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Influence of midgut bacteria on toxicity of *Bacillus thuringiensis* to pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

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Abstract
Gut bacteria play pivotal role in many aspects of insects' growth and development. They are generally beneficial but, can turn out to be harmful under certain conditions. Synergism/potentiation of *Bacillus thuringiensis* (Bt) efficacy in many insect species is one such instance, where it is harmful to insect but is of immense value for their management using Bt toxins directly or indirectly. In the present study, commercial formulation of Bt (DiPel®) and Cry1Ac and Cry2Ab toxins were evaluated using five concentrations against five-day old larvae of *Pectinophora gossypiella* with and without antibiotics (which removed the gut bacteria) by diet incorporation method. Lowest median lethal concentration (LC_{50}) was recorded in Cry2Ab (1.49 µg/gm of diet) followed by Cry1Ac (2.54 µg/gm of diet) and DiPel® (3.63 µg/gm of diet) without the antibiotics (AB). Whereas, highest LC_{50} (9.38 µg/gm of diet) was recorded with DiPel® + AB (chloramphenicol and tetracycline at 50 µg/gm of diet each) followed by Cry1Ac + AB (4.73 µg/gm of diet) and Cry2Ab + AB (3.66 µg/gm of diet). These results showed that DiPel® as well as both the Cry toxins along with antibiotics were less toxic to larvae of *P. gossypiella* thus indicates that gut bacteria potentially affect the susceptibility to *Bacillus thuringiensis*.

Keywords: *Pectinophora gossypiella,* midgut bacteria, symbiotic interactions, antibiotic sensitivity, Bt toxicity

Introduction
Cotton transformed with insecticidal *cry* gene(s), isolated from soil bacterium, *Bacillus thuringiensis* (Bt) is known as Bt cotton. It is an environment friendly alternative to conventional insecticides [1]. In India, since 2002 cultivation of Bt cotton expressing Cry1Ac gene (BG I) has led to the control of bollworm complex *viz.,* American bollworm *Helicoverpa armigera*, spotted bollworm *Earias vitella* and pink bollworm (PBW) *Pectinophora gosypiella* successfully till 2009. In 2010, Monsanto reported "unusual survival of pink bollworm" on Bt cotton producing Cry1Ac during 2009 and confirmed PBW resistance to Cry1Ac in four districts of Gujarat followed by Dhurua and Gujar [2]. To retain the sustainability of Bt cotton for bollworm control, second generation Bt cotton (BG II) expressing two Cry toxins (Cry1Ac + Cry2Ab) was introduced in 2006 in India. Earlier PBW presumed secondary importance as *H. armigera* has been the main cause of concern for the wide spread sub-continental epidemics in cotton and several other crops. To delay or counter resistance to first generation Bt cotton (BG I), farmers have swapped to BG II cotton hybrids producing two Bt toxins (Cry1Ac + Cry2Ab). Now, PBW is an endemic pest of Bt cotton in the South and Central India as it has developed resistance to BG II cotton also [3-5].
In many insect pests, gut bacteria play a major role in Bt toxicity [6-9] and this can vary with insect species [10-11]. Shift in bacterial structure or composition in insect gut [12-13] and production of xenobiotic degrading enzymes by the gut bacteria [13-15] can lead to resistance development. Present study was carried out with an aim to know the role of gut bacteria in Bt toxicity in an economic pest of cotton, *P. gossypiella*.

Materials and methods
Isolation of midgut bacteria of the pink bollworm larvae
Full grown larvae of *P. gossypiella* were collected from infested bolls of cotton belonging to eight different locations of India *viz*., Telangana [Adilabad (19.68° N 78.53° E),

Warangal (17.79º N 79.79º E)], Andhra Pradesh [Guntur (16.47º N 79.43º E), Kurnool (15.73º N 77.43º E)], Karnataka [Raichur (16.21º N 77.34º E)], Maharashtra [(Aurangabad (19.87º N 75.34º E)], Gujarat [Dantiwada (24.49º N 72.34º E)], Madhya Pradesh [Khandwa (21.83º N 76.38º E)]. Collected larvae were starved for two hours; surface sterilized with 70% ethanol followed by rinsing with sterile distilled water and gentle wiping with tissue paper. These larvae were dissected under binocular in 1x phosphate buffered saline (pH 7.4) under sterilized condition. Guts of 5-10 larvae were removed and macerated in 200µl of autoclaved double distilled water. This was replicated thrice and 10µl of suspension thus made was plated on Luria-agar plates followed by incubation at 30°C for 24 hours. Thirty seven culturable midgut bacteria were isolated from pink bollworm populations and identified based on 16S rRNA gene.

