

■ Original Article

## Investigation of whole body extract metabolites of *Lucilia sericata* larvae and potential antibacterial effects

### *Lucilia sericata* larvalarının tüm vücut ekstrakt metabolitlerinin araştırılması ve potensiyel antibakteriyel etkileri

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#### ABSTRACT

**Aim:** Complementary medicinal techniques have gained focus by modern medicine, recently. Maggot Debridement Therapy is a widely-used method worldwide. It is especially recommended for chronic wounds, and has serious advantages such as low cost, easily-applicability and rare adverse effects, but its effect mechanisms remains unclear. The aim of this study is to detect components and to investigate potential antibacterial effects of whole body extract metabolites of *Lucilia sericata* larvae.

**Material and Methods:** Due to potential antibacterial effects, agar well diffusion and flowcytometry methods were used against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* to do evaluation on whole body extracts of previously-cloned maggots in specialized climate room. After this antibacterial effect evaluation, 2-D PAGE analysis was done for protein investigation.

**Results:** Inhibition zones were observed for *S.aureus* (16mm), *E.coli* (22mm) and *E.faecalis* (14mm), but for *P.aeruginosa*, the extract could not provide any inhibition zone. In flow cytometry, different killing rates were detected in different extract dilutions, and for the lowest (1/64) dilution, killing rates were 51.9%, 75%, 80% and 98.7% for *P.aeruginosa*, *E.faecalis*, *E.coli* and *S.aureus*, respectively. 2-D PAGE showed various proteins with different molecular mass (<10-260kDa) and pI (3-9).

**Conclusion:** Antibacterial effects of maggot whole body extracts on tested strains are obviously detected. Many protein spots with widely variable molecular mass and isoelectric points were observed. As a result, this antibacterial effects may be caused by these proteins, but it is necessary that these proteins must be further evaluated via mass spectrometry and protein databases.

**Keywords:** *Lucilia sericata*; Chronic Wound Care; Larval Debridement; Biosurgery; Maggot Debridement Therapy

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## ÖZ

**Amaç:** Yakın dönemde, tamamlayıcı tıp uygulamaları modern tıbbın ilgi alanına girmiştir. Maggot Debritleme Tedavisi dünya çapında yaygın olarak kullanılan bir yöntemdir. Bu yöntem, özellikle kronik yaraların tedavisinde tavsiye edilmektedir ve düşük maliyet, kolay uygulanabilirlik ve nadir yan etkiler gibi avantajları bulunmaktadır, ancak yöntemin etki mekanizması henüz tam olarak ortaya konulamamıştır. Bu çalışmanın amacı, *Lucilia sericata* larvalarının tüm vücut ekstraktının metabolitlerini ortaya koymak ve bunların potansiyel antibakteriyel niteliğini araştırmaktır.

**Gereç ve Yöntemler:** Antibakteriyel etkinliği araştırmak için, önceden özel iklim odalarında üretilmiş larvalarının tüm vücut ekstraktları, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* ve *Enterococcus faecalis* bakterileri için agar difüzyon ve akan hücreölçer ile test edilmiştir. Antibakteriyel incelemeyi takiben, iki boyutlu elektroforez ile protein araştırılması yapılmıştır.

**Bulgular:** *S.aureus* (16mm), *E.coli* (22mm) ve *E.faecalis* (14mm) için inhibisyon alanı gözlenmiş ancak *P.aeruginosa* için alan oluşmamıştır. Hücre ölçer ile farklı dilüsyonlarda farklı öldürme oranları gözlenmiş ve en düşük dilüsyonda (1/64), *P.aeruginosa*, *E.faecalis*, *E.coli* ve *S.aureus* için sırasıyla %51,9, %75, %80 ve %98,7 oranları alınmıştır. İki boyutlu elektroforezde farklı moleküler ağırlık (<10-260kDa) ve izoelektrik noktada (3-9) proteinler tespit edilmiştir.

