

Full Length Research Paper

Physiological and mutagenic effects of pathogenic and non-pathogenic bacteria on the last larval instar of *Bombyx mori* (Lepidoptera: Bombycidae)

Abir A. Gad¹ and Ahmed A. Abdel-Megeed^{2,3*}

¹Department of Applied Entomology, Faculty of Agriculture (Elshatby), Alexandria University, Alexandria, Egypt.

²Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.

³Department of Plant Protection, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria 21513, Egypt.

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The present study investigated the physiological and genetical effects of non-pathogenic bacteria (G^-) *Escherichia coli* and entomopathogenic bacteria (G^+) *Bacillus thuringiensis* on the mulberry silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). We used bioassay and molecular markers linked to 5th instar larvae to inject bacterial and analyze the response of *B. mori*. Also, the effect of both bacteria on the total and differential haemocytes count of the 5th instar larvae of *B. mori* was evaluated. Results revealed that injection with *E. coli* increased the total haemocytes counts (THCs) to about 59.09% of the control at 24 h post-infection. In this respect, *B. thuringiensis* decreased the THCs by about 45.9, 58.39 and 69.4%, respectively, than control after 48 h post-infection at concentrations of 0.5, 1 and 1.5 ppm. Injection with *E. coli* increased the number of Pr, PI, Gr and Oe. On the contrary, injection with *B. thuringiensis* significantly decreased the number of Pr, PI and Gr and Oe. The mutagenic effect of the bacterial injection produced and/or affected several proteins that killed or caused larval deformation through the alteration of the physiological and genetic processes.

Key words: *Bombyx mori*, haemocytes, corpora allata, DNA structure, *Escherichia coli*, *Bacillus thuringiensis*.

INTRODUCTION

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) has been exploited as a silk producer in the silk industry for thousands of years. The recent success of transgenesis of the silkworm has opened new prospects for this insect species (Tamura et al., 2000). Silkworm diseases are the most important disease that inflict heavy loss to crops. Most losses in sericulture can be attributed directly to silkworm diseases. Among silkworm diseases, bacterial diseases are common, but in general, massive outbreaks are rare. Pathogenic bacteria of silkworm belong

to a wide variety of genera, including *Bacillus*, *Enterobacter*, *Serratia*, *Aeromonas*, *Streptococcus*, *Pseudomonas* and *Staphylococcus* (Tao et al., 2011). However, when silkworms are physiologically weak, bacterial diseases can attack them, eliciting a heavy toll on sericulture (Aruga, 1994). The bacterial diseases affecting silkworm are called flacherie because the cadavers of silkworms that have died of these diseases lose elasticity, soften, and rot. Bacterial diseases of silkworms are usually only secondary to virus diseases. The chief diseases affecting silkworm

*Corresponding author. E-mail: aamahmoud@ksu.edu.sa.

is flasherie caused by *Bacillus* bacteria. One casual bacillus of silkworm flasherie is *Bacillus thuringiensis* which is a widely distributed facultative entomogenous bacterium with as much as 34 varieties. It is a Gram positive spore forming bacterium widely distributed in the soils of various regions of the world. The endotoxin produced by *B. thuringiensis* is known to destroy the gut lining, causing paralysis and death in many insect species belonging to orders, Diptera and Lepidoptera including economically important insects in several kinds of silkworms (Aizawaza, 1971; Nataraju et al., 1991).

Insect innate immunity can be affected by juvenile hormone (JH) and 20-hydroxyecdysone (20E), but how innate immunity is developmentally regulated by these two hormones in insects and has not yet been elucidated. Fat body produces humoral response molecules and hence is considered as the major organ involved in innate immunity (Muramatsu et al., 2008). Tian et al. (2010) suggested that JH plays a positive role in the regulation of innate immunity in the larval fat body and the volume of *Corpora allata* (C.A.) was used as an indicator for its activity according to Pflugfelder (1948). Mulberry silkworm was selected as a model system for studying immunity responses against bacteria, because it is of great economic importance due to its unique white silk. Last larval instar of this silkworm is selected for the experiments; the main advantage is that in the last larval instar, the silk protein synthesis and most larval mortality was as a result of diseases caused in this stage.

