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

By

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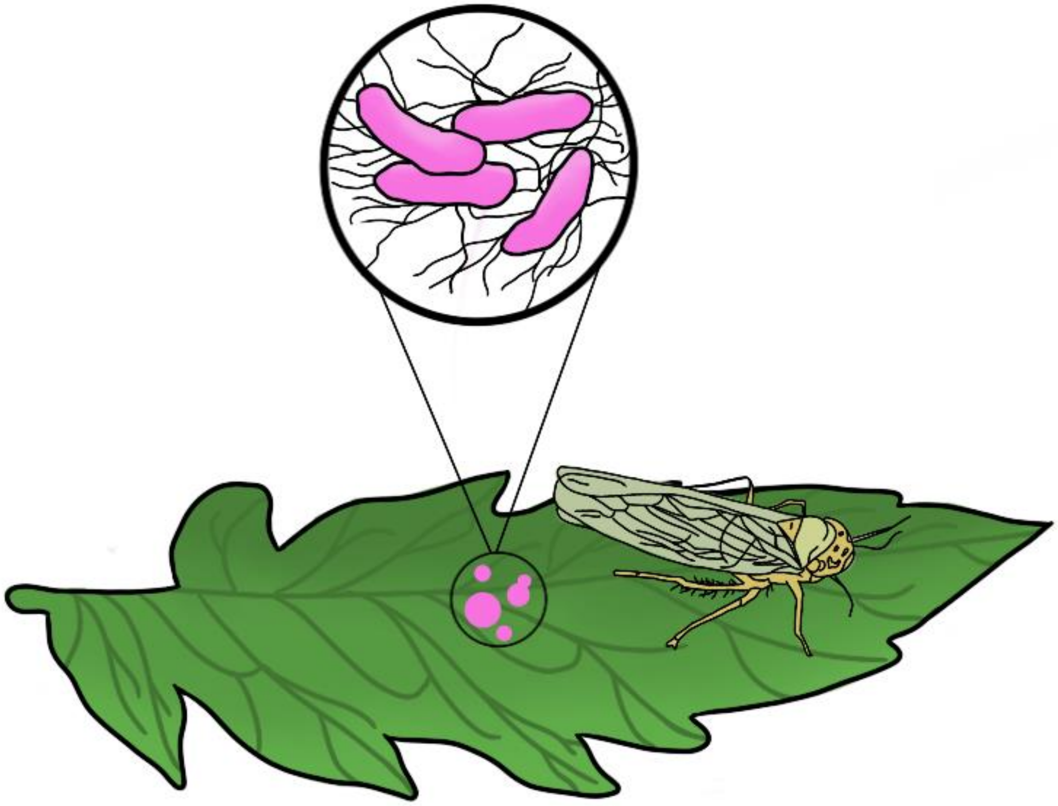
The dissertation is approved by the following members of the Final Oral Committee:
Russell L. Groves, Entomology
Jeri D. Barak, Plant Pathology
Lyric C. Bartholomay, Pathobiological Sciences
Shawn Steffan, Entomology

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 written 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:

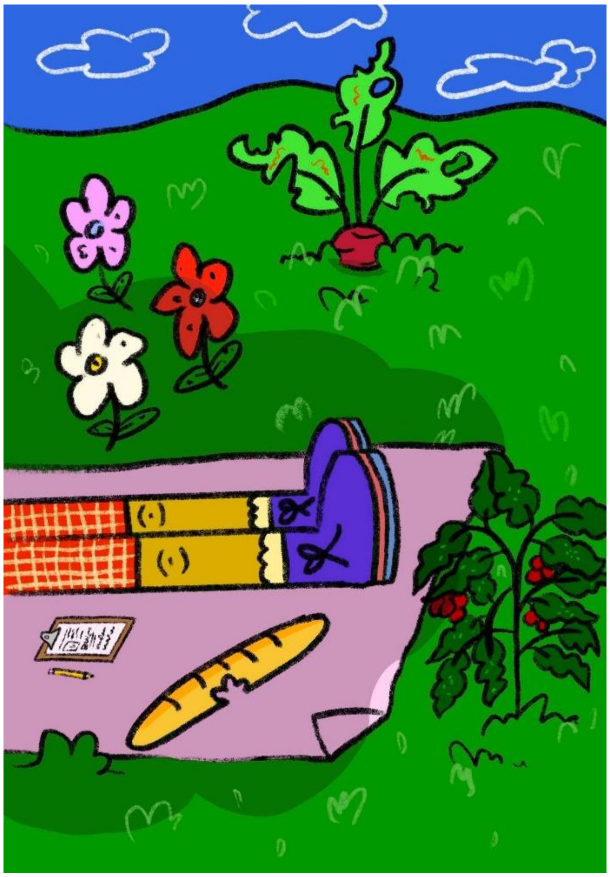
Harrod VL, Groves RL, Guillemette EG, and Barak JD. 2022. *Salmonella enterica* Changes *Macrosteles Quadrilineatus* Feeding Behaviors Resulting in Altered *S. enterica* Distribution on Leaves and Increased Populations. *Nature Scientific Reports* 12: 1–13. https://doi.org/10.1038/s41598-022-11750-3.

