EFFECTS OF STRESS, GENETICS, AND ENVIRONMENT ON MICROBIOME COMPOSITION IN WESTERN CORN ROOTWORM (*DIABROTICA VIRGIFERA VIRGIFERA* LECONTE)

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

EFFECTS OF STRESS, GENETICS, AND ENVIRONMENT ON MICROBIOME COMPOSITION IN WESTERN CORN ROOTWORM (*DIABROTICA VIRGIFERA VIRGIFERA* LECONTE)

Presented by Kyle J. Paddock, a candidate for the degree of Doctor of Philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

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Abstract

Increased study of microbiomes illuminated the diversity of roles microorganism play across ecosystems. For agricultural systems, microbiomes represent a new frontier to potentially improve plant, animal, and human health, while increasing agricultural sustainability. Insect pests represent a major threat to health and agriculture. Current management tactics aimed at addressing pest outbreaks may be improved through exploitation and adaptation of microbiomes.

Corn growers in the United States encounter several insect pests throughout the growing season. None may be as damaging as the subterranean larvae of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte. Transgenic corn producing insecticidal proteins derived from *Bacillus thuringiensis* Berliner (Bt) effectively controls WCR, but continuous use of Bt corn leads to the evolution of resistance and failure of the management tactic. Proper resistance management is necessary to prolong the efficacy of Bt-corn as new management tools are slow to reach the market. Insect microbiomes have been shown to enhance host nutrition, disrupt plant defenses, and protect against diseases, but are also capable of inducing mortality in certain situations. Thus, understanding WCR microbial communities may improve management of this serious pest.

The first objective of this investigation was to characterize WCR beetle-associated bacterial communities and describe their patterns of assembly across spatial scales. Communities were characterized from WCR adults collected across the United States using 16S rRNA sequencing. Results suggest dispersal dynamics shape bacterial communities at small spatial scales (~25 km apart) while host genetics or environment drive bacterial community composition at broad scales (>50 km apart). Findings provide

important information for understanding how determinants of insect microbiomes are influenced by deterministic and stochastic forces.

The second objective was to investigate the potential impact of Bt intoxication and resistance on the WCR larval microbiome. Comparisons of microbial communities of Bt-resistant and -susceptible WCR were made after feeding on Bt and non-Bt corn. Bt-resistant larvae harbor a less rich microbiome that is unresponsive to Bt ingestion. Selection for resistance to Bt produced heritable changes in the microbiome, potentially providing WCR an adaptive trait to mitigate stress.

In an effort to synthesize the previous findings of this work into a management application, the third objective asked how do sustainable management practices that boost soil microbial diversity affect WCR fitness. Soil microbes from fields planted with cover crops and no-till were applied to Bt and non-Bt corn seedlings and fed to Bt-resistant and -susceptible WCR larvae. Susceptible larvae were controlled by Bt regardless of soil microbiome treatment. Cover crop no-till soil microbes reduced Bt-resistant WCR larval dry weight. Transgenic crops can likely be integrated with the sustainable cropping practice of cover cropping and no-till to improve belowground pest management.

Food security in the future will rely on successful integration of sustainable management practices in agricultural systems to balance productivity and broader ecosystem functioning. Microbiomes represent a link between plant, animal, and human life that could help accomplish this goal. Documenting the ecological rules microbiomes follow serve as a foundation for application of these powerful systems.

Chapter 1: Introductory Literature

1.1 Introduction

Agriculturally active societies began ~10,000 years ago in the basin of the lower portions of the Tigris and Euphrates Rivers. Early growers likely had minimal pest management strategies and instead relied on growing crops in enough abundance to offset any losses. But by 2500 BC, Sumerian farmers were deploying sulfur containing compounds to control insect pests. Pest management in 300 AD China relied on ecosystem level control, through the cultivation of predatory ants and an understanding of the indirect negative impact birds play on aphids by reducing predatory lady beetles (Flint & van den Bosch, 1981). Modern agriculture, specifically in the United States, has followed a different pattern that ushered in one of the greatest agricultural productivity machines in the world but is not without shortcomings. Heavy use of chemical insecticides and disturbance laden practices are not sustainable (Bakker et al., 2020; DeBach, 1951; Weddle et al., 2009). Because agriculture has created societies that depend on intense food production that are unable to revert to hunter-gatherer practices, modern agriculture will require careful management to feed the estimated 9 billion people on Earth by 2050.

Adoption of broader ecosystem management can improve the sustainability of agricultural systems (Bommarco et al., 2013). These practices focus on promoting ecosystem services while minimizing environmental impacts incurred from traditional agricultural practices (i.e., synthetic fertilizers, pesticides, high disturbance tillage). Microbiomes provide ecosystem services across domains of life and thus have the potential to improve agriculture production sustainability. To properly leverage

microbiomes for human benefit, system specific research is required given the number of factors that influence microbiome composition and functioning. A silver bullet for pest management does not exist. Understanding how microbiomes interact with agricultural systems can provide another tool for growers in their food production system.

This book will address several questions related to insect microbiomes with the goal of improving sustainable pest management. In the United States, field corn (*Zea mays*) production covers over 90 million acres annually, representing 30% of the total percentage of acres of crops (USDA, NASS 2017). The most significant pest of corn is the western corn rootworm, (WCR; *Diabrotica virgifera virgifera* LeConte), a root feeding beetle with a propensity to circumvent management practices. What role does the WCR microbiome have in its success as a pest? Can the WCR microbiome be leveraged to improve management? Addressing these questions requires investigation into what factors influence WCR microbiomes, both in larvae and adults, before addressing its potential to be leveraged to improve management.

1.2 Key Players

1.2A Diabrotica

Beetles comprise nearly half of all herbivorous insect species in the world. The largest and most diverse clade among beetles is Phytophaga (McKenna et al., 2019), which contain the superfamilies Chrysomeloidea and Curculionoidea. Most beetles in Phytophaga feed on angiosperms (flowering plants), making this group a research priority given their propensity to impact human food systems. In the Americas, the native genus *Diabrotica* consists of over 350 described species of which several are damaging pests of

field crops. Two groups comprise most of the *Diabrotica* species, fucata and virgifera (Smith & Lawrence, 1967) and account for a large portion of the literature.

The divergence of these two groups coincides with a restriction of host plant usage. The common ancestor of the *Diabroticina* subtribe was likely a specialist of a single plant family, possible Cucurbitaceae. Broadening of host diet breadth may have led to the evolutionary radiation of the *Diabrotica* genus in the tropics (Eben & Espinosa, 2013). Species in fucata are typically polyphagous, feeding on plant species in Fabaceae, Cucurbitaceae, and Poaceae from tropic and sub-tropic regions. Virgifera spp. are oligo- or mono-phagous, often specializing on field crop species in Poaceae. Outside of several damaging pest species, little is known about host plant usage within *Diabroticina*.

The split of virgifera and fucata occurred roughly 30 million years ago (Eben & Espinosa, 2013), an event that coincided with an expansion in overall genome size (Lata et al., 2022). In general, fucata species are multivoltine, which is thought to be the ancestral trait of *Diabrotica*. The exploitation of host plants in Poaceae that adhere to seasonal patterns of availability may have driven life cycle changes leading to egg diapause and univoltinism in virgifera (Branson & Krysan, 1981). Diapause refers the arresting of development at a life stage in response to environmental or hormonal cues. After several generations in a year, species in fucata overwinter as adults in temperate regions. In the North American virgifera species, western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and northern corn rootworm (*Diabrotica barberi*) overwintering as eggs in diapause allows the synchronization of egg development and monocotyledon seed germination based on soil temperature. The diapause of western corn rootworm (WCR) contains both an obligate and facultative phase (reviewed in

Meinke et al. 2009). Oviposited eggs develop for 11-13 days before entering a time-sensitive diapause where the newly developed embryo will remain arrested for a period of 78 to 163 days. The facultative phase is triggered by temperature-sensitive signaling. Soil temperatures of less than 11ºC will keep eggs at the pre-diapause development stage. Once temperatures rise above 11ºC development recommences and ends in hatching. Post-diapause development may also be arrested under dry conditions, as eggs require water to complete development. The time to egg hatching is variable based on temperature, moisture, latitude of population origin, and soil characteristics. Egg diapause thus may have been a useful adaptation to cyclical weather patterns that mimic Poaceae lifecycles and allow virgifera to survive harsh winters in North America and beyond their tropical origins. North America now harbors several extant *Diabrotica* species that form the damaging corn rootworm complex: *D. virgifera virgifera, D. barberi, D. v. zeae,* and *D. undecimpunctata howardi*.

In the United States most of the economic damage inflicted by corn rootworms is to field corn (*Zea mays*), with western corn rootworm (*D. virgifera virgifera*; WCR) and northern corn rootworm (*D. barberi*; NCR) being the most severe pests. Prior to their discovery in the United States in the late 1800's, WCR and NCR were not corn pests. WCR was described in 1867 by John LeConte from adults collected on wild gourd (*Cucurbita foetidissima*) in far western Kansas (LeConte, 1868). However, economic damage on corn by western corn rootworm wasn't reported until 1909 and only in Colorado (Gillette, 1912). A hypothesis is that WCR followed the human-driven migration of corn northward from Mexico because of its high affinity for corn and documentation as an optimal host (Branson & Krysan, 1981; Branson & Ortman, 1967b,

1967a, 1970; Krysan & Smith, 1987). Yet, maps of the historical distribution of corn in the United States at that time show little to no corn in western Kansas (Weatherwax, 1954). Although WCR may have been using corn as a host in some of its range, it is more likely that WCR were using other prairie grass species as a host in the 1800s. Western wheatgrass was common there at the time and is a relatively good host for WCR that may be able to sustain populations (Oyediran et al., 2004). However, there is no evidence of WCR using hosts other than maize in this century.

Lombaert et al. (2018) analyzed the genetic structure of 21 WCR populations to determine the path and timeline of WCR migration. Allelic richness across 13 loci increased with increasing latitude and matched the hierarchical clustering of populations. The authors then built a model to simulate different scenarios of geographic origin and migration from the clustering results. The model estimates parameters for the most likely scenario based on genetic summary statistics from the input populations and determines the probability of recapitulating the original data set based on the variables of interests. Results from the models indicate WCR underwent a severe bottleneck in Mexico around 1100 years ago, which might represent a host shift or a dispersal event from the native population. WCR didn't reach the southwestern US until ~500 years ago, much later than when corn was originally planted in the area (Merrill et al., 2009). It is unclear what drove WCR northward from Mexico (or a more southern region) to western Kansas and Colorado, but it was likely not co-adaptation with corn. Instead, authors conclude WCR behaved more as an invasive pest. WCR exploitation of corn led to rapid population growth eastward from Colorado from the 1950's onward resulting in a lack of genetic structure across WCR populations (Kim & Sappington, 2005). NCR population genetics

have received less attention, but it is assumed they originated at a more northern latitude in the US and were relatively widespread. As corn spread north into the NCR native range, NCR may have begun to exploit corn as a host plant.

WCR lifecycles consist of a subterranean larval stage and an above-ground beetle stage. Larval hatch from eggs laid in soil is temperature dependent and commonly occurs between late May and early June in the US. Larvae begin searching for corn roots immediately by following CO_2 gradients released from roots (Arce et al., 2021; Strnad & Bergman, 1987). Since all plants release CO_2 from their roots, as larvae approach a potential host (8-10 cm away), they use a blend of corn specific volatiles to distinguish between host plants (Robert et al., 2012). Once corn roots are located, larval feeding begins in response to a common galactolipid, monogalactosyldiacylglycerol (MGDG; Bernklau et al., 2015). Larvae burrow below the epidermis and feed in the cortex of seminal and nodal roots (Strnad & Bergman, 1987). As the larvae develop, they move to newly emerging nodal roots as they emerge from the stalk. The pruned effect evident in the nodal root damage score is not due to eating a whole root but rather eating roots as they emerge from the stalk (Oleson et al., 2005). These newly developed roots may be required for development since older plants without fresh, nodal roots do not produce WCR adults (Hibbard et al., 2008). Larval development progresses through three instars over four to six weeks in which they continuously feed on corn roots. When ready to pupate, larvae crawl a short distance away from corn plants and form an earthen cell roughly 0-2 inches below the surface (Sechriest, 1968). Duration of larval development is variable and dependent on food quality and cumulative degree days (Hibbard et al., 2008). Hatch to adult emergence varies between ~20 to 45 days across different

temperatures with males tending to hatch (Ludwick et al., 2017b), develop, and emerge more quickly (Jackson & Elliott, 1988).

Peak emergence for WCR beetles typically occurs mid to late July in the US and can occur over 33.4 and 51.3 days for males and females, respectively. The early emergence of males allows for sexual development before sexually mature females emerge (Guss, 1976; Hammack, 1995). NCR emergence is usually later than WCR. Degree day accumulations required for NCR peak emergence totals 169 post-biofix whereas WCR require 118 degree days (Nowatzki et al., 2002). Adult WCR commonly feed on pollen, silk, young kernels, and to a lesser extent, corn leaves (Chiang, 1973). Economic damage rarely occurs but can be significant in cases where adults clip silk during pollination, limiting the development of kernels (Culy et al., 1992). They are strongly attracted to corn silk, which can influence their movement within and across fields and can result in non-corn host plant use when silk resources are low (Campbell & Meinke, 2006; Prystupa et al., 1988). However, NCR adults have weaker fidelity to corn as a diet than WCR (Ludwig & Hill, 1975). They have been found feeding on surrounding prairie forbs, squash blossoms, and various weeds in nearby fields (Hill & Mayo, 1980). This potentially is an artifact of their more recent encounter with corn given their more northern origin.

Mating typically occurs immediately following emergence of females, and once mated, females disperse within their original field and/or out to surrounding corn fields. Inter-field dispersal patterns are largely food driven where beetles respond to changes in food resources, such as maturing corn within fields and/or presence of developing corn and pollen-rich grasses in other fields (Moeser & Vidal, 2005). Short inter-field dispersal

events do not fully explain the rapid eastward expansion experience between 1945-1980, as average daily movement may only be 17 m for a total less than 1 km for their entire lifetime (Coats et al., 1986; Sappington and Kim, unpublished). The invasion front formed by inter-field dispersal events should progress at a continuous rate. Instead, rate of expansion varied over time and space. Adult WCR display a capacity for long distance dispersal that could deposit founder populations at a greater distance away from their natal field than the invasion front (Chiang, 1973; Ruppel, 1975). Medium to medium-high larval density during development can spur longer and further flight distance and may partly explain the non-uniform invasion front rate expansion (Yu et al., 2019). Long distance dispersal is likely exaggerated during storms too (Onstad et al., 2001). Dispersal allows for WCR invasion of new areas, but the greatest accessory to WCR spread was the massive increase in continuous corn planting across the Corn Belt in the 1950's onward as continuous corn is necessary for significant buildup of WCR populations (Metcalf, 1986).

Historically, WCR and NCR have been successfully managed by crop rotation with a non-host species such as soybean (Gillette, 1912). To this day, crop-rotation remains the most effective management option for most of the US and in Europe. The 1950's ushered in a new wave of management tactics consisting of synthetic insecticides. Benzene hexachloride was the first chemical to be documented as a rootworm larval insecticide both as a preplant broadcast and post-plant side dress (Hill et al., 1948). However, due to their persistence in the environment and toxicity to animals and humans, organochlorines were banned from use. Cyclodienes were proven to be effective shortly after the efficacy of organochlorines were documented. Aldrin, chlorodane, and

heptachol were all used from the 1950's onwards (Meinke et al., 2021). High market penetration of cyclodienes led to decreases in effectiveness of rootworm control by 1959 and worsened over the next two years (Ball & Weekman, 1962). The report by Ball and Weekman (1962) documented the first known case of field evolved resistance to insecticides (aldrin) by WCR. The spread of aldrin resistance spurred efforts to develop new insecticides with new modes of action, and as cyclodienes were removed from use, organophosphates and carbamates entered US fields (Mayo, 1986). Granular formulations of these chemicals helped lower the application rates and made treatments more targeted but often resulted in variable control outcomes due to interactions with the soil ecosystem or damage outside the banded application zone (Felsot, 1989; Gray et al., 1992). Prophylactic use of organophosphates and carbamate at planting time grew in use over the next several decades (Levine & Oloumi-Sadeghi, 1991), yet there are no documented cases of field evolved resistance to granular, in-row insecticides, likely due to their variable effect on adult emergence from untreated zones of corn roots. Liquid formulations exist but have been restricted from use in the US since the mid 1990s. The most recent two classes of insecticides registered for use against WCR and NCR are pyrethroids and neonicotinoids. As is the case with other previously deployed insecticides, WCR developed resistance to the pyrethroids (Pereira et al., 2015, 2017). Neonicotinoid seed treatments are widely used but are rather ineffective against high densities of WCR (Douglas & Tooker, 2015; Van Rozen & Ester, 2010). Neonicotinoids have substantial off-target effects (Wood & Goulson, 2017), which has led to their banishment from use in the European Union.

The life history of corn rootworms makes the subterranean larval stage difficult to manage, but by decreasing the adult egg-laying population in the field, a grower can limit the number of larvae present in the field the following year (Pruess et al., 1974). In addition, if densities are high, foliar application of insecticides can help limit the economic damage caused by silk clipping during pollination. Formulations of cyclodienes, organophosphates, and carbamates have all been used as foliar insecticides (Hill et al., 1948; Mayo, 1976; Pruess et al., 1974). Later, carbaryl and methyl parathion emerged as useful tools to control adult populations (Meinke et al., 1998). While both worked well for several decades, resistance began appearing in south-central Nebraska after 1990 and began spreading (Meinke et al., 1998). Growers now rarely rely solely on adult foliar insecticide as a management tactic, in part because of the wide success of transgenic crops producing insecticidal proteins derived from *Bacillus thuringiensis*.

Bacillus thuringiensis is primarily a soil-borne bacterium that displays toxicity to a wide range of hosts (Palma et al., 2014). During sporulation, Bt produces parasporal crystalline inclusions composed of pore-forming proteins, also known as Cry/Cyt proteins (δ-endotoxins) (Hofte & Whiteley, 1989). These proteins target and disrupt the midgut of insects once they are ingested. Early uses of Bt as a pest control tactic included sprays of bacterial spores (Roh et al., 2007). However, Schnepf and Whiteley (1981) discovered the pesticidal proteins could be cloned and expressed in bacterial plasmids, which opened the door for the discovery of a litany of new uses for Cry proteins. In 1987, a Bt toxin was expressed in tobacco, and a new generation of insect pest management began (Vaeck et al., 1987). For the past twenty years, transgenic crops expressing Bt proteins has been used to control various insect pests as its high degree of host specificity

makes it suitable for large scale integration (Estruch et al., 1996). The implementation of transgenic crops into agricultural systems has successfully reduced pest populations while simultaneously decreasing broad-spectrum, chemical insecticide use (Cattaneo et al., 2006; Lu et al., 2012). In turn, Bt fields experience higher natural enemy abundance and increased yields with lower off-target effects (Cattaneo et al., 2006; Wolfenbarger et al., 2008). In the US alone, over 100 million acres of Bt crops are planted annually (USDA, NASS 2019).

In 2003, the EPA approved the first transgenic maize line expressing a toxic Bt protein specifically targeting WCR, Cry3Bb1 (EPA, 2003). Soon after, two more traits were deregulated, Gpp34Ab1/Tpp35Ab1 (formerly named Cry34/35Ab1) in 2005 and mCry3A in 2006. Syngenta introduced a new transgenic maize line expressing eCry3.1Ab stacked with mCry3A in 2013 under the trade name Agrisure® Duracade (Syngenta, 2013). There are now four Cry toxins commercially available in transgenic corn lines today, either singly or in a pyramid. However, management has been complicated by the ability of WCR to evolve resistance to Bt.

In 2009, two field populations of WCR in Iowa were found to be resistant to Cry3Bb1 (Gassmann et al., 2011). These findings set off a decade of research to understand corn rootworm resistance to Bt. Instances of field evolved resistance to Cry3Bb1 increased in the years after 2009 (Calles-Torrez et al., 2019; Gassmann et al., 2014, 2020; Jakka et al., 2016; Wangila et al., 2015; Zukoff et al., 2016). In laboratory studies, the evolution of resistance progressed rapidly, with increased survival on Bt corn evident after three generations of selection on Cry3Bb1 (Meihls et al., 2008). Resistance extended to other proteins in subsequent years to where all commercially available toxins

have seen failures (Gassmann et al., 2014; Ludwick et al., 2017a). More troublesomely, field evolved resistance to Cry3Bb1 displayed cross-resistance to mCry3a and eCry3.1Ab (Zukoff et al., 2016). Like Cry3Bb1, laboratory selected resistance occurred quickly for eCry3.1Ab and Gpp34Ab1/Tpp35Ab1 (Frank et al., 2013; Lefko et al., 2008). Pyramid corn producing Gpp34Ab1/Tpp35Ab1 is recommended for populations resistant to other Cry3 toxins because of the lack of cross resistance between the two. Cry3 proteins consist of a three-domain structure with two possible sized protoxins, 65-kd and 130-kd (Pardo-Lopez et al., 2013), whereas Gpp34Ab1/Tpp35Ab1 is a two-domain protein belonging to the Toxin 10 family (Palma et al., 2014). As growers relied heavily on Gpp34Ab1/Tpp35Ab1, resistance began to appear more frequently (Calles-Torrez et al., 2019; Gassmann et al., 2020).

As adoption of Bt corn grew and resistance appeared, refuge mandates were put in place requiring non-Bt seed be planted within Bt fields to help stop the spread of resistance (Glaser & Matten, 2003). Refuge areas allow susceptible insects to survive to adulthood and thus allow susceptible alleles to persist in the population. In cases of recessive resistance, susceptible insects mating with resistant insects create heterozygotic offspring that will be killed by Bt plants and remove resistant alleles from the population (Carrière et al., 2010). Criteria for creating an effective refuge to delay resistance evolution include low levels of resistance alleles within the population, using a high dose transgenic plant, and the presence of a fitness cost associated with resistance. Estimates of initial resistance allele frequency from laboratory selection experiments were between 0.05 and 0.2 (Onstad et al., 2001), higher than found in other pests (Tabashnik et al., 2008). High dose requirements are defined as “25 times the toxin concentration needed to

kill susceptible larvae" (EPA [Environmental Protection Agency], 1998). Unfortunately, there are no high dose Bt-corn lines on the market for WCR. A fitness cost to resistance means a measurable fitness parameter in a resistant individual is lower when reared on non-Bt plants compared to susceptible insects on non-Bt plants (Gassmann et al., 2009). Without fitness costs, evolution of resistance can be more rapid in the presence of refuges (Caprio, 2001). For resistant populations of western corn rootworm, fitness costs are minimal or non-existent (French et al., 2015; Geisert & Hibbard, 2016; Meihls et al., 2008; Oswald et al., 2012; Paddock et al., 2021a).

