JOURNAL OF PURE AND APPLIED MICROBIOLOGY, September 2013. Vol. 7(3), p. 1707-1715

Physiological and Mutagenic Effects of Gram Negative and Positive Bacteria on the Last Larval Instar of *Bombyx mori* (Lepidoptera: Bombycidae)

Abir A. Gad[1] and Ahmed A. Abdel-Megeed[2,3]

[1]Department of Applied Entomology, Faculty of Agriculture (Elshatby), Alexandria University, Alexandria, Egypt.
[2]Department of Botany and Microbiology , College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.
[3]Department of Plant Protection, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria 21513, Egypt.

(Received: 27 January 2013; accepted: 02 March 2013)

The present study investigated the physiological and genetical effects of Gram negative bacteria (G^-) *Escherichia coli* and entomopathogenic Gram positive bacteria (G^+) bacteria *Bacillus thuringeinsis* on the mulberry silkworm *Bombyx mori* (Lepidoptera: Bombycidae) were studied. The author used bioassays and molecular markers linked to 5^{th} instar larvae to bacterial injection to analyze the response of *Bombyx mori.* Also, the effect of both bacteria on the total and differential haemocytes count of the 5^{th} instar larvae of *B. mori* was evaluated. Results revealed that injection with *E. coli* increased the total haemocytes counts (THCs) about 59.09% up to control at 24 h post-infection. In this respect, *B. thuringeinsis* decreased the THCs by about 45.9, 58.39 and 69.4 % , respectively less than control after 48 hrs post-infection at concentrations of 50, 100, 150 μg/ml. Injection with *E.coli* increased the number of Pr, Pl and Gr and Oe. On contrast, injection with *B. thuringeinsis* significantly decreased the number of Pr, Pl and Gr and Oe. The mutagenic effect of the bacterial injection produced and/or affected several proteins that enable it to kill or causing larval deformation through the alteration on the physiological and genetic processes.

Key words: *Bombyx mori*, Haemocytes, *Corpora allata*, DNA structure, *Escherichia coli, Bacillus thuringeinsis.*

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 being the most important those inflect heavy loss to the crop. 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 belonging 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

* To whom all correspondence should be addressed.

diseases lose elasticity, soften, and rot. Bacterial diseases of silkworms are usually only secondary to virus diseases. The main and important disease affected silkworm is flasherie caused by *Bacillus* bacteria. One casual bacillus of silkworm flasherie is *B. thuringeinsis* which widely distributed facultative entomologenous bacterium with as many as 34 varieties. It is gram positive spore formatting bacterium widely distributed in the soils of various regions of the world. The endotoxin produced by *B. thuringeinsis* 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 (Tao *et al*., 2011; 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 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 Pflugefelder (1948); Abd El-Aziz and Awad, (2010). 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 as a result of diseases caused in this stage (Tian *et al.* 2010).

In the present investigation we study the effect of some immunity responses of silkworm larvae such as, cellular immunity (total and differential haemocyte counts) against *E. coli* (G^-) and *B. thuringeinsis* (G^+) bacteria ,also study the effects of both *E. coli* and *B. thuringeinsis* at concentration 1.5ppm 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 the CA activity and its relation with total and differential haemocytes counts .

MATERIALS AND METHODS

Laboratory animals

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

Preparation of bacteria

Gram negative bacteria (G^-) *Escherichia coli* were obtained from Biology Department, University of Taibah was grown in the room temperature for 24h at 30 °C on nutrient agar (3 g of beef extract, 5g of Peptone and 20 agar in 1L Distilled water) , pH 7.0.

The commercial formulation of *Bacillus thuringeinsis (*Dipel®, Sumitomo Chemical Agro Europe (SCAE)) was offered from Applied Entomology department, Faculty of Agriculture, Alexandria University. Three serial dilutions of the tested product (50, 100, 150 µg/ml) of bacteria were used.

Bacterial injection

The newly molted 5th larval instar of *Bombyx mori* were injected with *E. coli* (G^-) (1.1 x 10^6 cell/ml) , *Bacillus thuringiensis* (G^+) (Dipel) was injected at concentrations of 50, 100, 150 µg/ml 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- flod 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 as described by Arnold and Hinks (1976) after 24, 36, 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 (1997) using fresh slid preparations.

