CATERPILLAR MICROBIOME IN NON-BT VERSUS WIDESTRIKE COTTON

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Abstract

Insecticide resistance in caterpillars has been attributed to many environmental and genomic changes, but the role of the microbiome in insects in comparison has barely been explored. In recent years, several studies suggest the microbiome might affect insect susceptibility to insecticides. The microbiome of an organism also can be affected by a number of variables including the insect's diet and potentially the introduction of transgenes into crop plants in which caterpillars feed. Often insects from the field are bioassayed using highly enriched artificial diet containing antimicrobial agents that might affect insecticide susceptibility tests. We report ongoing studies over two field seasons on the microbial diversity in the bollworm, *Helicoverpa zea*, collected from the field from non-Bt and WideStrike cotton. In years 2 and 3, the internal cultivable bacteria were higher in 2nd to 3rd stadium bollworms from WideStrike versus non-Bt cotton, even when the plants were grown in the field just a few rows apart. Amplification of the bacterial V3 region of the 16S rRNA gene suggested differences in bacteria diversity between WideStrike versus non-Bt cotton-collected bollworms, although additional work is needed to understand reduced PCR product amplification and a low number of OTUs in our Illumina sequencing work. Potential reasons for the differences in the bollworm microbiome between WideStrike and non-Bt cotton are discussed. Our findings argue for more research to understand the interactions between the bollworm and plant microbiomes and the impact of cotton variety and transfer to artificial diets on Bt susceptibility.

Introduction

Microorganisms are important to the survival and success of any higher organism including insects. The insect gut is a highly nutrient rich ecological niche in which various microbes survive and multiply (Rajagopal 2009). Insect and bacteria act in symbiont relationships, and absence of these microbes can affect adversely insect growth and development. For example, bacteria free crickets have a lower enzymatic hydrolytic activity as compared to their conventional counterparts, and these bacteria help in utilizing a wider range of food substrates (Domingo et al. 1998). Hemipterans, such as aphids, survive exclusively on plant sap which is deficient in essential nutrients. The sap feeding aphids are dependent on their association with gut symbionts to use nutrient deficient food sources for survival (Rajagopal 2009). Similarly, *Megacopta cribraria* without gut symbionts show developmental delays, reduced ability to reproduce, and die prematurely (Hosokawa et al. 2006). In addition to growth and development, microbial symbionts also are involved in their host's immunity. Axenic locusts are susceptible to infection by the entomopathogenic fungi, *Beauveria* and *Metarhizium* (Rajagopal 2009). Similarly, axenic silkworms, *Bombyx mori*, larvae were found to be more susceptible to *Serratia piscatorum* and baculovirus infection (Kodama and Nakasuji 1971). Flies with no gut microbes were more susceptible to *Gluconobacter*, compared to flies with its normal gut micro flora (Ryu et al. 2008). *Hamiltonella defensa* protects the aphid from attack by the parasitoid, *Aphididus ervi* (Oliver et al. 2005). Similarly, infection by the facultative symbiont, *Serratia symbiotica*, helps the aphid to tolerate higher temperatures (Russel and Moran 2006).

Symbionts not only play an important role in host nutrition, development, and immune responses, but they may also be important in developing insect resistance to insecticides (Berticat et al. 2002, Kikuchi et al. 2012, Xia et al. 2013, Gressel 2018). Insect resistance to pesticides is a worldwide problem, and the mechanisms of resistance include increased metabolism, target site modification, behavior modification, reduced penetration, increased excretion and sequestration (van Kretschmar et al. 2013). The role of symbionts in insect resistance to pesticides is an

understudied area, but in recent years several scientific papers highlight their role. Kikuchi et al. (2012) found that a soil specific bacteria from the genus *Burkholderia* when acquired by the bean bug, *Riptortus pedestris*, induced resistance to the insecticide, fenitrothion. Also, in the diamond back moth, *Plutella xylostella*, where resistance to different insecticides have been studied and many mutations have been identified related to resistance, the microbiome is different between resistant and susceptible strains for chlorpyrifos and fipronil (Xia et al. 2013). In another study, microbe-free mosquito larvae were more susceptible to *Bacillus thuringiensis* (Bt) toxin compared to larvae with their natural flora. Berticat et al. (2002) found a higher density of *Wolbachia* in mosquito, *Culex pipiens*, strains which are resistant to organophosphates. The microbiome of the Oriental fruit fly has been reported to confer elevated insecticide resistance (Cheng et al. 2017). Further, recent whole metagenome sequencing evidence found links between mosquito microbiota and insecticide resistance in malaria vectors (Dada et al. 2018).

Insect resistance to transgenic plant technologies threatens their long-term effectiveness (Oppert et al. 1997), and in recent years resistance towards Bt has also been documented. The first case of *H. zea* resistance toward Bt was documented in 2002 (Tabashnik et al. 2013) and in cotton by Reisig et al. (2018). *Spodoptera frugiperda* resistance towards Bt corn (Cry1F) has also been reported in the US (Hung et al. 2014). Development of resistance could at least in theory be attributed to microbial diversity in the larvae. For example, Bt protoxins need an alkaline pH for their activation (Wilson and Benoit 1993), and *Enterococcus faecalis*, found in higher pHs can acidify their environment through metabolite production (Manero and Blanch 1999). If this bacteria could invade the insect midgut, this could protect the insect from Bt toxicity (Broderick et al. 2003). There is also evidence that the microbiome can be propagated across generations. Jin et al. (2018) recently found a dominant point mutation in a tretraspanin gene associated with field-evolved resistance of the cotton bollworm, *Helicoverpa armigera*, to transgenic cotton, and Zhang et al. (2018) found decreased Cry1Ac activation by midgut proteases associated with Cry1Ac resistance in *Helicoverpa zea*

The two year focus of our laboratories was to examine potential difference in the bollworm microbiome from field collected caterpillars collected from non-Bt versus WideStrike cotton grown in the same area in North Carolina. Reported here is the sequencing results of the microbiome analysis of bollworms from Year 1 collections and results from Year 2 bollworm collections of differences between non-Bt cotton versus WideStrike cotton in the density of bacteria determined by culturing. Both Year 1 and 2 results are presented *in toto* for comparison purposes where the year 1 results (not including Illumina sequencing) were presented earlier at a Beltwide conference (Dhammi et al. 2017).

