis work to my graduate research committee. The oral presentation, while traditionally presented to a crowd of scientists, is meant for a wider audience; however, this work is only presented once. The Wisconsin Initiative for Science Literacy (WISL) graciously provides graduate students with a platform to present their data to the public, as with an oral presentation, but in a permanently present and writien format. With this chapter, my hopes are that it highlights just one of the many intricacies of insects and their significance on our everyday lives, and moreover, I hope this chapter emphasizes my love for the subject at hand. Having published papers myself, I'm well aware that finding and reading scientific manuscripts can come off as a challenging (yet rewarding) task, so if you wish to immerse yourself in my published work that covers this chapter in greater detail, please check out:

Harrod VL, Groves RL, Guillemette EG, and Barak JD. 2022. *Salmonella enterica* Changes *Macrosteles Quadrilineatus* Feeding Behaviors Resulting in Altered *S. enterica* Distribution on Leaves and Increased Populations. *Nature Scientific Reports* 12: 1–13. https://doi.org/10.1038/s41598-022-11750-3.

From the bottom of my heart, I'd like to dedicate this chapter to my parents (Maciej and Dorota Lason) and my aunt and uncle (Andrzej and Bozena Palczewski) and thank them for instilling a hardcore resiliency that's only found in Polish immigrants. I love you all dearly. Lastly, I'd like to extend my gratitude to the WISL Group for providing this opportunity.

ONE STRAW, TWO GUESTS

Aphids and leafhoppers utilize their stylets – a collection of straw-like mouthparts – to access the plant vasculature for sustenance. But what happens when a food borne pathogen, like *Salmonella enterica*, is present on the plant and joins in on the feast? In this article, we breakdown the hidden interactions between plants, sap-feeding insects, and *Salmonella*, and highlight the hidden implications it holds upon the safety of our food.



During the weekend, we swarm to the local farmers' market to indulge in the arrangement of rainbow produce, and on road trips we spoil ourselves to a personalized assortment of snacks – All in all, food is our greatest equalizer. We as consumers hold immense trust in the producers who grow our food and the corporations that pre-package them. While we might not consider it with every bite, our trust in those that handle our food transcends beyond being provided a tasty meal, but ventures into being provided food that's safe to eat. Despite food quality and preventative measures put into place, food borne outbreaks occur more often than you might think. *Salmonella enterica*, a human enteric bacterial pathogen (HEBP) and the causal agent of salmonellosis, is the primary source of foodborne illness in the United States leading to nearly 1.35 million infections and 26,500 hospitalizations every year (1). Typical symptoms of fever, diarrhea, and stomach cramps can onset as soon as 6 hours or as late as 6 days and persist from 4 to 7 days after infection. Although most associate salmonellosis with the consumption of raw animal products (such as raw cookie dough, or undercooked chicken), nearly 46% of salmonellosis outbreaks concern the consumption of contaminated fresh produce.

Considering fresh produce, there are several routes for *S. enterica* contamination pre- and post- harvest. Supported by a vast body of research, several living and non-living components, either naturally occurring or applied by humans, are known to support and facilitate the dispersal of *S. enterica* populations (2). Contaminated irrigation water, for instance, intensifies both the spread and magnitude of *S. enterica* contaminated produce, prompting bacterial populations to persist for weeks upon the surface of plants. Contaminated irrigation water can also spread bacteria across a multitude of fields (3, 4). Splash dispersal from water droplets (from rainfall or irrigation water) has similarly shown to spread *S. enterica* from its origin (5).

Further, in relation to animal agriculture, manure treated soils are commonly applied to maintain soil fertility, yet these treated soils threaten the safety of produce by enhancing bacterial growth (6, 7). When harvested and stored for only 24 hours, tomato fruits that were tightly packed exhibited a 5-fold increase of *Salmonella* populations, which again doubled after 48 hours. Although poor personal food safety practices may well increase one's chance of foodborne illness, other unseen agricultural practices and environmental conditions may similarly enhance *S. enterica* persistence.

