Journal of Medical Bacteriology

Lucilia sericata Maggot Extract: A Promising Tool against Biofilms of Antimicrobial Resistant Strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ARTICLE INFO

Article type:
Research Article

Article history:
Received: 03 Dec 2023
Revised: 20 Jan 2024
Accepted: 01 Feb 2024
Published: 06 Feb 2024

Keywords:
Antimicrobial resistance, Biofilm, Lucilia sericata, Pseudomonas aeruginosa, Staphylococcus aureus.

ABSTRACT

Background: This study explores the impact of *Lucilia sericata* maggots on the development and eradication of biofilms created by the pathogenic bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Methods: We assessed the influence of *Lucilia sericata* maggot extract on the viability of planktonic bacteria, the formation and disruption of biofilms, bacterial metabolic activity. Also the effect of simultaneous ES-antibiotic treatment in biofilm elimination was investigated. Additionally, the expression levels of genes associated with biofilm formation, namely *LasI*, *psLA*, *agrA*, and *icaD* was studied.

Results: The results showed that ES can reduce the viability of planktonic *S. aureus*, significantly. Furthermore, ES of larvae fed on *S. aureus*-infected meat displayed the most substantial inhibition of biofilm formation (62.11% and 75.04% inhibition for *S. aureus* and *P. aeruginosa*, respectively). A similar trend was observed in biofilm destruction, with values of 56.67% and 68.50% inhibition for *S. aureus* and *P. aeruginosa*, respectively. The simultaneous application of ES of larvae that fed on *S. aureus*-infected meat and the minimum inhibitory concentration (MIC) of gentamicin resulted in 100% inhibition of biofilm formation by *S. aureus*. Notably, the group treated with ES of larvae fed on *S. aureus*-infected meat exhibited the most significant reduction in metabolic activity, with values of 95.03% and 68.25% for *S. aureus* and *P. aeruginosa*, respectively. The expression of *LAsI* and *pslA* genes in *P. aeruginosa* and the expression of *agrA* and *icaD* genes in *S. aureus* has decreased

Conclusion: The findings of this study demonstrate that maggot extract has not only impacted the formation, but also eliminated the biofilms of *S. aureus* and *P. aeruginosa*.

- ***Please cite this paper as:*** Rashid S, Momeni-Moghaddam M, Ghavidel Z. *Lucilia sericata* Maggot Extract: A Promising Tool against Biofilms of Antimicrobial Resistant Strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *J Med Bacteriol.* 2024; **12** (1): pp.43-58.

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Introduction

Biofilms are often implicated in the etiology of chronic infections in humans. These biofilm-related diseases tend to resist the body's immune defenses and exhibit only temporary responses to antimicrobial therapy (1). Conditions associated with biofilms include cystic fibrosis, periodontitis, endocarditis, chronic otitis media, sinusitis, chronic osteomyelitis, and persistent wounds (2, 3). Infections related to medical implants and biomaterials, such as intravenous central lines, prosthetics, urinary tract catheters, prosthetic heart valves, and contact lenses, are frequently linked to the presence of biofilms (4).

Chronic wounds, in particular, often remain in an inflammatory state and tend to harbor substantial microbial populations (3). The detrimental impact of microbial infections on wound healing has been recognized for many years, and it is acknowledged that managing bioactivity is a crucial aspect of wound care (1). Microorganisms aggregate to form microbial communities encapsulated within an extracellular matrix (ECM) composed of polysaccharides, proteins, and glycoproteins, which is known as extracellular polymeric material (EPS). Microorganisms can exist in either a planktonic (free-living) state or a biofilm phenotypic state. Both of these states can significantly influence the progression of acute and chronic wound infections and the healing process, but biofilms are more commonly found in chronic wounds as compared to acute wounds (60% versus 6%) (3, 4).

A significant and serious complication during the healing of chronic wounds is the buildup of bacteria on the wound surface, leading to infection (1). This complication hinders the healing process, particularly when these bacteria are capable of forming biofilms. Biofilms are intricate bacterial aggregations encased in a glycocalyx layer that can adhere to mucosal surfaces (2). Within biofilms, bacterial or fungal cells are enmeshed in an extracellular matrix composed of hydrated polymers and residues. Water channels within the biofilm facilitate the transport of nutrients and the removal of metabolic byproducts (3). The size and thickness of these cell clusters within a biofilm can vary from a few microns to several millimeters (4).

Bacteria tend to attach to necrotic tissue in wounds, which becomes susceptible to infection due to a compromised host immune response (4). Biofilms created by bacteria are found widely in various environments, including extreme conditions. They are prevalent in nature, industrial materials, and the human body, where they can have either a symbiotic or pathogenic relationship (4).

Extensive research on biofilms has primarily focused on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These opportunistic pathogens, often associated with hospital settings, exhibit a high capacity for biofilm formation (5).

Since the 1980s, researchers have been actively seeking alternative methods to control and prevent infections, given the critical role of biofilms in infection development and the escalation of drug resistance (1, 2). Various strategies have been explored to target biofilms in wound care, including approaches like debridement and the use of antibiofilm and antimicrobial agents, such as disinfectants, antiseptics, and antibiotics (3). Additionally, it's possible to transform a pathogenic biofilm into a more typical biofilm through targeted therapies that specifically target the bacteria in the wound or by adjusting factors that influence the biofilm's structural arrangement (4).

One older approach that has resurfaced is Maggot Wound Therapy (MWT), originally used in the 1930s to treat chronic ulcers and infections. This technique employs the larvae of the insect species *Lucilia sericata* (5, 6). The secretions of these larvae contain antibacterial compounds, and research has indicated that maggot therapy is particularly effective against infectious wounds caused by Gram-positive bacteria such as *S. aureus* and Gram-negative bacteria such as *P. aeruginosa*

(5, 7-10). It has also been demonstrated to reduce biofilm formation (7, 11, 12).