Rearing of test insect

Pink bollworm infested cotton bolls were collected from infested cotton fields of Dantiwada (24.49º N 72.34º E) Gujarat, Central India and brought to the laboratory. Larvae were separated from cotton bolls and transferred to semi-synthetic diet and reared till pupation. The adult emerged were transferred to mating jars (15 cm diameter × 20 cm height), and kept at 27±1 °C, 65±5% RH and 14L: 10D photoperiod. Adults were provided with 10% honey solution fortified with 1-2 drops of multivitamin mixture in a cotton swab as food. Tender cotton twigs washed with water and air dried were placed inside mating jars to facilitate the egg laying. Cotton twigs were replaced after every two days, and the cotton twigs with eggs were transferred to the plastic jars separately. Newly hatched single larvae were transferred to the plastic container (2 cm diameter × 1 cm height) having small cubes of semi-synthetic diet. These larvae were used for further experiments.

Antibiotic sensitivity assay against pink bollworm gut microbes: *in vitro* assays

The sensitivity of isolated bacterial colonies towards different antibiotics was studied by disc diffusion assay. Antibiotic octa-disc containing ampicillin, tetracycline, gentamycin, kanamycin, co-trimoxazole, amikacin, streptomycin, and chloramphenicol with concentrations as per manufacturer's protocol (HIMEDIA) were placed on the plates inoculated with bacteria and allowed to incubate at 30 °C for 24 hours. Observation on zone of inhibition was recorded and effective antibiotics were selected for *in vivo* assay.

***In vivo* screening of potential antibiotics**

In vivo assay was carried out using semi-synthetic diet amended with antibiotics with two different concentrations *i.e*., 100 and 50µg each of chloramphenicol and tetracycline per gram of diet. No antibiotic was mixed in control. Five day old pink bollworm larvae were used for bioassay and three replicates each of 10 larvae were maintained for each treatment. Observations on developmental stage of insect were recorded after 16 days. Five larvae were randomly picked and their midguts were excised, homogenised and plated to check the presence of gut bacteria. Dose which cleared off the gut bacteria without affecting the physiology of *P. gossypiella* was selected for further study.

Effect of antibiotics on the toxicity of Bt-formulation and Cry1Ac and Cry2Ab toxins towards pink bollworm

Toxins, Cry1Ac (19.7% MVPII liquid formulation) and Cry2Ab (6 mg toxin/gm corn leaf powder) were obtained from Monsanto Research Centre, Bangalore. The Cry1Ac, Cry2Ab toxins and commercial formulation of Bt (DiPel®) were evaluated alone as well as along with two antibiotics (a mixture of chloramphenicol and tetracycline @ 50 µg/gm of diet each) at five different concentrations *viz*., 0.1, 0.5, 1.0, 2.0, 5.0 µg/gm of diet against five-day-old larvae of pink bollworm by diet incorporation method. The measured quantity of semi-synthetic diet was prepared, poured into beakers and kept in the water bath at 50-60°C, and pre-weighed toxins were added and mixed thoroughly. After cooling, approximately 5 gm of diet cube was placed in each plastic container (2 cm dia × 1 cm height). The diet without toxin/antibiotic was used as control. Single five-day-old larvae were transferred on to the respective toxin-incorporated and toxin + antibiotic incorporated diets with the help of fine camel hairbrush. Thirty larvae were used per concentration and minimum of 180 larvae were used for each bioassay. All the assays were conducted under controlled conditions at 27 ± 1 °C and 60-70% RH. Observations on mortality and developmental stage of survivors were recorded after 16 days. Larvae developing into 3rd instar (light pinkish colour) were considered as live; younger larvae, < 3rd instar were recorded as dead [2]. The LC_{50} values were calculated based on the mortality data on DiPel®, Cry1Ac, Cry2Ab and DiPel® + mixture of antibiotics, Cry1Ac + mixture of antibiotics and Cry2Ab + mixture of antibiotics by using Maximum Likelihood Programme [16].