Field-evolved and laboratory-selected resistance of WCR populations to Cry3 proteins (Cry3Bb1, mCry3A, and eCry3.1Ab) have driven efforts to better elucidate the underlying genetics, genomics, and biochemical mechanisms that enable resistance (Gassmann et al., 2014, 2016, 2020; Heckel, 2020; Ludwick et al., 2017a; Zukoff et al., 2016). The precise mode of action underlying Bt toxicity of Cry3 proteins is unclear, but the sequential binding and pore formation model of Bt proteins is assumed for WCR toxicity (Heckel, 2020). In this model, proteins on the brush border membrane of the CRW midgut serve as ligands, which allow binding of Cry3 toxins leading to the assembly of toxin monomers into oligomers that span the plasma membrane. Extensive leakage of cations into the cell through pores in the membrane ultimately results in midgut cell lysis. If not repaired, the resulting cell damage breaches the midgut, leading to fatal septicemia (Broderick et al., 2006; Caccia et al., 2016).

Molecular research has shed light on the complexity of disease progression, but questions remain unresolved in how insects overcome Bt toxicity (Liu et al., 2021). Resistance mechanisms in lepidopterans have largely focused on structural changes in the

Bt receptor protein, cadherin, leading to decreased binding (Gahan et al., 2010; Jin et al., 2018; Zhang et al., 2012), and the downregulation of processing enzymes alkaline phosphatase (Jurat-Fuentes et al., 2011) and aminopeptidase N (Coates et al., 2013). Midgut brush border membrane vesicles from resistant WCR larvae exhibited reduced mCry3A binding (Zhao et al., 2016) suggesting a down regulation in receptor expression. In other words, limiting damage to midgut tissues confers resistance. However, midgut tissue regeneration or repair presents a route to resistance (Castagnola & Jurat-Fuentes, 2016; Forcada et al., 1999; Martinez-Ramirez et al., 2010). Pathways to Bt resistance may involve heightened immune responses in insects. Paddock et al. (2021b) demonstrated that a rapid shift in the microbiome of WCR larvae follows eCry3.1Ab feeding. A disrupted midgut leads to disrupted microbiome. Moreover, multiple generations of selection on eCry3.1Ab leading to resistance also altered the microbiome in comparison to an isoline selected colonies (Paddock, et al., 2021b). Stress tolerance and apoptotic cell pathway transcripts significantly upregulated between resistant and susceptible insects fed Cry3Bb1 represent a variable path to resistance to be investigated further (Coates et al., 2021; Rault et al., 2018). Research into mechanisms paints a multifaceted picture of Bt resistance.

New management approaches targeting WCR are appearing (Paddock et al., 2021c). While Bt is still effective, managing resistance is important. Pyramids can help slow the spread of resistance by presenting insects with two modes of action that cause mortality. A new pyramided transgenic corn line has been developed to express a double stranded RNA (dsRNA) targeting DvSnf7 (Diabrotica virgifera {DV} + sucrose-non-fermenting {SNF} locus) along with Cry3Bb1 and Gpp34Ab1/Tpp35Ab1 (Head et al.,

2017). Resistance management guidelines may need to be revisited, especially since a laboratory population was selected for resistance to not only DvSnf7, but to all dsRNAs tested (Khajuria et al., 2018). Two other proteins originating from *Brevibacillus laterosporus* and *Pseudomonas chlororaphis* are being pyramided with existing Bt proteins to increase durability and effectiveness (Bowen et al., 2020; Yin et al., 2020). These traits have yet to be deregulated for use by the EPA. Outside of new transgenics, management of corn rootworms may benefit from a more holistic and sustainable approach, one incorporating microbiomes. Together this represents a unique opportunity to leverage microorganisms to improve pest management.

1.2.B Microorganisms

The ~3.7 billion years of life on Earth have been dominated by microorganisms. Comprising two of the three domains of life, microorganisms represent the most diverse and abundant organisms on Earth, second only to plants in total biomass (~77 Gt C; Bar-On et al., 2018). Bacteria facilitate the geochemical cycling of the major elements required for building all biological life. This extant capacity to use diverse nutrient sources for food is a result of their ability to rapidly evolve, both vertically, due to their short generation time (less than 20 minutes), and horizontally through the transfer of genes (Falkowski et al., 2008). In turn, bacteria occupy almost every niche on Earth, from deep sea hydrothermal vents in the East Pacific Ridge and rocks in Antarctica (Rothschild & Mancinelli, 2001), to inside animal guts and cells (Moran & Baumann, 2000). However, bacteria don't always exist in isolation under natural settings; they can live in diverse communities sharing space and resources. These communities are called microbiomes.

A microbiome is a collection of all the genetic material in a group of microorganisms living in a particular environment (Marchesi, 2017). Most commonly, microbiome refers to bacteria, as other names have appeared in reference to other communities of microorganism. For example, a collection of viruses is known as a viriome, and a collection of fungi is known as a mycobiome. Microbiomes are described in relation to the environment which they are associated or from which they originate. Through this framework, researchers have started to characterize microbiomes of numerous environments and illuminate the complexity of microbial systems on Earth. Advances in microbiome research have deepened the understanding that microorganisms play powerful roles in near every aspect of life.

Assigning taxonomy to bacteria historically relied on culturing an isolate and visually identifying morphological features indicative of previously described genera. However, most bacteria are not able to be cultured (Lloyd et al., 2018). The development of culture-independent methods targeting genetic material accelerated the cataloging of the diversity of bacteria in various niches. None have had a greater impact than next generation sequencing (NGS) targeting the hypervariable regions of the 16S rRNA gene present in bacteria. The full 16S rRNA gene is roughly 1500 base pairs (bp) in length and contains eight regions considered highly conserved and nine regions considered hypervariable. Primers targeting a conserved region upstream of the desired hypervariable region and containing a sample-specific tag are used to amply DNA through polymerase chain reaction (PCR). The resulting amplicons are pooled and then sequenced on a NGS sequencer. Afterwards, advanced bioinformatic pipelines conduct sample discrimination, taxonomic assignment, and read abundance calculations of

individual amplicon sequence variants (ASVs) or operational taxonomic units (OTUs). This high-throughput technology greatly expanded the field's capacity to characterize bacteria within communities (i.e., microbiomes). From that, the understanding the functionality of microbiomes also grew.

Ever since Louis Pasteur pioneered germ theory, microorganisms have largely been seen as disease-causing agents. Recent evidence has broadened our perception of microorganisms from one-dimensional pathogens to important mediators in plant and animal health. Of course, all known eukaryotes are descended from a common ancestor that associated with a bacterium that eventually led to mitochondria. Host cells and bacteria can interact across the cell membrane as well. In this case, bacteria provide functions that expand the suite of useable nutrients for the host without the costly evolution of novel pathways (Tremaroli & Bäckhed, 2012). Associations with bacteria can also provide host protection against stress, toxins, and pathogens (Dearing & Weinstein, 2022; H. Liu et al., 2020; Miller et al., 2021). Ultimately, microbiomes can increase reproductive success in certain conditions. Microbiomes have come to now represent a unifying aspect of health for Eukaryota.

1.2C Beetle microbiomes

The diversity of extinct and extant beetle species raises questions about what mechanisms could contribute to the Order's wide success. One significant morphological change, the development of sclerotized forewings that protect folded hindwings, allowed adults to occupy enclosed spaces and cryptic habitats. This niche expansion likely accompanied a necessity to exploit different resources for food, some of which were previously unfavorable. The capacity for the diet breadth diversity found in beetles

partially comes from microorganisms (Wybouw et al., 2016). Plant cell walls are composed mainly of recalcitrant polysaccharides (cellulose, hemicellulose, pectin, lignin) that provide support and defense against the external world. Animals have a minimal capacity to cleave the bonds of these polysaccharides, but bacteria and fungi have long utilized this resource in their metabolic pathways (Gilbert, 2010). Microorganisms possess a suite of plant cell wall degrading enzymes (PCWDE) that break down glycosidic bonds to release mono- or oligo-saccharides from a range of polysaccharides. Thus, glycosidic hydrolases (GH) would be a great benefit to insects, especially ones feeding on secondary cell walls (woody tissue). Across the beetle phylogeny, evidence of microbial cooperation exists and partially explains their adaptive radiation (Acuña et al., 2012; McKenna et al., 2019).

The co-option of microbial based PCWDE by insects occurs by two different means, symbiont-independent and symbiont-dependent. Insects possess only a couple of natively evolved genes able to digest recalcitrant plant material. The gene families GH1 and GH9 are present in a common Metazoan ancestor (Chang & Lai, 2018; Davison & Blaxter, 2005). For beetles, most PCWDE genes were incorporated into their genome through horizontal gene transfers from microorganisms. Phylogenetic alignment of PCWDE genes from 147 beetle species revealed a large expansion of the number of families incorporated in beetles that correlates to the appearance of specialized herbivory common in Buprestoidea and Phytophaga (McKenna et al., 2019). In total, 22 families of beetles were found to harbor PCWDE genes. Higher rates of diversification correlate to the increase in PCWDE found in beetle genomes. Chrysomeloidea, the superfamily containing *Diabrotica*, contains gene families GH32 invertase, CE8, GH28, GH5, GH10,

GH43, GH45, and GH48, which represent a suite of enzymes able to digest the extensive polysaccharides found in a range of plant tissue (McKenna et al., 2019). The WCR transcriptome possess several copies of predicted cellulases (GH45, GH48, GH16, GH27), hemicellulases (GH5), pectinases (GH28), and xylanases (GH11) that are likely of microbial origin (Eyun et al., 2014). Characterizing these genes revealed a diversity of functionality within a single gene family. For example, WCR encode seven different GH45 genes. Of those, only two are found to break down cellulose. Two other genes were xyloglycans, while others were inactive or uncharacterized (Busch et al., 2019). Some GH families are predicted to interact with the immune system of the host (Kim et al., 2000). HGT events likely occurred throughout the evolution of insects and contribute to a diversity of functions from defense to courtship (Li et al., 2022).

Insects also incorporate microbial genes into their repertoire by harboring microorganisms in their gut or in highly specialized organs, a symbiont-dependent mechanism. The microbes harbored within specialized organs exhibit tight associations with their hosts, often dictating the host's capacity to survive on a specific host plant (Salem et al., 2020). From the host's standpoint, the specialized organs are also tightly regulated, often limited to only one strain of a specific microbe. Endosymbionts can also be housed within specific cells called bacteriocytes (Buchner, 1965). Coevolution of host and symbiont has led to an obligate lifestyle for partners. To ensure the successful association in subsequent generations, female insects vertically transmit the microbe to their offspring through several routes. Females may coat eggs from which hatching larvae will acquire the symbiont or apply symbionts to a brood ball from which larvae will feed. In contrast, intracellular symbionts can be passed down directly during oogenesis

(Hosokawa & Fukatsu, 2010). For beetles, these symbionts have been shown to provide diverse nutritional benefits (Salem & Kaltenpoth, 2022).

Beetles also harbor diverse communities of microbes in their gut that help digest recalcitrant plant material (Douglas, 2009), detoxify plant secondary metabolites (Ceja-Navarro et al., 2015), and supplement nutrition (Salem & Kaltenpoth, 2022). Often, gut microbial communities are transmitted horizontally, originating from the environment or acquired from conspecifics (Adair et al., 2018). Relying on horizontally acquired microbial communities can be risky and may be why we see few instances of obligate symbiosis from horizontally acquired microbiomes. Still, harboring horizontally acquired gut microbes can be advantageous to beetles (i.e., facultative microbes).

Research into the microbial communities of *Diabrotica* show several consistent patterns. Some of the following text first appeared in (Paddock et al., 2021c). Of the species within *Diabrotica*, most studies have focused on the bacterial communities of WCR given its high economic impact. The WCR actively selects for microorganisms it harbors (Chu et al., 2013; Ludwick et al., 2019). Larvae reared in two different soils harbor similar bacterial communities even though the soil samples vary widely in community composition (Ludwick et al., 2019). Both culture-dependent and culture-independent methods have discovered similar genera in WCR larvae. The WCR larval bacterial community commonly consists of species of *Serratia*, *Pseudomonas*, *Klebsiella/Enterobacter*, *Streptomyces*, and *Tsukamurella* with other species appearing in high abundance but sporadically (Dematheis et al., 2012; Ludwick et al., 2019; Prischmann et al., 2008). At least some of the taxa appear to be vertically transferred. Sterilized eggs contain *Wolbachia*, *Pseudomonas sp.*, *Streptomyces sp.*, *Tsukamurella sp.*,

and *Duganella sp.*, which suggests bacteria reside within the egg (Dematheis et al., 2012; Ludwick et al., 2019). Moreover, a portion of the WCR larval microbiome is likely horizontally introduced from the corn rhizosphere (Dematheis et al., 2012). Yet, host species identity likely accounts for some control of the bacterial communities. For example, *Diabrotica speciosa* larvae reared on corn roots contained 73 culturable bacterial strains and comprise a similar set of taxa as WCR being mainly from the orders Pseudomondales and Enterobacteriales (Perlatti et al., 2017). *D. unidecimpata* reared on corn roots also contained taxa from Pseudomondales and Enterobacteriales (Tran & Marrone, 1988). Microbiomes of other species in the genus may also acquire a significant portion of their community from feeding (Schalk et al., 1987). Larvae of *D. balteata* fed on wheat did not yield culturable taxa found in other Diabrotica larvae, but it is unclear if that would change if larvae were reared on corn roots. The role diet plays in the composition of *Diabrotica* larval microbiomes is understudied.

Adult WCR harbor a distinct set of bacteria that reflects their different dietary niche and gut physiology compared to larvae. One study found WCR adults have a less taxonomically rich bacterial community compared to larvae from the same study (Ludwick et al., 2019). A few taxa were conserved across beetle samples: *Wolbachia,* an Enterobacteriaceae species, and an Acinetobacter species. Another study identified sets of conserved taxa belonging to Enterobacteriales, Lactobacillales, and Xanthomonadales (Chu et al., 2013). Of these, *Enterobacter* sp., *Lactococcus* sp., and *Enterococcus* sp., were most abundant with the lesser abundant *Klebsiella* sp. and *Stenotrophomonas* sp. present in a high proportion of samples. Wild collected beetles may harbor a higher diversity of bacteria, but this has not been thoroughly tested (Schalk et al., 1987).

Diabrotica as a group may follow the pattern in WCR and harbor different microbiomes across life stages. *D. balteata* show low overlap in culturable bacteria between larvae and adult (Schalk et al., 1987). The adult microbiome was also influenced by diet. However, how the diet or environment influence Diabrotica adult microbiomes is also understudied.

Studies have focused on surveying the bacterial community of WCR but have done little to try to characterize functionality of that community. Chu et al. (2013) identified alterations in the gut microbial community of rotation-resistant populations of WCR. These shifts in the bacterial community were accompanied by increased cysteine protease activity in the gut that facilitated adult survival on soybean foliage (Chu et al., 2013). In another study, bacterial isolates from abdomens of females influenced oviposition preference in choice tests (Lance et al., 1992). Robert et al. (2013) evaluated fitness of multigenerational, antibiotic-treated WCR and found no significant difference in weight gain or survival on conventional corn. Antibiotics were given to adults, and only the presence of *Wolbachia* was analyzed using PCR so it is unclear what other bacteria remained after antibiotic treatment, or what bacteria were acquired from the soil during larval feeding.

A majority of WCR populations carry a high proportion of the maternally transmitted endosymbiont, *Wolbachia* (Clark et al., 2001; Dematheis et al., 2012; Ludwick et al., 2019). *Wolbachia* can play an influential role in insect reproduction by inducing cytoplasmic incompatibility, parthenogenesis, feminization, and male killing (Werren et al., 2008). Reproductive isolation caused by cytoplasmic incompatibility can result in speciation events at a much greater speed than traditional genetic elements (Bordenstein, 2003; Werren, 1997). Two subspecies of *Diabrotica virgifera*, *D. v.*

virgifera (WCR) and *D. v. zeae* Krysan and Smith (Mexican corn rootworm), are a result of *Wolbachia*-induced cytoplasmic incompatibility that occurred after the ancestral population reached the area of modern-day Arizona less than 1100 years ago (Giordano et al., 1997; Lombaert et al., 2018). *Wolbachia* has also been shown to influence the composition of the host microbiome (Ye et al., 2017) and even protect the host from viral infection in populations of *Drosophila melanogaster* (Hedges et al., 2008). A role outside of reproductive incompatibility has not been found for *Wolbachia* in WCR. *Wolbachia* does seem to modulate plant gene expression, but this does not seem to impact major defenses or WCR resistance (Barr et al., 2010; Robert et al., 2013). Yet, it appears that some of the bacteria that inhabit WCR display functional capacity in overcoming plant defenses.

Mechanistic studies investigating the role of WCR gut microbiome in WCR ecological success are crucial to develop effective pest management strategies. To that end, documenting the rules of engagement for WCR and their microbiome is needed to provide a foundation to explore management tactics aimed at disrupting the link between the two.

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Chapter 2: Patterns of microbiome composition vary across spatial scales in a specialist insect

2.1 Introduction

Animals have evolved while in constant contact with microorganisms. Associations between hosts and microorganisms exist on a continuum from beneficial to detrimental. In insects, bacterial communities can improve host fitness by enhancing nutrition (Ben-Yosef et al. 2014), disrupting plant defenses (Chu et al., 2013), and protecting against disease (Miller et al., 2021), but are also capable of inducing mortality in certain situations (Mason et al., 2019; Caccia et al., 2016). Variation in bacterial communities within hosts may result in differential survival with direct implications for design and implementation of conservation and pest management strategies (Paddock et al., 2021).

Inter- and intra-species variation in the microbiome can be influenced by numerous factors. Host species identity can dictate what microbes survive within the host. Insect guts vary in morphology, pH, and immune response (Caccia et al., 2019), which serve as filters for specific microbes resulting in communities that vary between closely related species (Adair et al., 2020). Host diet partially determines the local species pool with which the host interacts. Different feeding modalities (e.g., chewing vs. sucking mouthparts) constrain access to food sources, which can affect which microbes are associated with insects (Huang et al., 2021). Furthermore, certain food substrates can be digested by microbes within insects, and thus can alter communities through resource limitation or niche partitioning (Mason et al., 2020; Brochet et al., 2021). The host's external environment can also generate variation between insect microbiomes by

affecting the local source pool of microbes. Such factors as temperature (Wang et al., 2020), landscape context (Park et al., 2019) and plant diversity (Cohen et al., 2020) may be associated with variation between insect microbiomes in different local habitats.

Host microbiomes across a biogeographic space can best be described through a metacommunity framework (Adair and Douglas, 2017; Miller et al., 2018), where host-associated communities in local environments are subsets of the larger environmental metacommunity and linked through dispersal. Spatial limits on microbial dispersal, or "dispersal limitation", can result in patterns of geospatial correlation in which microbial community similarity decays with increasing geographic distance (Finkel et al., 2012; Moeller et al., 2017). At different spatial scales, the determinants of microbiome composition may change and result in different communities. Both landscape composition and configuration can dictate the dispersal capacity of microorganisms within the metacommunity (Parajuli et al., 2020). For example, in humans, small scale dispersal events may be disrupted by barriers such as human-made structures or vegetation (Parajuli et al., 2020). At a continental scale, dispersal may be very limited and other factors such as lifestyle, diet, age, and genetics may account for most variation between hosts (Yatsunenko et al., 2012). Which microbes are associated with hosts is determined by interactions of both deterministic and stochastic processes (Adair and Douglas, 2017), but how these processes interact across spatial scales is not well understood.

Corn rootworms (genus *Diabrotica*; Coleoptera, Chrysomelidae) represent a useful system to investigate the influence of biogeographical arrangement on host associated microbiome composition. Many studies have investigated determinants of

microbiome composition in generalist feeding insects (Cohen et al., 2020; Jones et al., 2019; Adair et al., 2018). Studies with generalist insects have limitations on distinguishing between the influence of geographic location and the influence of diet on microbiome composition. In the United States, two rootworm species predominate east of the Rocky Mountains: the western corn rootworm (*D. virgifera virgifera* LeConte; WCR) and the northern corn rootworm (*D. barberi* Smith & Lawrence; NCR). Both species overlap in distribution, phenology, and host plant usage (Krysan and Miller, 1986), which allows us to better examine environmental factors influencing microbiome composition. In addition, they comprise the most damaging groups of corn pests in the United States, with management costs and yield losses combining for over $2 billion annually (Wechsler and Smith, 2018). Management continues to become more complicated as both species have evolved resistance to crop rotation and transgenic crops producing toxins derived from the bacterium *Bacillus thuringiensis* (Bt) (Calles-Torrez et al., 2019; Levine et al., 2002; Levine et al., 1992). In WCR, the microbiome may help beetles overcome plant defenses, and changes in larval microbiome composition are linked to resistance to Bt (Chu et al., 2013; Paddock et al., 2021). The geospatial consistency of bacterial community composition within WCR has yet to be investigated.

Here, we characterize the bacterial communities in two sister *Diabrotica* species and compare the patterns of assembly at different spatial scales. We collected WCR beetles from corn fields across their ranges in the United States using two sampling scales, regional scale (~12-50 km between sites along linear transects) and continental scale (>200 km). We examined correlations between bacterial community dissimilarity and distance between collection sites for WCR. We hypothesize that environment

contributes to variation in microbial communities, and thus, differentially influences the similarity of microbial communities across biogeographical space. Specifically, we predict that microbiome sequence similarity decreases with geographic distance (distance decay), and that the effect is stronger at the landscape level where dispersal limitation may be higher compared to local levels. Increased understanding of the factors influencing microbiome composition in insects can provide insight into how microbe-mediated effects on plant-insect interactions may have evolved.

2.2 Materials & Methods

Insects

WCR and NCR are univoltine species that emerge in late July into August. In this study, adult beetles were collected from corn fields between July and August in 2016, 2019, and 2020. Information including date of collection and location of the closest city or populated place to the collection site can be found at DOI: 10.6084/m9.figshare.17130035. WCR were collected at two spatial scales (Figure 2.1). A small, regional-scale sampling scheme consisted of field sites located ~25 km apart along two transects in eastern Colorado and western Kansas. On average, WCR can disperse ~17 m a day. Distances between any two sites ranged from 11.46 – 276.73 km. Beetles were collected in ethanol and stored at -20ºC at the Corn Insects and Crop Genetics Research Unit (CICGRU) in Ames, Iowa until DNA extraction. For the broad, continental-scale sampling scheme, field sites were ~250 km apart, with paired distances ranging from 248 – 3 122 km. Beetles were stored in 95% ethanol and shipped to Columbia, Missouri, where they remained until DNA was extracted. A total of 24 sites

across 11 states were sampled for WCR in 2016, 2018, and 2020. Within the small regional-scale sampling scheme, 14 city level sample sites were collected in 2016. 8 beetles were collected at each site for a total of 192 WCR beetles. NCR were collected only at a continental scale and were processed in the same manner as WCR. A total of four sites were sampled for NCR in 2020. Within Missouri, two city level samples were collected. 8 beetles were collected at each site for a total of 32 NCR beetles.