The biofilm state of bacteria is associated with the expression of specific genes and the suppression of planktonic genes (13). Biofilms differ from planktonic bacteria in terms of their structure, gene expression, antibiotic resistance, and interactions with the host (14). The biofilm state of bacteria is linked to the expression of certain genes and the inhibition of planktonic genes as well (13).

The formation of biofilms is primarily driven by the activity of an operon called *icaABCD*, which represents the most critical factor in the construction of the exopolysaccharide matrix constituting biofilm components. Furthermore, the agr system plays a pivotal role in the development of *S. aureus* biofilms (1, 2).

In this study, we explored the antibacterial impact of Lucilia sericata maggot extract (ES) on both the creation and disruption of biofilms, as well as on the planktonic state of bacteria. Our investigation involved combining larval excretions and secretions with sub-minimum inhibition concentrations of Tobramycin and gentamicin antibiotics, and we examined their effects on biofilm formation.

Materials and Methods

Preparation of maggot secretions

500 sterile *Lucilia sericata* maggots (first instar) were purchased from Science and Research Park, Jundishapour University of Medical Sciences, Ahvaz, Iran. In order to simulate the wound environment, the larvae were fed beef for three days and incubated in the dark at 25 °C. Four groups were categorized as follows: Group A) the Control group that was fed healthy meat under sterile conditions, Group B) This group was fed with meat which was already infected with *S. aureus*, Group C) This group was fed with meat which was already infected with *P. aeruginosa*. Group D) which was fed contaminated meat which was already infected with *S. aureus* and *P. aeruginosa*. For this purpose, 10 microliters of bacterial stock were mixed in 1 ml of fresh culture medium and cultured for 12 hours at 37 °C and rotation at 180 rpm, then to feed 500 larvae, 2 grams of fresh meat was mixed with 200 microliters of bacterial culture. Maggot secretions were collected according to the protocol established by van der Plas et al. (1).

Maggots extract preparation

The larvae (third instar) were washed with distilled water and incubated in sterile phosphate buffered saline (PBS: pH = 7.3) for 12 hours at 25 °C. The secretions (ES) were centrifuged (5000g/ 4 °C/ 45 min) and filtered through a 0.22 µm filter (Biofil, Canada), and preserved at -20 °C. According to the manufacturer's instructions, the concentration of ES protein was determined using the Pierce BCA Protein Assay Kit (KIAzist, Iran). Concentration of untreated *L. sericata* ES assesses 105 µg/ml, the concentration of treated larvae ES with *P. aeruginosa* and *S. aureus* was 95 µg/ml and treated larvae concentration with *S. aureus* was 120 µg/ml.

Bacterial cultures

Gram-positive *S. aureus* (ATCC 6538) and Gram-negative *P. aeruginosa* PAO1 (Donation from Bahonar University, Kerman, Iran) were grown in Tryptone Soy Broth medium (TSB, QUElab, Canada) at 37 °C, under dynamic shaking. The strains were preserved in TSB containing 10% v/v glycerol at -80 °C.

Antimicrobial susceptibility testing

This test was performed using the Kirby & Bauer method and according to the protocol of the Clinical and Laboratory Standards Institute (CLSI). Mueller-Hinton agar (MHA, Merck,

Germany) medium was used for the disk diffusion assay (2). Antibiotic discs were: (PADTAN TEB, Iran. Ampicillin (AM10), chloramphenicol (C30), ciprofloxacin (CP5), gentamycin (GM10), imipenem (IMP10), methicillin (ME5), Ofloxacin (OFX), Penicillin (P10), Tetracycline (TE30), Tobramycin (TOB10), vancomycin (V30). Microbial suspensions (with a turbidity of 0.5 McFarland and approximate/standard concentration (1/5 ×10 8 CFU/ml)) were prepared from 18-hours cultures. Microbial suspensions were used for inoculation of Mueller-Hinton agar plates. The diameter of the growth inhibition halo was measured with a caliper after 24 hours of incubation at 37 °C (2).

Minimum Inhibitory Concentration (MIC)

The Microbroth dilution method was used in this study according to M. Andrews protocol (3). Initially, Minimum Inhibitory Concentration (MIC) values were determined for cells grown under planktonic conditions. Above mentioned values were compared to tobramycin (300 mg/4 ml -DarmanYab darou, Iran) for Pseudomonas aeruginosa and gentamicin (20 mg/2 ml - Alborz darou, Iran) for *S. aureus*. Then, serial dilutions of the above antibiotics (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 512 µg/ml) were prepared and added to 96-well plate wells. Microbial suspensions with a final concentration of 10^5 CFU/ml, depending on the type of antibiotic, were added to each well containing Mueller-Hinton Broth (MHB-Merck, Germany) medium followed by incubation at 37 °C for 24 hours. Optical density was observed by spectrophotometry at 600 nm using an ELISA reader (powerWave XS2, Biotek, USA). Finally, the lowest concentration at which bacterial growth was inhibited, was considered as MIC (3).

The effect of maggot extract on planktonic state

To evaluate the inhibitory effect of the extracts on bacterial growth, the bacterial strains were cultured overnight, then diluted (1: 100) using fresh culture media. Following Bohova et al.'s (2014) protocol, 10 µl of cell suspension aliquots were added to 96-well plate wells (containing 90 µl of TSB culture medium and maggot extract from different experimental groups). At intervals of 0, 2, 4, 6, and 24 hours, the optical density trend was recorded based on absorption at 600 nm. Additionally, a well containing culture medium and maggot exract and a well containing culture medium and bacterial suspension were considered as controls.

