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Elimination of Gut Microbes with Antibiotics Confers Resistance to *Bacillus thuringiensis* Toxin Proteins in *Helicoverpa armigera* (Hubner)

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Abstract *Helicoverpa armigera* is one of the most important pests worldwide. Transgenic crops with toxin genes from *Bacillus thuringiensis* (*Bt*) have been deployed on a large scale to control this pest. The insecticidal activity of *Bt* is probably influenced by the insect midgut microbes, which vary across crop hosts and locations. Therefore, we examined the role of gut microbes in pathogenicity of *Bt* toxins in the *H. armigera*. Antibiotic cocktail was used for the complete elimination of the *H. armigera* gut microbes. Activated Cry1Ac, *Bt* formulation, and transgenic cotton resulted in larval weight loss and increase in mortality, but pretreatment of larvae with antibiotic cocktail significantly decreased larval mortality and increased the larval weight gain. Activated Cry1Ac and *Bt* formulation inhibited the activity of proteases in midgut of *H. armigera* larvae but showed no such effect in the larvae pretreated with antibiotic cocktail. Five protease bands in activated Cry1Ac and two in *Bt* formulation-treated larvae were inhibited but no such effect in the larvae pretreated with antibiotic cocktail. Cry1Ac protein was detected in *Bt*/Cry1Ac protoxin-fed larval gut extract in the absence of antibiotic cocktail, but fewer in larvae pretreated with antibiotic cocktail. The activity of antioxidant enzymes and aminopeptidases increased in larvae fed on *Bt* toxin, but there was no significant increase in antioxidant enzymes in larvae reared on toxin protein in combination with antibiotic cocktail. The results suggest that gut microbes exercise a significant influence on the toxicity of Cry1Ac and *Bt* formulation in *H. armigera* larvae. The implications of these results have been discussed in relation to development of insect resistance to *Bt* transgenic crops deployed for pest management.

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Introduction

Cotton bollworm/legume pod borer, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), is a polyphagous pest, which damages important crops such as cotton, tomato, sunflower, corn, pigeonpea, chickpea, and vegetable and field crops [1]. *Bacillus thuringiensis* (*Bt*), a Gram-positive bacterium, has been used as a biopesticide for many years. It produces crystalline parasporal inclusions containing insecticidal proteins called δ-endotoxins [2]. These δ-endotoxins are in inactive toxin form (protoxins), which are proteolytically cleaved in the insect midgut to smaller active toxins when ingested by the insect larvae under basic conditions (pH 8.0–10.0) [3]. The activated toxins bind to the specific receptors in the insect midgut epithelium and cause cell death [4].

Symbiotic relationships between insects and their gut microbes have been studied extensively in several insect systems, particularly in termites and aphids [5]. The microbial community facilitates nutrient availability and utilization and detoxification of environmental toxins. Composition of gut microbes is highly variable between and within species; however, there is little information on gut microbes and their role in Lepidoptera insects. Treatment of the aphid, *Acyrthosiphon pisum* (Harris), with antibiotics to eliminate the symbiotic microbes has no direct deleterious effects on the aphid biology [6]. However, the chitinase-producing gut microbes, *Serratia marcescens* (Bizio), enhances the growth and development of the diamond back moth, *Plutella xylostella* (L.) larvae [7]. *Acrosternum hilare* (Say) requires symbionts for development and survival, whereas symbionts may not be required for development and survival of *Murgantia histrionica* (Hahn) [8]. In velvetbean caterpillar, *Anticarsia gemmatalis* (Hubner), the gut symbionts when eliminated by antibiotics did not seem to play a major role in survival and development [9].

Transgenic crops with toxin genes from *Bt* have been deployed on a large scale for effective control of *H. armigera*. An understanding of the insect-gut symbiont interaction with *Bt* pathogenicity is very important, as these interactions might provide information for developing novel approaches for pest management. There exist varying controversies for the role of midgut microbes in the pathogenicity of *Bt* against insects. Tolerance to Cry1Ac in *H. armigera* might be due to the adaptation of *H. armigera* to *Bt* toxins through variation in midgut symbionts [10]. In the absence of midgut microbes, *Bt* does not kill the larvae of gypsy moth *Lymantria dispar* (L.), *Vanessa cardui* (L.), *Manduca sexta* (L.), and *Pieris rapae* (L.) and the contribution of gut bacteria to *Bt* susceptibility varies across a range of Lepidoptera [11, 12]. Presence of *Enterococci* in the larval gut halved Cry1Ac toxicity to *M. sexta* (L.), and gut bacteria are not required for the insecticidal activity of *Bt* [13]. Purified *Bt* toxin was pathogenic to *P. xylostella* reared aseptically and showed that, in the gut, antibiotics synergizes *Bt* infection by reducing the abundance of commensal gut microbes [14].). In view of the variation in insecticidal effects of *Bt* toxin toward insects vis-a-vis the gut microbes, the current study was aimed at understanding the role of gut microbes in biological activity of *Bt* toxins against *H. armigera*.

Materials and Methods

Insect Culture

The studies were carried out at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana State, India. The *H. armigera* larvae were reared on chickpea-based artificial diet under controlled laboratory conditions at 26±1 °C, 60–70 % relative humidity, and photoperiod of 16: 8 h (L/D) [15] in the insect-rearing laboratory at ICRISAT. The larvae used for antibiotic treatment were reared aseptically. Initially, *H. armigera* neonates were reared on sterilized (autoclaved) artificial diet treated with antibiotic cocktail for one generation. The eggs laid by first-generation adults were surface sterilized with 0.02 % sodium hypochlorite solution, dried, and allowed to hatch.