DNA extraction and 16S rRNA gene amplification

For each sample location, DNA was extracted from individual beetles (n = 8). Before the day of extraction, beetles in ethanol were removed and placed in individual 2-mL tubes to dry overnight. Beetles were transferred to beaded tubes (MP Biomedicals, Santa Ana, CA, Catalog No. 116913500), flash frozen in liquid nitrogen for 20 seconds, immediately placed in a single-tube bead beater, and shaken for 20 seconds to pulverize the sample. Bacterial DNA was extracted from pulverized beetles using PowerFecal® DNA Isolation Kit (QIAGEN, Catalog No. 12830-50) in accordance with the manufacturer's protocols (https://www.qiagen.com/us/resources/resourcedetail?id=00e4513c-597b-4bd5-a600-9259e6d62d07&lang=en). Frozen beetles processed at CICGRU were ground by mortar and pestle and then transferred to sterile 1.5-mL microcentrifuge tubes. Bacterial DNA was extracted using DNA Blood and Tissue kit (QIAGEN, Catalog No. 69504) in accordance with the manufacturer's protocols (https://www.qiagen.com/us/resources/resourcedetail?id=68f29296-5a9f-40fa-8b3d-1c148d0b3030&lang=en). The concentration of extracted DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). DNA was stored at -80 °C until further downstream processing. Prior to amplification, DNA concentration was standardized to

3.51 ng/μL. The V4 hypervariable region of the 16S rRNA gene was amplified using single indexed universal primers (U515F/806R) with Illumina standard adapter sequences. Dual-indexed forward and reverse primers were used in all reactions. PCR reaction steps were as follows: $98°C^{(3:00)}$+ 25 cycles of [$98°C^{(0:15)}$+$50°C^{(0:30)}$+$72°C^{(0:30)}$]. The resulting amplicons (5 μL) were pooled before sequencing on the Illumina MiSeq 2 × 250 bp platform (6). The construction and sequencing of 16S sequencing amplicon libraries were completed at the University of Missouri (MU) DNA Core facility.

16S rRNA community analysis

Sequence assembly and annotation were performed by the MU Informatics Research Core Facility. Primers were trimmed using Cutadapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200/479) in two rounds, first removing forward primers with an error rate of 0.11 mismatches and minimum length of 19 bp, followed by a second round of trimming from the 3' end executed with an error rate of 0.1 mismatches and minimum length of 20 bp. A minimal overlap of 3 with the 3' end of the primer sequence was required for removal. Untrimmed contigs were discarded between rounds of trimming. Using the Qiime2 plugin, DADA2 (Callahan et al., 2016) (version 1.10.0), forward and reverse reads were truncated to 150 bp and discarded if the number of expected errors was > 2.0. Chimeras were detected using the "consensus" method and removed. Resulting sequences were filtered to retain only sequences 249 – 257 bases long. Taxonomy was assigned to amplicon sequence variants (ASV) using the Silva.v132 database with the 'sklearn" classifier in Qiime2. ASVs were compiled into biom tables for data analysis. ASV tables with metadata and associated taxonomy were imported in to RStudio version 3.5.2 for downstream analysis.

ASVs matching chloroplast, mitochondria, and archaea sequences were filtered and removed using phyloseq::filter_taxa in RStudio (McMurdie and Holmes, 2013). Taxa labeled 'uncharacterized' at the phylum level were also removed. An extraction blank was used to remove contaminant sequences based on prevalence using decontam in RStudio. The resulting data set was rarefied to a read depth of 750 and subsequently used for analyses of alpha and beta diversity in RStudio.

Statistical analysis

To examine differences in microbial communities within host species, we removed *Wolbachia* from the data. *Wolbachia* can account for over 90% of the relative abundance in microbial communities (Paddock et al., 2021; Ludwick et al., 2019), and its association is nearly ubiquitous (Giordano et al., 1997). Conversely, *Wolbachia* infection in NCR populations exhibits strong geographic partitioning (Roehrdanz and Levine, 2007). Alpha diversity indices (Chao-1 and inverse Simpson's *D*) for both host species across sites were generated using estimate_richness in the phyloseq package. Analyses of beta-diversity were conducted using a permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis and Jaccard distances at the ASV level. Comparisons of differentially abundant taxa present between species were conducted using DESeq2 in RStudio using unrarefied data (Love et al., 2014).

We asked whether the state where a sample was collected from affected microbiome composition within the WCR and NCR. To do this, we analyzed alpha- and beta-diversity separately for each species. For WCR, we also compared microbiome composition across cities within the smaller spatial scale in Colorado and Kansas. Alpha diversity indices (Chao-1 and inverse Simpson's D) were log-transformed before analysis

of variance to correct for non-normal distributions, and significance considered as $p < 0.05$. Analyses of beta-diversity were conducted using a permutational multivariate analysis of variance PERMANOVA based on Bray-Curtis and Jaccard distances at the ASV level. Year of collection was treated as a random factor by restricting permutations within year. Beta dispersion as measured by the average distance to centroid of each group was calculated using vegan::betadisper and compared using a permutational test with vegan::permutest. Pairwise comparisons were made for significant differences observed in PERMANOVA using pairwiseAdonis::pairwiseadonis2 at corrected $p < 0.05$ (Martinez Arbizu, 2020).

To better understand the patterns of community composition at different scales in WCR, we examined the correlation between community dissimilarity and geospatial arrangement. First, we calculated microbiome dissimilarity between individual insects using both Bray-Curtis and Jaccard distances at the ASV level. Geographic distance between sample locations was calculated using the Haversine formula for distance (Robusto 1957). Resulting distance matrices were analyzed by mantel test using Spearman's rank-ordered correlation permuted 9999 times using vegan::mantel. Secondly, values for PC1 for the WCR, individual-species PCoA were extracted using the scores function and then used as the dependent variable in a linear model examining the relationship to geographic distance from a historically relevant origin source region defined as the sample site closest to Mexico along the invasion path (Arizona). To visualize differences between ASVs in WCR collected from different locations, we generated a heat map using the log10 abundance of 125 most abundant ASVs.

Data availability

Raw sequences can be found on the NCBI SRA database under the project accession number PRJNA785968. Raw data and accompanying metadata can be found at FigShare at DOI: 10.6084/m9.figshare.17130035. Code used in statistical analyses can be found at FigShare at DOI: 10.6084/m9.figshare.17130455.

2.3 Results

WCR and NCR bacterial community composition

Beetle microbiomes were mainly composed of bacteria from the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteriodetes, in order of decreasing relative abundance (Figure 2.2A). Overall, 230 ASVs (~12% of the total ASVs identified) were shared in at least one WCR beetle and one NCR beetle (Figure 2.2B). These shared ASVs composed 88.2% (± 1.5%) of reads in WCR and 91.2% (± 3.0%) of NCR reads on average. Seven ASVs were found in each species under a prevalence threshold of 50% presence across all samples per species. Four of those ASVs were shared between WCR and NCR: two from the family Enterobacteriaceae, and one each from the genera *Pantoea* and *Lactococcus*. ASVs from an unclassified genus in Enterobacteriaceae had the highest relative abundance in both WCR (54.3%) and NCR (40.4%). Other taxa with high relative abundance in WCR were in the genera *Lactococcus*, *Acinetobacter*, *Pseudomonas*, *Serratia*, *Pantoea*, *Microbacterium*, *Massilia*, *Sphingomonas*, and *Exiguobacterium* in order of decreasing relative abundance. In NCR, *Lactococcus*, *Pantoea*, *Serratia*, *Acinetobacter*, *Sphingomonas*, *Pseudomonas*, *Chryseobacterium*, *Stenotrophomonas*, and *Microbacterium* were found in high relative abundance (in decreasing order). Nevertheless, several taxa differed in relative abundance between the

two rootworm species. Alpha diversity measurements for WCR and NCR were averaged across sites (Table 2.1).

Biogeographical impact on bacterial community composition

Local environments have an impact on the composition of bacterial communities within WCR and NCR (Figure 2.3). We found bacterial communities differed at the city and state level in both host species based on the results of the PERMANOVA with Bray-Curtis and Jaccard distances (Table 2.2). In pairwise comparisons, WCR collected from New York, Pennsylvania and Michigan consisted of unique bacterial communities different from all other states. Arizona was significantly different from all other locations except Colorado and Illinois. Texas was also different from Kansas and South Dakota. For samples collected from smaller regional-scale sampling scheme in Colorado and Kansas (n = 14), overall variation between communities was less than at the landscape level (Table 2.2). Wild NCR collected from Minnesota harbored unique bacterial communities compared to Missouri and North Dakota. We found no differences between North Dakota and Missouri at the city or state level. Patterns of differential abundance of ASVs across sites were observed (Figure 2.4).

Pairwise comparisons do not account for geospatial correlation, however. We used mantel tests to investigate correlations between geographic distance and microbiome community dissimilarity between collection locations. The full WCR data set encompassing the continental scale showed no significant correlation between geographic distance and microbiome dissimilarity for Bray-Curtis ($p = 0.15$) or Jaccard ($p = 0.16$) distances. At a smaller geospatial scale (~25 km apart), microbiome dissimilarity increased with increasing distance between sample locations (Figure 2.5A; Bray-Curtis, p

= 0.03; Jaccard, p = 0.03). We suspected the historical geographic pattern of invasion may be correlated to microbiome similarity. Specifically, we examined the relationship between distance from Arizona, the most ancestral population sampled (Lombaert et al., 2018) and microbiome dissimilarity. The values from PC1 of the PCoA of WCR based on Jaccard distances revealed a significant correlation (Figure 2.5B; $p = 0.009$). This correlation explained 3% of the variation captured by PC1 (Figure 2.5B; $R^2 = 0.03$)

2.4 Discussion

In this study, we found bacterial communities of two insects, WCR and NCR, are significantly impacted by location of collection. However, the geospatial patterning of bacterial communities differed at different spatial scales. The factors governing organization of microbial communities associated with hosts vary in strength across time and space. Populations of WCR can disperse between locations, encountering and mixing microbial populations from isolated patches. If selective environmental forces are similar between locations, the host-associated microbiome may be more affected by dispersal and drift. Thus, community similarity would be expected to decrease over increasing geographical space (Finkel et al., 2012). Here we found, at smaller spatial scales, bacterial community similarity was correlated with geographic distance between WCR populations. Genetic analyses of the same WCR populations studied here revealed a weak but significant pattern of isolation by distance in Colorado (Kim and Sappington, unpublished data). Our results appear to follow this same pattern of decay by distance (Finkel et al., 2012). We conclude that the spatial turnover observed at the local level in WCR microbiomes is a result of dispersal limitation and ecological drift. However, at

broad spatial scales, differences in landscape diversity or host genetics may overshadow dispersal limitation and account for most of the differences in observed microbiome communities separated by long distances.

In *Drosophila*, rapid genomic evolution can occur in response to the microbiome and, at broad spatial scales, differences in microbiome community composition could be driven by local adaptation (Rudman et al., 2019). Previous genetic analysis found a lack of genetic structuring in WCR across most of the United States, presumably because of a lingering lack of genetic equilibrium after the eastward range expansion out of the western Great Plains beginning in the mid-20th century (Kim and Sappington, 2005; Flagel et al., 2014). This eastward expansion of WCR (Gray et al., 2009) may also partially explain the lack of distance decay in microbial community composition at the continental scale observed in this study. Populations in New York, Pennsylvania, and Michigan relatively near the eastern front of WCR expansion may exhibit residual effects of founder populations as evidenced by their disparate microbiome communities. Cropping patterns in agricultural landscapes in the eastern US are more diverse and include less continuously-planted corn than in the central US, increasing isolation between populations of insects and microbes by hindering dispersal between locations (Onstad et al., 2003). Correlation with distance from a historically relevant origin region (Arizona) for WCR and microbiome composition may reflect an interaction between host genetic structure and isolation by distance. Alternatively, because the agricultural landscapes of the eastern US differ markedly from those in the Corn Belt, local environmental selection of the microbial species pool could result in significantly different microbial communities found within the insect. It is not well understood what

influence the soil microbiome has on adult corn rootworm microbiome composition or which microbes persist through metamorphosis (Ludwick et al., 2019). Feedback between host genetics, the microbial species pool and dispersal limitation could account for the patterns found here. Further investigation is warranted to elucidate environmental effects (i.e., temperature, landscape composition, altitude) on host associated microbiomes in rootworms and other insects.

It is possible that differences in microbiome composition derive from management tactics used within a crop field. While the beetles in this study were collected from corn, we do not know what hybrids or management practices were used in the fields of collection. Macro-level changes in diet (i.e., different host plant species) can have pronounced effects on microbiomes across insect species (Mason et al., 2020; Jones et al., 2019), but differences seem to diminish at finer levels of host plant taxonomic resolution (i.e., plant genotypes) (Mason et al., 2021). Several corn genotypes harbor unique taxa, but it is unclear how they are affected by their local environment (Favela et al., 2021). Microbiomes of WCR larvae also respond to ingestion of transgenic crops producing *Bacillus thuringiensis* (Bt). Larvae resistant to Bt host a unique microbial community that is less taxonomically rich than susceptible insects (Paddock et al., 2021). We do not know if any populations we sampled are resistant to Bt. To that end, no studies have investigated whether adult WCR microbiomes also respond to Bt. The functionality of microbial communities in adult WCR are documented to involve adaptation to plant defenses and to influence oviposition sites (Chu et al., 2013, Lance, 1992). However, estimation of individual species functionality in the microbiome of WCR is lacking. Resolution provided by 16S rRNA sequencing does not allow for accurate prediction of

functionality and requires further study. While we observed differences between Colorado and other sites, we cannot distinguish the biological signal from a possible kit artefact. In addition, we did not examine microbial strain-level diversity in this study. The increased resolution may illuminate differences in community composition that could not be detected by 16S rRNA sequencing.

Differences in the ecological properties and requirements of microbes may generate variation in the species pool at different locations, which can then affect assembly of host-associated microbiomes. Sample location affects microbiome composition in both species of corn rootworm (Table 2.2). The local species pool of microbes available to colonize the host likely varies with collection location. For instance, communities within a state are more similar than across states (Table 2.2), which suggests a local environmental or geospatial effect on bacterial assembly. Microbes associated with a host's food comprise most of the local species pool for that host (Deb et al., 2019; Delsuc et al., 2014). Thus, specialist insects might have a more predictable microbiome (Gomes et al., 2020). Certain components of corn have a predictable and heritable microbiome (Walters et al., 2018). In our case, WCR adults are likely closely associated with the microorganisms colonizing their diet, sharing taxa found both in corn silk and leaves (Khalaf et al., 2021; Wagner et al., 2020). The most common family found in our samples, Enterobacteriaceae, is generally found in other insects feeding on corn plants as well (Jones et al., 2019), suggesting a close association with corn. Other microbial taxa may be more responsive to abiotic and biotic factors associated with the local environment or may vary in dispersal capacity. Future studies

examining the site-specific environmental pool of bacteria would improve the understanding of the impact of food source on microbiome composition in insects.

We found WCR and NCR harbor a small set of commonly occurring bacteria regardless of location. Commonly occurring bacteria (i.e., those represented in 50% of samples) totaled seven for both *D. v. virgifera* and *D. barberi*, with four being shared between the two species. This is consistent with other insects which host facultative microbes with high variation between individuals (Silver et al., 2021; Blankenchip et al., 2018; Hammer et al., 2020). The number of shared taxa between WCR and NCR was relatively small (~12%) but accounted for a high amount of the total sequences within the communities (~90%). Specific filtering by host species can structure distinct microbial communities (Adair et al., 2020; Jones et al., 2019), and while we documented distinct taxa within each host species, we cannot be certain they are due to host filtering. A more controlled study is necessary to distinguish environmental and host effects. Still, host species identity does not account for all the interindividual variation observed in host associated microbiomes (Colman et al., 2012; Yun et al., 2014). In our study, the commonly occurring bacteria may capture a large amount of the variation between sites. Consequently, the correlations between locations we observed may be signatures of smaller parts of the bacterial communities found within WCR and NCR, given the effect sizes (Figs. 2.5A and 2.5B). Generalist insects may exhibit weaker distance decay patterns due to variation in diet overshadowing local environmental influence.

Integrating landscape ecology, biogeography and metacommunity theory into host-associated microbiome studies has been complicated. What microbes an animal encounters in its life are influenced in part by the host diet and the environment in which

the host interacts (Littleford-Colquhoun et al. 2022). Both the diet and environment are impacted by selective and neutral forces. Host factors such as species identity or life stage also influence microbial community composition (Suarez-Moo et al., 2020). Specialist insects provide a unique opportunity to investigate factors affecting microbiome composition across geographical space. Dispersal limitation between geographically isolated environments results in unique microbial communities. This isolation can also impact host genetic factors that results in variation between microbiomes. Through a metacommunity framework, WCR can be thought of as "islands within islands", where microorganisms can disperse and establish (or not) on sessile corn plants whereby they interact with another layer of dispersing organisms (insect) that impart their own selective filtering and dispersal barriers onto the microbial communities. This nested structure accounts for variation in dispersal capacity across scales. Understanding how all determinants interact to shape microbiomes within animals is important to better leverage the beneficial effects microbiomes can provide.

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Table 2.1 Average alpha diversity metrics of western corn rootworm (WCR) and northern corn rootworm (NCR) collected from different locations across the United States

Species	*Chao-1*	*Inverse Simpson's D*
WCR	34.95 ± 2.25	4.37 ± 0.48
NCR	53.87 ± 6.65	5.06 ± 0.77

Table 2.2 Beta diversity metric comparisons between collection site within host species

Species	Response	Factor	df	F	R^2	*p*
WCR	Community	City	23,158	2.38	0.288	0.001
		State	10,158	3.35	0.184	0.001
		City (Colorado)	13, 91	1.45	0.19	0.009
NCR	Community	City	3,31	1.72	0.156	0.002
		State	2,31	2.11	0.127	0.001

Notes: Results of models for bacterial community composition (community) in WCR and NCR species collected from differing localities. Distance matrices used in PERMANOVA models were analyzed using Bray-Curtis distances on rarefied data.

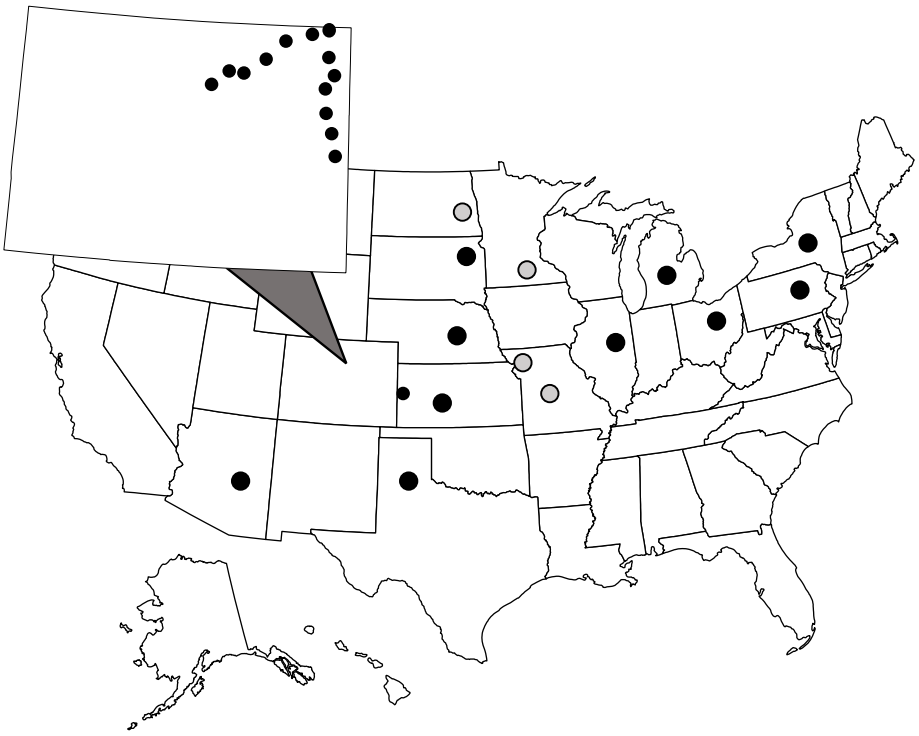

Figure 2.1 Sample locations of wild, adult *Diabrotica virgifera virgifera* (black dots) and *D. barberi* (grey dots). Beetles were collected from cornfields during late July to early August. Colorado is zoomed out with small circles showing close-proximity sampling locations (note one location is across the border in Kansas). Each dot consists of one sample site where 8 individual beetles were collected.

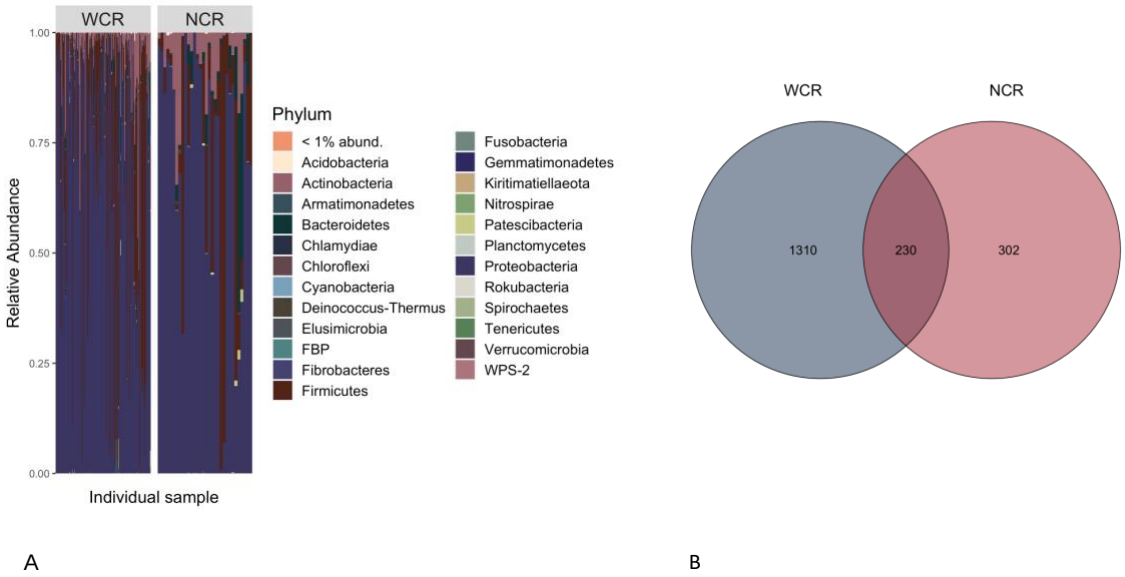

Figure 2.2 A) Phylum level stacked bar chart of average relative abundance of bacterial communities from adult *Diabrotica virgifera virgifera* (WCR) and *Diabrotica barberi* (NCR) collected from across the United States arranged in order of decreasing longitude (west to east). Each bar represents an individual beetle. **B**) Venn diagram comparing ASV overlap in WCR and NCR microbiomes using rarefied data. Data presented with *Wolbachia* removed from communities.

