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Characterization of Antibacterial Peptides in *Culex pipiens* (Diptera: Culicidae) in Response to *Bacillus sphaericus* infection

Doaa M. Mohamed [1], Nahla M. Wassim [1,] Tito N. Habib [2] and Howayda E. Khaled [1]

[1] Department of Zoology, Faculty of Science, Suez University, Suez, Egypt
[2] Department of Zoology, Faculty of Science, Sohag University, Sohag, Egypt

ABSTRACT

Antimicrobial peptides (AMPs) play a crucial role in defending mosquitoes against micro-organisms. The objective of this study was to identify AMPs produced in the hemolymph of *Culex* (Cx.) *pipiens* mosquitoes following bacterial infection. Mosquito larvae were collected from the Nahia drainage conduit in Giza Governorate and treated with *Bacillus sphaericus* strain 2362. The lethal concentration (LC50 and LC80) values were determined at concentrations of 0.042 and 0.174 ppm, respectively. The protein profiles of both treated and untreated *Cx. pipiens* mosquito larvae were analyzed using SDS-PAGE in a Bio-Rad cell. The analysis revealed the presence of several polypeptides, including Megacin β, α, and γ forms (15, 29.2, and 39.8 kDa), Gambicin (7.5 kDa), Diptericin (9.4 kDa), Attacin (23.8 kDa), and Subtilisin (27.6 kDa). Additionally, *B. sphaericus* double toxin Bin A (41.9 kDa) and Bin B (51.6 kDa) were observed, which are synthesized during sporulation and co-crystallize within bacteria. Furthermore, soluble mosquitocidal toxins Mtx1 (98.6 kDa) and Mtx2 (31 kDa) produced during vegetative growth were identified. Lastly, the two-component crystalline toxins Cry48Aa1 (135 kDa) and Cry49Aa1 (52.8 kDa) were detected. Understanding the mechanism behind mosquito resistance to bacterial pathogens is essential for developing strategies to combat this resistance.

Keywords: Antimicrobial peptides*; Bacillus sphaericus*2362; *Culex pipiens*

INTRODUCTION

Numerous *Bacillus* species are common soil dwellers that foster tight interactions that help plants flourish. Antimicrobial peptides that suppress plant-pathogenic fungus or bacteria are one of the relevant mechanisms behind these symbiotic partnerships (Abo El-Dahab *et al.*, 2023). The genus *Bacillus* is known for producing a wide range of antimicrobial compounds, including peptide and lipopeptide antibiotics as well as bacteriocins (Yahya *et al.*, 2021). In particular, certain strains of *Bacillus* that produce bacteriocins have potential applications in environmental settings such as mosquito larvae mana-gement. Among these, Toxin *B. sphaericus* has been extensively studied and effectively utilized as a pesticide against mosquitoes (Yuan, 2002).

Mosquitoes play a crucial role in transmitting hundreds of human diseases worldwide. According to Abdel-Shafi *et al.* (2016) and El Zayyat *et al.* (2017), the main vector of lymphatic filariasis has historically been the widely disseminated mosquito species *Cx. pipiens*. Additionally, it has been linked to the emergence of bancroftian filariasis and Rift Valley fever in Egypt's Nile Delta (Gad *et al.*, 1999).

In order to combat prokaryotic infections, insects have evolved a diverse array of immunological substances. These immunological proteins are produced by specialized hemocytes and the fat body (Lombardo and Christophides, 2016). According to Harikrishna *et al.* (2012), these peptides have demonstrated their ability to inhibit bacteria, fungi, and parasites in mosquitoes. Insects have proven to be a valuable source of medicinal compounds with extensive antimicrobial and anti-inflammatory properties. In fact, more than 900 such compounds including peptides, enzymes, and biogenic amines have been derived from insects (Bulet *et al.*, 2004). Given the importance of preventing microbial diseases for public health concerns, there is a need for the development of novel antimicrobial substances that are both new and enhanced in their efficacy.

In mosquitoes, antimicrobial peptides (AMPs) play a crucial role in preventing pathological harm that may result from the chronic spread of arboviruses that are present in their tissues (Ahmad *et al.*, 2012). The mosquito's innate immune system, which detects and reacts to harmful pathogens, produces these peptides. The peptides can cause oxidative stress in bacteria, alter microbial membranes, and decrease the functioning of enzymes (Bulet *et al.*, 2004). The discovery and characterisation of AMPs in mosquitoes can shed light on the mechanisms behind mosquito immunity and aid in the creation of fresh approaches to the management of mosquito-borne diseases (Gabrieli *et al.*, 2021).

