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Histological Effect of *Bacillus thuringiensis* Isolate against Pink Bollworm Larval Midgut, *Pectinophora gossypiella* (Saund.)

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ABSTRACT

Experiments were conducted to assess the impact of *Bacillus thuringiensis* isolate against the midgut of larvae for pink bollworm (PBW) *Pectinophora gossypiella* (Saund.). The data illustrated indicated that the LC_{50} value of the tested potent isolate was 3.77 CFU/ml; the confidence limits at (95%) were 2.93- 4.91CFU/ml. In addition, LC_{90} value also determined and it obtained 44.23 CFU/ml. and its confidence limits at (95%) were 23.18- 149.70 CFU/ml. The effects of the bacterial isolate on the midgut of 4th larval instar of the pink bollworm, treated with LC_{50}CFU/ml showed several histological changes some epithelial cells were disintegrated, vacuolated and their cell boundaries were destructed and separated from the basement membrane. The peritrophic membrane was also destructed and detached from the epithelial cells. In general, epithelium layer in untreated specimen was thicker than that in bacterial treated one. The mode and site of action of the active isolates on PBW larvae have been investigated with Transmission Electron Microscope. The Columnar epithelial cells of the midgut showed the muscle layer surrounding the basement membrane lost its normal appearance and degenerated. An increase of deeply infoldings of the basement membrane occurred and the mitochondria of the basal regions were transformed into a condensed form. Separation in columnar and goplet cells occurred and cleared scatting into the gut luman. Microvilli were disrupted showing non-altered appearance. Strong alterations of cytoplasmic structure and organelles occurred. Also, the cytoplasm was distinguished by numerous vacuoles and destroyed brush border. Fragmented chromatin inside the nucleus that has a terminal position destroyed the nucleus sheath.

INTRODUCTION

Bacillus thuringiensis is a gram-positive spore forming bacterium that produces a parasporal crystal protein inclusion during its sporulation. *B. thuringiensis* has become the leading biopesticide since the beginning of the 1960s. The toxicology of *B.t.* is complex and its potency against particular insects varies with the strain of *B.t.* used.

Bacterial infections in insects can be broadly classified as bacteremia, septicemia, and toxemia. Bacteremia occurs when the bacteria multiply in the insect's hemolymph without the production of toxins. This situation occurs in the case of bacterial symbionts and rarely occurs with bacterial pathogens (Durasula *et al*., 1997). Septicemia occurs most frequently with pathogenic bacteria, which invade the hemocoel, multiply, produce toxins, and kill the insect (Wang *et al*., 1993). Toxemia occurs when the bacteria are confined to the gut lumen and produce toxins (Garczynski *et al*., 1991).The spore forming bacilli have received the most attention as biological control agents. Many of them produce proteinaceous insect selective protoxins during sporulation. One member, (*B.t.*), has been used as a microbial pestside against several insect pests, particularly lepidopterans. *B. thuringiensis* is a gram-positive spore forming bacterium that produces a parasporal crystal protein inclusion during its sporulation. *B. thuringiensis* has become the leading biopesticide since the beginning of the 1960s. The toxicology of *B.t.* is complex and its potency against particular insects varies with the strain of *B.t.* used.

Pink bollworm larvae, (PBW) *Pectinophora gossypiella* (Saund.) burrow into cotton bolls to feed on the cotton seeds. In the process they destroy the cotton lint. This feeding damage allows other insects and fungi to enter the boll and cause additional damage. For a long time, pesticide application was the effective control method of this pest. Many problems have been encountered as a result of the extensive use of synthetic pesticides. Increasing problems concerning the application of such pesticides include pest resistance, residue contamination of human foods, mammalian toxicity and pollution of environment. So, many workers used the microbial control against this pest. Bacteria infect insects throughout the mouth and digestive tract, and less commonly through the eggs, integument, and trachea. They may also enter an insect by means of parasitoids and predators.

The experiments were conducted to assess the impact of *Bacillus thuringiensis* isolate on the midgut of larvae for pink bollworm (PBW) *Pectinophora gossypiella* (Saund.).