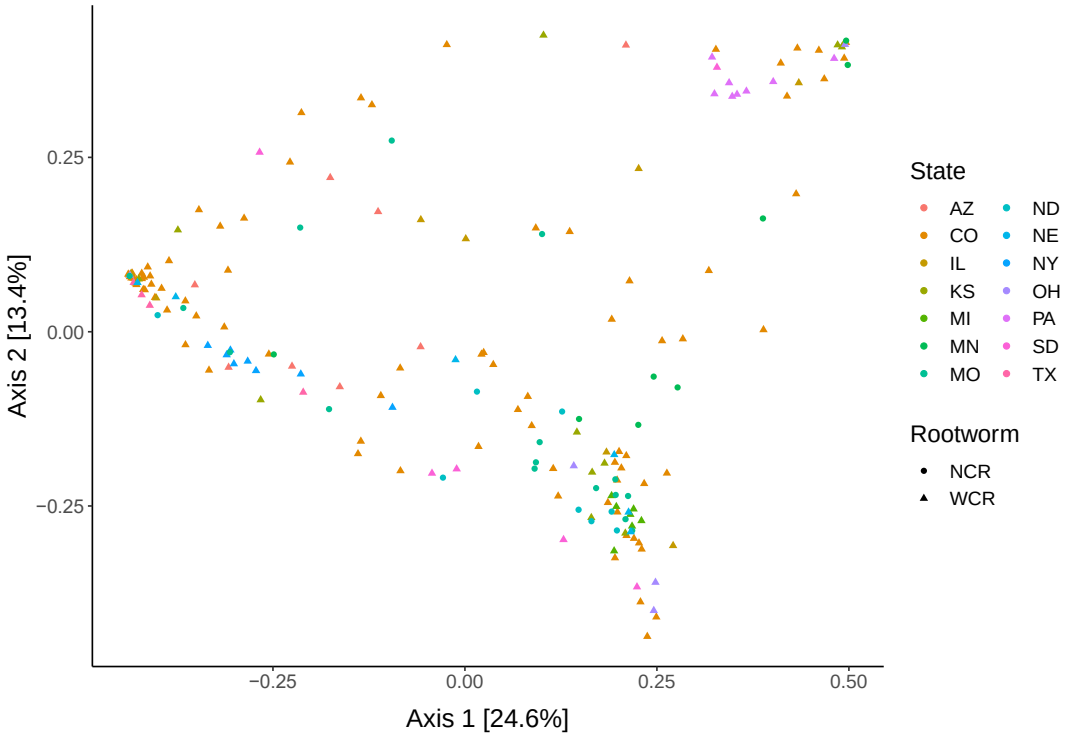

Figure 2.3 Principal coordinates analysis of bacterial communities in *Diabrotica virgifera virgifera* (WCR) and *Diabrotica barberi* (NCR) collected from their natural host plant corn in the wild based on Bray-Curtis distances. Data presented with *Wolbachia* removed from communities.

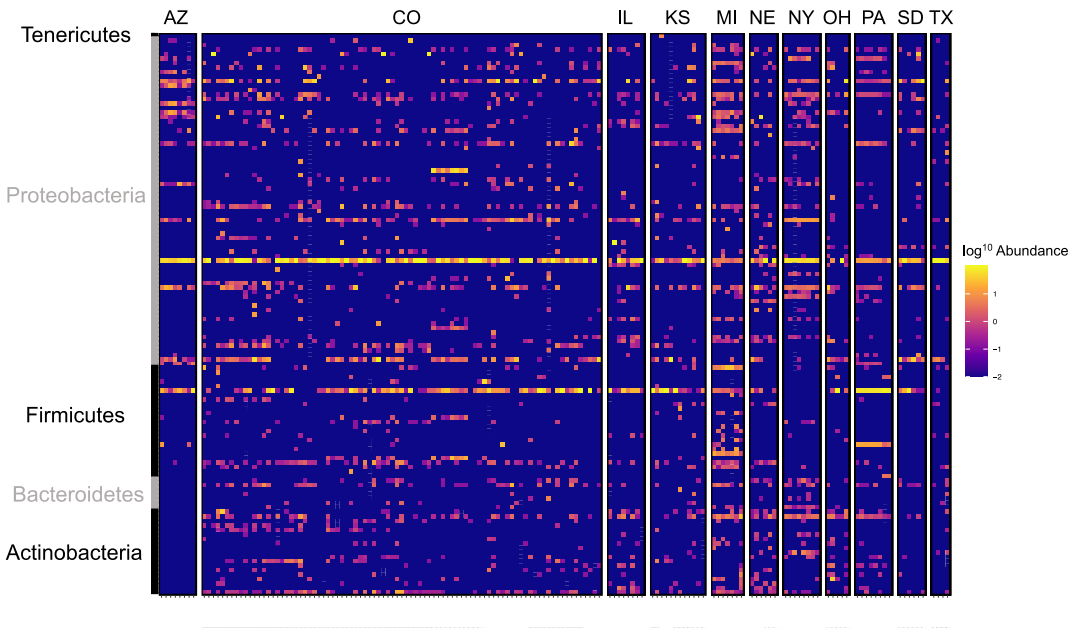

Figure 2.4 Heat map of log^{10} abundance of the top 125 most abundant ASVs in western corn rootworm (WCR) across sampling locations in the United States. Each row represents a single ASV, and each column represents one beetle microbiome collected from one site within the state. Colorado consisted of multiple sampling sites. Family level grouping of ASV is provided on the right of the figure. Data presented with *Wolbachia* removed from communities.

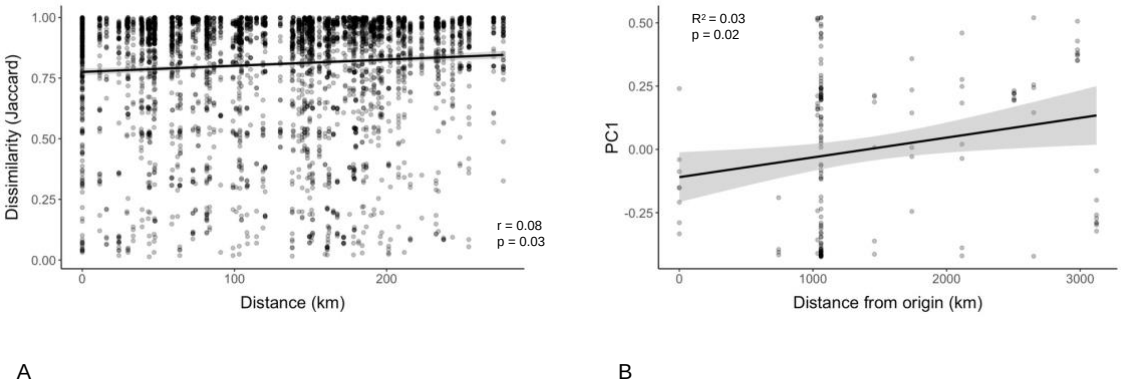

Figure 2.5 Correlations at various spatial scales between A) Haversine distance and microbiome community dissimilarity (Jaccard) and B) Haversine distance from ancestral population (Arizona) of *Diabrotica virgifera virgifera* and values from axis 1 of PCoA of microbiome community similarity based on Jaccard distance.

Chapter 3: Host resistance to *Bacillus thuringiensis* is linked to altered bacterial community within a specialist insect herbivore

3.1 Introduction

The intensification of agriculture has resulted in an increased reliance on large scale pest control, both chemical and biological. Transgenic crops expressing insecticidal toxins have been successful at managing pests but are not without limitations, as numerous species have evolved resistance (Tabashnik & Carrière, 2017). Studies aimed at characterizing resistance have largely focused on target-site or metabolic mutations in insects (Pardo-Lopez et al., 2013). However, microbial communities associated with insects can influence host fitness and susceptibility to pesticides, but are often overlooked when characterizing resistance (Douglas, 2018; Gressel, 2018). Understanding how the microbiota affect resistance and vice versa, how resistance affects the microbiota, is fundamental to the design and success of sustainable management tactics.

Few biological controls have obtained the commercial success of *Bacillus thuringiensis* (Bt) since its discovery in 1911 (Roh et al., 2007). A naturally occurring soil-borne bacterium, Bt displays toxicity in a diverse set of arthropods through the production of parasporal crystalline inclusions composed of pore-forming proteins, or Cry proteins (δ-endotoxins) (Hofte & Whiteley, 1989). Cry toxins target midgut columnar cells, where binding and insertion into the membrane leads to pore formation and eventually osmotic cell shock and death of the insect (Pardo-Lopez et al., 2013). Historically, applications of Bt have consisted of spore and crystal-containing sprays, which rely on ingestion and lysis inside the target pest, but success is limited by the

relatively quick UV degradation of proteins (Behle et al., 1997; Roh et al., 2007). However, the advent of transgenic crops expressing Cry toxins improved delivery and efficacy of Bt as a control tactic, especially for belowground pests, while simultaneously reducing the use of conventional insecticides (Benbrook, 2012; Sanchis, 2011). Now, transgenic crops expressing Cry proteins comprise roughly 80% of field crop acreage in the United States, with over 100 million hectares grown worldwide (ISAAA, 2017; USDA-NASS, 2019). Consequently, resistance to Bt has developed in a number of pest species with new instances continuing to appear (Tabashnik & Carrière, 2017). Resistance mechanisms characterized have largely been attributed to modifications of binding sites resulting in reduced toxin binding (Pardo-Lopez et al., 2013). However, the cause of death of the insect itself following Bt ingestion has been a heavily debated issue with uncertainty in regards to the extent endogenous bacteria are involved (Broderick et al., 2006, 2009; Hilbeck et al., 2018; Johnston & Crickmore, 2009; Mason et al., 2011; Paramasiva et al., 2015; Raymond et al., 2009; Visweshwar et al., 2015).

Previous work investigating the role of enteric bacteria in Bt susceptibility relied on curing the insect of bacteria prior to treatment with varying sources of Bt. Removal of enteric bacteria decreased larval susceptibility to Bt in some insect species but not others. Moreover, there was variability in how various bacterial species in the gut community interacted with Bt and with insect guts of different species. For example, susceptibility was restored after re-inoculation with an *Enterobacter* sp. (Broderick et al., 2009), but not with an *Enterococcus sp.* (Johnston & Crickmore, 2009; Raymond et al., 2009). Midgut bacteria can also influence the evolution of resistance to Bt. In selection experiments, resistance to Bt toxins only developed (within three generations) in the

presence of endogenous bacteria, yet no decrease in susceptibility was observed after curing the insect of its microbiota (Paramasiva et al., 2015). A role for gut bacteria in Bt susceptibility has been demonstrated (Broderick et al. 2006). However, the interpretations of some findings in other studies are mired in the confounding effects of the antibiotics on Bt itself and the effects of antibiotics on host nutrition and physiology (Raymann et al., 2017; Raymond et al., 2009; Van Der Hoeven et al., 2008). In addition, these studies are almost exclusively conducted using phytophagous caterpillars even though Bt is utilized against other Orders of insects. Past experiments across insect species have used different diets (artificial, food source), Bt sources (bacterial lysates, commercial formulations, in-plant toxins), and characterization methods (culturing, DGGE fingerprinting, 16S rRNA sequencing), further complicating interpretations.

More recently, additional evidence supporting septicemia as the killing mechanism of Bt in caterpillars has been reported (Broderick et al. 2006). After silencing a common immunosuppression gene involved in nodulation, enteric bacteria were observed passing through the midgut epithelium into the hemocoel, demonstrating commensal bacteria could become pathogenic upon entry into the hemocoel (Caccia et al., 2016). If microbiota are necessary for susceptibility to Bt, then resistance to Bt could induce changes in the microbial community. No studies to date have examined the associated microbial community as a whole (16S rRNA sequencing) in Bt-resistant and -susceptible insect species fed on their natural diet, nor how those communities change in response to ingestion of Bt. In our study, we address these issues using a below-ground specialist herbivore, the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae).

WCR is one of the most severe pests of maize in the United States Corn Belt, and recently it has become established in Europe through multiple introductions and range expansions (Miller et al., 2005). Root feeding by the larvae causes severe injury to maize, resulting in decreased nutrient uptake, increased plant lodging and increased susceptibility to pathogens (Hou et al., 1997; Kahler et al., 1985; Kurtz et al., 2010; Riedell, 1990; Spike & Tollefson, 1991). Annual estimates of the combined cost of management and yield loss due to this injury amounts to over two billion dollars (Wechsler & Smith, 2018). WCR is notorious for evolving resistance to management practices including crop rotation, chemical insecticides, RNAi and Bt toxins (Ball & Weekman, 1962; Gassmann et al., 2011, 2016, 2020; Khajuria et al., 2018; Levine et al., 2002; Ludwick et al., 2017; Meinke et al., 1998; Parimi et al., 2006; Pereira et al., 2015; Zhu et al., 2009; Zukoff et al., 2016). Resistance to Bt can develop quickly, in as few as three generations, with evidence of cross-resistance to multiple Cry proteins (Meihls et al., 2008; Zukoff et al., 2016). Yet, a complete understanding of the mechanisms of Bt resistance in WCR remains largely unknown.

WCR have a relatively conserved microbiome with documented phenotypic functionality. Bacterial communities associated with WCR influence oviposition preference (Lance, 1992), increase tolerance to plant defenses (Chu et al., 2013) and confer mating incompatibilities between subspecies (Giordano et al., 1997). The gut bacterial communities of WCR are both transmitted vertically and filtered from the larger regional species pool encountered as they move through the soil and feed on corn roots (Chu et al., 2013; Dematheis, Kurtz, et al., 2012; Ludwick et al., 2019; Perlatti et al., 2017; Prischmann et al., 2008). Other *Diabrotica* can vector plant pathogens and

evidence suggests some rhizosphere bacteria acquired from the environment can persist through pupation and for as long as two weeks in adults (Palmer & Kommedahl 1969; Snyder et al. 1998). This system provides a unique opportunity to investigate the interaction between Bt resistance and the bacterial community as a whole in an insect with a relatively conserved microbiome known to have phenotypic functionality.

In this study, we asked i) do the bacterial communities differ between resistant and susceptible insects when feeding on non-Bt maize, ii) do the bacterial communities in resistant and susceptible insects respond differently to feeding on Bt maize, and iii) does the presence of the soil alter the communities and their response to Bt. We hypothesized that resistance to Bt would produce changes in the associated bacterial communities, and upon ingestion of Bt, we would observe changes in the community of the susceptible insects reflective of intoxication that were not seen in the resistant insects. Understanding the processes that shape microbiome composition is important to understanding the overall fitness of the host and how they respond to biotic and abiotic stresses.

3.2 Methods

Neonate WCR larvae from Bt-resistant and -susceptible colonies were fed both Bt and non-Bt maize for one and three days. The bacterial communities associated with WCR fed different diets were characterized using 16S rRNA sequencing. Experiments were conducted twice, once in an environment with soil present and once in a soilless environment.

Insects and seeds

Eggs of susceptible insects were originally purchased from Crop Characteristics (non-diapausing WCR; Farmington, MN) and subsequently maintained as a colony in Columbia, MO. The resistant colony was the same line used in Frank et al. (2013) and Geisert and Hibbard (2016) (eCry3.1Ab-resistant). At the time of experimentation, resistant larvae had been continuously selected for resistance for 43 generations on Bt corn. Adults were housed in 30 cm^3 BugDorm cages (Megaview Science Co., Ltd., Taichung, Taiwan) and provided with young maize leaves, artificial diet (Frontier Agricultural Sciences, Newark, DE), zucchini slices (*Cucurbita pepo* L.) and an agar gel water source. Cages were kept at room temperature (25 °C) with a photoperiod of 14:10 (L:D). Petri dishes filled with moist, sieved soil were placed in cages to be used as oviposition sites for mated females. Each week, eggs were rinsed with water in an 80-mesh sieve to remove soil and then placed in new Petri dishes containing moist, sieved soil. Eggs were incubated at 25 °C until neonates started to emerge. At this point, the remaining eggs were rinsed with water in a 60-mesh sieve to remove soil and placed in a 50 mL glass beaker. Any floating debris was poured off. Using a sterile 1.5 mL transfer pipette, eggs were transferred onto a clean coffee filter in a uniform layer. The coffee filter was then placed inside a sterilized 16 oz. Solo® deli container (Solo Cup Company, Lake Forest, IL) with a lid that had been punctured with holes (#0 insect pin). Eggs inside the container were allowed to hatch inside an incubator at 25 °C with a photoperiod of 14:10 (L:D) and neonates were used within the same day of hatching. Non-Bt maize seeds were purchased from Albert Lea Seed (Viking 42-92; Albert Lea Seed, Albert Lea, MN). Maize seeds expressing Bt toxin eCry3.1Ab (event 5307) were provided by Syngenta AG.

Experimental set up

Soil environment

50 mL conical tubes (Thermo Fisher Scientific, Waltham, MA) were filled with ~30 mL of a 2:1 non-autoclaved, local topsoil:Promix mixture (Premier Horticulture Inc., Quakertown, PA). Approximately 3-4 maize seeds, either Bt or non-Bt, were then placed in each tube and covered with ~10 mL of the soil mixture. Tubes were watered with ~10 mL of water and lids were loosely attached to each tube. Larvae fed for either one or three days, and each time point (one or three days) had eight replicate tubes. Tubes were placed in growth chamber at 25 °C with a photoperiod of 14:10 (L:D) and lids were removed two days later. Four days after planting, 10 neonates emerging from unsterilized eggs in deli containers were transferred to each tube with a horsehair paint brush. Tubes were returned to the same growth chamber and allowed to grow for their designated amount of time (one or three days). On the day of collection, the contents of the 50 mL tube were emptied into a modified Berlese funnel with a glass jar containing 10 mL of water attached to the base and left for one hour. Insects that fell into the jar were collected using a paint brush, rinsed with sterile water, and placed in a 1.5 mL Eppendorf tube (three insects per tube). From the eight 50 mL tubes, insects were collected from only four. Tubes were stored at -80 °C until DNA extraction.

Soilless environment

Bt and non-Bt seeds were sterilized by soaking for three minutes in 5% bleach solution followed by a triple rinse with sterile water. To aid germination, autoclaved filter papers were moistened with sterile water and placed in the bottom of petri dishes. 3-4 maize seeds were then placed in Petri dishes, and dishes were wrapped with Parafilm. Dishes

were incubated at 25 °C with a photoperiod of 14:10 (L:D) until neonates had hatched (~ 4-5 days). Freshly hatched neonates from unsterilized eggs of either eCry3.1Ab-resistant or -susceptible colonies were placed on maize seedlings with a paintbrush at a density of 20 per Petri dish, rewrapped with Parafilm and returned to the incubator. Resistant and susceptible insects feeding on either Bt or non-Bt maize were grown concurrently in quadruplicate dishes for each time point. After one and three days, three living insects were collected, immediately placed in 1.5 µL Eppendorf tubes (three insects per tube, one tube per replicate, four replicates) and promptly frozen at -80 °C to preserve bacterial colonies.

DNA extraction and 16S rRNA gene amplification

Bacterial DNA was extracted from frozen, whole larvae (3 per tube) using PowerFecal® DNA Isolation Kit (QIAGEN, Catalog No. 12830-50) in accordance with manufacturer's protocols (https://www.qiagen.com/us/resources/resourcedetail?id=00e4513c-597b-4bd5-a600-9259e6d62d07&lang=en). Initial range finding experiments determined DNA yield and quality were optimal for samples containing between 2-4 insects. DNA concentration was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA), and extracted DNA was stored at -80 °C until further downstream processing was initiated. The construction of and sequencing of 16S sequencing amplicon libraries were completed at MU DNA Core. Prior to amplification, DNA was standardized to a concentration of 3.51 ng/µL. The V4 hypervariable region of the 16S rRNA gene was amplified using single indexed universal primers (U515F/806R) with Illumina standard adapter sequences. PCR reaction steps were as follows:

$98°C^{(3:00)}+[98°C^{(0:15)}+50°C^{(0:30)}+72°C^{(0:30)}]$ for 25 cycles. The resulting amplicons (5 µL)

were pooled before sequencing on Illumina MiSeq 2 × 250 bp platform (Ludwick et al., 2019).

16S rRNA community analysis

Sequence assembly and annotation were conducted at the MU Informatics Research Core Facility. Raw sequences are available at NCBI (Bioproject number PRJNA531879). Overlaps in sequences of paired-ends were joined using FLASH (Magoč & Salzberg, 2011) and filtered after trimming for base quality of less than 31. Minimum and maximum overlap was set to 200 bp and 225 bp. Primers were trimmed using Cutadapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200/479) in two rounds, first removing forward primers with an error rate of 0.11 mismatches and minimum length of 19 bp. After discarding untrimmed contigs, a second round of trimming from the 3' end was executed with an error rate of 0.1 mismatches and minimum length of 20 bp. Contigs were removed if errors were greater than 0.5 using USEARCH (http://drive5.com/index.htm) and the remaining contigs were trimmed to a length of 248 bp. Remaining contigs were clustered *de novo* into OTUs using uparse (http://drive5.com/uparse/) and detected chimeras were removed using Qiime v1.9 (Kuczynski et al., 2012). Clustering into OTUs was done *de novo* at a 97% nucleotide identity similarity. Annotation of OTUs was conducted using BLAST against the SILVA database of 16S rRNA sequences and compiled into OTU biom tables for data analysis (Quast et al., 2013). After creation of OTU biom tables, OTUs matching to chloroplast and mitochondria were filtered and removed using phyloseq::filter_taxa in RStudio version 3.5.2 (McMurdie & Holmes, 2013). Taxa were filtered based on prevalence across samples and 1,450 taxa found to be present in only one sample were filtered out

using phyloseq::prune_taxa in RStudio. Taxa labeled ‘uncharacterized’ at the phylum level were also removed. The resulting table containing data from soilless and soil environments was used for the analysis of alpha and beta diversity in RStudio.

Statistical analysis

Wolbachia, a common insect endosymbiont, had a very high relative abundance in the majority of WCR samples, which significantly impacted inverse Simpson’s D indices but had little impact on Chao-1. Therefore, we filtered *Wolbachia* before generation of inverse Simpson’s D diversity indices. Metrics of alpha-diversity (Chao-1, inverse Simpson’s D) were generated using phyloseq::estimate_richness function on raw, non-rarefied data sets (McMurdie & Holmes, 2014). To determine if bacterial communities of susceptible and resistant insects differ and whether soil influences those differences, comparisons of Chao-1 richness and inverse Simpson’s D indices between environments (soil and soilless), colony (susceptible on non-Bt, resistant on non-Bt) and days (1 and 3) were made using a linear mixed effects model with PROC GLIMMIX in SAS 9.4. Cohorts nested within environment were treated as a blocking variable in a randomized complete block design testing for main effects of environment, colony and day as well as all two- and three-way interactions. Alpha diversity indices were rank transformed to correct for non-random residuals. Two cohorts were used to increase the statistical power from the added variation within environments. To identify changes in alpha diversity within the colonies after ingestion of Bt, we analyzed each colony in separate models (susceptible on non-Bt vs. Bt, resistant on non-Bt vs. Bt). Again, analysis of differences in Chao-1 richness and inverse Simpson’s D indices were made with environment, trait (Bt and non-Bt) and days as main effects with cohort nested within environment as the

random effect in a linear mixed effects model with PROC GLIMMIX in SAS 9.4. Alpha diversity indices were rank-transformed to correct for non-random residuals. Pairwise comparisons for all models were considered significant at $p < 0.05$.