Although salmonellosis may be acquired from the consumption of raw produce, hostile environmental conditions, such as direct UV radiation, desiccation, and a lack of nutrient availability, are a few of the limiting factors prompting bacterial populations to decrease over time on leaves (8). While these populations naturally decline, the high proportion of *S. enterica* outbreaks on produce indicate that these bacteria have evolved to exploit several biological niches to successfully persist. When alone on the surface of healthy leaves, *S. enterica* populations concentrate around glandular trichomes and stomates, two abundant leaf structures that exude valuable nutrients and provide a route to the protective internal structures of the leaf, respectively, resulting in a beneficial niche for bacteria found on leaves (9, 10). *S. enterica* also successfully persists near regions of leaves previously damaged by bacterial plant pathogens, such as *Xanthomonas* species, which expose nutrients and provide direct access to the inside of the leaf (11). As previously mentioned, rainfall not only provides a means of splash dispersal, but also a means of moisture to prevent bacterial desiccation. In conjunction with these biological multipliers (biomultipliers), insects have been identified as additional promoters to *S. enterica* survival.

Previous literature demonstrates that insects can manipulate human enteric bacterial pathogen populations directly, and indirectly. Within poultry-dominated environments, several species of cockroaches may mechanically transmit *Salmonella* by traversing from contaminated egg surfaces to clean substrates, consequently facilitating the movement of bacteria between poultry eggs (12). Within proximity to humans and other animals, houseflies are capable of contaminating water, human food, and even mice via physical contact. Furthermore, *Salmonella* may persist within house flies for the duration of up to 4 weeks (13)! Seaweed flies, intimately associated with decaying and pathogenic seaweed beds, excrete viable bacterial populations within intertidal zones, enhancing the potential transmission of *E. coli* (14). Most recently, and of most interest to myself given their association with food crops, a group of sap-sucking insects belonging to the scientific order Hemiptera (more on Hemipterans in the following paragraph) have been identified as biomultipliers, specifically enhancing *S. enterica* populations. Notably, when exposed to tomato and lettuce plants, Aster leafhopper (*Macrosteles quadrilineatus*) infestations significantly promote *S. enterica* populations and persistence over a 6-day period (Figure 1). While the presence of green peach aphids (*Myzus persicae*), another sap-sucking hemipteran, did not similarly enhance *S. enterica* populations, green peach aphid honeydew (poop from insects which exclusively feed upon plant sap) contained viable *S. enterica* populations. While this tri-trophic relationship (between plants, sap-sucking hemipteran insects, and *S. enterica*) had been identified, the feeding behaviors by which the insect facilitates bacterial populations had not yet been explored, and ultimately laid the foundation for my doctoral thesis. Considering myself a classically trained entomologist and a

lover of all foods (especially seasonal produce), I found this hidden role of insects terrifyingly intriguing.

Comprised of nearly 80,000 species – including leafhoppers, and aphids – hemipteran insects are ubiquitous across a multitude of environments, especially agricultural ecosystems. Apart from their unique leathery wing structures (hemelytra), hemipteran

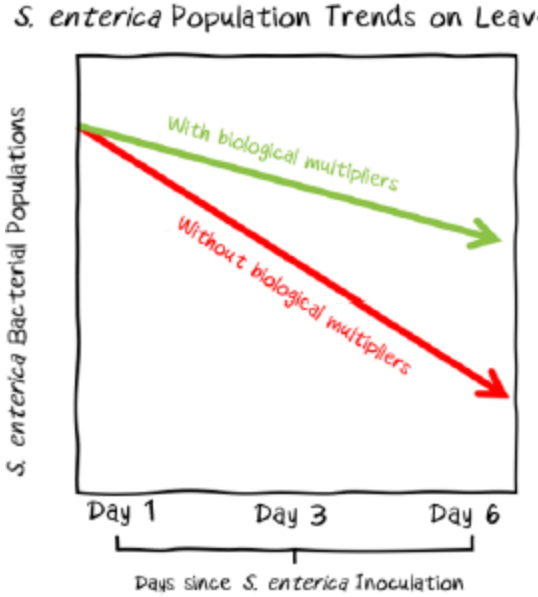


Figure 1. *The presence of biological multipliers (such as plant pathogens or sap-sucking hemipteran insects; green line) enhances S. enterica populations upon tomato and lettuce leaves.*