Corpora allata activity

Corpora allata (CA) volume was used as an indicator of the juvenile hormone (JH) level

(Pflugefelder, 1948). Larvae were dissected every 24 h and until prepupal stage after treatment with *E. coli* (G^-), *Bacillus thuringiensis* (G^+) at concentration 150 µg/ml 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 $MgC1_2$, and 0.02% (w/v) of $NH_4 (SO_4)_2$ 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 final concentration of 0.5% and 100 g ml^{-1}, respectively. The mixture was incubated for 1 hr 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-$CHCl_3$ 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 puffer. 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-Na_2-salt, 0.89 M Boric acid, pH 8.5). The run was performed at 77 volt. Gels were stained with Sybr[a] Green. Genomic DNA was restricted with *Eco*R1 according to the instructions described by Sambrook *et al*., (1989).

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(Pl), Granulocyte (Gr) and Oenocytoide (Oe).Grs were the most abundant haemocytes followed by Pls. Oecytoides were numerically less abundant.

E. coli

A marked increase in THC was noted at 48 h post- treatment as compared to control 69500 and 57690 cell/ mm^3, respectively (Table 1).Also, the present results revealed a slight increase in the THC at 24 h of *E.coli* treatment but still increase as compared to control value, 38500 cell/ mm^3. These results revealed that *E.coli* treatment increased the THC of *B. mori* by 59.09 , 33.1 and 20.47 % as compared with THC of control after 24, 36 ,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 total larval hemocyte counts (THC) was initially declined when the larvae were

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

Time Post infection (hrs)	Average no.		Haemocytes/mm^3		
	Control	*E.coli*	*B.* t_1	*B.* t_2	*B.* t_3
24	24200±15.01[b]	38500±16 [a]	18000±15.7[c]	17333.3±14.5[c]	14000±25[d]
36	32800±25.13[b]	43667±19.11 [a]	20333.3±23[c]	19334± 31 [c]	15000±11.5[d]
48	57690±22.5[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 significantly at $p \leq 0.05$ .according to the LSD test.

injected with protoplasts, growth medium (MGM), or *E. coli*.

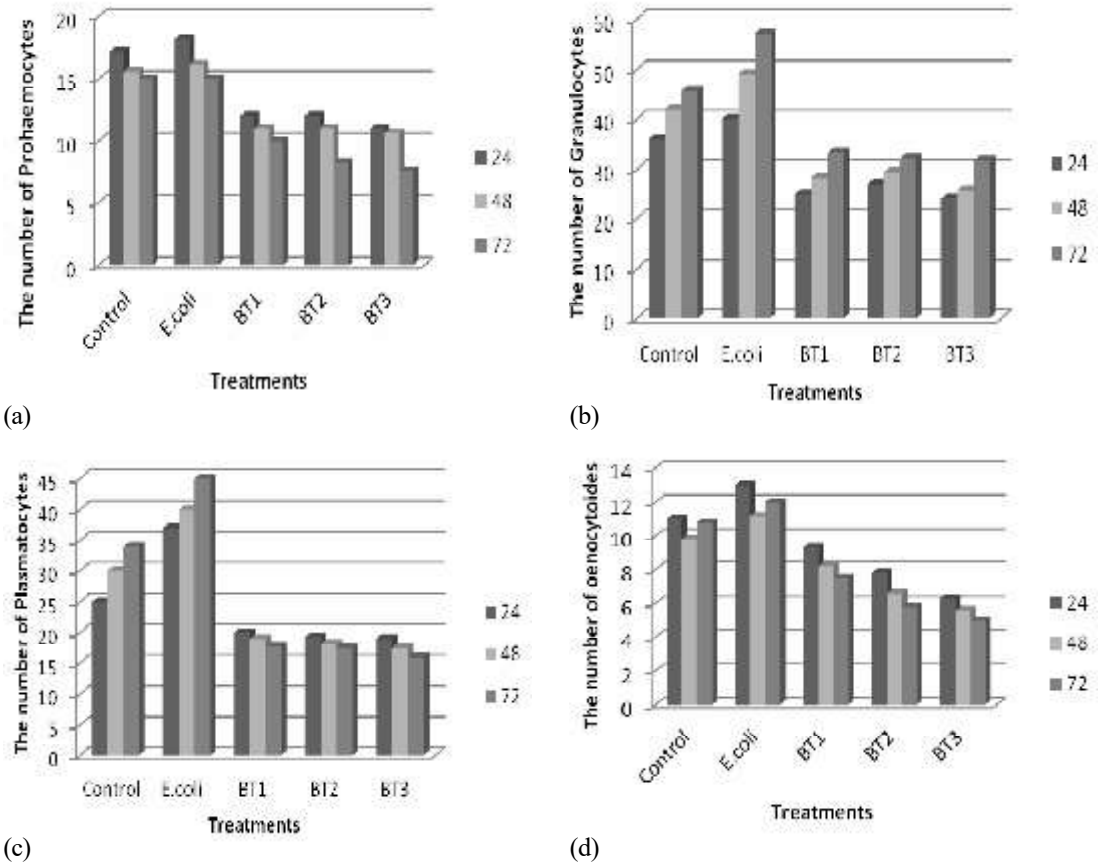
The % DHC of Pr increased at 24, 36 and 48 h post –infection, 5.8, 7.6 and 6.6 %, respectively. As shown in Figure 1 (a, b, c, d) the infection of *B. mori* fifth larval instar with *E.coli* markedly increased the % DHC of Pl and Gr at 48 h post-infection to about 16.7 and 35 %, respectively.

