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Antibacterial Potential of *Lucilia sericata* excretions/secretions in bovine cutaneous wounds

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Abstract

In the last decade, many patients worldwide have treated their injuries with maggots, so many doctors recognize maggot therapy as an influential supplement in traditional medicine. This study examined the excretions/secretions (ES) of *Lucilia sericata* larvae on fibroblast cells and isolated bacteria from bovine chronic wounds. The viability of bovine fibroblast cells was assessed using MTT assay. The results showed that the minimum inhibitory concentration (MIC) of larval ES on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* was 40 mg mL^{-1}. The disk diffusion MIC experiment revealed that larval ES can control the growth of bacteria from bovine chronic wounds. The MTT assay showed that 0.1, 0.05, and 0.025 mg mL^{-1} larval ES did not affect the survival of bovine fibroblasts. In comparison, concentrations equal to or greater than 0.25 mg mL^{-1} caused a significant decline in the proliferation and viability of the fibroblast cells ($p<0.05$). In conclusion, the maggot *L. sericata* ES showed control bovine chronic wound bacteria *in vitro* less than 0.25 mg mL^{-1}, but using more can produce side effects on living cells. *In vivo* studies are recommended for the evaluation of the maggot *L. sericata* ES on cutaneous bovine wounds.

1. Introduction

The skin is an immune protective organ. The dermal, epidermal, and hypodermic layers consist of populations of specialized non-immune cells such as fibroblasts, keratinocytes, and adipocytes, which perform immune surveillance functions and phagocytose invading pathogens in case of injury or infection (Dube et al. 2022). The peripheral nerve fibers of the skin are activated after the initial injury and release several neuropeptides in the wound environment. Skin integrity must be revived because it is important in managing homeostasis (Singh et al. 2023). The wound-healing process is very complex and requires the coordination of intracellular, intercellular, and

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extracellular elements. This process includes homeostasis (blood clotting), inflammation, cell proliferation (tissue growth), and cell maturation and differentiation (tissue regeneration) (Vyas &Vasconez 2014). Bacterial infections complicate the wound-healing process (Gjodsbol et al. 2006).

Delayed recovery of chronic wounds is a challenge for medics and medical techniques. In addition, it adds a great disease burden to the individual (Das et al. 2012). Chronic wound management is a challenge because of the formation of bacterial biofilms. Biofilms interact with the host's immune system through the arousal of pro-inflammatory neutrophils and macrophages and cause the collection of inflammatory cytokines (including TNF-α, IL-6, and MMPs). Nevertheless, chronic wound conditions lead to bacterial proliferation, followed by further biofilm development and continuous inflammation (Raziyeva et al. 2021). The main reason for biofilm formation is to protect bacteria from antibiotics (Gilbert et al. 2002). Following the inability of the host's immune cells to eliminate the infectious cause of inflammation, tissue destruction is created by reactive oxygen species (ROS) and proteases freed by active phagocytes (Wagner et al. 2004).

Maggot therapy is a traditional method of healing chronic wounds and is recognized as biosurgery (Hultmark et al. 1998). Although widely used in human medicine to treat chronic wounds, maggot debridement therapy (MDT) in veterinary medicine is limited. However, veterinary practices have reported that a variety of wounds have been treated with MDT in animals in recent years (Ugur et al. 2023). The larvae of *Lucilia sericata* (greenbottle fly) are used in the rapid treatment of necrotic chronic wounds that cannot be treated by conventional methods (Kerridge et al. 2005). The employment of sterile larvae of L. sericata is increasing widely in the therapy of chronic wounds (Grassberger et al. 2013, Mumcuoglu et al. 1998, Sherman 2009). The necrophagous feeding behavior of Lucilia spp larvae has made it possible to use them in forensic research (to determine the end of life) and in the therapy of necrotic and chronic wounds (Gazi et al. 2021). *In vitro* investigations of the antibacterial properties have demonstrated that the impacts of maggot therapy on microorganisms that cause chronic wounds have not yet been determined (Jaklic et al. 2008). Due to the increase of drug-resistant organisms (like Methicillin-resistant *S. aureus*), new therapeutic methods are required to treat wound biofilms (Kruglikova 2011).

In this investigation, for the first time, the antibacterial impact of maggot excretions/secretions (ES) was studied on samples isolated from bovine chronic wounds, and the viability of fibroblast cells was assessed to identify side effects of larvae ES on wound healing cells.