Antimicrobial peptides (AMPs) are distinct constituents of prokaryotic and eukaryotic species' inborn vulnerable defence complexes. Although some AMPs can range in length from 7 to 100 amino acids, they commonly comprise between 12 and 50 amino acids and are divided into numerous subgroups depending on amino acid substitutions (Yi *et al.*, 2014). More than half of the amino acid residues in their molecules are generally hydrophobic. According to their amino acid composition and structural characteristics, insect AMPs can be divided into three groups: (i) linear peptides that form helices but lack cysteine residues; (ii) cyclic peptides that contain cysteine residues; and (iii) peptides with an excess of proline

* *Corresponding author e-mail: DoaaMamdouh211@yahoo.com*

and/or glycine residues (Hetru *et al.*, 1998). AMPs are intriguing prospects for the creation of next- generation antimicrobial medicines because they offer broad-spectrum antibacterial action. The ongoing identification of natural AMPs from various sources will contribute to the growth of the AMP database and offer helpful tips for the creation of AMP variants with enhanced therapeutic characteristics as next-generation antibiotics. AMPs have anticancer, immunomodulatory, and wound-healing abilities in addition to their antibacterial action (Silva and Machado, 2012).

Gram-positive entomopathogenic bacteria produce a variety of protein toxins that target the insect midgut, leading to infection and eventual host death. These toxins belong to different homology groups and exhibit various protein structures and modes of action (Fischetti 2019). Many toxins have unique protein folds or novel combinations of domains in addition to well-known protein folds. Notably, most of these toxins are localized within parasporal crystals and are produced abundantly during sporulation (Ruud *et al.*, 2003). Extensive research has been conducted on protein toxins produced by entomopathogenic bacteria as potential biocontrol agents against insect vectors (Lacey *et al.*, 2015). The use of *Bacillus thuringiensis* (Bt) toxins in the creation of insecticidal sprays and genetically modified crops resistant to insect damage is a well-known example (Schnepf *et al.*, 1998). These toxins are considered ecologically friendly alternatives to chemical insecticides due to their high selectivity for specific insect orders. Recent investigations have revealed the discovery of new families of protein toxins produced by entomopathogenic bacteria. For instance, the Pdx1 toxin from *Photorhabdus luminescens* and the Yen-Tc toxin from *Yersinia entomophaga* have been identified as examples (Waterfield *et al.*, 2010; Chaston *et al.*, 2011). These novel toxins exhibit unique structures and modes of action, offering potential opportunities for controlling insect pests.

Crystalline inclusions that *B. sphaericus* produces during the sporulation stage of growth are what give rise to its larvicidal action. These crystals contain protoxins that affect the midgut epithelial cells of the larvae following consumption (Soberón *et al.*, 2007). The safety standards for mosquito control larvicides heavily rely on the selective mechanism of action of these toxins. The insecticidal crystals made by *B. sphaericus* must be ingested by the larvae in order for the toxicity to be effective. In the intestines, protoxins are transformed into active toxins. However, due to its effectiveness, specificity, and safety for non-target organisms, *B. sphaericus* has been extensively employed as a means to control mosquito populations (Charles *et al.*, 2019). However, the documented emergence of *B. sphaericus* resistance in mosquitoes highlights the need for alternative control methods to be developed (Dulmage *et al.*, 2020). One potential strategy involves enhancing the mosquito's innate immune system by inducing the synthesis of antimicrobial peptides (AMPs) in response to bacterial infection.

AMPs have proven their ability to suppress bacteria, fungi, and parasites in mosquitoes and play a crucial role as effectors within the innate immune system (Harikrishna *et al.*, 2012). Following an infection with *B. sphaericus*, AMPs are identified and characterized in mosquitoes. This valuable information can contribute to a better understanding of mosquito immune mechanisms and aid in the development of innovative strategies for preventing mosquito-borne diseases.

MATERIALS AND METHODS

Insect rearing

Mosquito larvae were collected from a drainage canal in Nahia, Giza Governorate, Egypt. *Cx. pipiens* larvae were reared in the laboratory under controlled conditions of temperature (25 ± 2 °C) and relative humidity (70-80%). Only late third and early fourth instar larvae were used in the study. The mosquitoes were reared in an insectary at the Research and Training Center on Vectors of Diseases at the Faculty of Science, Ain Shams University. The rearing conditions were based on established protocols for mosquito rearing in the laboratory (Reisen *et al.*, 1992; Alto *et al.*, 2005). Briefly, mosquito larvae were fed on a diet of ground fish food and yeast, and the water in the rearing containers was changed every two days to prevent bacterial growth and accumulation of waste products. The pupae were transferred to a separate cont-ainer until the emergence of adult mosquitoes.

