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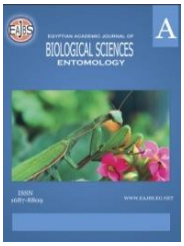

Microbial Stress Resistance of *Eristalis tenax* Rat-Tailed Maggots

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ABSTRACT

The drone fly maggots; *Eristalis tenax* (Diptera) have survived in habitats with sever microbial contamination. Despite this polluted environment, they avoid infection by microorganisms. In this paper, we have investigated the first physical barrier, the cuticle surface of *E. tenax* maggots collected from Alakrasha dain, Egypt, using scanning electron microscopy which revealed an array of high density and dimensions of nano and microstructures that narrow to sharp points and appear to prevent bacteria from colonizing its surface which interferes with the biofilms formation and potentially serves as a defence against the infection. This investigation leading us to more examine the antibacterial properties of the whole maggot extract naturally without any previous simulation, the results were promising against *Staphylococcus aureus* ATCC 6538, *Staphylococcus mutans* ATCC 25175, *Escherichia coli* ATCC 25922 and *Salmonella enterica* Serotype Typhimurium ATCC 14028 bacteria compared to Nitrofurantoin antibiotic. Such antibacterial properties of both the maggot cuticle surface and the whole maggot extract have applications in many different fields, including antibacterial surfaces and biofilms besides the future isolating and developing of antimicrobial peptides from the maggot crude extract which could be a breakthrough against antibiotic resistance.

INTRODUCTION

Eristalis tenax, the common drone fly belonging to family Syrphidae and order Diptera, with over than 5,000 recognized species worldwide (Rotheray, 1993). In contradiction of the flower-feeding habitats of adults, the immature stages of drone flies usually referred to as a rat-tailed maggot and are found in a wide variety of habitats. They prefer living in sluggish aquatic habitats with high organic and microbial pollution (Altincicek, & Vilcins kas 2007). Because *E. tenax* maggots prefer the sloppy waters with anaerobic conditions, they serve as trustworthy indicators in the biological assessment of the quality of water with sever pollution by organic materials (Chapman, 1996). Although rat tailed maggots are closely associated with filthy and highly polluted water, they're actually remarkably resistant to infection from all the bacteria that surround them, the case which attracts the attention of scientists to survive in such aquatic habitat which is

commonly do not colonized by larvae of other dipteran or any animal. Insects have antimicrobial properties and substances secreted on the surface or inside their blood to stop microbial infection (Hazlett and Wu. 2011), these antimicrobial properties and substances can be used for several defensive purposes, which comprise numerous antimicrobial peptides (AMPs) as efficient inhibitors against diverse pathogens (Brown *et al.*, 2008). AMPs, synthesized by the blood cells and the fat bodies and then secreted into the blood, are a major part of the immune defence (Manniello *et al*., 2021). They are hopeful candidates instead of traditional antibiotics, owing to their low toxicity to eukaryotic cells and their wide spectrum against bacteria, mycobacteria, viruses, fungi and cancer cells. Insect AMPs are among the richest sources than any other living organism, because of the high biodiversity of insects and their highly diversified living environments (Manniello *et al*., 2021). Recently, two cecropin-like peptides (EtCec1-a and EtCec2-a) and a diptericin-like peptide (EtDip) were identified under simulated physiological conditions which showed antibacterial efficiency against multidrug-resistant Gram-negative bacteria. However, the antibacterial activity of Rat-tailed maggot extract in natural conditions without any simulation has not yet been recorded. Accordingly, the purpose of this work is to reveal the adaptation to an environment with extremely high microbial pressure beginning from its surface as a first physical barrier, to examine the morphology of the drone fly maggots using scanning electron microscopy (SEM) and identify the configuration of micro-scale spikes on their body surface. Also, to screen the antimicrobial activity of the natural maggot extract without any inducement have against four bacterial species (two Gram-positive and two species of Gram-negative).

