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Response of Aquatic Bacterial Species Isolated from Contaminaed Water to Whole Body Extract of *Lucilia sericata* Larvae

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ABSTRACT

Drainage microbial contamination has been identified as a serious human health risk that have a detrimental impact on health issues brought on by the invasion of pathogenic organisms and are associated with wound infection. Ingestion or contact of polluted water are the two ways that these bacteria might infect humans. Maggot debridement is a commonly used treatment around the world to clean wounds. Based on these data, the aim of this work was to identify the microbial contamination, examining gene expression and possible antibacterial properties of metabolites from Extraction/Secretions (ES) of *Lucilia sericata* larvae. In this study, the antibacterial activity of the ES of *L. sericata* larvae was examined against Gram-positive *Streptococcus pneumoniae*, *Staphylococcus aureus*, and Gram-negative *E. coli*, *Pseudomonas aeruginosa* bacteria, both in their sterilized and multi-antibiotic-resistant forms. The agar well diffusion method was utilized to assess the maggots' ES in comparison with the strains. A 2-D PAGE protein analysis was performed. Inhibition zones were observed for *S.aureus* (18.3±2.1mm), *Streptococcus pneumoniae* (13.4±0.58mm) and *E. coli* (20.4±2.0mm); however, the extract was unable to produce an inhibiton zone for *P.aeruginosa*. Different proteins with varying molercular mass (<20-150kDa) and pI (3.3-7) were observed using 2-D PAGE. Following a series of antibiotic treatments, we assessed *lucifensin* and *attacin* gene expression changes in the bacteria. The antibacterial impact was investigated using antibiotic disk diffusion and optical absorption by analyzing the expression of the previously known genes. Using Fluorescence-activated cell sorting analysis, various extract dilutions showed varying killing rates for *S. aureus*, *S. pneumoniae*, *E. coli*, and *P. aeruginosa*, with killing rates of 76, 71, 89.2, and 49.1% for the lowest (1/64) dilution, respectively.

INTRODUCTION

The presence of water contaminants and aquatic bacteria, particularly those subjected to human interaction during cleaning practices, can significantly impact water quality and public health **(Alaidarous *et al*., 2017)**. It is obvious that there are risks of spreading microbial infections with commensal and pathogenic microbes through direct contact with water contaminants. In wound management, debridement is a recognized technique. Bacteria in the wound form biofilms and release substances that block the skin's defense mechanisms. As a result, the infection persists, and the production of new skin cells is halted because immune cells are unable to eradicate the bacteria. This can slow the healing process and may

ultimately lead to insufficient wound healing **(Kadam & Kaushik, 2020)**. Its elimination of foreign matter and devitalized or polluted tissue is supposed to lower the risk of infection and facilitate wound healing **(Zarchi & Jemec, 2012)**. Given that certain insect species and vertebrates excrete maggots, their excretion may contain antibacterial chemicals **(Selsted *et al.*, 1992; Weinbergl *et al.*, 1998)**. In addition to helping maintain homeostasis, the skin serves as a physical barrier against environmental harm, particularly infections. Any disruption in the skin's structure can lead to the loss of bodily fluids or the onset of pathological infections **(Baum & Arpey, 2005)**. Among these methods, biosurgery, larval therapy, and maggot debridement therapy (MDT) are the most widely researched, used, and frequently practiced in many countries **(Sherman *et al.*, 2013)**. It provides a healing advantage. Maggots may shorten treatment duration, however this is debatable, as is the treatment's cost-effectiveness and possible antimicrobial activity **(Moya-López *et al.*, 2020)**. Wound healing typically stops at the inflammatory stage and is unable to combat drug-resistant bacteria. Acute wounds can occasionally turn chronic since they don't always heal according to a predictable pattern **(Ousey & McIntosh, 2008)**. Therapeutic benefits include wound disinfection, accelerated wound healing, and debridement (removal of necrotic tissue) **(Nigam *et al.*, 2006)**. It seems that no single action can be identified due to the extensive research on the multiple actions occurring simultaneously, despite several studies focusing on their mechanisms of effect **(Fleischmann *et al.*, 2004)**. Several investigations have concentrated on the ES of larvae of *L. sericata*. Researchers discovered a number of components that might influence the healing process of chronic wounds **(Kruglikova & Chernysh, 2011; Valachova *et al.*, 2014)**. Antimicrobial peptides, as the most important components of the insect immune system, are known for their exceptional resistance to a wide range of diseases and parasites **(Hoffmann, 1995; Bulet *et al.*, 1999)**. Most of these peptides are characterized by their low molecular weight **(Hoffmann, 1995)**. The antibacterial properties and beneficial effects of maggots may influence the proteins of the excretions and secretions (ES). The molecular weight (MW) of an unidentified protein can be determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) **(Van der Plas *et al.*, 2008)**. A broad range of peptides and polypeptides have been shown to be active against fungi, bacteria, and both Gram-positive and Gram-negative organisms **(Hoffmann, 1995; Fehlbaum *et al.*, 1996)**. *In vitro* studies of antibacterial activity have demonstrated their effectiveness against numerous types of bacteria **(Robinson & Norwood, 1933; Simmons, 1935; Pavillard & Wright, 1957; Vistnes *et al.*, 1981)**.

