CATERPILLARS, COTTON PEST PROTECTION AND INSECT BIOASSAY: DON'T FORGET THE MICROBIOME

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Abstract

Insecticide resistance in insects has been attributed to many factors, namely target site modification, penetration, sequestration, metabolism, excretion and behavior. But the role of the microbiome present in insects in resistance is barely explored. In recent years, several studies suggest the microbiome might affect insect susceptibility to pesticides. The microbiome of an organism also can be affected by a number of variables including the insect's diet (often artificially produced) and food plants. Often insects from the field are bioassayed using artificial diet which contain antimicrobial inhibitors. In this study, changes in microbial diversity in the cotton bollworm, *Helicoverpa zea*, were investigated from conventional and WideStrike field cotton and which were also transferred to the laboratory on artificial diet. Microbial diversity was analyzed by the Tryptic Soy Agar (TSA) plate drop count method and by denaturing gradient gel electrophoresis (DGGE) after amplification of the bacterial V3 region of the 16S rRNA gene. The TSA analysis shows a lower number of culturable bacteria when field collected bollworms from conventional cotton were reared for 48 h on regular artificial diet (with a microbial inhibitor, methyl paraben (MPB)) as compared to bollworms collected directly from conventional cotton. The bacteria count was higher when they were reared on MPB free diet. There also were more culturable bacteria in bollworms from WideStrike as compared to conventional cotton. The DGGE studies show that the transfer of bollworms from conventional cotton to artificial diet with or without MPB changes the make-up of the bacterial community. Also the bollworms from WideStrike had a higher microbial diversity as compared to conventional cotton. These preliminary findings need further replication but at least 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, including the use of high throughput sequencing to better characterize the bacterial communities.

Introduction

Microorganisms have contributed a great deal towards 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 on insect growth and development. For example, germ free crickets have a lower enzymatic hydrolytic activity as compared to its conventional counterpart, and these bacteria help in utilizing a wider range of food substrates (Domingo et al. 1998). Hemipterans like aphids survive exclusively on plant sap which is deficient in essential nutrients. The sap feeding aphids are dependent on its association with gut symbionts to use its nutrient deficient food source for its survival (Rajagopal 2009). Similarly, kudzu bugs 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 help its host to maintain 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 may also be important in developing insect resistance to pesticides (Berticat et al.; Kikuchi et al. 2012; Xia et al. 2013). 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.

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). Fall army worm resistance towards Bt corn (CryF1) 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 though metabolite production (Manero and Blanch 1999). If this bacteria would invade the insect midgut, this could protect the insect from Bt toxicity (Broderick et al. 2003). Also since the microbiome could be affected by a change in diet, it is important to understand differences between the caterpillar microbiome in the field on different cotton varieties and what happens when caterpillars are transferred to the laboratory and reared and bioassayed on artificial diet (which most often contains anti-microbial agents). There is no current published research on this subject. The objective of this study is to investigate the impact of artificial diet on microbial diversity of the cotton bollworm. We also examined the microbial diversity of bollworms feeding in the field on conventional versus Bt cotton. In our work presented here, we found that changes in the diet lead to changes in the microbial composition of the larvae.

Materials and Methods

Insect collection, treatment and sample preparation

In this study, the impact of artificial diet and cotton variety on bacterial density and diversity was assessed. Cotton bollworms (CBWs) were collected from conventional and Bt (WideStrike) cotton located at the Upper Coastal Plain Research Station, Rocky Mount, NC (Fig. 1). CBWs (2^{nd} to 3^{rd} instars) collected from conventional cotton (2 different collection times, Fig. 2) were fed on artificial diet with (n=7) and without methyl paraben, MPB (an antimicrobial agent) (n=7) for 48 hours for each collection time. The microbiome was analyzed between the bollworm from conventional cotton (directly from the field) and when they were fed on artificial diet with and without MPB. Bollworms were also collected in the second collection time (Fig. 1) from WideStrike and were only transferred to artificial diet with MPB (n=4; because of the limited number of bollworms available in the field). The microbiome was investigated by estimating the number of culturable bacteria by the TSA plate count method and by analyzing the 16S rRNA gene profile using a DGGE analysis (described in more detail later). Each larva was surface sterilized with 95% ethanol (30 sec) followed by 1% bleach (30 sec) and finally washed 5 times with sterile water. Larvae were homogenized in 300 µl of sterile phosphate-buffered saline (PBS) and then used for the TSA plate count and DGGE analysis.