**Sonuç:** Maggot ekstraktlarının test edilen suşlar üzerine antibakteriyel etkisi net olarak gözlenmiştir. Farklı moleküler ağırlık ve izoelektrik noktada proteinler tespit edilmiştir. Antibakteriyel etkinin bu proteinler tarafından sağlanması muhtemel olsa da, proteinlerin kütle spektrometrisi ve protein veri bankaları ile ayrıca araştırılması gerekir.

**Anahtar Kelimeler:** *Lucilia sericata*; Kronik Yara Bakımı; Larval Debritleme; Biyocerrahi; Maggot Debritleme Tedavisi

## Introduction

Professionals have put a distance between complementary medicinal techniques and current medicine, but recently, scientific researches indicate that these methods may actually have utilities in medical care [1-3]. Among these techniques, maggot debridement therapy (MDT) or larval therapy or biosurgery, by far, is one of the most studied and accepted application, and is routinely performed in many country [4].

The main area for application of MDT is chronic wound care. Chronic wounds has become more frequent and cheap, effective, easily-applicable methods are actually needed, especially when patient comorbidities are also under consideration[5-9]. Venous stasis ulcers, pressure wounds, neuropathic ulcers (diabetic foot ulcer), traumatic and post surgical non-healing wounds were major indications. Many studies were published that focus on effect mechanisms, but it seems there is no "one" action to define, and there is a serious mesh consisting of serial activities working simultaneously. Although the modes of action have not been entirely enlightened yet, but it seems the result of the therapy is affected by maggot itself, patient immunity, wound type, infective microorganisms. *Lucilia sericata* larvae is by far the

most investigated and applied maggots worldwide [4,10,11].

Excretions/secretions (ES) and whole body extracts (WBE) of *Lucilia sericata* larvae have become topics of many investigations. Researchers found various components that may have impact on chronic wounds towards healing. They have different molecular mass, isoelectric points and structure, which indicate that the components may have different and multiple duties on wound debridement, antimicrobial effect, biofilm degradation and wound healing. Some studies stated potential homologies with "known" proteins and enzymes in databases, but unfortunately these studies actually focused on very limited components [12-20].

The aim of this study is to detect components and to investigate potential antibacterial effects of WBE metabolites of maggots. Flow-cytometry is recently used in antimicrobial susceptibility analysis, and this method was not previously used for maggot ES and WBEs. Since previous studies were generally performed on sterilized and/or pure maggots, we have focused on "provoked (encountered to pathogen)" maggots to see potential differences from previous studies to observe changes on components.



## Material and Methods

**Strains:** *P.aeruginosa* ATCC 27853, *E.coli* ATCC 25922, *E.faecalis* ATCC 29212 and *S.aureus* subsp. *aureus* ATCC 25923 strains were inoculated onto 5% sheep blood agar and cultured in 5% CO<sub>2</sub> atmosphere and 37 °C for 24 hours. Only fresh a maximum of one-day old colonies were used during the entire study.

***L.sericata* larvae and Climate Room:** The method of Tanyuksel et al [21] were based for special air and light conditioning (>50% moist, >25 °C temperature, 16 hours dark & 8 hours light). Caged adult *L.sericata* flies were fed with sugar, cow liver and water. After spawning eggs for 4-8 hours onto liver surface, the livers were taken into another cage with additional livers and sawdust at the bottom, and the cage was covered air-permeable clothing. When adult flies were observed in the cage, the same feeding process was applied and new eggs were obtained via liver again. This time the eggs were fed with additional fresh liver since instar 2 and 3 larvae were observed. These larvae were further collected and after cleaning with sterile saline, they were ready to use.

**“Liver Culture” and Maggot Application:** *E.coli* ATCC 25922 and *S.aureus* subsp. *aureus* ATCC 25923 strains were prepared in 0.5 McFarland turbidity, and these solutions were poured onto fresh livers as in two separate groups. Instar 2 and 3 maggots were inoculated onto livers and they were caged with air-permeable clothing. These boxes were incubated at 5% CO<sub>2</sub> atmosphere and 37 °C for 48 hours.