The aim of this work was to identify the microbial contamination, examining gene expression and possible antibacterial properties of metabolites from Extraction/Secretions (ES) of *Lucilia sericata* larvae.

MATERIALS AND METHODS

Insect used and rearing

The 3rd larval instar of *Lucilia sericata* was collected from the slaughterhouse near Al-Awaed, Alexandria Governorate, Egypt, and morphologically identified using identification keys **(Crosskey & Lane, 1993; Williams & Villet, 2014)**. The fly colony

was maintained in the Medical Entomology Laboratory, Animal House, Zoology Department, Faculty of Science, Al-Azhar University. Adult *L. sericata* flies deposited their eggs on fresh liver, which was replaced every one to two days. After hatching, the larvae were fed liver and placed in plastic boxes (25 x 15 x 10cm) containing fresh liver and covered with gauze. Once the larvae were ready to pupate, they moved the moist food to the top of the box, where sawdust was placed. The pupae were then transferred to wooden cages. Environmental conditions were carefully controlled with light/dark cycles of 14:10 hours and temperature and humidity maintained at 26 ± 2 °C and 75 ± 5%, respectively **(Sanei-Dehkordi *et al.*, 2016)**. The adult flies were fed a 1:1 mixture of powdered milk and sugar water soaked in cotton. To collect eggs, pieces of fresh cow liver (100-150g) were placed in the cage for one full day. The livers, along with others, underwent the same feeding procedure and produced fresh eggs after 4 to 8 hours of egg laying. Larvae in the 2nd and 3rd instars were detected and fed with more fresh liver. These larvae were then collected, washed with sterile saline, and prepared for use.

Preparation of ES of *L. sericata* larvae and sterilization

To extract 100 milliliters of the extract and remove extraneous matter, 350 3rd-stage larvae of *L. sericata* were collected from the colony. The larvae were placed in a 50ml Falcon tube, starved for eight hours, and then rinsed with saline solution before being cleaned with distilled water for ten minutes. The tube was covered with aluminum foil and incubated at 37°C for three hours **(Cruz-Saavedra *et al.*, 2016)**. Larval excretions and secretions (ES) were collected by pipetting and were centrifuged at 10,000 rpm for 12 minutes at 4°C. The supernatant fluid was then collected as the extract and sterilized using Millipore bacterial filters (0.22µm) **(Kawabata *et al.*, 2010)**.

Sample collection

Water samples were collected from Elsaff City, located along the Helwan-Alkurimat Road in Cairo, Giza. Aseptic practices were followed during the sampling process.

Bacterial isolation and characterization

After inoculating the collected samples on nutrient agar, blood agar, and MacConkey agar, they were cultured for 48 hours at 37°C. Following incubation, purification and subculturing were performed on the isolated colonies. Pure colonies were selected using nutrient agar and 5% sheep blood agar. Each isolate was initially identified using oxidase, catalase, and Gram stain tests **(Alaidarous *et al.*, 2017)**. If any isolates were suspected to be *E. coli*, selective media such as Eosin Methylene Blue (EMB) agar were then used.

Bacterial identification

GP ID REF21342 (for Gram-positive bacteria), including *S. pneumoniae* and *S. aureus*, and GN ID REF21341 (for Gram-negative bacteria), including *P. aeruginosa* and *E. coli*, were used in the identification process with the Vitek 2 Compact system (bioMérieux Inc., USA) **(Alaidarous *et al.*, 2017)**.

Determination of growth-inhibition zone

A standard diffusion method was used to evaluate microbial growth inhibition. This technique typically involves visually observing the inhibition of microbial growth on agar plates and measuring the diameter of the growth inhibition zone in millimeters (Mean ± SD) **(Bulet *et al*., 1999)**. The inhibition zones of ampicillin, a positive control for Gram-positive bacterial strains, and gentamycin, a positive control for Gram-negative bacterial strains, were used to standardize the diameter of the growth inhibition zone.