Bioassay

B. sphaericus strain 2362 was provided by Abbott Laboratories, North Chicago, IL, USA. The susceptibility of *Cx. pipiens* larvae to *B. sphaericus* was determined using bioassay tests (Zayed *et al.*, 2006). To initiate the experiment, we prepared a 1% stock suspension by dissolving 1 g of the granular formulation in 100 ml of distilled water, which contained 1.5 X 10^8 bacterial colony-forming units (cfu). Fresh suspensions were meticulously prepared for every individual experiment to ensure accuracy and consistency.

For the experimental design, disposable 200 ml polyethylene cups were used, each accommodating twenty larvae individually. Within each cup, 150 ml of tap water treated with varying concentrations of *B. sphaericus*, ranging from 0.01% to 0.1%, with each subsequent concentration representing a hundredfold dilution increment. To ensure reliability, triplicates from each treatment was carried out and reared under the condition. After a post-bacterial treatment period of 48 hours, mortality rates were assessed. Lethal concentrations resulting in 50% and 80% mortality levels were determined using log probit regression analysis as described by Mulla *et al.* (1988).

The bioassay protocol followed established guidelines for testing the efficacy of larvicides against mosquito larvae (WHO, 2005). The bioassay tests were performed in a controlled environment at a temperature of 25 ± 2 °C and a relative humidity of 70-80%.

Treatment with *B. sphaericus* bacteria

Two populations of *Cx. pipiens* larvae were reared under identical laboratory conditions, with one group subjected to bacterial treatment using *B. sphaericus*, while the other population was kept without any bacterial exposure as a control. To ensure an adequate number of

surviving individuals for subsequent large-scale experiments, approximately 600-700 late third and early fourth instar larvae were treated according to their respective methods. After a post-treatment period of 48 hours, the treated larvae underwent careful washing procedures, and the surviving individuals were collected and stored in Eppendorf tubes for subsequent protein extraction and hemolymph differentiation analyses.

Protein extraction from frozen larvae followed established protocols for insect hemolymph (Söderhäll and Smith, 1983; Jiang *et al.*, 2010). In brief, the frozen larvae were homogenized in ice-cold phosphate-buffered saline (PBS) and subsequently centrifuged to obtain the supernatant containing hemolymph proteins. Hemolymph differentiation was carried out following established protocols for insect hemolymph cells (Lavine and Strand, 2002; Dimopoulos *et al.*, 2002). Briefly, the hemolymph was diluted in PBS before undergoing centrifugation to separate the cells. The isolated cells were then washed with PBS and resuspended in an appropriate buffer suitable for downstream applications.

Protein extraction

Protein extraction was performed as follow in which samples were homogenized and extracted using a buffer of Tris and NaCl at pH 8.0. The homogenates were spinned for 10 minutes at 10,000 Xg and then supernatants were collected (HASSAN et al. (2018).

Quantification of Protein Concentration

The total protein concentration in hemolymph was estimated using the bicinchoninic acid (BCA) assay, which was calibrated with bovine serum albumin (BSA) standards (Smith *et al.*, 1985). Briefly, 2 mL of each sample (control, LC_{50}, and LC_{80}) was mixed with 48 mL of 1x phosphate-buffered saline (PBS) and six BSA standard solutions. The mixture was incubated at 37 °C for 30 minutes, and the absorbance was measured at a wavelength of 562 nm using a spectrophotometer, estimated by Thermoscientific Pierce ™ BCA Protein Assay Kit according to manufacturer instructions

SDS-PAGE analysis and protein profiling

SDS-PAGE analysis was performed to assess the protein profiles of both treated *Cx. pipiens* mosquitoes infected with bacteria and untreated control mosquitoes. The hemolymph samples were extracted and subjected to electrophoresis using a vertical Bio-Rad SDS-PAGE system (Laemmli, 1970). The gel was loaded with the samples as well as molecular size markers, consisting of a Blue Ultra Pre-stained Protein Ladder with molecular weights ranging from 6.5 to 270 kDa. Electrophoresis was carried out at a constant current of 140 V, and the gel was subsequently stained with Coomassie blue staining solution (Neuhoff et al., 1988) by shaking it for 30 minutes. To achieve a suitable background, the gel was destained, transferred onto filter paper, and dried under vacuum in a gel drier. The Molecular Imager Gel Doc XR system (Bio-Rad, USA) was used to analyze the gel. The identification of protein bands was accomplished by comparing their molecular weights with the protein ladder. To validate the results, the GenBank database (https://www.ncbi.nlm.nih.gov/protein) was accessed.

