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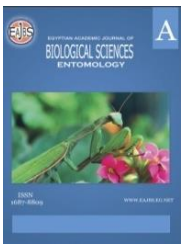

Histology and Ultrastructural Changes of Larvae Midgut Epithelium of *Phlebotomus papatasi* (Diptera, Psychodidae) Fed with Insect Growth Regulator and *Bacillus sphaericus*

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ABSTRACT

Sandfly, *Phlebotomus papatasi* (Diptera: Psychodidae) is the potential vector of leishmania in Egypt. In this study, an attempt was made to investigate the ultrastructure and histopathological alteration in the midgut of *Phlebotomus papatasi* post-treatment with methoprene (IGR) and *B. sphaericus* 6184 and 6446 using transmission electron micrographs (TEM).

After 48 h, the TEM of the midgut treated with methoprene (IGR) showed sloughing of the periepithelial layer, and overgrowth of the brush border of the midgut wall. After 48 h post-treatment, *B. sphaericus* 6184 spores were discovered in the lumen and the bacterium began to enter the microvilli. Certain ultrastructural abnormalities were found in the midgut of treated *P. papatasi* larvae after 48 h of treatment with *B. sphaericus* 6446, the cytoplasm was distinguished by many vacuoles and a broken brush border. Using the light microscope, IGR-treated third instar larvae showed brush boundary overgrowth and multifocal disintegration of the midgut wall. Focused areas were sloughed and epithelial cells were dissociated from each other in larvae treated with *B. sphaericus* 6184. The 3rd instar *P. papatasi* larvae treated with *B. sphaericus* 6446 showed clear alterations such as total epithelial cell degradation. These results demonstrated that *B. sphaericus* 6446 showed the best results and great effect on *P. papatasi* 3rd instar larvae. More research is needed to establish the appropriate control strategies for the many leishmaniasis vectors in their various ecological environments.

INTRODUCTION

Sand flies (Psychodidae) carry a variety of medically significant viruses, including the bacteria *Bartonella baciliformis* and, most critically, the protozoan parasites that cause leishmaniasis (WHO 2008).

Humans are bitten by sand flies, which induce acute dermatitis and delayed-type hypersensitivity reactions (Belkaid *et al.*, 2000). *Phlebotomus papatasi* is common in Egypt (Kamal *et al.*, 2003; El-Naggar *et al.*, 2006), where Leishmania major was isolated and identified (El-Naggar *et al.*, 2006).

Insecticide resistance in phlebotomine sandflies populations around the world poses a threat to the efficacy of initiatives aimed at halting the spread of leishmaniasis (Delinger *et al.,* 2016). Because phlebotomine species (Diptera, Psychodidae, Phlebotominae) are important vectors of leishmaniasis, it is crucial to research techniques for controlling them, particularly in their immature stages. Sandfly biological management offers a lot of potential for providing long-term and environmentally beneficial solutions.

Biological control strategies for sand flies have been utilized in the past e.g. essential oils; (Ahmed *et al.,* 2018) and entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Ahmed *et al.,* 2021). De Barjac *et al.* (1981) demonstrated the role of *Bacillus thuringiensis* var *israelensis* in the control of *Phlebotomus papatasi* and *Lutzomyia longipalpis* larvae. *Bacillus* species have been employed as an alternate control approach for some medically important Diptera, and the potential use of these entomopathogenic agents against phlebotomine vectors has been discussed (Pener & Wilamowski 1996; Robert *et al.,* 1997). Only one study evaluated the effect of *Bacillus sphaericus* (strain-2362) offered in the laboratory to *P. papatasi* in different concentrations (Wahba 2000).

Around the world, highly effective mosquitocidal strains of the microbial agent *B. sphaericus* have been used to suppress mosquito larvae (Mulla *et al.,* 2003 and Kong *et al.,* 2017). This entomopathogenic bacteria offers a number of advantages, including minimal environmental toxicity due to the great specificity of *B. sphaericus* toxins, high efficacy, environmental persistence, and the capacity to overcome resistance to commonly used (Nielsen-LeRoux *et al.,* 2001). BinA and BinB are the two main polypeptides found in *B. sphaericus* crystals, a 42-kDa polypeptide, and a 51-kDa polypeptide, respectively (Darboux *et al.,* 2007).

The use of methoprene (IGR) is based on its effect on the chitin synthesis process and interferes with the physiological processes of insects, eventually causing the death of these organisms. The main sub-groups of methoprene (IGR) are chitin synthesis inhibitors, juvenile hormone analogues and anti-juvenile hormone analogues. Chitin synthesis inhibitors interfere with larvae moulting, especially in the first several stages. Juvenile hormone analogues or Anti-juvenile hormone analogues affect the insects through their impacts on normal transformation processes (Jiang *et al.,* 2008).