Materials and Methods

Insect Collection, Treatment and Sample Preparation in Year 2 Studies

In this study, the effects of cotton variety on bacterial density and diversity in the cotton bollworm microbiome were assessed. Bollworms (2^{nd} to 3^{rd} instars), *Helicoverpa zea*, were collected in 2018 (Year 2 of the study) from non-Bt (also referred to as "conventional" cotton) and Bt (WideStrike) cotton located at the Upper Coastal Plain Research Station, Rocky Mount, NC and immediately returned to the laboratory for assays. Initial processing of the larvae was as follows: each larva was surface sterilized with 95% ethanol (30 sec) followed by 1% bleach (30 sec) and finally washed 5 times with sterile water. The larvae were then homogenized separately in 750 µL of sterile phosphate-buffered saline (PBS) and then used in cultivable bacteria density assays using plate count agar (PCA) bacterial growth media.

Bacterial Enumeration of Year 2 Collected Bollworms

To estimate the amount of total cultivable bacteria, homogenates from each sample were serially-diluted up to 10^{-6}. Volumes of 50 microliters of each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were applied (two drops of 25 µl each) to a PCA plate for CFU (colony forming unit) counts. Colonies were counted after 48h of incubation at 37 °C. CFUs/insect were calculated based on the maximum colony forming units corresponding to the dilutions.

DNA Extraction of Year 1 Collected Bollworms

Bollworms in Year 1 of our studies were collected from non-Bt (conventional) versus WideStrike cotton as described before by Dhammi et al. (2017). DNA was extracted from bollworms by a method described previously (Ponnusamy et al. 2014). Briefly, two hundred microliters of homogenate were transferred to a 1.5 ml Eppendorf tube, and 160 microliters of lysis buffer 1 was added along with 20 microliters lysozymes and 20 microliters of proteinase K to each sample. Samples were then incubated at 37 °C for 1h. Subsequently, 200 microliters of pre-

warm lysis buffer 2 were added with further incubation at 56 °C for 1h. DNA was recovered through phenol/chloroform extraction and ethanol precipitation, and the resulting DNA pellet was resuspended in 100 microliters of DNA grade water. Subsequently, crude DNA was purified with the WIZARD DNA Cleanup System (Promega Corporation, Madison, WI, USA).

DNA Amplification and Illumina Sequencing of Year 1 Collected Bollworms

Isolated and purified DNA from 14 bollworm larvae were used in PCR reactions to amplify the V4 region of the 16S rRNA gene to produce an Illumina library. Sample subjects included 6 bollworm larvae that had been collected on conventional (non-Bt) cotton and 8 larvae collected from WideStrike (Bt) cotton. The library development protocol followed the Illumina protocol (Illumina Preparation Guide) using universal bacterial primers 515F and 806R for paired-end sequencing on the Illumina MiSeq platform. Sequencing, sequence quality control, taxonomic assignment and analysis were performed at the UNC Metagenomics facility (Chapel Hill, NC).

Results and Discussion

Comparison of Methods Used for Characterization of the Bollworm Microbiome

The experimental design was to collect 2nd to 3rd stadium bollworms, *Helicoverpa zea*, from non-Bt (referred to as "conventional") versus Bt (WideStrike) cotton grown in the field in locations as close to each other as possible where the cotton fields *a priori* were being used for other research projects. This restriction on the original planting of cotton plant types was dictated by land availability, routine field usage, normal farming practices and economy of resources for conducting the research and not based on any biases associated with the research objectives. The research presented in this proceedings presentation resulted from research during two field seasons, i.e., Year 1 in 2016 and Year 2 in 2018. A previous presentation and proceedings paper for work completed in Year 1 was published before (Dhammi et al. 2017) and will be discussed again here for comparison purposes.

The bacteria characterization in bollworms collected from conventional versus Widestrike cotton was restricted to bacteria internal to the caterpillars. The insect surface was treated to remove any living bacteria and also any bacteria DNA. Three methods (Fig. 1) were used to characterize the bacteria: Method I to determine the cultivable bacteria density (Year 1, see Dhammi et al. 2017; Year 2, see Materials and Methods); Method II used denaturing gradient gel electrophoresis (DGGE) to characterize bacteria species variability (Year 1 only; see Dhammi et al. 2017); and Method III used Illumina sequencing as described in the Materials and Methods (Year 1 sample results reported here; Year 2 sample analysis not completed at the time of this report).

