

Article

Biochemical and Molecular Analysis of Gut Microbial Changes in *Spodoptera littoralis* (Lepidoptera: Noctuidae) to Counteract Cry1c Toxicity

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Abstract: *Bacillus thuringiensis* (Bt) represents one of the most economical biopesticides to date. It produces toxins with insecticidal activity against many agricultural pests, including members of the genus *Spodoptera*. However, Bt tolerance leads to inefficiency in biological control. To overcome this problem, discovering the hidden cause(s) for the evolution of insect tolerance against Bt is of great importance. We hypothesized that changes in the gut microbiota due to the frequent application of Bt is one of those hidden causes. To investigate this hypothesis, we studied the effect of Bt Cry1c application on the *Spodoptera littoralis* larval gut microbiota in both Bt-susceptible and Bt-tolerant populations. The results revealed changes in the diversity and abundance of gut bacterial composition between the susceptible and tolerant populations. A high abundance of *Enterococcaceae* was detected in the tolerant population. Interestingly, Cry1c tolerance eliminates the bacterial genera *Klebsiella* and *Serratia* from the larval midgut. These changes may confirm the mechanism developed by *Spodoptera* larvae to counteract Bt Cry1c toxicity. Understanding the *B. thuringiensis*–gut microbiota interaction may help in improving biocontrol strategies against agricultural pests to overcome the evolution of tolerance.

Keywords: biological control; *Bacillus thuringiensis*; *Spodoptera littoralis*; insect tolerance; gut microbiota



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1. Introduction

The noctuid cotton leafworm *Spodoptera littoralis* (Boisduval) is a major polyphagous insect pest that feeds on a wide variety of plant species [1–3]. In addition to its high reproductive capacity and the strong ability of adults to migrate, *S. littoralis* is able to adapt to various ecological conditions. Under favorable conditions, its population increases rapidly, leading to economic losses [4]. Chemical insecticides have been the main technique for managing this pest. It has been subjected to various insecticides throughout the years because of its polyphagous nature. Unfortunately, *S. littoralis* has developed different levels of resistance to various types of registered insecticide classes [5]. Additionally, these insecticides have harmful side effects that may pose risks to the environment as well as human, animals, and additional non-target organisms. Hence, an urgent requirement exists to find alternatives for managing this pest that are highly efficient and specific in their targeting, while also being safe for humans and ecofriendly. There is a growing focus on biopesticide-based microorganisms or botanicals. These microbial pesticides, including viruses, bacteria, fungi, and nematodes, are becoming more popular because they are highly specific to certain species and safe for the environment [6,7].

Formulations of entomopathogenic bacteria and the product proteins derived from them have proven successful as biological control agents [8]. Several strains of *Bacillus* species have been identified as effective insect pathogens [9,10]. *Bacillus thuringiensis* (Bt) is

the most widely used and effective method for managing the larvae of most Lepidoptera, Coleoptera, and many Diptera [11,12]. *B. thuringiensis* (*Bt*) is a critical bacterium that infects insects, and its toxins are commonly used in genetically modified plants [13]. *Bt* toxins undergo a process of hydrolyzation and activation by alkaline protease during insect digestion. This results in the formation of a small peptide that binds to a specific receptor on the membrane vesicles of the epithelial cells of the midgut, which leads to perforation of the cell membrane of the gut, followed by paralysis and eventually causing larvae death [14,15]. The toxins of *Bt* have been widely used around the world as a result of its very targeted pesticidal activity [16].

The insect gut harbors numerous microorganisms that are crucial for various metabolic and physiological functions. These microbes play a role in food digestion, nutrient absorption, lifespan, fertility, the regulation of larval development, and detoxification [17–19]. The intestinal bacteria in both *Plutella xylostella* and *Lymantria dispar* moths can detoxify secondary compounds like phenols [20,21].

Microorganisms residing in an insect's gut can enhance their ability to adapt to different environmental conditions by supplying essential nutrients, such as amino acids [22] and vitamins, that insects cannot synthesize themselves [23], as well as offer protection against harmful invaders [24]. Furthermore, symbiotic microbiota can increase insects' resistance to pesticides [25–27]. Many factors, including diet, the host environment, and evolutionary and ecological factors affect the structure of microbial community in the intestine [28,29]. Insects develop varying compositions of symbiotic microorganisms at different developmental stages in order to adjust to diverse environmental changes [30,31]. The presence of symbiotic bacteria in their intestines can be directly and indirectly influenced by their diet [32–34]. It was previously mentioned that the composition of *S. littoralis* gut bacteria varied significantly depending on the types of plants they were fed [35]. There have been limited published studies on how *Bt* toxins or *Bt* impact the microbiota in insect guts [36]. In *Galleria mellonella* and *P. xylostella*, both Cry toxins and *Bt* infection can dramatically decrease the variety and titer of gut microbes [37,38]. On the other hand, a research project carried out by Jiang et al. [39] involving honeybees demonstrated that the presence of genetically modified maize pollen expressing Cry *Bt* did not have a significant effect on the diversity of symbiotic bacteria in their gut.

Bt toxin's impact on the gut microbiota stimulates the immune response of the host. This leads to the activation of antimicrobial peptides, melanization, and stem cell growth as the host tries to combat the harm caused by *Bt* infection [40,41]. Exposure to harmful pathogens like *Bt* toxins leads to dysbacteriosis, which triggers the activation of antimicrobial peptide genes and oxidative stress [20,37,42]. Maintaining a balance in the gut microbiome is crucial, and these factors play a key role in achieving this [43]. When the gut barrier is compromised by *Bt* infection, gut bacteria can enter the hemolymph, cause perforation of the gut membrane, and worsen dysbacteriosis [44]. This dysbacteriosis, in turn, activates the immune system response [45,46]. Prior research stated that the connection between the amount of symbiotic bacteria in the intestinal tract of the insect host and *Bt* toxicity implies that higher levels of symbiotic bacteria can contribute to increased resistance to *B. thuringiensis* [47,48]. Although *B. thuringiensis* toxins have been employed in managing insect populations, the exact contribution of intestinal bacteria, particularly dominant ones, to *Bt* resistance remains obscure. Understanding how *Bt* toxins interact with the gut microbiota is a crucial step for developing an effective method to manage *Bt* resistance and for ensuring the effective utilization of *Bt* toxins [49]. In this current research, we compared the diversity and abundance of the intestinal symbiotic microbiota of *S. littoralis*, a serious agricultural pest. Our study helped in identifying *Bt*-induced alterations in the gut bacterial community to enhance the effectiveness of pest management strategies utilizing *Bt*.