Antibiotic Sensitivity Assay Against *H. armigera* Gut Microbes

Midguts were removed by dissecting the fourth-instar larvae and homogenized in 0.1 M phosphate buffer, pH 7.0. The gut homogenate was inoculated on Luria-Bertani (LB) media by the spread plate method. Different antibiotic discs (Table 1) were placed at the center of each half-strength LB media plate to determine the sensitivity of antibiotics toward gut microbes. Zones of inhibition were observed. All the antibiotics were pooled to make cocktail and screened to assess the effectiveness in inhibiting the colony growth of gut microbes. The midguts were removed from fourth-instar larvae and homogenized. The gut homogenates were serially diluted in 0.85 % saline solution (0.9 ml), and 0.1 ml of solution was spread on half-strength LB agar plates containing increasing concentration of antibiotic cocktail (20, 40, 60, 80, and 100 μg of each antibiotic/ml diet) and incubated at 30 °C for 24 h. The microbial counts were converted in colony-forming units per milligram of whole gut.

Bioassays of *Bt* Formulation, Activated Cry1Ac, and Transgenic and Non-Transgenic Cotton in Presence and Absence of Antibiotic Cocktail

To study the role of gut microbes on biological activity of *Bt* formulation, the early second-instar *H. armigera* larvae were reared on artificial diet containing varying concentrations of *Bt* formulation (Biolep®, Biotech International Ltd, New Delhi, India; composition: spores 5–8 %

Table 1 Zones of inhibition by antibiotics toward *H. armigera* gut microbes

Antibiotic (μg/disc)	Inhibition zone (radius in cm)[a]
Neomycin (30)	1.4
Chloramphenicol (10)	1.5
Gentamycin (10)	1.9
Streptomycin (10)	0.0
Rifampicin (5)	0.8
Penicillin (2 units)	0.0
Amphicillin (25)	0.8

The live fourth-instar larvae were used to isolate the gut microbes

[a] The values represent the mean of three replications

w/w and δ-endotoxins 5–8 % *w/w*) (0.005, 0.010, 0.025, 0.050, 0.10, and 0.20 %) and trypsin-activated Cry1Ac (obtained from Dr. Marianne P. Carey, Department of Biochemistry, Case Western Reserve University, Cleveland, OH, USA) (0.4, 0.8, 1.6, 3.2, 6.4, and 13 μg/ml diet). The bioassay with transgenic cotton cultivar BG I (JKCH), BG II (Siva 9 and MRC 7351), and non-transgenic cotton was conducted by detached leaf assay method, and the experiment was terminated after 5 days. There were three replications for each treatment and 24 larvae in each replication in a completely randomized design (CRD). The LC_{50} values (effective concentration to kill 50 % of the *H. armigera* larvae) were calculated for *Bt* formulation and trypsin-activated Cry1Ac toxin.

In another set, the *H. armigera* neonates reared on diet containing antibiotic cocktail were subsampled at early third instar and transferred to artificial diet treated with *Bt* formulation or Cry1Ac toxin at LC_{50} concentration and on transgenic and non-transgenic cotton leaves.

Preparation of Larval Midgut Extracts

The midguts were removed from fourth-instar larvae reared on different treatments in the presence and absence of antibiotics, weighed, and homogenized separately in two volumes of 0.1 M glycine-NaOH buffer (pH 10.0). The homogenates were centrifuged at 12,000 rpm at 4 °C for 20 min, and the supernatants were used for further work. Protein concentration of the gut homogenate was quantified by Lowry's method using BSA as standard protein [16].

Processing of Protoxin in the Midgut to Active Toxin and Its Detection

The presence of Cry1Ac protein in gut homogenates of larvae reared on Cry1Ac protoxin isolated from *Bt* 4D4 strain (supplied by Daniel R. Zeigler, Bacillus Genetic Stock Center, The Ohio State university, OH) according to [17] and *Bt* formulation-amended diet in the presence and absence of antibiotic cocktail was detected by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under same conditions [18]. Bands at 65 kDa were excised, and presence of Cry1Ac was detected by using *Bt*-Cry1Ac ELISA kit (Agdia®, India). Fecal matter collected from larvae fed on *Bt* proteins in presence and absence of the antibiotic cocktail was homogenized and centrifuged. The supernatant was used to detect Cry1Ac toxin protein using ELISA kit as per manufacturer's instructions (Agdia®, India).

Protease Activity Assay in the Midgut of *H. armigera* Larvae

Total protease activity was determined by using azocasein as a substrate [19]. The gut extract was mixed with the substrate and incubated for 50 min. Then, 200 μl of 5 % TCA was added and centrifuged. To the supernatant, equal volumes of 1 N NaOH were added and absorbance was read at 450 nm. Units for total protease activity (UA) were calculated by using the equation unit activity (UA)=ABS_{450} nm/[time (min)×volume of enzyme (ml)]. Trypsin activity was measured by incubating the gut extract with Nα-benzoyl-DL-arginine p-nitroanilide (Sigma-Aldrich), and chymotrypsin activity was measured by incubating the gut extract with *N*-glutaryl-L-phenylalanine p-nitroanilide (Sigma-Aldrich) [20]. Elastase activity of *H. armigera* gut proteases was measured by incubating the gut extract with *N*-succinyl-alanine-alanine-alanine p-nitroanilide (Sigma-Aldrich) [21]. The gut extract was mixed with respective substrates and incubated for 20 min at 37 °C. Then, 300 μl of 30 % acetic acid was

added and stand for 10 min. The samples were centrifuged, and absorbance was read at 410 nm using UV-visible spectrophotometer (Hitachi U-2900, Japan). One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol substrate per minute at 30 °C.

Zymogram Analysis of *H. armigera* Larval Midgut Proteases

The gut extracts, prepared from the live fourth-instar larvae reared on activated Cry1Ac-intoxicated diet with and without antibiotic cocktail, were subjected to 10 % SDS-PAGE under non-reducing conditions. After electrophoresis, the gel was treated with 2.5 % Triton X-100 for 10 min to remove the SDS. The gel was incubated with 2 % casein in 0.1 M glycine-NaOH buffer, pH 10, for 60 min, washed, and stained with Coomassie Brilliant Blue and destained [22]. Proteolytic bands will appear as clear zones against blue background.