In the present investigation, we studied the effect of some immunity responses of silkworm larvae such as, cellular immunity (total and differential haemocyte counts) against *E. coli* (G⁻) and *B. thuringiensis* (G⁺) bacteria and also studied the effects of both *E. coli* and *B. thuringiensis* at concentration of 1.5 ppm on the corpora allata (CA) activity during the last larval instar of *B. mori* to understand the effect of both bacteria on activation or inhibition of the CA activity and its relation with total and differential haemocytes counts.

MATERIALS AND METHODS

Laboratory animals

Silk worm, *B. mori* (PM X NB4D2) were reared on an artificial diet at 25 ± 2°C and RH 65 to 70%. Newly molted (day 0) fifth larval instar (weighing 1 to 1.5 g) was utilized for all experiments.

Preparation of bacteria

Non pathogenic bacteria (G⁻) *E. coli* obtained from Biology Department, University of Taibah was grown in the room temperature for 24 h at 30°C on nutrient agar (3 g of beef extract, 5 g of peptone and 20 agar in 1 L distilled water), pH 7.0.

The commercial formulation of *Bacillus thuringiensis* (Dipel®, Sumitomo Chemical Agro Europe (SCAE)) was taken from Applied Entomology Department, Faculty of Agriculture, Alexandria University. Three serial dilutions of the tested product (0.5, 1.0 and

1.5 ppm) of bacteria were used.

Bacterial injection

The newly molted 5th larval instar of *B. mori* were injected with *E. coli* (G⁻) (1.1 × 10⁶ cells/ml), *B. thuringiensis* (G⁺) (Dipel) was injected at concentrations of 0.5, 1.0 and 1.5 ppm and physiological saline (Sigma, UK), as control. Each larva was injected with 50 µL in the dorsal segment of larvae by BD Micro-fine™ plus syringe. Five replicates, 60 newly molted larvae were used for each treatment.

Studies of haemocytes

For total haemocyte counts (THC), fresh haemolymph was collected after 24, 36 and 36 h of treatment and diluted 10- fold with a cold physiological saline buffer containing 0.4% trypan blue (Horohov and Dunn, 1983). Cells in the diluted haemolymph were counted using a Thoma haemocytometer (Essawy et al., 1999). Under phase contrast optics was as described by Arnold and Hinks (1976) after 24, 36 and 48 h. The THC was estimated according to the formula suggested by Jones (1962). The differential haemocyte counts (DHC) were estimated according to the technique used by Akai and Sato (1979) using fresh slid preparations.

Corpora allata activity

Corpora allata (CA) volume was used as an indicator of the juvenile hormone (JH) level (Pflugfelder 1948). Larvae were dissected every 24 h and until prepupal stage after treatment with *E. coli* (G⁻), *B. thuringiensis* (G⁺) at concentration of 1.5 ppm and control. The method of Armstrong and Carr (1964) was used to calculate the CA surface area.

Larval DNA studies

Genomic DNA was extracted according to the protocol described by Pither et al. (1989) with the following modification: Whole larvae homogenized in 100 µl of extraction buffer (1X PCR Buffer) 0.08% (w/v) of Tris base, 0.06% (w/v) of MgCl₂, and 0.02% (w/v) of NH₄ (SO₄)₂ in 90 ml of HCl. The final volume was adjusted to 100 ml with distilled water. The ground larvae in solution were placed in a boiling water bath for 15 min. The tube is centrifuged at 6,000 rpm to get rid of debris. Larval tissues were centrifuged again at 6,000 rpm for 10 min at 4°C, resuspended in buffer (100 mM Tris-HCl (pH = 8.0), 100 mM EDTA, 250 mM NaCl). Sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.5% and 100 g ml⁻¹, respectively. The mixture was incubated for 1 h at 37°C. Precipitation was then performed by adding 0.8 M NaCl and 0.5% hexadecyltrimethyl ammonium bromide solution and incubating for 20 min at 65°C. The solution was extracted with an equal volume of Roti® phenol-CHCl₃ mixture. DNA was precipitated with 0.6 volume of isopropanol. The resulting DNA pellet was washed with 70% ethanol, centrifuged at 8,000 rpm for 30 min at 4°C and air dried. DNA pellet was then dissolved in TE buffer. Genomic DNA was analyzed and visualized by agarose gel according to the method of Sambrook et al. (1989). The gel was prepared with 0.8% (w/v) agarose dissolved in TBA buffer (0.89 M Tris, 0.02 M EDTA-Na₂-salt, 0.89 M Boric acid, pH 8.5). The run was performed at 77 V. Gels were stained with Sybr[®] Green. Genomic DNA was restricted with *Eco*R1 according to the instructions described by Sambrook et al. (1989).