MATERIALS AND METHODS

1- Soil Samples:

Soil samples were collected randomly from different fields in El-Bahariya Oases, Surface materials of the soil was removed; and with a sterile spatula, about 100 gm sample of soil was taken from at least 5 cm in depth. The soil samples were preserved in sterile plastic bags and stored for 2 - 12 months at 4°C until analyzed. The collection sites had no history of treatment with *B.t.*

2- Isolation Technique, Culturing and enumeration of Bacteria:

Based on the acetate selective method described by (Smith *et al*., 1991), The germinated colonies were fixed to clean slides and stained according to (Smirnoff 1962) stain method, then examined microscopically.

For culturing the obtained isolates, the method Shake Flask Fermentation described by (Morris *et al*. 1996). The method of enumeration of Bacteria described by (Dulmage1971).

3-Origin and maintenance of PBW culture:

The mass rearing of the PBW larvae occurred on the kidney bean diet that previously described by (Abdel-Hafez *et al.* 1982). The original colony of the PBW was supplied from the Plant Protection Research Institute, Agriculture Research Centre. Mass rearing was carried out in the laboratory of the Economic Entomology Unit, Plant Protection Department, Desert Research Center.

4-Method of application on pink bollworm larvae:

Equal weights of artificial media mixed with several concentrations of isolate (1.25, 2.5, 5, 10 CFU/ml) and Provide as food to starved newly hatched larvae of PBW. Food in water only was offered as a control, for each concentration, 10 replicates of 10 larvae each were tested. Numbers of alive and dead larvae were recorded daily till pupation.

5- Statistical Analysis and Assessment of Results:

- Data obtained in different tests were subjected to statistical analysis to evaluate the relative efficiency of the isolate. Mortalities were corrected for the natural mortality according to (Abbot's formula 1925).

The corrected percent = (Observed %-Control %) x 100/ (100-Control %)

Concentration / mortality regression lines were drawn on probit logarithmic graph according to the method developed by (Finney 1971). The LC_{50} and LC_{90} values were calculated according to probane program.

6- Histological Examination of Larval Midgut:

1- By Optical Microscope:

The histological technique and investigations were carried out at the Entomology Department, Faculty of Science, Ain Shams University. Histological sections of the midgut of normal and diseased 4th instar larvae of *Pectinophora gossypiella* (Saund.) larvae treated with LC_{50} of the tested *B.t.* isolate was made following the method of Gad (1951).

2- By Transmission Electron Microscope (T.E.M.):

This technique was carried out at the transmission electron microscope Lab., Faculty of Science, Ain Shams University. Following the method of Van Rie *et al*. (1990)

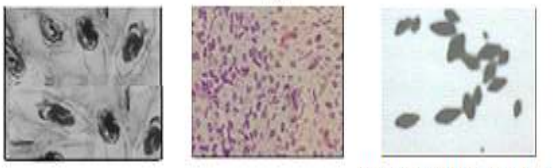
RESULTS AND DISCUSSION

The present study deals, the histological changes in the midgut of 4th larval instar of the pink bollworm larvae (PBW), *P. gossypiella*, treated with LC_{50} CFU/ml. of *B.t.* isolate were studied by using the optical microscope and the transmission electron microscope (T.E.M.).

1-Identification of *B. t.* Isolates:

Based on the acetate selective method described by Smith *et al.,* (1991) positive isolates with *B. thuringiensis* were identified. The germinated colonies obtained from the soil samples were examined microscopically after staining according to Smirnoff (1962). The crystals obtained from the positive isolates were characterized by their bipyramidal shape. The parasporal inclusion bodies (crystals) appeared blue in color; whereas the spores were oval and purple in color (Figure 1). Examination of different isolates of *B. thuringiensis* grown in nutrient broth medium by using the transmission electron microscope showed the release of spores and bipyramidal crystals from the sporangium of 24 hours cultures (Figure 2).

Fig (1):



Bipyramidal crystals | Stained smear of *B.t.* (100x) | Transmission electron micrograph of B.t. grown on nutrient broth medium at 28°C (500)

Enumeration of Bacteria:

After identification of *B.t.* isolate, the average number of bacteria per ml of water was determined. The results showed that the average number of bacterial isolate was (6.70×10^6 bacteria /ml); the colony forming units per ml (CFU) which measure the viable bacterial number.

