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SPECIAL FEATURE: RESISTANCE EVOLUTION, FROM GENETIC MECHANISM TO ECOLOGICAL CONTEXT

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

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Abstract

Evolution of resistance to transgenic crops producing toxins from *Bacillus thuringiensis* (Bt) threatens the sustainability of the technology. Examination of resistance mechanisms has largely focused on characterization of mutations in proteins serving as Bt toxin binding sites. However, insect microbial communities have the potential to provide host resistance to pesticides in a myriad of ways. Previous findings suggest the killing mechanism of Bt relies on enteric bacteria becoming pathogenic in the disrupted gut environment of the insect following Bt intoxication. Thus, here we hypothesized that resistance to Bt would alter the microbiome composition of the insect. Previous studies have manipulated the microbiome of susceptible insects and monitored their response to Bt. In our study, we characterized the associated bacterial communities of Bt-resistant and -susceptible western corn rootworms, a widespread pest of maize in the United States. We found resistant insects harbor a bacterial community that is less rich and distinct from susceptible insects. After feeding on Bt-expressing maize, susceptible insects exhibited dysbiosis of the associated bacterial community, whereas the community within resistant insects remained relatively unchanged. These results suggest resistance to Bt produces alterations in the microbiome of the western corn rootworm that may contribute to resistance. We further demonstrated that by itself, feeding on Bt toxin-expressing seedlings caused a shift in the microbiota. This work provides a broader picture of the effect stressors have on microbiome composition, and the potential heritable changes induced as a result of intense selection.

KEYWORDS
Bacillus thuringiensis, evolution, insect, microbiome, resistance, western corn rootworm

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 approximately 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 reinoculation 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 haemocoel, demonstrating commensal bacteria could become pathogenic upon entry into the haemocoel (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 defences (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 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.

2 | MATERIALS AND 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.

2.1 | Insects and seeds

Eggs of susceptible insects were originally purchased from Crop Characteristics (nondiapausing 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.) and provided with young maize leaves, artificial diet (Frontier Agricultural Sciences), 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) 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.

2.2 | Experimental set-up

2.2.1 | Soil environment

Conical tubes (50 ml: Thermo Fisher Scientific) were filled with ~30 ml of a 2:1 nonautoclaved, local topsoil:Promix mixture (Premier Horticulture Inc.). 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.

2.2.2 | Soilless environment

Bt and non-Bt seeds were sterilized by soaking for 3 min 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. Then, 3–4 maize seeds were 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.

2.3 | DNA extraction and 16S rRNA gene amplification

Bacterial DNA was extracted from frozen, whole larvae (3 per tube) using PowerFecal DNA Isolation Kit (Qiagen, catalogue 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). Initial range finding experiments determined DNA yield and quality were optimal for samples containing between 2–4 insects (Figure S1). DNA concentration was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific), 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).

2.4 | 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 1450 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.

2.5 | 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, nonrarefied 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 nonrandom 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 analysed 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 nonrandom residuals. Pairwise comparisons for all models were considered significant at $p < .05$.

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. Community level results without *Wolbachia* present can be found in the Tables S1–S4. 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 (Tables S5, S6) 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 nonsignificant ($p > .05$), 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 analyse 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 $p < .05$ (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 $p < .05$. 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 analysed separately.

3 | RESULTS

We compared the bacterial communities associated with Bt-susceptible and -resistant insects after feeding on Bt and non-Bt maize seed for one and three 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 ± 3218.29 sequences (Figures S2, S3).

3.1 | 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 1, 2). In comparisons of alpha diversity metrics between environments (soil vs. soilless), richness and diversity as estimated by Chao-1 index and inverse Simpson's 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: $p = .0271$; Simpson: $p = .0095$; Table 1; Figure 1). Additionally, there was a significant colony ×environment interaction for richness but not for diversity ($p < .0001$). 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 1). Susceptible and resistant insects showed no differences in diversity as measured by inverse Simpson's 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 (Table S7). WCR harbored significantly different bacterial communities when reared in different