RESULTS

Data obtained as an exploring the peptides present in larval hemolymph after subjecting them to an immune challenge with *B. sphaericus* strain 2362 showed that the median lethal concentration (LC50) was 0.042 ppm (with a range of 0.033-0.053, Fig. 1). Based on these findings, the experiment proceeded to investigate the effects of the calculated LC80 concentration of 0.174 ppm (with a range of 0.132-0.228) on *Cx. pipiens* larvae for further analysis and evaluation.

Mortality rate and protein quantification

The results represented in Figure 2 provide insights into the impact of *B. sphaericus* exposure on the mortality rate of *Cx. pipiens* larvae. Notably, the control group, which remained unexposed to *B. sphaericus*, exhibited no mortality. However, in the second group, which was exposed to the LC50 concentration of *B. sphaericus* and correspondingly released antimicrobial peptides (AMPs) at a concentration of 14.97 µg/mL, a mortality rate of 48% was observed. In the third group, exposed to the LC80 concentration of B. sphaericus and associated with the release of AMPs at a concentration of 20.87 µg/mL, a mortality rate of 77.5% was reported.

These findings suggest a direct relationship between dose exposure to *B. sphaericus* and subsequent increases in antimicrobial peptide concentrations (AMPs) as well as larval mortality rates. It is worth noting that employing the obtained LC50 and LC80 values from this experiment allows for estimating potential mortality rates, thereby aiding in assessing the severity of toxic effects induced by *B.sphaericus* exposure on tested larvae.

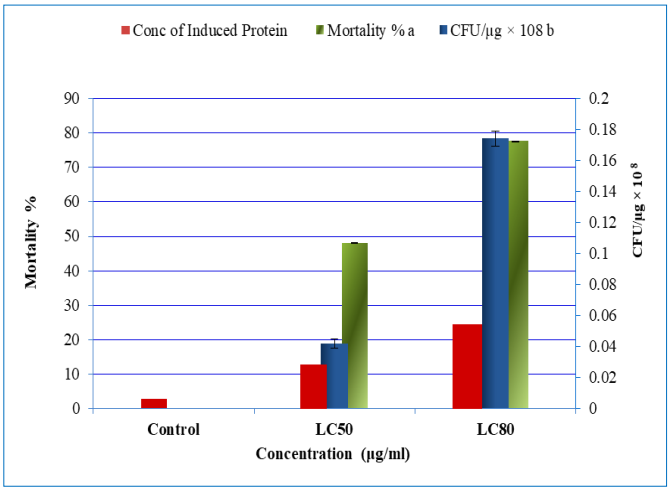


Figure (1): Toxicity and concentration of protein induced by *B.sphaericus* against third-instar larval stage of *Cx. pipiens* after 48 h post-treatment. a, LC50 and LC80 values calculated using Probit analysis to determine the lethal concentration required to kill 50% and 80% of the larval population (n=600), respectively. b, CFU, or colony forming unit.

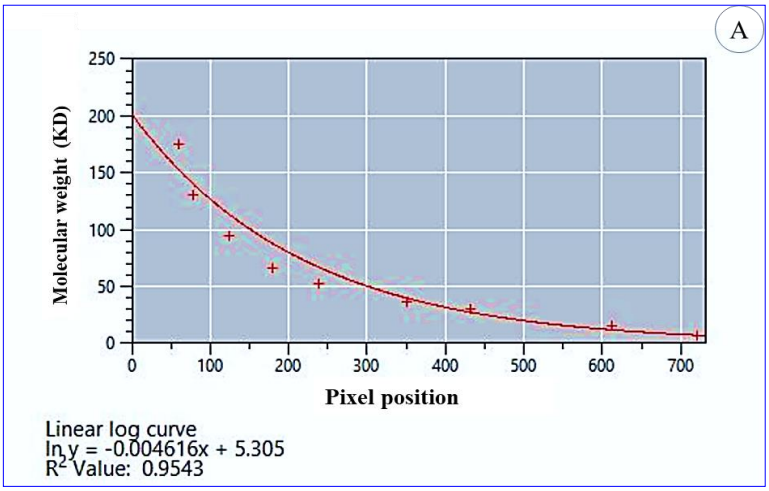
Protein profile

The hemolymph analysis of larvae subjected to bacterial treatment revealed distinct protein bands, as clearly depicted in Figures 2A and 2B. In contrast, these protein bands were absent in the control group. The SDS-PAGE analysis indicated that most of these proteins exhibited