Results

***In vitro* screening of antibiotics**

Zone of inhibition reflects the area around the antibiotic disc where bacterial growth is inhibited. Higher values represent greater sensitivity and vice versa. Perusal of data in Table 1 and Fig. 1 showed that two antibiotics *i.e*., chloramphenicol and tetracycline showed 100% sensitivity to all the bacterial colonies followed by amikacin (97.3%) and gentamicin (94.6%). Ampicillin and co-trimoxazole were effective up to 60% whereas, streptomycin was only 43% effective in inhibiting the growth of culturable gut bacteria of *P. gossypiella*. Antibiotics with 100% efficacy were mixed to make a cocktail and used for clearing off the gut bacteria *in vivo*.

***In vivo* screening of potential antibiotics**

Antibiotics, chloramphenicol and tetracycline were tested at two concentrations *viz*., cocktail of 50µg of each antibiotic and 100µg of each antibiotic to choose the dose which removes the gut bacteria without hampering the physiology of *P. gossypiella*. Both the concentrations managed to clear off the gut bacteria. However, higher dose (100µg) produced abnormalities in the growth stages of the insect. Larval growth was slowed in 100µg compared to 50µg and control. Higher dose resulted in malformed pupae and hindrance to adult emergence from the pupae resulting in malformed adults (Fig.2). Hence, cocktail of 50µg of each antibiotic was used for further *in vivo* assay.

Impact of gut bacteria on the efficacy of *Bacillus thuringiensis* in *Pectinophora gossypiella*

Preliminary single concentration (at 1.0 µg/g of diet) assays were conducted to evaluate the role of gut bacteria on the efficacy of Bt formulation and Cry toxins. Commercial formulation of Bt (DiPel®) and the pure Cry1Ac and Cry2Ab toxins were tested alone @ 1.0 µg/g of diet and along with the antibiotic cocktail (chloramphenicol and tetracycline at 50

µg/gm of diet each). Perusal of the data in Fig. 3 showed that, mortality percentage was less in treatments where antibiotics were used along with toxin (20-33%) whereas, comparatively higher mortality (30-47%) was observed in treatments where toxin alone was used (gut bacteria intact). Further, bioassays were carried out with different concentration of toxins alone and with antibiotics to know the role of gut bacteria in Bt efficacy.

Perusal of data in Table 2 showed that lowest median lethal concentration (LC_{50}) was recorded in Cry2Ab (1.49 µg/gm of diet) followed by Cry1Ac (2.54 µg/gm of diet) and DiPel® (3.63 µg/gm of diet) without the antibiotics (AB). Whereas, highest LC_{50} (9.38 µg/gm of diet) was recorded with DiPel® + AB (chloramphenicol and tetracycline at 50 µg/gm of diet each) followed by Cry1Ac + AB (4.73 µg/gm of diet) and Cry2Ab + AB (3.66 µg/gm of diet). There was difference in LC_{50} between the treatments of toxin alone and toxin + antibiotic mixture. However, statistically they were at par, as their fiducial limits were overlapping. Efficacy Ratio (LC_{50} of toxin + antibiotic/ LC_{50} of toxin alone) showed that LC_{50} of DiPel® + antibiotic increased by 2.58 fold from DiPel® alone followed by 2.45 in Cry2Ab and 1.86 fold in case of Cry1Ac.

Table 1: *In vitro* antibiotic susceptibility of midgut bacteria isolated from last instar larvae of *Pectinophora gossypiella* in terms of zone of inhibition (diameter in cm)