**Obtaining Whole Body Extract:** The maggots were collected and after cleaning with sterile saline, the *E.coli* and *S.aureus* subsp. *aureus* maggot groups were separately smashed in mortar. The collected body fluid were centrifuged in 13.000 rpm for 10 min, and supernatant fluid were used for further tests immediately without any delay to prevent protein destruction.

**Agar Well Diffusion Method:** The test was performed according to the same procedures in Kirby-Bauer disc diffusion method regarding Clinical and Laboratory Standards Institute (CLSI) [22] and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [23] guides and Dogandemir’s study[24]. Following inoculation of strains onto Mueller-Hinton agar (Biomérieux, France), the WBE fluids were dropped into 6-8 mm wells on the agar surface, and additionally, 10 µg meropenem (Oxoid Ltd, UK) and 10 µg colistin (Bioanalyse, Turkey) for Gram- negative bacteria, 30 µg vancomycin (Bioanalyse, Turkey) and 10 µg linezolid (Bioanalyse, Turkey) for Gram-positive bacteria were tested for susceptibility. The

plates were incubated in ambient atmosphere, separately at 30 °C and 37 °C temperature for 24 hours. Then, the inhibition zones were measured and noted.

**Flow-cytometry:** The test were based on Michelsen et al [25]. Two kinds of staining were performed (thiazole orange – TO for both living and dead cell DNA, propidium iodide – PI for only dead cell DNA) (Sigma Aldrich, MO, USA). Fresh bacterial colonies in tryptic soy broth (TSB) (Oxoid Ltd, UK) were incubated (max 2 hours) until 0.5 McFarland turbidity (5x10<sup>8</sup> cfu/ml) is provided [26]. According to Nuding et al [27] and manufacturer application notes [28], dilutions, mixtures and incubations were applied. Dilutions of WBEs were decided from 1/2 to 1/64, based on MIC levels in Dogandemir’s study[24]. The analysis were done with BD Accuri C6 flow-cytometry device (BD, Maryland, USA) and rates of living/dead cells were defined according to data from detectors and software applications. Thus, by comparing fluorescence of TO and PI, rates of bacterial cells killed by WBEs were detected.

**2D-PAGE:** Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the major step of protein analysis, which gives important structural data (molecular mass – kDa, isoelectric point – pI), but not functional information. Preparations, solutions and staining were done according to manufacturer’s instructions and previous studies [29-31]. The analysis were done on 12% separating gel with Protean IEF-Cell and Criterion SDS-PAGE electrophoresis cell devices (BioRad, CA, USA) and silver staining were performed to evaluate in optimal sensitivity. The spots were compared according to immobilized pH gradient (IPG) strip (BioRad, CA, USA) (pI values) and size marker.

## Results

The results of agar well diffusion and flow-cytometry analysis are summarized in Table 1. Inhibition zones were observed for *S.aureus* subsp. *aureus* (16mm), *E.coli* (22mm) and *E.faecalis* (14mm), but for *P.aeruginosa*, the extract could not provide any inhibition zone. In flow-cytometry, various data were found depending on tested strain and WBE dilution. Unfortunately, it was not possible to observe detectable fluorescence in dilutions 1/2 and 1/4 for all strains. The bacteria-killing rates in dilutions 1/8, 1/16, 1/32 and 1/64 were 66.6%, 52%, 61% and 80% for *S.aureus* subsp. *aureus*; 13.6%, 55.6%, 67%, 75% for *E.faecalis*; 11.9%, 36.4%, 78%, 98.7% for *E.coli*; 15.5%, 24.9%, 30.7% and 51.9% for *P.aeruginosa*, respectively. Despite of decreasing WBE fluid concentration, it is clear that killing rates showed an increasing trend, except dilution 1/8 of *S.aureus* subsp. *aureus*, which is a paradoxical situation for susceptibility testing.