Bacterial enumeration

To enumerate total cultivable bacteria, homogenate from each sample was serially-diluted up to 10^{-7}. Volumes of twenty five microliters of each dilution (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions) was applied to a TSA plate as a drop, and colonies were counted after 24 hours of incubation at 28 °C. Colony forming units (CFU)/insect was calculated based on the maximum colony forming units (CFUs) corresponding to the dilutions.

DNA extraction

DNA was extracted from CBWs by a method described previously (Ponnusamy et al. 2014). Briefly, two hundred microliters of homogenate was 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 proteinase K to each sample. Samples were then incubated at 37 °C for 1 hour. Subsequently, 200 microliters of pre-warm lysis buffer 2 was added with further incubation at 56

°C for 1 hour. 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, Madison, WI, USA).

DNA amplification

The V3 region of the 16S rRNA gene (specific to bacteria) was amplified by the universal primer **F357-GC** (5′-GC-clamp+ CCTACGGGAGGCAGCAG-3′) and **518R** (5′-ATTACCGCGGCTGCTGG-3′) with a GC-clamp added to the 5' end of the forward primer to prevent a complete denaturation of the double-stranded fragments. The touchdown PCR was performed as described by Ponnusamy et al. (2008).

Denaturing gradient gel electrophoresis

DGGE was performed using the DCode System (Bio-Rad) as described by Muyzer el al. (1993). Samples (15 microliters) were loaded onto 8 % polyacrylamide/bis (37.5:1) gels with denaturing gradients from 45–55 % [where 100 % is 7 M urea and 40 % (v/v) deionized formamide] in 0.5X TAE electrophoresis buffer. Electrophoresis was performed at 50 V at a temperature of 60 °C for 18 hours. The gel was stained with SYBR green I in 0.5X TAE buffer and digitally photographed using a ChemiDoc-It^{TS2} Imager (UVP, LLC Upland, CA).

Results and Discussion

Collection sites and collection times

The location of our two field sites is shown in Fig. 1, i.e., conventional cotton and WideStrike. We also conducted collections twice from the conventional cotton and once from WideStrike for the second collection period (Fig. 2).

Figure 1. Cotton bollworm collection site at Upper Coastal Plain Research Station, Rocky Mount, NC. Bollworms from conventional cotton were collected from plot C15 (red circle) and bollworms from WideStrike were collected from plot C19 (blue circle).

First Collection

Bollworms from conventional Cotton :

- Straight from the field (CF)
- On regular artificial diet (CA+)
- On artificial diet without methylparaben (MPB) (CA-)

Second Collection

Bollworms from conventional Cotton

- Straight from the field (CF)
- On regular artificial diet (CA+)
- On artificial diet without MPB (CA-)

Bollworms from Bt Cotton:

- Straight from the field (WF)
- On regular artificial diet (WA+)

Figure 2. Summary of experimental design, treatments and abbreviations. Regular diet contained MPB.

Estimation of culturable bacteria by TSA (Tryptic Soy Agar): the field to artificial diet

In the first collection the CFU analysis showed a lower number of live bacteria in CBW after feeding for 48 hours on artificial diet with MPB as compared to CBWs from conventional cotton (Fig. 3). CBWs fed on diet with no MPB had a higher number of culturable bacteria in comparison to the other treatments (Fig. 3). Similar trends were seen in the second run (Fig. 4). Interestingly, many bollworm larvae which were fed on artificial diet with MPB had no CFUs especially in the second collection where 3 of the 6 larvae demonstrated no culturable bacteria (Figs. 3 and 4). The differences between larvae could be a result of when the larvae start feeding on the artificial diet (with MPB present) with those insects that start feeding earlier having lower levels of bacteria. A longer feeding period than 48 hours should be examined in the future to see if the trend for no culturable bacteria becomes more prevalent.