high expression levels based on the detected signals. Among the identified proteins, Bin B, Bin A, and Migacin γ were observed in the 42-52 KDa range, while Megacine α, subtilisin, and Attacin proteins were expressed in the 23-30 KDa range. Additionally, Megacine ß, Diptericin, and Gambicin were detected in the 6.5-16 KDa range. This intriguing discovery suggests that the immune challenge posed by *B. sphaericus* strain 2362 stimulated the synthesis of specific peptides and proteins in *Cx. pipiens* larvae.

The identification of these protein bands indicates that the immune response activated by the bacterial treatment triggered a cascade of molecular events within the larvae. These events likely involved the activation of specific genes and subsequent production of corresponding peptides and proteins. The observed protein bands may represent the products of this immune response, potentially serving crucial roles in defending against the bacterial challenge. Further investigation is warranted to elucidate the exact nature and functions of these proteins.

Understanding the specific peptides and proteins synthesized in response to the immune challenge can provide valuable insights into the molecular mechanisms underlying the immune defense of *Cx. pipiens* larvae against *B. sphaericus* strain 2362. This knowledge may have implications for the development of novel strategies to combat bacterial infections in mosquito populations or even in the broader context of understanding host-pathogen interactions.



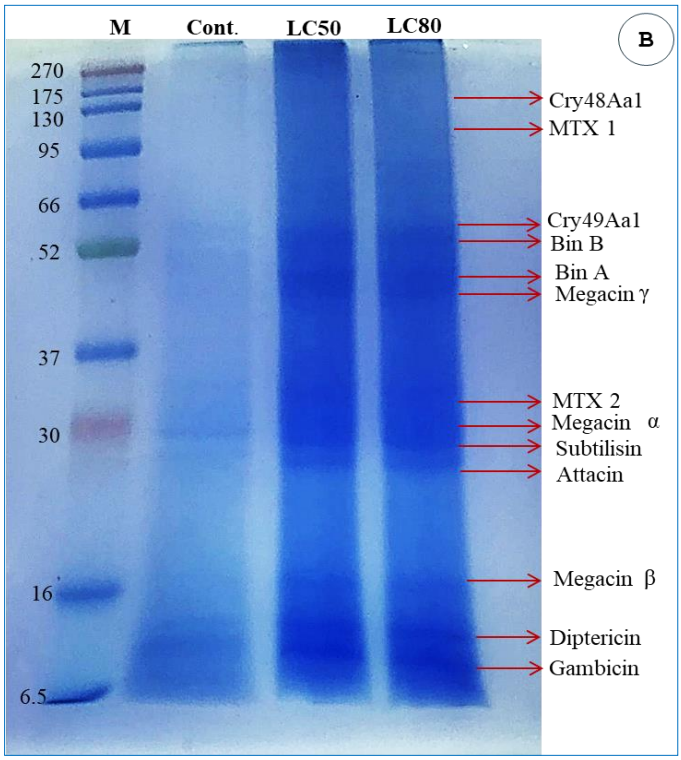


Figure (2): A, The calibration curve to assess protein molecular weight. B, SDS-PAGE of protein induced by *B. sphaericus* strain 2362 in *Cx. pipiens* larvae. Lane 1: protein molecular mass markers (6.5 to 270 KDa, Blue Ultra Pre-stained Protein Ladder); lane 2, protein extracted larvae in control group; lanes 3 and 4, protein extracted from larvae exposed to *B. sphaericus* at doses LC50 and LC80, respectively. Red arrows indicate the different pattern of proteins.

Discussion

The results of this study demonstrate that the immune challenge with *B. sphaericus* strain 2362 induces the synthesis of additional proteins in *Cx. pipiens* larvae. The total number of protein bands detected in both LC50 and LC80 samples was greater than in control samples, indicating that more proteins were produced when mosquito larvae were under the pressure of bacterial infection. The protein concentrations were 14.97 µg/mL with LC_{50} and 20.87 µg/mL with LC_{80}, which were higher than in the control group.