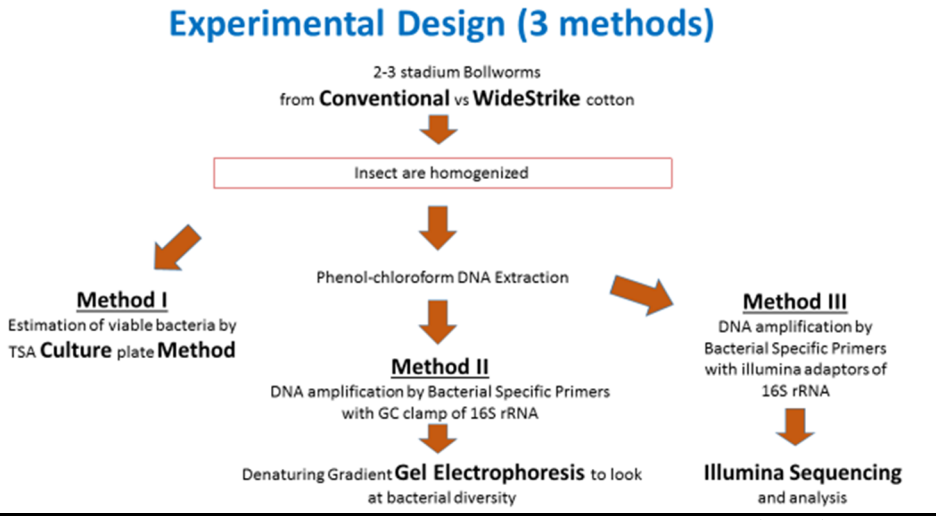


Figure 1. Three different methods were used to characterize the microbiome of 2nd to 3rd stadium bollworms, *Helicoverpa zea*, collected directly from the field from conventional (non-Bt) versus WideStrike (Bt) cotton.

Collection Sites for Bollworms from Conventional Versus WideStrike Cotton

The location of the conventional (non-Bt) and WideStrike (Bt) cotton field sites in Year 1 is shown in Fig. 2, top (Dhammi et al. 2017). Although the collection sites were in the same area, they were one field apart. In Year 2, the collection sites were just a few rows apart (Fig. 2, bottom) to further control for any possible field to field variations other than cotton type that that might account for differences in the caterpillar microbiomes.

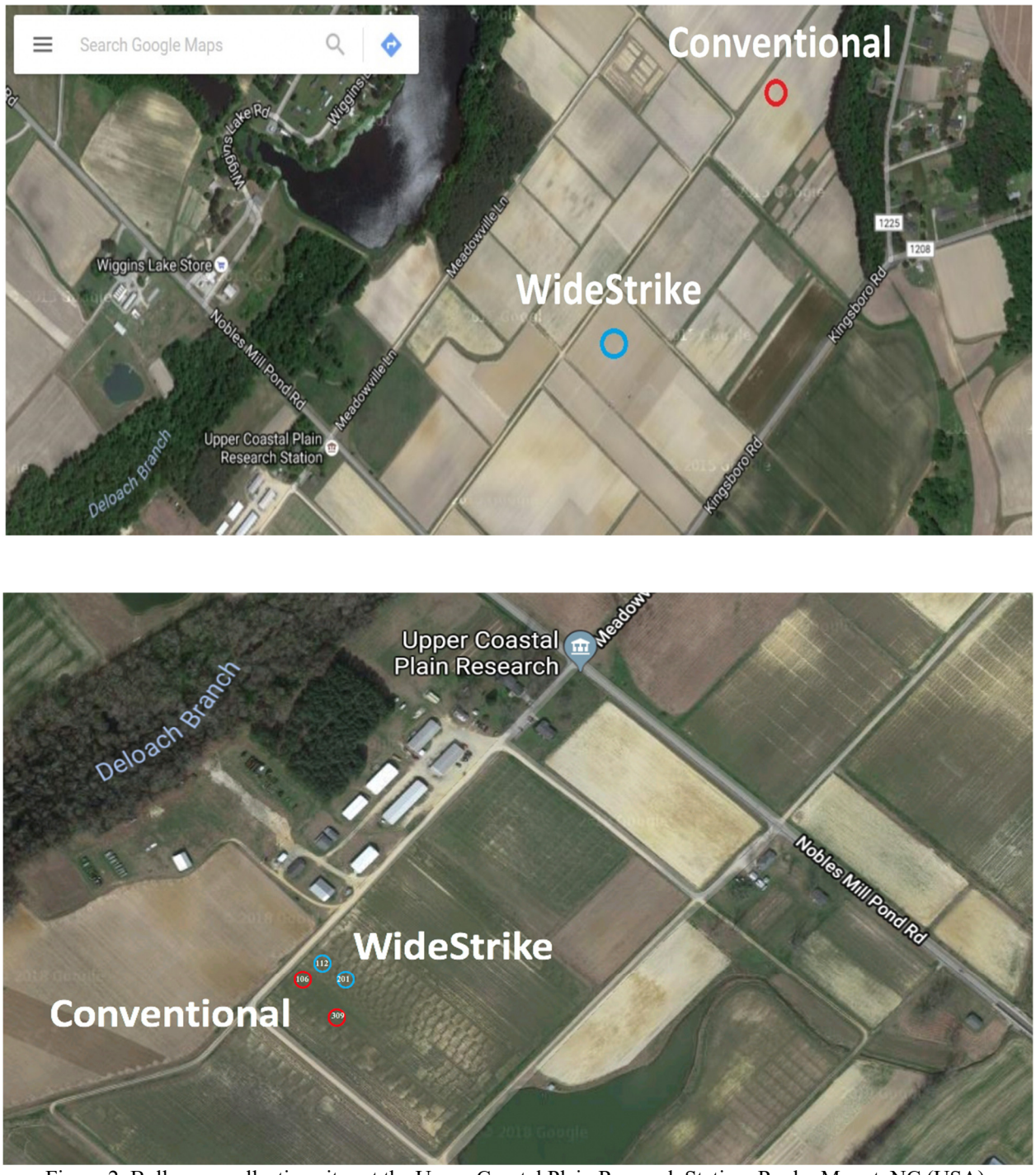


Figure 2. Bollworm collection sites at the Upper Coastal Plain Research Station, Rocky Mount, NC (USA). Bollworms from conventional (non-Bt) cotton were collected from plot C15 in Year 1 (top map) and from plots 106 and 309 (bottom) in Year 2. Bollworms from WideStrike were collected from plot C19 in Year 1 and from plots 112 and 201 in Year 2.