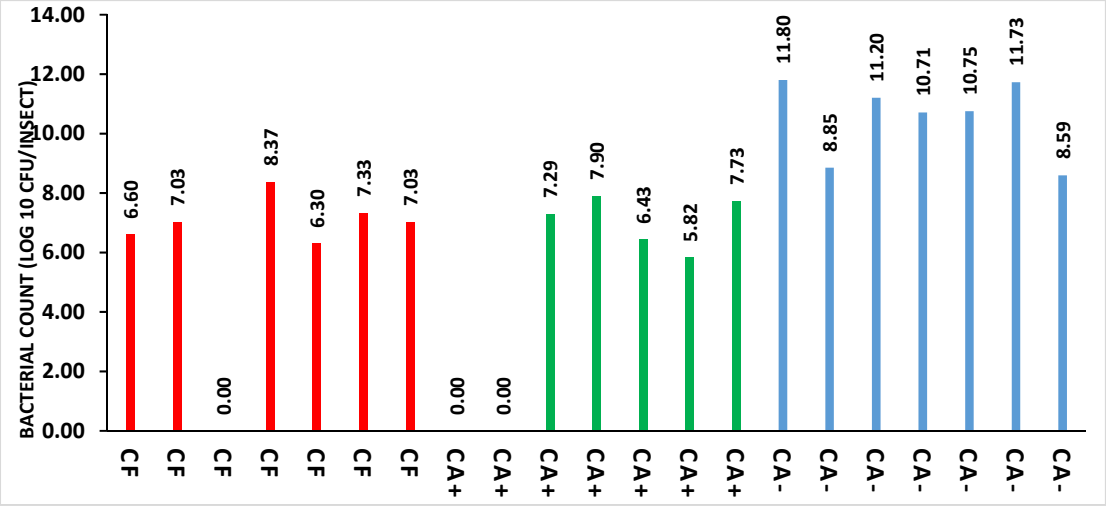

Figure 3. Prevalence of culturable bacteria in bollworms from conventional cotton from the field (CF), reared on artificial diet with (CA+) and without MPB (CA-) at the first collection time (Fig. 2). Each bar represents CFUs from a single larva.

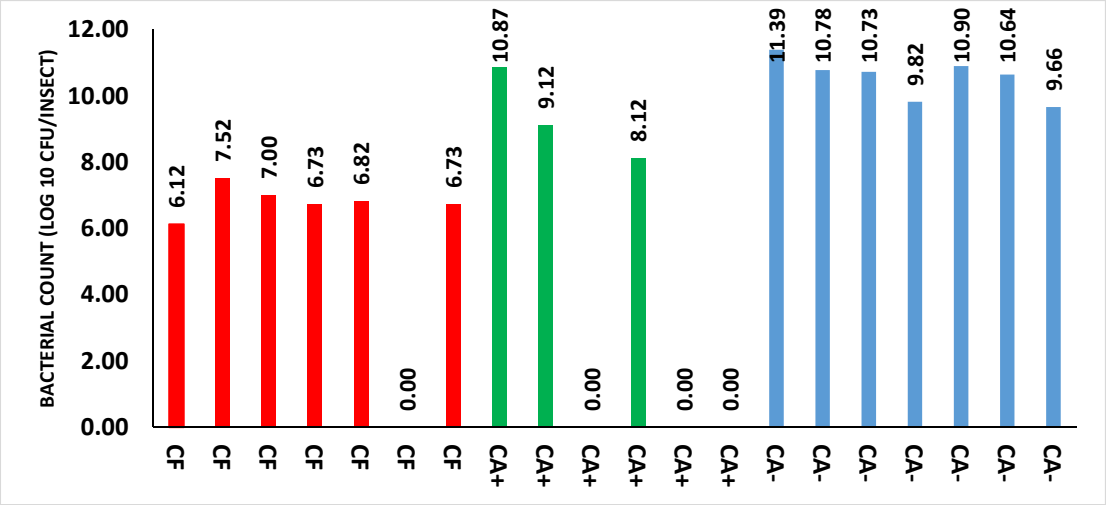

Figure 4. Prevalence of culturable bacteria in bollworms from conventional cotton from the field (CF) and reared on artificial diet with (CA+) and without MPB (CA-) at the second collection time (Fig. 2). Each bar represents CFUs from a single larva.

CBWs which were collected from WideStrike show a decrease in culturable bacteria from the field to those fed on artificial diet with MPB (Fig. 5) similar to conventional cotton (Figs. 3 and 4). Our data show that diet type impacts microbial density in CBWs (Figs. 3, 4 and 5).

