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Comparison of Gut Microbial Community between Bt-Resistant and Susceptible Strains of *Ostrinia furnacalis*

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Abstract: *Bacillus thuringiensis* is an effective entomopathogen, and its crystal toxin expressed in transgenic crops has been widely used for pest control. However, insect resistance risk is the main threat to the continued successful utility of Bt crops. Several studies reported the role of midgut microbiota in Bt resistance, but the mechanism remains controversial. In the present study, using high-throughput sequencing of the bacterial 16S ribosomal RNA gene, we surveyed the midgut bacterial flora of *Ostrinia furnacalis* from one Bt-susceptible (ACB-BtS) and two Bt-resistant (ACB-AbR and ACB-FR) strains and explored the mortality of *O. furnacalis* after eliminating the gut bacteria. Gut bacterial diversity in Bt-resistant strains was significantly lower in Bt-resistant than in Bt-susceptible strains. Ordination analyses and statistical tests showed that the bacterial community of ACB-AbR was distinguishable from ACB-BtS. The genus *Halomonas* was dominated in ACB-BtS, but the *unclassified_Enterobacteriaceae* was the most enriched genus in ACB-AbR and ACB-FR. Furthermore, interactions of the bacterial community are more complex in Bt-resistant strains than in Bt-susceptible strains. Moreover, the mortalities of ACB-AbR and ACB-BtS strains treated by the Cry1Ab toxin were significantly reduced after eliminating the gut bacteria. Our findings suggest that Bt stressors structured in the midgut bacterial community and the microbiota have the potential to regulate the Bt-induced killing mechanism.

Keywords: *Bacillus thuringiensis*; gut microbiota; *Ostrinia furnacalis*; Cry toxin

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1. Introduction

Bacillus thuringiensis (Bt) is a gram-positive insect pathogenic bacterium which produces highly specific insecticidal crystal δ-endotoxins during the sporulation phase [1]. The Cry toxins are activated by insect gut protease and then bind to specific receptors of the midgut epithelium, leading to cell lysis and subsequent death [2]. Transgenic Bt crops have been widely used to control insect pests; however, the evolution of resistance by pests mainly threatens the continued successful utility of Bt crops [3,4]. The Asian corn border (ACB), *Ostrinia furnacalis*, is a major pest in Asia, particularly in China, the Philippines, and Vietnam [5]. ACB can feed all parts of maize and cause serious yield losses. Field trials in China have demonstrated that Bt toxin-expressing maize can effectively control ACB [6]. However, ACB can develop more than 150-fold resistance and 1700-fold resistance to Cry1Ab and Cry1Ftoxins, respectively, after selection under laboratory conditions [7,8]. These may be a restrictive factor for the widespread application of genetically modified maize.

Investigations into the evolution of Bt resistance have proposed different potential mechanisms, and most of them were focused on the receptor proteins [4]. Compared to susceptible strains, the Cry1Ab resistant ACB strain (ACB-AbR) showed a reduction

of the specific binding of both Cry1Ab and Cry1Aa, suggesting that part of the binding sites were lost or altered [9]. Through RNA-seq technology, the different expressions of several Bt resistance genes, such as aminopeptidase Ns, cadherin protein, and ATP binding cassette transporter group gene, were found in Bt-resistant strains of ACB [10,11]. It was demonstrated that the cadherin protein was a functional receptor for Cry1Ac in ACB, and its disruption conferred a moderate Cry1Ac resistance [12]. The in vivo functional investigation demonstrated the causality of the OfABCC2 truncating mutation with high-level resistance to the Cry1Fa toxin in ACB [13]. However, the mechanism therein that leads to the resistance of ACB to Bt is not fully understood. The insect gut harbors abundant bacteria that may contribute to the survival and environmental suitability of insect hosts. Many studies reported that gut microbiota may influence the Bt susceptibility and potentially influence the insect resistance to Bt [14,15]. Cry toxin-induced mortality of *Vanessa cardui*, *Manducasexta*, *Pieris rapae*, and *Heliothis virescens* was reduced when reared in an antibiotics diet but restored high toxicity to them after reintroducing the resident bacteria *Enterobacter* sp. [16]. The activity of protease was not inhibited by activated Cry1Ac and *Bt* formulation, and fewer Cry1Ac protein was detected in Bt/Cry1Ac protoxin-treated larvae when *Helicoverpa armigera* pretreatment with antibiotics and the susceptibility of *H. armigera* pretreatment with antibiotics to activated Cry1Ac, Bt formulation, and transgenic cotton all significantly decreased [17]. Decreased susceptibility to Bt toxin in *Plodiainter punctella*, *Plutellaxylostella*, *Spodopteraexigua*, *Chilosuppressalis*, and *Plagioderaversicolora* were all found after removal of the gut bacteria [18–22]. These suggested that gut bacteria exercise an important role in the toxicity of Bt toxins to insects.

The microbial community associated with insects is dynamic and shaped by stress factors in nature conditions. Moreover, if the gut bacteria are necessary for insect resistance to Bt, the resistant evolution to xenobiotic Bt in insects could induce changes in the gut bacterial community. A less rich and distinct bacterial community was found in Bt-resistant western corn rootworms compared with susceptible strains [16], while higher microbiota diversity and more complex bacteria co-occurrence patterns in Bt-resistant strains than that in Bt-susceptible strains were found in *Chilosuppressalis* [21]. The field-collected population of *Helicoverpazea* from Bt cotton showed higher bacterial density and diversity than the population collected from non-Bt cotton [23]. Bt GS57 strain infection reduced the gut bacterial diversity and an increase in bacterial load in *S. exigua* [20].

To investigate the interaction between the Bt resistance of ACB and the bacterial community, we characterized the midgut microbiota from two high Bt-resistant strains and a Bt-susceptible strain of ACB using 16S rRNA Illumina sequencing. Moreover, the larval mortalities of ACB with Bt toxins were conducted after larval pretreatment with antibiotics to remove gut bacteria. This study aims to determine the role of gut bacteria in the process of resistance evolved in ACB and the Bt selective pressure on the dynamics of the bacterial community.