Table 1. Physiological effect of *E.coli* and *B. thuringiensis* on the total haemocyte counts.

Time post infection (h)	Average no.		Haemocytes/mm ³		
	<i>E. coli</i>	<i>E. coli</i>	<i>B. t₁</i>	<i>B. t₂</i>	<i>B. t₃</i>
24	38500±16 ^a	38500±16 ^a	18000±15.7 ^c	17333.3±14.5 ^c	14000±25 ^d
36	43667±19.11 ^a	43667±19.11 ^a	20333.3±23 ^c	19334±31 ^c	15000±11.5 ^d
48	69500±20.2 ^a	69500±20.2 ^a	31176.9±37 ^b	24000±36 ^c	17666±20 ^d

- Each value presents the mean ± SE. Statistical analysis between control and treatment; there are no significant differences among means with same letter. The different letter(s) are significant at $p \leq 0.05$ according to the LSD test.

Statistical methods

Data were statistically analyzed to check the significance of differences between treatments and control using F test and LSD (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Haemocyte counts

The total number of circulating haemocytes in an insect varies with developmental and physiological stages (Essawy, 1990). Four primary types of haemocytes were observed in the haemolymph of the *B. mori* fifth larval instar. There were prohaemocyte (Pr), plasmatocyte (PI), granulocyte (Gr) and oenocytoide (Oe). Granulocytes were the most abundant haemocytes followed by PI. Oeocytes were numerically less abundant.

E. coli

A marked increase in THC was noted at 48 h post-treatment as compared to control with 69500 and 57690 cells/mm³, respectively (Table 1). Also, the present results revealed a slight increase in the THC at 24 h of *E. coli* treatment but still increased as compared to control values, 38500 cells/mm³. These results revealed that *E. coli* treatment increased the THC of *B. mori* by 59.09, 33.1 and 20.47% as compared to THC of control after 24, 36 and 48 h, respectively. The results obtained are in contrast to those detected by Ericsson et al. (2009) who reported that there was significant reduction in the haemocyte count after the injection of *Trichoplusia ni* with *E. coli* and Dunphy and Nolan (1982) who found that THC was initially declined when the larvae were injected with protoplasts, growth medium (MGM) or *E. coli*.

The percentage of the number of Pr increased at 24, 36 and 48 h post-infection, 5.8, 7.6 and 6.6%, respectively. As shown in Figure 1a, b, c and d the infection of *B. mori* fifth larval instar with *E.coli* markedly increased the percentage of the number of PI and Gr at 48 h post-infection to about 16.7 and 35%, respectively.

These results may be due to the important role of plasmatocytes and granulocytes in the insect immunity against bacterial infection. The data also revealed that Pr,

Gr and PI increased at 24, 36 and 48 h post-infection, also Oe increased at the same time to 15.38, 17.6 and 16.6%, respectively. These results are in accordance with the findings of Horohov and Dunn (1983) who reported that bacterial injection into *Manduca sexta* larvae caused significant changes in the number of oenocytoides.

B. thuringiensis

The dynamic reactions of the 5th larval instar of *B. mori* against *B. thuringiensis* revealed marked decrease in the total haemocyte count (THC).

Quantitative analysis of THC of insects infected with *B. thuringiensis* gave the results shown in Table 1. Bacterial injection at a concentration of 1.5 ppm led to significant decrease in THCs after 24, 36 and 48 h post-injection reaching to 14000, 15000 and 17666 cells/mm³, respectively, while in the control THCs were 24200, 32800 and 57690 cells/mm³, respectively. Meanwhile, the injection of *B. mori* with *B. thuringiensis* at concentration of 0.5, 1 and 1.5 ppm decreased the THCs to about 45.9, 58.39 and 69.4% after 48 h post-infection, respectively, as compared to the control.