MATERIALS AND METHODS

Insects:

The maggots of Rat-tailed were collected in sufficient numbers (about a hundred) , (Fig 1), between March and April (2019), from an open drain in Alakrasha, Al Khankah city, Al Qalyubia Governorate, using a standard aquatic D-frame hand net. The third instar maggots of *Eristalis tenax* were identified using the suitable taxonomic keys established for the identification of hoverfly larvae. As diagnostic features, the following characters were observed: the lake of setae over the lower lateral margins and the last pair of prolegs with most of the outsized primary crochets opposite towards the body lateral margins. Primary crochets broad, strong, distinctly bent, their length barely exceeding their width at base; distal 2/5 of crochets intensely pigmented (Rotheray, 1993).

Fig.1: Larva of the Rat-tailed maggot, *Eristalis tenax* (Linnaeus).

Bacterial Strains:

We used the following human pathogenic bacterial strains, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Salmonella enterica* serotype Typhimurium ATCC 14028, and *Streptococcus mutans* ATCC 25175, were obtained from the Collection of American Type Culture.

Transmission Electron Microscopy:

Five Rat-tailed maggot samples were fixed, washed three times in phosphate buffer, osmicated, then washed, and dehydrated in alcohol. Then, the samples were sweetly re-suspended into hexamethyldisilazane (reagent grade >99%, Aldrich chemicals) and putted onto SEM stubs in a fume hood. The volatile hexamethyldisilazane vaporized quickly and kept the soft membranous structure of fragile specimens (Hayes *et al*., 2016).

Preparation of Rat-Tailed Maggot's Crude Extract:

Insect samples were washed thoroughly in clean sterile water, the whole bodies of 30 larvae were homogenized using a hand homogenizer. The homogenate was applied as a crude extract without any solvent.

Antibacterial Assay:

The selected human bacterial pathogens were subjected for antibacterial screening and their susceptibility patterns to Rat-tailed maggot extract using the standard disc diffusion method. In which holes with a diameter of 11 mm were punched into the agar and filled with 100 µl of crude extract. The diameters of the clear zones were measured after 24 h of incubation at 37°C, a standard antibiotic was used for comparison (Nitrofurantoin 300 µg) according to the standard procedures of the CLSI, 2020 and British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method [CLSI, 2020]. The experiment was done in triplicate and the mean diameter of a radius of clear inhibition of zones (mm) was calculated.

RESULTS AND DISCUSSION

Antimicrobial Susceptibility Testing:

The results of the antimicrobial susceptibility testing for the Rat-tailed maggot extract of the whole body as a crude extract show a high level of antibacterial activity against both Gram-positive and Gram-negative bacterial tested strains, zones of inhibitions are observed as indicators of antimicrobial activity. The obtained results revealed that the highest antibacterial activity after 24 hrs post-treatment against *Staphylococcus mutans* (46mm) followed by *Staphylococcus aureus* ATCC 6538(40), *Escherichia coli* ATCC 25922 (38mm), *Salmonella Enterica Ser. Typhi ATCC 14028* (35) compared to Nitrofurantoin 300 mcg (11mm) *for* Gram positives and 15 mm for Gram-negatives, Table 1 and Figure 2(A-D). All tested strains are resistant strains to Nitrofurantoin 300 µg except for *Salmonella Enterica* Ser. Typhi ATCC 14028 which not Applicable yet according to CLSI ,2020 interpretation.

Table 1. The antibacterial results of Rat-tailed maggot extractives/ mm.