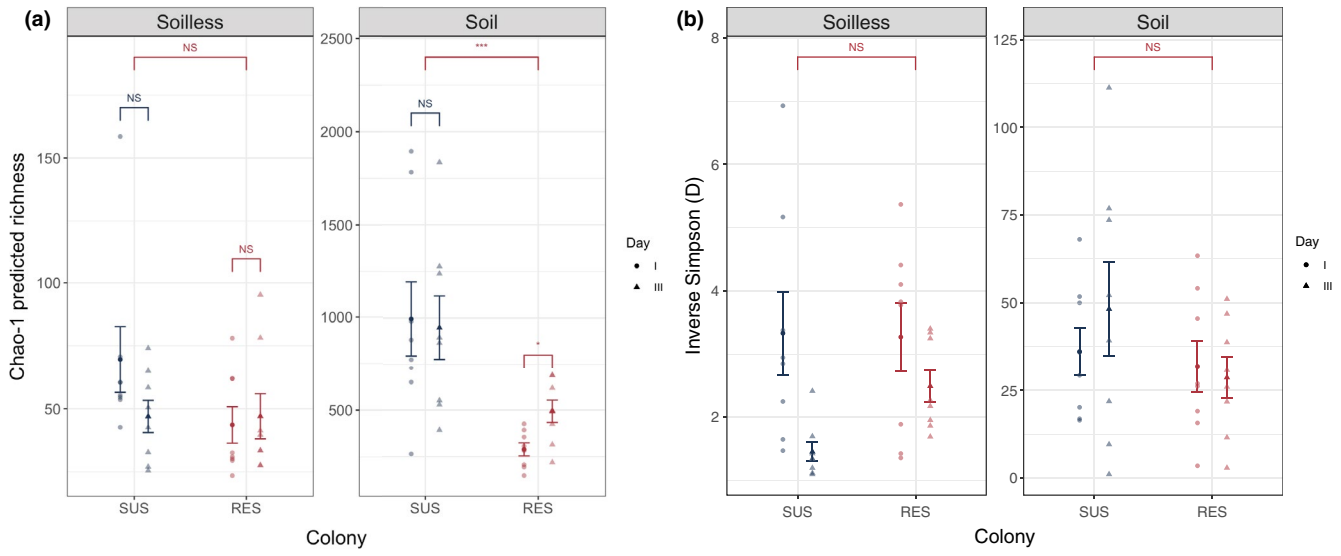

FIGURE 1 Comparisons of (a) richness (Chao-1) and (b) diversity (Inverse Simpson's D) of Bt-resistant and -susceptible western corn rootworm larval bacterial communities when fed on non-Bt maize in two different environments, with and without soil. Sample means are represented as a bold data point with accompanying standard error bars. Differences are pairwise comparisons from the three-way interaction with environment, trait and day using LSMeans at $p < .05$