Estimation of Cultivable Bacteria by TSA (Tryptic Soy + Agar) (Year 1) and PCA (Plate Count Agar) (Year 2): Conventional (Non-Bt) Versus WideStrike (Bt) Cotton

Comparison of the CFUs per insect in bollworms collected from conventional (non-Bt) versus WideStrike (Bt) cotton in Year 1 showed that the majority of bollworms from Bt cotton had a higher density of live bacteria as compared to their conventional counterparts (Fig. 3) (Dhammi et al. 2017). Bollworms on WideStike had a mean bacterial count (log 10 CFUs/insect) of 8.42 as compared to 5.85 (log 10 CFUs/insect) for conventional cotton, which is a 2.57 log (10) difference (Fig 4). Comparison of the CFUs per insect in bollworms collected from conventional versus WideStrike cotton in Year 2 showed again that the bollworms from Bt cotton overall had a higher density of live bacteria as compared to their conventional (non-Bt) counterparts (Fig. 5). Bollworms on WideStike had a mean bacterial count (log 10 CFUs/insect) of 5.36 as compared to 4.30 (log 10 CFUs/insect) for conventional (non-Bt) cotton (Fig 6). For both Years 1 and 2, the bacterial count was higher for bollworms collected from Widestrike versus conventional cotton, even though in Year 2 the insect collections were much closer between the two cotton types (Fig. 2).

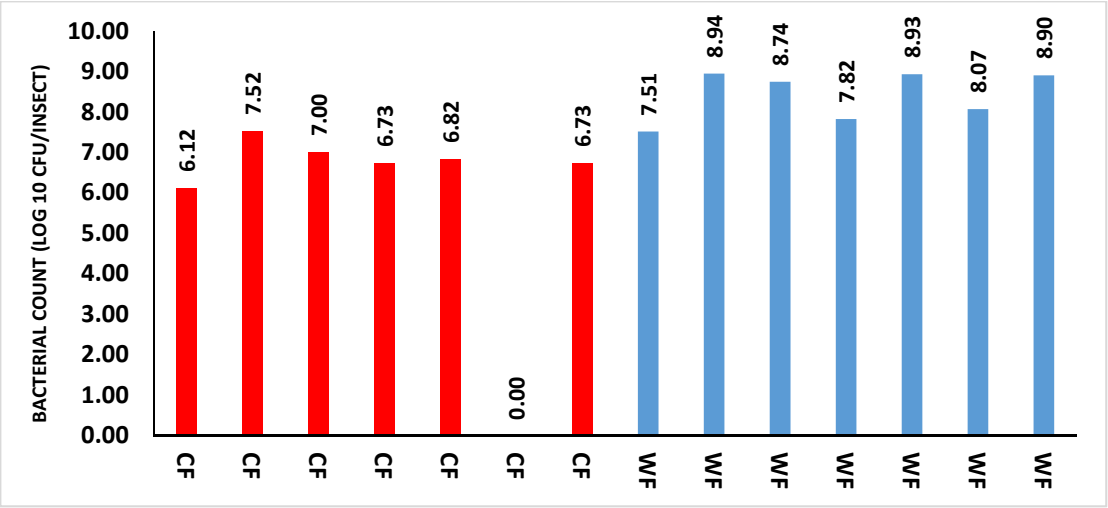


Figure 3. Prevalence of cultivable bacteria in bollworms from conventional (non-Bt) cotton (CF) versus WideStrike (WF) from the field in Year 1 (Fig. 2, top). Each bar represents CFUs from a single larva. Presented before by Dhammi et al. (2017).

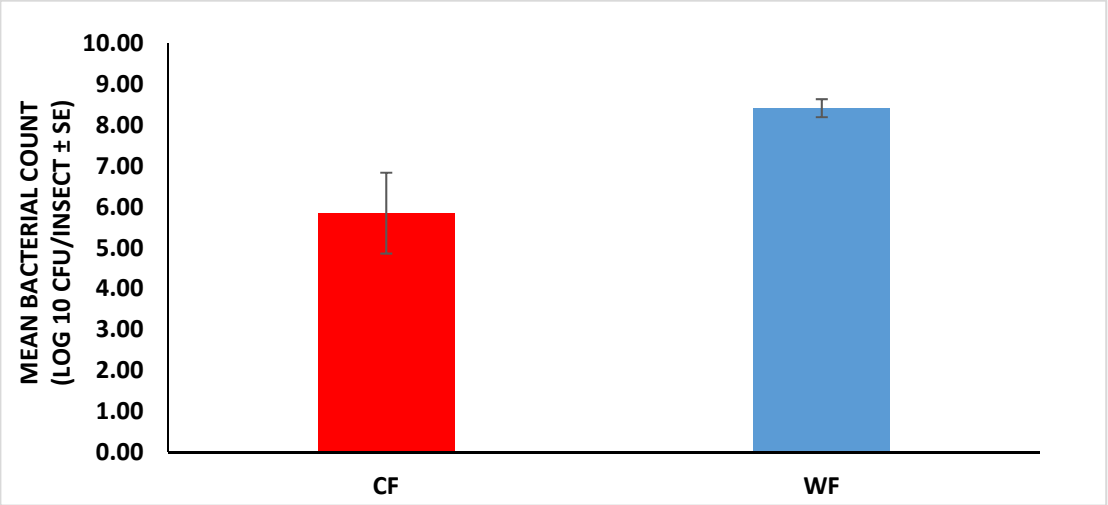


Figure 4. Mean prevalence of cultivable bacteria in bollworms from conventional (non-Bt) cotton (CF) versus WideStrike (Bt) cotton (WF) from the field in Year 1 (Fig. 2, top).