Sl. No	Bacterial Strain	Bacterial ID	Antibiotic : Zone of inhibition (cm)							
			AMP	TE	GEN	K	COT	AK	S	C
1	AdF1	*Ochrobactrum pseudogrignonense*	0.8	3.0	1.8	0.0	0.0	3.0	1.9	2.0
2	AdF2	*Paenochrobactrum glaciei*	2.0	3.0	2.1	1.5	0.0	1.8	1.7	2.0
3	AdM1	*Enterococcus casseliflavus*	0.0	3.0	2.0	2.0	3.0	1.6	1.5	1.5
4	AdM2	*Enterobacter bugandensis*	3.0	3.0	2.0	3.0	3.0	1.2	0.0	3.0
5	AdM3	*Enterococcus casseliflavus*	3.0	3.0	2.2	2.2	1.0	3.0	1.5	3.0
6	AdM4	*Bacillus* sp.	0.0	3.0	2.0	0.0	0.0	2.0	1.8	2.2
7	AuF	*Enterobacter hormaechei* subsp. *xiangfangensis*	3.0	3.0	2.5	2.1	0.0	0.0	1.0	1.6
8	AuM1	*Staphylococcus sciuri*	1.0	3.3	1.6	0.0	2.0	2.5	1.8	2.5
9	AuM2	*Staphylococcus sciuri*	3.0	3.6	0.0	0.0	0.0	2.5	2.0	2.0
10	AuM3	*Staphylococcus sciuri*	1.0	3.1	2.1	1.0	1.7	2.2	1.25	1.5
11	GF1	*Bacillus* sp.	0.0	3.3	2.5	1.6	1.2	2.3	0.0	1.5
12	GF2	*Enterococcus casseliflavus*	1.45	3.5	2.1	1.5	0.0	1.7	0.0	3.0
13	GM1	*Bacillus cereus*	0.0	3.2	2.2	1.3	1.0	2.4	0.0	1.5
14	GM2	*Pantoea dispersa*	1.2	3.8	2.5	1.2	1.1	2.1	0.0	2.0
15	GM3	*Staphylococcus gallinarum*	1.6	3.4	2.6	2.2	1.5	2.5	0.0	2.4
16	KF1	*Enterobacter cloacae* subsp. *dissolvens*	0.0	3.3	2.1	2.5	2.0	3.0	0.0	3.0
17	KF2	*Enterobacter cloacae* subsp. *dissolvens*	0.0	3.3	2.3	1.5	1.8	2.2	0.0	2.3
18	KM1	*Bacillus* sp.	0.0	3.0	1.8	1.2	0.0	2.2	0.0	2.0
19	KM2	*Staphylococcus sciuri*	1.05	3.5	2.0	1.8	1.5	2.0	0.0	2.6
20	KM3	*Enterobacter hormaechei* subsp. *xiangfangensis*	3.0	3.0	3.0	1.6	1.2	2.1	0.0	3.1
21	WF1	*Enterobacter hormaechei* subsp. *xiangfangensis*	1.2	3.0	2.3	2.1	1.2	2.2	0.0	2.3
22	WF2	*Serratia rubidaea*	0.0	3.0	2.1	2.1	1.6	2.1	0.0	2.2
23	WM1	*Citrobacter gillenii*	2.0	3.0	3.0	2.2	1.3	3.1	0.0	2.7
24	WM2	*Enterobacter hormaechei* subsp. *oharae*	1.5	3.4	2.2	2.2	1.6	2.2	0.0	3.2
25	DF	*Enterobacter hormaechei* subsp. *xiangfangensis*	1.7	2.8	2.0	2.0	0.0	1.6	0.0	1.9
26	DM1	*Pantoea dispersa*	3.0	3.0	2.4	2.2	1.0	1.6	0.0	2.8
27	DM2	*Bacillus cereus*	3.0	3.0	2.0	0.0	0.0	1.5	1.5	2.0
28	RF1	*Bacillus cereus*	1.5	3.0	2.2	0.0	2.4	1.9	1.0	2.2
29	RF2	*Klebsiella quasivariicola*	0.0	3.4	2.5	1.9	0.0	1.6	1.0	2.5
30	RM	*Klebsiella quasivariicola*	0.6	3.4	2.2	1.4	0.8	2.0	1.2	3.0
31	KhF1	*Burkholderia contaminans*	2.8	2.6	3.2	1.2	2.2	2.7	0.0	1.0
32	KhF2	*Bacillus* sp.	0.0	2.4	2.0	2.0	0.0	2.6	0.0	2.1
33	KhF3	*Brevundimonas aurantiaca*	1.0	3.0	3.0	2.5	2.5	2.8	0.0	1.0
34	KhF4	*Cupriavidus nantongensis*	0.0	2.6	0.0	0.0	1.7	1.0	0.0	1.9
35	KhM1	*Bacillus safensis* subsp. *safensis*	3.0	3.0	2.6	2.0	0.0	2.6	2.2	0.8
36	KhM2	*Bacillus* sp.	0.0	1.6	1.2	1.2	0.0	2.2	1.8	2.0
37	KhM3	*Ralstonia mannitolilytica*	1.2	2.6	2.6	1.8	0.0	3.4	2.6	2.4