The ultrastructure and histopathological changes in the midgut of *P. papatasi* larvae after methoprene (IGR) and *B. sphaericus* treatments are conducted in this research.

MATERIALS AND METHODS

Rearing Experimental Sand Fly:

Phlebotomus papatasi larvae were acquired from a laboratory colony at Ain Shams University in Cairo, Egypt's Department of Zoology, Faculty of Women for Arts, Science, and Education. Modi & Tesh (1983) colonization and raising protocols were followed. The sand flies were raised in an ecologically controlled, walk-in insectary with a photoperiod of 12:12 (L:D) and a temperature of 27± 2°C. Adults were given a 30 percent sugar solution and females were allowed to feed on mice as a blood source. The larval diet was made up of ground and autoclaved cow blood and dried rabbit pellets. Third-instar larvae were subjected to bioassay tests.

Bioassays Methoprene (IGR):

The granular formulation of methoprene (IGR) was ground to the uniformity of fine particles with a mortar and pestle and agitated for 1 h in distilled water. The IGR (ALTOSID® SBG II Single Brood Granule slowly releases effective levels of (S)-

methoprene insect growth regulator), were dissolved by w/v to make a stock solution of 1 g/L. This suspension was subjected to one concentration (1µl for 10 larvae).

Bacterial strain: Two strains of *Bacillus sphaericus* 6184 and 6446 were provided by Abbot laboratories. Suspension was prepared by suspending 1 g of the granular formulation in 1 Liter of distilled water.

Treatment Conditions:

All experiments were conducted at a temperature of 27 ± 2°C and a relative humidity of 70 ± 5%. *P. papatasi* 3rd instar larvae were fed a larvae diet mixture including one concentration (1µl /10 larvae) of the control agent. Larvae of the control group were fed a regular medium after a 20-minute mixing incubation at 33°C and humidification for 48 h. The concentrations that were chosen were based on Abo El-Mahasen et al., 2010. Four groups of laboratory *P. papatasi* larvae were used in the tests. simultaneously. The first group was fed a normal medium, the second group was fed methoprene (IGR), the third group was fed *B. sphaericus* strain 6184, and the fourth group was fed *B. sphaericus* strain 6446.

The following tests were carried out to assess the impact of methoprene (IGR) and two strains of *B. sphaericus* on the midgut epithelium of 3rd instar larvae of *P. papatasi*.

Histopathological Examination of Larval Midgut 48 h Post Treatment:

1- By light Microscope: Treated and untreated larvae of the *P. papatasi* were stained with Hematoxylin and Eosin according to the method of Bancroft and Stevens (1996).

2- By Transmission Electron Microscope (TEM): This technique was carried out through transmission electron microscope microscopy (TEM, JEOL 1000, Japan) at the laboratory of the Faculty of Science, Ain Shams University. following the method of Van Rie et al. (1990).

RESULTS

The present study deals with the changes that occurred in the midgut of 3rd larval instar of *P. papatasi* treated with IGR and two strains of *B. sphaericus* by using the transmission electron microscope (TEM) and the light microscope (LM).

TEM of the Larval Midgut of *P. papatasi*:

The normal midgut wall of *P. papatasi* larvae appears to be made up of a single layer of epithelial cells, including columnar and goblet cells, sitting atop a basement membrane linked to the muscular connective tissue. The columnar cells are cylindrical in shape and have a massive granular nucleus (N) at the cell's midpoint. On the apical surface of columnar cells (arrows), many microvilli emerge as a striated border (Fig. 1A). Numerous organelles, such as mitochondria and the endoplasmic reticulum, are found in the cytoplasm. The basement membrane is deeply folded, and mitochondria (M) are plentiful near the cell's base, which has a spherical or cylindrical shape (Fig. 1B).

The TEM of the midgut treated with methoprene (IGR) exhibited degeneration 48 h post-treatment, with an influence on appetite clear cells. The epithelial cells and peritrophic membrane, however, showed some differences in size, shape, and electron density. A solitary oval nucleus is found at the cell's apical portion (Fig. 2A). A well-developed granular endoplasmic reticulum was seen in the cytoplasm. Mitochondria are typically elongated and more abundant at the cell's basal area. Many microtubules and microfilaments can be seen in the cytoplasm, especially in the apical part of the cell. Multi-vesicular bodies were also seen quite frequently (Fig. 2B).