We then investigated the differences in bacterial community composition between resistant and susceptible insects reared on Bt and non-Bt maize in both environments. Since WCR bacterial communities contained very high relative abundances of *Wolbachia*, analyses were conducted with and without *Wolbachia* to properly assess robustness of any findings. For parametric multivariate analysis of between group differences, samples were log transformed to correct for high sparsity prior to analysis using the vegan::adonis function in R (Oksanen et al., 2019). Similar to alpha diversity, analysis of beta-diversity between colonies across days on non-Bt were conducted using a three-way permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis and Jaccard distances between environments, colony and day with cohort as the random variable specified by 'strata'. Bray-Curtis distances allow us to account for the presence and absence of bacteria, as well as their relative abundance within a sample, whereas Jaccard is based on presence/absence of taxa. As we were not directly interested in the three-way interaction, if it was found to be non-significant ($p > 0.05$), it was removed from the model. Interactions found to be significant between environment and colony were followed by models and ordinations (PCoA) restricted to one environment. A significant day by environment interaction led us to analyze models on separate days to investigate the differences driving the interaction. Centroids for use in PCoA were generated by extracting PCA1 and PCA2 using vegan::betadisper and scores function within treatment groups. Axis variance measurements were taken from

phyloseq::plot_oridination output. Beta dispersion between colonies was tested using a permutational test (vegan::permutest) of the distances to centroid of each colony generated with vegan::betadisper. Pairwise comparisons were made for significant differences observed in PERMANOVA using EcolUtils::adonis.pair at corrected p <0.05 (Salazar, 2020). Large differences observed in beta diversity between environments led us to restrict beta diversity analysis to within environments to better delineate the differences in colonies when feeding on Bt. Pairwise comparisons were made for significant differences using EcolUtils:adonis.pair at corrected p <0.05. Comparisons of differentially abundant taxa present between samples were conducted using DESeq2 in RStudio with the lowest taxonomic level being genus (Love et al., 2014). We compared taxa between and within resistant and susceptible colonies using data from both cohorts combined at the day level as a more conservative estimation. Each environment was analyzed separately.

3.3 Results

We compared the bacterial communities associated with Bt-susceptible and -resistant insects after feeding on Bt and non-Bt maize seed for 1 and 3 days in two different environments, with and without soil. Sequencing of the 16S rRNA libraries generated from bacteria associated with all insects regardless of environment, day, or maize type yielded an average (± SE) of 80,190.39 ± 3,218.29 sequences. Sequence data have been deposited on NCBI under BioProject #PRJNA531879. Original OTU table and metadata with accompanying R code are available at FigShare under doi: 10.6084/m9.figshare.12974621 and doi: 10.6084/m9.figshare.12974597.

Differences in bacterial communities between Bt resistant and susceptible WCR

We found bacterial communities differed between resistant and susceptible insects and changed over time, regardless of the environment in which the insects were reared (Figures 3.1, 3.2). In comparisons of alpha diversity metrics between environments (soil vs soilless), richness and diversity as estimated by Chao-1 index and inverse Simpson D, respectively, were significantly higher in insects reared on maize seedlings in soil compared to insects reared on germinated maize seedlings in soilless petri dishes (Chao-1: $p = 0.0271$; Simpson: $p = 0.0095$; Table 3.1; Figure 3.1). Additionally, there was a significant colony × environment interaction for richness but not for diversity ($p < 0.0001$). We found susceptible insects had a 2.5-fold higher predicted richness (Chao-1) compared to resistant insects when reared on non-Bt maize seedlings in soil but found no differences between colonies in the soilless environment (Figure 3.1). Susceptible and resistant insects showed no differences in diversity as measured by inverse Simpson D within either environment.

We then compared the composition of Bt-resistant and -susceptible WCR bacterial communities reared on non-Bt maize. The three-way interaction between environment, colony and day was not significant. We found a significant colony × environment interaction, with the differences seen in environment attributed to the magnitude of the effect alone. WCR harbored significantly different bacterial communities when reared in different environments (Figure 3.2; $p = 0.001$; Table 3.1), and resistant and susceptible insects harbored distinct bacterial communities that varied with day regardless of environment (Colony: $p = 0.001$; Day: $p = 0.005$; Figure 3.2). Beta dispersion was significantly different between environments with increased heterogeneity

in insects reared in the soil environment (p = 0.001; Figure 3.2). However, we found no differences in colony beta dispersion when testing within environments (soil: p = 0.757; soilless: p = 0.075). Beta dispersion between days nested within colony was not different in either environment. The significance of these results was unchanged when *Wolbachia* was excluded from analyses.

Phylogenetic classification of OTUs from insects reared on non-Bt maize resulted in assignment to 41 unique bacterial phyla with the most common across environments being *Proteobacteria*, *Actinobacteria*, *Firmicutes and Bacteroidetes*, in order of relative abundance. Resistant and susceptible insects reared in both soil and soilless environments were dominated by the classes Alphaproteobacteria and Gammaproteobacteria, followed by the less abundant Actinobacteria and Bacteroidia. In soil, DNA recovered from resistant insects was composed of 96.9% Alphaproteobacteria and 1.61% Gammaproteobacteria, while DNA from susceptible insects was composed of 74.13% Alphaproteobacteria and 15.87% Gammaproteobacteria. DNA recovered from resistant insects reared in a soilless environment were composed of 52.61% Alphaproteobacteria and 47.24% Gammaproteobacteria, and while DNA from susceptible insects were composed of 70.22% Alphaproteobacteria and 28.43% Gammaproteobacteria (Figure 3.3).

Alphaproteobacteria includes the genus of the common insect endosymbiont *Wolbachia*, which accounts for 98.35% of the class's composition across samples. Within the Gammaproteobacteria class, several genera were commonly found across environments and insects. The most relatively abundant genera were *Serratia*, *Acinetobacter*, *Rahnella*, *Pseudomonas*, *Burkholderia-Caballeronia-Paraburkholderia,*

Klebsiella, *Azotobacter*, *Aquabacterium*, *Massilia* and *Stenotrophomonas*. The largest number of differentially abundant taxa between resistant and susceptible insects were observed in the soil environment, with the majority of taxa being enriched in the susceptible insect. In the soilless environment, resistant insects were enriched in taxa from the genera *Rahnella*, *Pantoea*, and *Bradyrhizobium*, whereas susceptible insects were enriched in taxa from the genera *Mycobacterium* and *Tsukamurella*.

Effect of Bt ingestion on resistant and susceptible WCR bacterial communities

Overall bacterial communities of resistant and susceptible insects responded differently to Bt ingestion. In susceptible insects, there was a significant interaction of environment and maize type ($p = 0.0035$) and environment and day ($p = 0.0076$) for Chao-1 richness (Table 3.2). In soil, we found bacterial richness was significantly lower when susceptible insects fed on Bt maize compared to non-Bt maize (pairwise comparison, $p = 0.016$), but was not different in the soilless environment (pairwise comparison, $p = 0.0723$; Figure 3.4a;). However, we found richness of bacterial communities of resistant insects were not different when fed Bt or non-Bt regardless of environment (main effect: $p = 0.9311$; interaction: $p = 0.5007$; Figure 3.4b). We observed a significant interaction between environment and day in resistant insects with richness increasing with age only in the soil environment ($p = 0.0236$). We observed a decrease in richness over time in the soilless environment in susceptible insects. Bacterial diversity as measured by inverse Simpson's D exhibited a similar overall pattern. Diversity of bacterial communities of susceptible insects significantly decreased when insects fed on Bt maize compared to non-Bt maize in soil (pairwise comparison, $p = <0.0001$; Figure 3.4c), but was not different in the soilless environment (pairwise comparison, $p = 0.763$). Again, no differences were

observed in bacterial community diversity of resistant insects when fed Bt or non-Bt maize, regardless of environment ($p = 0.5007$; Figure 3.4d). Susceptible insects showed a decrease in richness with age only in the soilless environment (interaction: $p = 0.0176$).

Previously observed differences in community structure based on environment led us to analyze beta diversity separately within each environment. Regardless of environment, we found a significant interaction between colony and maize type (soil: $p = 0.027$; soilless: $p = 0.022$; Figure 3.5; Table 3.2). This implies associated bacterial communities of resistant and susceptible WCR are distinct in their response to Bt ingestion by the insect. As expected, when susceptible insects fed on Bt expressing maize, their bacterial communities were significantly different than when feeding on non-Bt expressing maize in both soil (pairwise comparison: $p = 0.017$) and soilless environments (pairwise comparison: $p = 0.036$). Yet, when resistant insects fed on Bt, the structure of their bacterial communities remained relatively unchanged (pairwise comparison, soil: $p = 0.36$; soilless: $p = 0.47$; Figure 3.5). Beta dispersion between colonies on Bt and non-Bt was not significantly different in either environment (soil: $p = 0.926$, soilless: $p = 0.167$). We were interested in the impact the number of days feeding would have on the bacterial communities of resistant and susceptible insects. Two separate models were used for one day or three days of feeding. We found a significant three-way interaction between environment, colony and maize type only after one day of feeding (Day 1: $p = 0.011$; Day 3: $p = 0.585$). Again, the significance of these results was unchanged when *Wolbachia* was excluded from analyses. These results demonstrate susceptible insects experience disruption of their bacterial communities (dysbiosis), whereas resistant insects prevent or contain bacterial community disturbances.

As expected, both resistant and susceptible insects feeding on Bt were dominated by Alphaproteobacteria and Gammaproteobacteria. However, the response of the resistant and susceptible communities to Bt was unique. When feeding on Bt-expressing maize, DNA recovered from susceptible insects was composed of 57.84% Alphaproteobacteria and 40.02% Gammaproteobacteria in the soilless environment, and 50.91% Alphaproteobacteria and 47.31% Gammaproteobacteria in the soil environment. This represents an 11.59% and 31.26% increase in Gammaproteobacteria relative abundance in the soilless and soil environments, respectively, when compared to communities within susceptible insects that had been reared on non-Bt maize (Figure 3.3). Resistant insects experienced much smaller perturbations. DNA recovered from resistant insects reared in the soilless environment and fed Bt maize were composed of 53.24% Alphaproteobacteria and 46.26% Gammaproteobacteria while soil reared insects were composed of 88.22% Alphaproteobacteria and 1.04% Gammaproteobacteria. We found a decrease in Gammaproteobacteria relative abundance of 0.98% in the soilless environment and 0.57% in the soil environment compared to resistant insects reared on non-Bt maize (Figure 3.3). To further investigate these shifts in Gammaproteobacteria, we compared differentially abundant genera between susceptible insects after feeding on Bt or non-Bt maize and found several OTUs were in higher relative abundance in Bt fed insects (Figure 3.6). Among these OTUs were the genera *Klebsiella*, *Citrobacter*, *Serratia*, and *Acinetobacter*. Several less abundant taxa decreased in relative abundance in Bt fed susceptible insects compared to non-Bt fed insects. In order of magnitude of decrease, these genera were *Lysobacter*, *Steroidobacter*, *Acidibacter*, *Haemophilus* and *Rhodanobacter* (Figure 3.6). We found no differentially abundant genera in the soilless

environment using the same method. Similarly, we found no differentially abundant taxa from the Gammaproteobacteria class in resistant insects after feeding on Bt in either environment. Specific taxa are changing in relative abundance in response to Bt ingestion in the susceptible insect, but these changes are minimal in resistant insects.

3.4 Discussion

Evolution of resistance to pesticides is an increasingly salient issue (Gould et al., 2018). In the case of Bt, elucidation of resistance mechanisms mainly focuses on alterations in toxin binding sites, upstream processing/activation of toxins or broad identification of genetic loci involved with resistance (Flagel et al., 2015; Pardo-Lopez et al., 2013). However, evidence of septicemia induced by endogenous bacteria exists and led us to consider whether resistance to Bt could affect the microbiome of insects (Broderick et al. 2006; Caccia et al., 2016). Using 16S rRNA amplicon sequencing, we characterized the bacterial communities associated with resistant and susceptible WCR larvae after feeding on Bt and non-Bt maize. We found Bt resistance is correlated with a simplified bacterial community that is unresponsive to Bt ingestion. In comparison, WCR susceptible to Bt experience disturbances in their bacterial community after feeding on Bt for one day, further implicating the role of septicemia in Bt induced mortality.

WCR larvae live in a rich, microbial landscape that has a direct impact on its bacterial community. We found significantly higher richness and diversity in bacterial communities of WCR reared in soil compared to without soil (Figure 3.1). Previous work has shown WCR selects for a conserved bacterial community regardless of the soil bacterial community composition reared in (Ludwick et al., 2019), yet it appears the

presence of the soil has a significant impact on the composition of the community. Bacterial communities of insects reared in soil were more heterogenous, more diverse and more taxonomically rich than when reared without soil (Figures 3.1, 3.2). The maize root rhizosphere is a diverse microbial community that is largely shaped by root exudates and the microbial bank provided by the soil, which can be influenced by numerous abiotic and biotic factors (Bais et al., 2006; Berg & Smalla, 2009). Emerging neonates encounter bacteria attached to the chorion of eggs as well as any bacteria colonizing the maize root rhizosphere or the maize root when feeding. In the soilless environment, access to bacterial inoculum is limited to the egg and any surviving endophytes on the maize root. These differences could be driving the divergence between the environments.

The differences between environments had little impact on the differences seen between resistant and susceptible colonies reared within them. We found bacterial communities of resistant and susceptible insects were compositionally distinct from each other in both the soil and soilless environments (Figure 3.2). The largest differences were seen in the soil environment where resistant insects harbored significantly fewer taxa compared to susceptible insects (Figure 3.1). Similar responses have been documented in mosquitos where Bt-tolerant larvae harbor a microbiome with significantly fewer species of bacteria and is less diverse overall (Tetreau et al., 2018). These findings suggest Bt-resistant insects are more selective of occupying bacteria.

Previous studies have documented intrusion of midgut luminal bacteria into the hemocoel of Bt intoxicated insects leading to septicemia (Caccia et al., 2016; Mason et al., 2011). We hypothesized this type of disruption would be less evident in resistant insects. Bacterial communities of susceptible insects when feeding on Bt are disrupted in

both the soil and soilless environments (Figure 3.5). Susceptible insects fed Bt harbored a bacterial community significantly reduced in richness and diversity in the soil environment. We found no such change in the community composition in resistant insects (Figure 3.4). This lack of change could be the result of reduced toxin binding or containment of dysbiosis, but the identification of the cause is outside the current scope of this study. However, as evidenced in the susceptible insects, Bt can induce dysbiosis in the WCR larvae. These changes were likely driven in part by the higher relative abundance of taxa in the genera *Klebsiella*, *Citrobacter*, *Serratia*, and *Acinetobacter* in Bt-fed insects (Figure 3.6). Plant-expressed Bt toxins do not induce 100% mortality in WCR larvae, and likely rely on secondary factors for killing the host (Binning et al., 2010; Hibbard et al., 2011). Many of the bacterial genera in higher relative abundance in susceptible insects feeding on Bt cause disease in WCR and other Coleoptera (Hamilton, 1968; Moore, 1971; Pu & Hou, 2016). Root herbivory affects the rhizosphere microbial community (Dematheis, Zimmerling, et al., 2012; Grayston et al., 2001), and previous work documents that maize roots infested with WCR promote the growth of certain *Acinetobacter* and *Serratia* species (Dematheis, Zimmerling, et al., 2012; Prischmann et al., 2008). A few of these species were isolated from diseased adult *Diabrotica* and can be found in higher relative abundance in intoxicated larvae in our study (Benitez et al., 2017; Dematheis, Zimmerling, et al., 2012; Prischmann et al., 2008). There exists a rich microbial community in the soil and the rhizosphere, and with increased contact with bacteria, WCR are highly likely to encounter species capable of becoming pathobionts under certain conditions, particularly those within the midguts disrupted by Bt toxins. Whereas it is possible changes observed in the susceptible insect are a result of starvation

and not Bt intoxication, the presence of chloroplast in susceptible insects alive at the time of collection suggests feeding still occurred. Bacteria present on the cuticle of the insect could contribute to the heterogeneity of the communities seen in the soil, but relative abundance of bacteria are generally higher than those of plant associated sequences suggesting higher level establishment inside the insect (Hammer et al., 2017). Additionally, the differences seen between resistant and susceptible insects would more likely reflect changes in internal filtering by the insect rather than external since insects were reared in identical substrates.

It is clear microorganisms harbored by invertebrates can influence nutrition, reproduction, insecticide susceptibly and interactions with predators and pathogens (Douglas, 2009; Kikuchi et al., 2012; Oliver et al., 2005; Salem et al., 2013; Vásquez et al., 2012). Selection for resistance to Bt could affect the microbiome in several ways. There could be an advantage to harboring a heightened immune system, inducible or constitutive, to reduce potentially pathogenic bacteria, especially in a continuously selective environment (Hamilton et al., 2008). Transcriptomic analyses following Bt ingestion have identified genes involved with immunity (Dubovskiy et al., 2016; Sayed et al., 2010; Zhao et al., 2019), and suppression of immune response can increase host susceptibility (Broderick et al., 2010; Caccia et al., 2016; Shrestha et al., 2010). In one caterpillar species, *Galleria mellonella*, resistant insects had constitutively higher expression of certain immune response genes, potentially priming the insect for ingestion of Bt (Dubovskiy et al., 2016). This priming can occur trans generationally in *Tribolium castaneum* with offspring from Bt infected host experiencing increased survival (Tate et al., 2017). As components of the immune response are believed to be responsible for

controlling endosymbiont and gut microbe communities (Login et al., 2011), resistance to Bt could indirectly limit the number of species of bacteria and their abundance within the insect.

Selection for Bt resistance could potentially favor bacteria known to degrade Cry proteins or alter the gut environment (i.e. biofilms, antimicrobials) to reduce binding or competitively exclude harmful bacteria (Patil et al., 2013; Shan et al., 2014; Vásquez et al., 2012). In addition, the harboring of nutrient-providing or plant-digesting bacteria could increase tolerance to Bt as nutritional differences in diet correlate to alterations in Bt susceptibility (Deans et al., 2017; Ludwick et al., 2018). Previous experiments involving combinations of bacterial spray formulations, spores and purified crystals, following antibiotic treatment on artificial diet have yielded conflicting results (Johnston & Crickmore, 2009; Raymond et al., 2009). Broderick et al. (2006) used an *E. coli* strain engineered to produce the Bt toxin and demonstrated that toxin alone is not sufficient to induce significant mortality. It is also possible to examine the effect of the Cry toxin alone by using Bt crops expressing the active toxin. In one such study, axenic insects had marginally higher survival compared to non-axenic ones reared on Bt-expressing maize (Hilbeck et al., 2018). In conjunction, they found mortality was delayed in axenic insects, a common finding in previous work using various sources of Bt other than genetically modified plant material (Broderick et al., 2006, 2009; Hilbeck et al., 2018; Johnston & Crickmore, 2009; Raymond et al., 2009). Bt has been shown to more severely affect younger larvae with evidence of older larvae losing binding sites altogether (Ali & Young, 1996; Rausell et al., 2000). By simplifying the bacterial community and

controlling large fluctuations in community composition, WCR may be able to evade or delay Bt induced mortality by outpacing/outgrowing the toxin.

The mode of action of Bt involves complex interactions between toxin binding, native bacteria, nutrition and the host immune response. We found that resistance to Bt in WCR can result in altered bacterial communities between resistant and susceptible insects. Furthermore, those communities in susceptible and resistant insects varied in their response to Bt. Dysbiosis was induced only in the susceptible insects after feeding on Bt expressing maize. Studies with other animals have shown that dysbiosis frequently precedes disease states (Kamada et al., 2013; Raymann et al., 2017). Indeed, toxin binding/activation is crucial to Bt intoxication, but additional routes of resistance are likely. These results add to the growing body of evidence of gut bacterial community involvement in Bt susceptibility. As such, while characterization of the WCR microbiome is increasing, elucidation of the biological role of specific taxa is lacking. Further investigation into the role of native bacteria in the WCR could deepen the understanding of possible resistance mechanisms and provide targets for new management strategies for this challenging pest.

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Table 3.1 Alpha and beta diversity differences between insect colonies, environments, days and their interaction

Response	*Factor*	*df*	*F*	*p*
Chao-1				
	Environment	1,2	35.44	**0.0271**
	Colony	1,54	22.76	**<0.0001**
	Day	1,54	0.06	0.8069
	Environment × Colony	1,54	5.2	**0.0266**
	Environment × Day	1,54	3.33	0.0737
	Colony × Day	1,54	5.2	**0.0266**
	Environment × Colony × Day	1,54	0	0.9443
Inverse Simpson's D				
	Environment	1,2	103.94	**0.0095**
	Colony	1,54	0.41	0.5249
	Day	1,54	3.69	**0.0602**
	Environment × Colony	1,54	2.59	0.113
	Environment × Day	1,54	1.81	0.1839
	Colony × Day	1,54	0.78	0.3813
	Environment × Colony × Day	1,54	0.94	0.3359
Community				
	Environment	1,63	42.417	**0.001**
	Colony	1,63	5.245	**0.001**
	Day	1,63	1.975	**0.006**
	Environment × Colony	1,63	5.698	**0.001**
	Environment × Day	1,63	1.38	**0.043**
	Colony × Day	1,63	1.091	0.159
Beta dispersion				
	Environment	1,62	39.44	**0.001**
	Colony	1,62	4.5659	**0.035**
	Day	1,62	0.2764	0.615

Note

Results of models for western corn rootworm bacterial community richness (Chao-1) and diversity (inverse Simpson's D) and composition from Bt-resistant and -susceptible colonies. Experimental data are from assays conducted in soil or soilless environment where insects fed on non-Bt expressing maize roots for one or three days (day). Richness

and diversity results are from ANOVA using rank transformed data with two replicated cohorts as a blocking variable. Community results are from multivariate PERMANOVA model using a Bray-Curtis distance.

Table 3.2 Alpha and beta diversity differences between maize trait, environments, days and their interaction

Condition	*Response*	*Factor*	*df*	*F*	*p*
Susceptible					
	Chao-1				
		Environment	1,2	128.44	**0.0077**
		Trait	1,54	0.21	0.6461
		Day	1,54	0.75	0.3914
		Environment × Trait	1,54	9.33	**0.0035**
		Environment × Day	1,54	7.68	**0.0076**
		Trait × Day	1,54	0.37	0.5439
		Environment × Trait × Day	1,54	1.53	0.2216
	Inverse Simpson's D				
		Environment	1,2	18.7	**0.0495**
		Trait	1,54	14.22	**0.0004**
		Day	1,54	2.31	0.1346
		Environment × Trait	1,54	11.17	**0.0015**
		Environment × Day	1,54	5.99	**0.0176**
		Trait × Day	1,54	3.19	0.0796
		Environment × Trait × Day	1,54	0.01	0.9433
Resistant					
	Chao-1				
		Environment	1,2	14.74	0.0616
		Trait	1,54	0.01	0.6461
		Day	1,54	6.44	**0.0141**
		Environment × Trait	1,54	9.33	0.5007
		Environment × Day	1,54	5.43	**0.0236**
		Trait × Day	1,54	1.7	0.1978
		Environment × Trait × Day	1,54	0.07	0.7953
	Inverse Simpson's D				
		Environment	1,2	37.36	**0.0257**
		Trait	1,54	3.91	0.0531
		Day	1,54	2.66	0.1085
		Environment × Trait	1,54	0.89	0.351
		Environment × Day	1,54	1.76	0.1908
		Trait × Day	1,54	0.09	0.7599

		Environment × Trait × Day	1,54	3.99	0.05009
Soil					
	Community				
		Colony	1,63	7.0224	**0.001**
		Trait	1,63	1.7772	**0.031**
		Day	1,63	3.143	**0.003**
		Colony × Trait	1,63	1.7279	**0.027**
		Colony × Day	1,63	1.0189	0.297
		Trait × Day	1,63	0.8895	0.463
Soilless					
	Community				
		Colony	1,63	9.4306	**0.001**
		Trait	1,63	1.5619	**0.024**
		Day	1,63	1.8561	**0.007**
		Colony × Trait	1,63	1.5219	**0.022**
		Colony × Day	1,63	1.5782	**0.024**
		Trait × Day	1,63	0.7149	0.533

Notes. Results of models for western corn rootworm bacterial community richness (Chao-1), diversity (inverse Simpson's D) and composition from Bt-resistant and -susceptible colonies. Experimental data are from assays conducted in soil or soilless environment where insects fed on Bt or non-Bt expressing maize (trait) for one or three days (day). Results for richness and diversity are from ANOVA using rank transformed data with two replicated cohorts as a blocking variable. Community composition results are from multivariate PERMANOVA model using a Bray-Curtis distance matrix with log-transformed data.