2. Materials and Methods

2.1. Insects

The laboratory population of *S. littoralis* was kindly provided by the insectary laboratory of the Agricultural Research Center, Giza, Egypt, where the population was kept in controlled laboratory conditions for many years. The larvae were cultured in plastic containers (23 × 10 × 7 cm) at 25 ± 1 °C and 70–80% relative humidity, and during a 14:10 h light/dark photoperiod, and they were fed on clean dry *Ricinus communis* leaves until they pupated. Thereafter, the pupae were collected and placed in 150 mL plastic containers where they were kept until adult emergence. Adults were reared in plastic containers and fed on a 10% sugar solution supplied through cotton pads. Adults were supplied with the leaves of *Nerium oleander* as a substrate for egg laying. The eggs were collected daily in plastic containers with a white covering until they hatched.

The Cry1C-tolerant strain of *S. littoralis* originated from the susceptible strain. Briefly, L1 larvae ($n = 200$) were exposed to a 0.05 µg/g Cry1C-supplemented diet throughout the larval stages. Surviving larvae were fed on castor leaves. Each subsequent generation of larvae were exposed to a sub-lethal concentration of the Cry 1C toxin higher than that used in the previous one. The increasing Cry 1C concentrations used for selection were as follows: 0.1, 0.2, 0.4, 0.8, 2.5, 4.0, and 6.0 µg/g in the 2nd, 3rd, 4th, 5th–7th, 8th–10th, 11th–13th, and 14th–15th generations, respectively, according to [50]. Selection pressure continued in the same manner until the mortality rate reached 40–60% of exposed insects.

2.2. Bt Cry1C Toxin Preparation

Bt Cry1C toxin purification was executed in accordance with [51]. Briefly, the bacterial cells were cultured in T3 medium. The mixture was kept in a shaking incubator at 30 °C with continuous shaking at 150 rpm for 3–5 days. The spores and crystals were collected by spinning them at 5500 rpm for 10 min at 4 °C, followed by washing six times with 50 mM EDTA by spinning at 9500 rpm for 10 min at 4 °C. The toxin concentration was determined using the Bradford method [52], and the integrity of the toxin was assessed on 10% SDS PAGE.

2.3. Toxicological Bioassay of Bt Cry1C

The dose response of *S. littoralis* to the *Bt* Cry1C toxin was determined as mentioned earlier [51]. Briefly, 10 recently hatched neonates from both the sensitive and *Bt* Cry1C-tolerant populations of *S. littoralis* were given their own separate semi-artificial diet containing the appropriate concentration of purified *Bt* Cry1C toxin. These concentrations were 0.0, 0.2, 0.4, 0.8, 1.6, and 3.2 µg/g for the sensitive population and 0.0, 2.0, 4.0, 8.0, 16.0, and 32.0 µg/g for the Cry1C-tolerant population. Three biological replicates of each concentration were conducted. Mortality rates were recorded daily for a week. The data were subjected to probit analysis to determine the lethal concentrations (LC₅₀) along with their corresponding confidence limits using the LC₅₀ in the EPA Probit analysis program (version 1.5).

2.4. Isolation and Identification of Bacterial Isolates

Recently molted third-instar larvae ($n = 5$) were randomly selected from each population and subsequently moved to Petri dishes where they were starved for a period of 24 h. Larvae were surface-sterilized in 70% ethanol for 1 min and rinsed in sterile water before dissection to remove foreign substances that had adhered to them, especially external microorganisms [53]. The larvae were carefully cut open in a sterile laminar-flow hood using sanitized dissection tools. The larvae were dissected by removing the head and final abdominal segment; then, we cut open the body along the middle to separate the gut, and the entire gut was taken out. Each specimen's gut was placed in a 1.5 mL centrifuge tube along with 0.5 mL of 10 mM PBS and then crushed individually using a plastic pestle. After briefly vortexing the mixture at a moderate speed for 30 s, 100 µL of the homogenate was transferred into a new sterile centrifuge tube for bacterial culture. Each homogenate

tube was diluted to 10^{-3} – 10^{-6} dilutions with sterile distilled water and then spread on nutritionally rich solid lysogeny broth (LB) medium plates and incubated in darkness at 25 °C for 3 days. Afterwards, single colonies displaying various characteristics, such as a certain size, shape, color, and opacity, were selected and cultured on new solid LB agar plates. Subsequently, the pure colonies were placed in LB media, mixed with 30% glycerol, and then stored at –80 °C.

Characterization of the isolated bacteria was carried based on the morphology of the colonies [54], Gram staining [55], a motility test [56], the activity of catalase and oxidase [57], urease [58], an oxidative fermentative test [59], methyl red and Voges–Proskauer tests [60], an indole test [61], the hydrolyzation of starch [62], a gelatin hydrolysis test [63], and the carbon utilization of sugars [64,65].

For the molecular identification of symbiotic gut bacteria, first, DNA was extracted separately from predominant isolates of the larval gut of the *Bt*-tolerant and susceptible populations using a Promga DNA Purification Kit (Madison, WI, USA, Cat. #A1120). The DNA was checked for quality according to the manufacturer's protocol by running it on 1% agarose gel, and then, its concentration was quantified using a Nano-Drop spectrophotometer.

The 16S rRNA gene was amplified using PCR. Briefly, 10 mg of genomic DNA served as a template. The forward primer (5' CCAGCAGCCGCGGTAATACG 3') and the reverse primer (5' ATCGGYTACCTTGTTACGACTTC 3'), where Y is C or T, were used [66]. The process of amplification was carried out in a thermocycler (Analytic JENA Model, Flex-Cycler2 PCR thermal Cycler, Radnor, PA, USA) for 35 reaction cycles. The PCR condition started with initial denaturation at 95 °C for 5 min. Then, 35 reaction cycles were carried out at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. Finally, the reaction cycle was terminated by 10 min incubation at 72 °C for the final extension step. The amplified products were visualized by gel electrophoresis, and then the products were purified by gel elution, using the Gene JET Gel Extraction Kit Thermo Scientific (Waltham, MA, USA, Cat. #K0691). The PCR products, each with a barcode, were sequenced by the Macrogen company (Seoul, Republic of Korea). The obtained sequence results were aligned with the GenBank database using the software BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). Phylogenetic analysis was conducted to demonstrate the relationships between isolates utilizing the Neighbor-Joining (NJ) approach and evaluated with 1000 bootstrap replicates using MEGA software (version 11.0.13), and MUSCLE software was used for aligning the sequences.