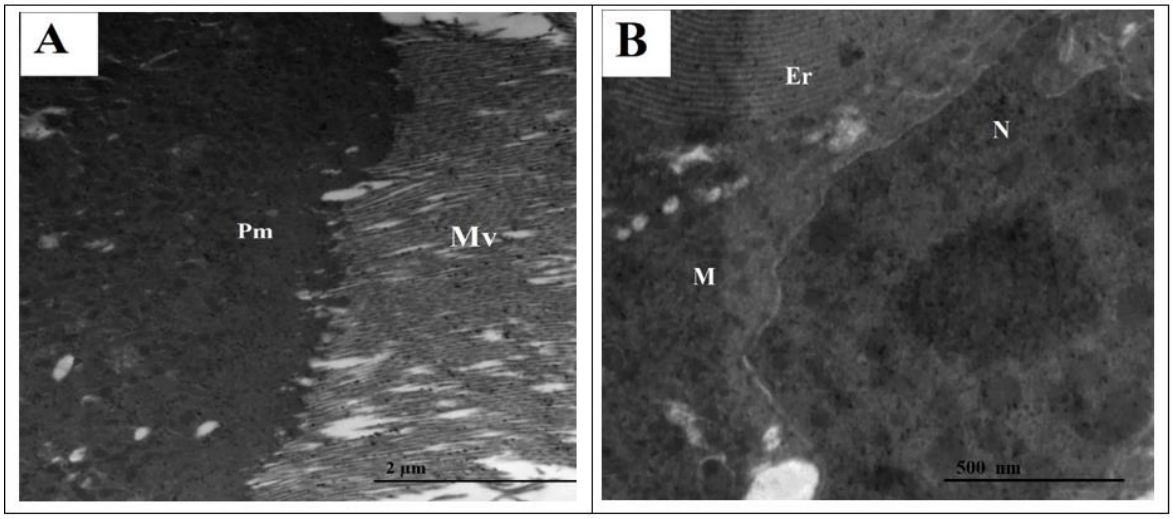

Fig. 1. Transverse ultrathin sections of untreated *P. papatasi* third larval instar. (A &B) the cell of untreated larva rich in microvilli (Mv), mitochondria (M), normal nucleus (N), and Endoplasmic reticulum(Er) (Scale bar= 2µm and 500 nm respective).

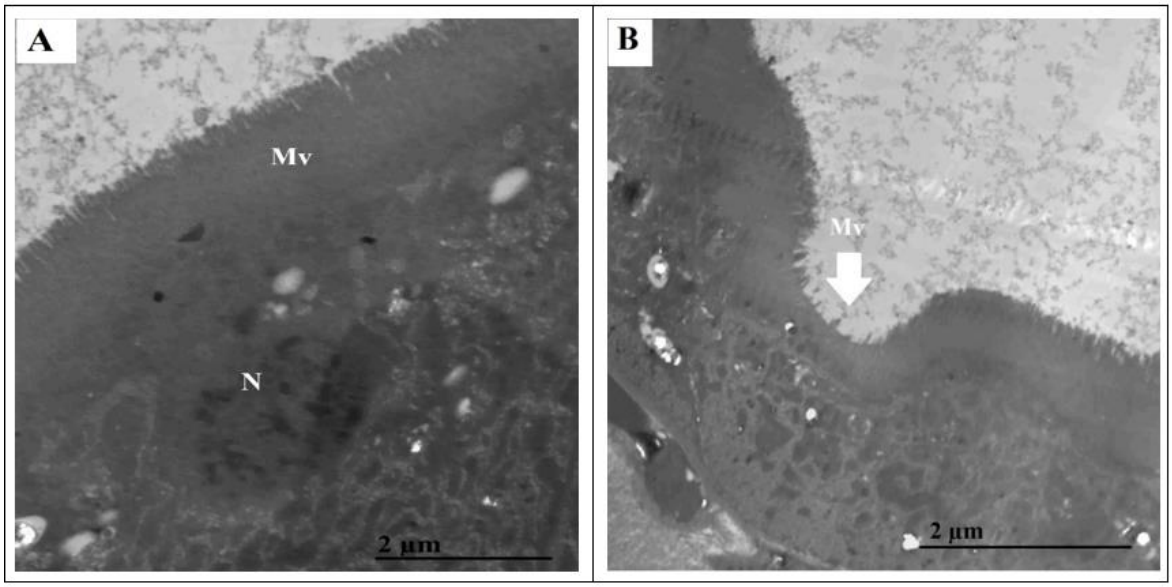

Fig. 2. Transverse ultrathin sections of treated *P. papatasi* third larval instar treated with methoprene (IGR). (A&B) cell from 48 h-treated larvae with preserved microvilli (Mv) and N: nucleus. (Scale bar= 2µm).

Bacillus sphaericus 6184 spores were found in the lumen 48 h post-treatment, microvilli were damaged (Fig. 3A). The bacterium, on the other hand, began to penetrate the microvilli. The presence of bacteria in the lumen was visible at this time. Bacteria started infiltrating the epithelial layer. Several cytopathology affecting the microvilli, mitochondria, and rough endoplasmic reticulum were reported in the midgut epithelial cells of sand fly larvae intoxicated with Bin. Still, the most dramatic feature of Bin intoxication was the appearance of abnormal, electron-clear vacuoles, indicating an important cellular stress (Fig. 3B).