2. Material and Methods

2.1. Collection of maggot L. sericata ES

L. sericata blowflies were raised in mesh cages under control following Kerridge et al.'s method at 25 ºC, stable relative humidity, and continuous provision of water and sugar at 16:8 h light-dark cycle. A sheep's liver was used to lay eggs for flies. After sterilization, the obtained eggs were seeded on blood agar plates (Merck, 1108860-500) with defibrinated horse blood (Accurate, ACL1500-100D) and kanamycin (Gibco, MMS-048). ES were accumulated from third-instar larvae of *L. sericata* (Probiotic Laboratory, IROST, IRAN) in aseptic conditions. Roughly 100 larvae were rinsed 3 times in distilled water (about 1 mL) for one and a half at 28 °C to recover ES. The sterility of the collected ESs was checked and stored at -20 ºC. Bovine serum albumin (BSA) (Sigma, A-7926) was utilized as a standard to determine the protein concentration. The curve of the standard was shown as follows: $y = 0.5795x + 0.1559$ (R^2= 0.9806) [Protein concentration is shown on the x-axis and optical density (OD) on the y-axis]. ES concentrations were reported based on the amount of their proteins (Kerridge et al. 2005).

2.2. Bacterial strains and culture conditions

The bacteria utilized in this research contained *E. coli* (PTCC 1399), *S. aureus* (PTCC 1764), and *P. aeruginosa* (PTCC 1310), which are typically

found in wound infections. Organisms were obtained from the Persian Type Culture Collection (PTCC, IROST, IRAN). *E. coli* and *P. aeruginosa* were expanded in Luria–Bertani medium (Merck) at 28 ºC, and *S. aureus* was developed in Tryptone Soya Broth (Merck) with vigorous shaking at 37 ºC.

2.3. Sample collection from infected bovine hooves and wounds

Samples were collected from the infected hooves and wounds from four Holstein cows. The infected area from every cow was cleaned, and sterile swabs were used to sample the area. The swabs were separately placed in sterile tubes containing brain-heart infusion media (BHI) (Merck) plus glycerol and refrigerated until plated. All bacteria were cultured in appropriate media and then identified by rapid tests (gram staining, bile solubility, catalase, oxidase, and coagulase) and biochemical tests (oxidation/fermentation, triple sugar iron, and nitrate reduction) (Marasini et al. 2015).

2.4. Minimum inhibitory concentration (MIC) analysis

Three bacterial samples (*E. coli*, *S. aureus*, and *P. aeruginosa*) were grown in BHI for 6 h. The turbidity test was done using the Johnson et al. (2011) method; briefly, the concentration (total count) of test bacteria was determined by nephelometry using the McFarland scale.

Then 1 mL of 1.5×10^8 cells mL^{-1} was inserted in tubes with 9 mL BHI enriched with various concentrations (5, 10, 20, 40 mg mL^{-1}) of the ES. Afterward, at 24 h and 37 °C, the MIC values of the samples were identified by calculating the OD values at 620 nm.

2.5. Zone-of-inhibition Test

This test was adjusted using Hultmark et al. (1998) with some modifications. Briefly, bacteria were developed for 48 h in 5 mL of BHI at 37 °C. BHI agar was prepared with 0.5 g agar to 50 mL broth and put in the water bath at 44 ºC. After mixing, the agar was quickly spread in 100 mm dishes. After solidification, 2×10^8 bacteria per mL were spread over the agar surface, and 100 µL of the sample was filled in the 7 mm diameter holes created in the agar. Penicillin (10 mg mL^{-1}) was used as a control. After incubating the plates overnight at 37°C, the inhibition's diameter of bacterial growth was checked the next day.

2.6. Bovine fibroblast cell culture

Bovine fibroblast cell lines were collocated in the stem cell at the transgenic animal lab, IROST, from bovine ear skin as described by Rajabalian et al. (2003) and Shah et al. (2008). In summary, sampling was done from the ear skin of adult cows under aseptic conditions and in sterile phosphate-buffered saline (DPBS) (Sigma-Aldrich, D8537). After extracting the skin tissues, the remaining tissues were divided into small fragments and placed in dishes including Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D5796), culture medium with 15% FBS, and10 µL mL^{-1} penicillin-streptomycin (Sigma, P4333) and were incubated at 37 °C and 5% CO_2. The cells were passaged upon reaching approximately 75-80% confluency by partial trypsinization. Cells at passage 5 were cryopreserved and used in this study.