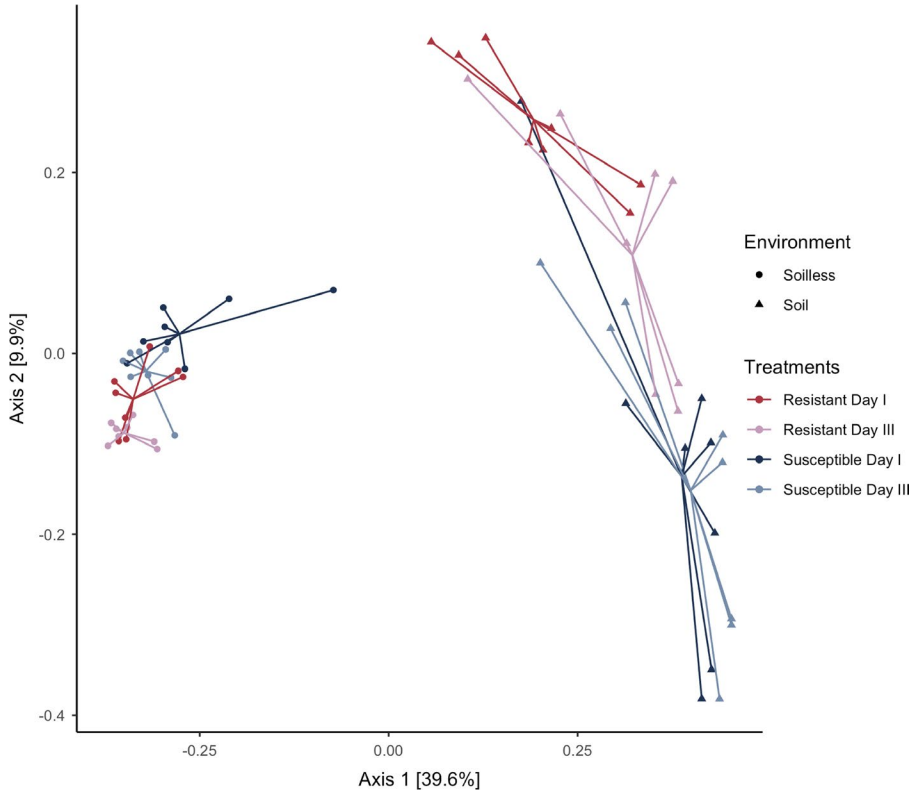

FIGURE 2 Principal coordinate analysis of bacterial communities in Bt-resistant and -susceptible western corn rootworm larvae reared on non-Bt maize roots in soilless and soil environments for one and three days. Centroids are based on beta-dispersion of colonies on each day in each environment

environments (Figure 2; p = .001; Table 1), and resistant and susceptible insects harbored distinct bacterial communities that varied with day regardless of environment (Colony: p = .001; Day: p = .005; Figure 2). Beta dispersion was significantly different between environments with increased heterogeneity in insects reared in the soil environment (p = .001; Figure 2). However, we found no differences in colony beta dispersion when testing within environments (soil: p = .757; soilless: p = .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 (Table S1, S2).

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).

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 was observed in the soil environment, with the majority of taxa being enriched in the susceptible insect (Figure S4). 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* (Figure S4).

3.2 | 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 = .0035) and environment and day (p = .0076) for Chao-1 richness

TABLE 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	**.0271**
	Colony	1,54	22.76	**<.0001**
	Day	1,54	0.06	.8069
	Environment ×Colony	1,54	5.2	**.0266**
	Environment ×Day	1,54	3.33	.0737
	Colony ×Day	1,54	5.2	**.0266**
	Environment ×Colony × Day	1,54	0	.9443
Inverse Simpson's D				
	Environment	1,2	103.94	**.0095**
	Colony	1,54	0.41	.5249
	Day	1,54	3.69	**.0602**
	Environment ×Colony	1,54	2.59	.113
	Environment ×Day	1,54	1.81	.1839
	Colony ×Day	1,54	0.78	.3813
	Environment ×Colony × Day	1,54	0.94	.3359
Community				
	Environment	1,63	42.417	**.001**
	Colony	1,63	5.245	**.001**
	Day	1,63	1.975	**.006**
	Environment ×Colony	1,63	5.698	**.001**
	Environment ×Day	1,63	1.38	**.043**
	Colony ×Day	1,63	1.091	.159
Beta dispersion				
	Environment	1,62	39.44	**.001**
	Colony	1,62	4.5659	**.035**
	Day	1,62	0.2764	.615

Notes: 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 matrix with log-transformed data. Beta dispersion results are from PERMANOVA model using average distance to centroid for groups.

Significant results are presented in bold

(Table 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 = .016), but was not different in the soilless environment (pairwise comparison, p = .0723; Figure 4a). However, we found richness of bacterial communities of resistant insects was not different when fed Bt or non-Bt regardless of environment (main effect: p = .9311; interaction: p = .5007; Figure 4b). We observed a significant interaction between environment and day in resistant insects with richness increasing with age only in the soil environment (p = .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 = <.0001; Figure 4c), but was not different in the soilless environment (pairwise comparison, p = .763). Again, no differences were observed in bacterial community diversity of resistant insects when fed Bt or non-Bt maize, regardless of environment (p = .5007; Figure 4d). Susceptible insects showed a decrease in richness with age only in the soilless environment (interaction: p = .0176).