Antioxidant Enzyme Assay in Midgut of *H. armigera* Larvae

The catalase (CAT) activity was estimated as rate of decomposition of hydrogen peroxide, and its activity was expressed in units (1U=1 mM of H_2O_2 reduced/min/mg of protein) using extinction coefficient of 0.0394/mM/cm [23]. Superoxide dismutase (SOD) activity was assayed by reduction in inhibition of rate of NBT with the superoxide anion [24]. Glutathione S-transferase (GST) activity was determined as a change in concentration of 5-(2, 4-dinitrophenyl)-glutathione, which is a conjugation product of 1-chloro-2,4-dinitrobenzene (CDNB) and the reduced glutathione [25], and its activity was expressed in units (1U=μM glutathione-CDNB conjugate formed/min/mg of protein) using extinction coefficient of 9.6/mM/cm. Lipid peroxidase activity was assayed in terms of malondialdehyde (MDA) [26]. The amount of MDA formed was calculated using a molar extinction coefficient of 1.56×10^5/M/cm.

Aminopeptidase Activity Assay in Midgut BBMVs of *H. armigera* Larvae

Brush border membrane vesicles (BBMVs) were prepared from *H. armigera* larval midgut [27]. The aminopeptidase activity in *H. armigera* BBMV was assayed using LpNA (Sigma) as substrate [20]. The BBMV was mixed with the substrate and incubated for 20 min. Then, 300 μl of 30 % acetic acid was added and centrifuged. The absorbance was read at 410 nm. One unit of enzyme activity was defined as the micromole of p-nitroaniline released per minute per milligram of gut protein.

Statistical Analysis

The data were subjected to analysis of variance using the Genstat [14th edition, Version 14.1.0.5943, VSN International Ltd, United Kingdom] software. The significance of differences between the treatments was judged by F test, while the treatment means were compared by least significant difference (LSD) at $P<0.05$. Duncan's multiple range test (DMRT) was used to know the difference between treatments. The LC_{50} values were determined by using EPA PROBIT analysis program (Version 1.5).

Results

Effect of Various Antibiotics on the Gut Microbes of *H. armigera*

The effect of different antibiotics on the growth of gut microbes of *H. armigera* is shown in Table 1. Of the seven antibiotics tested, gentamycin showed maximum inhibition (microbial growth inhibition radius 1.90 cm), followed by chloramphenicol, neomycin, amphicillin, and rifampicin (microbial growth inhibition radius 1.50, 1.40, 0.8, and 0.8 cm, respectively). Streptomycin and penicillin did not show inhibition. All the seven antibiotics were also pooled to prepare cocktail, and the cocktail was used to eliminate gut microbes from *H. armigera* and used for evaluating the interaction of gut microbes with biological activity of *Bt* toxin proteins against this pest. Antibiotic cocktail at 80 μg/ml concentration inhibited all the gut microbes on culture plates, so this concentration is sufficient to eliminate all the gut microbes from *H. armigera* larval midgut (Fig. 1).

Biological Activity of *Bt* Formulation, Activated Cry1Ac, and Transgenic and Non-Transgenic Cotton Leaves Toward *H. armigera* Larvae

Significant increase in weight loss percentage and mortality was observed in larvae reared on artificial diets with an increase in concentration of *Bt* formulation (22.6 to 68.0 and 4.9 to 60.0 %; weight: $y=219.7x+26.9$, $R^2=92.4$ %; and mortality: $y=274.4x+7.8$ and $R^2=97.4$ %) and Cry1Ac (24.6 to 72.4 % and 6.24 to 65.4 %; weight: $y=3.6x+30.6$, $R^2=87.2$ %; and mortality: $y=4.2x+14.4$ and $R^2=89.6$ %), respectively (Fig. 2a, b). Significant increase in larval weight (5.0 to 48.9 %; $y=0.5x-7.4$, $R^2=99.1$ %) and a slight decrease in mortality (5 to 0 %; $y=-0.04x+3.4$, $R^2=67.8$ %) were observed when the larvae were reared on diets containing antibiotic cocktail in a concentration-dependent manner (Fig. 3a, b). However, when the larvae were reared on diets containing increasing concentration of antibiotic cocktail and *Bt* formulation at a time, the larvae gained more weight (3.9 to 32.1 %; $y=0.3x-7.4$, $R^2=94.7$ %), and the larval mortality decreased (40 to 7 %; $y=-0.3x+38.8$, $R^2=98.9$ %). Significant increase in weight gain percentage (2.12 to 28.96 %; $y=0.3x-6.5$, $R^2=96.8$ %) and decrease in larval mortality (45 to 9 %; $y=-0.3x+42.1$, $R^2=97.5$ %) were observed when larvae reared on diet containing increasing concentration of antibiotic cocktail and activated Cry1Ac, where larvae pretreated with antibiotic cocktail. The LC_{50} for *Bt* formulation and

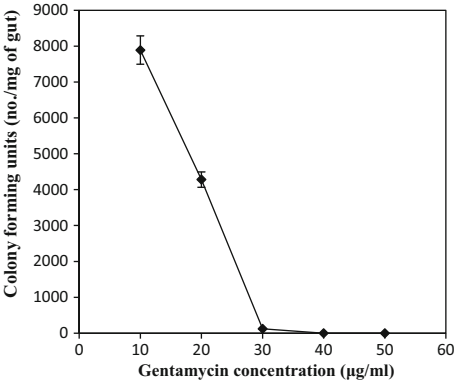

Fig. 1 Concentration-dependent inhibition of gut microbial growth in the presence of antibiotic cocktail. The fourth-instar larvae were used to isolate the gut microbes

Cry1Ac for *H. armigera* was 0.11 % and 3.32 μg/g diet, respectively. But, the LC_{50} concentration was increased in antibiotic cocktail-fed larvae, activated Cry1Ac-fed larvae (4.10 μg/g diet), and antibiotic cocktail and *Bt* formulation-fed larvae (0.17 %). The higher mortality and lower larval weights were observed in the larvae reared on transgenic cotton leaves than on non-transgenic cotton leaves. When compared to respected non-transgenic cotton, mortality rate and larval weights in transgenic cotton, JKCH (BG I), were 60 % and 5.1 mg/larva; in Siva 9 (BG II) were 70 % and 4.3 mg/larva; and in MRC 7351 (BG II) were 80 % and 2.8 mg/larva, respectively (Fig. 4). The mortality was reduced, and weights were gained in larvae when reared on antibiotic cocktail-treated cotton leaves of JKCH (35 %, 7.5 mg/larva), Siva 9 (42 %, 7.9 mg/larva), and MRC 7351(45 %, 5.2 mg/larva).