**Table 1:** The results of agar well diffusion and flow-cytometry analysis

Dilutons	Killing Rate (%)			
	<i>S.aureus</i>	<i>E.faecalis</i>	<i>E.coli</i>	<i>P.aeruginosa</i>
1:2	NA			
1:4	NA			
1:8	66,6	13,6	11,9	15,5
1:16	52	55,6	36,4	24,9
1:32	61	67	78	30,7
1:64	80	75	98,7	51,9
Inhibition Zone (mm)	16	14	22	None

Table 2 shows detected protein bands and spots in SDS-PAGE and 2D-PAGE. For each pI value, multiple spots with different molecular mass were detected, which indicates separate protein molecules. In total of 14 bands and 88 spots were observed in various pI (3-9) and molecular mass (<10-260kDa).

**Table 2:** Detected protein bands and spots in SDS-PAGE and 2D-PAGE

SDS-PAGE	2-D PAGE	
Molecular Weight (kDa)	Molecular Weight (kDa)	pI
260	260; 33; 72	3
250	12; <10	3.3
240	70	3.5
85	260; 105; 100; 70; 55; 48; 45; 42; 37; <10	3.7
75	30; 15; 8	4
67	260; 120; 87; 75; 70; 68; 60; 40; 35; 20; 15	4.5
60	65; 60; 23; 20; 15; 10	4.8
48	60; 42; 34; 15; 12	5.2
40	70; 45; 28; 12; 10	5.5
35	68; 38; 27; 15; 12; 7	5.8
30	68; 45; 37; 30; 25; 14; 13; 12	6.2
27	50; 35; 30	6.5
15	50; 47; 45; 30; 15; 12	6.8
12	50; 38; 30	7
	48; 34	7.5
	40; 34	8
	70; 48; 40; 35; 25	8.7
	30; 25; 23; 15; 14; 13; 12	9

## Discussion

Although several studies focused on MDT, a very limited data have been obtained until now. These researches are mainly based on ES and WBE of sterile and/or patient-applied maggots [24,32-38]. In this study, maggots are collected from laboratory conditions and differently from "liver culture" to

create a counterfeit environment that maggots suppose like they are on an infected wound. This method has never been performed before, but as we know from entomological studies, *L.sericata* larvae particularly chooses infected and dead tissue [4,10]. Previous researchers stated that obtaining ES and/or WBE is a thorny procedure, because too many maggots are necessary to gain enough material to analyse. With this method, we aim to create a standardized maggot pool, which is consisted of maggots grown in and encountered the same conditions and microorganisms, and also, we were able to get high number of maggots. In addition, in this way, we might have gotten a preliminary vision about specific and inducible antibacterial activity by encountering maggots with the same microorganism and testing the extract with different strains. Furthermore, testing patient-applied maggots may cause limitations such as inability in avoiding external factors (antibiotic consumption, additional hyperbaric oxygen therapies, etc), which causes deflections on study results. On the other hand, our study has some limitations. In this study, only one bacterial strain (*S.aureus* subsp. *aureus* and *E.coli*, in separate liver cultures) in each group was inoculated, but wound infections may be caused by multiple agents [39,40]. Also, there is no data that liver itself carries similar conditions with a chronic wound, so it is controversial whether maggots may have acted distinctly. Of note, quantitative cultures to observe bacterial death via MDT were not applied.

**Antibacterial Analysis:** Agar well diffusion is standardized method for susceptibility testing, but there is no reliable data to interpret inhibition zones. It is quite possible to comment as "no inhibitory effect" for *Paeruginosa*, whereas existence of inhibition zones for other strains does not prove any sufficient antibacterial effect. This output can be stated as "dose-dependent efficiency" or "potential antibacterial effect". It is impossible to compare size of inhibition zones to evaluate susceptibility results, since the strains are different. Additionally, diffusion test results may not be compatible with dilution tests and in vivo efficiency.