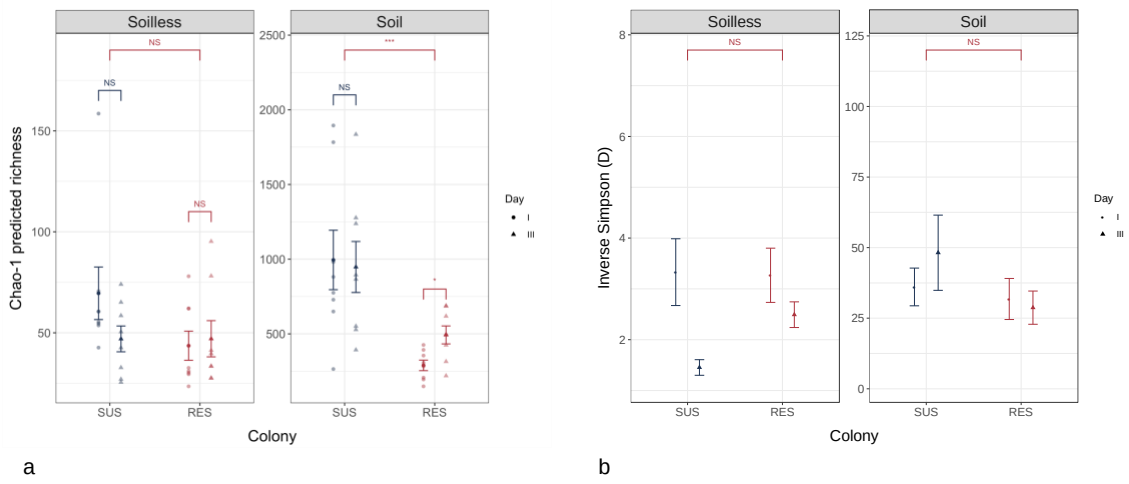

Figure 3.1 Comparisons of **a**) richness (Chao-1) and **b**) diversity (Inverse Simpson's D) of Bt-resistant and -susceptible western corn rootworm larval bacterial communities when fed on non-Bt maize in two different environments, with and without soil. Sample means are represented as a bold data point with accompanying standard error bars. Differences are pairwise comparisons from the three-way interaction with environment, trait and day using LSMeans at $p < 0.05$.

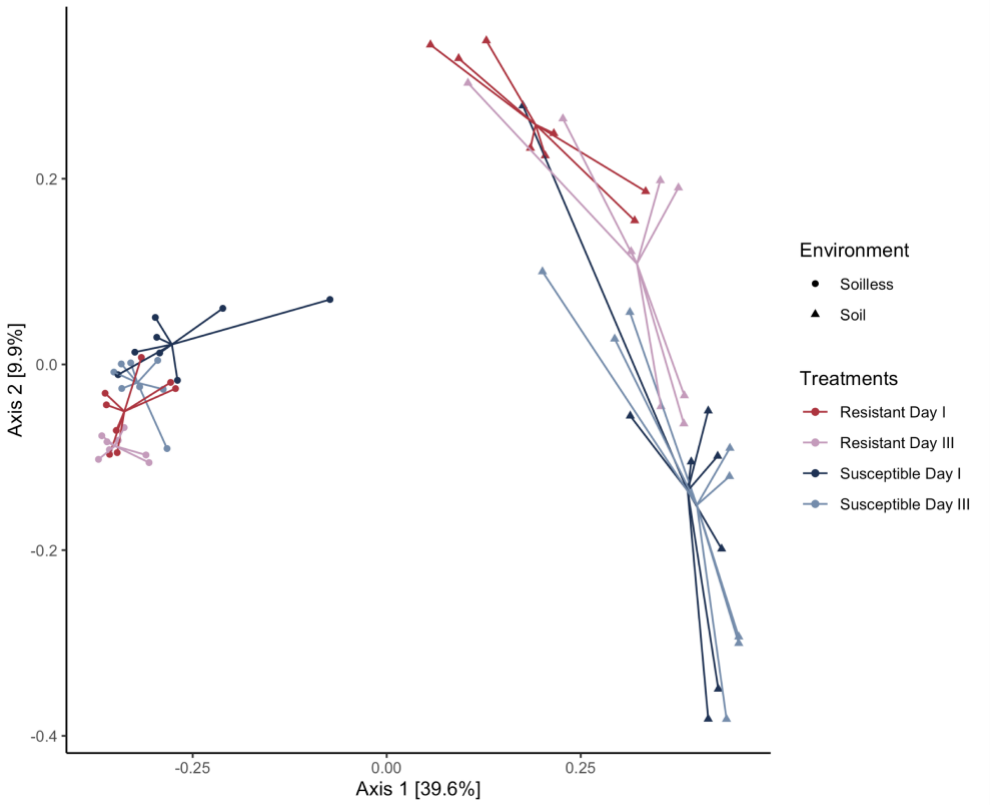

Figure 3.2 Principal coordinate analysis of bacterial communities in Bt-resistant and -susceptible western corn rootworm larvae reared on non-Bt maize roots in soilless and soil environments for one and three days. Centroids are based on beta-dispersion of colonies on each day in each environment.

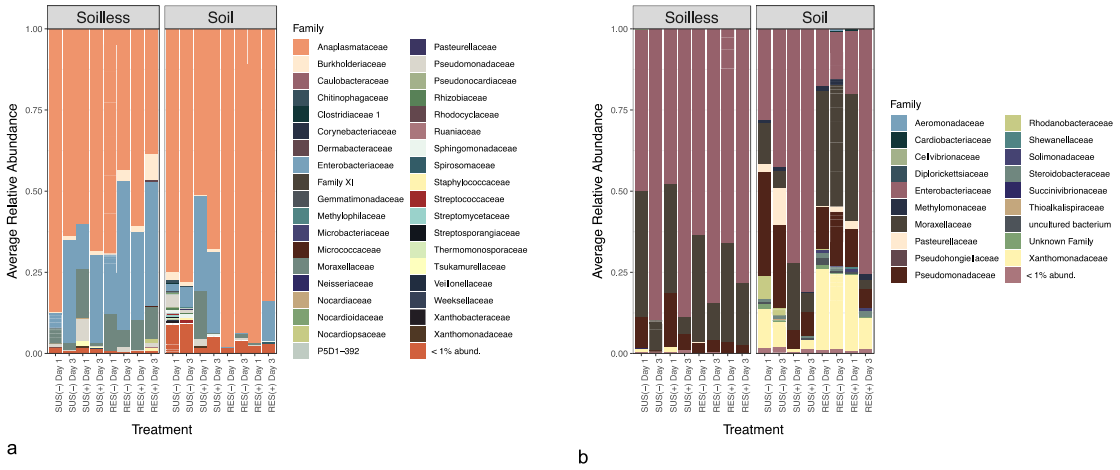

Figure 3.3 Family level stacked bar chart of average relative abundance of **a**) total bacterial communities and **b**) Gammaproteobacteria class from Bt-susceptible and -resistant western corn rootworm larvae reared in soil and soilless environments on Bt or non-Bt expressing maize roots for one and three days. Each bar is an average of eight samples from two replicated experiments. ((+) = Bt maize roots, (-) = non-Bt maize roots)).

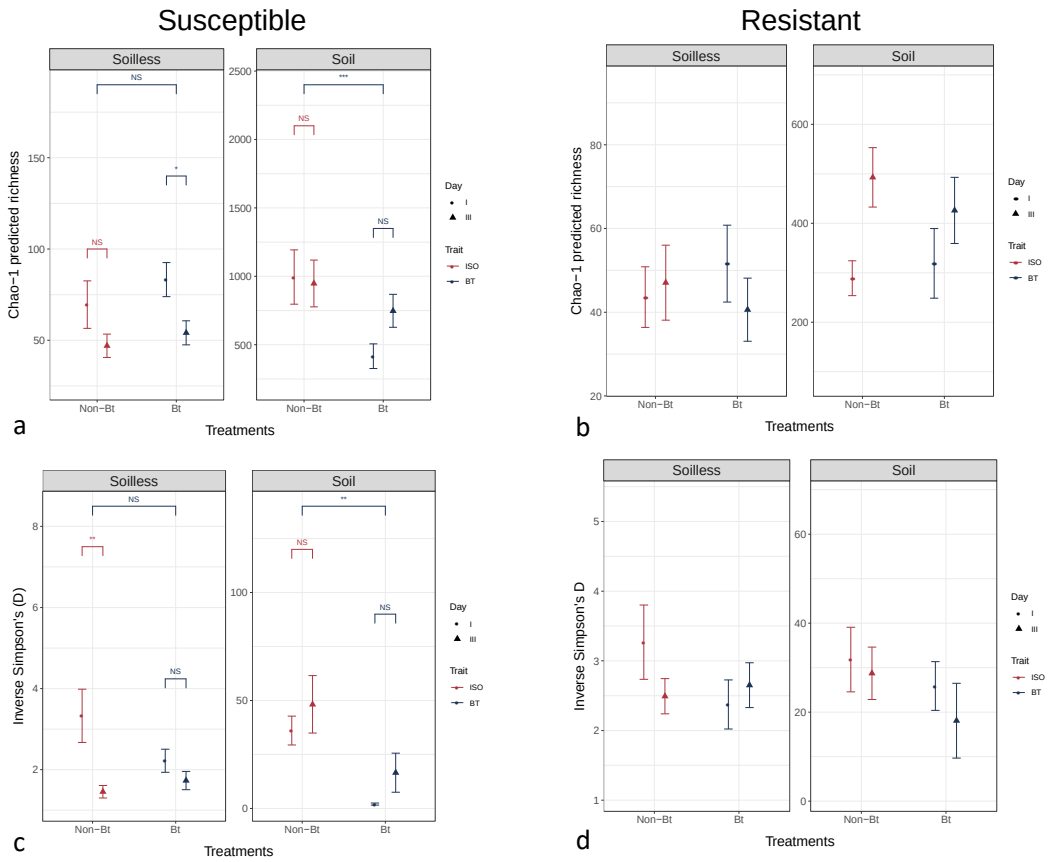

Figure 3.4 Comparisons of richness (Chao-1) and diversity (Inverse Simpson's D) of **a**) Bt-susceptible and **b**) -resistant western corn rootworm larval bacterial communities reared on Bt and non-Bt maize roots for one and three days in two different environments, with and without soil. Sample means are represented as a bold data point with accompanying standard error bars. Differences are based on pairwise comparisons from three-way interaction with environment, maize type and day for each colony using LSMeans at $p < 0.05$.

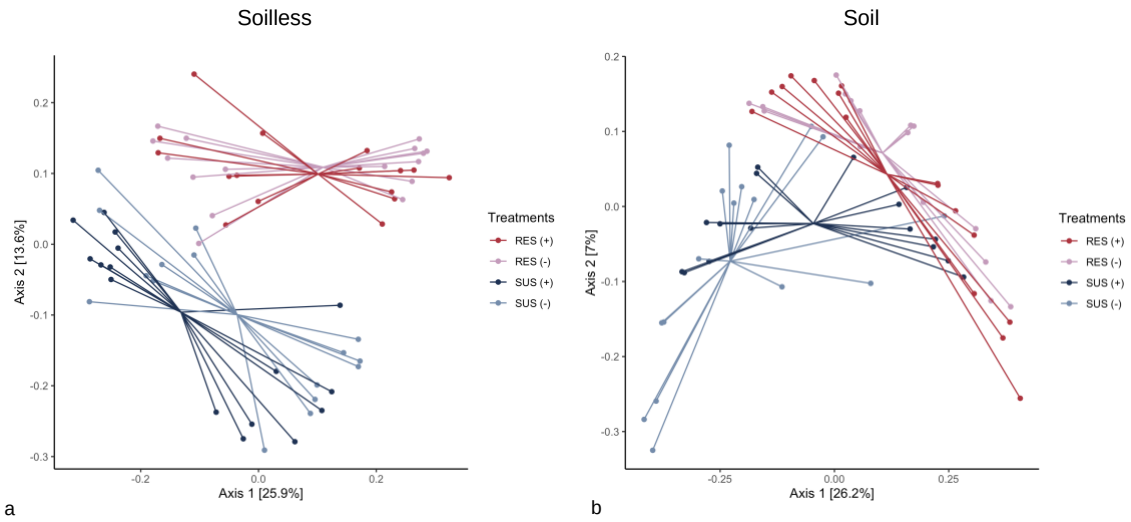

Figure 3.5 Principal coordinate analysis of bacterial communities in Bt-resistant and -susceptible western corn rootworm larvae reared on non-Bt and Bt maize roots in **a**) soilless and **b**) soil environments for one and three days.

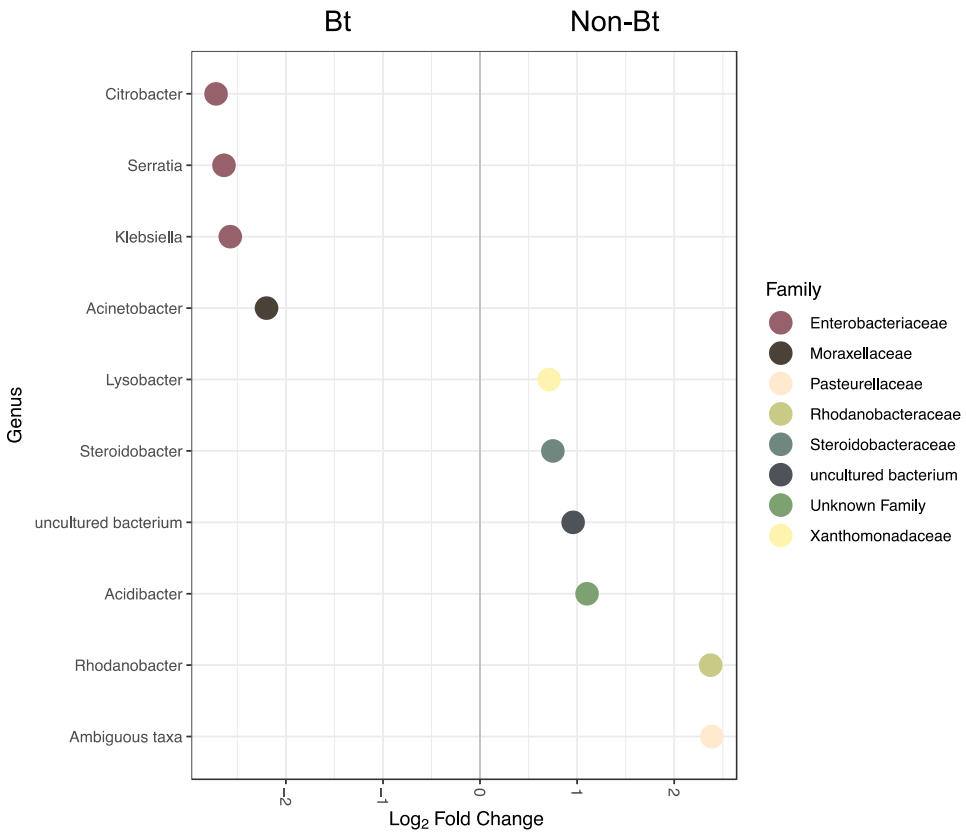

Figure 3.6 Differentially abundant genera in the class Gammaproteobacteria in Bt-susceptible larvae fed on either Bt or non-Bt maize roots in soil environment. Significant differences in abundance between treatments were calculated using a negative binomial distribution in a generalized linear model in the DESeq2 package (R Studio) with a $p < 0.05$.

Chapter 4: Soil microbes from conservation agriculture systems reduce growth of Bt-resistant western corn rootworm

4.1 Introduction

A major goal of sustainable cropping practices is to maintain or improve soil health, defined as, "the continued capacity of a soil to function as a vital living ecosystem that sustains plants, animals, and humans" (e.g., Karlen et al., 2019). Soil health can be modified through management practices that change soil disturbance, residue and amendment inputs, soil cover, and crop diversity (Doran, 2002; Veum et al., 2015; 2022). In turn, management systems integrating soil health conservation practices experience increased soil organic matter, reduced erosion, lower nutrient loss, higher pathogen suppression, and increased microbial abundance (Nunes et al., 2020a,b; Veum et al., 2015; 2022; Hartwig & Ammon, 2002; Kim et al., 2020).

Common practices aimed at improving soil health include planting cover crops, reducing tillage, and expanding crop rotations (USDA NASS, 2017; Wallander et al, 2021). Instead of leaving a field fallow with bare soil over the winter, a grower can seed cover crops in the fall immediately before or after harvest of the cash crop. The cover crop will overwinter and be terminated the following spring. Although a corn yield drag has been observed under some conditions (Liedgens et al., 2004), cover crops can potentially increase cash crop production by improving several soil health metrics (Hartwig & Ammon, 2002; Kim et al., 2020). Tillage operations are known to lead to dramatic losses in soil organic matter and soil health status, whereas reduced tillage or no-till systems can maintain or restore soil health (e.g., Nunes et al., 2020a,b; Veum et

al., 2015; 2022). Integrating cover crops into no-till systems can further boost surface soil carbon and water infiltration (Mitchell et al., 2017). Enhanced above-ground biodiversity, achieved through extended crop rotations, may contribute to improved crop productivity, nutrient cycling, pathogen suppression, and water dynamics (Tilman et al., 2014). In contrast, low diversity cropping systems, such as monoculture or two-crop systems, can reduce soil health and productivity (Schmer et al., 2020; Chahal et al., 2021). Growers are continuing to adopt practices increasing soil health; farmland acreage planted with cover crops totaled over 15 million in the US in 2017, an increase of 50% from 2012 (USDA NASS, 2017). Over 100 million acres of farmland practice no-till, and rates are 2-3 times higher in systems using cover crops (Wallander et al., 2021).

Soil management practices can also affect insect pest populations, an important concern for growers as insect pests reduce yields by 20% annually (Culliney, 2014). A majority of studies investigating the effects of cover crops and no-till on insect pests focus on the benefits of promoting arthropod predator populations as a top-down control tactic (Bowers et al., 2021; Lundgren & Fergen, 2010; Prasifka et al., 2006; Rivers et al., 2020), but these studies yield variable outcomes (Fox et al., 2016; Rowen & Tooker, 2021). Cropping systems that incorporate multiple conservation practices, such as extended rotations with cover crops and reduced tillage, increase soil health by supporting diverse microbial communities and enhancing nutrient cycling and availability (Lehman et al., 2015). Yet considerably less work focuses on bottom-up pest suppression through changes in the soil microbiome. Moreover, studies investigating the effect of soil microbes on plant-insect interactions rarely examine the insect microbiome and instead focus on changes in the plant and its microbiome. Insects can use microbes to overcome

plant defenses (Chu et al., 2013), supplement nutrition (Douglas, 2009), and resist insecticides (Kikuchi et al., 2012). Oftentimes, these microbes are acquired from the environment (Kikuchi et al., 2007; van den Bosch & Welte, 2017). Thus, changes in the plant or soil microbiome may disrupt insect microbiomes and impact insect pest management.

It is increasingly clear that soil microbiomes can improve plant health and alter plant defenses against pests (Chaparro et al., 2012; Pineda et al., 2010). Examples of microbe-induced pest resistance in cover crop systems exist (Blundell et al., 2020; Krey et al., 2020), but are often confounded with organic farming practices, of which cover crops are only one aspect of management. In reality, the vast majority of cover crop no-till acreage has been adopted on non-organic farms (USDA NASS, 2017). Despite the growing interest in plant-soil-microbial interactions, the effects of management on belowground insects are rarely investigated even though root herbivory has significant impact on ecosystems (Hunter, 2001). Therefore, it is necessary to investigate the effects of conservation management systems on belowground pest suppression via the soil microbiome in a more traditional agricultural setting.

One of the most damaging belowground crop pests in the U.S. is the western corn rootworm (WCR), *Diabrotica virgifera virgifera*. The immature larvae feed on corn roots causing severe damage that results in decreased plant nutrient uptake, decreased plant stability, and increased susceptibility to pathogens (Hou et al., 1997; Kahler et al., 1985; Kurtz et al., 2010; Riedell, 1990; Spike & Tollefson, 1991). The combined cost of damage and management of WCR accounts for an estimated $2 billion annually (Wechsler & Smith, 2018). WCR management relies heavily on transgenic corn that

produces toxins derived from *Bacillus thuringiensis* (Bt). Consequently, the continuous planting of Bt corn has led to the evolution of resistance. All four commercially available transgenic Bt products (Cry3Bb1, mCry3a, eCry3.1Ab, and Gpp34/Tpp35) have seen failures in the field while the number of acres planted with Bt continues to increase (Gassmann, 2021). Given the concurrent increase in cover crop utilization, it is important to understand how these two management strategies interact and how their interaction influences WCR management.

Current evidence suggests the WCR microbiome may be susceptible to management-driven disruption of microbial communities. It is likely that WCR are highly adapted to corn root microbes. There is overlap in corn root microbiomes and WCR microbiomes (Dematheis et al., 2012; Ludwick et al., 2019), but the extent to which larval WCR acquire microbes from the environment is not well understood (Ludwick et al., 2019). Larvae select for a relatively conserved bacterial community that is refined with age (Ludwick et al., 2019). Continuous selection on Bt alters the larval microbiome suggesting it may play a role in susceptibility and resistance to Bt (Paddock et al., 2021). As adults, WCR can use microbes to overcome plant defenses to exploit a new host plant (Chu et al., 2013), but their microbiome varies based on their geographical location (Paddock et al., 2022).

Here, we examined the impact of soil microbiomes from a long-term conservation management system [a corn (*Zea mays* L.)-soybean (*Glycine max* L. Merr)-wheat (*Triticum aestivum*) rotation under no-till with cereal rye (*Secale cereale*) cover crops] and a conventional management system (mulch till corn-soybean rotation without cover crops) on the microbiome of WCR feeding on two different corn lines (Bt and non-Bt).

Our hypothesis was that changes in soil microbiomes introduced through varying management practices would alter the corn root rhizosphere. These changes may then disrupt the microbiome of WCR larvae. Given the influence of microbiomes on host fitness, we sought to compare how these soil health management practices affect plant-insect interactions. We paired fitness assays with 16S rRNA gene sequencing to capture microbe-mediated changes to corn and WCR fitness. We hypothesized that rhizosphere community compositional changes would reduce WCR fitness in the presence of microbes from the conservation management system. Taken together, this study will inform future pest management decisions for growers integrating conservation management practices such as cover crops, no-till, and extended rotations into a traditional row crop setting.