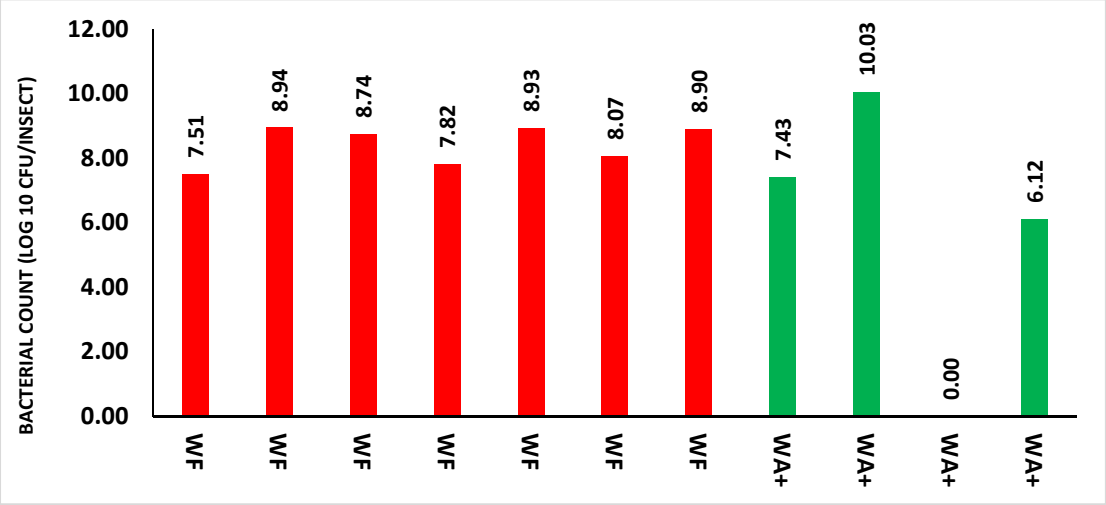

Figure 5. Prevalence of culturable bacteria in bollworms from WideStrike cotton from the field (WF) and reared on artificial diet with MPB (WA+) at the second collection time (Fig. 2). Each bar represents CFUs from a single larva.

Estimation of culturable bacteria by TSA (Tryptic Soy + Agar): conventional versus WideStrike cotton

Comparison of the CFUs per insect in bollworms collected from conventional versus WideStrike cotton shows that the majority of bollworms from Bt cotton had a higher density of live bacteria as compared to its conventional counterparts (Fig. 6). CBWs 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 7).

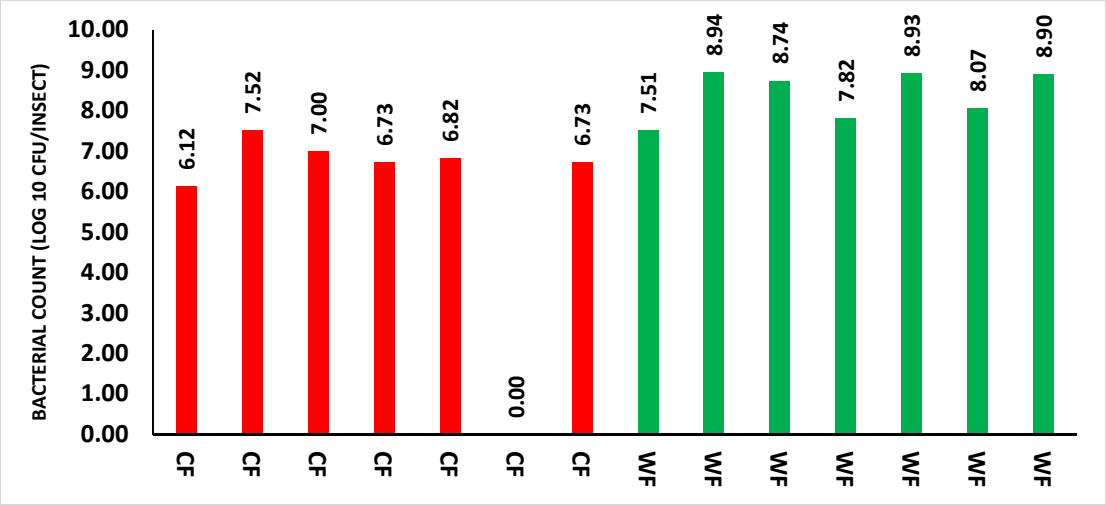

Figure 6. Prevalence of culturable bacteria in bollworms from conventional cotton (CF) versus WideStrike (WF) from the field at the second collection time (Fig. 2). Each bar represents CFUs from a single larva.