Detection of Cry1Ac Toxin Protein in *H. armigera* Larval Midgut and Fecal Matter

A 65-kDa band was detected in Cry1Ac protoxin- and *Bt* formulation-fed larval midgut extracts resembling the trypsin-activated Cry1Ac (65 kDa) toxin protein in polyacrylamide gels (Fig. 5). To confirm and quantify the activated Cry1Ac protein, we used the ELISA technique. Appearance of blue color is a positive indication for the presence of Cry1Ac. Cry1Ac protein was detected in Cry1Ac protoxin (0.30 μg/mg of gut protein) and *Bt* formulation (0.28 μg/mg of gut protein)-alone-fed larval midgut extracts, but fewer amount of Cry1Ac toxin protein was detected in larvae reared on Cry1Ac protoxin and *Bt* formulation-

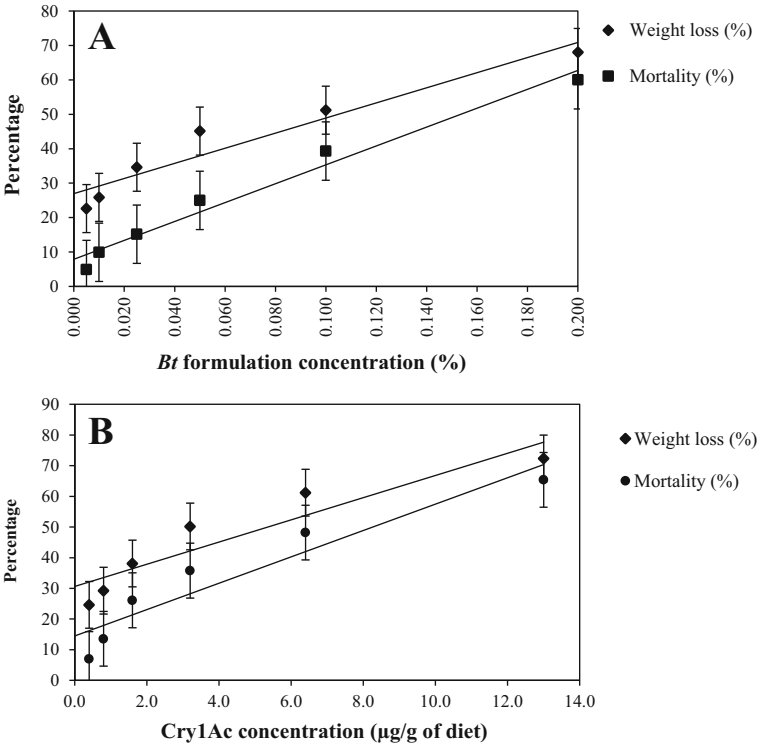

Fig. 2 Effect of *Bt* formulation (**a**) and activated Cry1Ac (**b**) on *H. armigera* larval development and survival. Early third-instar larvae were released on Bt formulation and Cry1Ac-treated diet. The data represented as mean± SE (n=3)

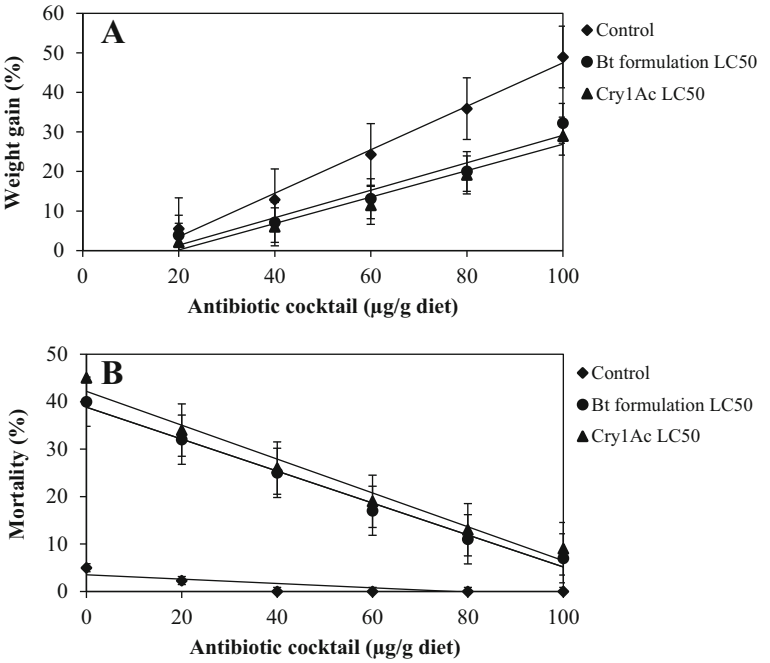

Fig. 3 Survival and development of *H. armigera* larvae on toxin *Bt* protein in the presence of antibiotic cocktail. Regression lines of percentage weight gain (**a**) and mortality (**b**) in *Bt* formulation and activated Cry1Ac-treated larvae in the presence of increasing concentration of antibiotic cocktail. Neonates were reared on artificial diet with antibiotic cocktail till second instar. The early third-instar larvae were transferred to the diet treated with antibiotic cocktail and *Bt* proteins. The data represented as mean±SE (n=3)

treated diet in the presence of antibiotic cocktail (0.20 and 0.19 μg/mg of gut protein, respectively) (Table 2). Cry1Ac was detected in the fecal matter of larvae reared on antibiotic cocktail and Cry1Ac protoxin (0.14 μg/g of fecal matter)/*Bt* (0.12 μg/g of fecal matter) formulation, but it is absent in fecal matter of the larvae reared on Cry1Ac protoxin and *Bt* formulation alone.