These findings are in line with those of De-Barjac (1990), who reported that there are three levels of toxicity for *B. sphaericus*. Based on the final entire concentration, 10^{-6}-10^{-8} was the LC50 value for the substance with the highest toxicity. High-toxicity strains of *B. sphaericus* include strains 2362, 1593, and LB24. An LC50 value of dilution of 10^{-4}-10^{-5} showed mild toxicity. *B. sphaericus* strains SSII-1, ISPC5, and LB29 have shown this toxicity. Low-toxicity *B. sphaericus* strains K and Q and their lowest values of dilution range from 10^{-2} to 10^{-3}. All of these LC values were obtained against *Culex* larvae.

The identification of specific proteins induced by *B. sphaericus* strain 2362 in *Cx. pipiens* larvae may provide valuable information for the development of more effective larvicides against this mosquito species. Further investigation of these proteins may also lead to a better understanding of the immune response of *Cx. pipiens* to *B. sphaericus* and the mechanisms underlying the toxicity of this bacterium.

In this study, we successfully isolated Gambicin using SDS-PAGE, revealing a molecular weight of 7.4-7.5 KDa. This finding aligns with the research conducted by Vizioli *et al*. (2001), who also reported the broad-spectrum antimicrobial activity of Gambicin against both Gram-negative and Gram-positive bacteria. Gambicin is considered a novel immune-responsive antimicrobial peptide (AMP) that has been exclusively discovered in mosquitoes. Further investigations by Zhang *et al*. (2017) demonstrated that Gambicin, similar to other AMP genes found in Anopheles gambiae, is induced in response to natural or experimental infections in the midgut and fat body. Moreover, Bartholomay *et al.* (2003) successfully isolated Gambicin from *Cx. pipiens* mosquitoes that had ingested a blood meal contaminated with *Wuchereria bancrofti*. These collective findings highlight the significance of Gambicin as a potent immune defense mechanism in mosquitoes, with the ability to combat a wide range of bacterial pathogens.

In addition to Gambicin, we also isolated Attacin using SDS-PAGE. Attacin exhibited a molecular weight range of 20.8 to 23.8 KDa. However, the specific number of amino acids composing Attacin was not specified in our findings. Interestingly, Mackintosh *et al.* (1998) conducted a pioneering study that unveiled the discovery of a novel insect antimicrobial peptide (AMP) family known as Attacins, originating from the big silk moth *Hyalophora cecropia*. The hemolymph of the moth yielded several isoforms of Attacin, characterized by molecular masses ranging from 20 to 23 KDa and isoelectric points spanning from 5.7 to 8.3. Attacins can be classified into two categories: basic (Attacins A-D) and acidic (Attacins E and F). Remarkably, both Gram-positive and Gram-negative bacteria are susceptible to the antimicrobial effects of Attacins. These Attacin isoforms, designated as Attacins A-F, represent a diverse set of proteins with varying sizes, as elucidated by Hultmark *et al*. (1983).

Attacins peptide may also be involved in controlling the microbiota in insects' guts, according to recent studies. For instance, Ryu *et al.* (2008) showed that Attacin A, which is expressed in the gut of *Drosophila melanogaster*, can kill some bacterial species preferentially while sparing others, These findings suggest that Attacin A may play role in shaping the composition of the gut microbiota. Subtilisin was separated at a molecular mass of 27.7 KDa in our SDS-PAGE. It was first synthesized by *B. subtilis* ATTC 6633 and is considered a very important antibiotic. Azrin *et al.* (2022) concluded that Subtilisin is a small peptide antibiotic with a chemical structure of 268-275 amino acid residues, and a molecular mass of 26.9-27.5 KDa. However, Megacins peptide were detected with molecular weights of 15, 29.2, and 39.8 KDa as reported in our SDS-PAGE, According to Kiss *et al.* (2008), The bioactive fraction contains three components: β, α, and γ chains (15, ~30, ~40 KDa), which correspond to the full-length protein, and two cleavage products.

Our study revealed the presence of two distinct proteins on SDS-PAGE analysis, with molecular masses of 41.9 and 51.6 KDa. These proteins were identified as the binary toxin components, Bin A (42 KDa) and Bin B (51 KDa), respectively. Interestingly, an additional protein with a molecular weight of 43 KDa was detected, which can be attributed to the release of protease enzymes in the midgut following the ingestion of B. sphaericus endospores. These enzymes play a crucial role in the conversion of the 51-KDa and 42-KDa proteins into the 43-KDa and 39-KDa proteins, respectively, while simultaneously breaking down the endospores into toxin subunit components (Guo et al., 2020).