Biofilm assay

Biofilm formation analysis was performed with microtiter plate assay based on O'Toole and Kolter protocol (4). First, an overnight culture of each bacteria was prepared in TSB medium at 37 °C. It was then diluted to a turbidity of 0.5 McFarland at 625 nm. 10 µl of bacterial suspension was added to 100 µl of TSB medium in 96-well plate wells and incubated for 24 h at 37 °C. Microplates were examined by Crystal violet staining. To quantify biofilm production, some 33% (v/v) acetic acid was added to each well. Finally, the results of 570 nm absorption were record. The biofilm formation ability is divided into four categories based on the spectrophotometry records: non-producing (OD (isolate) ≤ OD (control)), weak (OD (control) ≤ OD (isolate) ≤ 2OD (control)), moderate (2OD (control) ≤ OD (isolate) ≤ 4OD (control)), and strong-producing (4OD (control) ≤ OD (isolate)).

The effect of ES on biofilm formation

As previously described, 10 µl of microbial suspension was added to plate wells each contained 90 µl of treated and non-treated maggot extracts with a TSB medium. After incubation (24

hours at 37 °C), the microplates were examined with Crystal violet staining (5). The control groups were: 1) Extract control: this well was inoculated with TSB medium and untreated extract. 2) Negative control: this well was inoculated with bacterial cells without any extract. 3) Media control: Contains TSB only. The percentage of inhibition of biofilm formation in different concentrations of maggot extract was calculated using the following formula (6):

Formula 1.

$$\%\ \text{inhibition} = \frac{[(\text{OD negative control} - \text{OD media control}) - (\text{OD test} - \text{OD extract control})]}{(OD\ negative\ control - OD\ media\ control)} \times 100$$

The effect of ES on biofilm disruption

The effect of the extract on biofilm degradation was investigated according to the previous description. In summary, after 24 hours of biofilm formation, 100 µl of Magut extracts (groups; A, B, C and D) were added to the wells and incubated at 18 ° C for 18 hours. Finally, the Crystal violet staining method was used, and with Formula 1, the percentage of biofilm destruction was calculated. Also, control wells were defined as described in the previous section.

Assessing the ability of extracts to inhibit the metabolic activity of biofilm structures

A Dehydrogenase inhibition test was performed to assess the ability of the extract to inhibit the metabolic activity of cells isolated from the biofilm structure. Biofilm activity was measured via observation of dehydrogenase enzyme activity of the biofilms. After biofilm formation, three groups of extracts (with initial concentration) were added to the wells and incubated (24 hours / 37 °C). Then 50 µl of 0.1% triphenyl tetrazolium chloride solution was added to the contents of the wells and incubated (3 hours / 37 °C). Finally, the absorption at 490 nm was analyzed. Metabolic activity was calculated by comparing the absorbance of control bacterial cells and treating with each extract (6, 7).

Synergism between maggot excretions and antibiotics

To understand the effect antibiotic-extract combinations with extracts on biofilm formation, a microbial suspension of an overnight culture was prepared from each bacterial strain (turbidity 0.5 McFarland). To each well of microbial suspension, the given antibiotic solution and each isolated ES (groups; A, B, C and D) (equal amount, 50 µl) were added and incubated (24 hours/37 °C). Finally, after crystal violet staining, the absorbance of the wells was read at 570 nm, and the percentage of inhibition of biofilm formation was calculated (Formula 1) (8).

Investigation of gene expression

RNA extraction was performed using total RNA extraction kit (Dena Zist asia, Iran) for all treated groups. Nanodrop (Thermo scientific) was used to determine the concentration of extracted RNA. cDNA synthesis was performed using the Easy cDNA Synthesis kit (Pars Tous, Iran). For this purpose, the reaction components, including RNA, buffers, and RT enzyme, were mixed and carried out according to the company's protocols and placed in a thermocycler for 10 minutes at 27 °C, 60 minutes at 47 °C and 5 minutes at 85 °C.

Cyber-Green Real-Time PCR kit (Pars Tous, Iran) was used to study the expression of genes(LAsI and pslA genes in P. aeruginosa and the agrA and icaD genes in S. aureus) in bacterial groups before and after treatment using Real-Time PCR and reference genes. The specific primers for used in this study were synthesized by Macrogen Company (South Korea). All primers and sequences are listed in Table 1. The temperature

program of 35 reaction cycles, including 94 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 60 seconds, and finally melting curve analysis was performed (Corbett Research RG 3000).

Statistical analysis

All experiments of this study were performed with three replications. The differences between the data were investigated by SPSS.v18.0 software using the ANOVA test, and the significant level of data (P-value) was less than 0.05.

Result

Antimicrobial susceptibility

The effect of maggot extract on planktonic state

The effect of ES on the planktonic form of bacteria *P. aeruginosa* and *S. aureus* at intervals of 0, 2, 4, 6, and 24 hours after treatment is shown in Figure 1. The abovementiobned bacterial pathogens had the highest growth and proliferation in the negative control group. In comparison, the highest inhibitory effect was seen when *P. aeruginosa* planktonic cells were treated with ES of larvae fed on *S. aureus*-infected meat ($p<0.05$). Such a difference was not significant in the other groups (Figure 1A). Also, a similar detectable inhibitory effect was observed when *S. aureus* planktonic cells were treated with ES of larvae fed on *S. aureus*-infected meat ($p<0.0001$). This upward trend was also significant ($p<0.0001$) in the untreated extract group and the group treated with two bacterial strains (Figure 1B). According to the obtained results, it was found that in 24 hours after the treatment, the greatest growth inhibition effect is observed for both bacterial strains.

The effect of ES on biofilm formation

The results of the effectiveness of maggot extract and the antibiotics on the biofilm formation of *P. aeruginosa* and *S. aureus* strains are shown in Figure 2. According to the results of the data obtained from Formula 1, tobramycin inhibits biofilm formation by *P. aeruginosa*. Also, group A, B and D maggot extract (ES), affected the biofilm formation of *P. aeruginosa* by efficiencies of 62.12 %, 75.04 %, and 64.45%, respectively ($p<0.0001$) (Figure 2A). According to the results, gentamicin inhibits the biofilms formation of *S. aureus*. Also, untreated maggot extract (ES), maggot extract treated with *S. aureus* (ES+S), and maggot extract treated with both pathogens (ES+S+P), impacted the biofilm formation of *S. aureus* by efficiencies around 50.70%, 62.11%, 46.05%, respectively ($p<0.05$) (Figure 2B).