insects are notorious for their arrangement of multiple piercing and sucking mouthparts. Using this collection of mouthparts, otherwise known as stylets, hemipteran insects easily probe into plants, subsequently accessing all the dense assortment of sugars, water, and diverse organic compounds that the phloem and xylem (plant vasculature that carries sugars from leaves to other parts of the plant, and vasculature which delivers water from the roots throughout the plant, respectively) has to offer; Think of using a straw (stylet) to puncture a juice box (plant)! While hemipterans all have similar mouthpart structures, some species employ unique probing and feeding strategies that elicit distinctive plant responses. For instance, the stylet of an aphid reaches the phloem intercellularly by first puncturing the outermost layer of plants (the plant epidermis) and navigating between plant cells, prompting the increase of salicylic acid production. Production of salicylic acid is a plant's natural response to pathogens or the presence of sap-feeding insects. Conversely, leafhoppers feed intracellularly by probing and cutting through layers of plant cells to reach the phloem and xylem, leading to upregulation of both the salicylic and jasmonic acid pathways (Figure 2). Jasmonic acid is defensively produced when a plant is facing physical damage. Considering the suite of differences between leafhoppers and aphids, we first explored how probing behaviors, and thus the extent of plant damage, uniquely impact *S. enterica* populations.

Despite the documented behaviors of leafhopper and aphid probing (intracellular and intercellular, respectively), the extent of plant damage caused by feeding had not been documented. Much like plant pathogens that

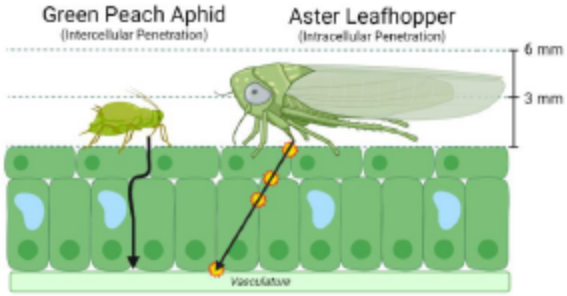


Figure 2. *These two sap-feeding, hemipteran insects (aphids and leafhoppers) utilize unique stylet penetration styles to reach the vasculature.* (Biorender.com)

expose a suite of plant nutrients to *S. enterica* by breaking down cellular walls, we suspected that insect-derived damage could benefit *S. enterica* in a similar manner. Using an electrical conductivity probe – A handheld device that

quantifies ions within a liquid by measuring an electrical current – I was able to determine the extent of plant leakage (damage) resulting from insect infestation. My results indicated that leafhopper-infested tomato leaflets elicited significantly greater magnitude of solute leakage and supported significantly higher populations of *S. enterica* than tomato leaflets infested by aphids. This finding suggests that the aggressive nature of leafhopper probing (or intracellular penetration) transforms the leaf surface into a more habitable environment for *S. enterica* by exposing a suite of plant nutrients that were previously unavailable to the bacteria.

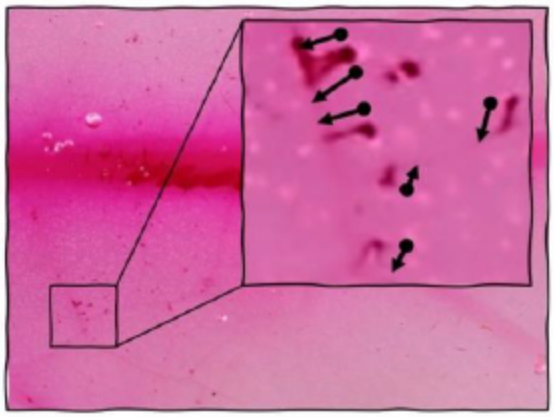


***Figure 3**. Leafhopper salivary sheathes (deep pink) reaching towards tomato veins. The black circles indicate the point of stylet insertion, whereas the arrows indicate the direction the stylets were pushed to reach the vasculature!*

