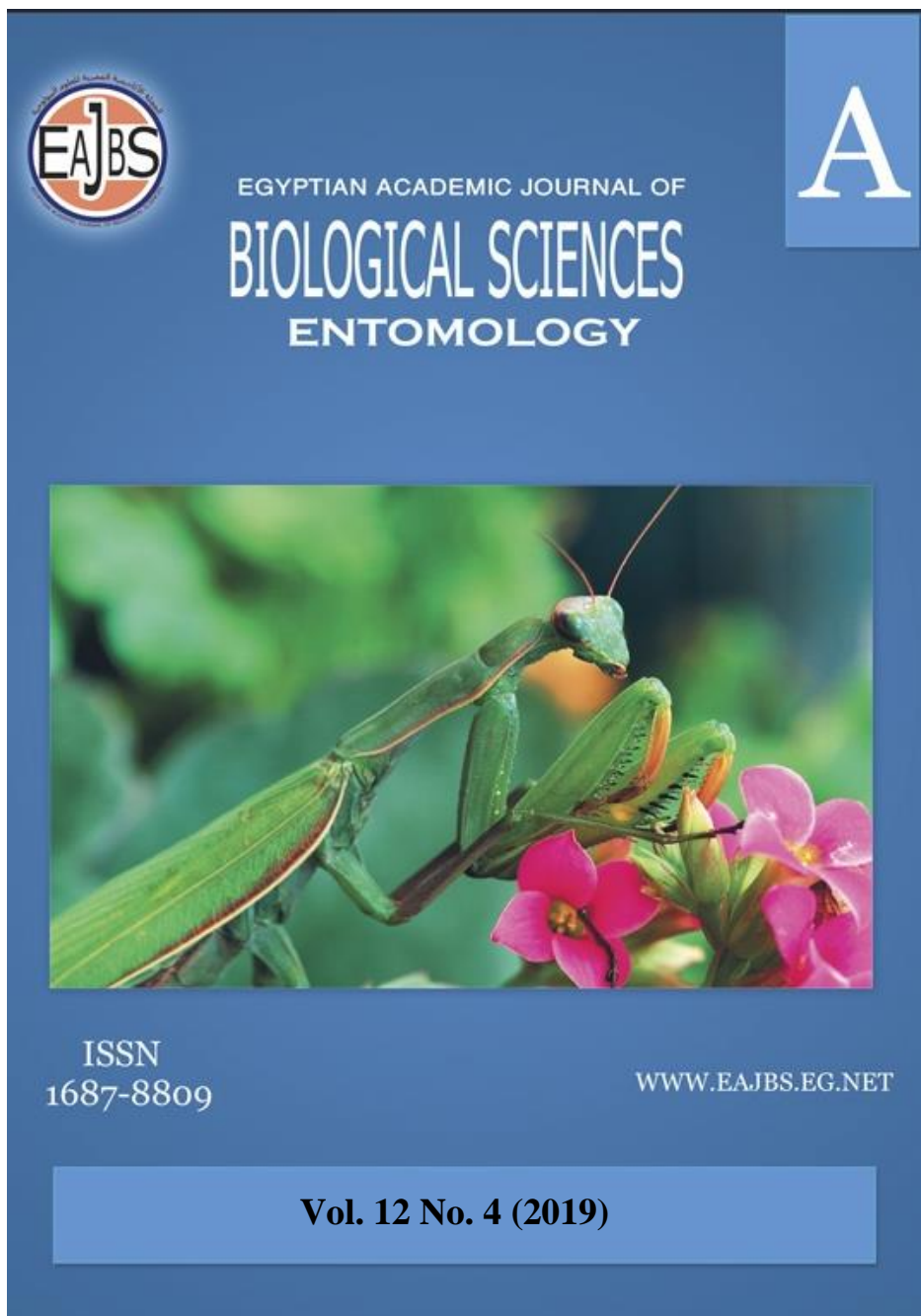


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Induction of Antimicrobial Peptides in the Hemolymph of *Spodoptera littoralis* Larvae Following Treatment with *Salmonella typhimurium*

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ARTICLE INFO

Article History

Received:29/5/2019

Accepted:15/6/2019

Keywords:

Spodoptera littoralis,
Salmonella typhimurium, LC₅₀,
immunization, cell-free hemolymph, electrophoresis, antimicrobial peptides, Gram-negative and Gram-positive bacteria

ABSTRACT

This study investigated the efficiency of intrahaemocoelic injection of sublethal concentration (LC₂₀ = 5.7×10³ cells/ml) of *Salmonella typhimurium* in the induction of immune response in 5th larval instar of *Spodoptera littoralis*. The role of antimicrobial peptides (AMPs) induced in the larval hemolymph was examined as natural antibiotics against different Gram-negative (G-ve) or Gram-positive (G+ve) bacteria. Where the larval plasma injected with *Salmonella* showed a significant antimicrobial activity against different strains of pathogenic bacteria (*Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Enterococcus faecalis*). The biochemical characterization of the immune hemolymph indicated drastic changes in both the total protein content and the protein banding patterns following bacterial injection. The total hemolymph proteins (THPs) decreased significantly 48 h post-*Salmonella*-injection. The hemolymph proteins profile was also qualitatively changed through the induction of new proteins and the disappearance of others simultaneously, which may be attributed to their participation in the immune reactions. The appearance of six new synthesized protein bands that were found to be specific for *Salmonella* injection, with a characteristic band of MW ~22 KDa predicting that this protein band may be Attacin-like AMP.

INTRODUCTION

Insects possess a qualified innate immune system, which allows rapid reactions to infectious factors. The infection process in insects relative to mammals is much faster, yielding effect more rapidly. Many studies have performed using insects as an alternative model host for investigating virulence factors of human pathogenic bacteria (Scully and Bidochka, 2006; Lionakis, 2011; Junqueira, and Mylonakis, 2019), and this substitution has several benefits.

One of the most global foodborne pathogens is *Salmonella*. It is a Gram -ve, rod-shaped (bacillus) facultative anaerobe, related to the family Enterobacteriaceae (Barlow and Hall, 2002). About 2600 serotypes have been identified within the genus *Salmonella*, most of them can adapt within most animal hosts, including humans (Allerberger *et al.*, 2003). Mostly all

Salmonella strains are pathogenic as they are able to invade, survive and replicate in human host cells, causing vigorous diseases.