4.2 Methods

Sample collection and preparation

The experimental site is located at the Goodwater Creek Experimental Watershed in Centralia, Missouri and managed by the USDA-ARS Cropping Systems and Water Quality Research Unit. The experimental plots were established in 1991 to study the productivity and environmental effects of conservation management practices. In 2012, the site was selected as one of the 10 initial ARS Long-Term Agroecosystem Research (LTAR) network sites and was named the Central Mississippi River Basin (LTAR-CMRB) site. Research conducted at this site has quantified the benefits of conservation management practices, including crop productivity, water quality, and soil health (Sadler et al., 2015; Veum et al., 2015). Ten management systems represent a continuum of

variable crop rotation, tillage, and cover crop practices. Research plots at the site are 18 by 189 m (59 by 620 ft) running east-west and the experimental design is a randomized complete block with three replications (Figure 4.1a). Soils at the site include Adco silt loam (fine, smectitic, mesic Vertic Albaqualfs) and Mexico silt loam (fine, smectitic, mesic Vertic Epiaqualfs). Cropping system treatments selected for this study included 1) a conservation management system in a corn-soybean-wheat rotation under no-till with cereal rye cover crops, and 2) a conventional management system in a corn-soybean rotation under mulch tillage with no cover crops (Figure 4.1b). Further details of the management practices and history can be found in Table 4.1.

In April 2021, soil cores were collected to 10 cm depth with a 3.18 cm diameter probe from the replicated conservation and conventional management system plots. Each system was in the corn phase of the crop rotation the year before. Crop residues were removed from the surface prior to sampling, and the soil probe was sterilized with 95% ethanol between locations. Five soil cores were collected and combined into a single composite sample at each of four locations within each plot (~150 ft apart) for a total of 12 soil samples per management system (Figure 4.1c). Samples were transported back to the laboratory at the University of Missouri-Columbia in a cooler with ice packs where they were placed in a 4°C refrigerator until further processing one week later.

Each soil sample was homogenized and passed through a steel sieve (1 cm) to remove any large debris. Sieved soil was homogenized by mixing, and 30 g was transferred to two separate 50 mL flip top tubes (Nalge Nunc International, Rochester, NY, USA). Three 1 mL soil samples were subsampled and stored at -80°C for subsequent DNA extraction and sequencing. Soil slurries were prepared based on previously

published methods (Walsh et al., 2021). To prepare microbial soil slurries, 37.5 mL of 1× PBS (7.4 pH) was added to each tube, and the tubes were shaken on their side on a platform shaker (New Brunswick Scientific, Edison, New Jersey) at a speed of 40 rpm for 30 minutes. Soil debris was separated from the microbial layer by centrifugation at 600 g for 4 min (Walsh et al., 2021; Allegra 25R, Beckman Coulter, Brea, CA, USA). The supernatant was transferred to a clean 50 mL tube. Three 1 mL inoculum samples were also collected and stored at -80°C for later DNA extraction and sequencing. Inoculum were used in bioassays the same day of preparation.

Bioassays

The experiment was designed as a 2×2×3 factorial design with 2 microbiome types (conservation and conventional), 2 corn types (Bt and non-Bt corn), and 3 rootworm infestation types (resistant, susceptible, and uninfested). Given the size of the experiment, a block was set up every other day over the course of one week for a total of four blocks matching the sampling location of the field. Thus, each sampling location within a replicated plot was treated as a block during the bioassay. Microbiome treatment and corn type were randomized within blocks. Paired infested and uninfested treatments were used to calculate relative root damage. Each experimental unit was run in duplicate to allow for destructive fitness and microbiome sampling, resulting in a total of 288 experimental units.

Assays were conducted in 50 mL flip top tubes (Nalge Nunc International, Rochester, NY, USA). First, tubes were filled with 30 mL of soil mixture. The soil mixture of two parts top-soil and one part Promix (Premier Horticulture Inc., Quakertown, PA, USA) was double-autoclaved, allowed to cool, and passed through a

sterile sieve (2mm) to remove large soil particulates and rock before use in bioassays. The day before planting, corn seeds of both genotypes were surface sterilized by soaking in 5% bleach solution, triple-rinsed with sterile DI water and left to soak overnight to speed germination in the tubes. Each 50 mL tube was seeded with two kernels of corn and then overlayed with 1 mL of microbial inoculum per seed. The seeds were then covered with 10 mL of soil, watered with 5 mL of DI water, and left for 30 minutes to allow water to permeate the soil. An additional 5 mL of water was then added to each tube, and caps were closed for two days to retain moisture until seeds germinated inside a growth chamber (16:8 L:D). Five days after planting seeds, tubes were infested with six neonate larvae of either Bt-resistant (Frank et al. 2013; Geisert and Hibbard 2016; Paddock et al. 2021) or Bt-susceptible WCR (non-diapausing WCR; Crop Characteristics, Farmington, MN, USA) by transferring living larvae with a horse-hair paintbrush. Tubes were returned to growth chamber for the duration of the bioassay and watered as needed throughout (~2 days).

Upon completion of the assay, tubes used for fitness measurements were emptied into Berlese funnels with a collection jar filled with water attached to the bottom to collect living larvae. Jars were collected after 24 hours, and larvae were counted and placed in 1.5 mL tubes containing ethanol. Larvae were dried at 40°C in an oven for one week. Plant fitness data were collected as follows. Aboveground tissue on corn seedlings was removed at the base of the mesocotyl. The remaining tissue was collected and rehydrated for 24 hours, washed to remove excess soil, and weighed. Roots were then allowed to dry in oven at 40°C for one week after which dry weights were recorded.

For microbiome sample collection, contents of the 50 mL tubes were emptied into a sterile metal container. Resistant larvae found moving were collected with a horse-hair paintbrush, surfaced sterilized with 70% ethanol, and transferred to sterile garnet beaded tubes (4 larvae max per tube). Tubes were frozen at -80°C. Roots were lightly disturbed to separate soil from the rhizosphere (~1mm surrounding roots). The roots and rhizosphere were transferred to sterile 50 mL flip top tube filled with 30 mL of 1× PBS. Tubes were fastened, shaken by hand, and then allowed to sit for 30 minutes to settle the rhizosphere soil before debris was removed. Rhizosphere samples in tubes were spun down at 3000 g for 15 min in a high-speed centrifuge (Allegra 25R, Beckman Coulter, Brea, CA, USA) to pellet the microbial layer. Supernatant layer was pipetted off leaving the rhizosphere sample undisturbed. Rhizosphere subsamples of equal weight (250mg) were transferred to sterile 1.5 mL beaded garnet tubes in triplicate and immediately frozen at -80 ^{o}C.

DNA extraction and 16S rRNA gene amplification

Bacterial DNA was extracted from the original soil samples, microbial inoculum, plant rhizospheres, and living, Bt-resistant insects using PowerFecal Pro DNA Isolation kits (Qiagen, catalogue No. 51804) in accordance with manufacturer protocols (https://www.qiagen.com/us/resources/resourcedetail?id=8896817a-253f-4952-b845-0aab796813ce&lang=en). The total sample list can be found with the metadata on FigShare at doi: 10.6084/m9.figshare.22229527. DNA concentration was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) to ensure equal concentrations of 3.51 ng/µL. Extracted DNA was stored at -80 ^{o}C until downstream processing began. 16S amplicon libraries were constructed and sequenced at the MU DNA Core in Columbia,

Missouri. The V4 hypervariable region of the 16S rRNA gene was amplified using single indexed universal primers (515F/806R; Caporaso et al., 2012) with Illumina standard adapter sequences. Forward and reverse, dual indexed primers were used in all reactions. PCR reaction steps were as follows: $98C^{(3:00)}$+[$98C^{(0:15)}$+$50C^{(0:30)}$+$72C^{(0:30)}$] for 25 cycles. The resulting amplicons were pooled before sequencing on Illumina MiSeq 2×250 bp platform.

16S rRNA sequence assembly

16S rRNA sequence processing was conducted using Qiime2 v2022.8 (Bolyen et al., 2019). Paired-end reads were demultiplexed prior to trimming primer sequences with Cutadapt (Martin, 2011). An error rate of 0.1 was allowed in the primer sequences, and any untrimmed reads were discarded. Low quality filtering and denoising was performed with DADA2 (Callahan et al., 2016). Reads were trimmed when the lower 25^{th} quartile range at a given base pair fell below a quality score of 30. Chimeras were detected using the "consensus" method and removed. Remaining sequences were filtered to retain those with lengths between 240 and 255. Taxonomy was assigned to amplicon sequence variants (ASVs) using the Silva.v132 database with the *sklearn* classifier in Qiime2 (Quast et al., 2012; Bokulich et al., 2018). ASVs were compiled into biom tables for downstream analysis in RStudio 4.0.4. Any ASV matching chloroplast, mitochondria, archaea, *Wolbachia*, or "uncharacterized" at the phylum level were filtered using *phyloseq:filter_taxa* (McMurdie and Holmes, 2013). For global comparisons across sample types, data were rarefied to even depth of 1300 reads.

Data analysis

All data analyses were conducted in RStudio 4.0.4. We first tested the effect that soil microbes from different management systems had on plant-insect interactions between Bt-resistant and -susceptible insects feeding on Bt and non-Bt corn. Larval fitness was estimated by measuring larval survival and average larval dry weight of surviving insects. All surviving insects collected from an experimental unit were weighed together and divided by the number of larvae to obtain the average weight per larvae. Dependent variables were checked for normality of variance using Levene's test and normality of distributions using Shapiro-Wilk test prior to evaluation in linear mixed effects models. Average larval dry weight data did not satisfy the assumptions of normality of residuals and were square-root transformed. The main effects of microbiome treatment, corn line, and colony and their interactions were examined using a linear mixed effects model with block, block × corn line, and block × colony interactions as the random effects. Post hoc comparisons of estimated marginal means were conducted on significant interactions using *emmeans* in the emmeans package based on a priori predictions (Russell 2022). Mortality was modeled using a generalized mixed effect model following a Poisson distribution with microbiome treatment, corn line, and colony and their interactions as main effects and a random slope of block and random intercept of corn line and colony. Type III sum of squares were compared using *Anova* in the car package (Fox & Weisberg, 2019), and post hoc tests on estimated marginal means were conducted on significant interactions using *emmeans* based on a priori predictions. To evaluate changes in plant growth, we compared the effect of soil microbes on root dry weight of uninfested control plants. Data were analyzed using a linear mixed effects model with treatment x corn line interaction as the main effects and block and block x corn line interaction as the

random effects. Post hoc comparisons of estimated marginal means were conducted on significant effects. Log response ratios for corn root dry weight were calculated by taking the natural log of the proportion of root dry weight of WCR infested plants to the paired uninfested control plant. Log response ratios were analyzed using a linear mixed effects model with treatment × corn line × colony interaction as the main effects and block, block × colony, and block × corn line interaction as the random effects. Post hoc comparisons of estimated marginal means were conducted on significant effects.

Next, we examined the overall effect of soil management system on the microbiome alpha and beta diversity of each sample type (soil, inoculum, rhizosphere, insect). Alpha diversity was calculated using *estimate_richness* in the phyloseq package. Estimates of richness and Inverse Simpson's *D* were evaluated using a linear mixed effects model with management tactic x sample type interaction with block as the random effect. Dependent variables were checked for normality of distributions using Shapiro-Wilk test prior to evaluation in linear mixed effects models. Post hoc comparisons of estimated marginal means were conducted on significant interactions using *emmeans* in the emmeans package. We analyzed beta diversity using a single PERMANOVA model with field block as the random effect and management tactic x sample type interaction with *adonis2* in the vegan package (Oksanen et al., 2019). A significant interaction was followed up with pairwise comparisons using *pairwise.adonis2* treating each treatment x sample type combination as an independent factor (Martinez Arbizu, 2020). Variance within a community was estimated using *betadisper* and compared using a permutational test of multivariate homogeneity of group dispersion treating each treatment x sample

type combination as an independent factor in the vegan package. Pairwise differences were compared using *TukeyHSD*.

We followed up the global tests with specific models investigating the differences between management tactics on rhizospheres of Bt and non-Bt corn fed upon by different insect strains (Bt-resistant, Bt-susceptible, and uninfested) using unrarefied data given the low variation in sampling depth between samples. We compared differences in overall community composition using Bray-Curtis and Jaccard distances in a permutational analysis of variance (PERMANOVA) with treatment × corn line × colony interaction as the main effects and field block as the random effect (specified through *permutation*). Significant interactions were tested using individual models across levels of an effect. NMDS ordinations based on Bray-Curtis distances were generated using *ordinate* in the phyloseq package. Alpha diversity metrics were calculated using *estimate_richness* in the phyloseq package. Estimates of richness and Inverse Simpson's *D* were evaluated using a linear mixed effects model with treatment, corn line, colony, and their interaction as fixed effects and block, block × corn line, and block × colony interaction as random effects. Dependent variables were checked for normality of distributions using Shapiro-Wilk test prior to evaluation in linear mixed effects models. Post hoc comparisons of estimated marginal means were conducted on significant interactions using *emmeans* in the emmeans package.

We examined bacterial taxa that were differentially abundant between treatments using analysis of compositions of microbiomes with bias correction (ANCOM-BC; Lin & Peddada, 2020). We made comparisons between those samples that had statistically different microbial communities based on PERMANOVA results, and each corn line was

analyzed separately to account for differences between the lines. Analyses were conducted at two different taxonomic ranks, family and ASV level. Resulting taxa that were found to be significantly differentially abundant between treatments were filtered to select the most abundant 125 ASVs across samples. The top 125 were used to visualize the log10 percent abundance across sample types using a heatmap. Recruitment from the soil microbiome by plants was estimated as the proportion of reads observed in the rhizosphere of each corn line in each soil microbiome treatment consistent with the field replication. Proportions were analyzed using beta regression with the treatment corn line interaction using *betareg* (Cribari-Neto & Zeileis, 2010).

Data availability

Sequence data have been deposited on NCBI SRA under Bioproject accession number PRJNA929989. Raw data and accompanying metadata can be found at FigShare at doi: 10.6084/m9.figshare.22229527. Code used for statistical analyses can be found at FigShare at doi: 10.6084/m9.figshare.22229638.

4.3 Results

To assess the impact of microbial communities on corn-WCR interactions, we first examined dry weight and larval survival of Bt-resistant and -susceptible WCR feeding on Bt and non-Bt corn inoculated with soil microbiomes from contrasting management systems. There were differences in how Bt-resistant and -susceptible WCR responded to the soil microbiome treatment (treatment × colony interaction: F-value = 6.643, p = 0.0123). Susceptible WCR dry weight was not affected by the soil microbiome treatment (Figure 4.2a). However, Bt-resistant WCR dry weight was significantly reduced when

reared on corn treated with soil microbes from the conservation management system (Figure 4.2b). Bt was equally effective in both soil microbiome treatments at controlling susceptible insects, both in terms of mortality and dry weight (weight; corn line × colony interaction: F-value = 28.31, $p < 0.001$; mortality; corn line x colony interaction: F-value = 18.51, $p < 0.001$; Figure 4.2a). We also examined the impact of the soil microbiome on plant fitness by comparing dry weight of corn roots. Overall, there was a marginally significant increase in root weight when inoculated with the conservation management soil microbiome (Figure 4.3a; $p = 0.058$). In addition, we found non-Bt roots to be heavier than Bt roots (Figure 4.3a; $p < 0.001$); however, these were not true isolines because these were not available. Whether roots were fed upon by Bt-resistant and Bt-susceptible insects did not influence root biomass. We observed only a main effect of soil microbiome treatment on the log response ratio of biomass of fed to unfed roots. There was a significantly greater difference between fed and unfed roots when reared in the presence of the conservation management microbiome (Figure 4.3b, F-value = 4.661, $p = 0.034$).

We then sought to examine how bacterial communities of soil, plant, and insect were impacted by management practices. Overall, we found evidence that the long-term use of conservation management significantly alters the soil microbiome composition. However, this effect was variable across rhizosphere and WCR samples. Rhizosphere samples were significantly influenced by the soil microbiome applied to the seedlings, both in terms of community richness, diversity, and composition (Figure 4.4). Rhizosphere richness was increased when corn seedlings were grown in the presence of the conservation management soil microbiome compared to the conventionally managed

soil microbiome (pairwise: $t = 5.176$, $p < 0.001$), despite there being no difference in the richness of the original soil microbiome. WCR larval microbiome composition did not vary based on the soil microbiome applied to their host plant (Figure 4.4a). Inverse Simpson's *D*, or the effective number of species, followed a similar pattern with the highest diversity in soil, followed by rhizosphere, and insect samples. Insects harbored the communities with the lowest diversity (Figure 4.4c). Beta diversity analyses revealed a significant interaction between sample type and microbiome treatment (type × treatment: $F = 2.54$, $p < 0.001$, $R^2 = 0.0214$). Pairwise comparisons between each combination resulted in significant differences in centroid location for all pairs except for WCR larval microbiomes reared in different soil microbiomes.

To specifically investigate whether changes in WCR fitness could be explained by rhizosphere communities, we analyzed data from rhizosphere communities of the two different corn lines, Bt and non-Bt, infested with either Bt-resistant WCR, Bt-susceptible WCR, or uninfested. We found a significant treatment by corn line interaction for richness and community composition. Diversity estimated by Inverse Simpson's D was not different across any of the conditions. For rhizosphere richness, the interaction was due to an observed increase in community richness in Bt corn rhizospheres grown in the presence of cover crop soil microbes, but no difference in non-Bt corn rhizosphere richness (Figure 4.5c; pairwise comparisons: Bt: p value < 0.001; non-Bt: $p = 0.18$). These differences in corn line may be driven by the increased variance in richness of non-Bt corn rhizospheres (Levene's test: corn line, $p = 0.03$). Soil microbes from different management systems significantly altered community composition based on Bray-Curtis and Jaccard distances in both Bt and non-Bt corn rhizospheres but by different

magnitudes (Figure 4.5b; B-C treatment × corn line: $p = 0.008$; Jaccard treatment × corn line: $p = 0.005$). Bt-corn rhizosphere communities varied more substantially between treatments compared to non-Bt corn rhizospheres (Table 4.2). When comparing corn lines within each soil microbiome treatment, we only found differences between corn lines when reared in the conventionally managed soil microbiome (Table 4.2). We did not detect differences in beta-dispersion between those groups. In addition, we found no evidence feeding by either insect strain altered rhizosphere communities compared to unfed corn rhizospheres.

Soil microbiomes from the two management systems were composed of similar classes of bacteria but in different relative abundance (Figure 4.5a). The conservation soil microbiome contributed a higher proportion of taxa from their bacterial communities to the corn rhizosphere ($p = 0.0144$). While the proportion was relatively small when accounting for only presence or absence, these taxa combined to account for around ~40% of the rhizosphere communities. Differentially abundant taxa between soil microbiome treatments in the rhizosphere of each corn line were concentrated across nine classes of bacteria (Figure 4.6a). A total of 118 ASVs overlapped in enrichment in the rhizosphere of both corn lines (Figure 4.6b). Some genera were found enriched in only one treatment, whereas other genera were represented in both management systems. For insects, we found an average of 127 ASVs also observed in the soil and rhizosphere samples, which represents an average of 14.64% of the total taxa observed across samples.

4.4 Discussion

Sustainable cropping practices can boost crop yield while maintaining broader ecosystem functioning. Practices promoting soil health such as extended rotations, cover crops, and reduced tillage, can increase plant health through changes in the soil microbiome (Lehman et al., 2015). Here, we document the soil microbiome of a conservation management system reduces Bt-resistant western corn rootworm fitness potentially through an increase in rhizosphere microbial richness. Changes in WCR fitness are likely plant-mediated effects as we detected no difference in the microbiome of WCR feeding on corn roots in conservation soil microbiomes compared to conventional soil microbiomes. Root weight for Bt plants was higher when grown in association with the conservation soil microbiome, further highlighting a correlation between rhizosphere richness and plant health. In addition, susceptibility to Bt was still high in Bt-susceptible insects regardless of the soil microbiome. Growers may achieve improved control of Bt-resistant WCR when using Bt corn in their conservation management system without sacrificing Bt effectiveness in non-resistant insects.

Conservation management practices can impact pest populations. One study that investigated the effects of cover crops on WCR found that WCR survival and plant damage decreased in a cover crop system compared to a traditional one (Lundgren & Fergen, 2010). The authors concluded that increased predator abundance in cover crop fields was responsible for reduced 3rd instar WCR abundance and plant damage. Interestingly, they also found that 2nd instar larvae were larger in traditionally managed fields, suggesting fitness decreased in cover crop fields, possibly to due to bottom-up effects via the soil microbiome. The conservation management practices significantly

altered soil microbiomes in our study, and these differences translated to distinct rhizosphere communities. Plants recruit mutualistic microbes with which they trade photosynthetic carbon for increased soil resource uptake and/or increased stress tolerance (Hu et al., 2018). In our study, conservation management soils contained a greater number of taxa that formed association with the corn rhizosphere. The rhizosphere community provides plants with important cues to stimulate defense against pathogens and pests. These changes are often associated with induced systemic resistance (ISR), a primed defense state that allows a plant to respond more quickly and/or strongly to attacks by pathogens and herbivores (Pieterse et al., 2014). Native corn root defenses against WCR can limit root damage and WCR fitness (Brkić et al., 2020; El Khishen et al. 2009, Hibbard et al. 2007), but how their expression is altered by microbial communities is relatively unknown. Our work builds on the understanding that conservation management practices can build soil health while influencing plant health and pest suppression.

Surprisingly, we found evidence that WCR herbivory on corn roots was greater under conservation management. The difference in root weight between infested and uninfested corn roots was smaller for plants inoculated with the conventional soil microbiome. The age and length of our experiment may skew the amount of damage observed. WCR often clip roots when feeding (Kahler et al., 1985), especially in new nodes of roots beginning to emerge from the stalk, which may compound the loss of root mass over time. This may explain the high level of variation within and between treatments in our study. It may be that the reduced ratio in conservation management systems is a result of altered defense or nutrition in the corn roots. Insects can

compensate for poor diets by increasing feeding rates (Lavoie & Oberhauser, 2004). One plant compound that negatively impacts insect nutrition is lignin (Campbell & Sederoff, 1996). Root lignin content can be altered by both soil microbial communities and WCR feeding (Bennett et al., 2015; Xue et al., 2012). Corn roots may be able to boost their lignin content in the presence of certain microbes when fed upon by WCR and reduce WCR fitness, a trade-off with overall growth of the plant. The reduced WCR growth could increase mortality over time (Benrey & Denno, 1997). Our study only investigated the impact on 1st instar WCR and thus did not capture the impact of the soil microbiome on later developmental stages of WCR.

We found no difference in the microbiome of the WCR when reared in different soil microbiomes or on different corn plants. Our findings corroborate previous work with WCR larval microbiome and the soil microbiome (Dematheis et al., 2012; Ludwick et al., 2019); WCR larvae exhibit tight control over their microbiome. Regulation of the larval microbiome may be especially beneficial when feeding on plants that display strong defense against WCR (Chu et al., 2013; Mason et al., 2019; Paddock et al., 2021). While we didn't observe whole community shifts, there may still be individual bacterial taxa altering WCR fitness. For example, other studies found feeding on corn infected with the fungus *Fusarium verticillioides* decreased larval fitness (Kurtz et al., 2010), and several *Serratia* strains identified on roots fed upon by WCR were also observed in diseased adults (Prischmann et al., 2008). In this study, we also did not find evidence of changes to the rhizosphere microbiome as a whole when fed upon by WCR. However, our experiment was conducted with neonate larvae feeding over a period of five days, which may not induce enough damage to stimulate whole community remodeling.