Note: AMP - Ampicillin, TE - Tetracycline, GEN - Gentamicin, K - Kanamycin, COT - Co- Trimoxazole, AK - Amikacin, S - Streptomycin, C – Chloramphenicol

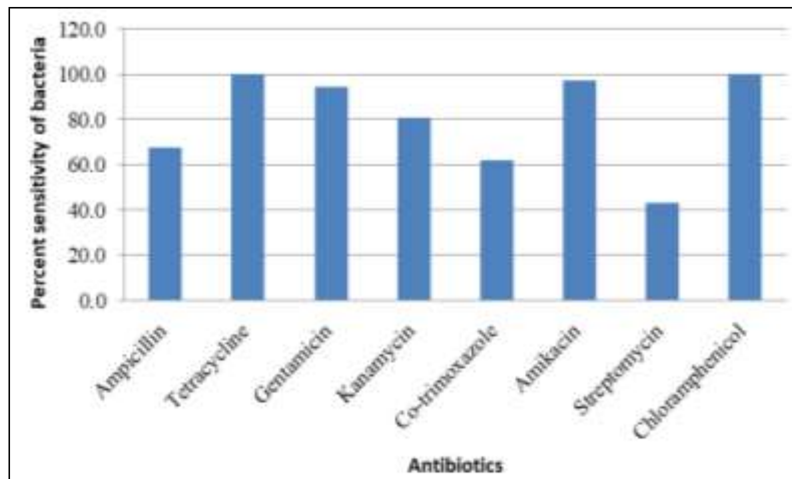

Fig 1: Sensitivity of culturable midgut bacteria of *Pectinophora gossypiella* larvae to different antibiotics

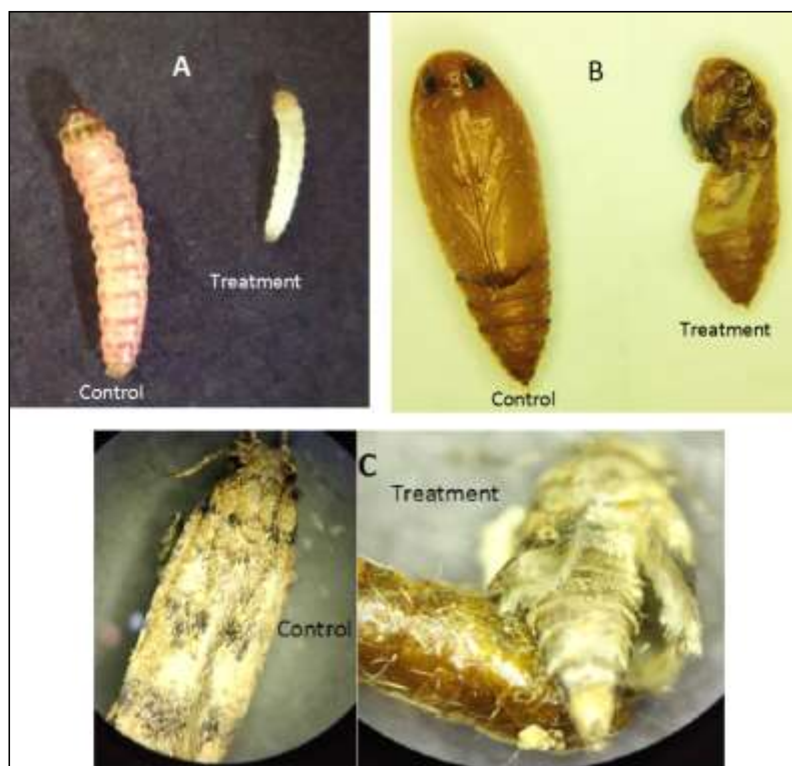

Fig 2: Abnormalities observed in different stages of *Pectinophora gossypiella* (A. larval B. pupal and C. adult stage) when five day old larvae were exposed to mixture of antibiotics (at 100µg of chloramphenicol and tetracycline each per gram of diet) for clearing off the gut bacteria.