2- Toxic effect of *B.t.* isolate on pink bollworm larvae:

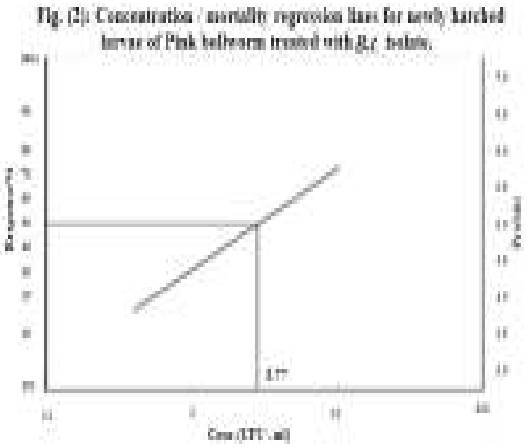
Feeding of newly hatched larvae of PBW to *B.t.* isolate (Table,1) revealed that adverse effects on the total percentage of larval mortality, which was concentration dependent. Whereas the total larval death recorded 5% in control trials, treatment revealed 35 and 74% of larval death at 1.25 and 10 CFU/ml, respectively.

The standard bioassay procedures were followed according to (Dulmage 1971). All bioassays were carried out using newly hatched larvae of PBW. The LC_{50} value of the tested potent isolate were computed from the data obtained on the percentage of larval mortality at each of the tested concentration through probit analyses within 95% confidence limits (Figure 2). The data illustrated indicated that the LC_{50} value of the tested potent isolate was 3.77 CFU/ml; the confidence limits at (95%) were 2.93-4.91CFU/ml. In addition, LC_{90} value also determined and it obtained 44.23 CFU/ml. and its confidence limits at (95%) were 23.18- 149.70 CFU/ml.

Table 1. The total mortality of newly hatched larvae of PBW treated by B.t. isolate

Conc. (CFU/ml)	Total mortality %	
	Obs.	Corr.
0	5	0
1.25	35	31.57
2.5	41	37.89
5	55	52.63
10	74	72.63

100 larvae in 10 replicates were tested



According to the recorded data all applied concentrations of *B.t.* isolate reduced the larval population of pink boll worm. Larval mortality, according to (Yoshinori and Kaya 1993), is probably due to either the septicemia in which the bacterial spores invade the hemocoel, multiply, produce toxin and subsequent kill the insect; or due to the toxemia in which the bacteria produce toxin and confined to the gut lumen. (Abdel-Aziz 2000) attributed the larval mortality to such septicemia case. Mortality in infected larvae may also be due to the deficiency in the excretory system due to Malpighian tubules infection (Lotfy, 1988). These factors individually or together may explain larval mortality. Such results are in harmony with (Abou-Bakr 1997) on *Spodoptera littoralis*. (Desuky 1998) found that when the 2nd larval instar of the cotton leafworm was fed on both clover and cotton leaves, accumulative mortality percents increased by time elapsed after spraying by Delfin till 24 hrs, then decreased, whereas in case of the 4th larval instar the accumulative mortality percents decreased. (El-Sayed *et al.* 1999) found that the tolerance of *Autographa gamma* (Linnaeus) to the pathogen slightly or highly increased as the larvae developed from the 2nd to the 3rd or the 4th instars, respectively.The high percentage of larval mortality, three days post treatment, revealed that, higher susceptibility level of early larval instars of *P. gossypiella* (Saund.) to different concentrations of *B.t.* isolate. The higher susceptibility of young larval instars may be either due to the binding of the bacterial endotoxin to the brush border membrane of the midgut epithelium (Van Rie *et al.*, 1990) or due to certain physiological differences between the early and late instars, where in late instars certain enzymes are secreted due to which tolerance to the bacterial infection may be developed (Goldberg *et al.*, 1974).