SDS-PAGE, 2D-PAGE

Protein analysis was performed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). While these methods do not provide functional information, they offer essential structural data, such as molecular mass (kDa) and isoelectric point (pI). Preparations, solutions, and staining procedures followed product instructions and previous studies **(Garfin, 2003; Friedman *et al*., 2009; Righetti *et al*., 2013)**. Silver staining was used to achieve the highest possible sensitivity during the study, which was conducted on a 12% separating gel using the Protean IEF-Cell and Criterion SDS-PAGE electrophoresis cell devices. For spot comparison, the immobilized pH gradient (IPG) strip (pI values) and size markers were utilized. Bioassays were generously conducted at the RCMB Antimicrobial Unit at Al-Azhar University, Cairo, Egypt.

Molecular studies

GAPDH is a housekeeping gene commonly used as a reference for quantifying gene expression, therefore the reference gene was chosen. Based on the mRNA sequence, forward and reverse primers for GAPDH, *Attacin* and *Lucifesin* were created, as the following primers:

Forward GAPH: 5/ACATCATCTGGGCTAG-CG-3/
Reverse GAPH: 5/TGTATAGATCCCGATCTGC-CC-3/
Forward attacin: 5/TGGTACTCCCGAACA-CAATC-3/
Reverse attacin: 5/ACCATGAGGGCTTG-TGTTA-3/
Forward Lucifensin: 5/TCTGCTTGGCTTT-GAGCTTT-3/
Reverse Lucifencin: 5/AGACGAACCGAAAC-TCGAAA-3/

The method used to extract tRNA was Trypure (Bioneer). To make cDNA, the amount of total RNA was extracted from every treatment at a 1µg concentration (using the Bioneer ready to use kit). Gene expression changes were quantified using Real-Time PCR.

Fluorescence-activated cell sorting analysis (FACS)

ES dilutions were selected between 1/2 and 1/32, based on the MIC values from Dogandemir's study **(Dogandemir, 2010; Michelsen *et al*., 2014)**. Two different staining methods were used: propidium iodide (PI) for dead cell DNA and thiazole orange (TO) for both living and dead cell DNA. To achieve 0.5 McFarland turbidity ($5x10^8$ cfu/ml), fresh bacterial colonies were cultured for up to two hours in tryptic soy broth (TSB) **(Faria-Ramos *et al*., 2013)**. We followed the manufacturer's application notes **(Biosciences, 2002; Nuding *et al*., 2013)** for dilutions, mixtures, and incubations. The analysis was performed using a BD Accuri C6 flow cytometry device, and the rates of

living and dead cells were determined using software and detectors. The rates of bacterial cell destruction were assessed by contrasting the fluorescence of TO and PI, allowing the determination of ES.

RESULTS

Studies of anti-microbial effect

The antibacterial effectiveness of larval excretions/secretions against the pathogens under investigation is shown in Tables (1, 2). As illustrated by the zones of growth inhibition in Figs. (2, 3), the larval excretions/secretions appear to inhibit the growth of Gram-positive bacteria, such as *Staphylococcus aureus* and *S. pneumoniae*, as well as the Gram-negative bacterium *Escherichia coli*. However, they showed only minimal inhibitory activity against *P. aeruginosa*, a Gram-negative bacterium.

The antimicrobial properties of ES of third larval instar against Gram-positive bacteria

As shown in Table (1) and Fig. (2), the results indicate that ES exhibits a strong growth-inhibitory effect against Gram-positive bacteria, represented by *S. aureus*. For ampicillin (control), the mean growth inhibition was 18.3±2.1 mm, compared to 27.4±1.5 mm for ES. *S. pneumoniae* showed a mean growth inhibition zone of 13.4±0.58 mm, compared to 24.7±1.2 mm for the control.

The antimicrobial properties of ES of third larval instar against Gram-negative bacteria

Extraction and secretions from the third larval instar showed the strongest growth-inhibitory (antibacterial) efficacy against *E. coli*. The average zone of growth inhibition was 20.4±2.0 mm, compared to 22.3±0.72 mm for gentamicin (control). However, very weak inhibition was observed against the Gram-negative bacterium *P. aeruginosa*, as shown in Table (2) and Fig. (3).