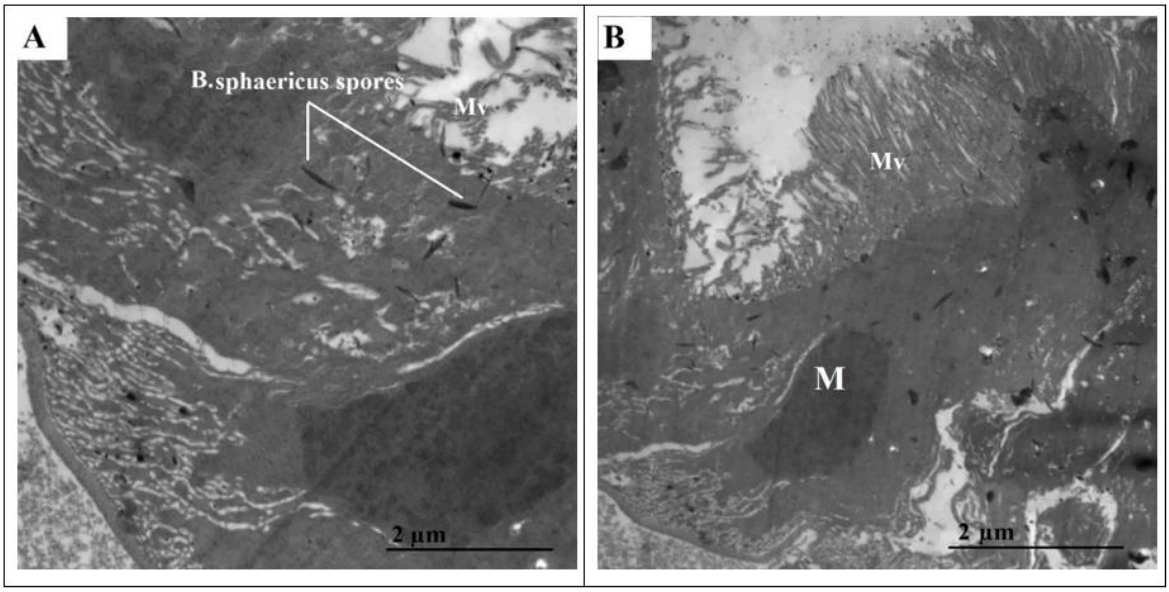

Fig. 3. Transverse ultrathin sections of treated *P. papatasi* third larval instar with 6184 *B. sphaericus*. (A&B) cell from 48h-treated larvae showing *B.sphaericus* spores, damage of microvillus (Mv)**,** and mitochondria(M) (Scale bar= 2µm).

After 48 h of treatment with *B. sphaericus* 6446, certain ultrastructural alterations were observed in the midgut of treated *P. papatasi* larvae, which deteriorated. The basement membrane began to fold more deeply, and the mitochondria in the basal areas were compressed. Columnar and goblet cells separated, resulting in scatting into the gut lumen. Microvilli were disturbed, but their appearance remained unchanged. Organelles and cytoplasmic structures were severely altered. Numerous vacuoles and a damaged brush border differentiated the cytoplasm. The nucleus sheath was disrupted by fragmented chromatin in the nucleus (N) with a terminal location. (Fig. 4 A). In the midgut epithelial cells of treated *P. papatasi* larvae, ultrastructural changes revealed lyses of epithelial cells and a change in nuclear structure with clumping of chromatin material. Cellular vacuolization occurs in the cytoplasm. Lysosomes and mitochondria were degraded. Peritrophic membrane separation from epithelial cells causes malformation. The intercellular connection that connects the cells vanished as a result of the treatment. The endoplasmic reticulum was divided into smaller vascular structures. The mineralized material is deposited in multi-vesicular structures in the cytoplasm of epithelial cells. They took up a significant percentage of the cytoplasmic space (Fig. 4 B). Spores of *B. sphaericus* were found in the lumen (Fig. 4 C).