2.5. Statistical Analysis

Mortality was analyzed using probit analysis to determine the lethal concentrations (LC_{50}) along with their corresponding confidence limits (CLs) utilizing the EPA Probit analysis software (version 1.5). Bray–Curtis similarity and Jaccard similarity based on abundance data were used to calculate the degree of similarities between *Bt*-susceptible and *Bt*-tolerant bacterial communities, and the Shannon–Wiener diversity index (H') and Simpson index (D) were computed using the software package PAST for paleontological data analysis V4.08 [67]. The protocol for the classification of dominance according to Engelmann [68] was followed. All data analysis was conducted employing IBM SPSS Statistics for Windows, Version 27, in conjunction with Microsoft Excel 365 (Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Toxicological Bioassay

To confirm obtaining a *Bt*-tolerant population, a toxicological bioassay of *Bt* Cry1C was performed against the susceptible and the tolerant populations. The susceptibility of the *Spodoptera* *Bt*-tolerant population to the Cry 1C toxin was significantly increased ($p < 0.05$) up to 6.5-fold compared to the susceptible population, and the 95% confidence intervals did not overlap. The LC_{50} values are presented in Table 1.

Table 1. Toxicological effect of Cry 1c against *Bt*-susceptible and *Bt*-tolerant strains of *S. littoralis* larvae.

Strain	LC50 (95% FL *) (µg/g Diet)	Slope ± SE	RR	χ ² (df)
Susceptible	1.8950 (1.3193–3.77)	1.489249 ± 0.290369	-	1.761 (3)
Tolerant	12.263 (9.433–16.692)	2.029307 ± 0.311965	6.5	1.212 (3)

* 95% FL fiducial limits, SE—standard error, RR—resistance ratio, χ²—chi-square, df—degree of freedom.

3.2. Identification of Bacterial Isolates

The dominant isolates of larval midgut bacteria from both susceptible and tolerant populations were used for Gram staining, the morphological characterization of bacterial shape, and the motility and biochemical activity tests (Table 2). The 16S rRNA gene sequences demonstrated strong similarities ($\geq 98\%$) to the GenBank database through BLAST searching (Supplementary Table S1). A phylogenetic tree of taxonomically related bacterial species and their maximum identity percentages is presented in Figure 1. The sequence of the predominant isolates was related to four different genera, *Staphylococcus*, *Bacillus*, *Enterococcus*, and *Enterobacter*. Three bacterial phyla, namely *Proteobacteria*, *Firmicutes*, and *Actinomycetota*, in the gut of susceptible and tolerant populations were identified (Figure 2A,B). In the susceptible populations, the highest number of bacteria was annotated to *Proteobacteria* (48.89%), followed by *Firmicutes* (46.67%), while *Bt* tolerance reversed the percentage of the two bacterial phyla, meaning that the highest number of bacteria found belonged to the *Firmicutes* phylum (71.11%), followed by *Proteobacteria* (22.22%). The lowest percentage of sequences was annotated to the phylum *Actinomycetota* in susceptible and tolerant populations (4.44% and 6.67%, respectively). Regarding bacterial classes, *Bt* tolerance increased the percentage of *Bacilli* to 62.22% compared to 42.22% in the susceptible population. Meanwhile, the class *Gammaproteobacteria* decreased in the *Bt*-tolerant population to 22.22% compared to 48.89% in the susceptible population (Figure 2C). The differentially abundant bacterial orders of the susceptible and tolerant populations were identified. *Bt* tolerance decreased the percentage of the order *Enterobacterales* to 20% compared to 42.22% in the susceptible population (Figure 2D). At the family level, *Bt* tolerance increased the percentage of *Enterococcaceae*, *Bacillaceae*, *Clostridiaceae*, and *Micrococcaceae* and decreased the percentage of *Staphylococcaceae*, *Enterobacteriaceae*, *Erwinaceae*, and *Moraxellaceae*. The bacterial family *Yersiniaceae* completely disappeared from the gut of the *Bt*-tolerant population (Figure 2E). At the genus level, the results revealed an increase in the percentage of *Enterococcus* and *Bacillus* in the *Bt*-tolerant compared to the susceptible population. Additionally, the complete disappearance of bacteria belonging to the genera *Klebsiella* and *Serratia* because of *Bt* tolerance was revealed (Figure 2F). Collectively, 11 genera of bacteria were recorded in the susceptible population, with a Shannon diversity index of 2.29 and evenness of 0.89 (the Simpson diversity index (1-D) was 0.89 with equitability of 0.96). The number of genera in the *Bt*-tolerant strain was 9, with a Shannon diversity index of 1.92 and evenness of 0.76 (Simpson diversity index (1-D) = 0.82 and equitability = 0.87). Diversity *t*-tests revealed a statistically significant difference between the two populations (for the Shannon diversity index, $t = 3.79$, p -value < 0.001, and for the Simpson diversity index, $t = 2.98$, p -value = 0.003).

Table 2. Identification of susceptible strain (N = 45) and 15th generation of *Bt* Cry1C-tolerant (N = 45) strain of *S. littoralis* larvae gut bacterial isolates based on morphological and biochemical parameters.