The effect of ES on biofilm disruption

The results of the effectiveness of maggot extract and the antibiotics on the biofilm disruption of the studied strains of *P. aeruginosa* and *S. aureus* are shown in Figure 3. According to Formula 1, tobramycin destruct the biofilm of *P. aeruginosa*. Also, untreated maggot extract (ES), maggot extract treated with *S. aureus* (ES+S), and maggot extract treated with both pathogens (ES+S+P), disrupt the biofilm of *P. aeruginosa* by efficciecies about 56.73%, 68.50%, 58.78%, respectively ($p<0.0001$) (Figure 3A). According to the results, gentamicin is 100% effective in disruption of S. *aureus* biolim. Also, untreated maggot extract (ES), maggot extract treated with *S. aureus* (ES+S), and maggot extract treated with both pathogens (ES+S+P), destruct the *S. aureus* biofilm by efficiencies of 51.05%, 56.67%, and 43.76%, respectively ($p<0.0001$) (Figure 3B).

Assessing the ability of extracts to inhibit the metabolic activity of biofilm structures

The effect of the extract on the inhibition of the metabolic activity (inhibition of the dehydrogenase enzyme) of the studied strains of *P. aeruginosa* and *S. aureus* is illustrated in Figure 4.

In the positive control group, tobramycin for *P. aeruginosa* and gentamicin for *S. aureus* have had 100% inhibitory effects on the metabolic activity ($p<0.05$). Also, untreated maggot extract (ES), maggot extract treated with *S. aureus* (ES+S), and maggot extract treated with both pathogens (ES+S+P), resulted in inhibition of the metabolic activity of *P. aeruginosa* by efficiencies around 63.27%, 68.25%, and 64.38%, respectively ($p<0.05$). Furthermore, untreated maggot extract (ES), maggot extract treated with *S. aureus* (ES+S), and maggot extract treated with both pathogens (ES+S+P), led to metabolic activity reductions in S. aureus by efficiencies of 88.47%, 95.03%, about 87.53%, respectively, ($p<0.05$).

In general, the treated ES with *S. aureus* had the strongest effect on inhibition of the metabolic activity of the biofilms of the studied strains compared to the positive control (antibiotics and bacterial cells), significantly ($p<0.05$).

Synergism between maggot excretions and antibiotics

Figure 5 shows the simultaneous effect of maggot extract and antibiotics on the strains of *P. aeruginosa* and *S. aureus*. First, the minimum inhibitory concentration (MIC) of tobramycin (64 mg/ml) for *P. aeruginosa* and gentamicin (2 mg/ml) for *S. aureus* was determined in planktonic conditions.

The effects of MIC of tobramycin and treated ES against *P. aeruginosa* were evaluated. The results showed that the MIC of tobramycin and treated maggot extract completely (100%) prevented the formation of biofilms in *P. aeruginosa*. Also, MIC of tobramycin, untreated ES, and treated ES separately had inhibitory effects of about 89%, 62.30%, and 74.77%, respectively, on the biofilm formation of *P. aeruginosa* ($p<0.0001$) (Figure 5A).

Also, the effects of MIC of gentamicin and treated ES against S. *aureus* were evaluated. The results showed that the MIC of the gentamicin and treated maggot extract 99% prevented the formation of biofilms in *S. aureus*. Also, MIC of gentamicin, untreated ES, and treated ES separately prevented 76%, 51.87%, and 63.03% of biofilm formation in *S. aureus*, respectively ($p<0.0001$) (Figure 5B).

Therefore, the ES-antibiotics combinations have shown synergistic effects against the biofilm formation by the studied strains of *P. aeruginosa* and *S. aureus*.

Investigation of gene expression

According to the results of real-time PCR, genes involved in biofilm formation were investigated for *P. aeruginosa* and *S. aureus*. rPsl for *P. aeruginosa* and 16SrRNA for *S. aureus* were consider as reference genes and PslA and LasI for *P. aeruginosa* and *agrA* and *icaD* for *S. aureus* were consider as target genes. Real-Time PCR results showed that all target genes in this study have been downregulated after treatment by *L. sericata* extract (Figure 6).

Discussion

Chronic wounds are often at high risk of infection with antibiotic-resistant bacteria and subsequently, biofilm formation, leading to increased and persistent infection. Bacteria in the biofilm phase are less likely to be targeted by antibiotics because of the low diffusion, dense extracellular matrix, and the simultaneous presence of antibiotic-degrading bacteria (9-11). Moreover, high doses of antibiotics should be used to eradicate biofilms, which would have its own side effects (9, 12).

P. aeruginosa and *S. aureus* are among the well-studied biofilm-forming bacterial pathogens which infect the wounds. Antibiotic resistance is reportedly frequent in these two pathogenic species (11, 13-16). Hence, researchers have been long looking for new approaches to deal with wounds infected by such resistant pathogens.

Research on biological strategies has become commonplace due to the problems of chemotherapeutic compounds. Maggot therapy is gaining more attention as an efficient tool to fight wound infections (6). Today, maggot therapy is a well-known treatment for healing necrotic wounds. In fact, maggot extract have some effective properties such as antimicrobial peptides tat control infection (17, 18). Seeking for an alternative biological approach to battle wound infection, the effect of maggot extract on the biofilm formed by *P. aeruginosa* and *S. aureus*, was investigated.

Larval extract of *Cochliomyia macellaria*, *Chrysomya albiceps*, *Sarcophaga peregrine*, and *Musca domestica* have been shown to impact the growth of MRSA (19-21). *Lucilia cuprina* is one of such maggots for which the antimicrobial properties of larval extract against the growth of S. aureus, *P. aeruginosa* ,and *E. coli* have been reported (20, 21). The antibacterial activity of *Lucilia sericata* whole-body extracts, hemolysis and excreta have been demonstrated in several studies (22-24). A wide range of bacteria, including *S. aureus* and *P. aeruginosa*, have been reported as susceptible pathgens to *L. sericata* (25, 26). Studies have shown that antibacterial protein from maggot can inhibit S. aureus growth (27).