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.

Vicky Lason | WISL

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.

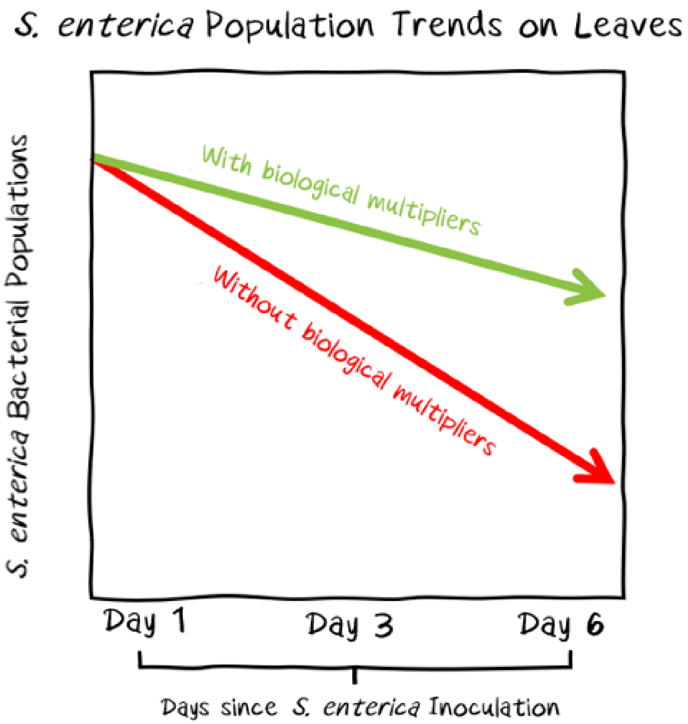


***Figure 1**. The presence of biological multipliers (such as plant pathogens or sap-sucking hemipteran insects; green line) enhances S. enterica populations upon tomato and lettuce leaves.*

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 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 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

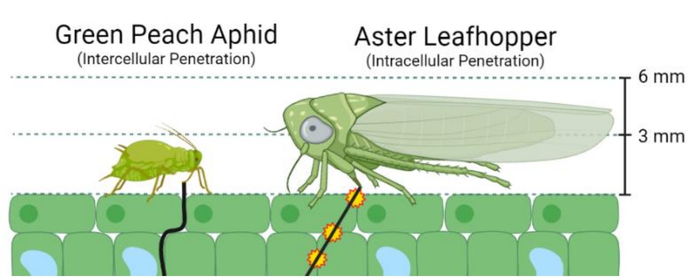


***Figure 2.** These two sap-feeding, hemipteran insects (aphids and leafhoppers) utilize unique stylet penetration styles to reach the vasculature.*

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.

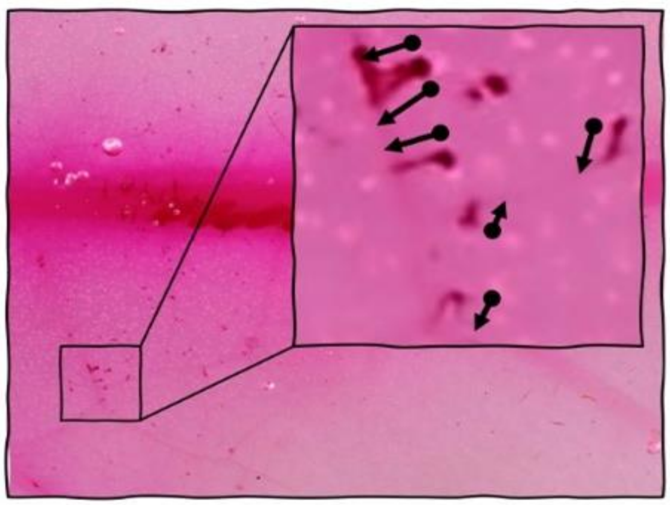


Figure 3. *Leafhopper salivary sheathes (deep pink) reaching towards tomato veins. The black circles indicate the point of stylet insertion, whereas the arrows indicate the direction the stylets were pushed to reach the vasculature!*