Isolate No.	Colony Color	Morphology	Motility	Gram	Biochemical Test												Bacterial Type
					Starch Hydrolysis	Catalase	Oxidase	Gelatin Hydrolysis	Indole Production	Methyl Red Test	Voges-Proskauer Test	Urease Production	Sucrose	Xylose	Lactose	Dextrose	
S.L. S. 1	White	rod	+	+	+	+	-	+	-	-	-	+	+	+	+	+	<i>Bacillus</i> sp.
S.L. S. 2	Lemon yellow	rod	+	-	+	+	-	-	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. S. 3	Pale yellow	s. rod	-	-	-	+	-	-	-	-	-	+	-	+	-	+	<i>Acinetobacter</i> sp.
S.L. S. 4	Creamy white	rod	+	-	-	+	-	+	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. S. 5	Grey	cocci	-	+	-	-	-	-	-	+	+	-	+	+	+B	+	<i>Enterococcus</i> sp.
S.L. S. 6	Yellow	cocci	-	+	+	+	-	+	-	-	+	+	+	+	+	+	<i>Staphylococcus</i> sp.
S.L. S. 7	Pale yellow	s. rod	-	-	-	+	-	-	-	-	-	-	-	+	-	+	<i>Acinetobacter</i> sp.
S.L. S. 8	Yellow	cocci	-	+	-	+	-	+	-	+	+	+	+	-	+	+	<i>Staphylococcus</i> sp.
S.L. S. 9	Yellow	cocci	-	+	-	+	-	+	-	+	-	-	+	-	+	+	<i>Staphylococcus</i> sp.
S.L. S. 10	Creamy white	rod	+	-	-	+	-	-	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. S. 11	Lemon yellow	rod	+	-	+	+	-	+	-	-	+	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. S. 12	Grey	cocci	-	+	-	-	-	-	-	+	-	-	+	+	+	+	<i>Enterococcus</i> sp.
S.L. S. 13	Grey	cocci	-	+	-	-	-	-	-	+	+	-	+	+B	+	+B	<i>Enterococcus</i> sp.
S.L. S. 14	Grey	rod	+	-	-	+	-	-	-	+	+	+	+	+	+	+	<i>Enterobacter</i> sp.
S.L. S. 15	Grey	rod	+	-	-	+	-	-	-	+	+	-	+	+	+	+	<i>Enterobacter</i> sp.
S.L. S. 16	Lemon yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. S. 17	Grey	rod	+	+	-	-	-	+	-	+	-	-	-	+	-	+	<i>Clostridium</i> sp.
S.L. S. 18	White	rod	+	+	-	+	+	+	-	-	+	-	+	+	+	+	<i>Bacillus</i> sp.
S.L. S. 19	Slightly yellow	rod	+	+	-	+	+	+	-	-	+	-	+	+	-	+	<i>Bacillus</i> sp.
S.L. S. 20	Yellow	cocci	-	+	-	+	-	+	-	-	+	+	+	+	+	+	<i>Staphylococcus</i> sp
S.L. S. 21	Lemon yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	-	+	<i>Pantoea</i> sp.
S.L. S. 22	Pale yellow	s. rod	-	-	-	+	-	-	-	-	-	-	-	+	-	+	<i>Acinetobacter</i> sp.
S.L. S. 23	Grey	rod	+	-	-	+	-	-	-	+	+	-	+	+	+	+	<i>Enterobacter</i> sp.

Table 2. Cont.

Isolate No.	Colony Color	Morphology	Motility	Gram	Biochemical Test												Bacterial Type
					Starch Hydrolysis	Catalase	Oxidase	Gelatin Hydrolysis	Indole Production	Methyl Red Test	Voges-Proskauer Test	Urease Production	Sucrose	Xylose	Lactose	Dextrose	
S.L. S. 24	Grey	cocci	-	+	+	-	-	+	-	+	+	-	-	+	+	+	<i>Enterococcus</i> sp.
S.L. S. 25	Grey	cocci	-	+	-	-	-	-	-	+	+	-	+	+	+	+	<i>Enterococcus</i> sp.
S.L. S. 26	Grey	rod	+	-	-	+	-	-	-	+	+	-	+	+	+	+	<i>Enterobacter</i> sp.
S.L. S. 27	Yellow	rod	-	+	+	+	+	+	-	+	-	+	+	-	+	+	<i>Micrococcus</i> sp.
S.L. S. 28	White	rod	+	+	-	+	+	-	-	-	+	-	+	+	+	+	<i>Bacillus</i> sp.
S.L. S. 29	Creamy white	rod	+	-	-	+	-	+	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. S. 30	Lemon yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. S. 31	White	rod	+	+	+	+	+	+	-	-	+	+	+	+	+	+	<i>Bacillus</i> sp.
S.L. S. 32	Grey	cocci	-	+	+	-	-	-	-	+	+	-	+	+	+	+	<i>Enterococcus</i> sp.
S.L. S. 33	Grey	rod	+	-	-	+	-	-	-	-	+	-	+	+	+	+	<i>Enterobacter</i> sp.
S.L. S. 34	Yellow	rod	-	+	+	+	+	+	-	+	-	+	+	-	-	+	<i>Micrococcus</i> sp.
S.L. S. 35	Creamy white	rod	+	-	-	+	-	-	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. S. 36	Grey	rod	+	+	-	-	-	+	-	+	-	-	-	+	-	+	<i>Clostridium</i> sp.
S.L. S. 37	Grey	cocci	-	+	+	-	-	+	-	+	+	+	+	+	+	+	<i>Enterococcus</i> sp.
S.L. S. 38	Slightly yellow	rod	+	+	+	+	+	+	-	-	+	+	+	+	+	+	<i>Bacillus</i> sp.
S.L. S. 39	Lemon yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	+	+B	<i>Pantoea</i> sp.
S.L. S. 40	White	rod	+	+	+	+	+	+	-	-	+	+	+	+	+	+	<i>Bacillus</i> sp.
S.L. S. 41	White	rod	+	+	+	+	+	+	-	+	+	+	-	-	-	+	<i>Bacillus</i> sp.
S.L. S. 42	Lemon yellow	rod	+	-	+	+	-	-	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. S. 43	Bluish-white	rod	+	-	-	+	-	+	-	-	+	-	+	-	-	+	<i>Serratia</i> sp.
S.L. S. 44	Grayish-white	rod	-	-	-	+	+	-	-	-	+	+	-	+	-	+	<i>Klebsiella</i> sp.
S.L. S. 45	Bluish-white	rod	+	-	-	+	-	+	-	-	+	+	+	-	-	+	<i>Serratia</i> sp.
S.L. T.1	White	rod	+	+	-	+	+	+	-	-	+	-	+	+	-	+	<i>Bacillus</i> sp.
S.L. T.2	White	rod	+	+	-	+	+	+	-	-	+	-	+	+	-	+	<i>Bacillus</i> sp.
S.L. T.3	Lemon-yellow	rod	+	-	+	-	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.

Table 2. Cont.