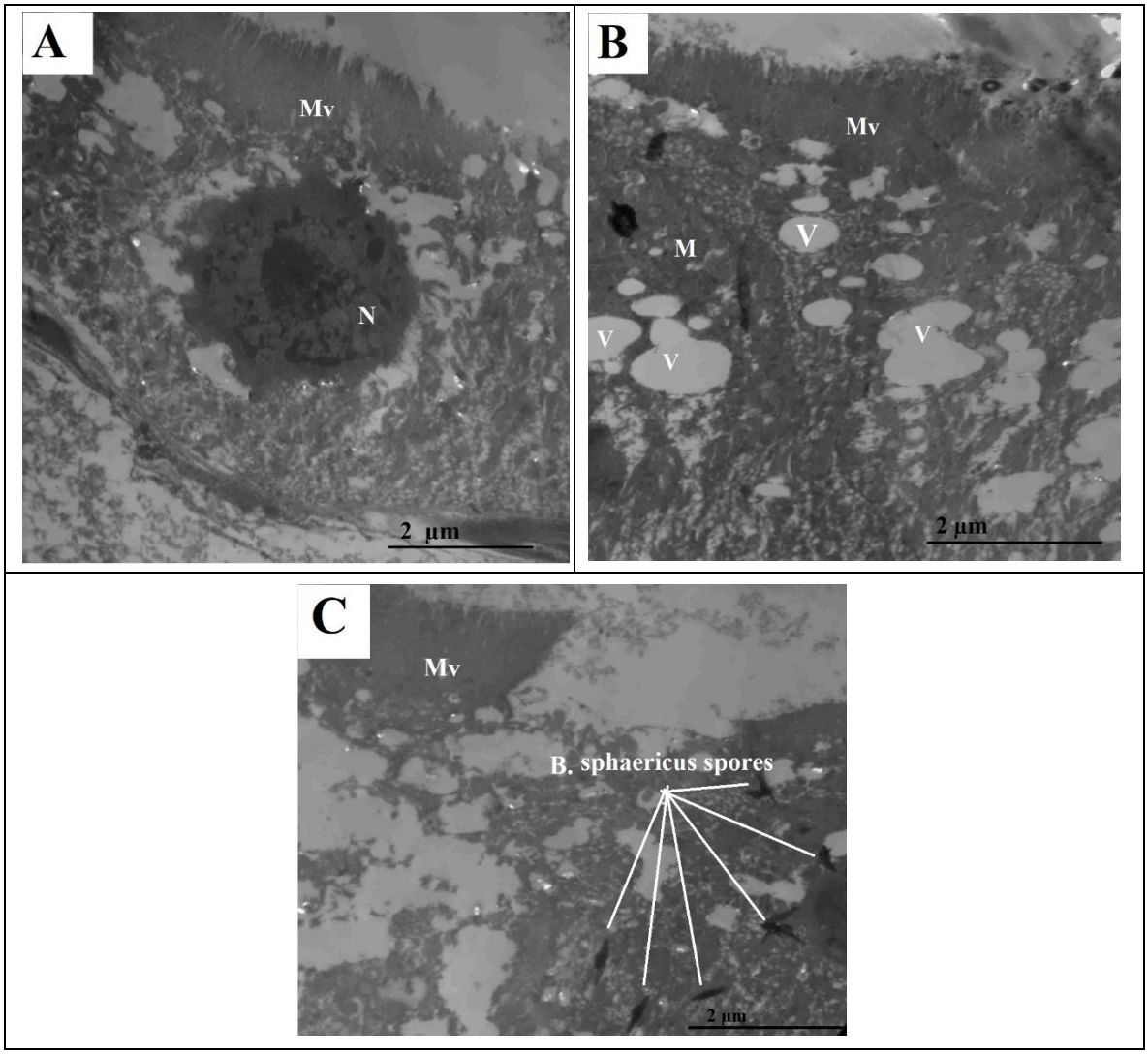

Fig. 4. Transverse ultrathin sections of treated *P. papatasi* third larval instar with *B. sphaericus* 6446 (A&B&C) cell from 48h-treated larvae showing evident microvillus damage (Mv), large cytoplasmic vacuoles (V), mitochondria (M) and *B. sphaericus* spores (Scale bar= 2µm).

Histopathological Examination of 3rd Larval Midgut of *P. papatasi* 48 h Post-Treatment:

The alimentary canal of the third instar larva of the sandfly is formed of three familiar regions i.e., foregut, midgut, and hindgut. The midgut is functionally responsible for digestion and absorption of nutrients. The anterior midgut is lined with a single continuous layer of columnar cells with clear cytoplasm, oval-shaped nuclei, and a well-developed brush border (Fig. 5 A). The transverse section in the anterior midgut region of the 3rd instar larva treated with methoprene (IGR) showed slight changes such as overgrowth of the brush border and multifocal destruction of the midgut wall with sloughing of the periepithelial coat (Fig. 5 B). The larvae that were treated with *B. sphaericus* 6184 showed that the focal area was sloughed and dissociation of epithelial cells from each other and epithelium cell contents passed into the midgut lumen (Fig. 5 C). Finally, the effects of *B. sphaericus* 6446 after being ingested into the 3rd instar *P. papatasi* larvae midgut were shown to be disruption, separation, and complete degeneration of epithelial cells (Fig. 5 D).