S. aureus has also been reported to be a gram-positive bacterium with a monolayer cell wall, so it is more susceptible to maggot extract than *P. aeruginosa*, a gram-negative bacterium (28). Whereas one study in 2010 showed that *L. sericata* ES inhibited the growth of gram-negative *E. coli* more than that of *S. aureus* (29). In our study, the effect of larvae extract on inhibiting the growth of *P. aeroginosa* was more than that of *S. aureus*.

Antimicrobial activity of non-sterile insects was found to be higher than that of sterile insects, which indicates that insects produce more amounts of antibacterial compounds in the presence of bacteria (12, 24) In the continuation of this research, to investigate the effect of a contaminated environment, group B of maggots were fed with *S. aureus*-infected food. The results indicated that as compared to the sterile extract(group A) and the extract infected with both bacteria (group D), these infected maggots had an increased antibacterial effect on destroying and inhibiting the formation of *P. aeroginosa* biofilm. While in Kawabata et al. study, the antibacterial activity of the contaminated extract was reported only against *S. aureus* (16). According to the findings of Pöppel et al. exposure of larvae to bacteria strengthens the antibacterial effect and increases the synergistic activity of the peptides in the extract (30). Also, it has been shown that the secretions of larvae grown in an environment contaminated with *E. coli* can inhibit the growth of *E. coli* strains for about 3 hours, and the secretions of larvae grown in an environment contaminated with *Bacillus subtilis* it also hinders the growth of *B. subtilis* for about 5 hours (12). As Kohan et al. found that if maggots expose to *S. aureus* and *E. coli*, the extracts of maggots can not affect the growth of *P. aeruginosa* (31).

In the present study, the antibacterial activity of maggot extract infected with *S. aureus* was shown within 6-24 hours after being exposed to the bacteria in a planktonic state and in another study, this antibacterial activity was observed within 12-24 hours following exposure to *P. aeroginosa* and *S. aureus* and disappeared after 36 hours of incubation (16).

It has also been shown that larval extract was most effective within the first four hours and caused an 85% reduction in *P. aeruginosa* growth. A decrease in the growth rate of *S. aureus* after 6 hours was reported to be 91.5%, while a reduction to 100% after 8 hours has also been reported (17, 23, 32). This antibacterial activity decreases after 24 hours compared to the initial hours, but it is steady (32). It appears that the main reason for the inactivation of larvae extract is due to inactivation of protein and peptids after several hours (23, 33).

A study conducted on *S. aureus* bacteria found that treatment with group B extract inhibited biofilm formation by 62.11% and destroyed it by 56.67%. Furthermore, biofilm formation was also inhibited by 75.04% and destroyed by 68.50%

when *P. aeruginosa* was treated with group B extract ($p<0.0001$, $p<0.05$). Other studies also confirm our report about inhibiting the formation and destruction of *S. aureus* and *P. aeruginosa* biofilm by maggot ES (34-36). According to van der Plas' research, ES exhibits different effects against biofilm of *S. aureus* and *P. aeruginosa*. Different concentrations of maggot extract inhibit the formation and destruction of S. aureus biofilm (34). Compounds such as serine proteases, chymotrypsin, fatty acid and phormicin C are derived from maggot extract and have been introduced as anti-biofilm compounds. These compounds have also negative effects on the metabolic activity of bacterial cells, membrane, cell wall which lead to cell death (10, 37-39).

Based on TTC method, it was found that the synergistic effect of FLIP7 present in the maggot extract of *Calliphora vicina* along with antibiotics (meropenem, ampicillin, amikacin, kanamycin) inhibits metabolic activity of *S. aureus* and *P. aeroginosa* (35). In this study, maggot extract infected with *S. aureus* inhibited the metabolic activity of *S. aureus* and *P. aeroginosa* by 95.03% and 68.25%, respectively.

Bacterial growth is normally stopped by aminoglycosides such as gentamycin and tobramycin, which hinder protein synthesis and disrupting their cell wall structure (28, 40). In our analysis the maggot extract combined with gentamycin and tobramycin for *S. aureus* and *P. aeroginosa* resulted in a complete inhibition of biofilm formation compared to that of only MIC of gentamycin 76%, and MIC of Tobramycin 89%.

Synergism between gentamicin and maggot ES has intensified the antibacterial activity and reduced the number of MRSA (33). In another study, the synergistic effect of gentamicin/ES and flucloxacillin/ES have been determined against *S. aureus*, but the growth of *Streptococcus pyogenes* and *P. aeruginosa* was not affected in the presence of ES (28). Although gentamycin has more effective on *S. aureus* than larvae extract (33), our data showed that minimum inhibitory dose of antibiotic (in planktonic mode) coupled with larvae extract can prevent bacterial biofilm formation. Previous studies have shown that *Lucilia sericata* and *Calliphora vicina* maggot extract work synergistically with antibiotics as interfered from the reductions in crystal violet binding to *S. aureus*, *P. aeroginosa* , and *E. coli* biofilms (35, 38). The use of the antibiotic ciprofloxacin at levels below the MIC and the combination of daptomycin and ES have also eradicated the antibiotic resistant infection (38, 41).