Electrophoretic proteins of ES

Table (4) displays the bands and spots of identified proteins in SDS-PAGE. Multiple spots with distinct molecular masses were observed for each pI value, indicating the presence of different protein molecules. A total of 71 spots and 12 bands were identified, with molecular weights ranging from 20 to 145 kDa (21-150 kDa) and pI values ranging from 3.3 to 7.

Examination of gene expression

The expression of each gene relative to the reference gene was investigated in samples treated with bacteria and controls, based on data from the effectiveness of each gene's primer and the results of real-time PCR using the pFaffl technique. The findings showed that, compared to the control, the expression of the *attacin* gene increased in larvae treated with *S. aureus*, *S. pneumoniae*, and *E. coli* (Fig. 4). However, the expression of this gene in larvae treated with *S. aureus*, *S. pneumoniae*, and *E. coli* was not significantly different from that in the control. In contrast, compared to the control, larvae

treated with *S. aureus* and *E. coli* showed increased expression of the *lucifensin* gene (Fig. 5). The increase in *lucifensin* gene expression was slightly higher in *E. coli*-treated larvae compared to *S. aureus*-treated larvae.

Fluorescence-activated cell sorting (FACS) determination

Regretfully, for all strains, visible fluorescence could not be seen at dilution 1/2. The dilutions 1/4, 1/8, 1/16, and 1/32 had the following bacteria-killing rates: 56.3, 44, 52.5 and 76% for *S. aureus*; 11.7, 47.6, 55, 71% for *S. pneumoniae*; 13.6, 22.9, 26.7, 49.1% for *P. aeruginosa*; 10.8, 31.4, 69, 89.2% for *E.coli*, respectively (Table 4 & Fig. 6).

Table 1. The antibacterial activity of extraction and secretions of third larval instar resistance of *L. sericata* to Gram-positive bacteria is demonstrated by the growth-inhibition zone

Tested micro-organisms (Gram+ve)	Inhibition Zone /mm	Control (Mean±SD)* *(Ampicillin)*
***Staphylococcus aureus* (RCMB 010028)**	18.3±2.1	27.4±1.5
***Streptococcus pneumoniae* (RCMB 010013)**	13.4±0.58	24.7±1.2

Table 2. The antibacterial activity of extraction and secretions of third larval instar Resistance of *L. sericata* to Gram-negative bacteria is demonstrated by the growth-inhibition zone

Tested micro-organisms (Gram+ve)	Inhibition Zone /mm	Control (Mean±SD)* (*Gentamicin*)
***E. coli* (RCMB 010052)**	20.4±2.0	22.3±0.72
***Pseudomonas aeruginosa* (RCMB 010043)**	-	21.3±1.4

Fig. 1. Location of water sample isolated from the drainage with longitude and latitude, 29.581533, 31.284109, respectively.

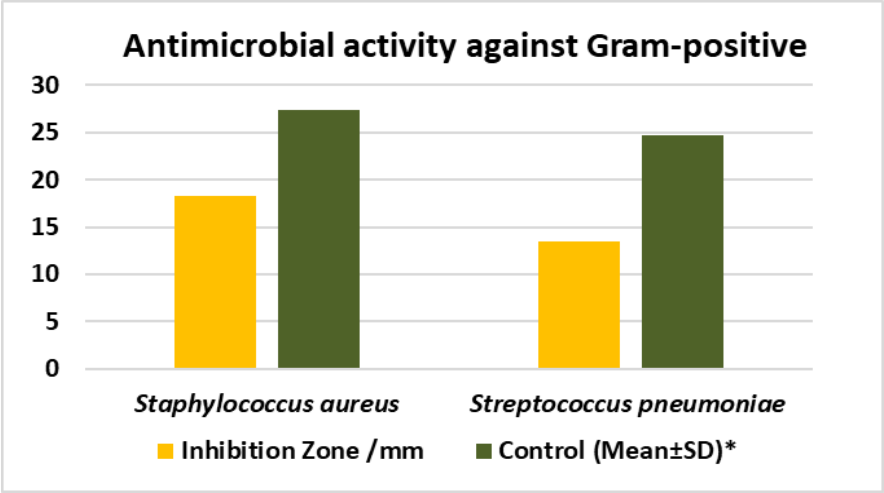

Fig. 2. Growth-inhibition zone resulting by *L. sericata* larval extraction/secretions against Gram-positive bacteria indicates antimicrobial activity