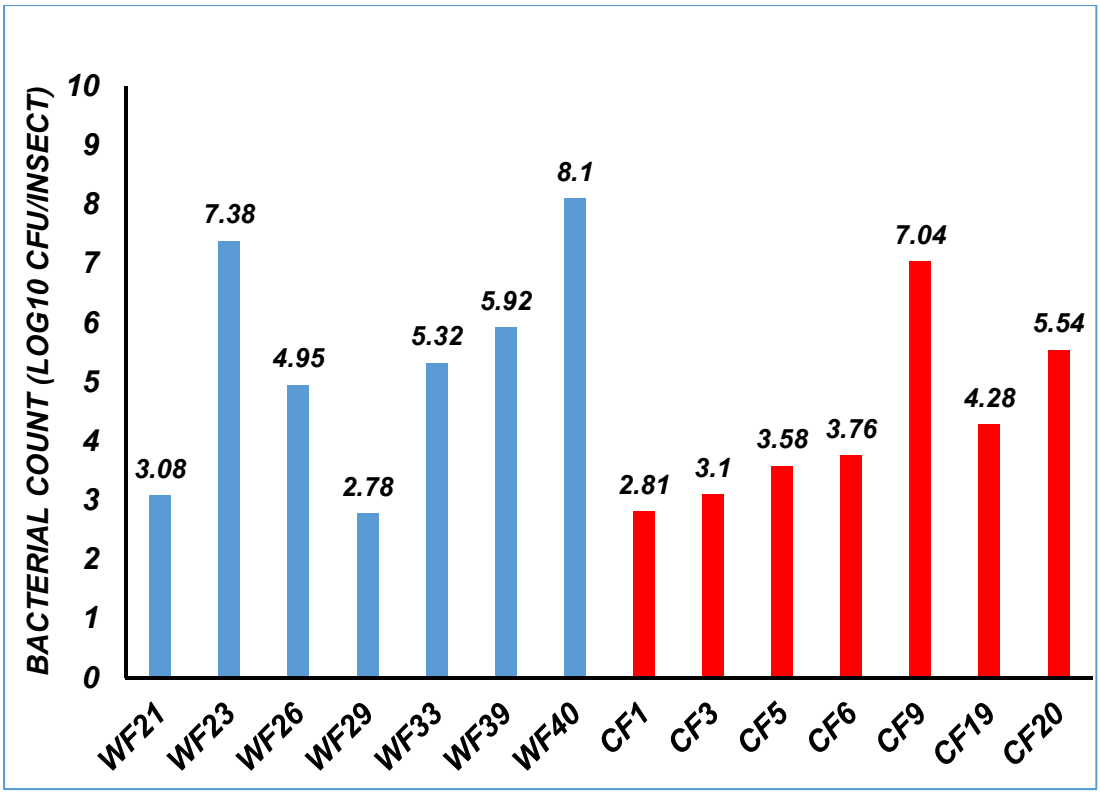


Figure 5. Prevalence of cultivable bacteria in bollworms from conventional (non-Bt) cotton (CF) versus WideStrike (Bt) cotton (WF) from the field in Year 2 (Fig. 2, bottom). Each bar represents CFUs from a single larva.

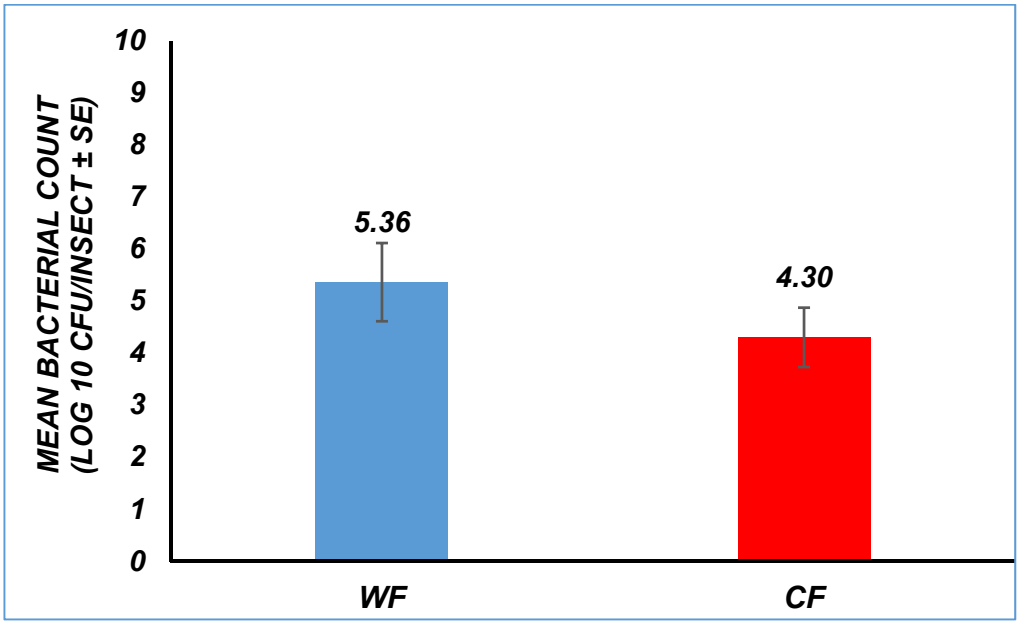


Figure 6. Mean prevalence of cultivable bacteria in bollworms from conventional (non-Bt) cotton (CF) versus WideStrike (Bt) cotton (WF) from the field in Year 2 (Fig. 2, bottom).