2.7. MTT assay

The MTT viability assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was accomplished according to Shabani et al. (2017), with some changes. The MTT test converts water-soluble MTT to insoluble formazan, then the formazan is dissolved, and the concentration is measured by OD at 570 nm. The MTT test applies the transformation of the water-soluble MTT to an insoluble formazan, then the formazan is solubilized, and the concentration is measured by OD at 570 nm. In summary, after 24 h of cell culturing (5000 cells $well^{-1}$) in the presence of different concentrations (0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2 mg mL^{-1}) of ES in 96 well plates, the culture medium was substituted with 100 µL of the renewed culture medium and 10 µL of MTT was added (5 mg mL^{-1} in PBS) to each of the wells. The cells were incubated for 4 h at

37 °C. Then, 100 µL of SDS-HCl solution (10 mL of 0.01 M HCl was added to 1 g of SDS) was added to each of the wells, and after mixing with a pipette, they were kept in an incubator for 16 h and at 37 °C, and the absorbance was measured with a spectrophotometer.

2.8. Statistical analysis

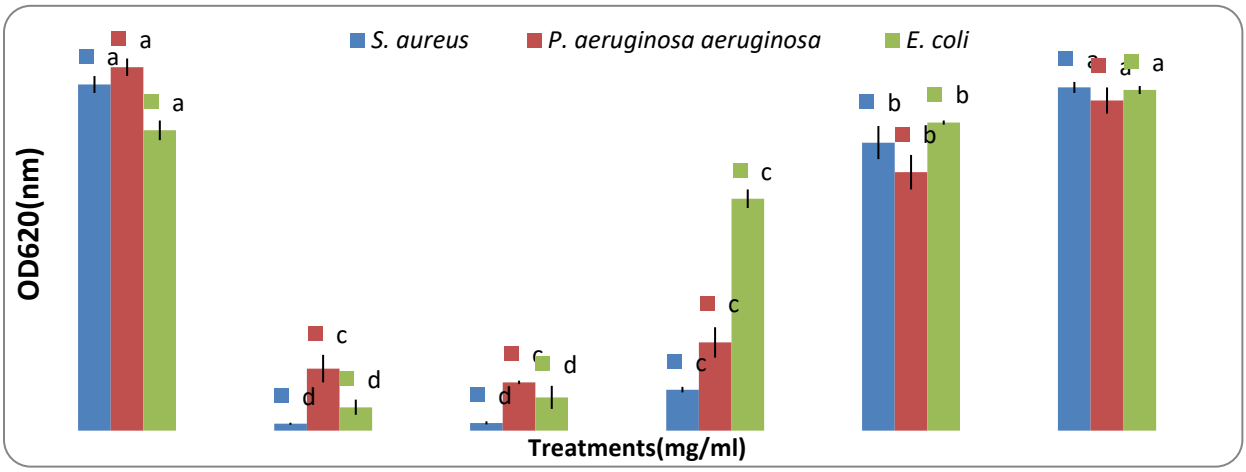
The data was statistically analyzed using SPSS version 16.0 statistical software and using analysis of variance (ANOVA). Duncan's multiple range test measured specific differences between pairs of means after analysis of the variance. The results were described as mean ± standard error of the mean, and significance was accepted for a P value < 0.05.

3. Results and Discussion

3.1. MIC assay of maggot *L. sericata* ES

To identify the minimum effective concentration of maggot *L. sericata* ES, concentrations of 0, 5, 10, 20, and 40 mg mL^{-1} of ES on *S. aureus*, *P. aeruginosa*, and *E. coli* were investigated. Results showed that 40 mg mL^{-1} of ES significantly inhibited the growth of bacteria under study compared to other concentrations ($P<0.05$), and there was no significant difference compared with penicillin (10 mg mL^{-1}). However, no significant differences were observed between 40 mg mL^{-1} and 20 mg mL^{-1} of ES on *P. aeruginosa* (Fig. 1).

Figure 1: Minimum Inhibitory Concentration of L. sericata larvae ES on S. aureus, P. aeruginosa, and E. coli.

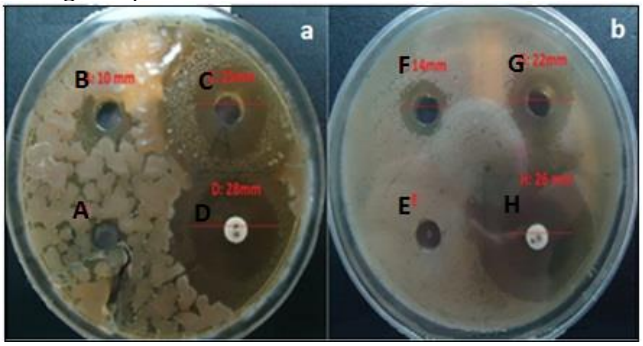


Note. *Different Levels for each of Bacteria Represented Significant Differences at p<0.05*