We've now established that probing behaviors by leafhoppers enhance *S. enterica* populations – But does the presence of *S. enterica* impact leafhopper behaviors in any way? Previous studies had identified that fruit flies explore, yet avoid *E. coli* contaminated surfaces, highlighting an insect's potential to recognize surfaces contaminated by bacteria. As you might remember from earlier, this finding also highlights the potential for insects to act as a vehicle for bacteria, as they move from contaminated regions and subsequently seek out 'clean' areas. To our excitement, these aversive behaviors had not yet been identified (or explored) within aphid or leafhopper systems, prompting us to explore this insect and bacterial interaction within a new system (hemipteran insects, *S. enterica*, and food crops). During our primary experiment, we had subjected tomato leaves to a series of *S. enterica* inoculations at unique locations across a leaflet and released a suite of leafhoppers to actively move and feed wherever they chose. Over a 24-hour period, a clear pattern emerged: Although leafhoppers explored the entire region of a contaminated leaf, they preferred to rest upon water-inoculated plant surfaces over regions that were contaminated with *S. enterica*. Despite belonging to a different taxonomic classification than fruit flies, we've established that this avoidance behavior is conserved across a variety of insects.

Although we've identified where leafhoppers prefer to explore, we have not yet identified the frequency or location of their feeding attempts. One other unique attribute of hemipteran, sap-sucking insects, is their ability to produce salivary sheaths. As the name suggests, salivary sheathes are made of spit-out compounds which immediately harden and protect the insect stylet as it reaches towards the vasculature of a plant – Simply put, it's armor for their straw-like mouthparts. Moreover, salivary sheathes occur with every probing attempt, making it a useful and dependable visual of where leafhoppers love to feed (Figure 3). Through a series of bacterial inoculations, insect infestations, and chemically clearing and staining insect-damaged tomato leaves, we were able to highlight the exact distribution of salivary sheathes. We found that while leafhopper salivary sheathes were found across the entire leaflet, leafhoppers prefer to feed upon the middle – So what happens if you