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 Pl increased at 24, 36 and 48 h post-infection, also Oe increased at the same time estimated by 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.

Bacullus 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. thuringiensi*s gave the results shown in Table (3) & Fig. 1 (a, b, c, d). Bacterial injection at a concentration of 150 µg/ml led to significant decrease in THCs after 24, 36 and 48 h post-injection reaching to 14000, 15000 and 17666 cell/mm^3, respectively, while in the control THCs were 24200, 32800 and 57690 cells/mm^3, respectively. Meanwhile, the injection of *B. mori* with *B. thuringiensi*s at concentration of 50, 100, 150 µg/ml decreased the THCs about 45.9, 58.39 and 69.4 % after 48hrs post-infection, respectively, as compared to the control.



where; (BT1 & BT2& BT3): *B. thuringiensis* at concentration 50, 100, 150 µg/ml respectively.
a) Prohaemocyte; b) Granulocyte; c) Plasmatocyte; d) Oenocytiode .

Fig. 1. Effect of *E.coli* and *B. thuringiensis* on the different haemocyte count of the *B. mori* 5th larval instar

Similar results were reported by Ericsson *et al*., (2009) who studied the immune response to *B. thuringiensi*s 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 amrericanus* with bacteria. Also, the present data are in agreement with those obtained for *Agrotis ipsilon* larvae infected with *B. thuringiensi*s 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 (a, b, c, d), it is clear that the injection with *B. thuringiensis* at all concentrations decreased the DHC of Pr , Gr and Pl counts especially at 48hr post-infection. The maximum decrease was observed at concentration of 1.5%. The DHC of Prs decreased at 24, 36 and 48 hrs post -infection about 20, 37.3 and 42 %, respectively. Furthermore, the injection of the 5th

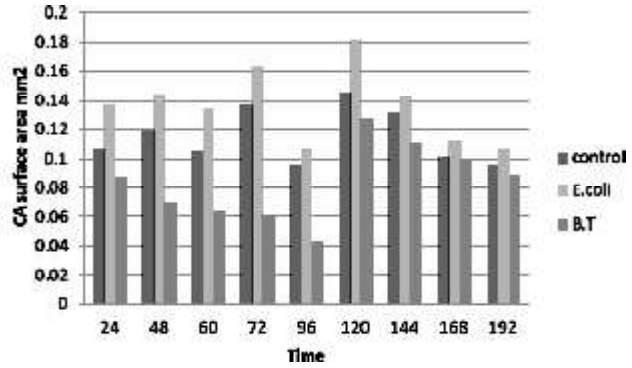


Fig. 2. Physiological effect of *E.coli* and *B. thuringiensis* on the corpora allata surface area

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

These results were 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 Pls. Also, Chain and Anderson (1982) reported a direct demonstration that *B. cereus* injection can cause a selective removal of plasmatocytes from the circulating haemolymph of *Galleria mellonella.* Perhaps the injection with bacteria in some way causes Pls to clump together or to cling to the lining of the haemocel (Abd El-Aziz and Awad, 2010).