Sample ID	Inhibition zone diameter (mm) on ager plate			
	***Staphylococcus aureus* ATCC 6538**	***Staphylococcus mutans* ATCC 25175**	***Escherichia coli* ATCC 25922**	***Salmonella Enterica* Ser. Typhi ATCC 14028**
Rat tailed maggots extract	40	46	38	35
Nitrofurantoin 300 µg	11	11	15	15
Interpretation to Nitrofurantoin 300 µg (CLSI ,2020)	Resistant if ≤ 14	Resistant if ≤ 14	Resistant if ≤ 14	Not Applicable

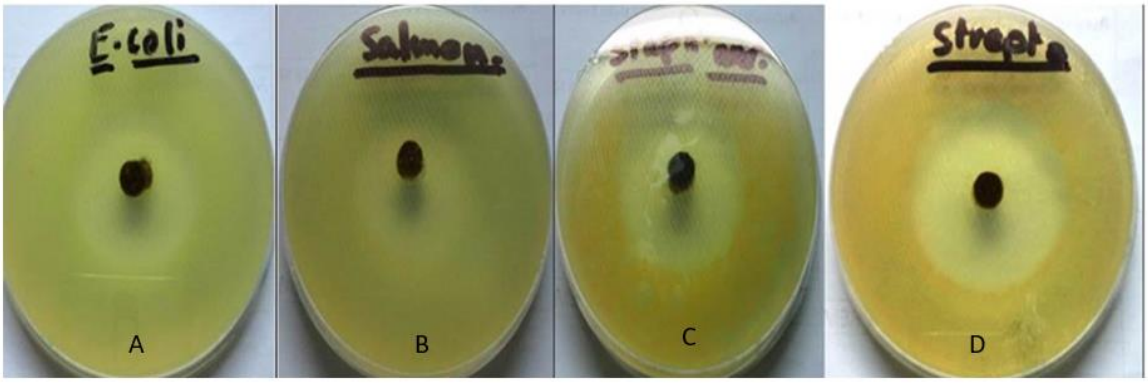

Fig.2: Antibacterial activity of Rat-tailed maggots crude extracts against (A)*Escherichia coli* ATCC 25922, (B) *Salmonella Enterica* Serotype Typhimurium ATCC 14028 (C)*Staphylococcus aureus* ATCC 6538, (D) *Staphylococcus mutans* ATCC 25175.

The antibacterial activity results showed that, tested extracts induced a high activity against both Gram-positive and Gram-negative comparing to many insects crude extracts as reported by Thomas *et al.* (1999), Leem *et al.* (1999), Yamauchi,(2001) and Amer *et al. (*2019). Positive bacterial strains were susceptible to the tested maggots' extracts more than Gram-negative bacterial strains. The obtained antibacterial activity of the crude extract in natural conditions without any simulation to explain the amazing interdependency of ecological adaptation.

However, a physiological induction to immune transcripts in *Eristalis tenax* maggots was performed by Altincicek & Vilcinskas(2007) and followed by Hirsch et al, (2020). .Altincicek & Vilcinskas(2007) identified thirty novel genes of *E. tenax* that were induced in response to septic injury including novel putative AMPs.Hirsch *et al,* (2020) tested the antibacterial activity under induced physiological conditions, a bacterial lipopolysaccharide was injected into *Eristalis tenax* maggots to enhance immunity-related AMPs that are produced in response to the injection, they are identified twenty-two AMPs and selected nine for larger-scale synthesis to explore their activity against multidrug-resistant Gram-negative bacteria. Two cecropin-like peptides (EtCec1-a & EtCec2-a) and diptericin-like peptide (EtDip) displayed potent activity against the pathogens.

The ecological niche with this extremely harsh environment enables *Eristalis tenax* immune system to overcome infection by a wide-ranging and rapid response to changed invading organisms, similarly, the larvae of *Hermetia illucens*, the black soldier fly, shows a surprising ability to live in severe environments, as it forages on decaying substrates, which are rich in diverse microbial colonies, and is one of the most promising sources for AMPs. The related physicochemical properties were resulted in identification of 57 active peptides appropriate for later investigational and validation studies (Moretta *et al.*, 2020).