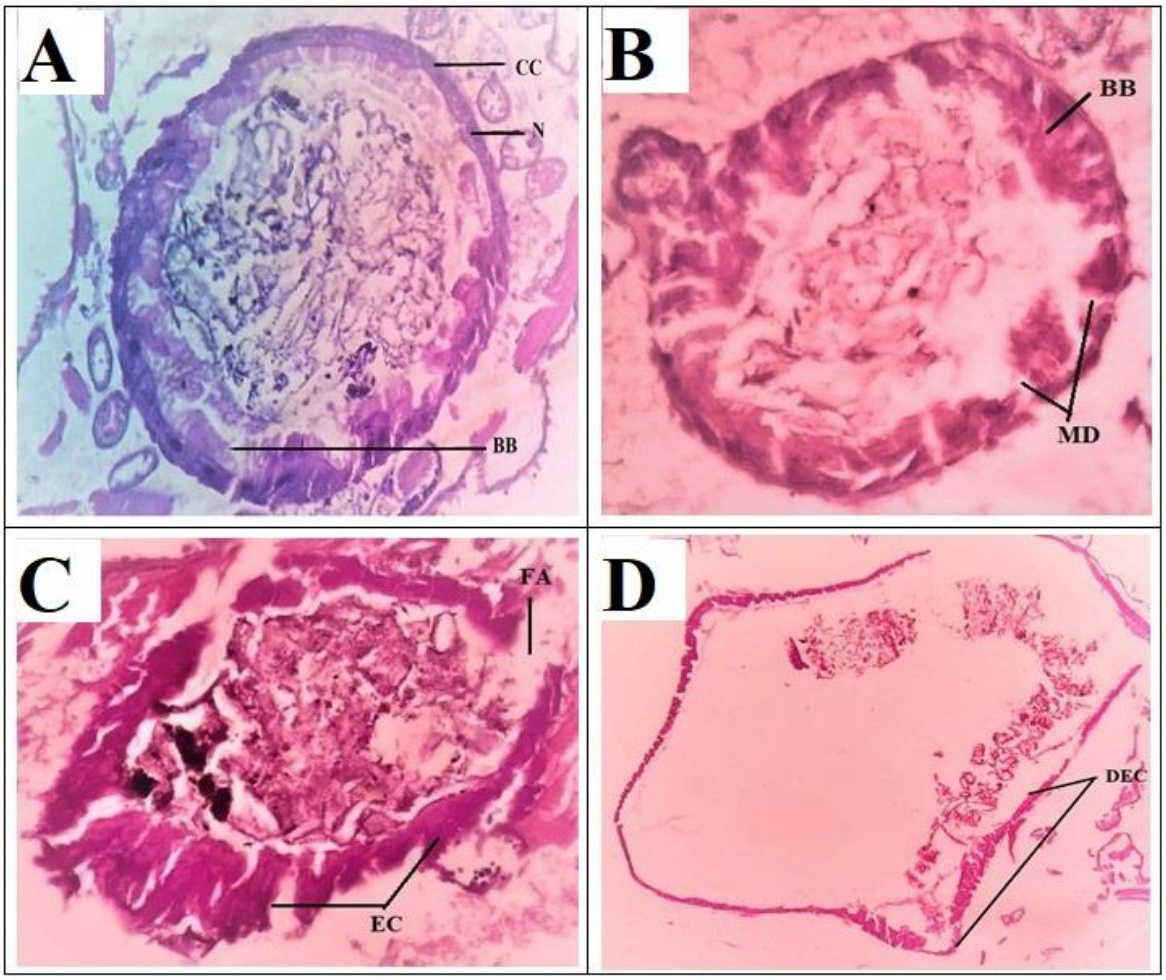

Fig. 5. Histopathological effect of methoprene (IGR) and *B. sphaericus* on the midgut of 3rd instar larvae of *Phlebotomus papatasi*, 48h post-treatment. (A) represent cross-sections in the midguts of untreated larvae. (B) represent cross-sections in midguts of treated larvae with methoprene (IGR). (C) represent cross-sections in midguts of treated larvae with *B. sphaericus* 6184. (D) represent cross-sections in midguts of treated larvae with *B. sphaericus* 6446.

DISCUSSION

Many scientific trials have been conducted to identify the compounds that effectively control disease-carrying insects. Chemical pesticides have been discovered to have negative consequences on humans, biomass, and the environment.

Using the transmission electron microscope (TEM) and histopathological examinations, the current work used methoprene (IGR) and two *Bacillus sphaericus* (6184 and 6446) species to suppress the sand fly *P. papatasi*, which is the vector of cutaneous leishmaniasis in Egypt. Despite the importance of histological evaluation of methoprene (IGR) against sand fly larvae, there have been few studies on this topic. The main aberrations generated in *P. papatasi* midgut larvae as a result of methoprene (IGR) were described in this work. Microvilli were damaged and certain places lost their microvilli 48 h post treatment.