Activities of Midgut Proteases, Aminopeptidase, and Antioxidant Enzyme

Significant decreases in activities of total protease, trypsin, chymotrypsin, and elastase were observed in activated Cry1Ac-treated larvae (0.12 AU, 0.79, 0.28, and 0.12 U, respectively), and significant increases in total, trypsin, and chymotrypsin activities were observed in Cry1Ac protoxin (0.46 AU, 1.48, 0.62, and 0.32 U) and *Bt* formulation (0.40 AU, 1.41, 0.59, and 0.30 U)-treated larvae as compared to untreated larvae (0.30 AU, 1.26, 0.62, and 0.32 U) (Table 3). No significant decrease in protease activities was observed in larvae treated with antibiotic cocktail and activated Cry1Ac (0.24 AU, 1.16, 0.38, and 0.22 U). There was no change in protease activities in cocktail and Cry1Ac protoxin (0.33 AU, 1.18, 0.43 and 0.25 U)/*Bt* formulation (0.31 AU, 1.18, 0.44 and 0.24 U)-treated larvae as compared to the control. Aminopeptidase activity increased significantly in BBMV of *H. armigera* larvae fed on diets intoxicated with Cry1Ac protoxin and *Bt* formulation in the presence and absence of antibiotic cocktail as compared to the untreated control larvae (1.01 U) (Table 4). However, there was no significant increase in activity of aminopeptidase in BBMV of *H. armigera* larvae fed on diets with antibiotic cocktail and Cry1Ac protoxin/*Bt* formulation-fed larvae (1.72 and 1.60 U, respectively) as

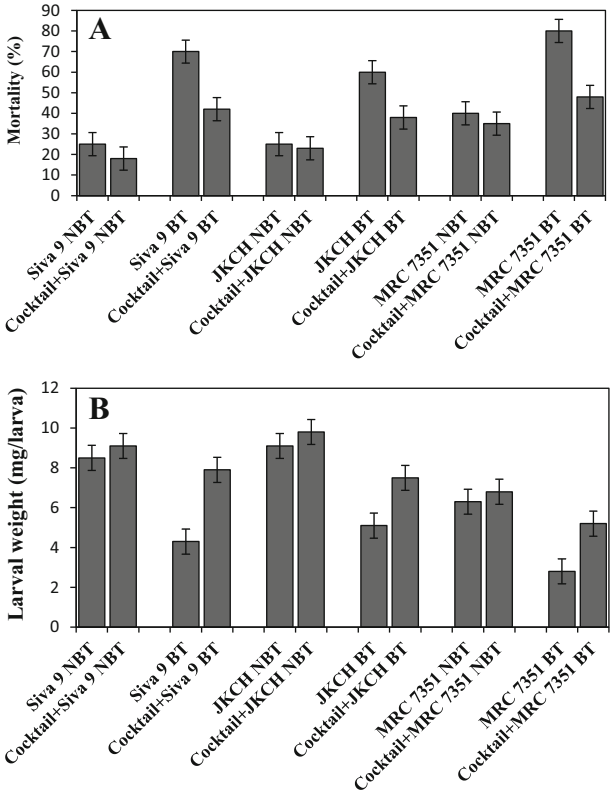

Fig. 4 Survival and development of *H. armigera* larvae on transgenic and non-transgenic cotton leaves. Percent mortality (**a**) and larval weights (**b**) were recorded on transgenic and non-transgenic cotton leaves in the presence and absence of antibiotic cocktail. The neonates of *H. armigera* larvae were reared on artificial diet with and without antibiotic cocktail till second instars, and the early third-instar larvae were transferred to transgenic and non-transgenic cotton leaves

compared larvae reared on Cry1Ac protoxin/*Bt* formulation in the absence of antibiotic cocktail (2.55 and 2.22 U). Antioxidant enzymes SOD, GST, and CAT activities and lipid peroxidation increased significantly in *H. armigera* larvae reared on diets containing Cry1Ac protoxin and *Bt*

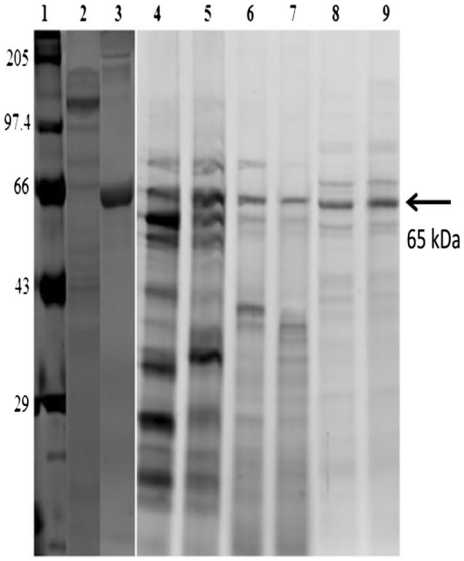

Fig. 5 Detection of Cry1Ac protein in the gut extract of *H. armigera* larvae by SDS-PAGE. Cry1Ac (65 kDa) was detected in polyacrylamide gels in gut extracts of *Bt* formulation and activated Cry1Ac-fed larvae in presence and absence of antibiotic cocktail. SDS-PAGE conditions were same for all treatments. In each well, 30 μg of protein was loaded. *Lane 1*—molecular markers, *lane 2*—Cry1Ac protoxin, *lane 3*—trypsin-activated Cry1Ac, *lane 4*—control larval gut extract, *lane 5*—antibiotic cocktail fed, *lane 6*—antibiotic cocktail + Cry1Ac protoxin fed, *lane 7*—antibiotic cocktail + *Bt* formulation fed, *lane 8*—*Bt* formulation fed, *lane 9*—Cry1Ac protoxin-fed larval gut extract