Transmission Electron Microscopy of Rat-Tailed Maggot Surface:

Rat-tailed maggot surface observed by SEM exhibited with specific micron and nanoscale features like pillars, spikes and strips with various topographies Figure 3 (a, b, c & d), these features showed a significant overall reduction of adherent bacteria compared to the flat surfaces (Wu, *et al.*, 2018). Patterns of topography are larger than bacteria in every dimension and hence influencing the development of multicellular structures on surfaces. Depending on both the shape and the size of these larger surface patterns, it is possible to promote or hinder bacterial adhesion and therefore biofilm formation. Even if any microbes especially bacteria could fit between micro-structures

and attach to the surface; the presence of neighbouring microstrips, Figure 3c may interfere with creation of biofilms due to absence of enough space for the bacteria to divide or to form productive interactions with other colonizing microbes.

Nanopillars are grouped together in lattice-like arrays on the external surface of the maggot cuticle each about 10 μ thickness projected from the insect cuticle as illustrated in Figure 3 (a &b). Areas of the maggot cuticle on which the nanopillars are highly abundant are completely free of adherent bacteria, and other microbes like fungi and algae Figure 3 (a & b) (Hayes *et al.,* 2016). At the side where there are no nanopillars, a chain of bacilli bacteria is capable to adhere to the surface beside the center-to-center gap Figure 3a. the center- to center gap measuring 120 μ represents a separation track between nanopillars. A closer analysis of the mechanism discloses that nanopillars with their tapered pointed ends able to penetrate the membranes of adjacent bacterial cells and rupture them, leading to their departure. Flat and smooth surfaces cannot penetrate the bacteria cells, and thus lack antibacterial activity (Wu *et al.,* 2018). Spines and nanopillars are almost entirely absent from the siphon Figure 3 e. This may be due to scratching especially; the origin of each previous spine is present. There are trimmed nanopillars on the cuticle surface (hayes *et al.,* 2016).

All surface projections present random size, shape, and spatial distribution Figure 3 (a,c,d) resulting in more than one form of Topography, roughness and shape. Future work is required to set an actual correlation between surface topography and bacterial adhesion. It was concluded that the topography and surface chemistry are the critical features to consider in attaining potent bactericidal surfaces, and the slope presented by these surfaces can allow machine learning to design functional Antibacterial surfaces, (Dickson, *et al.,* 2015).

Cicada wings of different species, dragonfly, as well as a damselfly, possess topographies of mechano-bactericidal nanospike or nanopillar (Mainwaring, *et al.,* 2016). *Psaltoda claripenni*, known as Clanger cicada, whose wings have nanopillars (Pogodin *et al*,2013). The wings surfaces introduce a natural defense mechanism against bacteria, so it was expected that manmade nanopillars would exhibit comparable antibacterial properties. To this end, a study by (Dickson *et al*.,2015) showed that after *Escherichia coli* cells were grown on the nano-pillared surface, the nanopillars have a negative on the growth of the *E. coli* cells. The study concluded that nano-pillar surfaces killed more bacterial cells than smooth and flat surfaces did.