Quorum-Sensing (QS) is the most important regulatory system of biofilm formation in *P. aeroginosa* and *S. aureus*. Previous studies have shown that interfering with the quorum sensing system prevents the formation of bacterial biofilm or its destruction. (10, 42). The *las* and *agrA* genes play a critical role in QS system (43) and extracellular polysaccharides are necessary for establishment and maintenance of biofilms by *P. aeroginosa* and *S. aureus* (44, 45). *P. aeruginosa* produces at least three extracellular polysaccharides: Pel, Psl, and alginate. Non-mucoid *P. aeruginosa* strains use Pel and Psl polysaccharides to promote mature biofilm formation (45, 46). The ica operon contains *icaADBC* and the transcriptional repressor *icaR* (10). *icaA* and *icaD* genes play a serious role in the synthesis of exopolysaccharide structures in maintaining the bacterial biofilm structure (47). As part of this study, genes associated with *P. aeruginosa* (LasI and *psl A*) and *S. aureus* (*agrA* and *icaD*) were examined in the treatment with ES infected with *S. aureus* (for significant effect on biofilm destruction and formation). Results showed that there was a decreased expression of these genes in both pathogens, suggesting that the infected extract reduce biofilm formation through the QS

Table 1. Specific sequences of primers.

Gene	organism	Name	Sequence (5'-3')	References
rPsl	*P. aeruginosa*	Housekeeping gene	F: GCAAGCGCATGGTCGACAAGA' R : CGCTGTGCTCTTGCAGGTTGTGA	(8)
LasI	*P. aeruginosa*	QS-associated genes	F: GGCTGGGACGTTAGTGTCAT R : AAAACCTGGGCTTCAGGAGT-	(43)
psl A	*P. aeruginosa*	Polysaccharide synthesis locus	F: GTTCTGCCTGCTGTTGTTCATG R : AGGTAGGGAAACAGGCCCAG	(45)
16SrRNA	*S. aureus*	Housekeeping gene	F: TGTTTGACGATGTTTGAGCA R : CCTTCCTCCAGTTCAGATGC	(44)
agrA	*S. aureus*	Quorum-sensing regulator A	F: TGATAATCCTTATGAGGTGCTT R : CACTGTGACTCGTAACGAAAA	(44)
icaD	*S. aureus*	Intercellular adhesion D	F: ATGGTCAAGCCCAGACAGAG R : AGTATTTTCAATGTTTAAAGCAA	(44)

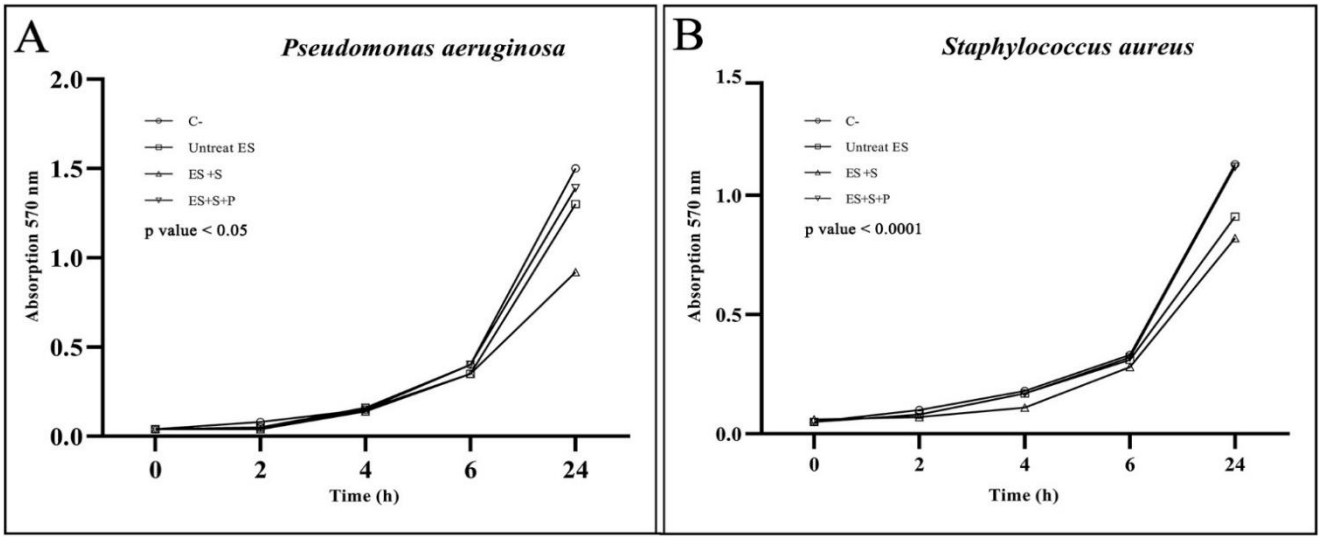

Figure 1. The effects of maggot extract on the planktonic forms of *P. aeruginosa* and *S. aureus* at intervals of 0, 2, 4, 6 and 24 hours, in 4 groups: control (culture medium and bacterial suspension , untreated maggot extract, maggot extract treated with S. aureus (ES+S) and maggot extract treated with both of *S. aureus* and *P. aeruginosa* (ES+S+P).

