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 *Paeruginosa, E.faecalis, E.coli* and *S.aureus*, respectively. 2-D PAGE showed various proteins with different molecular mass (<10-260kDa) and pl (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 tip uygulamaları modern tibbin ilgi alanına girmiştir. Maggot Debridman 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 potensiyel 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 ekstraklarının 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 olusa da, proteinlerin kütü spektrometrisi ve protein veri bankaları ile ayrıca araştırılması gereklidir.

Anahtar Kelimeler: *Lucilia sericata*; Kronik Yara Bakımı; Larval Debridman; Biyocerrahi; Maggot Debridman 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% CO₂ 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 Tanyukel 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 seperate groups. Instar 2 and 3 maggots were inoculated onto livers and they were caged with air-permeable clothing. These boxes were incubated at 5% CO₂ 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 seperately 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 (Biomerieux, 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, seperately at 30 °C and 37 °C temperature for 24 hours. Then, the inhibiton 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 (5x10⁸ 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 MIClevels 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 – pl), but not functional information. Preperations, solutions and staining were done according to manufacter’s instructions and previous studies [29-31]. The analysis were done on 12% seperating gel with Protean IEF-Cell and Criterion SDS-PAGE elektrophoresis 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) (pl 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 inhibiton 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 paradoxal situation for susceptibility testing.
Table 1: The results of agar well diffusion and flow-cytometry analysis

| Dilutions | Killing Rate (%) | S.aureus | E.faecalis | E.coli | Paeruginosa |
|-----------|------------------|----------|------------|--------|-------------|
| 1:2       | NA               | 66.6     | 13.6       | 11.9   | 15.5        |
| 1:4       |                  |          |            |        |             |
| 1:8       | 52               | 75       | 80         | 80     | 51.9        |
| 1:16      | 55.6             | 78       | 78         | 30.7   |             |
| 1:32      | 36.4             | 67       | 78         | 24.9   |             |
| 1:64      | 11.9             | 67       | 78         | 30.7   |             |

Table 2 shows detected protein bands and spots in SDS-PAGE and 2D-PAGE. For each pl 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 pl (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) | pl |
| 260 | 260; 33; 72 | 3 |
| 250 | 70; 42; 34; 15 | 3.3 |
| 240 | 70; 42; 34 | 3.5 |
| 85 | 70; 42; 34; 15; 12; 10 | 4.5 |
| 75 | 70; 42; 34; 15; 12; 10 | 5.5 |
| 67 | 70; 42; 34; 15; 12; 10 | 6.2 |
| 30 | 70; 42; 34; 15; 12; 10 | 6.8 |
| 27 | 70; 42; 34; 15; 12 | 7 |
| 15 | 70; 42; 34; 15; 12 | 7.5 |
| 12 | 70; 42; 34; 15; 12 | 8 |

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 premilinary vision about specific and inducable 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.aureussubsp. 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.

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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 seperated 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 diptericin (8882 ve 9025 Da) and anti-gram positive peptides (129-700 Da, 6466 and 6633 Da). Krugligova 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 poteases, phenil
mellitoprotease, 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 automated analysis devices, which we did not used. 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.pneumonaie-, Paeruginosa-, 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.

For a chronic wound treatment, the main attempts are debridement, antimicrobial action, provoking wound healing and biofilm distruction. 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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**References**

1. Fischer FH, Lewith G, Witt CM et al. High prevalence but limited evidence in complementary and alternative medicine: guidelines for future research. BMC complementary and alternative medicine 2014; 14: 46.

2. World Health Organization (WHO), WHO traditional medicine strategy: 2014-2023. Geneva, Switzerland: World Health Organization; 2013.

3. Barnes PM, Bloom B, Nahin RL. Complementary and alternative medicine use among adults and children: United States, 2007. National health statistics reports; No: 12. Hyattsville, MD: National Center for Health Statistics, 2008.

4. Sherman RA, Mumcuoglu KY, Grassberger M, Tantawi TI. Maggot Therapy. In: Grassberger M, Sherman RA, Gileva OS, Kim CMH, Mumcuoglu KY (eds). Biotherapy-history, principles and practice: A practical guide to the diagnosis and treatment of disease using living organisms. Springer Science & Business Media, Amsterdam 2013; 5-29.
5. Sherman RA, Hall M, Thomas S. Medicinal maggots: an ancient remedy for some contemporary afflictions. Annu Rev Entomol 2000; 45: 55-81.

6. Sherman RA, Pechter EA. Maggot therapy: a review of the therapeutic applications of fly larvae in human medicine, especially for treating osteomyelitis. Med Vet Entomol 1988; 2: 225-30.