Based on clinical diagnosis in human salmonellosis, *Salmonella* strains grouped into typhoid *Salmonella* and non-typhoid *Salmonella* (NTS). The antimicrobial resistance in *Salmonella* strains is a global serious health problem (Chiu *et al.*, 2002), stimulated by the usage of antibiotics in animals feed to enhance the growth of food animals, and their use in veterinary medicine for treating bacterial infections in these animals (Hyeon *et al.*, 2011).

Recently antimicrobial peptides represent a new field of antibiotics that have motivated research and clinical awareness as new therapeutic choices for many infections caused by multidrug-resistant bacteria (Giuliani *et al.*, 2007). The induced AMPs in insects mediates a momentary humoral immune response which lasts more than the initial cellular responses, and which is supposed to function as a back-up against persistent infections (Makarova *et al.*, 2016). Developed studies have led to recognizing many types of host defense peptides, including cecropins (Boman, 2000), defensins (Lehrer, 2004), and others with dissimilar structures and bioactivity profiles (Wang, 2017). Moreover, many AMPs show great effect against antibiotic resistant bacteria and possess low probability for developing resistance (Kendurkar and Sengupta, 2018).

Novel AMPs can be recognized by their similarity to known peptides, or by other features (Vilcinskas, 2011; Vogel *et al.*, 2011). Scientists classmate insect AMPs into three major structural classes according to their structure or function which are; linear α -helical peptides without any cysteine residues such as cecropins, peptides with a β -sheet globular structure stabilized by intramolecular disulfide bridges necessary for AMP activity such as defensins, and peptides containing high numbers of specific amino acid residues, such as glycine or proline (Brogden, 2005; Hancock and Sahl, 2006; Wiesner and Vilcinskas, 2010 and Yi *et al.*, 2014). According to specific mechanism of action of insect AMPs some possess a wide range, whereas others show varying degrees of specificity towards Gram-negative (G^{-ve}) or Gram-positive (G^{+ve}) bacteria, parasites, fungi, and even viruses (Vilcinskas, 2011; Pretzel *et al.*, 2013).

The interaction of those AMPs with the lipophilic, negatively charged membranes of bacterial cells is a result of that insect AMPs possess a net positive charge and contain up to 50% hydrophobic residues. So, AMPs are attached to bacterial cell membranes electrostatically, and just contact is established the hydrophobic residues enhance integration, causing the membrane outer leaflet to extend and become thinner, subsequently forming pores or even causing lysis (Bulet and Stocklin, 2005; Brown and Hancock, 2006; Wiesner and Vilcinskas, 2010).

For several past decades, antimicrobial resistance increased threats to the effective treatment of many infections caused by parasites and disease agents. Such resistance results in reducing the efficacy of antiparasitic, antibacterial, antifungal and antiviral drugs, making patients treatment more difficult, expensive, or mostly impossible. If governments fail to act on antimicrobial resistance, 10 million additional lives would be lost per year because of drug-resistant strains of malaria, human immunodeficiency virus (HIV), tuberculosis (TB), and other certain bacterial infections (O'Neill, 2014). Therefore, AMPs may act as a novel antibiotic generation and can complement traditional antibiotic therapy, as they are active in animal models, neutralize endotoxin and show synergy with conventional antibiotics. Minimal bactericidal concentrations (MBC) and minimal inhibitory concentration (MIC) often coincide (with less than a two-fold difference), indicating that killing is mainly the most desirable bactericidal mode of action (Toke, 2005).

This study aimed to investigate the efficiency of intrahaemocoelic injection of sublethal concentration of *S. typhimurium* in the induction of immune response in *S. littoralis* larvae. Biochemical characterization of the immune hemolymph and the role of AMPs induced in

the larval hemolymph were also examined as natural antibiotics against different G +ve and G -ve bacteria.

MATERIALS AND METHODS

Experimental Insect:

The cotton leaf worm, *Spodoptera littoralis* used in this study was obtained from the Cotton Leaf-worm Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. The colony was reared and established in our laboratory as reported by Rivnay and Meisner (1966). All the experiments outlined below were performed using the fifth-instar larvae with a group of 20 larvae and repeated using larvae from another different batch.

Bacterial Pathogens:

Bacteria used in this study; *Staphylococcus aureus* ATCC[®] 6538, *Salmonella enterica* subsp. *enterica* serotype *Typhimurium* ATCC[®] 14028, *Escherichia coli* NCTC 13127 VTEC / STEC O157 and *Enterococcus faecalis* ATCC[®] 29212 were obtained from Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food (QCAP), Giza, Egypt. The tested bacteria were grown aerobically at 37 °C in nutrient broth tubes for 24 - 48 h, cultured on Tryptic Soy Agar (TSA) (Oxoid CM0131) media by applying streaking dilution method, and then identified under the light microscope after applying Gram-staining (Gram, 1984). For long-term bacterial storage, cryobeads (CryoBank[®] Red, code CRYO80R) were used according to the method described by Jones *et al.* (1991).

Concentration Mortality Assay:

A stock suspension of *S. typhimurium* was adjusted to a concentration of 1.5×10^8 cells/ml by using the pour plate count technique according to Sutton (2011). From the stock, serial concentrations; 1.5×10^3 , 1.5×10^4 , 1.5×10^5 , 1.5×10^6 , 1.5×10^7 and 1.5×10^8 cells/ml were prepared and 5 μ l of each were injected into each of 20 *S. littoralis* larval groups. Control insects were injected with equivalent volumes of distilled water only. Final mortality percentages were recorded 48 h post-injection. For bioassay test, the LC₅₀ value was estimated according to Finney *et al.* (1971).

Insect Immune Challenge:

A bacterial suspension of *S. typhimurium* that produces 20% larval mortality was prepared to be injected into the hemocoel of the experimental larvae. Three groups of fifth larvae instar of *S. littoralis* were divided into; normal insects, (negative control, kept without any manipulation), control insects (positive control, injected with sterile distilled water to get the impact of physical shock), insects injected with G-ve bacteria (*S. typhimurium*). A Larval injection was made using a 10 μ l Hamilton micro-syringe fitted with a 26-gauge needle according to Meylaers *et al.* (2007).

Hemolymph Collection:

Samples of hemolymph from (normal, control and immune challenged larvae) were collected 48 h after injection. Fifth instar larvae were chilled for 15 min on ice, to slow down hemolymph coagulation and reduce the larval activity, and then dried on a piece of absorbent paper. Chilled insects were surface sterilized with 70% ethanol. Larvae were bled by puncturing the cuticle on the first proleg with a fine sterile dissecting needle, taking care not to puncture the gut or other organs, and the hemolymph was collected by capillary suction with a fine-tipped calibrated glass capillary and immediately transferred into sterile and chilled Eppendorf tubes containing 1 mg of phenylthiourea (Sigma chemical) to prevent melanization, which was kept at -20°C until further analyses.