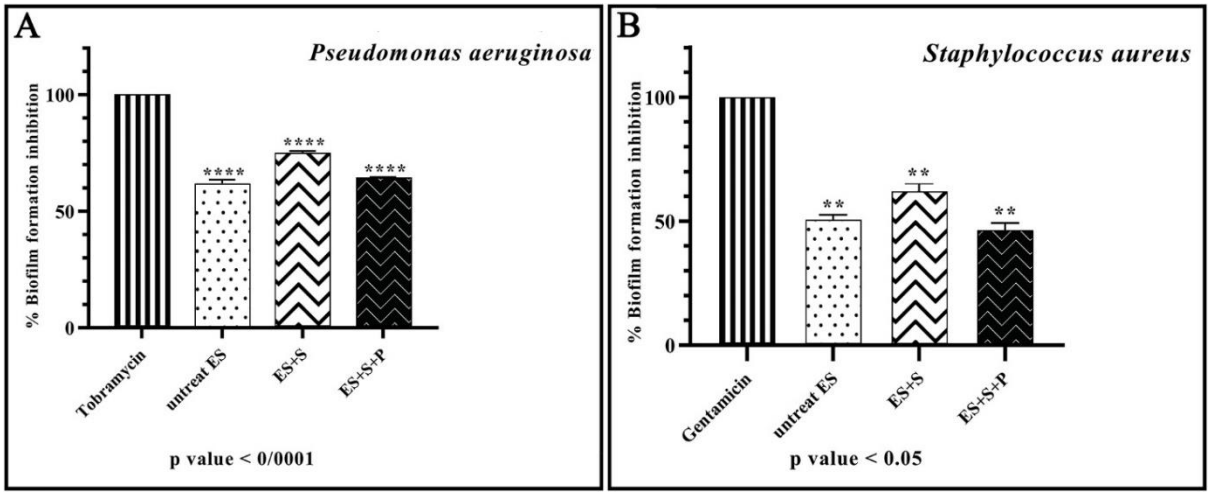

Figure 2. The effect of ES on biofilm formation inhibition in *P. aeruginosa* and *S. aureus* in 4 groups treated with antibiotics gentamicin and tobramycin, untreated maggot extract, maggot extract treated with S. aureus (ES+S) and maggot extract treated with both of *S. aureus* and *P. aeruginosa* (ES+S+P).

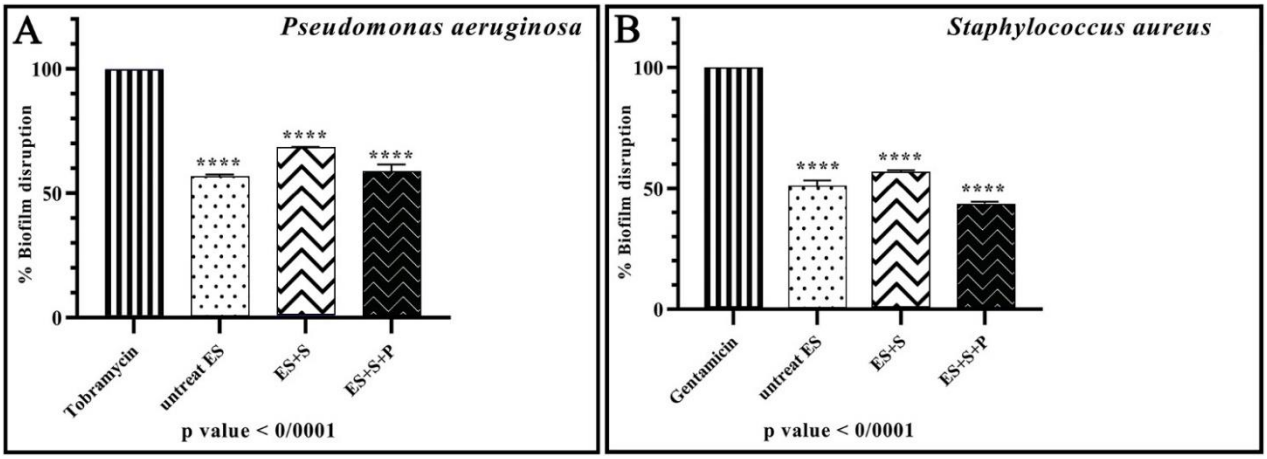

Figure 3. The effect of ES on biofilm disruption in *P. aeruginosa* and *S. aureus* in 4 groups treated with antibiotics tobramycin and gentamicin, untreated maggot extract, maggot extract treated with S. aureus (ES+S) and maggot extract treated with both of *S. aureus* and *P. aeruginosa* (ES+S+P).

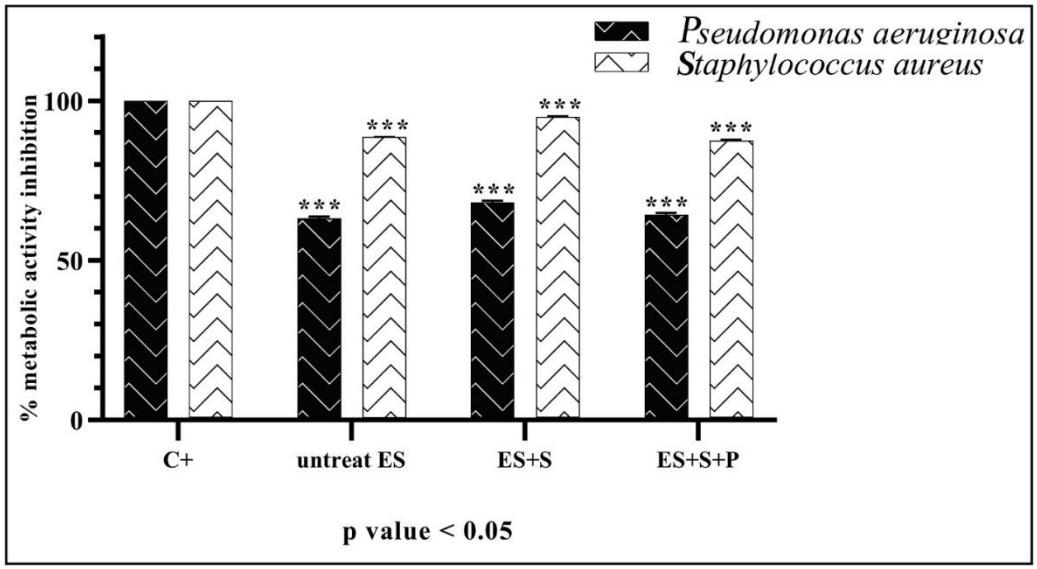

Figure 4. The ability of extracts to inhibit the metabolic activity of biofilm structures in *P. aeruginosa* and *S. aureus* in 4 groups treated with antibiotics tobramycin and gentamicin, untreated maggot extract, maggot extract treated with *S. aureus* (ES+S) and maggot extract treated with both of *S. aureus* and *P. aeruginosa* (ES+S+P).