3- Histological examination of larval midgut

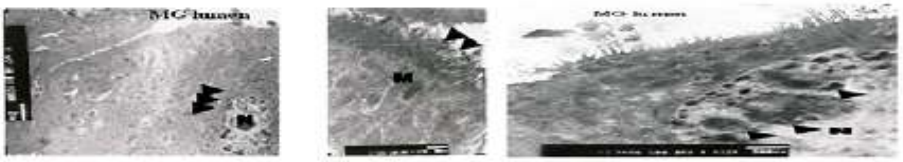
The midgut of the normal 4th larval instar of PBW as shown in Figure (3a) consists histologically of the outer sheath of the intestine, which includes two types of muscles, the outer one is a layer of longitudinal muscles, and the inner one is a layer of circular muscles. The musclosa is followed internally by a thin basement membrane (Bm) upon which the epithelium rests. The epithelium consists of a layer of epithelial cells (Ep), which are elongated and columnar in shape. Each cell contains a dark round nucleus occupying the middle part of each cell. There are other types of epithelial cells, the regenerative (Re) or the imaginal cells, which are small in size, found between the bases of the columnar cells. The regenerative cells are present individually or in clusters of few cells, each cell contains a large nucleus surrounded by a granular cytoplasm. The lumen (Lu) of the ventriculus is surrounded by the peritrophic membrane (Pm), which envelops the food-materials and protects the epithelial cells from contact with the food mass. The midgut of treated *P. gossypiella,* with LC_{50} CFU/ml. of the *B.t*. isolate showed several histological changes compared to the control (Figure, 3 b,c). Some epithelial cells disintegrated and others were vacuolated and their cell boundaries were destructed leaving synthetium-like structure. Also, the peritrophic membrane was destructed and detached from the epithelial cells. In general, epithelium layer in untreated specimen was thicker than that in bacterial treated one.

By using the Transmission Electron Microscope, it appears that the normal mid gut wall of Pink bollworm larvae consists of a single layer of epithelial cells including columnar and goblet cells, lying on a basement membrane, which is attached to the muscle connective tissue. The columnar cells are cylindrical in shape; contain a large granular nucleus (N) occupying the mid base of the. Numerous microvilli appear as striated border on the apical surface of the columnar cells (arrows) Figure. (4a,b). The Cytoplasm is packed with numerous organelles as mitochondria, endoplasmic reticulum and ribosomes. The basement membrane is deeply infolded with mitochondria (M) in a normal form, which is abundant at the base of the cell having a round or cylindrical shape.

Bm: basement membrane Ep: epithelial cells Lu: gut lumen
Pm: peritrophic membrane Re: regenerative cells V: vacuole

After 4th larval instar of *P. gossypiella*, treatment with LC_{50} CFU/ml. of *B. thuringiensis* isolate, some ultrastructural changes were detected in the mid gut of diseased larvae. The muscle layer surrounding the basement membrane lost its normal appearance and degenerated. An increase of deeply infoldings of the basement membrane occurred and the mitochondria of the basal regions were transformed into a condensed form. Separation in columnar and goblet cells occurred and showed scatting into the gut luman. Microvilli were disrupted showing non-altered appearance. Strong alterations of cytoplasmic structure and organelles occurred. Also, the cytoplasm was distinguished by numerous vacuoles and destroyed brush border. Fragmented chromatin inside the nucleus (N) that has a terminal position destroyed the nucleus sheath. (arrows) (Figure, 4c).

Fig.(4): Micrograph (TEM) of the mid gut of full-grown larva of Pink bollworm



a b c

In the present study, several histological changes occured in the midgut tissue of 4th PBW larval instar treated with the tested *B.t.* isolate. the majority of changes are almost similar to those reported for other insect larvae as given by Askary *et al.* (1993) in the European corn borer; Atwa and Abdel-Rahman (1976) and Hong *et al.* (1987) in the cabbage worm; Kinsinger and Mcgaughey (1979) in the Indian meal moth almond moth; Endo and Nishiitsutsuji (1980) in the silk worm; Ingle *et al.* (1997) and Tripathi and Singh (2004) in the cotton bollworm. According to some authors as Van Rie *et al.* (1990), and Zidan *et al*. (1998), the brush border membrane of the midgut epithelium in the susceptible insect is the main site of the bacterial toxic action (binding receptors of the toxin in the mid gut).

The histopathological changes in the midgut of the 4th instar larvae of PBW due to intoxication with *B. t.* could be explained as that; the ingested bacteria consisted of vegetative cells, developed endospores and some librated spores, pass to the foregut without the induction of any pathological action in the foregut epithelium, where according to Nishiitsutsuji and Endo (1980) and Van Rie *et al.* (1990) the midgut epithelium is the main site of *B. t.* action. On reaching midgut both vegetative cells and spores stimulate the gut lumen leading to extra-cellular secreted enzymes (metabolites). These influences the mechanism performed by the peritrophic membrane leading to its detachement and reaches the epithelial cells facing the lumen. The interaction between digestive enzymes secreted by midgut cells and those excreted as a result of *B.t.* secretion form a sort of apportion complex due to which the plasma membrane can permit the bacterial toxin to enter the epithelial cells; and accordingly, disruption, vacuolization and more consequence disintegration of epithelial cells are recorded and which are dose dependent. Such mode of *B. t.* action is also recorded by El-Sayed *et al.* (1999).

