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REVIEW ARTICLE

# The effect of *Lucilia sericata* larval excretion/secretion (ES) products on cellular responses in wound healing

U. GAZI[1], A. TAYLAN-OZKAN[1,2] and K. Y. MUMCUOGLU[3]

[1]Department of Medical Microbiology and Clinical Microbiology, Faculty of Medicine, Near East University, Nicosia, Cyprus, [2]Department of Medical Microbiology, Faculty of Medicine, Hitit University, Corum, Turkey and [3]Parasitology Unit, Department of Microbiology and Molecular Genetics, The Kuvin Center for the Study of Infectious and Tropical Diseases, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

**Abstract.** Chronic wounds are still regarded as a serious public health concern, which are on the increase mainly due to the changes in life styles and aging of the human population. There are different types of chronic wounds, each of which requires slightly different treatment strategies. Nevertheless, wound bed preparation is included in treatment of all types of chronic wounds and involves tissue debridement, inflammation, and infection control, as well as moisture balance and epithelial edge advancement. Maggot therapy (MT) is a form of biological debridement which involves the application of live medical grade *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) larvae. Whereas it was initially thought to act mainly through debridement, today MT is known to influence all four overlapping physiological phases of wound repair: homeostasis, inflammation, proliferation, and remodelling/maturing. During MT, medical-grade larvae are applied either freely or enclosed in tea-bag like devices (biobag) inside the wounds, which suggests that larva excretion/secretion (ES) products can facilitate the healing processes directly without the need of direct contact with the larvae. This review summarizes the relevant literature on ES-mediated effects on the cellular responses involved in wound healing.

**Key words.** Chronic wound, excretion, healing, *Lucilia cuprina*, *Lucilia sericata*, maggot therapy, secretion.

## Introduction

The genus *Lucilia* belongs to the family Calliphoridae (blow flies) in the order Diptera, which is one of the main groups in the class of Insecta. The genus is a small group of blow flies which are also known as green bottle flies due to their shiny green appearance. It contains carrion-breeders as well as obligate and facultative ectoparasites, among which the latter group includes as the most notable members, the Australian blow fly, *Lucilia cuprina* (Wiedemann, 1830) and the common green bottle fly, *Lucilia sericata* the primary myiasis producers of sheep (Owings & Picard, 2018). Representatives of the genus *Lucilia*, are one of the most abundant insect scavengers on carrion and can arrive within few minutes after death (Joseph *et al.*, 2011; Martin & Verheggen, 2018).

Under natural conditions, green bottle flies lay eggs on decaying organic material such as animal/human corpses and wounds, which then hatch to produce larva, called maggots, giving rise to myiasis on living tissues (Sunny *et al.*, 2016). *Lucilia* spp. larvae primarily feed on necrotized organic material and pass through three stages or instars before reaching the full size. At this point, they stop feeding and usually move off the host to pupariate, before metamorphosis and emergence as adult flies (Joseph *et al.*, 2011). This necrophagous feeding behaviour of *Lucilia* spp. larvae has enabled their use in forensic investigations e.g., determination of the time of death, as well as in the treatment of necrotized, chronic wounds (Amendt *et al.*, 2007; Nigam *et al.*, 2010).

Non-healing wounds still pose a significant threat to public health. In a recent study, the prevalence of chronic wounds of

Correspondence: Assoc. Prof. Dr. Umut Gazi, Department of Medical Microbiology and Clinical Microbiology, Faculty of Medicine, Near East University, Near East Boulevard, Nicosia, Cyprus. Tel.: + (90) 392 675 10 00/3037; Fax: + (90) 392 675 10 10; E-mail: umut.gazi@neu.edu.tr

mixed aetiology and leg ulcers was estimated to be 2.2 and 1.5 per 1000 population, respectively, especially in developed countries (Martinengo *et al.*, 2019). The prevalence has been increasing worldwide due to modern life-style changes that are associated with increased incidences of cardiovascular diseases, obesity and diabetes, and is thought to further increase in the future as people over 65 years of age are considered to be the fastest growing age group in the world (Sen *et al.*, 2009; Järbrink *et al.*, 2017; World Population Prospects, 2019; Stadler, 2020). Chronic wound treatments also impose a serious economic burden on health-care systems and were estimated to correspond to 2–4% of total health-care expenditure in Europe (Probst *et al.*, 2014).

Chronic wounds require several years, or even decades, to heal and patients often suffer from psychological problems such as lack of social contact, depression, and low will power (Herber *et al.*, 2007). This creates physical as well as emotional stress not only in the patients but also in their families (Järbrink *et al.*, 2017). In cases of lack of access to specialized wound care or failure of all intervention strategies, chronic wounds may also lead to amputation, after which the mortality rate can be up to 50% in the first 3 years and 70% in the first 5 years following amputation (Sen, 2019).