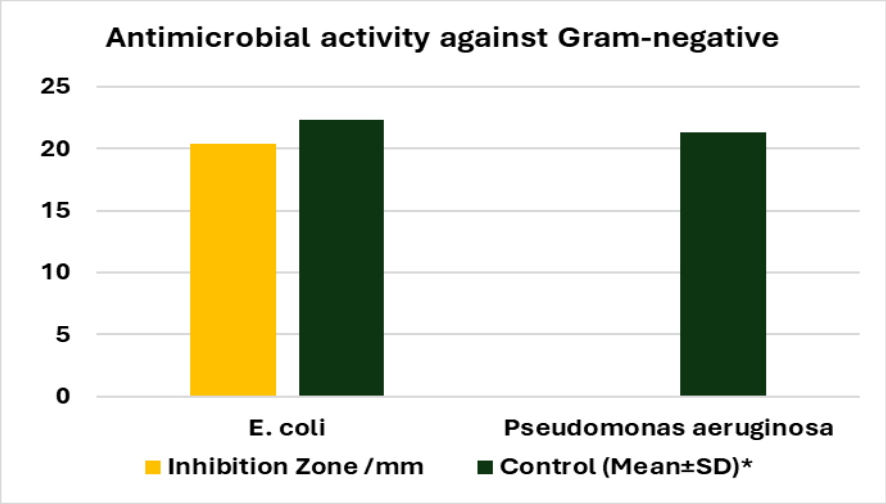

Fig. 3. Growth-inhibition zone resulting by *L. sericata* larval extraction/secretions against Gram-negative bacteria indicates antimicrobial activity

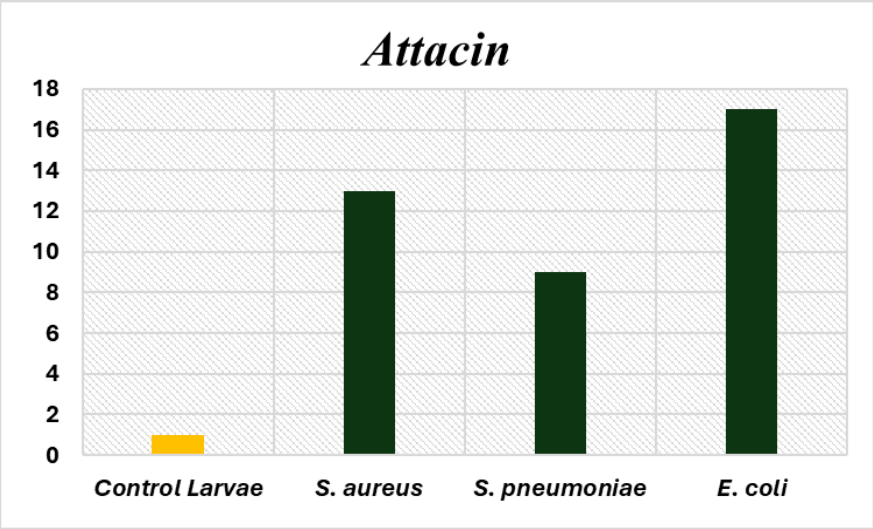

Fig. 4. The variation in *attacin* gene expression in larvae treated with *S. aureus*, *E. coli* and *S. pneumoniae* control

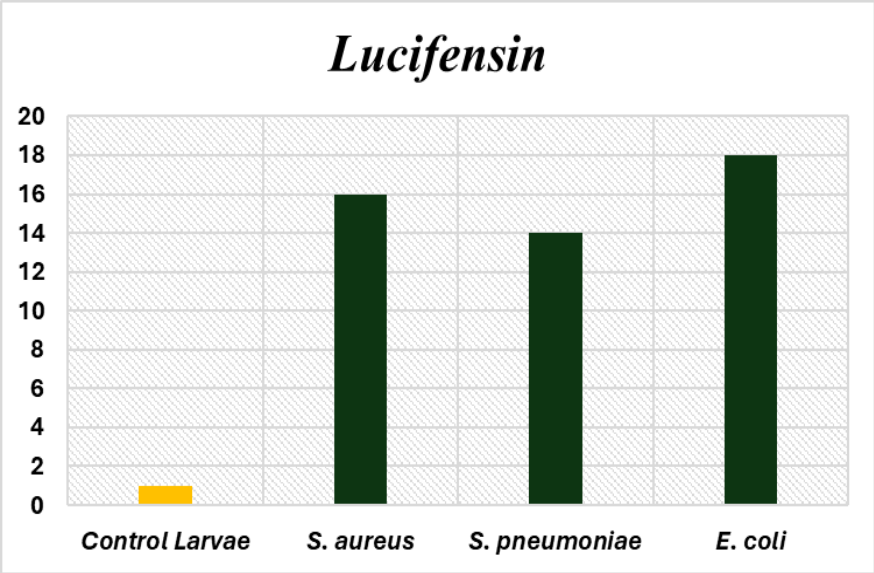

Fig. 5. The variation between the *lucifensin* gene expression in bacterial-treated and control