Remarkably, the larva experiences mortality within a few hours of ingesting *B. sphaericus* endospores. This can be attributed to the higher toxicity of the 39-KDa protein when compared to the 42-KDa protein, as observed in cell cultures derived from mosquitoes (Guo et al., 2020). Furthermore, the binding of this toxin to a specific receptor on the midgut epithelial cells of Culex mosquitoes leads to the development of pores and subsequently results in larval death (Wirth et al., 2007).

The potential of the binary toxin of *B. sphaericus* as a biopesticide for managing mosquito populations has been thoroughly investigated. The effectiveness of it

against *Culex pipiens, Aedes aegypti*, and *Anopheles gambiae* mosquito species has been demonstrated (Charles *et al.*, 2019). In both urban and rural contexts, the application of *B. sphaericus*-based biopesticides is beneficial in lowering mosquito populations (Lacey *et al.*, 2015; Silva-Filha *et al.*, 2014).

Upon conducting SDS-PAGE analysis, we observed the presence of specific protein bands at different concentrations. At the LC50 concentration, a single band with a molecular mass of 98.8 KDa was detected. In contrast, at both the LC50 and LC80 concentrations, two distinct bands with molecular masses of 28.8 and 29.2 KDa were identified. These proteins exhibit similarity to the mosquitocidal toxins, namely Mtx1 and Mtx2. The Mtx toxin, produced by *B. sphaericus* during the vegetative stage of bacterial growth, is independent of sporulation (Yuan, 2002). It consists of three subunits: Mtx1 (100 KDa), Mtx2 (32 KDa), and Mtx3 (36 KDa) (Chan *et al*., 1996; Yuan, 2002). It is important to note that unlike the binary toxin, the mosquitocidal toxin does not interact with receptors in the larval midgut. Furthermore, it only exhibits a minor antilarvicidal effect on *B. sphaericus* (Yuan, 2002).

Mtx1 has a molecular weight of 100 KDa and is a soluble toxin. It can be further broken down by gut proteases into two pieces that are 27 and 70 KDa in size (Thanabalu *et al.*, 1992a). The molecular weights of the Mtx2 and Mtx3 toxins are 31.8 and 35.8 KDa, respectively (Yuan, 2002).Various mosquito species, particularly *Culex* mosquitoes, are known to be very susceptible to the Mtx toxin's powerful mosquitocidal effects (Wang *et al.*, 2021).

In our study, AMP bands were detected with molecular masses of 52.8 KDa in both LC50 and LC80 samples. Additionally, a 135 KDa AMP band was observed on SDS-PAGE. Interestingly, these proteins share similar molecular masses with the Cry49Aa1 and Cry48Aa1 pathogenic proteins produced by *B. sphaericus*, as reported by Soberón *et al*. (2010). It is worth noting that bioinsecticides often contain insecticidal proteins derived from *B. sphaericus*. The Cry49Aa1 and Cry48Aa1 proteins belong to the Crystal (Cry) protein family and consist of three domains, as described by Williamson et al. (2022).

The analysis of resistant strains of mosquitoes has revealed the presence of a previously unknown type of two-component toxin. This toxin, Cry48Aa1, is related to the three-domain crystal toxins produced by *B. sphaericus and B. thuringiensis*. However, in this case, the presence of a second accessory protein, Cry49Aa1, is required for insect toxicity. Cry49Aa1 is related to both the binary toxin of *B. sphaericus* and the *B. thuringiensis* Cry35 and Cry36 proteins. The requirement for both Cry48Aa1 and Cry49Aa1 components for pathogenicity indicates an unprecedented interaction capable of producing toxicity. This new toxin combination may have arisen through a chance meeting between members of the gene families that encode 3-domain Cry toxins and Binary-like toxins, allowing for the "mix-and-match" evolution of a new component in the mosquitocidal arsenal (Jones *et al.*, 2007).

Recycled spores were determined to exhibit toxicity within 48 hours following treatment, suggesting that the larvae possessed all the necessary components for vegetative multiplication and toxin synthesis associated with the sporulation process (Labib and Mohamad, 2003). This discovery provides valuable insights into the ecological dynamics of *B. sphaericus* and its interactions with mosquitoes. Further exploration of the mechanisms underlying spore germination and recycling holds great potential in advancing the development of more effective and long-lasting mosquito control methods. By gaining a deeper understanding of how spores germinate and recycle, researchers can identify key targets for intervention and develop innovative strategies to disrupt the mosquito life cycle. This knowledge can aid in the development of enhanced insecticides or biological control agents that specifically target the spore germination and recycling processes, leading to more efficient and sustainable mosquito control techniques. Additionally, this research may contribute to the identification of novel molecular targets for the development of specific and environmentally friendly interventions against mosquito-borne diseases.