Preparation of Cell-Free Hemolymph:

The collected hemolymph was cold centrifuged at 6000 rpm for 20 min at 4°C for

separating the hemocytes (Human Centrifuge, TGL-16XYJ-2, 16000 rpm, Korea). The supernatant (referred to as plasma) was removed from the hemocyte pellet, and immediately transferred into sterile and chilled Eppendorf tubes and stored at -18°C until use. Under such conditions, the pure plasma did not show any sign of coagulation for several days at room temperature, but coagulation could be triggered if hemocytes were added.

Antimicrobial Susceptibility Test:

To determine the appearance of antibacterial activity in the hemolymph of injected insects, and the role of the induced antimicrobial peptides against different bacteria, the disc diffusion technique was used (Matuschek *et al.*, 2014).

Muller Hinton agar (Oxoid) is used for the tested bacteria as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Agar was distributed in sterile Petri dishes to reach a depth of 4.0 ± 0.5 mm. To prepare the inoculum suspension, several morphologically similar colonies were selected from an overnight growth (16–24 h of incubation on tryptic soy agar (Oxoid), a non-selective medium) with a sterile cotton swab and were suspended in sterile saline (0.85% NaCl w/v in water). The density of the bacterial suspension was compared visually to a McFarland 0.5 turbidity standard and adjusted by the addition of saline or more bacteria. Then a sterile cotton swab was dipped into the suspension, the excess fluid was removed by pressing and turning the swab against the inside of the tube. The agar plates were swabbed in three directions to make sure that the inoculum was spread regularly over the plate's entire surface leaving no gaps. 4mm diameter Whatman No. 1 filter paper disks were saturated with 30 μl of the tested plasma. Tetracycline antibiotic disks (30 $\mu\text{g}/\text{ml}$) (Sigma) were used as a positive control for the experiment. These disks were applied firmly to the agar surface within 15 min of the inoculation time. The plates were inverted and incubated for 16–20 h at $35 \pm 1^{\circ}\text{C}$ within 15 min of the disks application. When the plates were held obliquely to transmit light, a distinct zone clear of bacterial growth was seen surrounding the disks that contained immune plasma. The actual zone width was measured as:

$$\text{Zone width} = \text{zone diameter} - \text{disk diameter}$$

Estimation of the Hemolymph Total Protein Content:

The total protein content of plasma was measured spectrophotometrically at 595 nm (UNICO Spectrophotometer, SP2100 UV, China) according to the method described by Bradford (1976). The total protein content was estimated as mg/ml using the following formula derived from the equation obtained from the standard calibration curve of the Bovine serum albumin solution (BSA);

$$\text{Protein concentration} = (\text{Absorbency} - 0.1466) / 0.6854 \text{ (mg/ml)}$$

Electrophoretic Analysis of the Hemolymph Proteins:

One-dimensional gel electrophoresis was carried out for the hemolymph proteins in vertical polyacrylamide gels. Electrophoresis conditions and procedures were as reported by Laemmli (1970).

RESULTS

Microscopical Identification of *S. typhimurium*:

Microscopically, Gram stain of *S. typhimurium* showed G^{-ve} bacilli with pink to red color. They appeared in an irregular arrangement, arranged singly or found in pairs (Plate I, A). Bacterial colonies were cultivated on tryptic soya agar medium for 24 h at $37 \pm 2^{\circ}\text{C}$. *Salmonella typhimurium* appeared in medium sized, smooth colonies, beige in color. Most colonies appeared opaque, but some are translucent. The grown colonies also showed entire margin (Plate I, B).

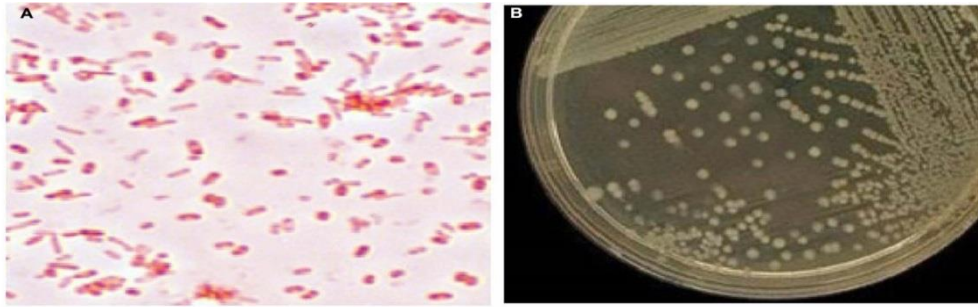


Plate I: Photomicrographs of A) Bacterial cells of *Salmonella typhimurium*.
B) *Salmonella typhimurium* colonies cultivated on tryptic soya agar media.

Susceptibility of *S. littoralis* Larvae to *Salmonella typhimurium*:

Data gained were illustrated in Table 1. The estimated LC₅₀ value, at 95% probability, were 2.4×10^5 cells/ml, while the estimated LC₂₀ were 5.7×10^3 cells/ml. These concentrations were found to enhance the immune response of the cotton leaf worm larvae and at the same time did not cause high mortality rates.

Table 1: Results obtained by Ldp analysis for bioassay test of 5th instar *S. littoralis* larvae to *S. typhimurium* injection

Concentration (cells/ml)	Dead/ total	Observed mortality (%)	Expected mortality (%)
1.5×10^3	2.7/20	13.50	12.68
1.5×10^4	5.3/20	26.50	26.67
1.5×10^5	9.0/20	45.00	45.84
1.5×10^6	13.3/20	66.50	66.06
1.5×10^7	16.0/20	80.00	82.45
1.5×10^8	18.9/20	94.50	92.66
Control	0/20	-	-
Chi ²	0.202		
Slope	0.5186 ± 0.0861		
LC ₅₀	2.4×10^5 cells/ml		
LC ₂₀	5.7×10^3 cells/ml		

Antimicrobial Susceptibility Test:

The bactericidal activity of the cotton leaf worm plasma was performed against *S. aureus*, *S. typhimurium*, *E. coli* and *E. faecalis* bacteria. Results were demonstrated graphically in Figure 2 and photographically in Plate II. A weak antibacterial activity was observed in the hemolymph of the 5th instar larvae, against the tested bacteria. While the inhibition zone in water-injected larvae did not show any significant difference with the normal larval serum. In addition, a significant induction of antimicrobial activity in the larval serum injected with *Salmonella* against *S. aureus* and *S. typhimurium* as compared with the positive and negative control (7.50 ± 0.50 and 7.20 ± 1.15), respectively. While the bactericidal activity in *E. coli* and *E. faecalis* bacteria showed no significant difference.