Many of the histopathological abnormalities revealed in the present study for the midgut of *P. papatasi* larvae treated with methoprene (IGR) are comparable to those reported by Thabit *et al.* (2010) which are based on the effect of methoprene (IGR) on the chitin production process. In addition, Federici (2003) discovered that when insects eat a toxicant,

a toxic peptide is released, which binds to locations on the microvillar membrane of the midgut, causing cytolysis, which leads to the usage of methoprene (IGR) based on their influence on the chitin manufacturing process.

The morphological effects of two strains of *B. sphaericus* toxin on midgut epithelial cells of *P. papatasi* larvae, on the other hand, are documented in this study. Cytoplasmic vacillation, mitochondrial fragmentation, and microvillus disruption were among the major changes identified 48 hours post-*B. sphaericus* exposure. After treatment with Bin toxin, similar results have been reported (de Melo *et al.,* 2008). However, mitochondrial dilation, which was characterized by internal matrix breakdown and organelle distortion without rupture of the exterior membrane, was a unique result not seen before with the Bin toxin (de Melo *et al.,* 2008). Mitochondrial injuries were a significant side effect of two *Bacillus* strain treatments. These organelle malfunctions can be dangerous because they create an increase in reactive oxygen species, which can harm important cellular components (Andreassen, *et al.,* 2000).

Cavados *et al.*, 2004; Luthy and Wolfersberger 2000 have previously reported changes in mitochondrial morphology (mitochondrial swelling and inner matrix destruction) following *B. thuringiensis* toxin action on mosquito and lepidopteran larvae midgut epithelia. Other significant alterations detected, such as the induction of cytoplasmic vacuoles and the dense aspect of the cytoplasm, are similar to Bin toxin effects and suggest that Bin toxin may act in a synergistic manner with Cry11Aa in cells lacking the Cqm1 receptor, consistent with studies showing that the combination of Bin toxin and Cry11Aa can overcome resistance (Wirth *et al.,* 2004). The initial step of delta-endotoxin activity was thought to be binding to particular receptors on the apical microvilli membrane (Ravoahangimalala *et al.,* 1993). Cry4 generated by *B. thuringiensis israelensis* was also found to bind to the microvilli of *Culex pipiens* posterior midgut and stomach caecae epithelial cells (Yamagiwa *et al.,* 2001). Microvilli of midgut epithelial cells of *Streptomyces griseus*-treated *Culex pipiens autogenicus* larvae, on the other hand, were unaffected, and there were no alterations in cell arrangement in the tissues (Zizka *et al.,* 1989). In addition, *B. sphaericus* spores were seen in the lumen and the bacterium began to invade the microvilli (Tewfick and Soliman 2018).

The histological structure of the third instar larvae of the sand fly *P. papatasi* was investigated in this work. The third larval instar midgut, according to Dow (1986), is the most functionally essential component of the digestive system.

The present study can conclude that such treatment caused significant histopathological alterations in the insect's midgut 48 h post-treatment, including multifocal damage, brush boundary overgrowth, columnar cell unbinding, and the loss of the peritrophic matrix.

Toxin complex a (Ta) from the bacterium *Photorhabdus luminescens* induced swelling of the apical regions of columnar cells in the anterior midgut of the Tobacco hornworm *Manduca sexta*, and these cells extruded large cytoplasmic vesicles into the gut lumen and formed apical blebs, according to Blackburn *et al.* (1998).

Finally, such cells fragmented, leaving huge gaps along the basement membrane that was empty of cells. In another investigation, *Bacillus thuringiensis* and *Bacillus sphaericus* isolate caused significant rupturing and sloughing of the columnar epithelium of the midgut of Sandfly *P. papatasi* larvae, as well as degeneration of their microvilli.

The anterior midgut area of the 3rd instar larva treated with methoprene (IGR) showed minor alterations such as brush boundary overgrowth and multifocal disintegration of the midgut wall with sloughing of the periepithelial layer in a transverse section.

Abd-El Wahed *et al.* (2011) used five insect growth regulators methoprene (IGR) to increase the susceptibility of the cotton leaf worm, *Spodoptera littoralis* (Boisd.)

(Lepidoptera: Noctuidae), and also tested the histopathological effects on the midgut, such as loss of the compact appearance of the muscularis layer, vacuolation, and exfoliation of the columnar cells. The larvae treated with *B. sphaericus* 6184, on the other hand, showed sloughing of localized areas and detachment of epithelial cells from one another 48 hours post-bacterial treatment. Midgut cells of *Aedes aegypti* larvae treated with *B. thuringiensis* toxins had the same impact (Tjokropranoto *et al.,* 2016 and Kong *et al.,* 2017).