From Morris *et al.* (1996) viewpoint, these changes are due to the pathway of *B. t.* produced crystal protein which is dissolved by the alkaline protease within the

insect midgut to toxic form. These proteins bind to specific sites in the gut epithelium generating pores in the cell membrane. Also, the connections between the epithelial cells are loosing by the separation of the cell membrane.

REFERENCES

Abbott, W. S. (1925): A method of computing the effectiveness of an insecticide. Jour. Econ. Entomol., 18: 265.

Abd El-Hafez, Alia , A. G. Metwally, and M.R.A. Saleh (1982) Rearing pink bollworm Pectinophora gossypiella (Saund). on kidney bean diet in Egypt. Res. Bull., Fac. Agric., Zagazig Univ., 576: 1-10.

Abdel-Aziz, S. Hanan (2000): Physiopathological studies on bacterial infection of cotton leaf worm, *Spodoptera littoralis*. M. Sc. Thesis, Fac. Sci., Ain Shams Univ., Egypt.

Abou-Bakr, H. (1997): Efficacy of different *Bacillus thuringiensis* formulations against the cotton leafworm, *Spodoptera littoralis* Boisduval. Egypt. Jour. Biolog. Pest Control, 7 (1): 7-11.

Askary, H.; Kharazi-Pakdel, A.; Behbudi, S. B. and Moazami, N. (1993): Histopathology of midgut of European corn borer (*Ostrinia nubilalis persica* Mutuura & Munroe, 1970) (Lep-P Pyraustinae) Larvae treated with *Bacillus thuringiensis* var. *kurstaki.* [Persian] Jour. of Entomological Society of Iran, 12&13: 14-15 (En).

Atwa, W. A. and Abdel-Rahman, H. A. (1976): Histopathological effect of *Bacillus thuringiensis* in larvae of *Pieris rapae*. Z. angew. Entom., 79: 326-331.

Desuky, H. M. W. (1998): Susceptibility of *Spodoptera littoralis* larvae to the microbial insecticide Delfin in the cotton and clover fields. Jour. Appl. Sci., 13 (1): 242-249.

Dulmage, H. T. (1971): Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. alesti. Jour. Invertebr. Pathol., 15: 232–239.

Durasula, A. N.; Heimpel, A. M. and Angus, T. A. (1997): The site of action of crystalliferous bacteria in Lepidoptera larvae. Jour. Insect Pathol., 1: 152-170.

El-Sayed, M. F. A.; Abdel-Latif, A. and El-Lakwah, F. A. (1999): Toxicity and joint action of the botanical insecticide NeemAzal W with malation to the cowpea weevil, *Callosobruchus maculatus* F. Egypt. Jour. Agric. Res., 77 (3): 1023-1034.

Finney, C. E. (1971): Probit Analysis, A statistical treatment of the sigmoid response curve 7th Ed. Cambridge Univ. Press, Cambridge, England FEMS Microbial. Lett. 231: 45–52.

Gad, A. M. (1951): The head capsule and mouthparts in the Ceratopogonidae (Diptera: Nematocera) Bull. Soc. Entomol. Egypt, 35:17-75.

Garczynski, U. J. and Endo, Y. (1991): Mode of action of Bacillus thuringiensis endotoxin: General characteristics of intoxicated Bombyx larvae. Jour. Invertebr. Pathol., 35: 219-228.

Goldberg, L. J.; Ford, I. and Singer, S. (1974): *Bacillus thuringiensis* var. *fusiformis* as a potential pathogen against *Culex trasalis* and *Culex pipiens*. Cal. Mosq. Cont. Assoc., 42: 81-82.

Hong, H.; Su, P. and Xu, H. (1987): Observations on the histopathology of *Pieris rapae* infected with *Bacillus thuringiensis*. Chinese Jour. of Biological control, 3 (1), 27-29.

Ingle, S. S.; Shah, M. P.; Vaidya, A.; Chhatpar, H. S. and Rao, K. K. (1997): Effect of delta-endotoxin of *Bacillus thuringiensis* var. *kurstaki* on midgut of *Helicoverpa (=Heliothis)* armigera. Indian Jour. of Experimental Biology, 35 (1): 83-85.