Similar results were reported by Ericsson et al. (2009) who studied the immune response to *B. thuringiensis* Kursraki. (Btk) in susceptible (Bt-Rs) and resistant (Bt-R) *Trichoplusia ni* after exposure to low doses of Btk. They reported a reduction in haemocyte counts after exposure to Btk. Also, Johnson (1981) investigated that haemocytes were lost from the circulation by their incorporation into aggregations or by lyses of individual cells after injection of *Homarus americanus* with bacteria. Also, the present data are in agreement with those obtained for *Agrotis ipsilon* larvae infected with *B. thuringiensis* (Abd El-Aziz and Awad, 2010). On the other hand, the obtained results were in contrast to those detected by Horohov and Dunn (1983) who found a marked increase in THC of *M. sexta* larvae injected with *Pseudomonas aeruginosa*.

As shown in Figure 1 a, b, c and d, it is clear that the injection with *B. thuringiensis* at all concentrations decreased the percentage of the number of Pr, Gr and PI counts especially at 48 h post-infection. The maximum decrease was observed at concentration of 1.5 ppm. The percentage of the number of Pr decreased at 24, 36 and 48 h post-infection to about 20, 37.3 and 42%, respectively. Furthermore, the injection of the 5th larval instar of

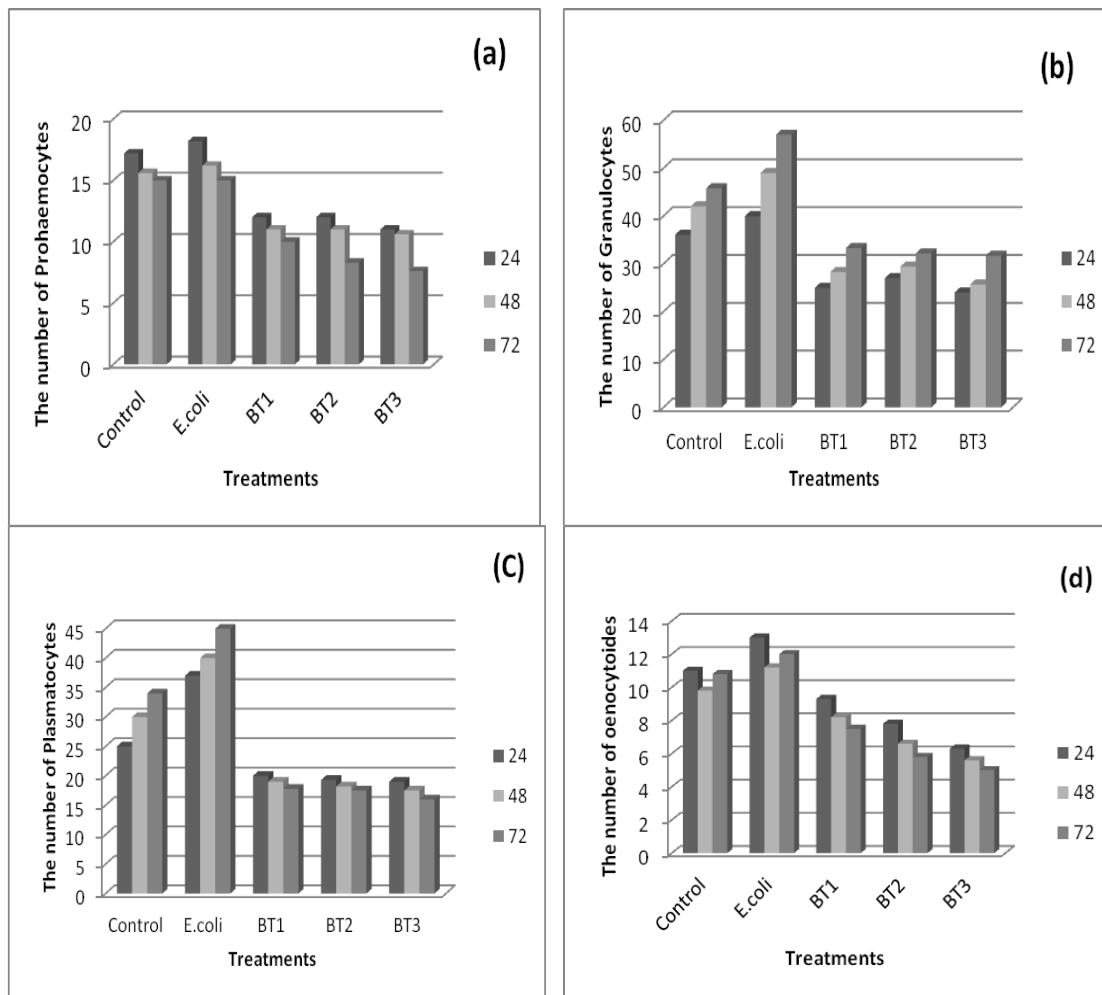


Figure 1. Effect of *E. coli* and *B. thuringiensis* on the different haemocyte count of the *B. mori* 5th larval instar. Where; (BT1 & BT2 & BT3): *B. thuringiensis* at concentration 0.5, 1 and 1.5 p.p.m, respectively. a) Prohaemocyte; b) Granulocyte; c) Plasmotocyte; d) Oenocytoid.

B. mori with *B. thuringiensis* at the same concentration decreased the Gr, PI counts to about 27.4 and 37% as compared to the control, respectively, at 48 h post-infection. The same trend was observed in the Oe counts which decreased to about 52.6%, less than control.