Previously observed differences in community structure based on environment led us to analyse beta diversity separately within each environment. Regardless of environment, we found a significant interaction between colony and maize type (soil: p = .027; soilless: p = .022; Figure 5; Table 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 = .017) and soilless environments (pairwise comparison: p = .036). Yet, when resistant insects fed on Bt, the structure of their bacterial communities remained relatively unchanged (pairwise comparison, soil: p = .36; soilless: p = .47; Figure 5). Beta dispersion between colonies on Bt and non-Bt was not significantly different in either environment (soil: p = .926, soilless: p = .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 = .011; Day 3: p = .585; Table S3). Again, the significance of these results was unchanged when *Wolbachia* was excluded from analyses (Table S4). 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). Resistant insects experienced much smaller perturbations. DNA recovered from resistant insects reared in the soilless environment and fed Bt maize

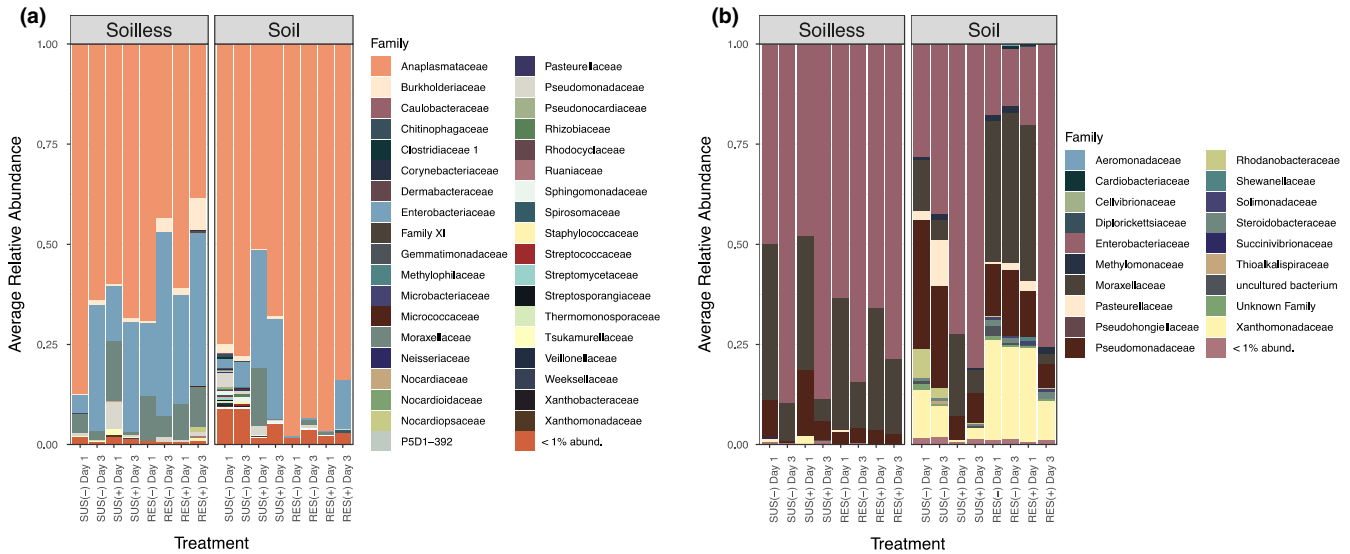

FIGURE 3 Family level stacked bar chart of average relative abundance of (a) total bacterial communities and (b) Gammaproteobacteria class from Bt-susceptible and -resistant western corn rootworm larvae reared in soil and soilless environments on Bt or non-Bt expressing maize roots for one and three days. Each bar is an average of eight samples from two replicated experiments. ((+) = Bt maize roots, (-) = non-Bt maize roots)

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). 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 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 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.

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 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 1 and 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

TABLE 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	**.0077**
		Trait	1,54	0.21	.6461
		Day	1,54	0.75	.3914
		Environment ×Trait	1,54	9.33	**.0035**
		Environment ×Day	1,54	7.68	**.0076**
		Trait ×Day	1,54	0.37	.5439
		Environment ×Trait × Day	1,54	1.53	.2216
	Inverse Simpson's D				
		Environment	1,2	18.7	**.0495**
		Trait	1,54	14.22	**.0004**
		Day	1,54	2.31	.1346
		Environment ×Trait	1,54	11.17	**.0015**
		Environment ×Day	1,54	5.99	**.0176**
		Trait ×Day	1,54	3.19	.0796
		Environment ×Trait × Day	1,54	0.01	.9433
Resistant					
	Chao−1				
		Environment	1,2	14.74	.0616
		Trait	1,54	0.01	.6461
		Day	1,54	6.44	**.0141**
		Environment ×Trait	1,54	9.33	.5007
		Environment ×Day	1,54	5.43	**.0236**
		Trait ×Day	1,54	1.7	.1978
		Environment ×Trait × Day	1,54	0.07	.7953
	Inverse Simpson's D				
		Environment	1,2	37.36	**.0257**
		Trait	1,54	3.91	.0531
		Day	1,54	2.66	.1085
		Environment ×Trait	1,54	0.89	.351
		Environment ×Day	1,54	1.76	.1908
		Trait ×Day	1,54	0.09	.7599
		Environment ×Trait × Day	1,54	3.99	.05009
Soil					
	Community				
		Colony	1,63	7.0224	**.001**
		Trait	1,63	1.7772	**.031**
		Day	1,63	3.143	**.003**
		Colony ×Trait	1,63	1.7279	**.027**
		Colony ×Day	1,63	1.0189	.297
		Trait ×Day	1,63	0.8895	.463
Soilless					
	Community				
		Colony	1,63	9.4306	**.001**