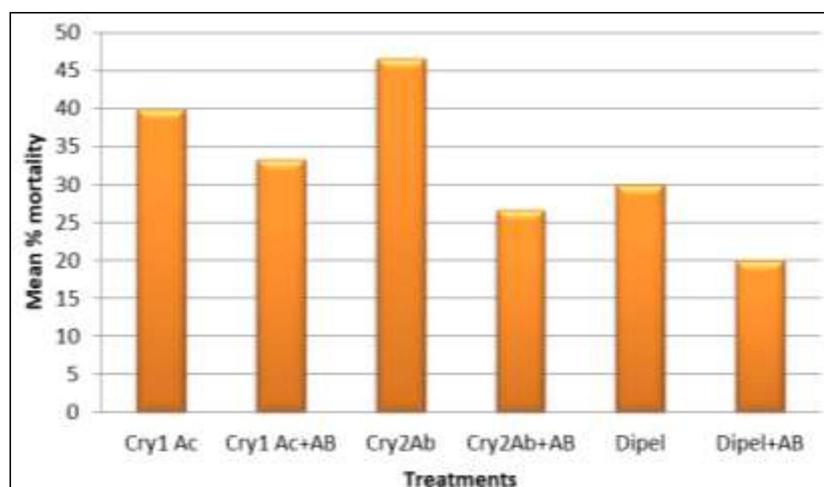

Fig 3: Efficacy of different treatments against five day old larvae of *Pectinophora gossypiella* (AB: Mixture of chloramphenicol and tetracycline antibiotics each at 50µg/g diet).

Table 2: Median lethal concentration of Cry toxin alone and in combination with antibiotics against five day old larvae of *Pectinophora gossypiella*

Treatment	LC_{50} µg/gm of diet	Fiducial limits		Slope ± SE	χ^2	DF	Efficacy Ratio*
		Lower	Upper				
Cry1Ac	2.54	1.22	12.95	0.66 ± 0.19	0.591	3	1.86
Cry1Ac+AB	4.73	2.14	39.99	0.72 ± 0.20	0.628	3	
Cry2Ab	1.49	0.75	4.18	0.71 ± 0.19	0.878	3	2.45
Cry2Ab+AB	3.66	2.00	12.31	0.93 ± 0.21	0.969	3	
DiPel®	3.63	1.79	18.36	0.76 ± 0.20	2.495	3	2.58
DiPel®+AB	9.38	3.86	132.19	0.83 ± 0.23	1.002	3	

AB: Mixture of chloramphenicol and tetracycline antibiotics each @ 50µg/gm of diet
* Efficacy Ratio = LC_{50} of toxin + antibiotic/ LC_{50} of toxin alone

Discussion

Microbiota residing in the gut plays various beneficial roles in insects. They are the hidden players in many aspects such as, thriving on suboptimal diets [17], digesting the indigestible [18], protection from pathogens, parasites and predators [19-20], detoxification and resistance to pesticides and other chemicals [21]. In present study, role of gut bacteria in Bt efficacy was studied in *P. gossypiella* by removing the midgut bacteria using antibiotics. Among the eight antibiotics tested, chloramphenicol and tetracycline found to be effective against all the gut bacteria isolated from different populations of PBW thus exhibited 100% efficacy in controlling the growth of all the gut bacteria *in vitro*. Amikacin and gentamicin showed 97.3 and 94.6% efficacy, respectively. Ampicillin and co-trimoxazole were effective up to 60% whereas, streptomycin was only 43% effective in inhibiting the growth of culturable gut bacteria. Similarly, gentamicin, chloramphenicol and neomycin managed to inhibit the growth of gut bacteria of *Helicoverpa armigera in vitro* [9]. Antibiotics; rifampin and streptomycin at the rate of 100µg/ml reduced the gut microbiota of *Manduca sexta* below detectable limits [11]. In *Plutella xylostella*, rifampicin and streptomycin sulfate at 3mg/mL significantly inhibited the growth of gut microbiota *in vivo* and their efficacies increased with increase in the concentration and the effect was maintained over time. Conversely, chloramphenicol, ampicillin and tetracycline were not efficient in inhibiting the growth of gut microbes and their efficacy reduced with time [22]. However, in an *in vivo* assay, penicillin, gentamicin, rifampicin and streptomycin each at the rate of 500 mg/l diet managed to reduce the gut bacterial community below detectable levels in five lepidopteran species *viz*., *Lymantria dispar* [6], *Vanessa cardui*, *M. sexta*, *Pieris rapae* and *P. gossypiella* [10]. Hence, it can be hypothesized that the efficacy of antibiotics varies with the insect from which the gut bacteria is isolated. There is a possibility that the gut bacteria are resistant to certain antibiotics to which they are previously exposed either from the host plant [23] or during laboratory rearing [24].