Kinsinger, R. A. and Megaughey, W. H. (1979): Histopathological effects of *Bacillus thuringiensis* on larvae of the Indian meal moth; *Plodia interpunctella* and the almond moth; *Ephestia cautella*. Annals of the Entomological Society of America, 72 (6): 787-790.

Lotfy, M. Nadia (1988): Pathogenesis of *Bacillus thuringiensis* toxic crystals in larvae of the silkworm, *Bombyx mori*. Ph. D. Thesis, Fac. Sci., Ain Shams Univ. Egypt.

Morris, O. N.; Trottier, M.; Converse, V. and Kanagaratnam, P. (1996): Toxicity of *Bacillus thuringiensis* subsp. *aizawai* for *Mamestra configurata* (Lepidoptera: Noctuidae). Jour. Econ. Entomol., 89 (2): 359-365.

Nishiitsutsuji, U. J. and Endo, Y. (1980): Mode of action of *Bacillus thuringiensis* endotoxin: General characteristics of intoxicated *Bombyx* larvae. Jour. Invertebr. Pathol., 35: 219-228 .

Smirnoff, W. A. (1962): A staining method for differentiating spores, crystals and cells of *Bacillus thuringiensis* Berliner. Jour. Invertebr. Pathol., 4: 384-385.

Smith, R. A. and Couche, G. A. (1991): The phylloplanc as a source of *Bacillus thuringiensis* variants. Applied and Environmental microbiology, 57: 311-315.

Tripathi-Rashrni and Singh, N. P. (2004): Bioefficacy of commercial BTK and the histopathology in fourth instar of *Helicoverpa armigera.* Jour. of Experimental Zoology, 7 (2): 349-352.

Van Rie, J.; McGaughey, W. H.; Johnson, D. E.; Barnett, B. D. and Van Mellaert, H. (1990): Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Sci., 247: 72-74.

Wang, N.; Hong, H.; and Xu, H. (1993): Observations on the histopathology of Pieris rapae infected with *Bacillus thuringiensis*. Chinese Jour. of Biological control, 3 (1), 27-29.

Yoshinori, T. and Kaya, H. K. (1993): Bacterial infections: Bacillaceae. In: "Insect pathology." Published by Academic press INC., 83-146.

Zidan, Z. H.; Abdel-Megeed, M. I.; Abdel-Hafez, A.; Hussein, N. M.; El-Gemeiy, H. M. and Shalaby, M. M. (1998): Toxicological and histological studies of *Bacillus thuringiensis*, MVPII against larvae of pink and spiny bollworms., Ann. Agri. Sci., 1: 319-332.

التغيرات الهستولوجيه الناتجه من تأثير عزله من البكتريا الممرضه للحشرات علي المعي الاوسط لدودة اللوز القرنفليه

إيمان ابراهيم إمام

قسم وقاية النبات ـ وحدة الحشرات الإقتصادية ـ مركز بحوث الصحراء

أجريت هذه الدراسة لتقييم تأثير سلالة من البكتيريا الممرضة للحشرات *باثليس ثيرونجينسيس* علي المعي الاوسط ليرقات دودة اللوز القرنفلية ومن البيانات يتضح ان قسمه الجرعة قاتلة النصف LC_50 للعمر اليرقي الرابع كانت 3.77 CFU/ml، في حين كانت الجرعه التي تميت 90% من الحشرات المستخدمه 44.23 CFU/ml. أظهرت معاملة يرقات العمر الرابع لدودة اللوز القرنفلية بالعزله البكتيرية العديد من التغيرات الهستوباثولوجية في المعي الاوسط حيث تهتكت بعض الخلايا الطلائية، وظهربها بعض الفجوات، وانفصلت عن الغشاء القاعدي، كما انفصلت أيضا عن الغشاء حول الغذائي الذي تحطم أيضا، وأجمالا كانت الطبقة الطلائية أكثر سمكا في اليرقات غير المعاملة عنها في تلك المعاملة بالبكتريا. وعند الفحص بأستخدام المجهر الإلكتروني النافذ أظهرت الخلايا الطلائية للمعي الاوسط لليرقات المعاملة بالبكتريا كروماتيد مفتت داخل النوايا، مع تغير شديد في عضيات السيتوبلازم، وأحتوائه علي فجوات، وتحطم شعيرات المحيط الداخلي. وظهور تهتك واضح في خملات الامتصاص الخاصة بالخلايا الطلائية.