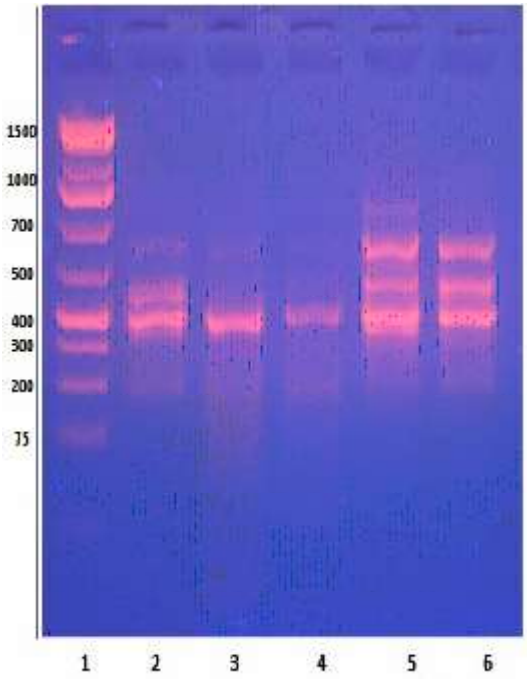
The hormonal regulation of immunity during the last larval instar of *B.mori*

Results reported in Fig., (2) depict that the infection of *B.mori* fifth larval instar with *E.coli* markedly increases the CA surface area at 72 and 120 h post –infection about 18.9 and 24.9 %, respectively folds 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 Freitak *et al.* (2007) who demonstrated that injection of Gram negative bacteria *E. coli* can induce the immune response of lepidopteran larvae.

On contrast, injection of *B.mori* fifth larval instar with *B. thuringiensis* caused sharp decrease in the CA surface area up to 96 h post-infection to reach the minimum value about 54.5 % less than control which followed by a marked increase of CA surface area at 120 hr about 12.18 % over control. Perhaps the infection with bacteria in some way caused inhibition of CA activity and the increase of the CA surface area 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 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 control of immune function in humoral. A number of studies in *Drosophila* imply that 20E induces AMP mRNA expression and acts as an immune-activator Meister *et al*. (1996) and 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 forgoing results, it could be concluded that the fluctuations occurred in THC and CA surface area (as an indicator of its activity)



Lane 1: Marker 1500 bp
2: Control
3: Treatment with *E. coli*
4: Treatment with *B. thuringiensis* (50 µg/ml)
5: Treatment with *B. thuringiensis* (100 µg/ml)
6: Treatment with *B. thuringiensis* (150 µg/ml)

Fig. 3. Detection and localization of the affected *B. mori* larval DNA treated with *E. coli* and *B. thuringiensis*

are ongoing on the same direction. These results were similar to those obtained previous studies (Gad, 1996) who noted a positive correlation between the CA volume and THC.

It was important to go deeply to explain the side effects and the mode of action by which the injection of the bacteria can effect on the physiology and genetic of *Bombyx mori*. Molecular marker of *Bombyx mori* DNA is the most important method for determination 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). *Bacillus thuringiensis* is the most widely used microbial pesticide. 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 on the Physiological processes (Brown *et al*., 2004).

Bacillus 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 *Bombyx mori* (silk worm). In fact it was found in previous studies that *Bacillus 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 *Bombyx 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. thuringeinsis* and *Escherichia*

coli against *Bombyx mori*. The change of a portion in specific regions of *Bombyx mori* DNA is an indicator of mutagenic effects occurred in the protein.

Our results confirm that this the genotoxicity occurred in *Bombyx mori*, in DNA band region leads us to predict that this region is composed of several structural domains that are disrupted by the toxin secreted by both *B. thuringeinsis* and *Escherichia coli* against *Bombyx 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 *Bombyx mori* require an unidentified PKA isotype to produce cecropins (antibacterial proteins) Choi HK *et al*., 1995; Shimabukuro M *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* (Hyrsl *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* play an important role as a key role in phagocytosis in normal larvae (Akai and Sato, 1973).

CONCLUSION

In conclusion, *E. coli* injection has lead to a significant increase in THCs 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 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 were significantly modulated. Furthermore, genes of juvenile hormone synthesis and metabolism related 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 *Bacillus thuringiensis* (Bt), Bb can also induce a silkworm poisoning-related response.

ACKNOWLEDGMENTS

The authors extend their appreciation to Deanship of Scientific Research at King Saud University for funding the work through the research group project No: RGPVPP- 010.