7. Sherman RA, Tran JMT, Sullivan R. Maggot therapy for venous stasis ulcers. Arch Dermatol 1996; 132: 254-56.

8. Sherman RA., Wyle FA. Low-cost, low-maintenance rearing of maggots in hospitals, clinics, and schools. Am J Trop Med Hyg 1996; 54: 38-41.

9. Stoddard S, Sherman R., Mason B, Pelsang D, Sherman R. Maggot debridement therapy. An alternative treatment for nonhealing ulcers. J Am Podiatr Med Assoc 1995; 85: 218-21.

10. Fleischmann W, Grassberger M, Sherman RA. Maggot therapy: A handbook of maggot-assisted wound healing. Thieme Pubfication, London, 2004.

11. Game FL, Apelqvist J, Attinger C et al. IWGDF Guidance on use of interventions to enhance healing of chronic ulcers of the foot in diabetes. International Working Group on Wound Healing Document, Amsterdam, 2015.

12. Andersen AS, Sandvang D, Schnorr KM, Kruse T, Neve S, Joergensen B, Karlsmark T, Krogfelt KA. A novel approach to the antimicrobial activity of maggot debridement therapy. J Antimicrob Chemother 2010; 65: 1646-54.

13. Kruglikova A, Chernysh S. Antimicrobial compounds from the excretions of surgical maggots, Lucilia sericata (Meigen) (Diptera, Calliphoridae). Entomological Review 2011; 91: 813-19.

14. Chernysh SI, Gordja NA, Simonenko NP. Diapause and immune response: induction of antimicrobial peptides synthesis in the blowfly, Calliphora vicina R.-D. (Diptera: Calliphoridae). J Entomol Sci 2000; 3: 139-44.

15. Čeřovský V, Žďárek J, Fučík V, Monincová L, Voburka Z, Bém R. Lucifensin, the long-sought antimicrobial factor of medicinal maggots of the blowfly Lucilia sericata. Cell Mol Life Sci 2010; 67: 455-66.

16. El Shazely B, Ververka V, Fučík V, Voburka Z, Žďárek J, Čeřovský V. Lucifensin II, a defensin of medicinal maggots of the blowfly Lucilia cuprina (Diptera: Calliphoridae). J Med Entomol 2013; 50: 571-78.

17. Valachova I, Majtan T, Takac P, Majtan J. Identification and characterisation of different proteases in Lucilia sericata medicinal maggots involved in maggot debridement therapy. J Appl Biomed 2014; 12: 171-77.

18. Valachova I, Takac P, Majtan J. Midgut lysozymes of Lucilia sericata—new antimicrobials involved in maggot debridement therapy. Insect Mol Biol 2014; 23: 779-87.

19. Pöppel AK, Koch A, Kogel KH et al. Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly Lucilia sericata. Biol Chem 2014; 395: 649-56.

20. Pöppel AK, Vogel H, Wiesner J, Vilcinskas A. Antimicrobial peptides expressed in medicinal maggots of the blow fly Lucilia sericata show combinatorial activity against bacteria. Antimicrob Agents Chemother 2015; 59: 2508-14.

21. Tanyuksel M, Araz E, Dundar K et al. Maggot debridement therapy in the treatment of chronic wounds in a military hospital setup in Turkey. Dermatol 2004; 210: 115-18.

22. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard — Eleventh Edition, CLSI document M02-A11, 2012.

23. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Antimicrobial susceptibility testing EUCAST disk diffusion method, version 5.0, Jan 2015.

24. Dogandemir G. Investigation of antimicrobial activities of Lucilia sericata against microorganisms colonising on chronic wounds. Medical Microbiology Thesis, Gulhane Military Medical Academy, Ankara, Turkey, 2010.

25. Michelsen CF, Christensen AMJ, Bojer MS, Haiby N, Ingmer H, Jelsbak L, Staphylococcus aureus alters growth activity, autolysis, and antibiotic tolerance in a human host-adapted Pseudomonas aeruginosa lineage. J Bacteriol 2014; 196: 3903-11.

26. Faria-Ramos I, Espinar MJ, Rocha R, Santos-Antunes J, Rodrigues AG, Cantón R, Pina-Vaz C. A novel flow cytometric assay for rapid detection of extended-spectrum beta-lactamases. Clin Microbiol Infect 2013; 19: 8-15.

27. Nuding S, Zabel LT. Detection, identification, and susceptibility testing of bacteria by flow cytometry. J Bacteriol Parasitol 2013; S5:005.

28. Becton Dickinson (BD) BioSciences. Application Note: Bacterial Detection and Live/Dead Discrimination by Flow Cytometry [Available at http://www.bdbiosciences.com/us/home, Date of Access: 15 May 2016].

29. Friedman DB, Hoving S, Westermeier R. Isoelectric focusing and two-dimensional gel electrophoresis. Methods in Enzymology 2009; 463: 515-40.