CONCLUSION

This study successfully identified several antimicrobial peptides (AMPs) isolated from *Cx. pipiens* larvae infected with *B. sphaericus*. These AMPs exhibited molecular weights of 7.5, 9.4, 15, 23.8, 27.6, 29.2, and 39.8 KDa. The identified peptides include Gambicin, Diptericin, Attacin, Subtilisin, Megacin (α, β, and γ forms), and Megacin. Notably, different strains of B. sphaericus demonstrated varying levels of virulence against mosquito larvae, with *B. sphaericus* 2362 emerging as the most potent strain and serving as the active ingredient in commercial products. This particular strain was found to produce a combination of three distinct types of toxins: the binary toxin (Bin), the soluble mosquitocidal toxins (Mtx1 and Mtx2) produced during vegetative growth, and the two-component crystal toxin (Cry48Aa1/Cry49Aa1). Understanding the mechanisms underlying mosquito resistance to microorganisms is crucial in the develop-pment of effective strategies to combat such resistance. The discovery of natural AMPs from diverse sources, including insects, offers a promising avenue for the development of next-generation antibiotics with enhanced therapeutic properties. AMP-based antib-iotics have emerged as highly promising candidates for combating antimicrobial resistance, and the continuous exploration of natural AMPs will further enrich the human AMP database. To summarize, this study provides valuable insights into the pathogenicity mechanisms of *B. sphaericus* and its interactions with mosquitoes. This knowledge may facilitate the

development of more efficient and sustainable methods for mosquito control. Additionally, the discovery of natural AMPs from various origins holds great potential for the advancement of novel and improved antimicrobial compounds.

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توصيف الببتيدات المضادة للبكتيريا فى *Culex pipiens* (Diptera: Culicidae) المستحثة ببكتيريا *Bacillus sphaericus*

دعاء ممدوح[1] ، نهلا وسيم[1] ، تيتو حبيب[2]، هويده خالد[1]
[1] قسم علم الحيوان - كلية العلوم - جامعة السويس - السويس – مصر
[2] قسم علم الحيوان - كلية العلوم - جامعة سوهاج - سوهاج - مصر

الملخص العربـــــي

تلعب الببتيدات المضادة للميكروبات (AMPs) دورًا حاسمًا في الدفاع عن البعوض ضد الكائنات الحية الدقيقة. اجريت الدراسة بهدف تحديد الببتيدات المضادة للميكروبات المنتجة في دم يرقات بعوض كيولكس بيبينزبعد العدوى البكتيرية. فقد تم جمع يرقات البعوض من قناة تصريف ناهية في محافظة الجيزة ومعالجتها بسلالة بكتيريا عصوية سفيريكوس 2362. و تم معالجتها بتركيزين LC50و LC80 هما 0.042 و 0.174 جزء من المليون على التوالي. كما تم تحليل البروتينات من يرقات بعوض المعالجة وغير المعالجة بالبكتيريا باستخدام تقنية الفصل الكهربى SDS-PAGE. لوحظ أربعة أنواع من الببتيدات الناتجة: 1.ميجاسين β ، α،γ ذات الاوزان الجزيئية (15، 29.2، 39.8 كيلو دالتون) على التوالى، جامبيسين (7.5كيلو دالتون)، ديبتريسين (9.4كيلو دالتون)، اتكين (23.8كيلو دالتون)، سبتليسين (27.6 كيلو دالتون) ؛ 2. اثنان من سموم البكتيريا العصوية التى يتم تصنيعها أثناء التبوغ والتحوصل وتتبلور داخلها وهى Bin A وBin B ذات الأوزان الجزيئية (41.9و51.6 كيلو دالتون) على التوالى ؛ 3. السموم القابلة للذوبان المنتجة أثناء النمو الخضري للبكتريا العصوية المستخدمة في الدراسة Mtx1 (98.6kDA) و Mtx2 (31kDA)؛ 4. وبالإضافة إلى السموم البلورية المكونة من مكونين Cry48Aa1 (135كيلودالتون) وCry49Aa1 (52.8 كيلودالتون). كما يعد فهم الآليات الكامنة وراء مقاومة البعوض للبكتيريا أمرًا ضروريًا لتطوير استراتيجيات المكافحة ضد هذه المقاومة.