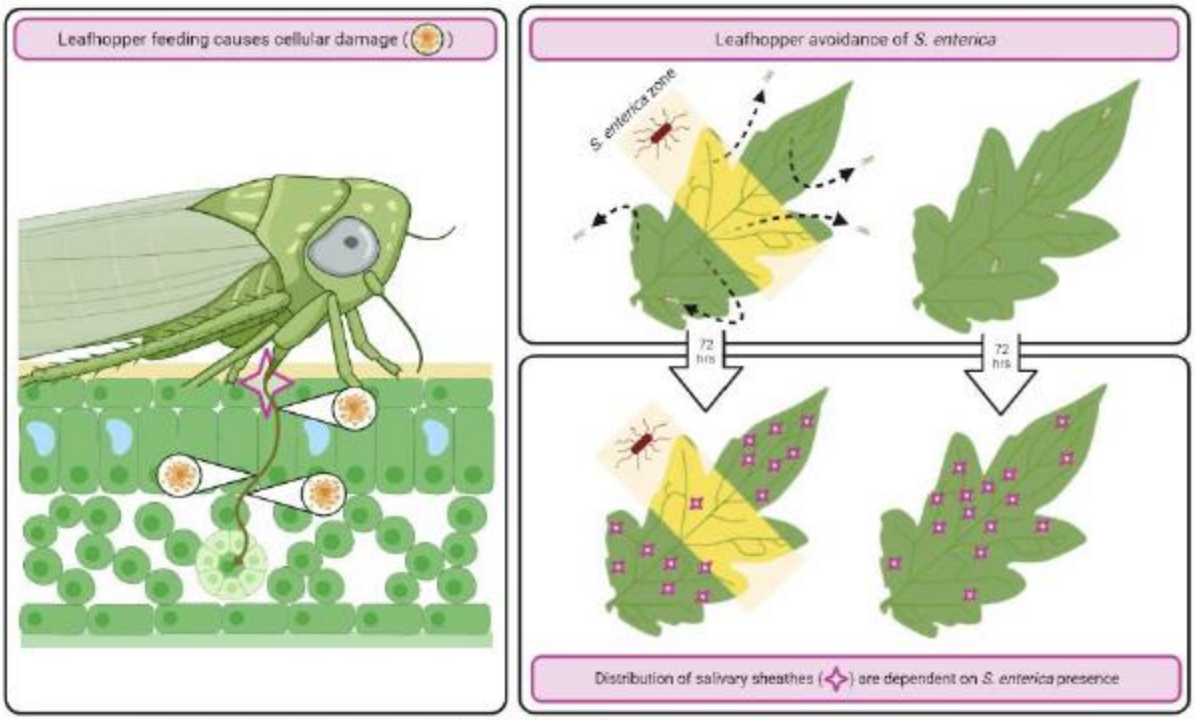


Figure 4. The presence of S. enterica alters the feeding behaviors of Aster leafhoppers. (Biorender.com)

add *S. enterica* to the middle of leaflets? Does the presence of *S. enterica* override their proclivity for their favorite feeding spot? Through a complementary experiment, we found that the presence of *S. enterica* at the middle of leaflets resulted in a new distribution of salivary sheathes. In fact, significantly more salivary sheathes were located at the tips and bases of leaves than at the middle! Moreover, leafhoppers exposed to *S. enterica* contaminated leaves for 72 hours migrated away from the plant and towards the experimental arena in an attempt to escape to non-contaminated host plants. While this avoidance tactic seems counterintuitive towards enhancing *S. enterica* – as more plant damage leads to a more conducive environment for the bacteria – it highlights the potential of leafhoppers as bacterial vehicles for *S. enterica*, and thus leafhoppers' effectiveness as biological multipliers (Figure 4).

Results from this study, along with our recent findings, have led us to a more comprehensive understanding of the entomological mechanisms involving *S. enterica*-associated food borne outbreaks. Characterizing and determining whether sap-sucking insects affect *S. enterica* by eliciting cellular damage or adjusting typical probing behaviors translates to a wider understanding of how ubiquitous sap-sucking insects impact the success of *S. enterica* dissemination and growth within agricultural ecosystems. By broadening our understanding of insect behavior, future actions can be taken to implement integrated pest management programs, thereby diminishing the prevalence of insect-supported food borne outbreaks within agricultural ecosystems.

While my experimental contributions end here, there are numerous lines of research that future scientists can pursue! Emphasizing the importance of insect probing behaviors, utilizing an electronic penetration graph (EPG) would be an excellent next step to further understand hemipteran feeding behaviors. Through a circuit connecting the feeding insect, the subject plant, and a resistor/amplifier, an EPG records electronic wavelengths. By deciphering these wavelengths, scientists can identify when the insect effectively probes into the plant, excretes saliva, and even when it ingests or egests the plant contents. Investigating the microscopic feeding behaviors of insects when exposed to a *S. enterica* inoculated surface would further highlight the behavioral (stylet probing, vasculature access, salivary production, etc.) shift that bacteria hold over insects. On the topic of stylet probing, it would also be exciting to identify the specific plant nutrients that emerge from insect-damaged leaves that directly benefit *S. enterica*. In conjunction with this experiment, one could use a mutant strain of *S. enterica* tagged with Green Fluorescent Protein (GFP). Turning bright green under light within the blue to ultraviolet range, the use of *S. enterica* with GFP would enable us to visualize the exact location of bacteria across a leaflet after insect infestation. While my graduate study described here primarily concerns lettuce and tomato host plants, the impact of insects that employ different feeding styles (ripping-sucking, chewing, etc.) should be explored on other plant systems to expand our understanding of the role insects play in foodborne outbreaks.