Isolate No.	Colony Color	Morphology	Motility	Gram	Biochemical Test												Bacterial Type
					Starch Hydrolysis	Catalase	Oxidase	Gelatin Hydrolysis	Indole Production	Methyl Red Test	Voges-Proskauer Test	Urease Production	Sucrose	Xylose	Lactose	Dextrose	
S.L. T.4	Yellow	cocci	-	+	+	+	-	+	-	-	+	+	+	+	+	+	<i>Staphylococcus</i> sp.
S.L. T.5	White	rod	+	+	-	+	+	+	-	-	+	-	+	+	-	+	<i>Bacillus</i> sp.
S.L. T.6	Slightly yellow	rod	+	+	-	+	+	+	-	-	+	-	+	+	-	+	<i>Bacillus</i> sp.
S.L. T.7	White	rod	+	+	+	+	-	+	-	+	+	+	+	-	-	+	<i>Bacillus</i> sp.
S.L. T.8	Creamy-white	rod	+	-	-	+	-	-	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. T.9	Lemon-yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. T.10	Grey	rod	+	+	-	-	-	+	-	+	-	-	-	+	-	+	<i>Clostridium</i> sp.
S.L. T.11	Creamy white	rod	+	-	-	+	-	-	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. T.12	White	rod	+	+	+	+	-	+	-	+	+	+	+	-	-	+	<i>Bacillus</i> sp.
S.L. T.13	Grey	cocci	-	+	-	-	-	-	-	-	+	-	+	+	+	+	<i>Enterococcus</i> Sp.
S.L. T.14	Slightly yellow	rod	+	+	+	+	-	+	-	+	+	+	+	-	-	+	<i>Bacillus</i> sp.
S.L. T.15	Lemon yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. T.16	White	rod	+	+	-	+	+	-	-	-	+	-	+	-	-	+	<i>Bacillus</i> sp.
S.L. T.17	Yellow	cocci	-	+	+	+	-	+	-	-	+	+	+	+	+	+	<i>Staphylococcus</i> sp.
S.L. T.18	Yellow	rod	-	+	-	-	+	+	-	+	-	+	+	-	+	+	<i>Micrococcus</i> sp.
S.L. T.19	White	rod	+	+	+	+	-	+	-	+	+	+	+	-	-	+	<i>Bacillus</i> sp.
S.L. T.20	Grey	cocci	-	+	-	-	-	-	-	+	-	+	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.21	Slightly yellow	rod	+	+	+	+	-	+	-	+	+	+	+	-	-	+	<i>Bacillu</i> sp.
S.L. T.22	Lemon yellow	rod	+	-	+	-	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. T.23	Grey	rod	+	+	-	-	-	+	-	+	-	+	-	-	-	+	<i>Clostridium</i> sp.
S.L. T.24	Grey	cocci	-	+	-	-	-	-	-	+	-	+	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.25	Grey	cocci	-	+	-	-	-	-	-	+	-	+	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.26	Grey	rod	+	+	-	-	-	+	-	+	-	-	-	-	-	+	<i>Clostridium</i> sp.
S.L. T.27	White	rod	+	+	-	+	+	+	-	-	+	-	+	+	-	+	<i>Bacillus</i> sp.
S.L. T.28	White	rod	+	+	+	+	-	+	-	+	+	+	+	-	-	+	<i>Bacillus</i> sp.

Table 2. Cont.

Isolate No.	Colony Color	Morphology	Motility	Gram	Biochemical Test												Bacterial Type
					Starch Hydrolysis	Catalase	Oxidase	Gelatin Hydrolysis	Indole Production	Methyl Red Test	Voges-Proskauer Test	Urease Production	Sucrose	Xylose	Lactose	Dextrose	
S.L. T.29	Grey	cocci	-	+	-	-	-	-	-	-	+	-	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.30	Grey	cocci	-	+	-	-	-	-	-	+	+	+	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.31	Grey	cocci	-	+	-	-	-	-	-	-	+	-	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.32	Lemon yellow	rod	+	-	+	+	-	+	-	-	-	-	+	+	+	+	<i>Pantoea</i> sp.
S.L. T.33	Grey	cocci	-	+	-	-	-	-	-	+	+	-	+	+	+B	+	<i>Enterococcus</i> sp.
S.L. T.34	Creamy white	rod	+	-	-	+	-	-	-	+	-	+	+	+	+	+	<i>Citrobacter</i> sp.
S.L. T.35	Grey	cocci	-	+	+	-	-	-	-	+	+	-	-	+	+	+	<i>Enterococcus</i> sp.
S.L. T.36	Yellow	rod	-	+	+	+	+	+	-	+	-	+	+	-	+	+	<i>Micrococcus</i> sp.
S.L. T.37	Grey	cocci	-	+	+	-	-	+	-	-	+	-	-	+	+	+	<i>Enterococcus</i> sp.
S.L. T.38	Grey	rod	+	-	-	+	-	-	-	+	+	-	+	+	+	+	<i>Enterobacter</i> sp.
S.L. T.39	Grey	cocci	-	+	+	-	-	+	-	-	+	-	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.40	Grey	cocci	-	+	-	-	-	-	-	+	+	+	+	+	+	+	<i>Enterococcus</i> sp.
S.L. T.41	Pale yellow	s.rod	+	-	-	+	-	+	-	-	-	-	-	+	-	+	<i>Acinetobacter</i> sp.
S.L. T.42	Yellow	rod	-	+	-	-	+	+	-	+	-	+	+	-	+	+	<i>Micrococcus</i> sp.
S.L. T.43	Grey	Cocci	-	+	-	+	+	+	-	+	-	+	+	-	+	+	<i>Enterococcus</i> sp.
S.L. T.44	Grey	rod	+	+	-	-	-	-	-	+	-	-	-	-	-	+	<i>Clostridium</i> sp.
S.L. T.45	Yellow	Cocci	-	+	-	+	-	+	-	+	-	+	+	+	+	+	<i>Staphylococcus</i> sp.

(+) indicates positive reaction; (-) indicates negative reaction; (B) indicates formation of bubbles.