Antibacterial efficiency of maggot ES was previously studied with flow-cytometry. Bexfield et al [33] found strong bacteriostatic act on *S.aureus* subsp. *aureus* and bactericidal effect on *E.coli*. However, they did not show any dilutional alteration on antibacterial action. Our study, despite of Bexfield et al [33], depends on WBEs. Because this is the first attempt for WBEs with flow-cytometry analysis, there is no standardization, so MIC values from Dogandemir's study [24] were referenced.



Dogandemir [24] did not find a MIC value lower than 1/64 for every tested strain, thus this level was accepted as a threshold for our flow-cytometry analysis. Paradoxically, we found an arising trend of antibacterial efficiency due to increased bacterial death rates during decreasing status of WBE concentration. Despite of Dogandemir's data [24], for dilution 1/64, there was the highest bacterial-killing rate for each of every strain. This is a major limitation for our study that MIC levels lower than 1/64 should have been tested. This paradoxical condition can be explained with "the autofluorescence effect", which is previously reported on flow-cytometry analysis. Cellular autofluorescence due to mostly NADH, riboflavins, and flavin coenzymes negatively impact on sensitivity of flow-cytometry [41]. We believe this effect may have caused a false assessment (shading) because of inability of cytometry device to detect the actual fluorescence from living and dead bacteria in high concentrations of WBE. Following the dwindling concentrations, this so called "shading effect" may have disappeared that caused ability of observing the bacterial cells. However, to prove this explanation, lower dilutions should be evaluated and a peak curve of killing rates must be observed. On the other hand, in overall, even in the 1/64 dilution, killing rates were reached at least 51.9% (*P.aeruginosa*), which indicates a highly effective antibacterial action even in low dilutions of WBE.

Among tested species, WBE showed the lowest activity against *P.aeruginosa* strain. This species is frequently isolated from chronic wounds, especially from ICU patients and its ability of biofilm formation is another problem [42]. Huberman et al [35], Cazander et al [43,44], Brown et al [45], Jiang et al [46] and Masiero et al [47] showed strong activity of maggot ES and body fluids against *P.aeruginosa* and its biofilms. Pöppel et al [20], found various peptides and genetic arrangements of *L.sericata*, particularly against this species. They also stated synergistic effects of these peptides. However, clinical efficiency of MDT on *Pseudomonas* or *Acinetobacter*-infected wounds is controversial [48]. Dogandemir [24] used patient-applied maggots and activity against *P.aeruginosa* was seriously limited. Among all these arguments, researchers have a consensus that antibacterial activity of maggots is strongly related with so called "provocation". This issue is about maggots showing specific and specialized activity against the encountered pathogen. Huberman et al [36] and Kerridge et al [38] claimed that following the first encountering, maggots secrete low-molecular weight proteins immediately, but after a while, high-molecular weight complex proteins are secreted

in greater amounts than sterile larvae. Data of Pöppel et al [20] also supported this information, which indicates that maggots do somehow adapt and fight in a particular way against what the "enemy" is. So, this *in vitro* undetectable antipseudomonal effect may be a result of facing with *P.aeruginosa* and/or synergistic activities of secreted peptides. Since, in our study, we used only *S.aureus* subsp. *aureus* and *E.coli* strains to provoke maggots, this could be the reason of low activity against *P.aeruginosa*. The controversial data about *P.aeruginosa* and *E.faecalis* may be also because of studies that are performed with different fluids (ES, WBE, etc) and various methods to obtain the fluids. That's why the studies should be expanded towards including different bacterial strains and various types of maggot materials. Furthermore, Van der Plas et al [49] reported an extended *P.aeruginosa* biofilm degradation effect, Masiero et al [47] and Daeschlein et al. [50] stated a dwindling antibacterial activity in time, so it is important to observe the "Time-Kill Analysis", which gives minimum bactericidal concentration, can be very beneficial to understand the actual antibacterial activity alterations [51].