REFERENCES

1. Abd El-Aziz, N.M., Awad, H. Changes in the haemocytes of *Agrotis ipsilon* larvae (Lepidoptera: Noctuidae in relation to dimilin and *Bacillus thuringiensis* infections. *Micron,* 2010; **41:** 203–209.
2. Aizawaza, K., Strain improvement and preservation of virulence of pathogens. In microbial control of insects and mites, H.D. Burges and N.W. Hussy Eds., *Academic press London*. 1971; 655-672.
3. Akai, H.S., Sato, S., Ultrarstructure of the larval haemocytes of the silkworm *Bombyx mori* L. Lepidoptera: Bombycidae. *Int. J. Insect. Morphol. Embryol.* 1973; **2:** 207-231.
4. Akai, H.S., Sato, S., Surface and internal ulterstructure of haemocyte of some insects .In insect Haemocytes, Development, Forms, Functions and techniques Ed.: Gupta A. P. Cambridge Univ. Press. London, New York 1979.
5. Anderson, R. S., Holmes, B., Good, R.A. Comparative biochemistry of phagocytosing insect hemocytes. *Comp. Biochem. Physiol.* 1973; **46B:** 595–602.
6. Armstrong, W.D., Carr, C.W. Physiological chemistry laboratory directions 3rd ED. *Burges publishing Co., Minneapolis, Minnesota*, 1964.
7. Arnold, J.W., Hinks, C.F. Haemopoiesis in Lepidoptera I. The multiplication of circulating

haemocytes. Cannadian *J. of Zool*., 1976; **54:** 1003-1012.

8. Aruga, H. Principles of sericulture. *CRC Press, Rotterdam,* 1994; **pp:** 209–212.
9. Brown, S. E., Cao, A.T., Eric, R., Hines, N., Akhurst, R. J., East, P .D. A novel secreted protein toxin from the insect pathogenic bacterium *Xenorhabdus nematophila*. *Journal of Biological Chemistry*, 2004; **279:** 14595-14601.
10. Chain, B.M., Anderson, R.S. Selective depletion of the plasmatocytes in *Galleria mellonella* following injection of bacteria. *J. Insect Physiol*., 1982; **244:** 377-384.
11. Choi, H.K., Kato, K. , Kadono, Y., Yamamoto, O.K., Chowdhury, M., Xu, S., Sugiyama, J, Choi, M., Miyanoshita, S.K., Debnat, A.H., Asaoka, N.C., Ishii, A., Yamakawa, T. Induction of activity of proteinkinase C and A by bacterial lipopolysaccharide in isolatedhemocytes from silkworm, *Bombyx mori*. *J. Sericultural Sci. Jpn*; 1995; **64:** 450–56.
12. Dettloff, M., Kaiser, B., Wiesner, A. Localization of injected apo-Lp-III in vivo: new insights into the immune activation process directed by this protein. *J. Insect Physiol*.; 2001; **47:** 789–97.
13. Ericsson, J.D., Janmaat, A.F., Lowenberger, C., Myers, J.H. Is decreased generalized immunity a cost of Bt resistance in cabbage loopers *Trichoplusia ni* . *J. Invertebr. Pathol*., 2009; **100:** 61-67.
14. Essawy, M.M., Changes in different haemocyte counts of the last larval instar of *Spodoptera littoralis* Boised. during wound healing. *Alex. Sci. Exch*. 1990; **114:** 151-176.
15. Essawy, M.M., El dakhakhini, T.N., Saad, I.A. The effect of chlorfluazuron IKI- 7899 as chitin synthesis inhibitor on some biological and physiological parameters of larval instar of *Philosamia ricini*. Boisd. *J. Agric. Res., Tanta Univ.*, 1999; **2:** 269-291.
16. Flatt, T., Heyland, A., Rus, F., Porpiglia, E., Sherilock, C., Yamamoto, R., Garbuzov, A., Palli, S.R., Tatar, M., Silverman, N. Hormonal regulation of the humoral innate response in *Drosophila melanogaster*. *J exp Biol.*; 2008; **211:** 2712–2724.
17. Flatt, T., Tu, M.P., Tatar, M. Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. *Bioessays.,* 2005; **27:** 999–1010.
18. Freitak, D., Wheat, C.W., Heckel, D.G., Vogel, H. Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni.* BMC Biol. 2007; **5:** 56.
19. Gad Abir, A., Physiological study on some lepidopterous insect. M.Sc. Thesis, Fac. Agric., Alex. Univ., Egypt. 1996.
20. Gillespie, J.P., Kanost, M.R. Biological mediators of insect immunity. *Annu. Rev. Entomol*.; 1997; **42:** 611–43.
21. Horohov, D.W., Dunn, P.E. Phagocytosis and nodule formation by hemocytes of *Manduca sexta* larvae following injection of *Pseudomonas aeruginosa*. *J. Invertebr. Pathol*., 1983; **41:** 203–213.
22. Huang, L., Tingcai, C., Pingzhen., X., Daojun, C., Ting F, Qingyou, X. A Genome-Wide Survey for host Response of silkworm, *Bombyx mori* during pathogen *Bacillus bombyseptieus* infection. *PLoS One*.; 2009; **12:** 80-98.
23. Hyrsl, P., Kubala, M.Z., Lojek, A. Silkworm *Bombyx mori* Hemocytes Do Not Produce Reactive Oxygen Metabolites as a Part of Defense Mechanisms. *Folia Microbiol*. 2004; 49 3, 315–319.
24. Johnston, P.T., Stewart, J.E. Histopathology of *Aerococcus viridians* var. **homari injection** Gaffkemia in the lobster, *Hommmarus americanus*, and a comparison with histological reactions to a gram –negative species, *Pseudomonas perolens* . *J. Invertebr, Pat*hol. 1981; **38:** 127-148.
25. Jones, J. C. Current concepts concerning insect haemocyes. Amer. zool. 1962. 2 2: 209-246.
26. Muramatsu, D., Kinjoh, T., Shinoda, T., Hiruma, K. The role of 20-hydroxyecdysone and juvenile hormone in pupal commitment of the epidermis of the silkworm, *Bombyx mori*. *Mech Dev* 2008; **5:** 411-420.
27. Nataraju, B., Balavenktasubbaiah, M., Baig, M., Singh, D.B. A report on the distribution of B. thuringiensis in seri cultural areas of Karnataka. *Indian Jn. Seric*., 1991; **1:** 56-58.
28. Pflugefelder, O. Volumetrissche untersuchugen an den corpora allata der Honigbiene Apis mellifera. *Biol.Zbl*. 1948; **66:** 211-235.
29. Pither, D.G., Saunders, N.A., and Owen, R .J. Rapid extraction of bacterial genomic DNA with guanidinum thiocyanate. *Lett. Appl. Microbiol*. 1989; **8:** 151–156.
30. Riddiford, L.M. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Advanc. Insect Physiol*., 2003; **24:** 213–274.
31. Sambrook, J., Fritsch, E. and Maniatis, T. Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratory. 1989. Cold Spring Harbour, N Y.
32. Shimabukuro, M., Xu U., Sugiyama, M., Taniai, K., Kadonookuda, K., Kato, Y., Yamamoto, M., Chowdhury, S., Choi, S.K, Choi H.K.,