Tjokropranoto *et al.,* 2016 looked at the combined effect of *B. thuringiensis* var. israelensis (Bti) and *B. sphaericus* (Bs) 2362 on *Cx. quinquefasciatus* larvae's midgut. Furthermore, Kong *et al.* (2017) investigated the effect of *B. subtilis* on *Aeromonas hydrophila*-induced damage and inflammation of the intestinal mucosal barrier function in grass carp. The peritrophic matrix lining of the midgut cells was also shown to be distorted. When *Heliothis armigera* larvae were treated with Txp40 toxin protein from *Xenorhabdus* and *Photorhabdus* bacteria, all of these tissues were destroyed (Brown *et al.,* 2006). *P. papatasi* larvae in the third instar treated with *B. sphaericus* 6446, on the other hand, showed evident modifications such as entire epithelial cell disintegration.

The results of the current study's histopathology investigation of *B. sphaericus* show that the findings are identical to those seen in Bti-infected midguts in prior studies (Bravo *et al.,* 2007, Soberon *et al.,* 2007, Al-Roba *et al.,* 2011). This could add to the evidence that these bacteria are viable candidates for use as biocontrol agents against the sand fly vector. These findings suggest that *B. sphaericus* 6446 was the best type for controlling 3rd *P. papatasi* larvae because it generated significant histological alterations in the midgut of sand fly larvae, which are responsible for their morbidity.

The principal aberrations caused in *P. papatasi* midgut larvae as a result of swallowing methoprene (IGR) and *B. sphaericus* were investigated in this work (6184 and 6446). The sand fly, *P. papatasi* larvae, was better controlled by *B. sphaericus* 6446.

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Declarations:

Ethical Approval: Not applicable.

Authors Contributions: Dr. Belal A. Soliman made a great effort in the technique of the experiment. Dr. Maha Moustafa Ahmed made great efforts to breed different stages of Phlebotomus papatasi used in this experiment. Dr. Heba Yehia and Dr. Maha Moustafa were a major contributor to the manuscript's writing. All authors read and approved the final manuscript.

Competing Interests: The authors declare that they have no competing interests.

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ARABIC SUMMARY

التغيرات النسيجية والتركيبية الدقيقة في أمعاء يرقات الطور الثالث من حشرة ذبابة الرمل من نوع *فليبوتوماس بباتاسي* بعد معالجتها بالميثوبرين وسلالتين من بكتيريا *باسيليس سفريكس*

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تهدف هذه الدراسة إلى بحث التغيرات فوق التركيبية والمرضية في أمعاء يرقات الطور الثالث من حشرة ذبابة الرمل من نوع فليبوتوماس بباتاسي *Phlebotomus papatasi* بعد معالجتها بالميثوبرين (منظم نمو الحشرات) و باسيليس سفريكس *Bacillus sphaericus* من سلالتي 6184 و 6446 بإستخدام المجهر الإلكتروني النافذ (TEM) والمجهر الضوئي.

قد أظهر الفحص بالمجهر الإلكتروني النافذ بعد مرور 48 ساعة من المعالجة بالميثوبرين (IGR): تقشر الطبقة فوق الظهارية ونمو مفرط للزوائد المبطنة لجدار الأمعاء. بينما بعد مرور 48 ساعة من المعالجة ببكتيريا سفريكس من سلالة6184 تم اكتشاف الأبواغ في تجويف الأمعاء وبدأت البكتيريا في دخول الخلايا الدقيقة. وقد أوضحت النتائج بعد مرور 48 ساعة من المعالجة ببكتيريا سفريكس من سلالة 6446 تغيرات واضحة في أمعاء اليرقات مثل التحلل الكامل للخلايا الظهارية، مع وجود العديد من الفجوات في السيتوبلازم وانكسار الزوائد المبطنة لجدار الأمعاء. قد أوضحت النتائج المستخلصة من الفحص المجهري الضوئي أن: يرقات الطور الثالث المعالجة بـ بالميثوبرين (منظم النمو للحشرات): أظهرت تغييرات طفيفة مثل نمو مفرط للزوائد المبطنة للمعي الأوسط وتدمير متعدد البؤر لجدار الأمعاء بينما اليرقات المعالجة ببكتيريا سفريكس من سلالة 6184: أظهرت مناطق انفصال في الطبقة الطلائية في حين اليرقات المعالجة ببكتيريا سفريكس من سلالة6446: أظهرت تحلل كامل للخلايا الطبقة الطلائية المحيطية.

تخلص الدراسة إلى أن المعالجة ببكتيريا سفريكس من سلالة 6446 كان له التأثير الأكبر على أمعاء يرقات الطور الثالث من حشرة ذبابة الرمل من نوع فليبوتوماس بباتاسي، حيث تسبب في تلف خلوي واسع النطاق، مما يشير إلى أنه عامل مكافحة بيولوجي أكثر فعالية مقارنةً بالميثوبرين وبكتيريا سفريكس من سلالة6184. مما يشير الي الحاجة الهامة لإجراء أبحاث مستقبلية لتحديث استراتيجيات المكافحة المناسبة لمختلف نواقل الليشمانيا في بيئاتها المختلفة.