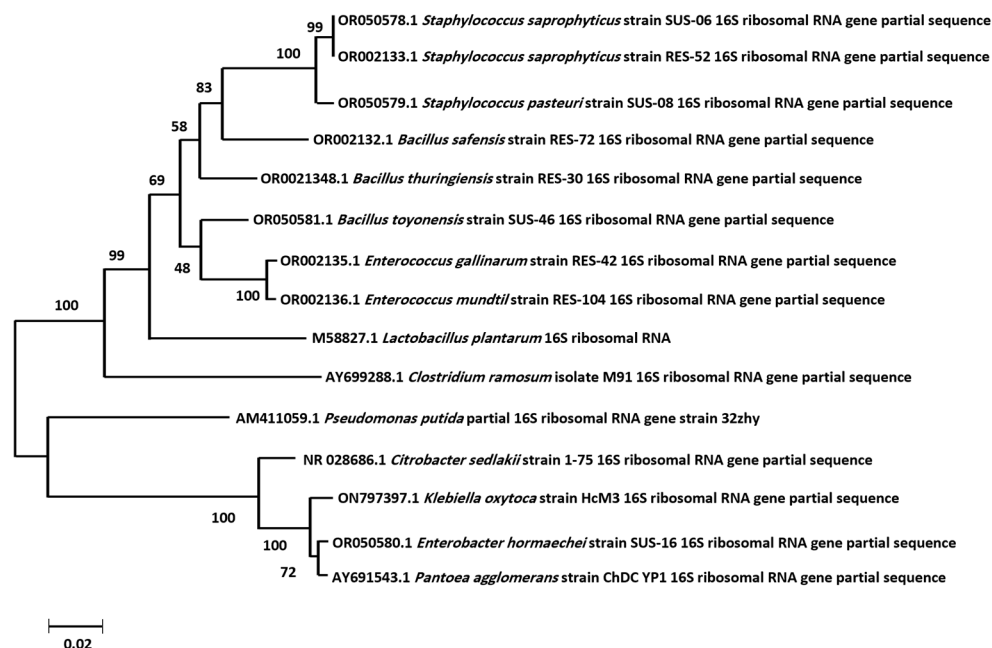


Figure 1. Phylogenetic tree of bacteria of *Bt*-susceptible and *Bt*-tolerant *Spodoptera littoralis* larval midgut based on 16s rRNA multiple sequence alignment.

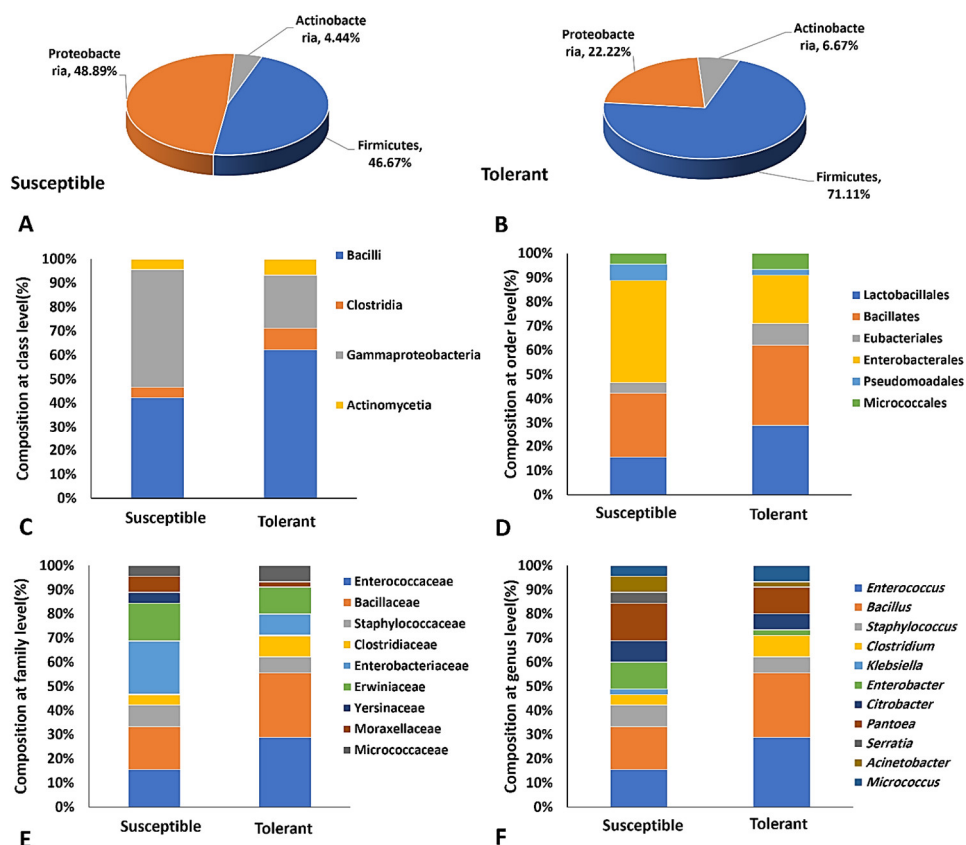


Figure 2. Effect of Cry1C toxin on load and composition of midgut bacteria of *Bt*-susceptible and *Bt*-tolerant *Spodoptera littoralis* at phylum level (A,B), at class level (C), at order level (D), at family level (E), and at genus level (F).

To further analyze the relationship between gut bacterial composition as a complex community and *Bt* susceptibility /tolerance, statistical analyses of similarity /dissimilarity,

namely the Bray–Curtis and the Jaccard dissimilarity tests, were used. The results revealed that the Bray–Curtis similarity percentage (Figure 3) based on the abundance of gut microbiota was 71.1%, while the Jaccard similarity percentage based on the presence/absence of bacterial types in *Bt*-susceptible and -tolerant populations was 81.82% indicating that most of the bacterial types were detected in both populations.

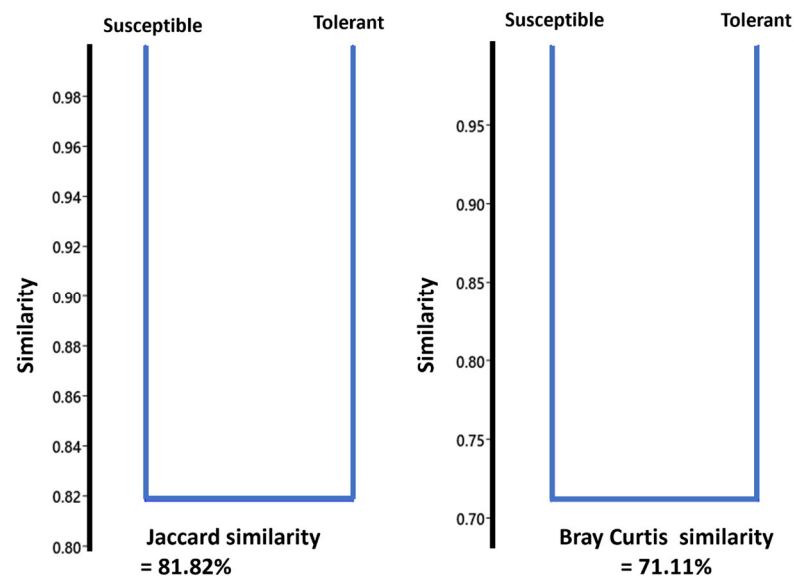


Figure 3. Jaccard and Bray–Curtis similarity coefficients computed from presence/absence and abundance data of larval midgut bacteria of *Bt*-susceptible and *Bt*-tolerant *Spodoptera littoralis*.