Illumunia sequencing and microbiome analysis

Previous characterization of bollworm microbiome diversity. Dhammi et al. (2017) investigated the species composition of the bacterial community (species richness and evenness) for bollworms collected in Year 1 from the field as shown in Fig. 2 (top) using a DGGE analysis of the 16S rRNA gene products amplified by PCR (Fig. 7). Each band (OTU = bacterial species) represents at least one unique phylotype. The richness of bacterial species in a sample in this analysis is reflected in the number of DGGE-DNA bands, the intensity of a band reflecting the relative abundance of the bacterial species. This approach provides a rough estimation of the diversity of the bacterial communities but is not as precise as a sequencing approach to identify species. This DGGE approach is less expensive and faster and is a reasonable first look at our samples before a more precise and costly sequencing method is conducted.

Bollworms from the conventional cotton had 1-2 prominent bands and 4-6 weak bands in different replicates. On the other hand, bollworms from WideStrike (Bt) cotton had 2-3 prominent bands but 10-12 weak bands. Bollworms in Year 1 from WideStrike had a higher density of bacterial (discussed earlier) than conventional cotton. Similar results were found in another study, where resistant *Heliothis virescens* had a higher bacterial diversity when fed on Bt cotton as compared to conventional (non-Bt) cotton (Dhammi et al. 2014).

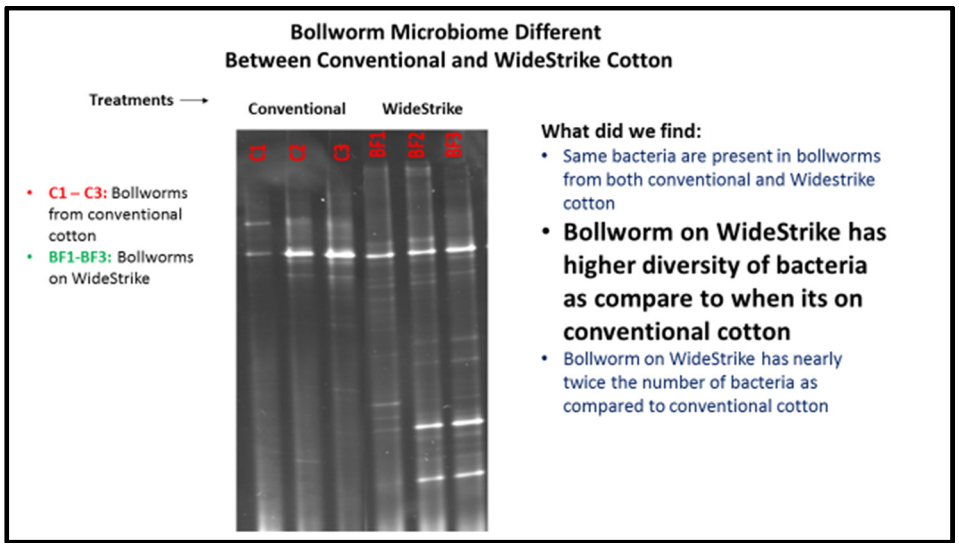


Figure 7. DGGE profile of bacterial communities from bollworms from conventional (non-Bt) cotton (C1-3) versus WideStrike (Bt) cotton (BF1-BF3) collected from the field in Year 1 (Fig. 2, top). Each lane represents a sample (one insect), and each band represents a possible single bacterial species. Presented before by Dhammi et al. (2017).

Illumina sequencing analysis (Method III). Since the DGGE analysis of our bollworm samples from Year 1 collections suggested a difference in bacteria diversity between conventional versus WideStrike cotton, this was a reasonable justification to conduct a more detailed analysis of species diversity of these same samples using a high throughput, Illumina sequencing approach. The method is described in the Materials and Methods, Fig. 8 shows the general work flow for this analysis, and Fig. 9 shows the species diversity. For some unknown reason, the 16S rRNA gene amplifications for these samples produced low amounts of PCR product. In trouble shooting the problem, the low amplification levels seem to be specific to caterpillars collected from the field. Because of this low product level from PCR, the number of sequences (operational taxonomic units, OTUs) obtained from Illumina sequencing was lower than what would be considered valid for microbiome characterization. Since the analysis was conducted and the data available and since a similar analysis for Year 2 was in progress but not completed at the time of this report, we present the results obtained in Year 1 (Fig. 9). Interestingly, we also are finding the same issue with amplification problems for Year 2 samples; we do not see any of these issues with genomic bacteria DNA from other sources including from other insects and chiggers, and we do not see this problem when we add exogenous bacteria genomic DNA to our caterpillar DNA.