2. Materials and Methods

2.1. Insect Rearing and Bt-Resistant Strain Selection

Three ACB laboratory strains were used in the current study, including a Bt toxin susceptible strain (ACB-BtS) and two laboratory selected resistant strains: Cry1Ab-resistance strain (ACB-AbR) and Cry1F-resistance strain (ACB-FR). All colonies were kindly provided by Professor Kanglai He. ACB-BtS was originally collected from summer maize fields in central China and reared on an artificial diet [24] without exposure to any Bt toxins. Resistant strains were selected as described previously for ACB-AbR [25] and ACB-FR [8]. Resistance to Cry1Ab and Cry1F was estimated between ACB-AbR and ACB-FR larvae, respectively, using a 7d exposure to diet incorporated dose-response assays. The ACB-AbR and ACB-FR strains have developed more than 700 and 900-fold resistance, respectively. All larvae were maintained at 70–80% relative humidity (RH), a photoperiod of 16:8 h (L:D) and 27 ± 1 °C.

2.2. Dissection of Gut Tissues and Extraction of DNA

To remove body surface contaminations, the fifth instar larvae were immersed in 70% ethanol, washed for 3 min, and then rinsed three times with sterile water. The midgut was separately dissected under sterile conditions and immediately frozen in liquid nitrogen for subsequent DNA extraction. Three biological replicates for each strain with twenty 5th instar larvae midgut per replicated were prepared. Total DNA was isolated from the midgut using a TGuide S96 Magnetic Soil/Stool DNA Kit (Tiangen Biotech Beijing Co., Ltd., Beijing, China) following the manufacturer's instructions. The concentration of all DNA samples was measured with the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Eugene, OR, USA) and the quality was assessed by agarose gel electrophoresis.

2.3. 16S rRNA Amplicon Amplification and Sequencing

The 16S rRNA amplicon of the V3-V4 regions was amplified using the primer pair (338F, 5′-ACTCCTACGGGAGGCAGCA; and 806R, 5′-GGACTACNNGGGTATCTAAT) with a barcode. All PCR amplifications were performed in a 10 µL reaction mixture containing approximately 10 ng template DNA. PCR products were mixed in the same volume with 1× loading buffer, and bright bands between 400 and 450 bp were chosen for further experiments. The target bands of the PCR products were purified with AgencourtAMPure XP Beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA). The library was constructed and sequenced on an Illumina NovaSeq 6000 platform (Illumina, Santiago, CA, USA), and 250 bp paired-end reads were generated.

2.4. Sequencing Data Analysis

Raw data was primarily filtered by Trimmomatic (v0.33) [26] and the primer sequences were removed by Cutadapt (v1.9.1) [27]. Paired-end reads were assembled by USEARCH (v10.0) [28] and followed by chimera removal using UCHIME (v8.1) [29]. Sequences were clustered into operational taxonomic units (OTU) at 97% similarity by USEARCH (v10.0) [30] and the OTUs with abundance < 0.005% were filtered. The most abundant sequence was picked as a representative sequence for each OTU to annotate taxonomic information with the Naïve Bayes classifier in QIIME2 [31] based on the SILVA 132 database [32]. The OTU abundance of each sample was rarefied to the value corresponding to the minimum sum of OTU sequences across all the samples to mitigate the differences in the sequencing effort. Then, the relative abundance was calculated based on these rarefied abundance data by dividing the number of sequences per OTU by the total number of sequences for a given sample. Subsequent diversity analyses were performed based on this rarefied abundance data or relative abundance data.

2.5. Diversity Analysis

Analyses were executed with R software (v4.2.2) [33]. To evaluate the alpha diversity, four indices, including ACE, Chao1, Shannon, and Simpson, were calculated using the phyloseq package [34] based on the OTU table. Alpha-diversity indices and relative abundance of microbiota between Bt-susceptible and Bt-resistant strains were compared by Student's *t*-test.

The dissimilarities of the microbial communities were quantified by calculating the Bray-Curtis dissimilarities using the vegan package [35]. The microbial communities among different strains were clustered using principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities in the vegan package [35] and plotted by the ggplot2 package [36]. Permutational multivariate analysis of variance (PERMANOVA) was performed in the vegan package to assess statistically significant differences of microbial communities between Bt-susceptible and Bt-resistant strains.

Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was conducted to identify biomarkers that differed significantly between Bt-susceptible and Bt-resistant strains with the 4.0 threshold for the LDA score [37].

Network analysis was performed on the co-occurrence patterns of the midgut microbial communities from Bt-resistant and Bt-susceptible strains using BMKCloud (www.biocloud.net (accessed on 21 November 2022)). Spearman rank correlation analysis was processed on the difference in the relative abundance of the top 60 genera with $|r| \geq 0.6$ at the 0.05 significant level. The characters of the network, including the number of nodes and edges (positive edges and negative edges), average degree, average path length, diameter, density, and modularity, were calculated. The ratio of positive to negative edges (P/N ratio) in the network was calculated to reflect the balance between cooperative or competitive interactions among bacteria [38].

2.6. Gut Bacteria Remove and Bioassay

Axenic larvae are generated by oral antibiotics. Neonates from the ACB-BtS and ACB-AbR strains were reared on an artificial diet for 7 days containing 500 µg/mL antibiotic units, including chlortetracycline, gentamicin, penicillin, rifampicin, and streptomycin with a ratio of 1:1:1:1:1. Neonates from the two strains reared on an artificial diet without antibiotic were used as control. Seven days after antibiotic treatment and control group, larvae were transferred to a 24-well plate containing an artificial diet incorporated with the LC_{50} dose of Cry1Ab toxin for bioassay, and all tests were replicated five times. All plates were kept under the same conditions as insect rearing. Mortalities were recorded 7 days after treatment and were compared using the Kruskal-Wallis test. To verify the removal efficiency of oral antibiotics against gut bacteria, five larvae from antibiotic-treated and control groups were surface sterilized and homogenized. The homogenates were cultured on a Tryptone Soya Agar (TSA) plate at 37 °C, and bacterial colonies on the plate were observed after 72 h.

3. Results

3.1. Sequencing Data

Sequencing of the 16S rRNA V3-V4 amplicons of nine samples yielded 718,315 raw reads. After quality filtering and removal of chimeric sequences, a total of 670,340 effective tags were obtained. The sequences were classified into 262 to 1140 OTUs for each sample at 97% similarity.

3.2. Gut Bacterial Diversity between Bt-Susceptible and Bt-Resistant Strains

The Shannon indices between ACB-BtS and ACB-AbR strains ($t = -3.09$, $df = 4$, $p = 0.04$; Figure 1a) and between ACB-BtS and ACB-FR strains ($t = -3.57$, $df = 4$, $p = 0.02$; Figure 1b) were significantly different. The Chao1 ($t = -3.11$, $df = 4$, $p = 0.04$; Figure 1c) and ACE ($t = -3.14$, $df = 4$, $p = 0.03$; Figure 1d) indices between ACB-BtS and ACB-FR strains also differed significantly.