Principal component analysis (Figure 4) of the relative contribution of bacterial composition in the *Bt*-tolerant and -susceptible strains revealed that principal components 1 and 2 accounted for 92.96% and for 7.04% of the total variation, respectively. The bacterial genera *Pantoea*, *Enterobacter*, *Bacillus*, *Staphylococcus*, *Citrobacter*, and *Acinetobacter* were correlated more with the susceptible strain; on the other hand, *Enterococcus*, *Micrococcus*, and *Clostridium* were more associated with the tolerant strain.

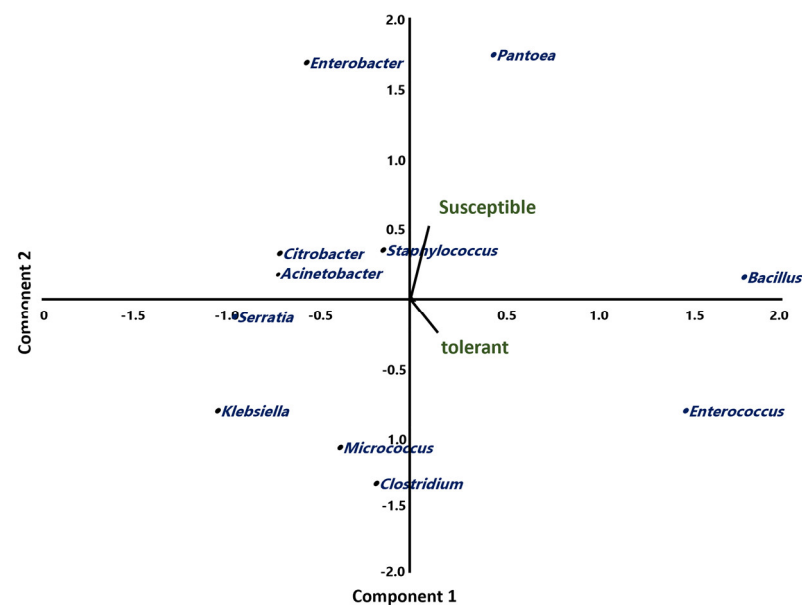


Figure 4. Principal component analyses of bacteria of *Bt*-susceptible and *Bt*-tolerant *Spodoptera littoralis* larval midgut at genus level.

4. Discussion

Microbes residing in the insect midgut are essential for various functions, such as helping with nutrition and development, adapting to the environment, processing dietary toxins, immunity to pathogens, and maintaining gut hemostasis [69–72]. These endosymbiont bacteria can serve as mediators or sensitive indicators of the different environmental conditions experienced by the insect host [73]. Specifically, domestic microbiota can serve as a protective barrier against harmful invaders, and they can collaborate with pathogens synergistically or additively [11,44,74,75] and transform their relationship from commensal to pathogenic by entering the insect's hemocoel [76,77]. Both the structure and variety of bacteria within host guts dynamically change in response to shifts in the environmental factors of the insects [78]. Prior research has shown that the relationship between the enterobacteria and insect hosts can impact the sensitivity or immunity of certain lepidopterans to the endotoxin of *B. thuringiensis* (*Bt*) [44,79–81] and *Enterobacter* sp. and increase the susceptibility of the axenous insect *Lymantria dispar* to *Bt* [74].

Insects gradually develop resistance to pesticides [82]. Research on how *Bt* resistance develops primarily investigates changes in the binding sites for the toxin and its activation or specific identification of genes that are associated with immunity [83,84]. Hernández-Martínez, P. et al. [48] stated that resistance to *Bt* in *Spodoptera exigua* was associated with a high microbiota load. Resistance to *Bt* results in the promotion of bacteria that are capable of breaking down proteins of *Bt* or changing the physiological environment in the gut by forming biofilms or producing antimicrobials in order to decrease or rigorously eliminate harmful bacteria through competition [85,86]. Evidence of septicemia caused by internal bacteria [44,74] prompted us to explore whether the influence of *Bt* on the microbiota in the midgut could contribute to resistance against *Bt*. To achieve this, we biochemically and molecularly characterized gut bacterial composition in *Bt*-susceptible and *Bt*-tolerant populations. We found that the intestinal symbiotic bacteria community structure was significantly altered by the *Bt* Cry1C toxin. The diversity analysis revealed a reduction in the diversity and richness of intestinal bacteria in the *Bt*-tolerant larvae compared with the susceptible strain. Similarly, Dubovskiy et al. [37] revealed a decrease in both the variety and quantity of microorganisms in the intestines of a *Bt*-resistant strain of the Greater wax moth, *G. mellonella*, which are vast and plentiful. Exposure to Cry1Ab/2Ab toxins resulted in a significant alteration in the makeup of the intestinal bacteria with a decrease in the overall load of symbiotic bacteria in *Locusta migratoria* [87]. It was previously mentioned that Cry1Ac treatment increased *P. xylostella* gut symbiotic bacteria load and decreased bacterial diversity [38]. The intestine of the *Bt*-resistant line of the rice stem borer, *Chilo suppressalis*, displayed higher microbiota diversity compared to strains susceptible to *Bt* [88]. *Bt* can stimulate the immune system to produce antimicrobial peptides, leading to a reduction in the number and variety of endosymbionts bacteria. However, the effects of high doses of *Bt* have a contrasting effect. *Bt* toxins can damage gut cells, leading to immune system issues that allow certain harmful gut bacteria to move from the gut to the hemocoel, where they can quickly increase in number [38,44]. Reports indicate that mosquito larvae, which hosts the lowest variety of gut bacteria, display strong resistance to *Bt israelensis* [36]; this suggests that lower variation in the composition of gut bacteria can benefit the host in defending against *Bt* infection, and is consistent among various insect species [38].

Interestingly, we found differences in gut microflora composition due to *Bt* tolerance. In the tolerant group, there was a greater presence of the Firmicutes phylum, while the susceptible group had a higher abundance of Proteobacteria, which is consistent with previous findings in *P. xylostella* [89]. In the resistant brown planthopper *Nilaparvata lugens*, Vijayakumar et al. [90] noticed a consistent pattern of Firmicutes being more abundant compared to its susceptible counterparts. Furthermore, their research showed a significant rise in the percentages of the Lactobacillales and Enterobacteriales orders. In the present study, a comparison at the order level showed a rise in the percentages of the Bacillales and Lactobacillales orders and a commensurate reduction in Enterobacteriales in the tolerant

population of *S. littoralis*. The tolerant population also had a high abundance of other bacterial orders, such as Eubacteriales (Clostridiales) and Micrococcaceae. Eubacteriales are involved in breaking down lignocelluloses and are believed to contribute to the nutritional physiology of the insect hosts [91–93]. Micrococcaceae play a role in the creation of antimicrobial peptides that exhibit a mechanism of protection [94]. Similar to our results, Enterobacteriales, Bacillales, and Lactobacillales were found in higher abundance in the susceptible population of various insect species [95–98].