(Continues)

TABLE 2 (Continued)

Condition	Response	Factor	df	F	p
		Trait	1,63	1.5619	**.024**
		Day	1,63	1.8561	**.007**
		Colony ×Trait	1,63	1.5219	**.022**
		Colony ×Day	1,63	1.5782	**.024**
		Trait ×Day	1,63	0.7149	.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.

Significant results are presented in bold

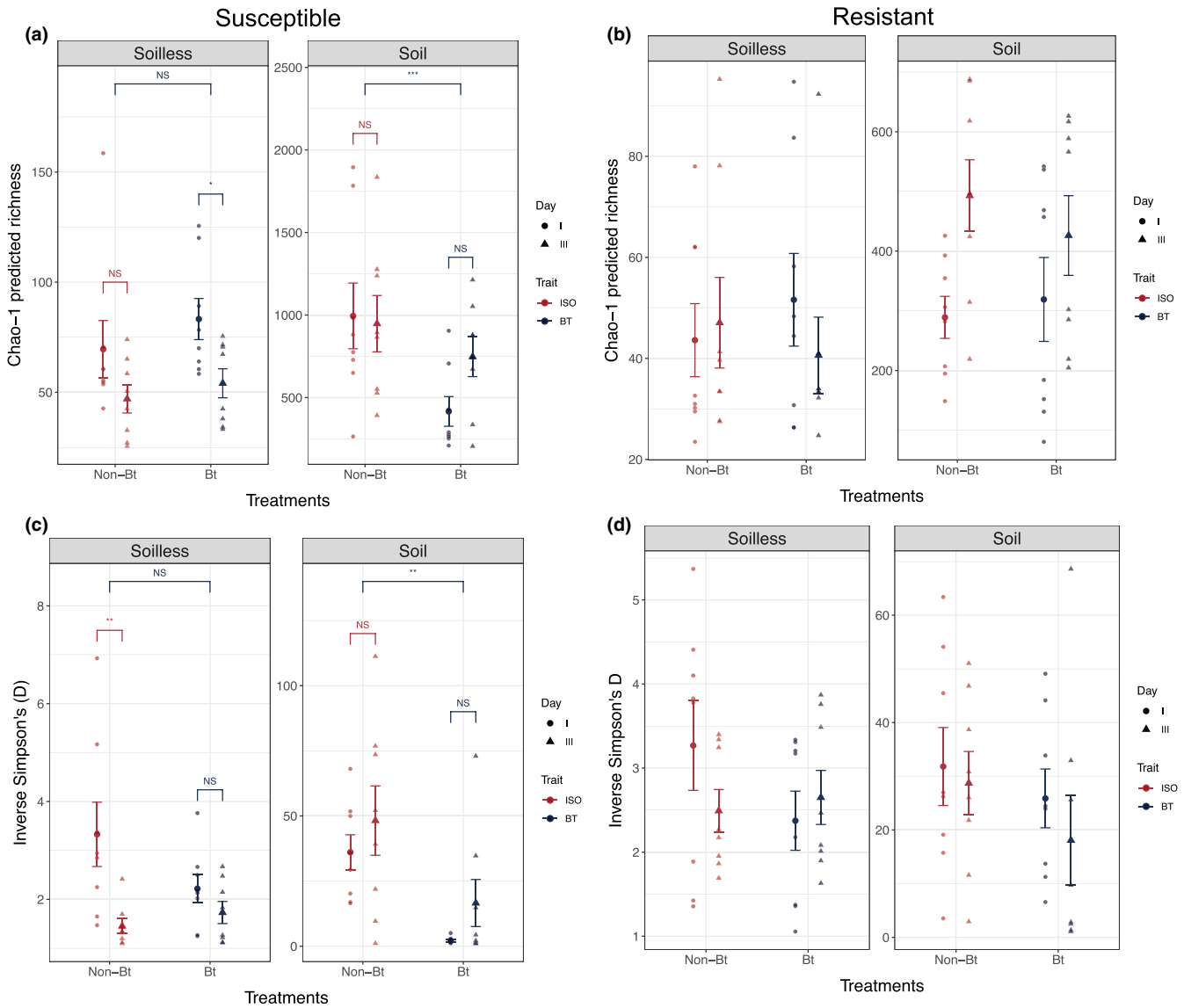

FIGURE 4 Comparisons of richness (Chao-1) and diversity (Inverse Simpson's D) of (a) Bt-susceptible and (b) -resistant western corn rootworm