For the composition of the midgut microbiota, in PCoA analysis (Figure 2), the ACB-AbR strains tended to position near each rather than away from the ACB-BtS strains, and PERMANOVA revealed significant differences in bacterial communities between these two strains ($F_{1,4} = 4.93$, $R^2 = 0.55$, $p = 0.001$). The composition of the microbiota in ACB-BtS and ACB-FR strains did not form distinct clusters, and no significant differences were found between the two strains ($F_{1,4} = 2.75$, $R^2 = 0.41$, $p = 0.10$).

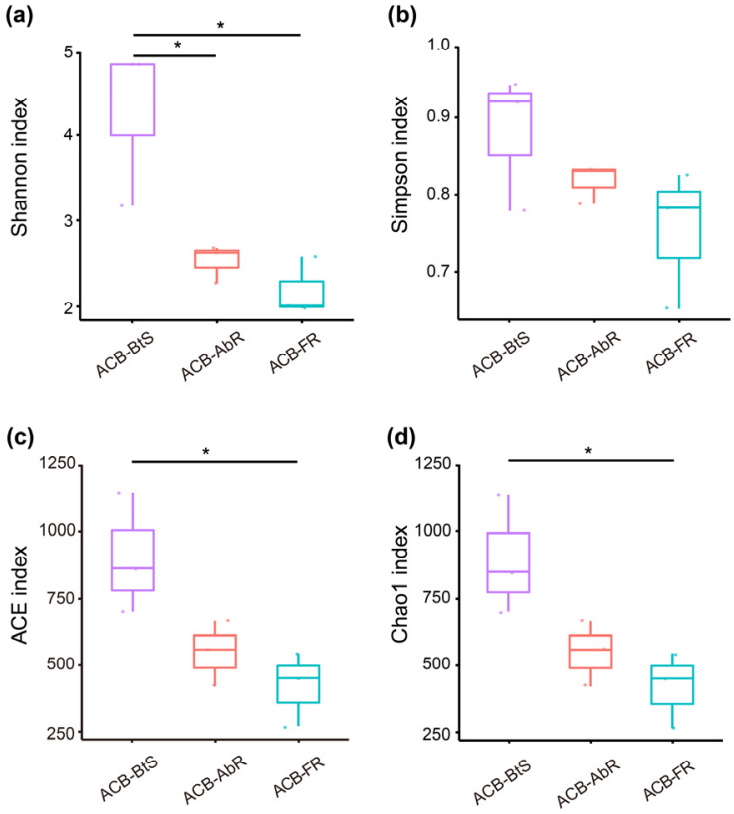

Figure 1. Comparison of alpha diversity indices between Bt-resistant strains (ACB-AbR and ACB-FR) and Bt-susceptible strains of *Ostrinia furnacalis*. Comparison of (**a**) Shannon index, (**b**) Simpson index, (**c**) ACE index, and (**d**) Chao1 index between ACB-AbR and ACB-BtS, ACB-FR and ACB-BtS. * $p < 0.05$.

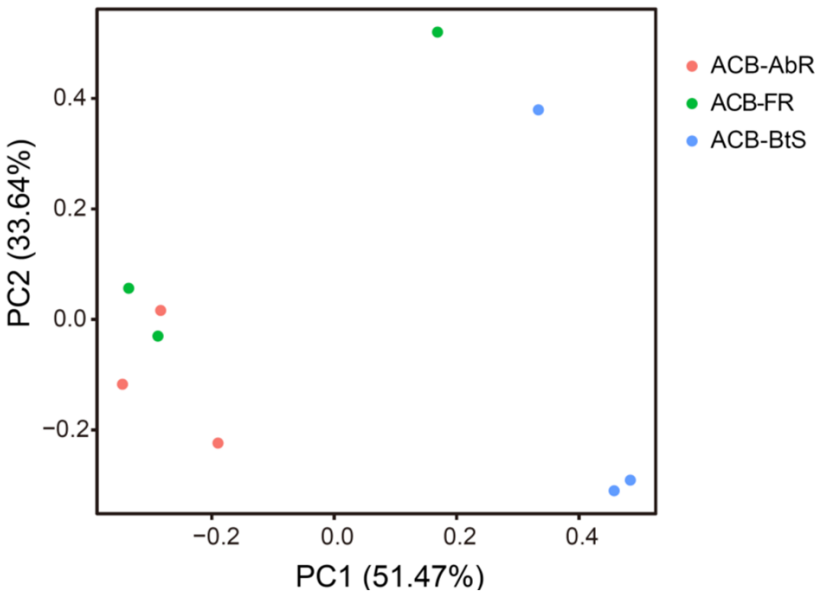

Figure 2. Principal coordinates analysis plot illustrating the separation of samples based on differences in midgut bacterial community structure between Bt-resistant and Bt-susceptible strains of *Ostrinia furnacalis*.

3.3. Gut Bacterial Abundance from Bt-Susceptible and Bt-Resistant Strains

In total, 5106 OTUs of the midgut microbiota from Bt-susceptible and Bt-resistant strains of *O. furnacalis* were annotated to 37 phyla and 944 genera. Overall, 23.87% of these OTUs were attributed to Proteobacteria, 19.98% to Firmicutes, and 15.67% to Bacteroidota. The most abundant phyla across the three strains of ACB were Proteobacteria (18.47–68.66%), Firmicutes (26.94–59.39%), and Bacteroidota (1.17–6.95%) (Figure 3a). These sequences accounted for more than 55% of the bacterial sequences in each strain. Proteobacteria was dominated in the Bt-resistant strains (68.66% for ACB-AbR and 50.71% for ACB-FR), while Firmicutes was the dominat phylum in ACB-BtS (59.39%) (Figure 3a). The relative abundance of Proteobacteria ($t = 11.07$, $df = 4$, $p < 0.001$) and Firmicutes ($t = -4.14$, $df = 4$, $p = 0.01$) were significantly different between ACB-AbR and ACB-BtS.