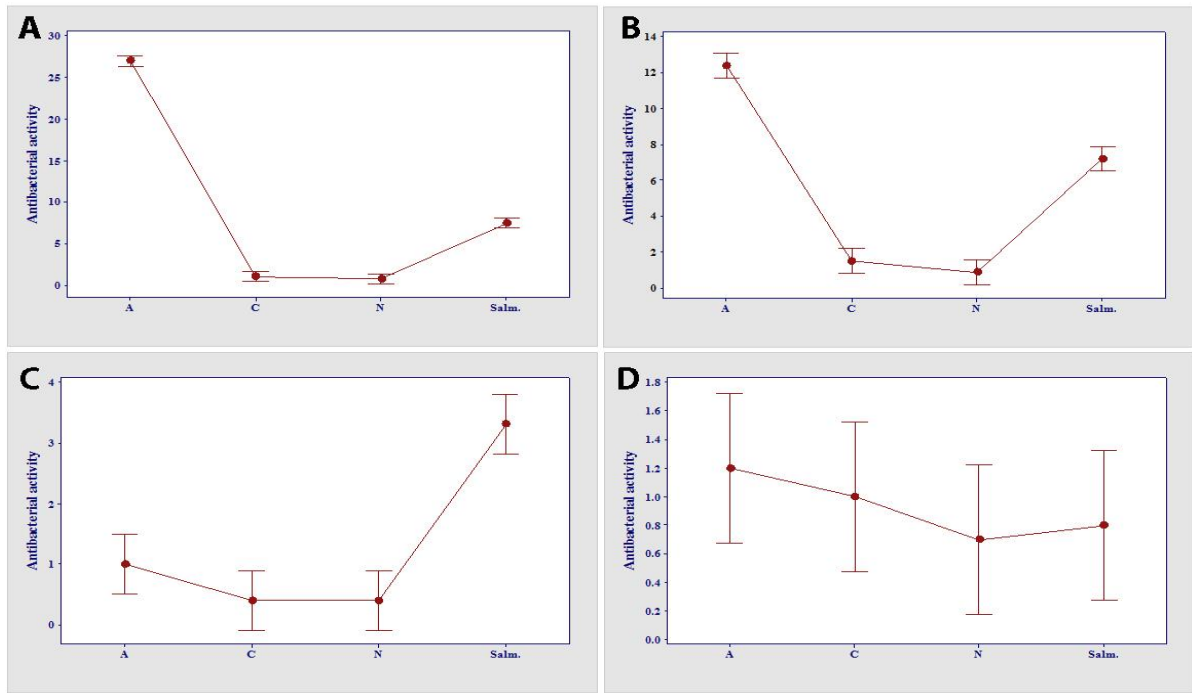


Fig. 2: Antimicrobial activity test against *Salmonella typhimurium*

(A) *S. aureus*; (B) *Salmonella typhimurium*; (C) *E. coli* and (D) *Enterococcus faecalis*.

A: antibiotic disc; C: Control; N: normal plasma; salm: plasma of *Salmonella* injected larvae

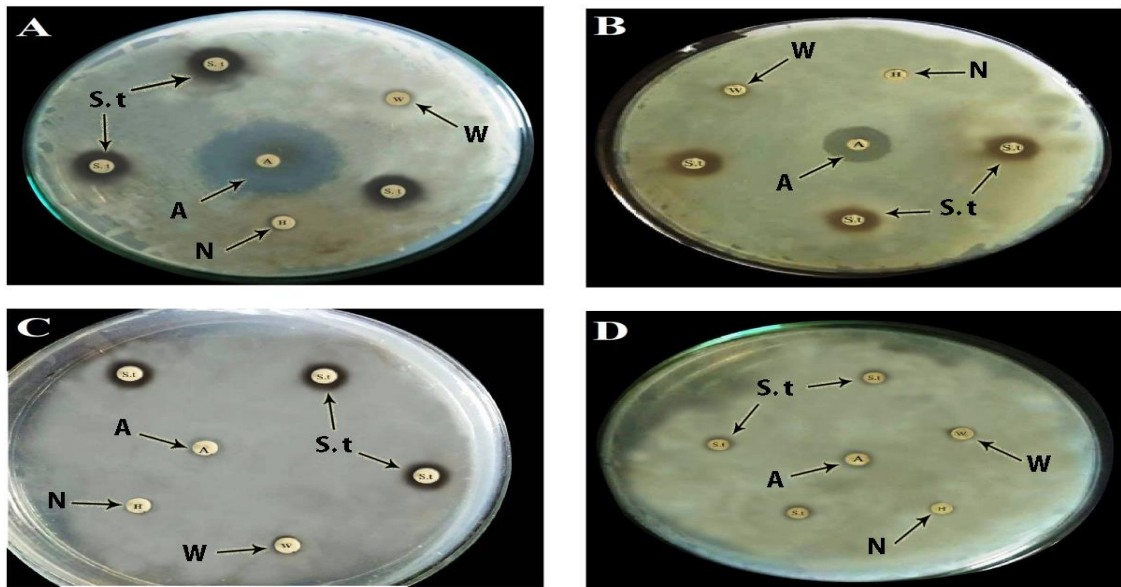


Plate II: Photomicrograph of inhibition zone of antibacterial activity test of different treatments against different bacteria; (A) *S. aureus*; (B) *Salmonella typhimurium*; (C) *E. coli* and (D) *Enterococcus faecalis*.

Disc-A (antibiotic disc; tetracycline); Disc-N (normal plasma); Disc-W (plasma of water-injected larvae) Disc-S.t. (plasma of *Salmonella typhimurium* injected larvae)

Effect of *S. typhimurium* on *S. littoralis* Total Haemolymph Proteins (THPs):

Results of the total hemolymph protein content of the 5th larval instar of the cotton leaf worm (normal, water-injected and *S. typhimurium*-injected larvae (48 h post-injection) were graphically assembled in Figure 3. No significant change ($P=0.09$) in the hemolymph protein

content of water-injected insects was observed as compared with normal insects, while the total haemolymph protein content of *salmonella* injected larvae was decreased significantly ($P=0.002$) than that of control.