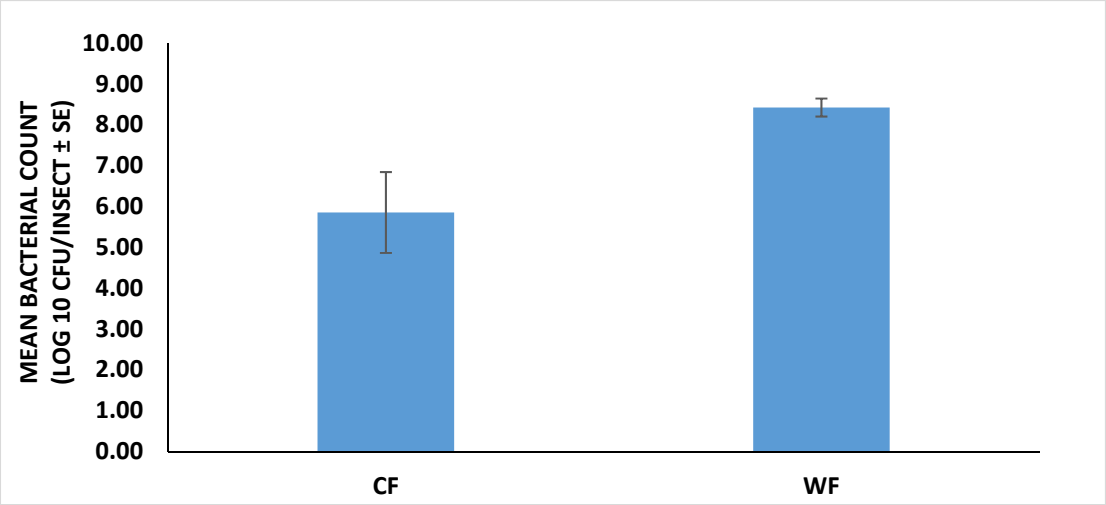

Figure 7. Mean prevalence of culturable bacteria in bollworms from conventional cotton (CF) versus WideStrike (WF) from the field at the second collection time (Fig. 2).

DGGE analysis: field versus artificial diet

We also investigated the species composition of the bacterial community (species richness and evenness) for each of the treatments shown in Fig. 2 using a DGGE analysis of the 16S rRNA gene products amplified by PCR. Each band shown (OTU = bacterial species) represents at least one unique phylotype. Therefore, the richness of bacterial species in a sample was reflected in the number of DGGE-DNA bands; the intensity of a band reflected the relative abundance of the bacterial species in a sample. 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 reasonable as a first look at our samples before a more precise and costly sequencing method.

Bollworms from conventional cotton had 1-2 prominent and about 4-6 weak bands (Fig. 8). On diet with no MPB, there were a higher number of bands but with some shared with bollworms taken directly from conventional cotton (Fig. 8). Bollworms on artificial diet with MPB also had a different microbial diversity (Fig. 8). Even though some OTUs are similar in all treatments, it is clear that food source affected the microbial diversity. In summary, a change in the food source changes the microbial diversity of CBWs.