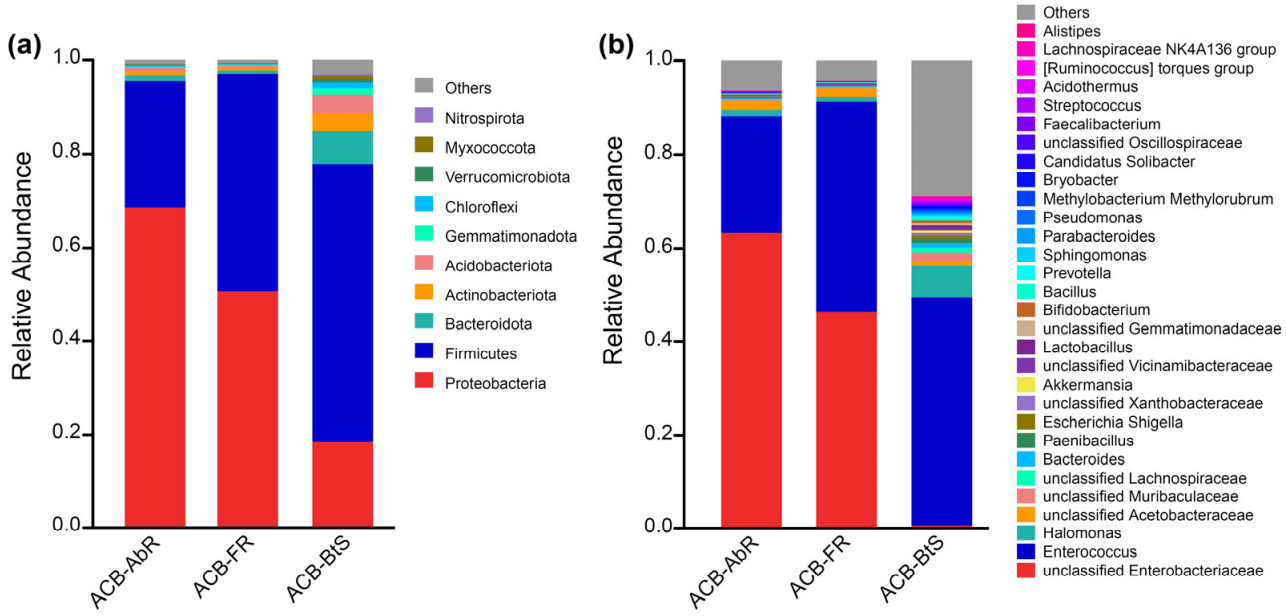

Figure 3. Relative abundance of the top 10 most abundant phylum (**a**) and top 30 most abundant genera (**b**) of the gut microbiota from Bt-resistant (ACB-AbR and ACB-FR) and Bt-susceptible strains of *Ostrinia furnacalis*.

The most abundant genera across the three strains were *unclassified_Enterobacteriaceae* (0.6–63.28%), *Enterococcus* (24.94–48.78%), and *Halomonas* (0.99–6.81%) (Figure 3b). The *unclassified_Enterobacteriaceae* was dominated in Bt-resistance strains (63.28% for ACB-AbR and 46.26% for ACB-FR), while *Enterococcus* was dominated in the susceptible strain, ACB-BtS (48.78%), followed by the *Halomonas* (6.81%). There was a significant difference in the relative abundance of *unclassified_Enterobacteriaceae* between ACB-AbR and ACB-BtS ($t = 16.36$, $df = 4$, $p < 0.001$). The relative abundance of *Halomonas* was significantly different between Bt-resistance strains and Bt-susceptible strains ($t = -3.47$, $df = 4$, $p = 0.03$ for ACB-AbR vs. ACB-BtS; $t = -3.60$, $df = 4$, $p = 0.02$ for ACB-FR vs. ACB-BtS).

The abundance of genus *Enterococcus* belonging to Firmicutes phylum and Actinobacteriota phylum were significantly different between ACB-BtS and ACB-AbR in LEfSe analysis (LDA score = 5.01 > 4.0 for *Enterococcus* genus, LDA score = 4.15 > 4.0 for Actinobacteriota phylum; Figure 4a). The abundance of genus *Halomonas*, belonging to Pseudomonadales order, was significantly higher in the susceptible strain ACB-BtS than in the resistant strains, ACB-AbR (LDA score = 4.44 > 4.0; Figure 4a) and ACB-FR (LDA score = 4.48 > 4.0; Figure 4b). The abundance of the Lachnospiraceae family classified as Clostridia class, Bacteroidales order classified as Bacteroidota phylum, and Acidobacteriota phylum were enriched in ACB-BtS strain, which was significantly higher than those in the resistant strains, ACB-AbR (LDA score = 4.05 > 4.0 for Lachnospiraceae family, LDA score = 4.40 > 4.0 for Bacteroidales order, LDA score = 4.22 > 4.0 for Acidobacteri-

ota phylum; Figure 4a) and ACB-FR (LDA score = 4.12 > 4.0 for Lachnospiraceae family, LDA score = 4.36 > 4.0 for Bacteroidales order, LDA score = 4.20 > 4.0 for Acidobacteriota phylum; Figure 4b). The abundance of unclassified Enterobacteriaceae, belonging to the Proteobacteria phylum, was enriched in the ACB-AbR strain and was significantly higher than in the ACB-BtS susceptible strain (LDA score = 5.47 > 4.0; Figure 4a). The Actinobacteria class, belonging to the Actinobacteriota phylum, and unclassified Enterobacteriaceae, belonging to Enterobacterales order, were significantly different in ACB-BtS and ACB-FR (LDA score = 4.02 > 4.0 for Actinobacteria class, LDA score = 5.36 > 4.0 for unclassified Enterobacteriaceae; Figure 4b).