In our study, the activity against other species (*S.aureus* subsp. *aureus*, *E.faecalis*, *E.coli*) was very promising. Kruglikova et al [13] and Chernysh et al [14] reported that *L.sericata* larvae ES had bacteriostatic act on *E.coli*, bactericidal act on many gram negative and positive bacteria and finally fungicidal activity. Similar results were stated by Cazander et al [43,44] and Van der Plas et al [49]. In overall, there is an opinion that maggot fluids are more effective against gram positives [12,52]. Despite of this, we found a strong activity against *E.coli*, which indicates that bacterial cell wall is not the only target and multiple mechanisms are on the move.

**Protein Analysis:** In our study, protein analysis was limited with 2D-PAGE. The peptides were separated according to their isoelectric points and molecular mass.

Proteins of maggot ES and WBE was previously topics of some studies. Chernysh et al [14] identified dipterocin (8882 ve 9025 Da) and anti-gram positive peptides (129-700 Da, 6466 and 6633 Da). Kruglikova et al [13] defined many peptides with various molecular mass (174-904 Da; 1014-9025 Da). Čeřovský et al [15] identified "lucifensin" (4,113.89 Da) and this was followed by isolation of "Lucifensin II" (4,127.93 Da) [16]. One of the widest studies was performed by Andersen et al. [12] that they found many proteins via BLAST protein bank such as lectin, dephencin, attacin and chitin binding protein. Valachova et al [17,18] defined three different serine proteases, phenil

metalloprotease, signal peptide protease, chymotrypsin and midgut lysozyme. Differently, Pöppel et al [19] isolated an antifungal protein, "lucimycin" (8,2 kDa). Recently, Pöppel et al [20] reported 47 different genes encoding antimicrobial peptides and they recombinantly produced 23 of them such as "cecropin", "cecropin like", "proline rich", "stomoxyn", "dephencin". Additionally, they detected proteins called "elevated during infection – edin" that are coded in case of infection. As previously stated, they also showed synergistic and additive effects of these proteins. As understood, the studies on maggot ES and WBE is just on a preliminary phase that there is a huge black hole to explore.

In our study, SDS-PAGE (1D-PAGE) showed many protein bands (12-260 kDa). Since the band intensities were different from each other, it can be noted that protein concentrations may have varied. This interpretation might also be valid for SDS-PAGE, but this kind of quantitation can be stated by automatized analysis devices, which we did not use. In 2-D PAGE analysis, many protein spots with various pI were observed. Table 2 gives detected bands and spots. In previous studies, proteins were mainly isolated separately, thus low-molecular weight peptides could be purified [33-36]. As previously stated, Huberman et al [36] and Kerridge et al [38] reported that high-molecular weight proteins were secreted following an "enemy-encountering". As seen, our results indicated high-molecular weight proteins. It should be noted that we investigated on WBE and used "provoked" maggots. Since we incubated maggot on a virtual infected wound, this was actually expected. However, WBE may have contained structural proteins, so it is a major limitation that functional analysis was not applied.

In this study, "liver culture" was infected by *S.aureus* subsp. *aureus* and *E.coli*. As noticed, we found the highest antibacterial activities against these agents. We believe that protein analysis should be performed to sterile and *S.aureus*-, *E.coli*-, *K.pneumoniae*-, *P.aeruginosa*-, *Proteus* spp.-, *Enterococcus* spp.-, *Acinetobacter* spp.-provoked maggots, and finally comparison should be made. This analysis will uncover main differences between sterile and provoked maggots, and it may also prove "specific and specialized antibacterial action". In addition, WBEs and ES of these maggots should also be tested with functional analysis with mass spectrometry and Protein ID. But, synergistic and additive effects should not be forgotten.

In conclusion, MDT is a very effective method as a part of multidisciplinary approaches in treatment of chronic wounds.

For a chronic wound treatment, the main attempts are debridement, antimicrobial action, provoking wound healing and biofilm destruction. In our study, it was obvious that there is a certain antibacterial effect, and various proteins may have a role on this. Furthermore, these proteins may also act in other attempts, which is in need of further studies. Dilutional antimicrobial tests, time-kill analysis and advanced functional protein identifications should be performed to clarify actual effect mechanisms of MDT.

### Declaration of conflict of interest

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