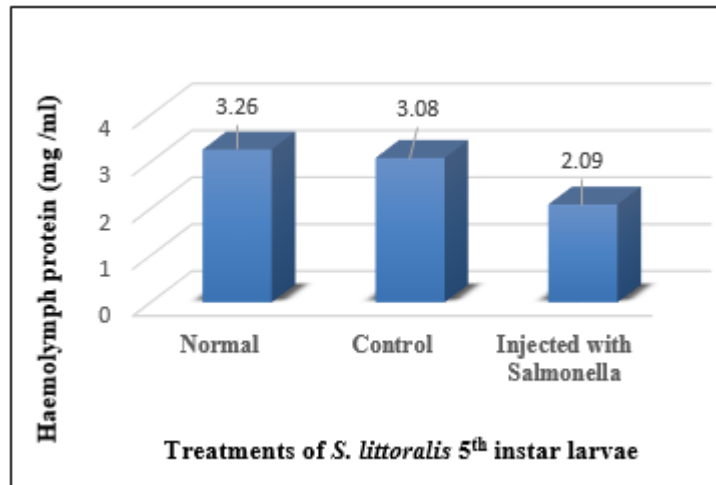


Fig. 3: Total protein content (mg/ml) of haemolymph of *S. littoralis* 5th instar larvae determined post-injection with *Salmonella typhimurium*.

Electrophoretic Separation of *S. littoralis* Hemolymph Proteins:

Protein profile of hemolymph plasma of 5th instar *S. littoralis* larvae were carried out for normal larvae as well as control and treated ones. Data are represented in Plate III, where the protein markers were separated into 10 bands with molecular weights (MW) ranged between 315 and 10 kDa. The hemolymph of the un-injected larvae was separated into eleven protein bands. Some proteins were disappeared or at least had different band percentage post injection with water or bacteria. Moreover, new bands were induced to synthesize, where seven new protein bands were detected as a response of water injection and six new synthesized protein bands were found to be specific for *Salmonella* injection. Plasma proteins from larvae injected with *Salmonella typhimurium* were separated into 14 bands with MW ranging from 350 to 15 kDa, with a major band of MW 22 KDa. Results also showed that there are three major bands were common between normal, control and treated larvae. Three bands were common between control and treated larvae, appeared to be specific for injection; and four bands were found to be specific for *Salmonella* injection

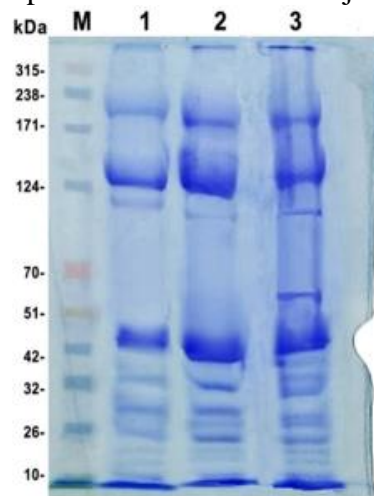


Plate III: Changes in the plasma protein banding patterns of *S. littoralis* normal, control and *Salmonella* injected larvae M: protein molecular weight marker; 1: plasma from normal larvae; 2: plasma from control larvae and 3: plasma from *Salmonella* injected larvae.

DISCUSSION

The global wide spreading of antibiotic resistant bacteria is the main threat to human health. Even with the great progress in the knowledge of the resistance mechanisms, the solution to this problem is still elusive. Recently much attention has been paid to AMPs as natural antibiotics, which are presumably protected from resistance development in bacteria. This study aimed to investigate the efficiency of intrahaemocoelic injection of sublethal dose of *S. typhimurium* in the induction of immune response in *S. littoralis* larvae. It forms a part of a wide study aims to obtain more information about the probability of considering insect AMPs as promising class of therapeutics that can be alternative to traditional antibiotics.

Salmonella is one of the most frequently isolated foodborne pathogens. It is a major global public health problem, predominantly found in eggs, poultry and dairy products (Silva *et al.*, 2011), fresh fruits and vegetables (Pui *et al.*, 2011). Transmission of zoonotic disease with multidrug resistant *Salmonella* strains from infected animals to humans through the ingestion of water or food contaminated with animals' faeces or through eating infected food animals (Eng *et al.*, 2015). In most human infections, four different clinical manifestations occur, which are enteric fever, bacteraemia, gastroenteritis and other extraintestinal complications (Sheorey and Darby, 2008). Due to the evolution of antimicrobial resistance in *Salmonella* strains, stimulated by the usage of antibiotics in animals feed to enhance the growth of food animals, and their use in veterinary medicine for treating bacterial infections in these animals (Hyeon *et al.*, 2011), *S. typhimurium* was chosen in this study as inducer to obtain a complete induction of AMPs, in the target insect hemocoel at sublethal dose.

Firstly, microscopic examination of the studied *S. typhimurium*, bacterial cells with Gram stain did not hold the crystal violet stain giving a pink to red color being G -ve bacilli. These results from the formation of an insoluble crystal violet-iodine complex inside the cell can be easily extracted by alcohol from G -ve bacteria. Also, alcohol penetrates the lipid-rich outer layer easily since they possess a thin layer of peptidoglycan cannot prevent solvent passage, thus, the stain complex is easily removed, and they hold the counter stain, safranin giving the red to pink color (Henderson *et al.*, 2016).

Spodoptera littoralis was the insect chosen in this research, since it had proved a great success in this kind of immunological studies a long time ago till now (Paterson *et al.*, 1987; Seufi *et al.*, 2011; Basiouny *et al.*, 2016). This comes from the fact that they can be easily reared with inexpensive media and materials, can be maintained in large numbers, easily identified, have a fast lifecycle and they possess large blood volume.

Since bacteria can produce septicemia in insects when invading the hemocoel through the gut, the pathogen used in the present study was placed in a direct contact with the hemocoel by injection, so that variations induced by dose losses and invasion irregularities was avoided and controlled (Barakat, 1997). Several researchers intended to use this technique particularly for the study of insect immunity and the observation of physiological and biochemical changes induced by pathogenic infection: Mukherjee *et al.* (2010), Momen *et al.* (2012), de Viedma and Nelson (2017) and Parthuisot *et al.* (2018) using different insects and different pathogenic bacteria.