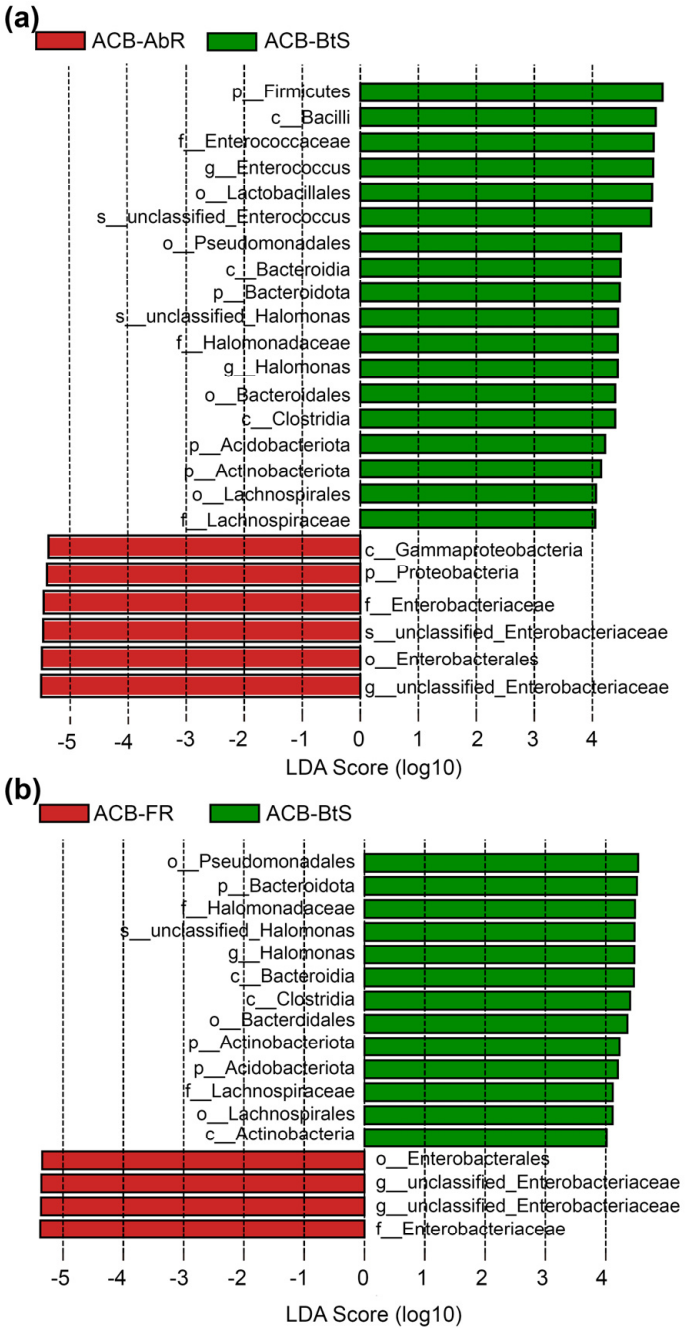

Figure 4. Significantly different biomarkers between ACB-AbR and ACB-BtS (**a**) and between ACB-FR and ACB-BtS (**b**) using linear discriminant analysis effect size (LEfSe) analyses with an LDA score > 4.0.

3.4. Network Analysis of the Gut Microbiota between Bt-Resistant and Bt-Susceptible Strain

There were 53 and 60 nodes in the networks of Bt-resistant and Bt-susceptible strains, respectively (Table 1). The average degree, average path length, graph diameter, density, clustering coefficient, and modularity were 3.77, 3.95, 26.31, 0.07, 0.59, and 0.64 in the Bt-resistant strains, which were higher than those of 3.33, 1.83, 12.00, 0.06, 0.18, and 0.59 in the Bt-susceptible strain (Table 1). There were 100 edges in the two networks, with 78 positive edges and 22 negative edges in the Bt-resistant strains, and 64 positive edges and 36 negative edges in the Bt-susceptible strain. The P/N ratio was 3.55 in the Bt-resistant strains and 1.78 in the susceptible strains, which suggested a more cooperative interaction between bacteria in Bt-resistant strains. The genus *Enterococcus* had complex correlations with other genera in the ACB-BtS strain, which had 19 strong negative correlations with other genera. While it had only three positive correlations and one negative correlation with other genera in Bt-resistant strains. The genus *Halomonas* had 21 strong positive links and 3 negative links to other nodes in ACB-BtS strains, while it had only 4 positive links and 1 negative link to other nodes in Bt-resistant strains (Figure 5).

Table 1. Key topological parameters of co-occurrence networks of the midgut microbiota from Bt-susceptible and Bt-resistant strains of *Ostrinia furnacalis*.

Strains	No. of Nodes	P/N Ratio	Average Degree	Average Path Length	Graph Diameter	Density	Clustering Coefficient	Modularity
Bt susceptible strain (ACB-BtS)	60	3.55	3.77	3.95	26.31	0.07	0.59	0.64
Bt resistant strains (ACB-AbR, ACB-FR)	53	1.78	3.33	1.83	12.00	0.06	0.18	0.59

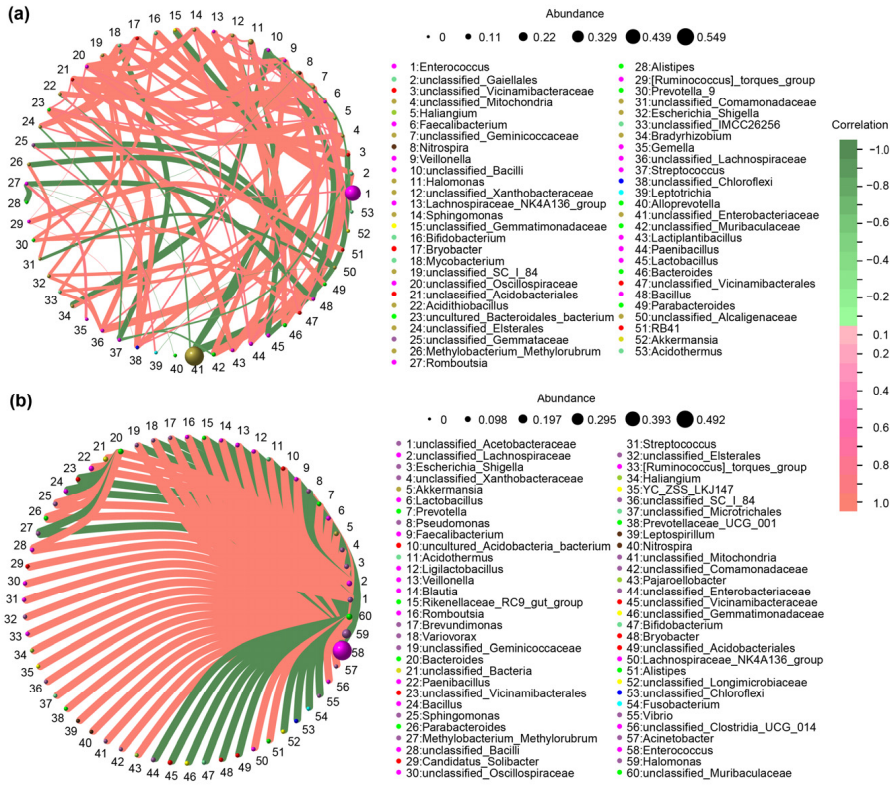

Figure 5. The co-occurrence networks of the gut microbial community from (**a**) Bt-resistant (ACB-AbR and ACB-FR) and (**b**) Bt-susceptible (ACB-BtS) strains of *Ostrinia furnacalis* at the genus level.