The varying gut bacteria composition between susceptible and tolerant populations could be a result of the microbiota adapting to distinct gut environments. Our results revealed that *Enterococcus mundtii* and *Enterococcus gallinarum*, belonging to the family Enterococcaceae, which form the core bacteria associated with *S. littoralis*, were found in more abundance in *Bt*-tolerant compared to *Bt*-susceptible individuals. *E. mundtii* and *E. gallinarum* were previously detected within the intestines of different insect species [88,99,100]. Both bacterial species were reported to be involved in insect degradation capacity for organic compounds [101,102]. Consequently, agricultural pests commonly consume both types of bacteria [103] to enhance their defense system [35,97]. *E. mundtii* was identified as having antimicrobial activity against various types of bacteria [11,104]. Additionally, in our study, the methyl red test for *E. mundtii* yielded a positive result, indicating that *E. mundtii* has the ability to produce acidic substances to lower the pH in the intestine. Similarly, Mead et al. [105] stated that *Enterococcus* can produce acetate, which results in a drop in the pH levels of gastrointestinal fluid in the intestine. This reduction in the acidity level in the intestines can directly reduce the toxicity of *Bt* [93] as it is only toxic under alkaline conditions.

The nitrogen-fixing bacteria *Citrobacter* were more abundant in the *Bt*-susceptible than the *Bt*-tolerant population. They can break down chitin and cellulose, reflecting their metabolic diversity [106–109]. Feeding Colorado potato beetle larvae with *C. freundii* and *B. thuringiensis* resulted in an alteration in the tissue that weakened both cellular and humoral immunity, ultimately enhancing their susceptibility to *Bt* [81]. Our sequence similarity results revealed that *Enterobacter hormaechei*, a member of the family Enterobacteriaceae, was found in greater numbers in the susceptible individuals compared to the tolerant ones. The association of *Enterobacter* with insects, especially from lepidopteron, has been widely recorded [11,104]. Members of *Enterobacter* play a crucial role in the biosynthesis of essential vitamins and pheromones, breaking down plant secondary compounds through processes such as cellulose catabolism and nitrogen fixation [110–112]. An investigation into the composition and interaction of intestinal bacteria in house fly larvae indicated that *E. hormaechei* suppressed the proliferation of injurious bacteria, like *Providencia stuartii*, *Pseudomonas aeruginosa*, and *Providencia vermicola*, while enhancing the proliferation of beneficial bacteria. The dominance of *E. cloacae* within the midgut of *P. xylostella* enhances the breakdown of foreign substances and contributes to the process of digesting food and acquiring nutrients [113]. The housefly larvae gut microbiota underwent changes when they were fed *E. hormaechei*, leading to a reduction in the abundance of *Klebsiella* and *Bacillus*. Similarly, we found that bacteria belonging to the genus *Micrococcus* were more abundant in the tolerant population than the susceptible one, perhaps attributed to their role in producing antimicrobial peptides that serve as protective agents against insect pathogens [94].

The levels of gut symbionts of proteolytic bacteria *Staphylococcus pasteurii* belonging to the Staphylococcaceae family, among the genera, were similar between the susceptible and tolerant populations. It was previously reported that under certain conditions, *Saprophyticus undecan* can cause a lethal infection in fully engorged ticks [96]. It can thus be considered as an alternative approach for the management of cattle tick *Rhipicephalus microplus* infestation [114,115].

Pantoea, a member of the Erwiniaceae family, is a common genus that is more prevalent in the susceptible population. A close relationship between *pantoea* and the eggs and females of insects suggested a vital role of *pantoea* in the morphogenesis, development,

and reproduction of their insect hosts [116]. It can produce diverse enzymes involved in plant polymer degradation and the utilization various kinds of plant materials [93,117].

Klebsiella and *Serratia* were among the other relevant genera identified in the present investigation. The discovery of *Klebsiella* in both male and female *S. littoralis*, as well as within the reproductive organs of the beetle *Phyllophaga obsoleta* and oriental fruit flies *Bactrocera dosalis*, suggested that they likely have important roles in the biological functions, physiological developments, and digestion processes within the insect midgut [93,118,119]. However, *Serratia*, usually seen as an opportunistic or a facultative pathogen because it is typically not harmful to insects in their digestive tract, only becomes lethal when it crosses the gut walls and enters the insect's hemocoel [120,121].

We also found that *Acinetobacter* is found in both populations. It has been found before within the midgut of different insect species. *Acinetobacter* sp. can help the host break down harmful secondary compounds produced by plants. The presence of *Acinetobacter* in the *G. mellonella* caterpillar helps it to break down the polyethylene and polystyrene that it has consumed [122,123]. These bacteria may help *S. littoralis* in protecting their gut from harm inflicted by those compounds when eating foliage.

5. Conclusions

The present research demonstrated that *Bt* influences gut microbiota composition and may participate in reducing *Bt* efficacy in controlling *S. littoralis*. The diverse and intricate structure of the gut microbiome in the *Bt*-susceptible population was significantly higher compared to the *Bt*-tolerant strain. Changes in the community of bacteria in the gut of the *Bt*-tolerant population were possibly linked to the advancement of insect tolerance to *Bt* in the insect. Additionally, a high abundance of Enterococcaceae (essentially *Enterococci*) was detected in the gut of the *Bt*-tolerant samples. Research has demonstrated that *Enterococcus* spp. can enhance tolerance to conventional *Bt*, and certain species within this genus can acidify their environment, potentially heightening their tolerance to *Bt* by reducing its activation. Therefore, the functional potential of midgut bacterial community changes needs to be assessed. In general, this research explores potential strategies for developing techniques to control insect pests and their resistance, which is essential for effective management through the interplay of the *Bt* toxin and midgut bacteria. Comparing the efficiency of *Bt* with and without specific anti-*Enterococcus* against agricultural insect pests is a task we will undertake in the future.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microbiolres15020062/s1>, Table S1: Blast results of alignment of 16s rRNA nucleotide sequences.

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