Miyanoshita, A., Debnath, N.C., Yamakawa, M. Signal transduction of cecropin B gene expression in adherent hemocytes of the silkworm, *Bombyx mori* Lepidoptera: Bombycidae. *Appl. Entomol. .Zool*.; 1996; **31:** 135–43.

33. Steel, R.G., Torrie, J.H. Principles and procedures of statistics. Abiometrical approach, 1980; 2nd ed. Mc. Graw-Hill Book Co., New York.
34. Tamura, T., Thibert, C., Royer C., Kanda, T., Abraham, E., Kamba, M., Komoto, N., Thomas, J.L., Mauchamp, B., Chavancy, G., Shirk, P., Fraser, M., Prudhomme, J.C., Couble, P. Germline transformation of the silkworm using a piggyback transposon-derived vector. *Nat. Biotechnol.,* 2000; **1:** 81-84.
35. Tao, H., Shen, Z., Zhu, F., Xu, X., Tang, X., and Xu, L. Isolation and Identification of a Pathogen of Silkworm *Bombyx mori* . Curr Microbiol, 2011; **62:** 876–883.
36. Tian, L., Enen, G., Yupu D., Shun, Z., Qin, P., Yang, C., Erjun, L. and Sheng, L. Genome-wide regulation of innate immunity by juvenile hormone and 20-hydroxyecdysone in the *Bombyx* fat body. *BMC Genomics*, 2010; **1:** 549.
37. Wago, H.. Phagocytic recognition in Bombyx mori. In: Gupta AP ed Immunology of insects and other arthropods. *CRC Press, Boca Raton*, 1991; **pp**:215–235.
38. Whitten, M.M., Ratcliffe, N.A.. In vitro superoxide activity in the hemolymph of the West Indian leaf cockroach, Blaberus discoidalis. *J. Insect Physiol*. 1999; **45:** 667–675.
39. Williams, JG, Kubelik, AR, Livak, KJ, Rafalski, JA. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990; **18:** 6531-6535.
40. Yao, Q., Lim W., Wang, Y., Wang, W.B., Lu, J., Dong., Y,Chen, K.P. Screening of molecular markers for NPV resistance in *Bombyx mori* L. Lep., Bombycidae. *J. Appl. Entomol*. 2003; **127:** 134-136.