Community composition of conservation soil microbiomes can be influenced through edaphic factors introduced by the practice (Hartmann & Six, 2022), the cover crop plant species used (Nevins et al. 2018), and the timing of cover crop termination (Nevins et al. 2018). By using a replicated, long-term experimental site where climate and edaphic characteristics are controlled, we account for the edaphic factors that exist following microbial isolation. However, we do not know how our results would change under varying cover crop management practices, such as cover crop species/mix, seeding rate and method, or termination timing and method. The increased community richness observed in rhizosphere samples may attenuate community composition variation from these factors. In other words, rhizosphere richness reduces WCR weight. Fungi can also impact plant health in similar ways to bacteria and have been shown to alter bacterial communities themselves. We have not characterized fungal communities, but they likely are involved in the plant-insect interactions observed here. Genetic differences in corn lines can manifest as distinct rhizosphere communities (Meier et al., 2022; Walters et al., 2018). The interplay between plant genetics and rhizobiomes can results in distinct phenotypes in corn (Wagner et al., 2021). The two corn lines used in this study are genetically distinct in addition to the presence of transgenic Bt toxin production. The impact Bt toxins have on the microbial ecosystem in largely unknown. How cover crops may affect yield in the field is highly variable and dependent on the system (Marcillo & Miguez, 2017).

Modern agriculture, typified by monoculture row crops or simple two-crop rotations managed with heavy chemical inputs, fallow periods, and high levels of soil disturbance, negatively impacts microbial communities (French et al., 2021). Ecological

intensification of agriculture, which utilizes management practices that minimize negative environmental impacts while maintaining yield, offers a more sustainable approach (Bommarco et al., 2013). However, our understanding of how sustainable conservation management practices affect microbiomes and, vice versa, how microbiomes affect management, is lacking (French et al., 2021). Unified microbiome research across climates, soils, and crops may provide insight into the sustainable management of pests.

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Table 4.1 Overview of management practices at the USDA Long Term Agroecosystem Research Central Mississippi River Basin site in Centralia, Missouri.

Management System	Crop Rotation	Tillage	Cover Crop	Fertilizer Input
Conservation	Corn (*Zea mays* L.)–soybean (*Glycine max* L. Merr.)–wheat (*Triticum aestivum*) rotation since 1991.	Mulch till from 1991-1995. No-till since 1996.	Variable cover crop spp. Including red clover (*Trifolium pratense*) or cereal rye (*Secale cereale*) since 1994.	150 kg N ha^{-1} for corn; 101 kg ha^{-1} for wheat; lime, P, and K by soil test.
Conventional	Corn-soybean rotation since 1991.	Mulch till since 1991	None	190 kg N ha^{-1} for corn; lime, P, and K by soil test.

Table 4.2 Model results investigating individual effects between treatments and corn lines using separate models testing for differences in overall microbiome community composition based on Bray-Curtis distances.

Treatment effects within corn lines

Condition	*Response*	*Factor*	*df*	*F*	*p*
Bt corn					
	Community				
		Treatment	1,71	14.9624	0.001
		Colony	2,71	0.7487	0.833
		Block	3,71	6.3113	0.001
		Treatment × Colony	2,71	0.7106	0.864
		Treatment × Block	3,71	2.1857	0.001
		Colony × Block	6,71	0.719	0.979
		Treatment × Colony × Block	6,71	0.6529	0.996
non-Bt corn					
	Community				
		Treatment	1,71	6.6946	0.001
		Colony	2,71	0.7995	0.792
		Block	3,71	5.2595	0.001
		Treatment × Colony	2,71	0.692	0.925
		Treatment × Block	3,71	1.8718	0.005
		Colony × Block	6,71	0.7312	0.983
		Treatment × Colony × Block	6,71	0.5517	1

Corn line effects within treatments

Condition	*Response*	*Factor*	*df*	*F*	*p*
Conservation					
	Community				
		Treatment	1,71	0.9912	0.4
		Colony	2,71	0.8507	0.657
		Block	3,71	8.2215	0.001
		Treatment × Colony	2,71	0.8981	0.571
		Treatment × Block	3,71	0.8228	0.761
		Colony × Block	6,71	0.668	0.992
		Treatment × Colony × Block	6,71	0.7296	0.963
Conventional					
	Community				
		Treatment	1,71	3.3517	0.004

	Colony	2,71	0.6494	0.938
	Block	3,71	5.6586	0.001
	Treatment × Colony	2,71	0.589	0.978
	Treatment × Block	3,71	0.9714	0.481
	Colony × Block	6,71	0.6415	0.999
	Treatment × Colony × Block	6,71	0.6174	1

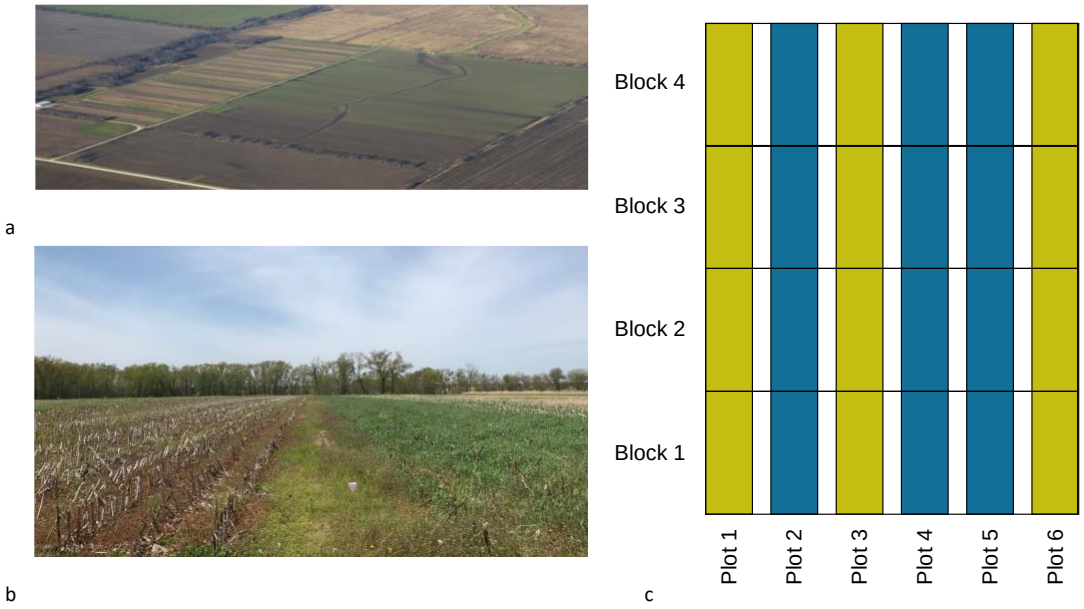

Figure 4.1 a) Aerial photo of Long-Term Agroecosystem Research network site at Central Mississippi River Basin in Centralia, Missouri managed by the Agricultural Research Service of the United States Department of Agriculture. **b**) Side by side comparison of research field plots under conservation management (left) and conventional management (right) at the time of soil sampling in April. **c**) Schematic of example plot layout and sampling design for soil sampling in the field.

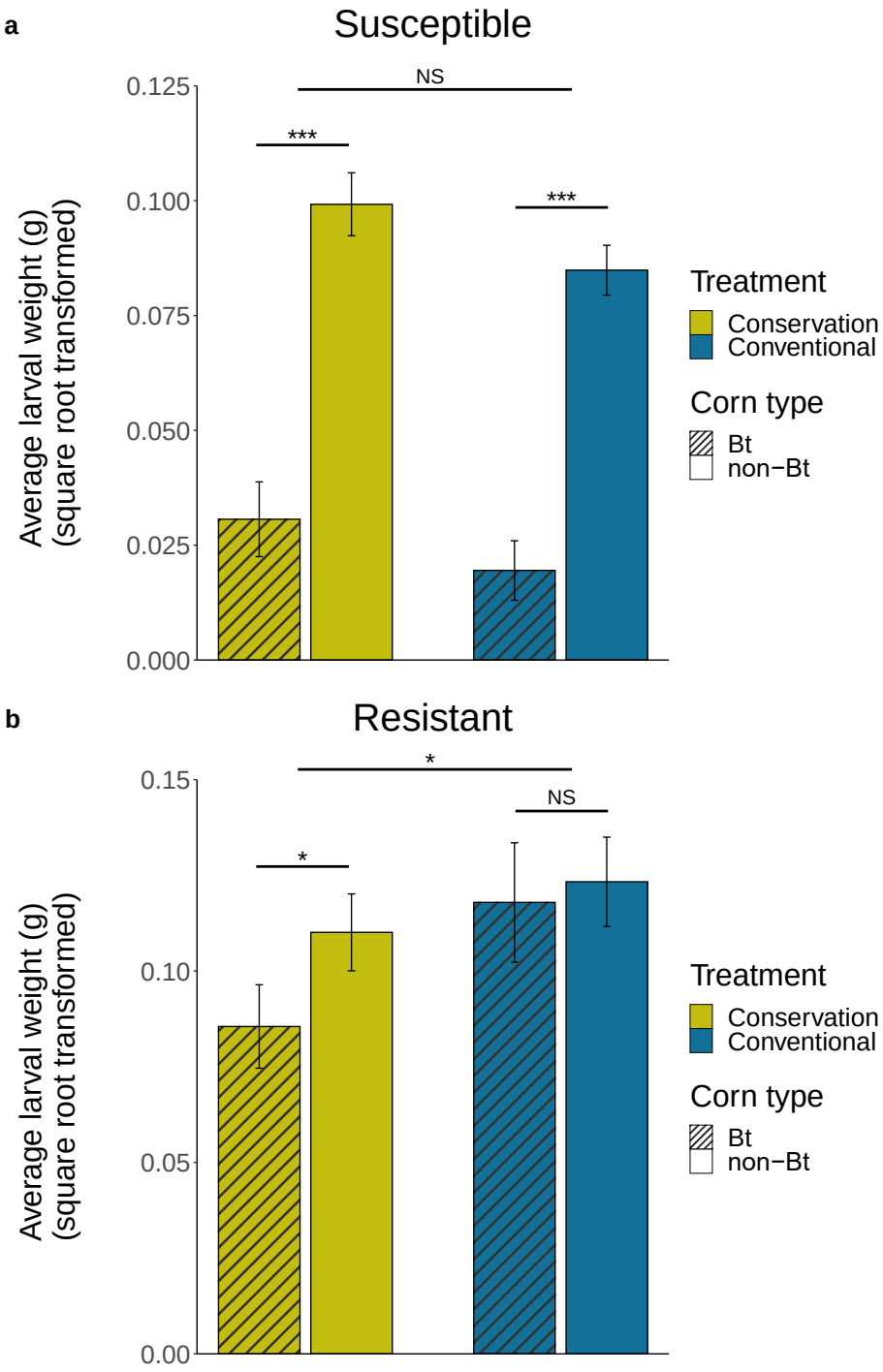

Figure 4.2 Average larval weight of **a**) Bt-susceptible and **b**) Bt-resistant western corn rootworm (*Diabrotica virgifera virgifera* LeConte) when reared for five days on either Bt or non-Bt corn treated with soil microbiomes from conservation and conventional

management systems. Each bar represents the average for each treatment across blocks. Error bars represent the standard error for each treatment combination across blocks. Pairwise differences are based on estimated marginal means from linear models where significance was considered for factors with $p < 0.05$.

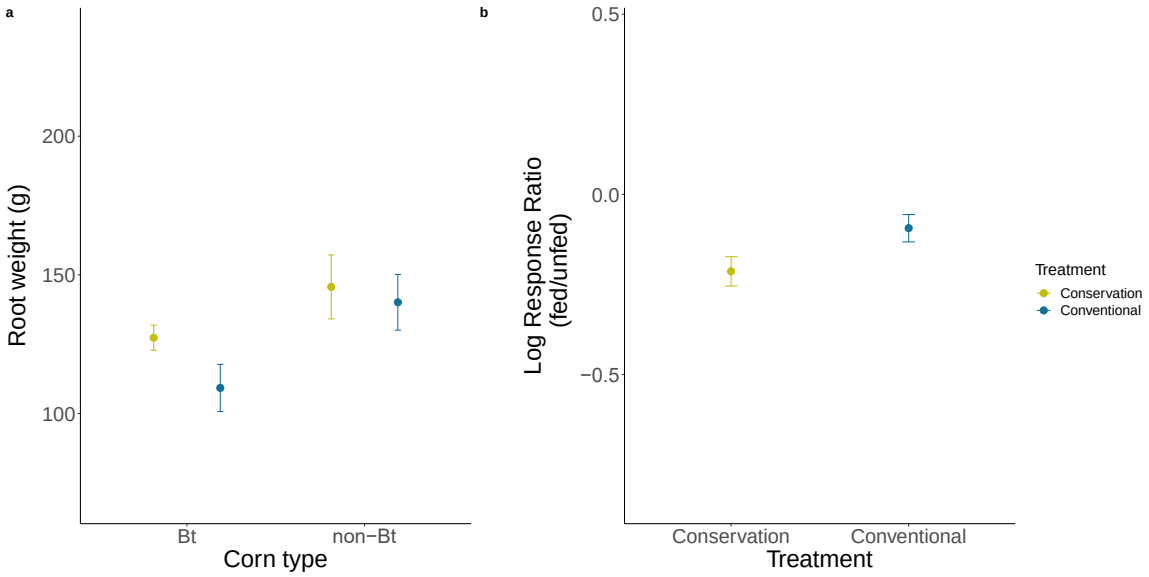

Figure 4.3 a) Dry root weight of corn lines (Bt or non-Bt) grown for 10 days after inoculation with soil microbiomes from either conservation or conventional management systems. **b**) Log response ratio of dry root weight between WCR infested and uninfested corn plants after 5 days. Sample means are presented as a bold point with accompanying standard error bars. Pairwise differences are based on estimated marginal means from linear models where significance was considered for factors with $p < 0.05$.

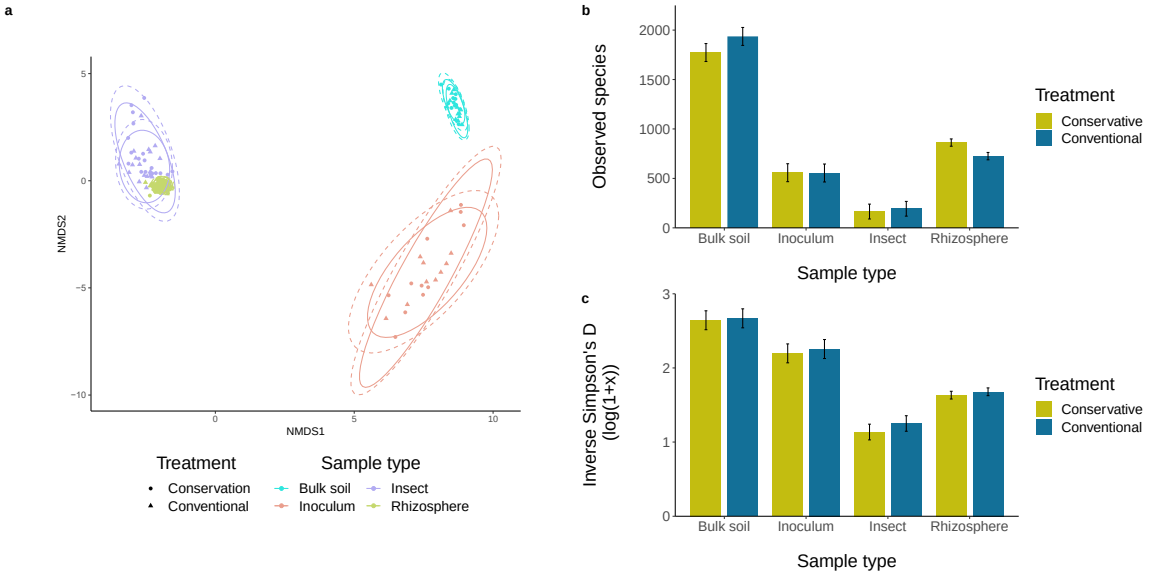

Figure 4.4 a) Non-metric multidimensional scaling of each sample type collected in the study. Sample data was rarefied to 1200 reads to account for uneven sampling depth. **b**) Observed number of taxa and **c**) inverse Simpson's diversity found between microbiome treatments within each sample type collected in the study. Error bars are 95% confidence intervals from the output of the model using emmeans.

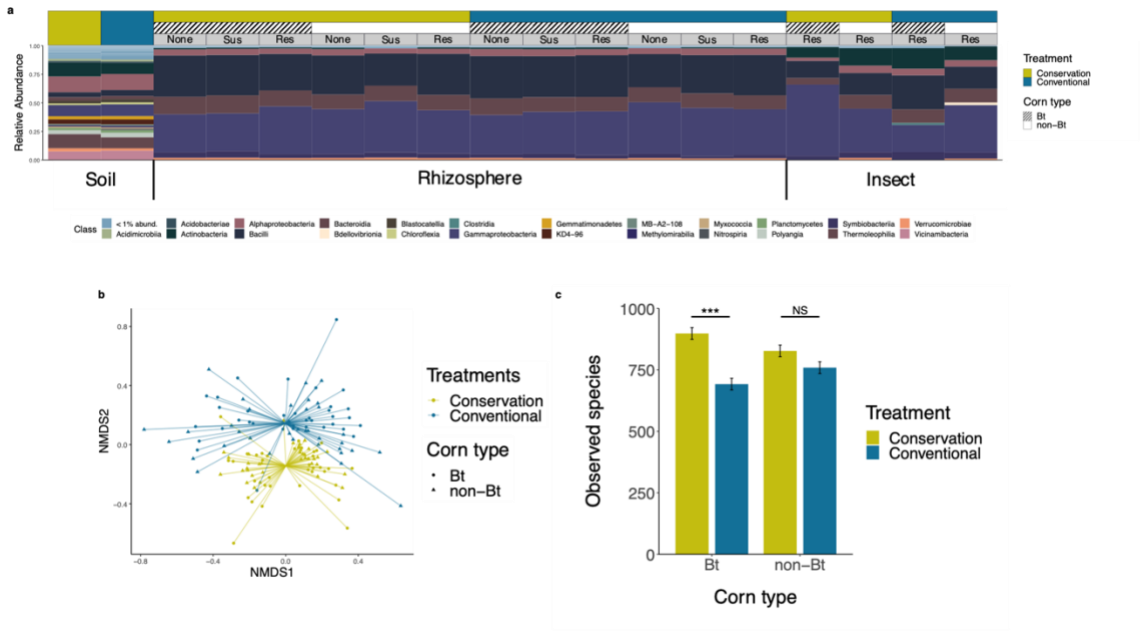

Figure 4.5 a) Relative abundance of different classes of bacteria found in different sample types. Each bar represents the combined abundances of all samples within the specified treatment group rarefied to 1300 reads. Conservation and conventional management systems are distinguished by colors, corn lines are distinguished by stripes, and insect colonies are distinguished by labels. **b**) Non-metric multidimensional scaling of 10-day old Bt or non-Bt rhizosphere bacterial communities inoculated with conservation or conventional management system soil microbiomes with unrarefied data. **c**) Comparison of number of observed species present in the rhizosphere of Bt and non-Bt corn inoculated with bacterial communities from conservation or conventional management system soils.

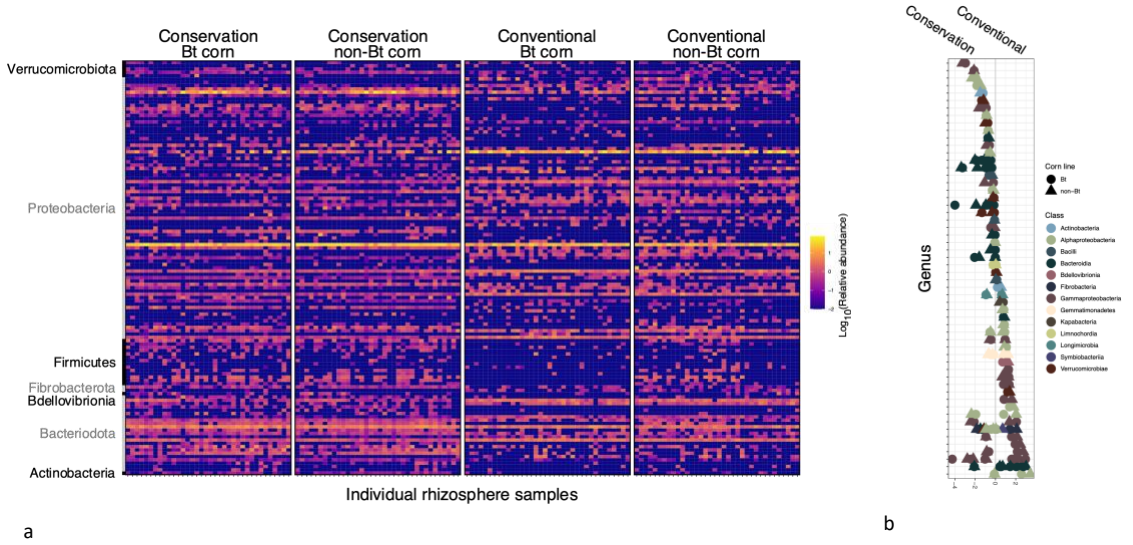

Figure 4.6 a) Heatmap of the top 125 most abundant ASV found to be significantly differentially abundant between treatments in both corn lines. Each column represents an individual rhizosphere sample from the corresponding corn line and microbiome treatment and each row represents an individual ASV grouped by class. The log10 relative abundance was calculated as a proportion of the ASV in the individual sample divide by the total sum of all reads in that sample. **b**) Differentially abundant genera between Bt- corn rhizospheres inoculated with conservation or conventional management system soil microbiomes. Significant differences in abundance between treatments were calculated using an analysis of compositions of microbiomes with bias correction (ANCOM-BC) with significance at $p < 0.05$.

Vita

Kyle John Paddock was born to Steven and Peggy Paddock in 1990. Kyle and his older sister Stephanie were raised in Clinton, a small farming town in central Illinois. His father was a farmer, his mother was a nurse, and his interests followed in the science of the natural world. He graduated from Clinton Community High School in 2009. The following fall, Kyle enrolled in University of Missouri in Columbia until he graduated in 2013 with a Bachelor of Science degree in biochemistry. After graduation, Kyle became a research technician in Dr. Gary Stacey's lab studying genetics of agronomic traits in soybeans. He then accepted a staff scientist position in Columbia with the start-up company, AgBiTech to study baculoviruses as a pest management tactic against Lepidopteran species. In 2017, Kyle moved to Dallas with AgBiTech where they opened a new production and research facility. His passion for entomological research grew over the next year, and he returned to the University of Missouri to pursue a PhD under the mentorship of Drs. Bruce Hibbard and Deborah Finke. In winter of 2021, Kyle was awarded a NIFA USDA Predoctoral Fellowship to fund his research for the final two years of his PhD. Throughout his degree, Kyle gained experience in insect ecology, microbiome science, and sustainable agriculture. He went on to fulfill the requirements for his Doctor of Philosophy degree set forth by the University of Missouri in May of 2023. He married his wife, Andrea, that April.