Table 3. SDS-PAGE and 2D-PAGE protein band and spot detection with varying molecular masses for every pI value

SDS-PAGE	2-D PAGE	
Molecular weight (kDa)	**pI**	**Molecular weight (kDa)**
145	**3.3**	**145; 97; 91; 69; 48; 37; 32;**
81	**3.1**	**39; 30; 21;26; 19; 12; <10**
72	**4.2**	**145; 87; 71; 72; 67; 61; 55; 32;30; 25; 15; 13**
63	**4.7**	**61; 58; 19; 16; 12; 10**
59	**4.9**	**56; 40; 27; 14;10**
41	**5.1**	**61; 36; 18; 12; 10**
38	**5.3**	**59; 31; 22; 13; 12; <10**
29	**5.5**	**62; 38; 30; 27; 23; 16; 14; 12**
27	**6.1**	**44; 35; 23; 18**
25	**6.4**	**43; 38; 31; 26; 15; 12**
13	**6.6**	**39; 28**
20	**7**	**41; 32; 27**

Table 4. The results of fluorescence-activated cell sorting (FACS) analysis

Dilutions	Killing rate %			
	S. aureus	***S. pneumoniae***	***P. aeruginosa***	***E. coli***
1/2	**0**	**0**	**0**	**0**
1/4	**56.3**	**11.7**	**13.6**	**10.8**
1/8	**44**	**47.6**	**22.9**	**31.4**
1/16	**52.5**	**55**	**26.7**	**69**
1/32	**76**	**71**	**49.1**	**89.2**