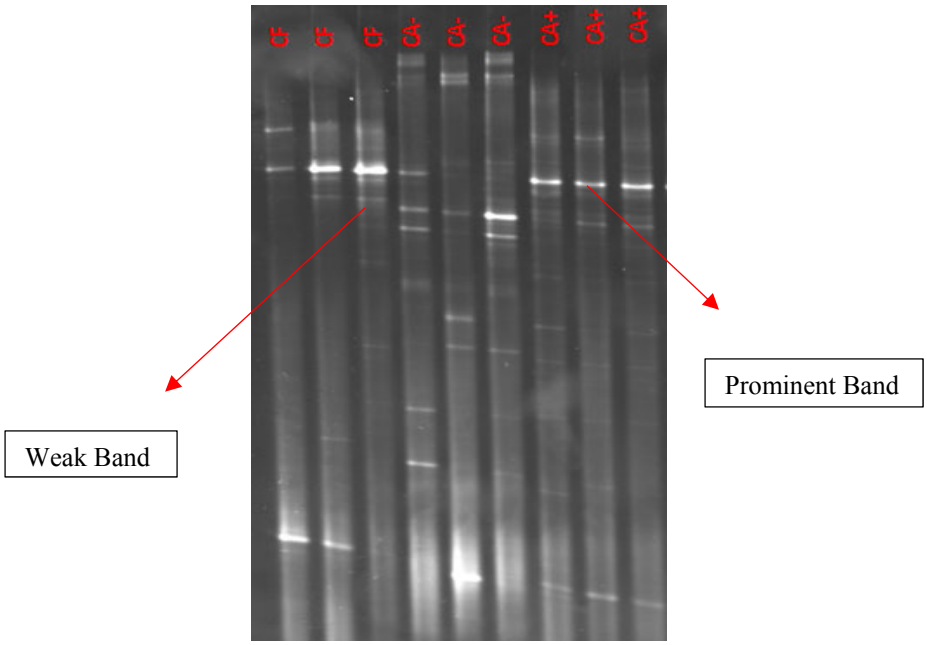

Figure 8. DGGE profile of bacterial communities from cotton bollworm fed on conventional cotton from field (CF) and on artificial diet without (CA-) and with MPB (CA+). Each lane represents a sample (one insect) and each band represents a single bacterial species.

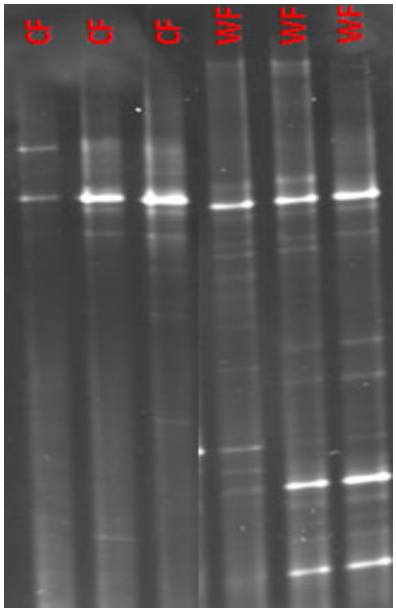

Figure 9. DGGE profile of bacterial communities from the cotton bollworm from conventional cotton (CF) versus WideStrike cotton (WF). Each lane represents a sample (one insect) and each band represents a single bacterial species.

DGGE analysis: conventional versus WideStrike cotton

CBWs from conventional cotton have 1-2 prominent bands and 4-6 weak band in different replicates. On the other hand, CBWs from WideStrike have 2-3 prominent bands but 10-12 weak bands. CBWs from WideStrike had a higher density of bacterial (discussed earlier) and a different diversity of species than conventional cotton. Similar results were found in our previous study, where resistant tobacco budworm had a higher bacterial diversity when fed on Bt cotton as compared to conventional cotton (Dhammi et al. 2014).

Conclusions and Future Directions

Preliminary analysis of the microbial diversity of CBWs by the TSA plate colony count method and DGGE suggests that their bacterial communities are affected by the type of diet. Our study shows that once CBWs are taken from the field and fed on artificial diet, microbial diversity is changed in as little as 48 hours. In the presence of antimicrobial agents in the artificial diet, there also was a trend in 48 hours toward the elimination of living bacteria in some insects. This finding suggests, if we want to mimic field conditions with a minimal impact on the insect's microbial community, new bioassay methods are needed. The current most common method to determine susceptibility is to transfer insects to artificial diet with antimicrobial agents and score mortality 7 days later. Also, in some cases, insects are reared on artificial diet with antimicrobial agents for multiple generations before testing. In addition, we need to examine the impact of microbiome changes on Bt susceptibility in caterpillars like the bollworm. Finally, our study found that the CBWs from WideStrike had a higher number of bacterial and a different microbial diversity compared to conventional plants.

The results reported here should be considered preliminary but argues that the cotton-insect microbiome ecosystem should be further evaluated to better understand its impact on the future sustainability of cotton production. Research is needed to identify the bacteria in the microbiome of CBW in different diets and its potential role if any in Bt resistance. An artificial diet with minimum impact on the microbiome in combination with a short assay time would be best to eliminate any microbiome bias for lab bioassays. Our 4 h FDT test (Bailey et al. 1998; Roe et al. 2000, 2002; Cabrera et al. 2010, 2011) could be a good alternative to the 7 day bioassays most commonly used to assess Bt susceptibility. Also for a short assay time like 4 h, antimicrobial agents are not needed in the diet to prevent diet spoilage.

Acknowledgements

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