These results are in accordance with the findings of Horohov and Dunn (1983) who reported that the bacterial injection into *Manduca sexta* larvae caused a significant decrease in the percentage of Grs and PIs. Also, Chain and Anderson (1982) reported a direct demonstration that *B. cereus* injection can cause a selective removal of plasmotocytes from the circulating haemolymph of *Galleria mellonella*. Perhaps the injection with bacteria in some way causes PIs to clump together or to cling to the lining of the haemocoel (Abd El-Aziz and Awad, 2010).

The hormonal regulation of immunity during the last larval instar of *B. mori*

Results reported in Figure 2 depicted that the infection of

B. mori fifth larval instar with *E. coli* markedly increased the CA surface area at 72 and 120 h post-infection to about 18.9 and 24.9%, respectively of the control. This increase may be due to the effect of *E. coli* on the immunity response of larvae. These results are in accordance with findings of Freitag et al. (2007) who demonstrated that injection of Gram negative bacteria *E. coli* can induce the immune response of lepidopteran larvae.

In contrast, injection of *B. mori* fifth larval instar with *B. thuringiensis* caused a sharp decrease in the CA surface area up to 96 h post-infection to reach the minimum value of about 54.5% less than the control which is followed by a marked increase of CA surface area at 120 h to about 12.18% over the control. Perhaps, the infection with bacteria in some cases caused inhibition of CA activity during few days of infection and the increase of the CA activity after that may be due to the release of antibacterial peptides as an immunity response against bacteria which helped the gland to reprogramme itself in its cycle during the last larval instar. The presented results are consistent