Based on qualitative descriptions on the way *S. littoralis* larvae were infected with the chosen bacteria *S. typhimurium* and the resulting mortality patterns, this study clearly demonstrated that the 5th instar larvae of *S. littoralis* are highly susceptible to *Salmonella* infection ($LC_{50} = 2.4 \times 10^5$ cells/ml), this may be due to G -ve bacteria are of great pathogenicity. So, a smaller number of *Salmonella* bacterial cells can easily induce infection (Beveridge, 1999). This may be attributed to the outer membrane in G-ve bacteria which is made up of lipopolysaccharide (LPS) which acts as an endotoxin, one of the most important virulent bacterial components in pathogenicity contributing to many inflammatory processes

(Raetz and Whitfield, 2002; Henderson *et al.*, 2016; Monteiro *et al.*, 2017).

Results obtained using antimicrobial susceptibility test, proved that normal insects exhibit a very weak antibacterial activity towards virulent bacteria without receiving any antigenic challenge. In this study, larval hemolymph was collected by centrifuging the insects after incising them through their bodies. Considerable cellular damage resulted from such treatment may explain the weak humoral antibacterial response appeared in normal insects without receiving any antigenic challenge. This result is supported by Yoon *et al.* (2018) who found a weak antibacterial activity in the studied insects as the fatty acids, lipids of sterol type and monoglycerides are known to exert antibacterial activity *in vitro*. These substances are liberated upon cells destruction or any change in the cell's natural environment. They act upon bacteria causing destabilization of bacterial cell membranes leading to a wide range of indirect and direct inhibitory effects consequently; the amount of antibacterial activity detected in the blood of normal insects should be proportional to the degree of cell destruction or to the degree of alteration of the normal environment.

Another important part in the study of immune induction is the control injection, a phenomenon known to all investigators working on insect immunity. Earlier studies indicated that the appearance of antibacterial activity and synthesis of new immune proteins are not only induced by bacteria, but also through the injection of sterile saline solutions (Barakat *et al.*, 2002 and Meshrif, 2008) or distilled water (Mo'men *et al.*, 2012).

The present investigation affirmed that injection of a bacterial virulence mediates a higher activation of *Spodoptera* immune response. Where larval plasma injected with *Salmonella* showed significant induction of antimicrobial activity against *S. aureus* and *S. typhimurium* as compared with the positive and negative control. This may be attributed to the fact that *Salmonella* is a more virulent pathogen though enhances the insect immune system to produce more and more antimicrobial peptides. This virulent effect comes from LPS which is one of the great virulence determinants of possessing a specific structure that might be a reliable indicator of virulence potential. This structure is composed of 3 domains: lipid A (endotoxin), O antigen and an oligosaccharide core (Murray *et al.*, 2003). Rahman (1997) stated that the O antigen group in the LPS has the main role in the bacterial pathogenicity.

Hemolymph of *S. typhimurium*-injected *S. littoralis* larvae recorded drastic changes in both the total protein content and the protein banding patterns following injection. Results achieved in our study confirmed that after larval microbial immune challenge, the total hemolymph proteins (THPs) decreased significantly 48 h post-injection. This can be attributed to the intensive consumption of plasma proteins during multiplication and growth of bacteria. Also, some hemolymph sticky proteins and soluble proteins may be involved in the attachment of the injected pathogen to the hemocytes or some native proteins might be converted into glycoproteins or lipoproteins after injection. The same explanation was also reported by Abdeen *et al.* (1986), Sabbour (2001), Meshrif (2008) and Beament *et al.* (2011).

There were great variations in the number, kinds and percentage of protein fractions of *S. littoralis* larval plasma, where they totally lie within MW ranged between 14 and 350 kDa. Water and bacterial injection into the larval hemocoel were capable of changing the hemolymph proteins profile qualitatively through the induction of new proteins and the disappearance of others simultaneously, which may be attributed to their participation in the immune reactions.

Protein band of MW 22 kDa was found to be a characteristic band in the plasma of *Salmonella*-injected larvae. From the literature data, we deduced that it may be a glycine-rich AMP (Attacin). This confirmed by the results obtained in many lepidopterous species, including *Trichoplusia ni* (Tamez-Guerra *et al.*, 2008), *Heliothis virescens* (Ourth *et al.*, 1994), *Helicoverpa armigera* (Wang *et al.*, 2010), *Bombyx mori* (Sugiyama *et al.*, 1995), *Spodoptera exigua* (Bang *et al.*, 2012), *Manduca sexta* (Rao and Yu 2010), *S. cynthia*

(Kishimoto *et al.*, 2002), and *Hyphantria cunea* (Kwon *et al.*, 2008). It has a molecular mass of ~22 KDa (Yi *et al.*, 2014).

Acknowledgement:

The authors are grateful to Dr. Sara El Khateeb, Department of Cotton Leaf-worm Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. Having provided us the source of insect colony

REFERENCES

- Abdeen, S. A. O., Saleh, W. S., Hossein, N. M., Moaued, G. M., & Godallah, A. I. (1986). Some toxicological and biochemical effects of bacterium *Bacillus thuringiensis* on the American bollworm *Heliothis armigera* (Hbn.). *Annals of Agricultural Science, Moshtohor (Egypt)*.
- Allerberger, F., Liesegang, A., Grif, K., Khaschabi, D., Prager, R., Danzl, J., Höck, F., Öttl, J., P. Dierich, M., Berghold, C. & Neckstaller, I. (2003). Occurrence of *Salmonella enterica* serovar Dublin in Austria. *Wiener Medizinische Wochenschrift*, 153(7-8), 148-152.
- Bang, K., Park, S., Yoo, J. Y., & Cho, S. (2012). Characterization and expression of attacin, an antibacterial protein-encoding gene, from the beet armyworm, *Spodoptera exigua* (Hübner)(Insecta: Lepidoptera: Noctuidae). *Molecular biology reports*, 39(5), 5151-5159.
- Barakat, E. M. S. (1997). A comparative study on the immune response of the wax moth, *Galleria mellonella* (L.) to some biotic and abiotic materials. Ph. D. Thesis, Ain Shams Univ.
- Barakat, E. M., Meshrif, W. S., & Shehata, M. G. (2002). Changes in the haemolymph of the desert locust, *Schistocerca gregaria* after injection with *Bacillus thuringiensis*. *J. Egypt. Acad. Soc. Environ. Dev*, 2, 95-115.
- Barlow, M., & Hall, B. G. (2002). Origin and evolution of the AmpC β -lactamases of *Citrobacter freundii*. *Antimicrobial agents and chemotherapy*, 46(5), 1190-1198.
- Basiouny, A., Ghoneim, K., Tanani, M., Hamadah, K. H., & Waheeb, H. (2016). Disturbed protein content in Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.)(Lepidoptera: Noctuidae) by some novel chitin synthesis inhibitors. *International Journal of Advanced Research in Biological Sciences*, 3(3), 1-12.
- Beament, J. W. L., Treherne, J. E., & Wigglesworth, V. B. (2011). *Advances in insect physiology* (Vol. 1). Academic Press.
- Beveridge, T. J. (1999). Structures of gram-negative cell walls and their derived membrane vesicles. *Journal of bacteriology*, 181(16), 4725-4733.
- Boman, H. G. (2000). Innate immunity and the normal microflora. *Immunological reviews*, 173(1), 5-16.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254.
- Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. *Nature reviews microbiology*, 3(3), 238.
- Chiu, C. H., Wu, T. L., Su, L. H., Chu, C., Chia, J. H., Kuo, A. J., Chien, M.S., & Lin, T. Y. (2002). The emergence in Taiwan of fluoroquinolone resistance in *Salmonella enterica* serotype Choleraesuis. *New England Journal of Medicine*, 346(6), 413-419.
- de Viedma, M. G., & Nelson, M. L. (2017). Notes on Insect Injection, Anesthetization, and Bleeding. *The Great Lakes Entomologist*, 10(4), 12.
- Eng, S. K., Pusparajah, P., Ab Mutalib, N. S., Ser, H. L., Chan, K. G., & Lee, L. H. (2015).