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

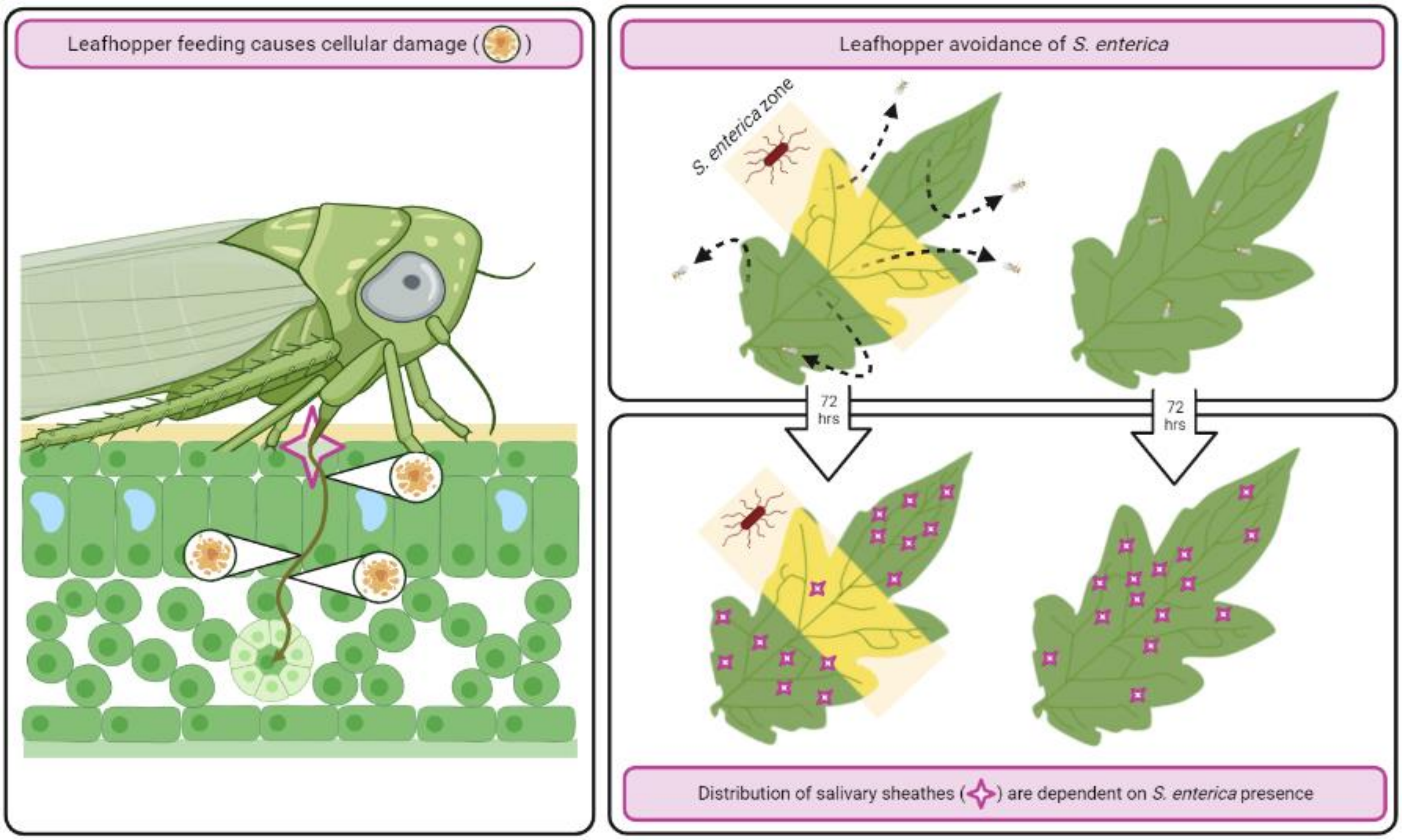


Figure 4. *The presence of S. enterica alters the feeding behaviors of Aster leafhoppers.*

across the entire leaflet, leafhoppers prefer to feed upon the middle – So what happens if you 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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