Table 2 Detection of Cry1Ac protein in the larval gut extracts and fecal matter using ELISA

Treatment	Cry1Ac (μg)/mg gut protein	Cry1Ac (μg)/g fecal matter
Artificial diet[a]	–	–
Cry1Ac protoxin[a]	0.30±0.03	–
Bt formulation[a]	0.28±0.03	–
Cocktail[b]	–	–
Cocktail + Cry1Ac protoxin[b]	0.20±0.02	0.14±0.01
Cocktail + *Bt* formulation[b]	0.19±0.02	0.12±0.03

Neonates were reared on diet without antibiotic cocktail and with antibiotic cocktail till second instar; then, early third-instar larvae were reared on respected compound-treated artificial diet till fourth instar. LC_{50} of *Bt* formulation (0.11 %), protoxin (50 μg), and antibiotic cocktail (150 μg) was used per gram of diet. The data represented as mean±SE (n=3)

[a] Without antibiotic cocktail

[b] With antibiotic cocktail

formulation individually as compared to the untreated controls (Table 5). However, there was no significant increase in the activities of SOD, GST, and lipid peroxidation when *H. armigera* larvae were reared on diets containing antibiotic cocktail and Cry1Ac protoxin/*Bt* formulation as compared to the larvae reared on Cry1Ac protoxin/*Bt* formulation without antibiotics.

Zymogram of Midgut Proteases

Zymogram analysis has shown that nine protease bands were observed in the gut extracts of untreated control larvae. However, out of nine proteases, eight proteases were observed in antibiotic

Table 3 *H. armigera* larval gut protease activities in the presence and absence of antibiotic cocktail and activated Cry1Ac or *Bt* formulation

Treatment	Total protease activity (UA)	Trypsin (U)	Chymotrypsin (U)	Elastase (U)
Artificial diet[a]	0.30±0.01 b	1.26±0.03 c	0.46±0.03 c	0.26±0.03 c
Cry1Ac protoxin[a]	0.46±0.02 c**	1.48±0.02 d**	0.62±0.04 d**	0.32±0.02 d
Bt formulation[a]	0.40±0.01 c**	1.41±0.04 d**	0.59±0.02 d**	0.30±0.01 d
Activated Cry1Ac[a]	0.12±0.01 a**	0.79±0.05 a**	0.28±0.05 a**	0.12±0.02 a**
Cocktail[b]	0.26±0.03 b	1.20±0.02 c	0.40±0.01 c	0.24±0.02 c
Cocktail + Cry1Ac protoxin[b]	0.33±0.01 b	1.18±0.03 c	0.43±0.03 c	0.25±0.03 c
Cocktail + *Bt* formulation[b]	0.31±0.02 b	1.18±0.02 c	0.44±0.03 c	0.24±0.02 c
Cocktail + activated Cry1Ac[b]	0.24±0.02 b	1.16±0.02 b	0.38±0.04 b	0.22±0.02 b

Neonates were reared on diet without antibiotic cocktail and with antibiotic cocktail till second instar; then, early third-instar larvae were reared on respected compound-treated artificial diet till fourth instar. The fourth-instar larvae were used to assess the protease activities. LC_{50} of activated Cry1Ac (3.32 μg), *Bt* formulation (0.11 %), protoxin (50 μg), and antibiotic cocktail (150 μg) was used per gram of diet. The data represented as mean±SE (n=3). Values with different letters in the columns are significantly different

**Significantly different from control at $p \leq 0.01$

[a] Without antibiotic cocktail

[b] With antibiotic cocktail

Table 4 Aminopeptidase activity in *H. armigera* larval gut BBMV

Treatment	Aminopeptidase activity (U)
Artificial diet[a]	1.01±0.03 a
Cry1Ac protoxin[a]	2.55±0.03 c**
Bt formulation[a]	2.22±0.04 c**
Cocktail + Cry1Ac protoxin[b]	1.72±0.02 b**
Cocktail + *Bt* formulation[b]	1.60±0.01 b**

Neonates were reared on diet without antibiotic cocktail and with antibiotic cocktail till second instar; then, early third-instar larvae were reared on respected compound-treated artificial diet till fourth instar. The live fourth-instar larvae were used to prepare the brush border membrane vesicles. *Bt* formulation (0.11 %), protoxin (50 μg), and antibiotic cocktail (150 μg) were used per gram of diet. The data represented as mean±SE (n=3). Values with different letters in the columns are significantly different

**Significantly different from control at $p \leq 0.01$

[a] Without antibiotic cocktail

[b] With antibiotic cocktail

cocktail-treated larvae (P5 band was inhibited); four proteases were observed in activated Cry1Ac-treated larvae (P1, P2, P5, P8, and P9 bands were inhibited) (Fig. 6). But, eight proteases were observed in larvae reared on both antibiotic cocktail and activated Cry1Ac (P8 was inhibited).

Discussion

The 130-kDa Cry1Ac δ-endotoxins undergo proteolysis in the alkaline environment of insect midgut at both the C and N termini to produce a toxic moiety that has a molecular mass of approximately 65-kDa, which binds to specific receptors in the insect midgut epithelium leading to

Table 5 Effect of Cry1Ac protoxin and *Bt* formulation in *H. armigera* larval SOD, GST, CAT activities and lipid peroxidation in presence and absence of antibiotic cocktail

Treatment	U/mg midgut protein			Lipid peroxidation (μM of MDA /min/ mg gut protein)
	SOD	GST	Catalase	
Artificial diet[a]	23.30±0.57 a	12.21±0.19 a	10.12±0.20 a	0.37±0.03 a
Cry1Ac protoxin[a]	42.60±0.85 c**	27.73±0.28 d**	22.08±0.28 d**	1.31±0.04 c**
Bt formulation[a]	38.20±0.53 d**	19.29±0.11 c**	21.63±0.16 c**	1.45±0.04 d**
Cocktail[b]	24.37±0.42 a	14.13±0.46 a	11.03±0.26 a	0.39±0.01 a
Cocktail + Cry1Ac protoxin[b]	29.99±0.28 b**	17.95±0.47 b	16.58±0.23 b	0.55±0.01 b**
Cocktail + *Bt* formulation[b]	28.45±0.32 b**	17.55±0.49 b	16.01±0.17 b	0.52±0.04 b**