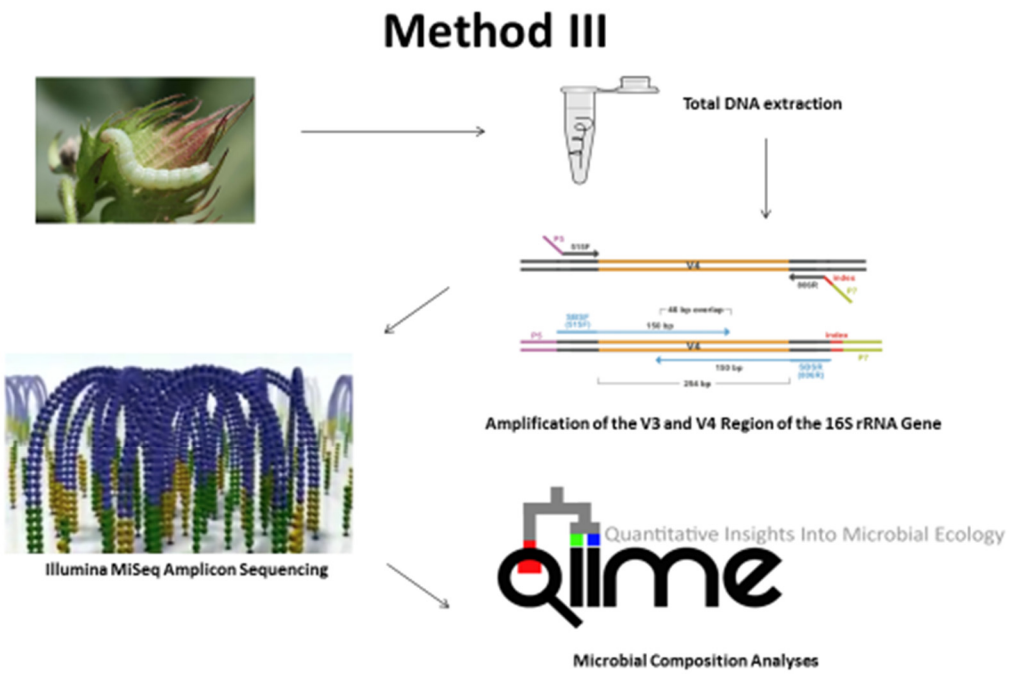


Figure 8. Method III (Fig. 1), outline of Illumina sequencing method used for characterization of the microbiome from bollworms from conventional versus WideStrike cotton. See Materials and Methods for details of procedure.

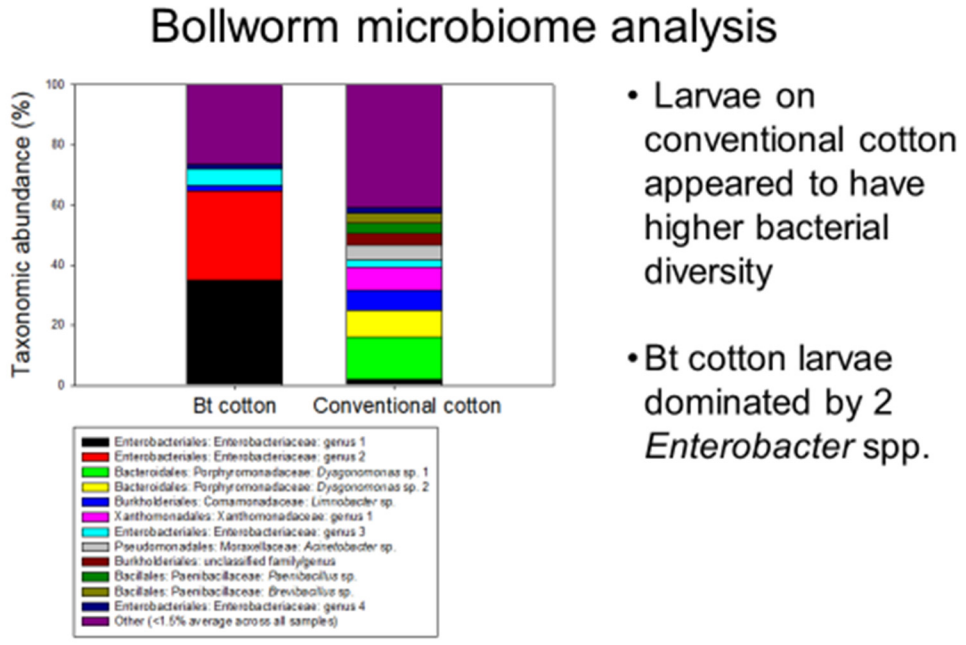
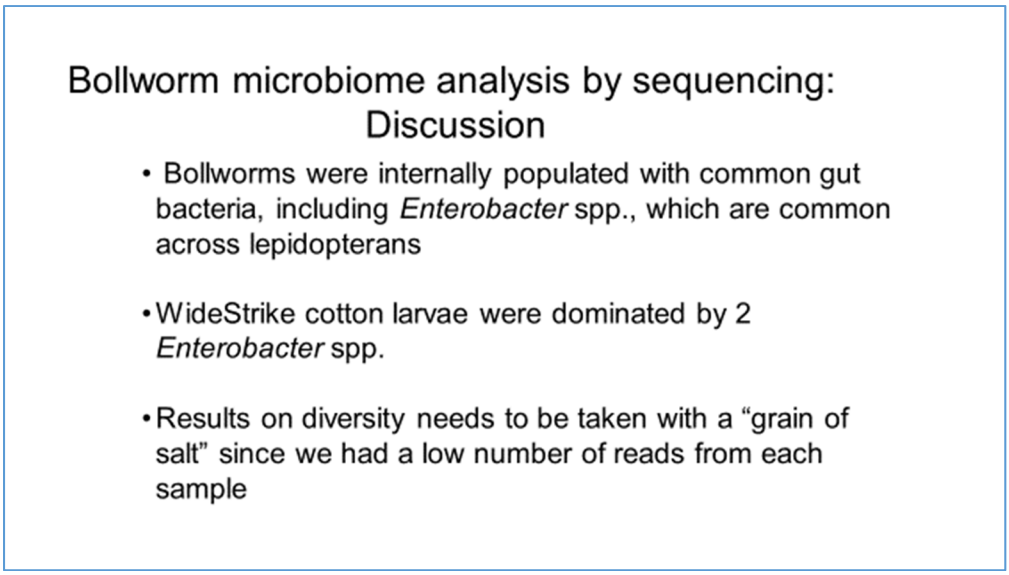


Figure 9. Bacteria diversity determined by Illumina sequencing (Figs. 1 and 8; see Materials and Methods for details) for the internal microbiome for bollworms, *Helicoverpa zea*, collected from conventional (non-Bt) cotton versus WideStrike (Bt) cotton in Year 1 (Fig. 1, top).