3.2. The effect of maggot *L. sericata* ES on infected bovine hooves and wound samples

The samples collected from infected cow hooves and wounds produced *S. aureus* and P. *aeruginosa*, which were isolated and characterized based on the study's diagnostic tests. Results indicated that 40 mg mL^{-1} of ES significantly controlled the bacterial culture from infected hooves and wound samples compared to 10 and 20 mg mL^{-1} of ES (23.5±1.5 mm vs. 0±0 mm and 12±2 mm, respectively) ($P<0.05$). Also, no statistically significant difference was observed compared to the control (23.5±1.5 mm vs. 27±1 mm, respectively) (Fig. 2).

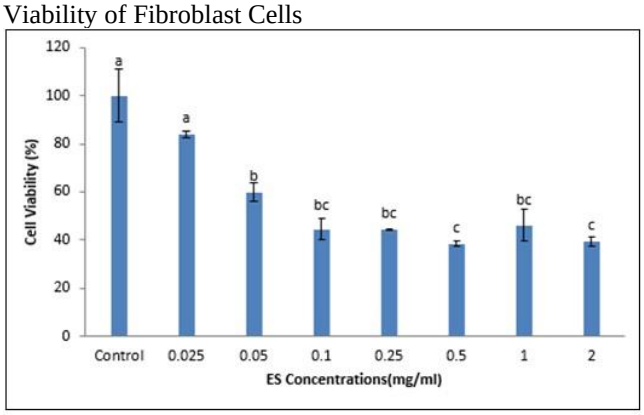
Figure 2: *Zone of Inhibition Assay for Bacterial Culture from Infected Hooves and Wound* Samples, a and b figures, respectively; A and E: 10 mg mL^{-1} larvae ES, B and F: 20 mg mL^{-1} larvae ES, C and G: 40 mg mL^{-1} larvae ES, D and H: 10 mg ml^{-1} penicillin.



3.3. The effect of maggot *L. sericata* ES on the viability of fibroblast cells

The results of the MTT assay using different concentrations of maggot *L. sericata* ES on bovine fibroblast cells showed that 0.025, 0.05, and 0.1 mg mL^{-1} of ES did not affect the viability of fibroblast cells ($P>0.05$). However, ≥0.25 mg mL^{-1} of ES significantly declined the viability of fibroblast cells compared to the control group ($P<0.05$) (Fig. 3).

Figure 3: The Effect of Maggot *L. sericata* ES on the Viability of Fibroblast Cells



Note. *Control: medium with fibroblast cells without ES.*

L. sericata larvae are utilized for the development of biological treatment, and every year, they gain many supporters among clinicians (Bazaliński et al. 2019). Larva therapy is especially useful for treating infections induced by Gram-positive bacteria (e.g., *S. aureus*) (Zare et al. 2021). In agreement with other studies on a wide range of gram-positive and negative bacteria (Andersen et al. 2006, Daeschlein et al. 2007), this study confirmed the bactericidal activity of *L. sericata* ES on *S. aureus* and *P. aeruginosa* and that *E. coli* and *S. aureus* was more sensitive than *P. aeruginosa* and *E. coli*. Reports show that maggot therapy is more useful and efficient in the case of wounds caused by Gram-positive bacteria, such as *S. aureus*, compared to wounds induced by Gram-negative bacteria, including *P. aeruginosa* and that wounds caused by Gram-negative bacteria require more larvae (van der Plas et al. 2008). The usefulness of the larva is due to its capacity to decrease pro-inflammatory factors (Bazaliński et al. 2019).

Amino acids have been recognized in larval secretions, including 3-guanidinopropionic acid, L-histidine, and L-valinol. These isolated components have been shown to specifically increase the rise of human endothelial cells *in vitro* (Bazaliński et al. 2019). Kruglikova showed that hemolymph antimicrobial compounds and exocrine secretions are the two main protective systems released into the environment by larval feeding (Kruglikova 2011). Although compounds of the maggot larval excretions have antibacterial effects, the maggot quality and physical-chemical situations *in vivo* conditions have also been cited as influential elements (Zare et al. 2021).