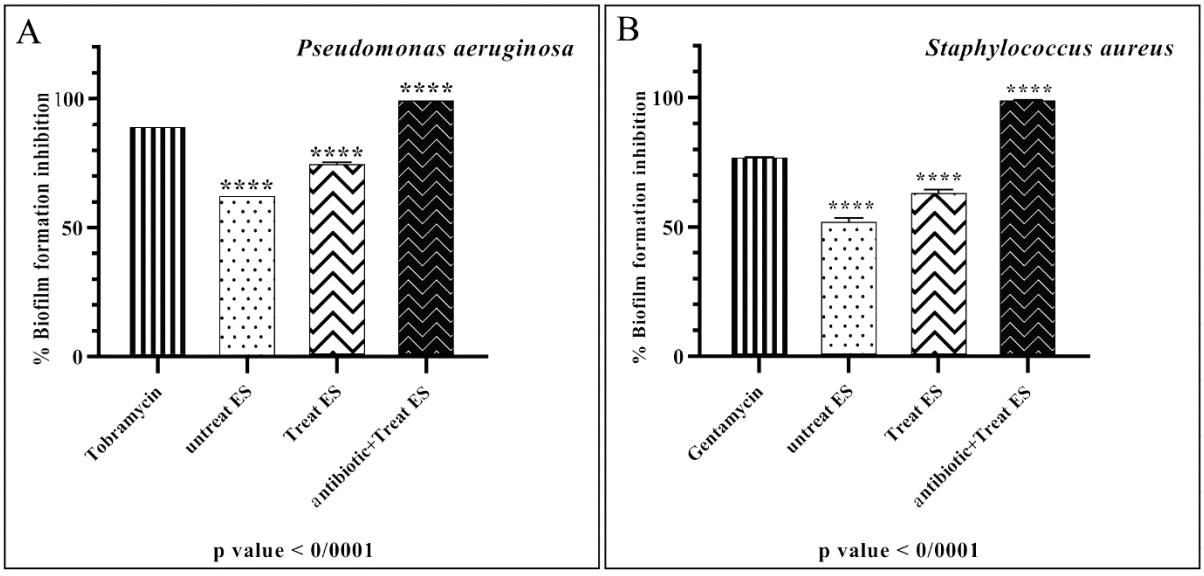

Figure 5. Synergism between Combination of maggot excretions and antibiotics Compared with tobramycin and gentamicin antibiotics, untreated maggot extract and treated maggot extract, in *P. aeruginosa* and *S. aureus*.

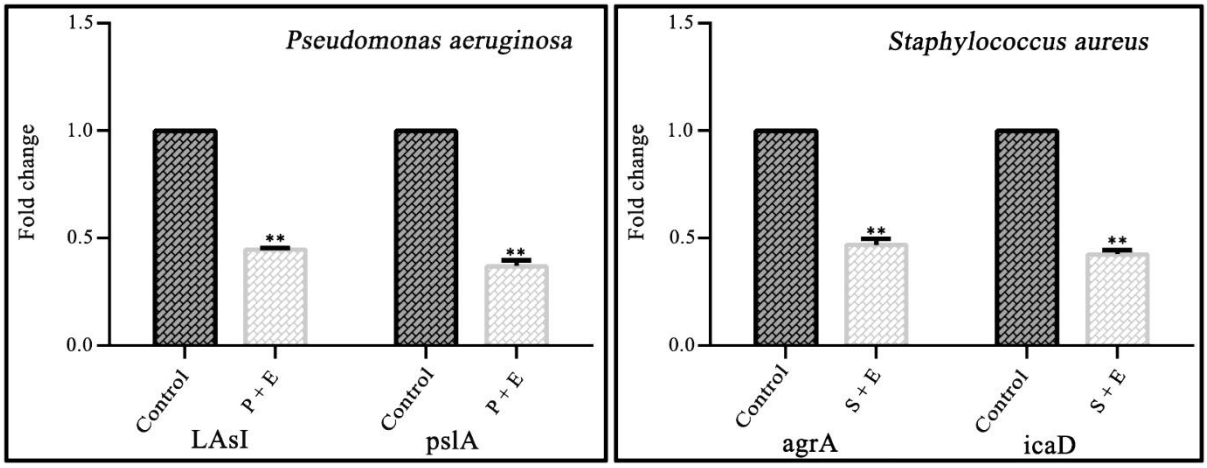

Figure 6. Real time PCR diagram and fold change data comparing to the expression of 4 genes related to the formation of biofilms of *P. aeruginosa* and *S. aureus* in the group treated with maggot extract infected with *P. aeruginosa* compared to the control group. The expression of *LAsI* and *pslA* genes in *P. aeruginosa* and the expression of *agrA* and *icaD* genes in *S. aureus* bacteria has decreased.

system and the structure of extracellular polysaccharides.

L. sericata maggot extract together with coumarin (a phenolic compound derived from the plant) reduced the expression of key genes involved in *las, rhl, PQS* and *IQS* systems in *P. aeruginosa* biofilm (48). Interestingly, Anderson et al. observed that maggot extract suppressed the expression of *rhlA* in *PAO1*,and interferd with the lasB QS system (42). The expression of genes related to biofilm formation (*altA*, *rbf*, *hla*, *hld*, *geh* and *psma*) was also downregulated in MRSA and *S. aureus* 29213 (10, 49).

Conclusion

In summary, when covering factors such as biofilm are present, the effects of antibiotics on the inhibition of microorganisms are reduced compared to non-biofilm conditions, it is therefore possible to use maggot therapy along with antibiotics as an option used in low concentrations to reduce severe side effects and treat infections. Identifying antibacterial compounds against biofilm is a major challenge in this field, and these studies will allow the development of new antibiotics.

Acknowledgements

The authors of this article are grateful to Hakim University for providing laboratory facilities.

Funding Information

This research did not receive any funding support.

Ethics approval and consent to participate

This article does not include human and animal studies. However, ethical and environmental issues have always been considered during high-precision scientific experiments.

Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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