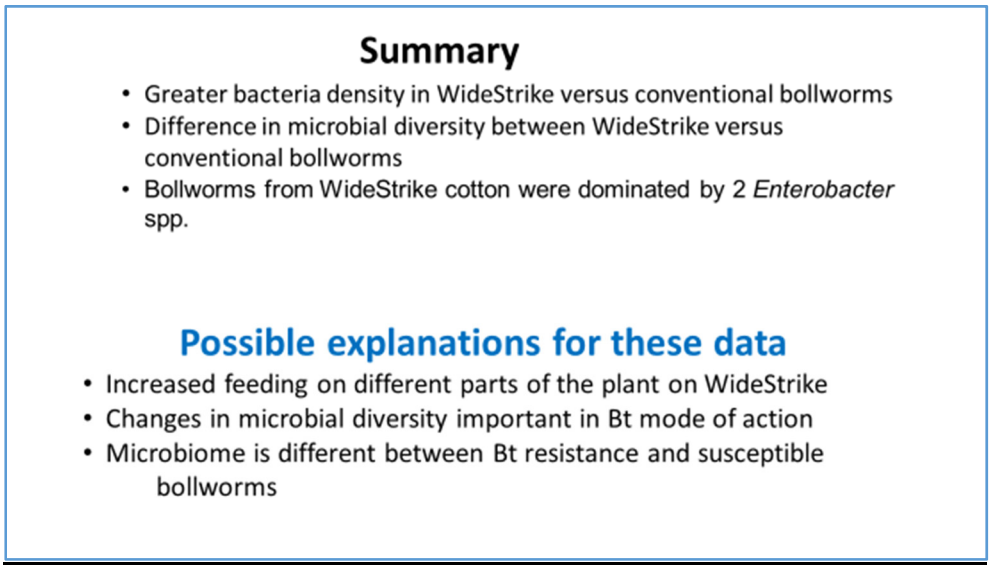
WideStike versus conventional bollworm microbiome diversity by sequencing. The microbiome diversity analysis by sequencing (Fig. 9) of bollworms collected directly from the field from conventional (non-Bt) versus WideStrike (Bt) cotton in Year 1 (Fig. 2, top) were consistent with the findings from the same collections analyzed by DGGE (Fig. 7). The microbiome of bollworms from WideStrike was dominated by 2 *Enterobacter spp.* unlike that from conventional cotton (Fig. 9). Overall bollworms were internally populated with common gut lepidopteran

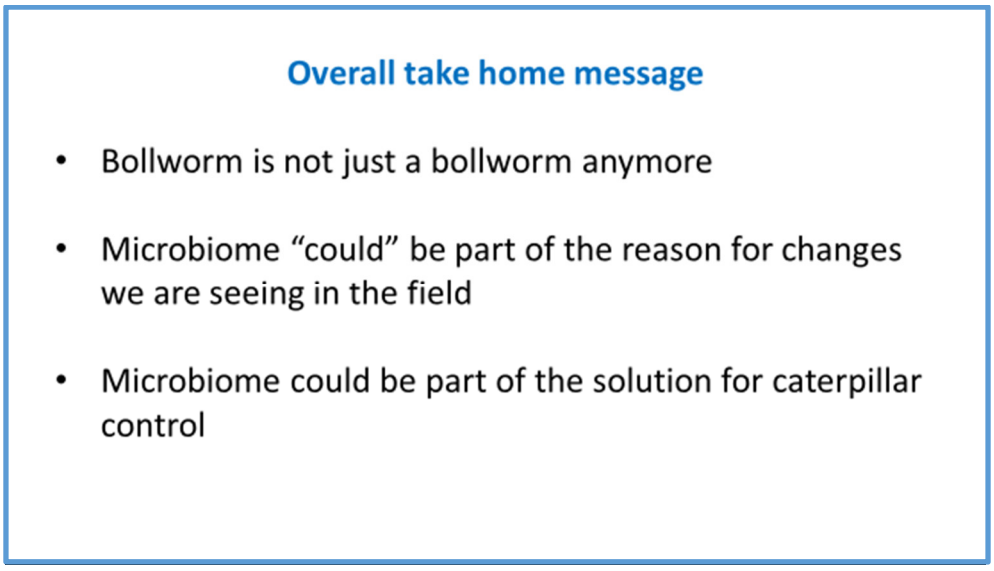


Enterobacter spp. Because of the domination of two bacteria species in WideStrike bollworms and the low number of sequences obtained from the Illumina sequencing run, the level of diversity in WideStrike could not be fully assessed. Our hypothesis is that the lack of other bacteria which were found by DGGE (Fig. 7) in the WideStrike bollworms but not in the same samples by Illumina sequencing (Fig. 9) resulted from the dominance of *Enterobacter* genus 1 and genus 2 bacteria and the low number of OTUs obtained for the latter.

Conclusions and Future Directions

In summary, greater bacteria density was found internally in 2nd-3rd stadium bollworms, *Helicoverpa zea,* collected from WideStrike (Bt) versus conventional (non-Bt) field cotton over two field seasons. There was a difference





in microbial diversity between bollworms collected from WideStrike versus conventional cotton as determined by two different methods from samples analyzed in Year 1 of the project. Bollworms from WideStrike were dominated by two *Enterobacter spp.* unlike that of conventional (non-Bt) bollworms. When considering changes in caterpillar susceptibility to Bt and other toxins, differences in the microbiome should be considered. The microbiome could be an important factor in the evolution of Bt toxin resistance in caterpillars, but also important, the microbiome could be a long-term solution to caterpillar control.

Acknowledgements

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