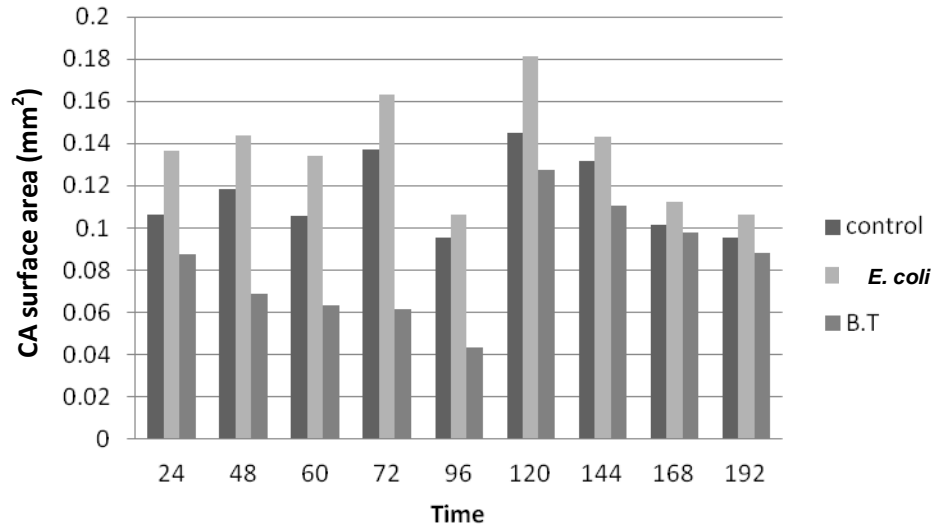


Figure 2. Physiological effect of *E. coli* and *B. thuringiensis* on the corpora allata surface area.

with those detected by Tian et al. (2010) who suggested that JH plays a positive role in the regulation of innate immunity in the larval fat body. Also, Riddiford (2003) suggested that JH has a significant role in the control of immune humoral function. A number of studies in *Drosophila* imply that 20E induces AMP mRNA expression and acts as an immune-activator (Meister et al., 1996; Silverman et al., 2000) while JH acts as an immune-suppressor by antagonizing 20E signaling (Flatt et al., 2008). Also, Flatt et al. (2005) found that juvenile hormone regulate the immunity of *Drosophila* by inhibiting phenoloxidase (Po) synthesis and prevents cuticular melanization.

In the light of the foregoing results, it could be concluded that the fluctuations that occurred in THC and CA surface area (as an indicator of its activity) are going in the same direction. These results are similar to those obtained in previous studies of Gad (1996) who noted a positive correlation between the CA volume and THC.

It is important to go deep to explain the side effects and the mode of action by which the injection of the bacteria can effect the physiology and genetics of *B. mori*. A molecular marker of *B. mori* DNA is the most important method for determining the affected region on DNA (Williams et al., 1990) since they reveal DNA polymorphisms among genetically related individuals. A similar strategy has been used to identify the nucleopolyhedrovirus (NPV), another important silkworm genotype virus (Yao et al., 2003). *B. thuringiensis* is the most widely used microbial pesticides. The biochemical basis of the pesticide is an insecticidal crystal protein (ICP), which is produced by the bacterium as a 133-kDa protoxin that requires proteolytic cleavage in the insect gut for activation. The mutagenic effect of the bacterial injection produced several proteins that enable it to kill insects through the alteration of the physiological processes (Brown et al., 2004).

B. thuringiensis Cry1Aa insecticidal protein is the most active known *B. thuringiensis* toxin against the forest insect pest *Lymantria dispar* (gypsy moth), unfortunately it is also highly toxic against the non-target insect *B. mori* (silk worm). In fact, it was found in previous studies that *B. thuringiensis* produces different types of insecticidal crystal proteins (ICPs) or delta-endotoxins. The type A protein from *B. thuringiensis* var. kurstaki HD-1 was found to be 400 times more active against *B. mori* than type C protein from *B. thuringiensis* var. kurstaki HD-244 (Brown et al., 2004). To locate the specificity domain of the type A protein for *B. mori*, site-directed mutagenesis was used to introduce or remove restriction enzyme sites, facilitating the exchange of regions of the two genes. The hybrid genes were overexpressed, and purified ICP was used in bioassays. The *B. mori* specificity domain for the ICP A toxin is located in the amino-terminal portion of the hypervariable region between amino acids 332 and 450.

It may be assumed that differences in restriction sites are due to amino acid differences in proteins of *B. thuringiensis* and *E. coli* against *B. mori*. The change of a portion in specific regions of *B. mori* DNA is an indicator of mutagenic effects occurring in the protein.

Our results confirm that this the genotoxicity occurred in *B. mori*, in a DNA band region leading us to predict that this region is composed of several structural domains that are disrupted by the toxin secreted by both *B. thuringiensis* and *E. coli* against *B. mori* (Figure 3).