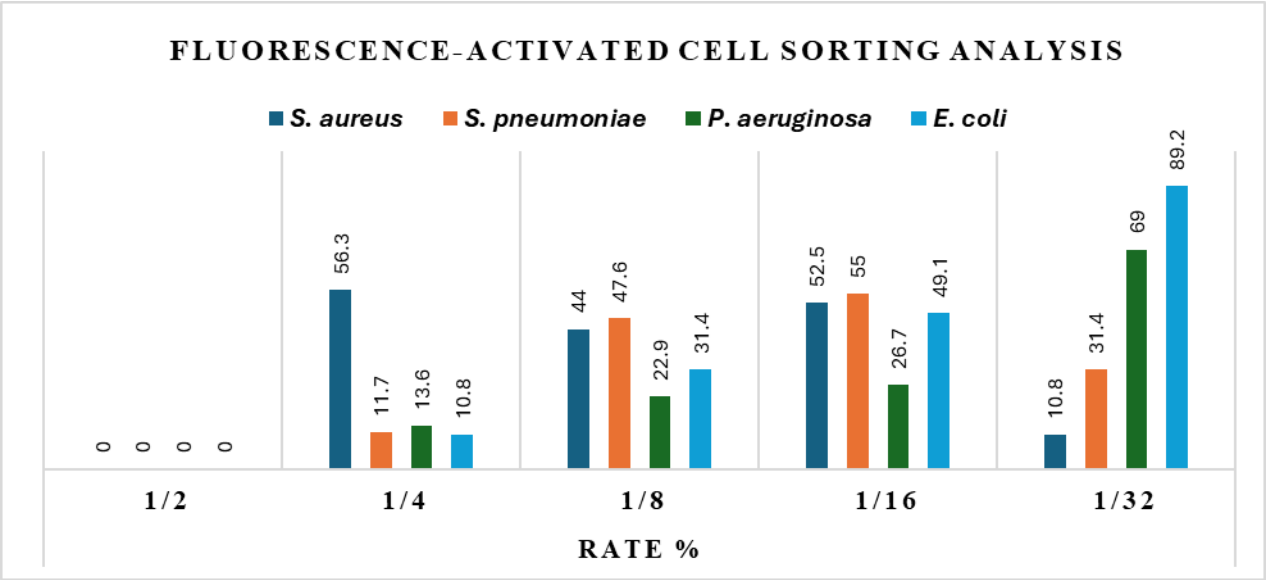

Fig. 6. Diagram of fluorescence-activated cell sorting (FACS) analysis

DISCUSSION

The present study revealed bacterial contamination with several pathogenic bacteria and waterborne bacteria that can act as pathogens. The results showed aquatic bacterial contamination with Gram-positive bacteria, including *S. pneumoniae* and *S. aureus*, and Gram-negative bacteria, including *P. aeruginosa* and *E. coli*. The results of this investigation are consistent with those of **Hassan *et al.* (2014)**, who observed that *Lucilia sericata* maggots can be used to treat debridement wounds, whether they are diabetic or not. Similarly the results of **Leem *et al.* (1999)**, showed the broad antibacterial spectrum of *Acantholyda parki* saw fly extract against both Gram-ve and Gram+ve bacteria. **Miyanoshita *et al.* (1996)** demonstrated the antibacterial activity against Gram-positive bacteria such as *S. aureus* and *B. subtilis*, but not against Gram-negative bacteria like *E.*

coli, *P. aureus*, and *B. subtilis*. The current study's results are not comparable with their findings. The present study revealed bacterial contamination of drainage with several pathogenic bacteria and waterborne bacteria that can act as pathogens, and this agrees with the findings of **Alaidarous *et al.* (2017)**. The results of the current study are comparable with those of **Steenvoorde and Jukema (2004)** since the current study was conducted *in vitro* and revealed that there is a highly effective effect against Gram- positive more than Gram-negative infections. *In vivo* maggots appear to be less effective against Gram-negative.

The current study's findings are similar to those of **Jaklic *et al.* (2008) and Bohova *et al.* (2014)**, who conducted quantitative research *in vitro* and *in vivo* to evaluate the impact of *Lucilia sericata* larval ES on various bacterial strains. Our results differ from those of **Cazander *et al.* (2009)**, who demonstrated that maggots and/or their excretions/secretions (ES) have no direct antibacterial activity *in vitro*. However, our findings are consistent with those of **Barnes *et al.* (2010)**, who investigated the antibacterial effectiveness of ES against various bacteria, including *P. aeruginosa*, *S. aureus*, and *E. coli*, and observed strong antibacterial activity against certain pathogenic strains.

Evaluation of antimicrobial agents

Agar well diffusion is a standard method for susceptibility testing. In the present study, Gram-positive bacteria *S. aureus* and *S. pneumoniae*, as well as Gram-negative bacteria *P. aeruginosa* and *E. coli*, were isolated from drainage samples. These organisms are commonly found in diabetic and skin structure infections, as reported by **Rennie *et al.* (2003)**, suggesting that the presence of these contaminants may result from water subjected to human interaction during cleaning practices **(Alaidarous *et al.*, 2017)**.

Regarding *P. aeruginosa*, there was "no inhibitory effect." However, inhibitory zones observed for other strains suggest a "potential antibacterial effect" or "dose-dependent efficiency." Given the variability between strains, comparing inhibitory zone sizes may not be a reliable method for assessing susceptibility data. Furthermore, diffusion testing outcomes did not align with those of dilution tests and *in vivo* efficacy.

Previous studies, such as those by **Bexfield *et al.* (2008)**, investigated the antibacterial efficacy of maggot excretion/secretion (ES) using fluorescence-activated cell sorting. They found that *S. pneumoniae* exhibited bactericidal effects, while *S. aureus* and *E. coli* showed strong bacteriostatic activity. However, acquiring sufficient material for such tests can be challenging, as maggot ES requires a large number of maggots for adequate examination. Studies on sterile and patient-applied maggots **(Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Huberman *et al.*, 2007a, 2007b; Barnes *et al.*, 2010)** have indicated significant antibacterial activity, though data remain limited. Entomological research highlights that *L. sericata* larvae specifically target dead and infected tissue **(Fleischmann *et al.*, 2004; Sherman *et al.*, 2013)**.

Van der Plas *et al.* (2008) and others **(Daeschlein *et al.*, 2007; Masiero *et al.*, 2017)** reported that *P. aeruginosa* biofilms degrade over time, with antibacterial activity diminishing. Time-Kill Analysis is crucial to better understand antibacterial changes, as it identifies the minimum bactericidal concentration **(Barry, 1999)**. In our study, larval ES

demonstrated promising activity against *S. aureus*, *S. pneumoniae*, *P. aeruginosa*, and *E. coli*.

Studies by **Chernysh *et al.* (2000)** and **Kruglikova *et al.* (2011)** also reported bactericidal and bacteriostatic effects against *E. coli* and other bacteria, with *L. sericata* larvae ES being notably effective against *S. aureus* and *E. coli* despite previous beliefs that maggot fluids are more efficient against Gram-positive bacteria **(Jaklič *et al.*, 2008; Andersen *et al.*, 2010)**.