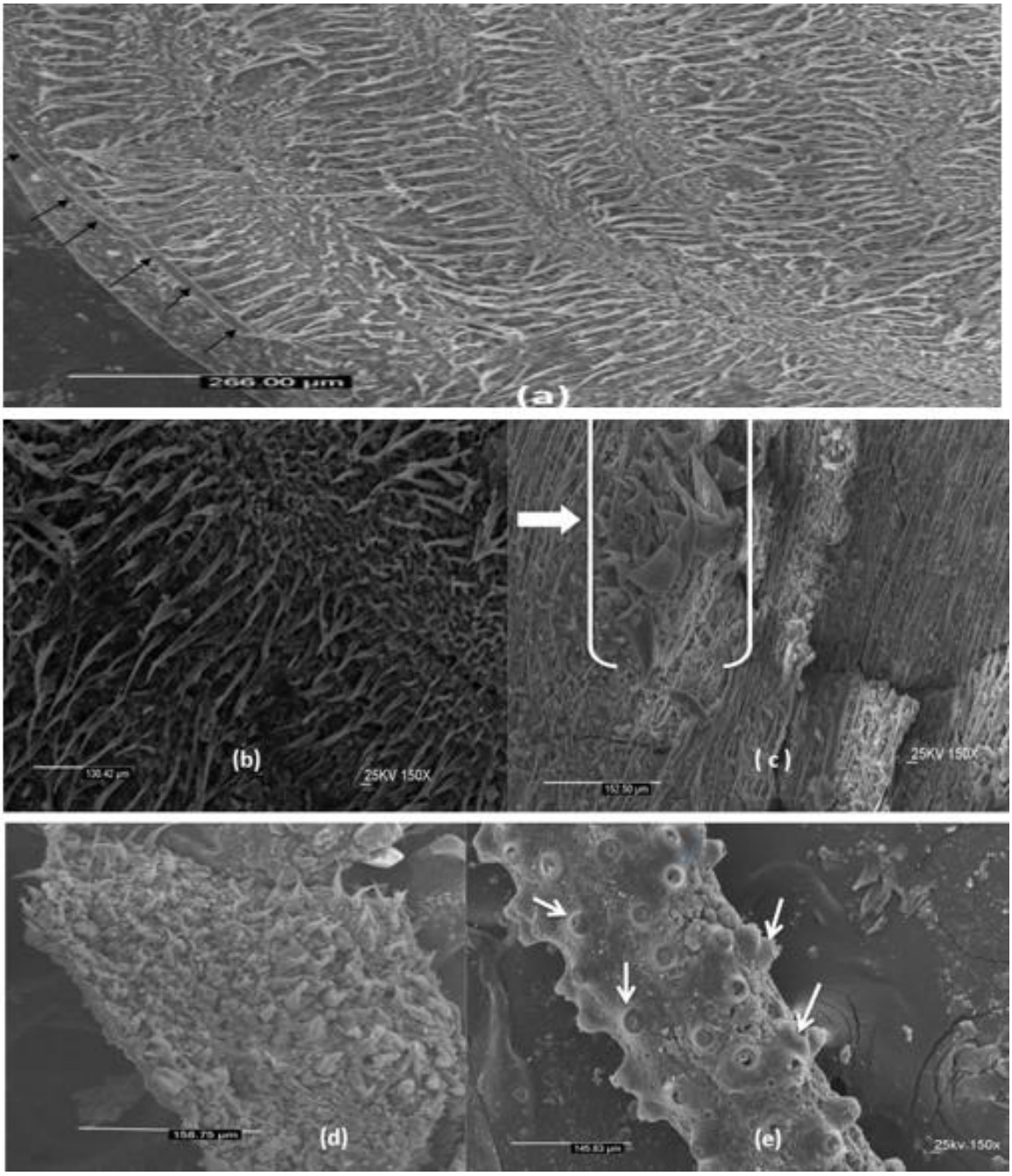

Fig .3: SEM image showing Rat-tailed maggot cuticle surface **(a)**: Nanopillar grouped together in lattice-like arrays, the white arrows refer to a chain of bacilli bacteria at side areas lacking nanopillars. **(b):** A higher enlargement image to Nanopillars, (25KV 150X) the diameter at the base and top, 100 and 10 µ respectively, the height of nanopillars is 120 µm. (c): Different topographies in the same section demonstrating Micro stripes (length of 50 µm and thickness of 2-10 nm and other projections, scale bar 152. 50 µm. (d): SEM image showing Strong Topography (microscale) spike-like microstructure with pointed tapering ends. Scale bar 158.75 µ m. (e) The siphon with truncated nanopillars.Scale bar 145.83 µm .

Conclusion

Screening the antibacterial activity of *E.tenax* maggots crude extract and the antimicrobial properties of their cuticle surface demonstrating that *E .tenax* maggot prove useful in the fight against invading pathogenic bacteria and can potentially be a source of novel antibiotic-like compounds for infection control beside inspiration to artificial antimicrobial surfaces.

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