3.5. Effect of the Gut Microbiota on the Toxicity of Bt Toxin

The efficacy of the antibiotic elimination of gut microbiota was confirmed by inoculating the homogenates of ACB larvae in the TSA plate (Figure S1). After 7 days of oral antibiotic, neonates developed into the third or fourth instar larvae, and the larvae were transferred into a 24-well plate with a Cry1Ab diet (LC_{50} dose) for bioassay. The larvae mortalities of the ACB-BtS (χ^2 = 6.99, *df* = 1, p = 0.008) and ACB-AbR (χ^2 = 6.82, *df* = 1, p = 0.009) strains treated with antibiotic were both significantly decreased compared with those that were not treated (Table 2).

Table 2. Analysis of larval mortality of ACB-AbR and ACB-BtS strains of *Ostrinia furnacalis* treated with an LC_{50} dose of Cry1Ab toxin after treating the larvae from these strains with 500 μg/mL antibiotic cocktail for 7 days.

Strains	Mortality ± SE (%)		
	Larvae Pre-Treated by Antibiotic	Control	χ^2, p Value
ACB-AbR	43.17 ± 0.02	59.50 ± 0.03	6.82, 0.009 *
ACB-BtS	20.83 ± 0.05	49.17 ± 0.03	6.99, 0.008 *

Note: Asterisks indicate significant differences according to Kruskal-Wallis test (* $p < 0.05$).

4. Discussion

Transgenic *Bacillus thuringiensis* plants provide an effective means to control insect pests. However, the evolution of resistance by target pests is a great threat to the continued success of Bt toxins used in insecticide formulations or expressed by transgenic plants. Bt cotton has been planted since 2003 in India, and the resistance of the target pest, *Pectinophoragossypiella*, to the plant was detected in 2008 [39]. After Bt cotton expressing Cry1Ac was planted in China in 2000, the resistance of *Helicoverp aarmigera* [40] and *P. gossypiella* [41] to the cotton were successively detected. Transgenic maize expressing Cry1F protein was planted in Puerto Rico in 2003, and the commercial sales of this maize were stopped in 2007 because of the resistance of *Spodoptera frugiperda* to Cry1F [42]. The resistance mechanisms to Bt crops mainly focus on mutations and altered expressions of the toxin receptors and variations in toxin activation [43–45]. During recent years, gut microbiota participated in susceptibility or resistance of host insects to Bt toxin have been reported [46]. In this study, using 16S rRNA amplicon sequencing and eliminating the bacterial flora by antibiotic methods, we investigated the effect of gut bacteria in ACB. The results showed that bacterial diversities of Bt-resistant strains were significantly higher than Bt-susceptible strains, and a more complex occurrence network in Bt-resistant strains was found. In addition, larval mortalities treated by LC_{50} values of Cry1Ab protein were reduced significantly after eliminating the gut bacteria. These results indicated that midgut bacteria are involved in the process of Bt-induced mortality in ACB, and Cry toxin structured the composition and abundance of midgut bacterial flora.

In the present study, the richness (Chao1 and ACE index) and diversity (Shannon index) of ACB-BtR strains were significantly lower than that in ACB-BtS strains. Ordination analysis and statistical tests revealed that the bacterial community of ACB-AbR was distinct from that of other strains. These findings are consistent with previous studies in *Diabrotica virgifera* [16] and *C. suppressalis* [21]. However, the microbiota diversity was significantly lower in ACB-AbR in the present study and *D. virgifera* Bt-resistant strains [16], but was higher in *C. suppressalis* Cry1Ca resistant strains [21]. In addition, Bt strain infection significantly reduced the richness and diversity of the gut microbiota in the *Galleria mellonella*-resistant strains [47]. The Cry1Ac treatment significantly reduced the bacterial diversity and changed the composition of the microbiota in *P. xylostella* [19]. It suggested that the low diversity of gut bacteria may contribute to the host resistance to Bt.

The relative abundances of some midgut bacteria exhibited significant differences between Bt-resistant and Bt-susceptible strains. The phylum Firmicutes, gram-positive

bacteria, was enriched in ACB-BtS, while the phylum Proteobacteria, gram-negative bacteria, was enriched in ACB-AbR. Similarly, post- *B. thuringiensis* subsp. *galleriae* treatment, the gram-negative Enterobacteraceae was prevalent in the midgut of Bt-resistant *Galleria mellonella*, while the gram-positive *Enterococcus* and *Bt* were enriched in the susceptible strain, which indicated that clearing the midgut from gram-positive bacteria may contribute to the resistance in *G. mellonella* [48]. Furthermore, the larval mortalities of both ACB-BtS and ACB-AbR treated by Cry1Ab protein were reduced significantly after eliminating the gut bacteria. The similar results were reported in other insects, including *Plodiainter punctella* [18], *P. xylostella* [19], *Spodoptera exigua* [20], *Chilo suppressalis* [21], and *Plagiodera versicolora* [22]. Broderick et al. [14] revealed that *B. thuringiensis*-mediated killing for gypsy moths relied on the presence of indigenous midgut bacteria. Moreover, reintroducing the indigenous gut bacteria to axenic larvae restored host sensitivity to Bt [16]. These provide clear evidence that midgut bacteria play an important role in the killing mechanism of Bt against pest insects.

Bt treatment can lead to the disruption of the insect midgut epithelium, and gut bacteria can enter the hemocoel through the damaged areas [20,23,49]. The translocation of bacteria from the midgut to hemocoel may become from commensal bacteria to pathogens, thus accelerating host mortality. Mason et al. [50] showed that *E. faecalis* is a commensal gut bacterium in *Manduca sexta* but becomes a pathogen in the hemocoel and accelerates the larvae mortality. The higher relative abundance of *Enterococcus* was found in ACB-BtS in the present study. We speculate that *Enterococcus* spp. could play an important role in the process of Bt treatment death, which may be similar to that in *M. sexta*. However, the killing process of septicemia introduced by gut bacteria was lost in axenic larvae for the absence of gut bacteria, and the death of axenic larvae is probably only due to the Bt toxicity. Moreover, the study found that the level of gut lesions was different in axenic and nonaxenic larvae and that epithelium was severely damaged in nonaxenic larvae [20]. The reason for this difference is unclear. However, the dysbiosis of midgut bacteria exaggerates the host immune response [20,49], and an over-activated gut immune response may be detrimental to the gut epithelium. Additionally, the exacted interactions between insects, gut bacteria, and immune response need to be further explored in the future.

In terms of microbiota, the ACB-AbR was separately clustered from t ACB-BtS; however, the ACB-FR could not be separated from ACB-BtS. In *C. suppressalis*, the bacterial flora of Ab-R strains was difficult to separate from that of the corresponding susceptible strains, while the Cry1Ca-resistant strain was separately clustered from the other strains [21]. These imply that the composition of the gut bacteria community of ACB from different Bt-resistant strains displayed different patterns.