larval bacterial communities reared on Bt and non-Bt maize roots for one and three days in two different environments, with and without soil. Sample means are represented 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 $p < .05$

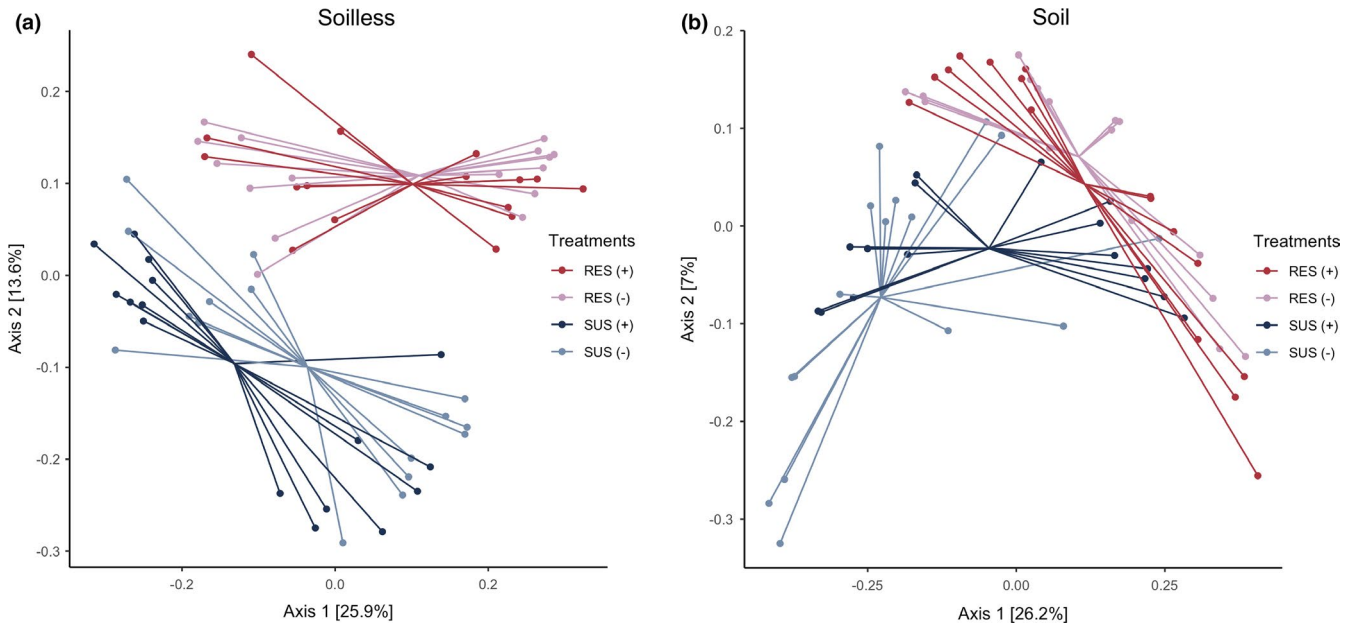

FIGURE 5 Principal coordinate analysis of bacterial communities in Bt-resistant and -susceptible western corn rootworm larvae reared on non-Bt and Bt maize roots in (a) soilless and (b) soil environments for one and three days