Chloramphenicol and tetracycline when used at the dose of 100µg each, abnormalities in the growth stages were observed and hence 50µg of each were used to clear off the gut bacteria *in vivo*. Lin *et al.* [22] also reported that increasing concentrations of antibiotics efficiently removes the gut bacteria however; toxic effects such as, reduced larval growth and development, high mortality, malformed pre-pupae, hindrance in pupation and adult emergence are more prominent.

In the present study, mortality of *P. gossypiella* larvae was reduced when antibiotic cocktail was added along with the toxins in all the treatments compared to experiments involving toxin alone. Likewise, LC_{50} values were high in treatments involving antibiotics along with toxins as compared to toxin alone. In some insects, resident midgut bacteria contribute to Bt efficacy by increasing the mortality. But this is not true with all the insects and this effect can vary with same insect species from different locations. Midgut bacteria are required for Bt insecticidal activity in *L. dispar* [6] *V. cardui*, *M. sexta*, *P. rapae*, *Heliothis virescens* [10] *H. armigera* [8, 9] and in *Spodoptera littoralis* [7]. However, role of gut bacteria in Bt efficacy is contradictory in *M. sexta* [11], *P. gossypiella* [10] and *L. dispar* [24]. In *Choristoneura fumiferana*, midgut bacteria do not have any obligatory role in Bt efficacy [24]. Based on the present knowledge, conflicting results involving the same insect can be attributed to; dominant bacterial species at the time of experimentation than on host species per se [24]. In the experiment conducted by Broderick *et al.* [10] *Enterobacter* sp. NAB3 and *Pseudomonas putida* constituted the gut bacteria of *L. dispar* however; *Enterococcus* and *Staphylococcus* dominated the gut bacteria of *L. dispar* and lacked *Enterobacter* in the study conducted by van Frankenhuyzen *et al.* [24]. In present study, gram positive bacteria *viz*., *Enterobacter* and *Pantoea* dominated the *P. gossypiella* larval midgut whereas, in the conflicting results published by Broderick *et al.* [10], gram negative bacteria *viz*., *Enterococcus* was the sole bacteria inhabiting the *P. gossypiella*. Difference in experimental results can also be due to difference in stage of the insect used for bioassay and difference in experimental methodology. Bacterial diversity of insect gut is determined by various factors such as diet, developmental stage, physiological conditions and host phylogeny [25-26].

Conclusion

All the isolated culturable midgut bacteria of *P. gossypiella* were susceptible to chloramphenicol and tetracycline *in vitro*. However, higher doses of antibiotics caused physiological abnormalities. It is important that, while considering the dose for *in vivo* administration, care must be taken not to use higher dose as, it can affect the physiology of the insect leading to false results. Symbiotic bacteria enhance the toxicity of Bt, as a result, treatment with gut bacteria intact (without antibiotics) has lower LC_{50} compared to the treatment devoid of gut bacteria (with antibiotics). Present studies were carried out using *P. gossypiella* population from a single location; however results can vary with locations, as pink bollworm from different locations constituted different gut bacteria. Results on the role of gut bacteria can be validated by feeding the GFP-tagged isolated gut bacteria followed by bioassay. Targeting the gene responsible for immunity in insects and elucidating the precise role of gut bacteria (RNAi) in Bt efficacy can throw some light on this phenomenon without exposing the insects to indirect effects of the antibiotics.

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