Neonates were reared on diet without antibiotic cocktail and with antibiotic cocktail till second instar; then, early third-instar larvae were reared on respected compound-treated artificial diet till fourth instar. The fourth-instar larvae were used to assess the antioxidant enzyme activities. Cry1Ac protoxin (50 μg), *Bt* formulation (0.11 %), and antibiotic cocktail (150 μg) were used per milliliter of diet. The data represents mean±SE (n=3). Values with different letters in the columns are significantly different

**Significantly different from control at $p \leq 0.01$

[a] Without antibiotic cocktail

[b] With antibiotic cocktail

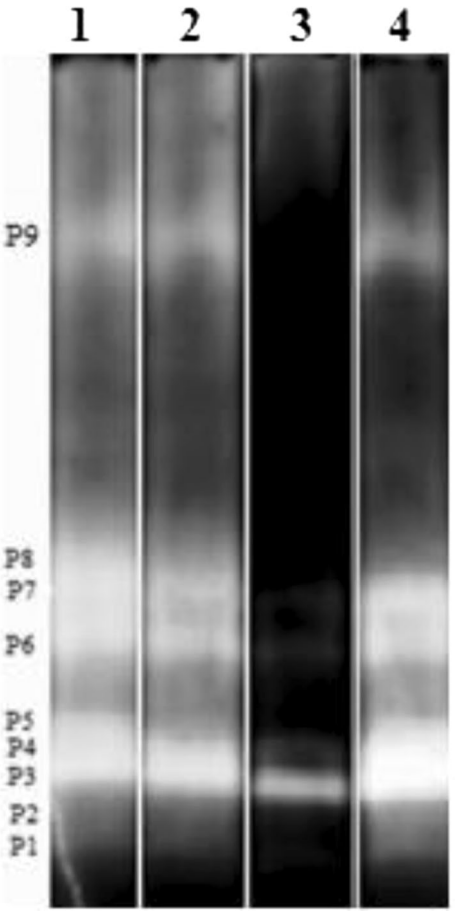

Fig. 6 Zymogram analysis of midgut proteases of *H. armigera*. Midgut proteases were determined by 10 % SDS-PAGE at constant voltage in activated Cry1Ac-fed larvae in presence and absence of antibiotic cocktail. Gels of all treatments were treated with 2 % casein for 60 min and then stained with CBB. In each well, 30 μg of protein was loaded for all treatments. Clear zones were identified as proteolytic bands. *Lane 1*—control, *lane 2*—antibiotic cocktail fed, *lane 3*—activated Cry1Ac fed, *lane 4*—antibiotic cocktail + Cry1Ac fed larval gut extract. *P* protease band

the pore formation and eventual death of the target insects. Understanding the basis of resistance to Cry1Ac in insects has been associated with the changes in cadherin gene, a receptor protein for Cry1Ac, and reduced binding of the toxin to the midgut epithelium [28, 29], variations in the expression pattern of proteases [30, 31], improper processing of protoxin [32], lack of major gut protease involved in the toxin activation [33], existence of an esterase-mediated resistance [34], and the presence of midgut microbes [11]. In the present study, *H. armigera* larvae were exposed to activated Cry1Ac and *Bt* formulation in the presence or absence of antibiotics, which used to eliminate midgut microbes, to assess the role of gut microbes in *Bt* biological activity. The various antibiotics tested for eliminating the gut microbes and antibiotic cocktail at 80 μg/ml concentration were most effective and inhibited the complete growth of insect gut microbes; hence, cocktail at 150 μg/g diet concentration was used for the complete elimination of gut microbes throughout the experiment. The larvae fed on diets with antibiotics were killed after the completion of experiment. Fresh culture of *H. armigera* neonates was used in each experiment, and thus, there was no possibility of development of resistance in gut microbes to the antibiotics. The *Bt* formulation and activated Cry1Ac toxin resulted in weight loss and mortality of *H. armigera* larvae in a dose-dependent manner. Active Cry toxin via a complex mode of action results in the cytolytic pores in the midgut epithelium and causes death of insects, whereas the weight loss in the survived larvae

was due to the inhibition of proteases and improper digestion of the ingested food. However, exposure of *H. armigera* larvae to *Bt* formulation and Cry1Ac when pretreated with antibiotic cocktail resulted in weight gain and reduced larval mortality. Similar trends on *Bt* toxin-induced larval mortality in the presence of antibiotics have been reported earlier in *L. dispar* [11, 12, 35]. Survival of *H. armigera* larvae fed on diets with *Bt* proteins was directly proportional to the concentration of antibiotics in the artificial diet [11, 36]. It was observed that the larvae gain weight when fed on diet containing antibiotic cocktail, and similar trend observed in *L. dispar* [10] and *M. sexta* raised on antibiotics attained more weight than insects reared on regular diet [37]. The increase in larval weight was probably due to elimination of microbes resulting in nutrient deficit, so that food intake was more to fulfill the nutrient loss. The reduced mortality in larvae fed on diets containing *Bt* toxins and antibiotics was due to the lesser binding of activated Cry1Ac toxin to the receptors in the midgut epithelium. The results suggested the role of gut microbes in biological activity of *Bt* toxins. Similar observations were reported earlier that addition of antibiotics in artificial diet decreased the toxicity of Cry toxins to the Lepidopteran larvae [38]. However, some reports have also suggested that purified *Bt* toxin was pathogenic to *P. xylostella* and *M. sexta* larvae reared aseptically [13, 14]. The Cry1Ac toxin was detected in the midgut extract of larvae fed on diet containing Cry1Ac protoxin and *Bt* formulation. But, when we quantified the formed activated Cry1Ac protein, fewer amounts were found in the larvae fed on *Bt* toxin proteins pretreated with antibiotic cocktail; this may be because of the absence of gut microbial proteases which are involved in the conversion of protoxin to active Cry1Ac toxin. But, when antibiotic cocktail was incubated with solution containing gut extract and Cry1Ac protoxin in vitro, there was no decrease in the quantity of activated Cry1Ac protein (results were not shown), indicating that there was no direct effect of antibiotics on protoxin-toxin conversion.