- Salmonella: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*, 8(3), 284-293.
- Finney, D. J. (1971). *Profit Analysis* Cambridge university press. Cambridge, 333.
- Giuliani, A., Pirri, G., & Nicoletto, S. (2007). Antimicrobial peptides: an overview of a promising class of therapeutics. *Open Life Sciences*, 2(1), 1-33.
- Hancock, R. E., & Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature biotechnology*, 24(12), 1551.
- Henderson, J. C., Zimmerman, S. M., Crofts, A. A., Boll, J. M., Kuhns, L. G., Herrera, C. M., & Trent, M. S. (2016). The power of asymmetry: architecture and assembly of the Gram-negative outer membrane lipid bilayer. *Annual review of microbiology*, 70, 255-278.
- Hyeon, J. Y., Chon, J. W., Hwang, I. G., Kwak, H. S., Kim, M. S., Kim, S. K., Choi, I.S., Song, C.S., Park, C., & Seo, K. H. (2011). Prevalence, antibiotic resistance, and molecular characterization of Salmonella serovars in retail meat products. *Journal of food protection*, 74(1), 161-166.
- Junqueira, J. C., & Mylonakis, E. (2019). Current Status and Trends in Alternative Models to Study Fungal Pathogens. *Journal of fungi*, 5(1), 12.
- Kendurkar, S. V., & Sengupta, D. (2018). *U.S. Patent Application No. 15/503,899*.
- Kishimoto, K., Fujimoto, S., Matsumoto, K., Yamano, Y., & Morishima, I. (2002). Protein purification, cDNA cloning and gene expression of attacin, an antibacterial protein, from eri-silkworm, *Samia cynthia ricini*. *Insect biochemistry and molecular biology*, 32(8), 881-887.
- Kwon, Y. M., Kim, H. J., Kim, Y. I., Kang, Y. J., Lee, I. H., Jin, B. R., Han, Y.S., Cheon, H.M., Ha, N.G. & Seo, S. J. (2008). Comparative analysis of two attacin genes from *Hyphantria cunea*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 151(2), 213-220.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*, 227(5259), 680.
- Lehrer, R. I. (2004). Primate defensins. *Nature Reviews Microbiology*, 2(9), 727.
- Lionakis, M. S. (2011). *Drosophila* and *Galleria* insect model hosts: new tools for the study of fungal virulence, pharmacology and immunology. *Virulence*, 2(6), 521-527.
- Makarova, O., Rodriguez-Rojas, A., Eravci, M., Weise, C., Dobson, A., Johnston, P., & Rolff, J. (2016). Antimicrobial defence and persistent infection in insects revisited. *Phil. Trans. R. Soc. B*, 371(1695), 20150296.
- Matuschek, E., Brown, D. F., & Kahlmeter, G. (2014). Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection*, 20(4), O255-O266.
- Meshrif, W. S. (2008). Defense reactions and biochemical changes in the haemolymph of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) following infection with entomopathogenic hyphomycete fungi Ph.D Thesis, Sci. Fac., Tanta Univ.
- Meylaers, K., Freitag, D., & Schoofs, L. (2007). Immunocompetence of *Galleria mellonella*: sex-and stage-specific differences and the physiological cost of mounting an immune response during metamorphosis. *Journal of insect physiology*, 53(2), 146-156.
- Momen, S. A., Salem, A. M., Barakat, M. S., & Salama, S. (2012). Activation of Prophenoloxidase during Bacterial Injection into the Desert Locust, *Schistocerca gregaria*. *World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, 6(8), 585-594.
- Monteiro, S., Santos, R., Bláha, L., & Codd, G. A. (2017). Lipopolysaccharide Endotoxins. *Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis*, 165-172.

- Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskis, A., & Chakraborty, T. (2010). *Galleria mellonella* as a model system for studying *Listeria* pathogenesis. *Appl. Environ. Microbiol.*, 76(1), 310-317.
- Murray, G. L., Attridge, S. R., & Morona, R. (2003). Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *Molecular microbiology*, 47(5), 1395-1406.
- O'Neill, J. (2014). Antimicrobial resistance: tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist*, 20, 1-16.
- Ourth, D. D., Lockey, T. D., & Renis, H. E. (1994). Induction of cecropin-like and attacin-like antibacterial but not antiviral activity in *Heliothis virescens* larvae. *Biochemical and biophysical research communications*, 200(1), 35-44.
- Parthuisot, N., Rouquette, J., & Ferdy, J. B. (2018). A high-throughput technique to quantify bacterial pathogens' virulence on the insect model *Galleria mellonella*. *Journal of microbiological methods*, 152, 69-72.
- Paterson, R. R. M., Simmonds, M. S. J., & Blaney, W. M. (1987). Mycotoxic effects of characterized extracts of *Penicillium* isolates and purified secondary metabolites (including mycotoxins) on *Drosophila melanogaster* and *Spodoptera littoralis*. *Journal of invertebrate pathology*, 50(2), 124-133.
- Pretzel, J., Mohring, F., Rahlfs, S., & Becker, K. (2013). Antiparasitic peptides. In *Yellow Biotechnology I* (pp. 157-192). Springer, Berlin, Heidelberg.
- Pui, C. F., Wong, W. C., Chai, L. C., Nillian, E., Ghazali, F. M., Cheah, Y. K., Nakaguchi, Y., Nishibuchi, M. & Radu, S. (2011). Simultaneous detection of *Salmonella* spp., *Salmonella Typhi* and *Salmonella Typhimurium* in sliced fruits using multiplex PCR. *Food Control*, 22(2), 337-342.
- Raetz, C. R., & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual review of biochemistry*, 71(1), 635-700.
- Rahman, M. M., Guard-Petter, J., & Carlson, R. W. (1997). A virulent isolate of *Salmonella enteritidis* produces a *Salmonella typhi*-like lipopolysaccharide. *Journal of bacteriology*, 179(7), 2126-2131.
- Rao, X. J., & Yu, X. Q. (2010). Lipoteichoic acid and lipopolysaccharide can activate antimicrobial peptide expression in the tobacco hornworm *Manduca sexta*. *Developmental & Comparative Immunology*, 34(10), 1119-1128.
- Rivnay, E., & Meisner, J. (1966). The effects of rearing conditions on the immature stages and adults of *Spodoptera littoralis* (Boisd.). *Bulletin of Entomological Research*, 56(4), 623-634.
- Sabbour, M. (2001). Biochemistry of haemolymph of *Earias insulana* larvae treated with *Bacillus thuringiensis* and *Beauveria bassiana*. *Journal-Egyptian German Society Of Zoology*, 36(E), 19-28.
- Scully, L. R., & Bidochka, M. J. (2006). Developing insect models for the study of current and emerging human pathogens. *FEMS microbiology letters*, 263(1), 1-9.
- Seufi, A. M., Hafez, E. E., & Galal, F. H. (2011). Identification, phylogenetic analysis and expression profile of an anionic insect defensin gene, with antibacterial activity, from bacterial-challenged cotton leafworm, *Spodoptera littoralis*. *BMC molecular biology*, 12(1), 47.
- Sheorey, H., & Darby, J. (2008). Searching for salmonella. *Australian family physician*, 37(10), 806.
- Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., & Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Frontiers in microbiology*, 2, 200.
- Sugiyama, M., Kuniyoshi, H., Kotani, E., Taniai, K., Kadono-Okuda, K., Kato, Y., ... &

- Choi, S. K. (1995). Characterization of a Bombyx mori cDNA encoding a novel member of the attacin family of insect antibacterial proteins. *Insect biochemistry and molecular biology*, 25(3), 385-392.
- Sutton, S. (2011). Accuracy of plate counts. *Journal of validation technology*, 17(3), 42-46.
- Tamez-Guerra, P., Valadez-Lira, J. A., Alcocer-González, J. M., Oppert, B., Gomez-Flores, R., Tamez-Guerra, R., & Rodríguez-Padilla, C. (2008). Detection of genes encoding antimicrobial peptides in Mexican strains of *Trichoplusia ni* (Hübner) exposed to *Bacillus thuringiensis*. *Journal of invertebrate pathology*, 98(2), 218-227.
- Toke, O. (2005). Antimicrobial peptides: new candidates in the fight against bacterial infections. *Peptide Science: Original Research on Biomolecules*, 80(6), 717-735.
- Vilcinskas, A. (2011). Anti-infective therapeutics from the Lepidopteran model host *Galleria mellonella*. *Current pharmaceutical design*, 17(13), 1240-1245.
- Vogel, H., Altincicek, B., Glöckner, G., & Vilcinskas, A. (2011). A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host *Galleria mellonella*. *BMC genomics*, 12(1), 308.
- Wang, G. (Ed.). (2017). *Antimicrobial peptides: discovery, design and novel therapeutic strategies*. Cabi.
- Wang, Q., Liu, Y., He, H. J., Zhao, X. F., & Wang, J. X. (2010). Immune responses of *Helicoverpa armigera* to different kinds of pathogens. *BMC immunology*, 11(1), 9.
- Wiesner, J., & Vilcinskas, A. (2010). Antimicrobial peptides: the ancient arm of the human immune system. *Virulence*, 1(5), 440-464.
- Yi, H. Y., Chowdhury, M., Huang, Y. D., & Yu, X. Q. (2014). Insect antimicrobial peptides and their applications. *Applied microbiology and biotechnology*, 98(13), 5807-5822.
- Yoon, B., Jackman, J., Valle-González, E., & Cho, N. J. (2018). Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. *International journal of molecular sciences*, 19(4), 1114.

ARABIC SUMMERY

استحداث الببتيدات المضادة للميكروبات في دم يرقات الاسبودوبترا ليتورالس بعد المعاملة بالسالمونيلا التيفيموريوم

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تحققت هذه الدراسة من كفاءة الحقن البكتيري بتركيز $10^3 \times 5,7$ خلية / مل من السالمونيلا التيفيموريوم المحقق لنسبه اماته 20 في المائه في تحفيز الاستجابة المناعية في الطور الخامس ليرقات الاسبودوبترا ليتورالس. وقد تم فحص دور الببتيدات المضادة للميكروبات المستحثه في دم اليرقات كمضادات حيوية طبيعية ضد البكتيريا سالبة الجرام و موجبة الجرام المختلفة. حيث أظهرت بلازما اليرقات التي تم حقنها بالسالمونيلا نشاطاً مضاداً للميكروبات ضد سلالات مختلفة من البكتيريا المسببة للأمراض (المكورات العنقودية الذهبية ، السالمونيلا التيفيموريوم ، الإشريكية القولونية والمكورات المعوية). اشار التوصيف البيوكيميائي للدم إلى تغييرات جذرية في كل من محتوى البروتين الكلي وأنماط النطاق البروتيني بعد الحقن الجرثومي. حيث انخفض مجموع البروتينات بشكل ملحوظ بعد 48 ساعة من حقن السالمونيلا. تم تغيير ملف التعرف علي بروتينات الدم نوعياً من خلال استحداث بروتينات جديدة واختفاء بروتينات أخرى في وقت واحد ، مما قد يعزى إلى مشاركتها في التفاعلات المناعية. قد ظهر ستة نطاقات جديدة من البروتين المركب تبين أنها محددة لحقن السالمونيلا ، مع وجود نطاق مميز عند الوزن الجزيئي 22 و تنتبأ بأن هذا النطاق البروتيني قد يكون اتاسين شبيه الببتيدات المضادة للميكروبات.