Electrophoretic protein analysis

Insects respond rapidly to bacterial and fungal threats by producing potent peptides and polypeptides effective against both Gram-positive and Gram-negative bacteria **(Hoffmann, 1995; Hoffmann *et al.*, 1996)**. Our findings align with those of **Tsuji *et al.* (1998)**, who identified a protease with a molecular weight of 26 kDa exhibiting antibacterial activity. In this study, antibacterial activity was observed at 20 kDa, with the strongest effects against the investigated bacterial strains exhibited by low-molecular-weight protein bands.

Leem *et al.* (1999) hypothesized that these low-molecular-weight proteins play a role in insect defense. The present study's 2D-PAGE analysis separated peptides based on molecular mass and isoelectric points. Previous studies **(Chernysh *et al.*, 2000; Kruglikova *et al.*, 2011)** identified multiple peptides with varying molecular masses exhibiting antibacterial activity, particularly against Gram-negative bacteria.

The study's results are consistent with findings by **Tsuji *et al.* (1998)**, who identified a 26-kDa protease in *Sarcophaga peregrina* with antibacterial activity. Similarly, **Van der Plas *et al.* (2007)** found that ES from third-instar *L. sericata* larvae contained molecules responsible for its beneficial effects. Our study confirms that the antibacterial activity of larval ES is attributed to proteins with molecular weights around 20 kDa, which aligns with findings by **Abraham *et al.* (1995)** and **Taha *et al.* (2010)**.

Examination of gene expression

The lucifensin gene did not show the same changes in expression as the attacin gene in response to bacterial infection. This discrepancy may be explained by the combined functions of insect antibacterial compounds, which can have synergistic effects, making them more efficient at combating bacterial infection **(Wollina *et al.*, 2002)**. Increased expression of attacin in response to infection likely compensates for deficiencies in other immune responses.

Our results align with the findings of **Baumann *et al.* (2015)**, who reported increased expression of the defensin-1 gene in response to Gram-negative bacteria. This suggests that lucifensin, a member of the defensin family, plays an effective role in combating Gram-negative bacterial infections, despite the restricted antibacterial properties of defensins against Gram-positive bacteria.

Fluorescence-activated cell sorting analysis

In this study, we used the minimum inhibitory concentration (MIC) values from **Dogandemir's** work **(2010)** as a reference, as there are no current standards for ES using fluorescence-activated cell sorting analysis. We observed a surprising increase in antibacterial efficacy with declining ES concentrations, with the highest bacterial mortality

rates noted at a dilution of 1/32. This contrasts with Dogandemir's findings, where MIC values did not fall below 1/32 for any of the strains tested.

A limitation of our study is that MIC values below 1/32 were not explored. High ES concentrations may have led to false evaluations due to the "autofluorescence effect" caused by cellular NADH, riboflavins, and flavin coenzymes, which can reduce flow cytometry sensitivity **(Mosiman *et al.*, 1997)**. It is important to examine lower dilutions to verify the peak of the killing rate curve. Even at the 1/32 dilution, overall killing rates for *P. aeruginosa* were as high as 49.1%, demonstrating the strong antibacterial effect of ES at low dilutions.

CONCLUSION

Our study highlighted the presence of aquatic microbial contamination in Elsaf-Drain, with a variety of pathogenic bacteria identified. Larval therapy, particularly using larval excretion/secretion (ES), proved to be an effective method for eradicating infections associated with aquatic contaminants and promoting the healing of chronic wounds. We observed numerous protein spots with distinct molecular weights and isoelectric points, suggesting that these proteins may contribute to the observed antibacterial activity.

A significant advantage of using larval ES in therapy is that it eliminates the need for live larvae, reducing the risk of harming healthy tissue while still retaining the beneficial effects of larval secretions. This approach could offer a safer alternative to traditional larval therapy, where live larvae are directly applied to wounds. Despite the promising results, the precise mechanism of action and the specific response of larval ES to the factors present in chronic wounds remain unclear. Further studies are needed to determine how the ES interacts with these elements, as well as its full potential in wound healing and infection control. Importantly, the presence of larvae in a contaminated environment appears to enhance the expression of antibacterial compounds, contributing to a more effective antimicrobial response. This study also confirmed the efficacy of larval ES in eliminating infections and inhibiting the growth of several antibiotic-resistant bacteria. Therefore, placing larvae in infected environments may significantly increase the potency of their ES, potentially developing it into a valuable treatment option for a range of antibiotic-resistant infections.

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