So, what's next for me? Having grown attached to the world of food safety and holding onto my longstanding fascination with insects, I've accepted a position as a Food Safety Entomology Consultant with HACCP Assurance Services. This Pennsylvania based company hires scientists with unique doctoral backgrounds (mine, of course, being insect-plant-*S. enterica* interactions) to provide first-hand food safety guidance to small and medium-sized companies or farmers selling food products. As the lead entomologist on the team, I'll specifically be working to form a new pest control division, focusing on preserving the safety of our food by controlling insect populations. I'm grateful to begin working with farmers and producers, and even more excited to continue my work within the field of entomology.

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Conclusions/Next Directions

Within this thesis, I characterized entomological mechanisms which influence *S. enterica* population dynamics upon plant hosts. Investigations into this interaction have expanded our understanding of the role of insects as biological multipliers of *S. enterica* within the context of agricultural ecosystems. While I've uncovered a suite of findings, I'd like to summarize some major points:

I. **Exhibiting avoidance of contaminated leaf surfaces, the distribution of Aster leafhopper probing attempts shifted in the presence of epiphytic *S. enterica*.**

Insect behavioral studies, specifically involving attachment to or avoidance of human enteric bacterial pathogens, have been limited. Here, I demonstrated that insects besides *Drosophila* exhibit similar avoidance tactics of areas contaminated by *S. enterica*. Migration away from *S. enterica* inoculated plants and avoidance of feeding upon contaminated leaf regions had highlighted that while the presence of leafhoppers impacts *S. enterica* population dynamics, the presence of *S. enterica*, in turn, impacts leafhopper behaviors. To further understand how *S. enterica* affects insect feeding patterns, behavioral waveforms associated with probing, salivation, and ingestion should be assessed using Electrical Penetrative Graphing (EPG) techniques. Apart from determining whether an insect successfully reaches the vascular structure, utilizing an EPG will additionally provide a temporal set of data to consider, including the time from the first attempt of probing to subsequent phloem ingestion.

II. **Insect honeydew benefits in-vitro populations of *S. enterica*, and honeydew derived from previously infested plants contains higher concentrations of salicylic acid than honeydew from fresh plants.**

Viable populations of *S. enterica* had previously been recovered from Aster leafhopper and green peach aphid honeydew; however, the influence of honeydew upon *S. enterica* populations had not been established. Honeydew was collected from a variety of plant host

species facing two levels of damage (fresh plant or previously infested plant) by *M. persicae* (feeding on celery, or turnip) or *M. quadrilineatus* (feeding on celery, or oat). All forms of honeydew had significantly enhanced in-vitro *S. enterica* populations over a 24-hour period. This established that aphid or leafhopper honeydew may act as an effective nutritional reservoir for *S. enterica*. Moreover, honeydew derived from plants facing higher rates of damage contained higher concentrations of SA, likely reflecting the elevated phytohormonal defenses employed by the host plant in response to elevated rates of insect damage. Investigating the concentration and presence of other sugars, amino acids, and remnant phytohormones using mass spectrometry technology would expand our understanding of how insect, plant host, or plant damage influences the composition of honeydew. Furthermore, it would highlight available components of honeydew for *S. enterica* to metabolize.

III. **Insect honeydew enhances *S. enterica* populations on tomato leaflets yet does not significantly impact *S. enterica* upon celery leaflets.**

Despite providing a nutritional reservoir for *S. enterica*, the effectiveness of honeydew as a biological multiplier for epiphytic bacterial populations is host plant dependent. Used frequently within traditional medicine, our findings further suggest anti-microbial properties within celery leaves that could act as an effective deterrent for *S. enterica* growth. Future studies would be informative in identifying the anti-microbial mechanisms within celery leaves that prevent *S. enterica* growth, even in the presence of a nutritional reservoir like honeydew.