Maggot movements, tissue scraping, and arginase secretion reduce the debridement process. Some released enzymes are collagenase, leucine aminopeptidase, and chymotrypsin-like proteases. The mentioned enzymes have different roles in the wound. Enzymes often liquefy dead tissue or are consumed by maggots. Researchers in several recent publications have pointed to chemicals that improve the healing process. The major step in wound healing is the migration of epidermal keratinocytes and local skin cells from the wound margin to the wound bed (Bazaliński et al. 2019). Although maggot therapy has been successfully used in dogs, cats, rabbits, donkeys (Choudhary et al. 2016), buffalo (Iversen 1996), bulls (Dickb 1953), and horses (Morrison 2005), this treatment method has problems such as the time required for the larvae to arrive after ordering (Jones & Wall 2008). While maggot therapy is based on three mechanisms (automatic removal of necrotic tissue, bactericidal and bacteriostatic activity, and improvement of the therapeutic process) (Bazaliński et al. 2019), Prete (1997) showed that natural extracts emanating from *Phaenicia sericata* hemolymph and alimentary secretions caused wound recovery and that maggot extract increases the total number of human fibroblasts.

William Baer reported the treatment of osteomyelitis with maggots during World War I, and maggot therapy was extensively practiced until World War II. In the 1940s, the availability of penicillin and other antimicrobial drugs significantly reduced the use of maggot therapy (Baer 1931, Romeyke 2021). Our results reveal that the bacterial culture from infected hooves and

wounds was controlled using ES treatment, and no significant difference was observed compared with penicillin (10 mg mL^{-1}). Antibiotic resistance also brings negative aspects regarding time and economic cost due to longer treatment periods and high treatment costs. The larvae usage reduces or eliminates the cost of treatment, hospitalization, and antibiotics usage. This finding introduced *L. sericata* ES as an innovative therapy to control bovine chronic wound bacteria. However, it should be noted that the viability of fibroblast cells was negatively affected by a higher concentration of *L. sericata* ES. Consistent with our results, Horobin et al. used ES in a dose-dependent manner to alter fibroblast migration and showed that it led to altered cell morphology and inhibited cell migration (2006).

Fibroblasts play a critical role in the formation of granulation tissue. They make cytokines and extracellular matrix elements, penetrate the fibrin clot, and proliferate to direct cell migration into the wound area (Polakovicova et al. 2015). However, the matrix is perhaps degraded by proteolytic enzymes derived from inflammatory cells (macrophages and neutrophils). Residual fibrin deposits (slough) inhibit cellular responses. Molecules inside larval secretions (such as chymotrypsin) with sericase affect the fibrinolytic system and may lead to chronic wound healing by removing slough (Cazander et al. 2013). The production of recombinant enzymes from *L. sericata* larvae is very effective as a new method in chronic wound treatment due to the lack of pathogenicity. Considering the application of collagenase in medical science, Alipour et al. (2019) cloned the *L. sericata* collagenase (MMP-1) gene in an insect cell line to develop a manner to express and purify *L. sericata* collagenase (MMP-1). Zare et al. (2021) demonstrated that maggot secretion/secreta is a rather functional therapy for testing keratitis induced by *S. aureus*.

Moreover, Polakovicova et al. showed a beneficial impact of salivary gland extract from *L. sericata* larvae on the proliferation of human fibroblasts in collagen hyaluronan membrane in laboratory conditions (2015). Other studies have shown that larval secretions enhance cell motility without any mitogenic effect (Smith et al. 2006). Thus, *in vivo* studies evaluating a two-step therapy for chronic wounds to rule out bacterial infections and fibroblast activation using different concentrations of *L. sericata* ES is suggested.

4. Conclusion

In conclusion, our results reveal that maggot *L. sericata* ES was able to control bacterial culture from infected bovine hooves and wounds. 40 mg mL^{-1} of ES was suitable as an anti-infection reagent and sanitiser. However, an increased concentration of ES had a significant negative impact on the viability of fibroblast cells, so it is recommended that more than 0.25 mg mL^{-1} of maggot *L. sericata* ES should not be used directly on the skin, live cells, or tissues.

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Authors' Contributions

Asghar Shariatinia: Taking samples, laboratory work, data collection; Mohammad Zandi: Supervisor, Conceptualization, Formal Analysis, Methodology, Writing – review & editing; Mohammad Reza Sanjabi: Supervisor, Validation; Annahita Ghaedrahmati: Writing – review & editing.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

The study procedures complied with the ethical guidelines of the Declaration of Helsinki, 2013. Moreover, this study was approved by the Ethics Committee of the Karaj Branch, Islamic Azad University (Code: IR.IAU.K.REC.1396.88). All study participants were ensured that their data would be confidentially managed and solely used for the present study.

Conflict of interest

The authors declare that they have no competing interests

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Not applicable

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