In conclusion, the present study provided evidence that midgut bacteria participated in regulating the Bt toxin-killing mechanism in *O. furnacalis*. The Bt-resistant strains of *O. furnacalis* exhibited a significantly lower diversity of bacterial flora than that from susceptible strains and a distinct composition of the bacterial community. Elimination of the gut bacteria significantly affects the susceptibility of *O. furnacalis* to Bt toxin.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071923/s1, Figure S1: Bacterial growth by inoculating the homogenates of *Ostrinia furnacalis* larvae in TSA plate.

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References

1. Hofte, H.; Whiteley, H.R. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **1989**, *53*, 242–255. [CrossRef] [PubMed]
2. Pardo-Lopez, L.; Soberon, M.; Bravo, A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: Mode of action, insect resistance and consequences for crop protection. *FEMS Microbiol. Rev.* **2013**, *37*, 3–22. [CrossRef]
3. Tabashnik, B.E.; Carriere, Y. Surge in insect resistance to transgenic crops and prospects for sustainability. *Nat. Biotechnol.* **2017**, *35*, 926–935. [CrossRef]
4. Xiao, Y.; Wu, K. Recent progress on the interaction between insects and *Bacillus thuringiensis* crops. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2019**, *374*, 20180316. [CrossRef]
5. Nafus, D.M.; Schreiner, I.H. Review of the biology and control of the Asian corn borer, *Ostrinia furnacalis* (Lep: Pyralidae). *Trop. Pest Manag.* **1991**, *37*, 41–56. [CrossRef]
6. He, K.; Wang, Z.; Zhou, D.; Wen, L.; Song, Y.; Yao, Z. Evaluation of transgenic Bt corn for resistance to the Asian corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* **2003**, *96*, 935–940. [CrossRef] [PubMed]
7. Wang, D.Y.; Wang, Z.Y.; He, K.L.; Cong, B.; Bai, S.X.; Wen, L.P. Temporal and spatial expression of CrylAb toxin in transgenic Bt corn and its effects on Asian corn borer, *Ostrinia furnacalis* (Guenee). *Sci. Agric. Sin.* **2004**, *37*, 1155–1159.
8. Wang, Y.; Wang, Y.; Wang, Z.; Bravo, A.; Soberon, M.; He, K. Genetic basis of Cry1F-resistance in a laboratory selected Asian corn borer strain and its cross-resistance to other *Bacillus thuringiensis* toxins. *PLoS ONE* **2016**, *11*, e0161189. [CrossRef]
9. Pinos, D.; Wang, Y.; Hernández-Martínez, P.; He, K.; Ferré, J. Alteration of a Cry1A sharedbinding site in a cry1ab-selectedcolony of *Ostrinia furnacalis*. *Toxins* **2022**, *14*, 32. [CrossRef]
10. Xu, L.N.; Wang, Y.Q.; Wang, Z.Y.; Hu, B.J.; Ling, Y.H.; He, K.L. Transcriptome differences between Cry1ab resistant and susceptible strains of Asian corn borer. *BMC Genom.* **2015**, *16*, 173. [CrossRef]
11. Zhang, T.; Coates, B.S.; Wang, Y.; Wang, Y.; Bai, S.; Wang, Z.; He, K.L. Down-regulation of aminopeptidase N and ABC transporter subfamily G transcripts in Cry1Ab and Cry1Ac resistant Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Crambidae). *Int. J. Biol. Sci.* **2017**, *13*, 835–851. [CrossRef] [PubMed]
12. Jin, W.; Zhai, Y.; Yang, Y.; Wu, Y.; Wang, X. Cadherin protein is involved in the action of *Bacillus thuringiensis* Cry1Ac toxin in *Ostrinia furnacalis*. *Toxins* **2021**, *13*, 658. [CrossRef] [PubMed]
13. Wang, X.; Xu, Y.; Huang, J.; Jin, W.; Yang, Y.; Wu, Y. CRISPR-mediated knockout of the ABCC2 gene in *Ostrinia furnacalis* confers high-level resistance to the *Bacillus thuringiensis* Cry1Fa Toxin. *Toxins* **2020**, *12*, 246. [CrossRef] [PubMed]
14. Broderick, N.A.; Raffa, K.F.; Handelsman, J. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 15196–15199. [CrossRef] [PubMed]
15. Broderick, N.A.; Robinson, C.J.; McMahon, M.D.; Holt, J.; Handelsman, J.; Raffa, K.F. Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. *BMC Biol.* **2009**, *7*, 11. [CrossRef] [PubMed]
16. Paddock, K.J.; Pereira, A.E.; Finke, D.L.; Ericsson, A.C.; Hibbard, B.E.; Shelby, K.S. Host resistance to *Bacillus thuringiensis* is linked to altered bacterial community within a specialist insect herbivore. *Mol. Ecol.* **2021**, *30*, 5438–5453. [CrossRef] [PubMed]
17. Visweshwar, R.; Sharma, H.C.; Akbar, S.M.; Sreeramulu, K. Elimination of gut microbes with antibiotics confers resistance to *Bacillus thuringiensis* toxin proteins in *Helicoverpa armigera* (Hubner). *Appl. Biochem. Biotechnol.* **2015**, *177*, 1621–1637. [CrossRef]
18. Orozco-Flores, A.A.; Valadez-Lira, J.A.; Oppert, B.; Gomez-Flores, R.; Tamez-Guerra, R.; Rodriguez-Padilla, C.; Tamez-Guerra, P. Regulation by gut bacteria of immune response, *Bacillus thuringiensis* susceptibility and hemolin expression in *Plodia interpunctella*. *J. Insect Physiol.* **2017**, *98*, 275–283. [CrossRef]
19. Li, S.; Xu, X.; De Mandal, S.; Shakeel, M.; Hua, Y.; Shoukat, R.F.; Fu, D.; Jin, F. Gut microbiota mediate *Plutella xylostella* susceptibility to Bt Cry1Ac protoxin is associated with host immune response. *Environ. Pollut.* **2021**, *271*, 116271. [CrossRef]
20. Li, Y.; Zhao, D.; Wu, H.; Ji, Y.; Liu, Z.; Guo, X.; Guo, W.; Bi, Y. Bt GS57 interaction with gut microbiota accelerates *Spodoptera exigua* mortality. *Front. Microbiol.* **2022**, *13*, 835227. [CrossRef]
21. Chen, G.; Li, Q.; Yang, X.; Li, Y.; Liu, W.; Chen, F.; Han, L. Comparison of the co-occurrence patterns of the gut microbial community between Bt-susceptible and Bt-resistant strains of the rice stem borer, *Chilo suppressalis*. *J. Pest Sci.* **2023**, *96*, 299–315. [CrossRef]
22. Lei, X.; Zhang, F.; Zhang, J. Gut microbiota accelerate the insecticidal activity of plastid-expressed *Bacillus thuringiensis* Cry3Bb to a leaf beetle, *Plagiodera versicolora*. *Microbiol. Spectr.* **2023**, *11*, e0504922. [CrossRef]