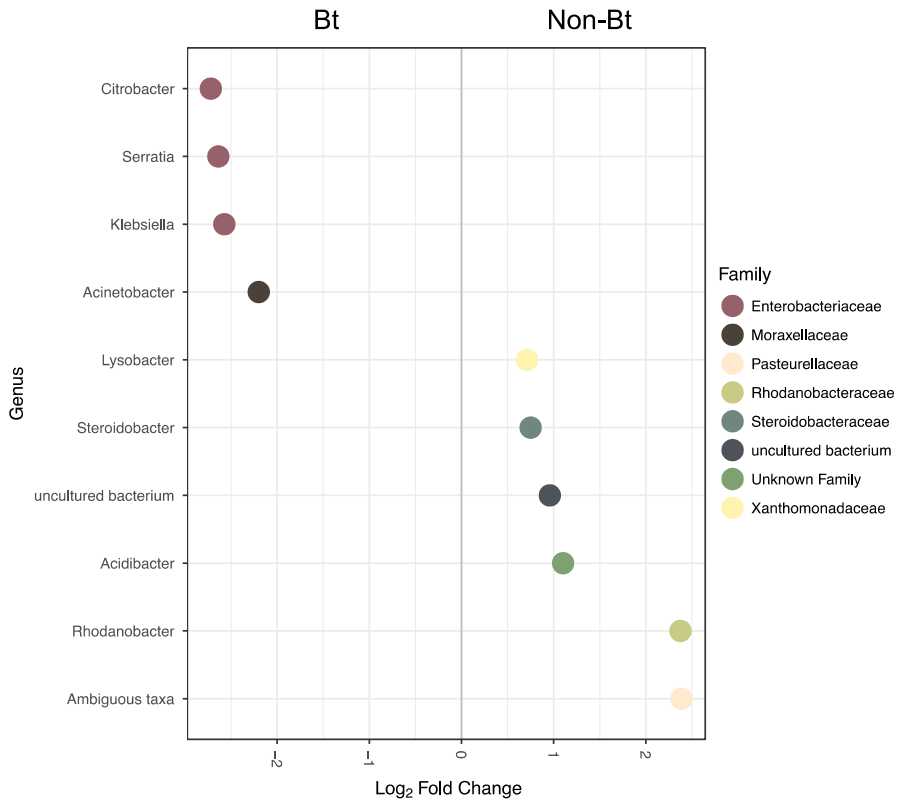

FIGURE 6 Differentially abundant genera in the class Gammaproteobacteria in Bt-susceptible larvae fed on either Bt or non-Bt maize roots in soil environment. Significant differences in abundance between treatments were calculated using a negative binomial distribution in a generalized linear model in the DESeq2 package (R Studio) with a $p < .05$

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 2). The largest differences were seen in the soil environment where resistant insects harbored significantly fewer taxa compared to susceptible insects (Figure 1). Similar responses have been documented in mosquitos where Bt-tolerant larvae harbour 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 haemocoel of Bt intoxicated insects leading to septicaemia (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 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 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 probably driven in part by the higher relative abundance of taxa in the genera *Klebsiella*, *Citrobacter*, *Serratia*, and *Acinetobacter* in Bt-fed insects (Figure 6). Plant-expressed Bt toxins do not induce 100% mortality in WCR larvae, and probably 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 susceptibility 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 favour 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 harbouring 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 nonaxenic 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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AUTHOR CONTRIBUTIONS

Kent S. Shelby and Bruce E. Hibbard conceived the study; Kyle J. Paddock, Adriano E. Pereira, Deborah L. Finke, Aaron C. Ericsson, Bruce E. Hibbard, Kent S. Shelby designed the experiments; Kyle J. Paddock and Adriano E. Pereira performed the experiments; Kyle J. Paddock analysed the data; Kyle J. Paddock and Deborah L. Finke interpreted results; Kyle J. Paddock wrote the manuscript with feedback from all authors.

DATA AVAILABILITY STATEMENT

Sequence data have been deposited on NCBI under BioProject #PRJNA531879. Original OTU table and metadata with accompanying R code are available at FigShare under https://doi.org/10.6084/m9.figshare.12974621 and https://doi.org/10.6084/m9.figshare.12974597.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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