The protease zymogram showed that five proteases were inhibited by activated Cry1Ac, but only one protease was inhibited in the presence of antibiotics. *Bt* formulation and Cry1Ac protoxin increase the protease activities, indicating that more proteases were involved in the proteolytic conversion of protoxin to active toxin. But, activated Cry1Ac decreased the protease activity. The midgut proteases of *Bt*-resistant *P. xylostella* population exhibited lower specific activity as compared with midgut proteases of susceptible population [39]. Luminal gut extracts from the Cry1Ab-resistant *Ostrinia nubilalis* colony exhibited reduced hydrolysis of azocasein compared with the susceptible colony [40]. The protease activities were increased in Cry1Ac protoxin-treated *H. armigera* [20]. However, the increase in protease activities was not observed when the larvae were fed on *Bt* protoxins in combination of antibiotic cocktail, indicating that gut microbial proteases are also involved in the activation of protoxin to active toxin. The protease inhibition was not significant in both antibiotics and activated Cry1Ac-treated larvae, indicating that only *H. armigera* proteases were inhibited but not gut microbial proteases in which the microbes were eliminated. Changes in the activity of the gut proteases responsible for toxin activation could lead to reduced susceptibility to *Bt* toxins.

Increased levels of SOD, GST, and CAT activities and lipid peroxidation were observed in the larvae reared on diets containing Cry1Ac protoxin and *Bt* formulation as compared to the untreated controls, indicating that the oxidative stress is increased in the presence of *Bt* toxin proteins. Increase in oxidative stress includes the formation of free radicals, and antioxidant enzymes were involved in clearing the free radicals, so the activity increased. *Bt* infection increased the level of oxidative stress in the *Galleria mellonella* larval midgut [41, 42], and higher SOD activities were observed in *Bt*-fed *Spodoptera exigua* than in non-*Bt* fed individuals [43]. However, there was not that much increase in activities of antioxidant in both antibiotic- and *Bt* toxin-fed larvae, indicating that, in microbes, eliminated *H. armigera* larvae *Bt* did not induced oxidative stress.

The toxicity of activated Cry1Ac is through binding to receptors on the midgut epithelium. To determine the binding of activated Cry1Ac protein to the larval midgut BBMVs, aminopeptidase activity was determined in *Bt* toxin-fed larvae in presence and absence of antibiotic cocktail, since aminopeptidase has been identified as a *Bt* toxin receptor in the midgut epithelium of *H. armigera* larvae [44]. Active Cry1Ac toxin, formed from protoxin by larval gut proteases, binds to the receptor aminopeptidase on BBMVs and increased the aminopeptidase activity. δ-Endotoxin by itself does not have aminopeptidase activity. The δ-endotoxin which binds to the receptor enhances the aminopeptidase activity, and inactive toxin did not show any increase in aminopeptidase activity [45]. In antibiotic cocktail and *Bt* protoxin-fed larvae, no such significant increase in the activity of aminopeptidase was observed, indicating that gut microbes were involved. When antibiotic cocktail incubated with the *Bt* protoxins treated larval gut extract in vitro, there was no change in the aminopeptidase activity (results not shown), indicating that antibiotic cocktail is not directly altering the binding efficiency of activated Cry1Ac to the receptors. Cry1Ac protein was detected in the fecal matter of larvae fed on toxin protein in the presence of antibiotics and *Bt* toxin, which may be due to lesser binding of toxin protein to the receptors on BBMVs. The work should be carried out on how the elimination of gut microbes influences the aminopeptidase activity and binding of Cry1Ac to its receptors on BBMVs. In our study, we observed that the toxicity of Cry1Ac was by inhibition of proteases, increased oxidative stress, and increased toxin binding to its receptor. However, as a result of elimination of gut microbes from *H. armigera* by antibiotics, both *Bt* formulation and the activated Cry1Ac resulted in reduced mortality and increased larval weights. Earlier studies argued that the reduced mortality in rifampicin-treated gypsy moth, *L. dispar*, larvae was due to direct effects of antibiotic and that midgut microbes were not required for the pathogenicity of *Bt* [14], but in our study, there was no direct effect of antibiotics on the *Bt* toxin biological activity. It has been proposed that *Entercoccus* spp. is a commensal in the gut and a pathogen in hemocoel. In presence of toxins like *Bt*, *Enterococcus faecalis* is translocated from gut to hemolymph inducing sepsis in *M. sexta* [46], thus demonstrating the role of gut microbes in *Bt* pathogenicity. We are continuing the studies on isolation and characterization of midgut microbes responsible for potentiation and/or detoxification of *Bt* toxin. Protease-producing midgut bacterial species belong to *Bacillus* sp., and the studies on their role in potentiation and/or detoxification of *Bt* toxins are under progress. Composition of gut microbes varies across crop host plants and locations [10]; however, there are some bacterial strains which are common in most of the populations of *H. armigera*. Information on bacterial strains involved in potentiation and/or detoxification of the *Bt* toxins will be useful globally to develop strategies to delay the development of resistance in the insect pests to *Bt* transgenic crops.

Conclusions

Midgut microbes influence the biological activity of *Bt* toxins against the larvae of *H. armigera*. Bioefficacy of *Bt* toxins against insect pests depends on the crop host and the bacterial communities residing in the midgut of insect pests. A better understanding of the role of midgut microbes in potentiation/ detoxification of *Bt* toxins will be useful to develop strategies to minimize the risk of development of insect resistance to *Bt* transgenic crops for sustainable crop production.

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