23. Deguenon, J.M.; Dhammi, A.; Ponnusamy, L.; Travanty, N.V.; Cave, G.; Lawrie, R.; Mott, D.; Reisig, D.; Kurtz, R.; Roe, R.M. Bacterial microbiota of field-collected *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic Bt and non-Bt cotton. *Microorganisms* **2021**, *9*, 878. [CrossRef] [PubMed]
24. Song, Y.; Zhou, D.; He, K. Studies on mass rearing of Asian corn borer: Development of a satisfactory non-agar semi-artificial diet and its use. *J. Plant Prot.* **1999**, *26*, 324–328.
25. Xu, L.; Wang, Z.; Zhang, J.; He, K.; Ferry, N.; Gatehouse, A.M.R. Cross-resistance of Cry1Ab-selected Asian corn borer to other Cry toxins. *J. Appl. Entomol.* **2010**, *134*, 429–438. [CrossRef]
26. Bolger, A.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef]
27. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10. [CrossRef]
28. Edgar, R.C.; Flyvbjerg, H. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* **2015**, *31*, 3476–3482. [CrossRef]
29. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]
30. Edgar, R.C. Search and clustering orders of magnitude faster than blast. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef] [PubMed]
31. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
32. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glockner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
33. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2022; Available online: https://www.R-project.org/ (accessed on 15 November 2022).
34. McMurdie, P.J.; Holmes, S. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef] [PubMed]
35. Oksanen, J.; Simpson, G.L.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'Hara, R.B.; Solymos, P.; Stevens, M.H.H.; Szoecs, E.; et al. Vegan: Community Ecology Package. R Package Version 2.6-4. 2022. Available online: https://CRAN.R-project.org/package=vegan (accessed on 15 November 2022).
36. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016.
37. Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W.S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12*, R60. [CrossRef]
38. Ma, Z.S. The P/N (positive-to-negative Links) ratio in complex networks-a promising in silico biomarker for detecting changes cccurring in the human microbiome. *Microb. Ecol.* **2018**, *75*, 1063–1073. [CrossRef] [PubMed]
39. Dhurua, S.; Gujar, G.T. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. *Pest Manag. Sci.* **2011**, *67*, 898–903. [CrossRef]
40. Liu, F.; Xu, Z.; Zhu, Y.C.; Huang, F.; Wang, Y.; Li, H.; Li, H.; Gao, C.; Zhou, W.; Shen, J. Evidence of field-evolved resistance to Cry1Ac-expressing Bt cotton in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in northern China. *Pest Manag. Sci.* **2010**, *66*, 155–161. [PubMed]
41. Wan, P.; Huang, Y.; Wu, H.; Huang, M.; Cong, S.; Tabashnik, B.E.; Wu, K. Increased frequency of pink bollworm resistance to Bt toxin Cry1Ac in China. *PLoS ONE* **2012**, *7*, e29975. [CrossRef] [PubMed]
42. Storer, N.P.; Babcock, J.M.; Schlenz, M.; Meade, T.; Thompson, G.D.; Bing, J.W.; Huckaba, R.M. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *J. Econ. Entomol.* **2010**, *103*, 1031–1038. [CrossRef]
43. Peterson, B.; Bezuidenhout, C.C.; Van den Berg, J. An overview of mechanisms of Cry toxin resistance in lepidopteran insects. *J. Econ. Entomol.* **2017**, *110*, 362–377. [CrossRef]
44. Jurat-Fuentes, J.L.; Karumbaiah, L.; Jakka, S.R.K.; Ning, C.; Liu, C.; Wu, K.; Jackson, J.; Gould, F.; Blanco, C.; Portilla, M.; et al. Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from *Bacillus thuringiensis*. *PLoS ONE* **2011**, *6*, e17606. [CrossRef] [PubMed]
45. Adang, M.J.; Crickmore, N.; Jurat-Fuentes, J.L. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. *Adv. Insect Physiol.* **2014**, *47*, 39–87.
46. Takatsuka, J.; Kunimi, Y. Intestinal bacteria affect growth of *Bacillus thuringiensis* in larvae of the oriental tea tortrix, *Homona magnanima* Diakonoff (Lepidoptera: Tortricidae). *J. Invertebr. Pathol.* **2000**, *76*, 222–226. [CrossRef] [PubMed]
47. Dubovskiy, I.M.; Grizanova, E.V.; Whitten, M.M.A.; Mukherjee, K.; Greig, C.; Alikina, T.; Kabilov, M.; Vilcinskas, A.; Glupov, V.V.; Butt, T.M. Immuno-physiological adaptations confer wax moth *Galleria mellonella* resistance to *Bacillus thuringiensis*. *Virulence* **2016**, *7*, 860–870. [CrossRef]
48. Grizanova, E.V.; Krytsyna, T.I.; Kalmykova, G.V.; Sokolova, E.; Alikina, T.; Kabilov, M.; Coates, C.J.; Dubovskiy, I.M. Virulent and necrotrophic strategies of *Bacillus thuringiensis* in susceptible and resistant insects, *Galleria mellonella*. *Microb. Pathog.* **2023**, *175*, 105958. [CrossRef] [PubMed]

49. Caccia, S.; Di Lelio, I.; La Storia, A.; Marinelli, A.; Varricchio, P.; Franzetti, E.; Banyuls, N.; Tettamanti, G.; Casartelli, M.; Giordana, B.; et al. Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 9486–9491. [CrossRef]
50. Mason, K.L.; Stepien, T.A.; Blum, J.E.; Holt, J.F.; Labbe, N.H.; Rush, J.S.; Raffa, K.F.; Handelsman, J. From commensal to pathogen: Translocation of *Enterococcus faecalis* from the midgut to the hemocoel of *Manduca sexta*. *mBio* **2011**, *2*, e00065-11. [CrossRef] [PubMed]