30. Garfin DE. Gel electrophoresis of proteins. In: Davey J, Lord M. (eds) Essential Cell Biology Volume 1: Cell Structure, A Practical Approach. Oxford University Press, Oxford, UK 2003; 197-268.

31. Righetti PG, Sebastiani R, Citterio A. Capillary electrophoresis and isoelectric focusing in peptide and protein analysis. Proteomics 2013; 13: 325-340.
32. Barnes KM, Dixon RA, Gennard DE. The antibacterial potency of the medicinal maggot, *Lucilia sericata* (Meigen): variation in laboratory evaluation. J Microbiol Methods 2010; 82: 234-37.

33. Bexfield A, Bond AE, Roberts EC et al. The antibacterial activity against MRSA strains and other bacteria of a < 500Da fraction from maggot excretions/secretions of *Lucilia sericata* (Diptera: Calliphoridae). Microbes Infect 2008; 10: 325-33.

34. Bexfield A, Nigam Y, Thomas S, Ratcliffe NA. Detection and partial characterisation of two antibacterial factors from the excretions/secretions of the medicinal maggot *Lucilia sericata* and their activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbes Infect 2004; 6: 1297-1304.

35. Huberman L, Gollop N, Mumcuoglu KY, Block C, Galun R. Antibacterial properties of whole body extracts and haemolymph of *Lucilia sericata* maggots. J Wound Care 2007; 16: 123-27.

36. Huberman L, Gollop N, Mumcuoglu KY et al. Antibacterial substances of low molecular weight isolated from the blowfly, *Lucilia sericata*. Med Vet Entomol 2007; 21: 127-31.

37. Bexfield A, Bond AE, Morgan C et al. Amino acid derivatives from *Lucilia sericata* excretions/secretions may contribute to the beneficial effects of maggot therapy via increased angiogenesis. Br J Dermatol 2010; 162: 554-62.

38. Kerridge A, Lappin-Scott H, Stevens J. Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata*. Med Vet Entomol 2005; 19: 333-37.

39. Nunan R, Harding KG, Martin P. Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. Dis Model Mech 2014; 7: 1205-13.

40. Daeschlein G. Antimicrobial and antiseptic strategies in wound management. Int Wound J 2013; 10: 9-14.

41. Mosiman VL, Patterson BK, Canterero L, Goolsby CL. Reducing cellular autofluorescence in flow cytometry: an in situ method. Cytometry 1997; 30: 151-56.

42. Trøstrup H, Bjamsholdt T, Kirketerp-Møller K, Hoiby N, Moser C. What Is New in the Understanding of Non Healing Wounds Epidemiology, Pathophysiology, and Therapies. Ulcers 2013; 8: 1-6.

43. Cazander G, Van de Veerdonk MC, Vandenbroucke-Grauls CM, Schreurs MW, Jukema GN. Maggot excretions inhibit biofilm formation on biomaterials. Clin Orthop Relat Res 2010; 468: 2789-96.

44. Cazander G, van Veen KE, Bouwman LH, Bernardis AT, Jukema GN. The influence of maggot excretions on PAO1 biofilm formation on different biomaterials. Clin Orthop Relat Res 2009; 467: 536-45.

45. Brown A, Horobin A, Blount DG et al. Blow fly *Lucilia sericata* nuclease digests DNA associated with wound slough/eschar and with *Pseudomonas aeruginosa* biofilm. Med Vet Entomol 2012; 26: 432-39.

46. Jiang KC, Sun XJ, Wang W et al. Excretions/secretions from bacteria-pretreated maggot are more effective against *Pseudomonas aeruginosa* biofilms. PLoS one 2012; 7: 49815.

47. Masiero FS, Aquino MFK, Nassu MP, Pereira DiB, Leite DS, Thyssen PJ. First Record of Larval Secretions of *Cochliomyia macellaria* (Fabricius, 1775)(Diptera: Calliphoridae) Inhibiting the Growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Neotrop Entomol 2017; 46: 125-29.

48. Čeřovský V, Bém R. Lucifensins, the insect defensins of biomedical importance: the story behind maggot therapy. Pharmaceuticals 2014; 7: 251-64.

49. Van Der Plas MJ, Jukema GN, Wai SW et al. Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. J Antimicrob Chemother 2008; 61: 117-22.

50. Daeschlein G, Mumcuoglu KY, Assadian O, Hoffmeister B, Kramer A. In vitro antibacterial activity of *Lucilia sericata* maggot secretions. Skin Pharmacol Physiol 2006; 20: 112-15.

51. Clinical and Laboratory Standards Institute (CLSI). Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline CLSI document M26-A, 1999.

52. Jakič D, Lapanjie A, Zupančič K, Smrke D, Gunde-Cimerman N. Selective antimicrobial activity of maggots against pathogenic bacteria. J Med Microbiol 2008; 57: 617-25.