Gillespie et al. (1997) and Dettloff et al. (2001) reported that haemocyte responses were triggered by antigens adhering to two types of receptor: pattern recognition and promiscuous non-pattern recognition receptors. The receptors must transfer information through signal transduction pathways into the haemocytes to continue the anti-antigen responses; that is, the release of opsonins and/or adhesive proteins. Haemocytes of the silkworm *B.*

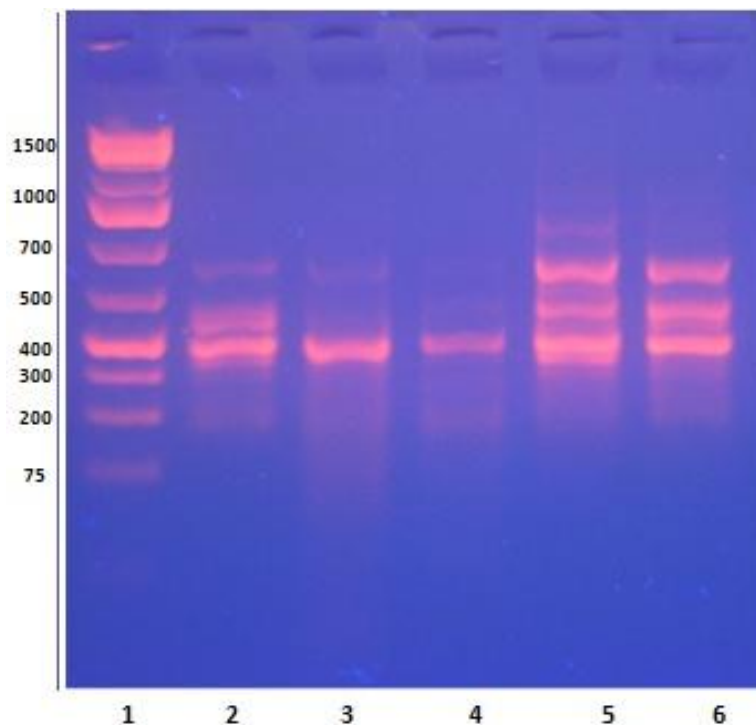


Figure 3. Detection and localization of the affected *B. mori* larval DNA treated with *E. coli* and *B. thuringiensis*. Lane 1: Marker 1500 bp; 2: Control; 3: Treatment with *E. coli*; 4: Treatment with *B. thuringiensis* (0.5 ppm); 5: Treatment with *B. thuringiensis* (1 ppm); 6: Treatment with *B. thuringiensis* (1.5 ppm).

mori require an unidentified PKA isotype to produce cecropins (antibacterial proteins) (Choi et al., 1995; Shimabukuro et al., 1996). Based on the use of H-89, an inhibitor of PKA isotypes, a type of PKA may limit using several experimental approaches (luminometry, spectrophotometry, fluorimetry); we found no increase in ROS production in the hemolymph of *B. mori* (Hyrsi et al., 2004). Research conducted by Anderson et al. (1973) showed that the hemocytes of *Blaberus craniifer* (Blattodea) did not reduce NBT in response to zymosan. Whitten and Ratcliffe (1999) provided evidence for the existence of an immune response resembling the respiratory burst in the hemolymph and hemocytes of the cockroach, *Blaberus discoidalis* (Blattodea). The granulocytes of *Bombyx*, which play a key role in phagocytosis in normal larvae was reported by Akai and Sato (1973).

Conclusion

In conclusion, *E. coli* injection has led to a significant increase in THCs as compared to the control. The injection effect of *E. coli* improved the immune system of the larvae as a result of the larval immune response when compared with the injection effect of *B. thuringiensis*. This was observed in the increase in the total number of haemocytes, the activity of Corpora allata gland and Juvenile hormone secretion as an immune stimulant.

The results demonstrated that *B. thuringiensis* induced the host strong response. Huang et al. (2009) suggested that injection of *B. mori* with *Bacillus bombysepticus* (*Bb*) caused a lot of basal metabolic pathways which were significantly modulated. Furthermore, genes of juvenile hormone synthesis and related metabolism showed up regulation, suggesting that juvenile hormone participate in host modulation during the infection. Moreover, host cellular and systemic immune responses are also induced. Similar to *B. thuringiensis* (*Bt*), *Bb* can also induce a silkworm poisoning-related response.

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