Wound healing can be divided into four overlapping physiological phases of repair: Haemostasis, inflammation, proliferation, and remodelling phases (Koh & DiPietro, 2011; Cazander *et al.*, 2013; Parker *et al.*, 2013; Sinno & Prakash, 2013; Kumar *et al.*, 2015; Thiruvoth *et al.*, 2015; Landén *et al.*, 2016; Nigam & Morgan, 2016; Young *et al.*, 2016; Werner & Grose, 2017; Larouche *et al.*, 2018). These four phases usually progress smoothly, however, in some cases healing may be trapped in one of these phases, usually the inflammatory phase, leading to exacerbated scarring and neoplastic progression (Zhao *et al.*, 2016).

Although chronic wound treatment strategies may slightly differ depending on the wound type such as venous ulcers, diabetic ulcers, and pressure ulcers, wound bed preparation (WBP) plays an essential role in all cases (Frykberg & Banks, 2015; Harries *et al.*, 2016). WBP involves management of a wound to maximize its potential to heal naturally or by alternative methods such as skin grafting, and is composed of four components which are summarized by the TIME acronym (Tissue debridement; inflammation and infection control; moisture balance; and epithelial edge advancement) (Demidova-Rice *et al.*, 2012; Pritchard *et al.*, 2016).

Regular tissue debridement is the main component, as it can lower the risk of infection and reduce level of inflammatory response to maintain a healthy wound bed in most chronic wounds (Wolcott *et al.*, 2009; Leaper *et al.*, 2012; Han & Ceilley, 2017). Today, there are five main types of tissue debridement available, i.e., surgical, mechanical, autolytic, enzymatic, and biological methods (Demidova-Rice *et al.*, 2012).

Maggot therapy (MT), also known as Maggot Debridement Therapy, is a biological debridement modality and involves the application of live fly larvae on the wound (Mumcuoglu, 2001; Pritchard *et al.*, 2016; Naik & Harding, 2017). Whereas the wound healing effects induced by MT were initially thought to be mediated by debridement, it is known today that application of live larvae is also associated with anti-microbial activity; growth stimulation, granulation initiation and extracellular matrix (ECM) remodelling; and decreasing the levels of pro-inflammatory immune response (Sherman, 2014; Yan *et al.*, 2018).

MT was shown to facilitate tissue repair and wound healing in several studies where the success rate ranged between 70% to 80% (Sherman, 2014; Choudhary *et al.*, 2016; Gazi *et al.*, 2019). Patients who received MT also had reduced hospitalisation days and amputation rate (Sherman *et al.*, 2007; Paul *et al.*, 2009; Davydov, 2011; Naik & Harding, 2017). Additionally, MT was also used in veterinary medicine, albeit less frequently than in human medicine. It was used in the treatment of horses and small animals, less often in pets, while overall, the outcomes were positive (Jones & Wall, 2008; Lepage *et al.*, 2012; Choudhary *et al.*, 2016). The importance of MT in human and veterinary medicine is thought to be further elevated in the near future, due to the increased global prevalence of antibiotic resistant bacteria, and demand for organic husbandry and residue-free meat and milk (Jones & Wall, 2008; Yan *et al.*, 2018).

Usually, disinfected larvae of *L. sericata*, rather than *L. cuprina* are used in MT since the former is considered to be safer to use, despite recent studies demonstrating safety and effectiveness of MT with *L. cuprina* (Paul *et al.*, 2009; Tantawi *et al.*, 2010). Medical-grade larvae are applied either freely ('free-range' technique) or contained within a bag ('contained' technique), which is preceded by surrounding of the wound by a hydrocolloid pad and covering of the top with a netting. Larvae are applied inside the wounds before the netting is closed hermetically. Gauze is then applied on top of the net to allow absorption of draining fluids from the wound (Steenvoorde *et al.*, 2005; Davydov, 2011). In the United States, *L. sericata* larvae are regarded as a prescription-only, single-use medical device, whereas they are regulated as a drug in some other countries, including in Europe.

Even though MT was shown to facilitate healing of chronic wounds by various clinical studies (Sherman, 2014; Choudhary *et al.*, 2016; Gazi *et al.*, 2019), its use under clinical conditions has some limitations. Factors such as patient and clinician anxiety of using live larvae, especially due to the possibility of larvae escaping the wounds, as well as irritation, pain, itching, and risk of hypersensitivity during treatment, were suggested to hinder its use (Collier, 2010; Mumcuoglu *et al.*, 2012; Choudhary *et al.*, 2016). The discomfort and feeling of pain may lead to removal of the larva dressing from the wound, which is the most commonly reported reason for failure of MT in human and veterinary medicine (Sherman, 2002; Jones & Wall, 2008; Mumcuoglu *et al.*, 2012). Phantom pain, possibly through nerve nourishment and nerve regeneration, has also been suggested as an adverse effect of MT by a recent case study (Lipiński *et al.*, 2020).

Furthermore, in a case report that involved treatment of critical limb ischaemia, iatrogenic pressure ulceration has been reported due to increased skin pressure that was developed every time the ankle was flexed during physical activity of the patient during the treatment and therefore, placement of dressing in the joint motion should be avoided (Nishijima *et al.*, 2017). Treatment with live larvae is also not recommended for treatment of wounds which are located in the body openings and in those with connection to vital organs (Sherman, 2009).

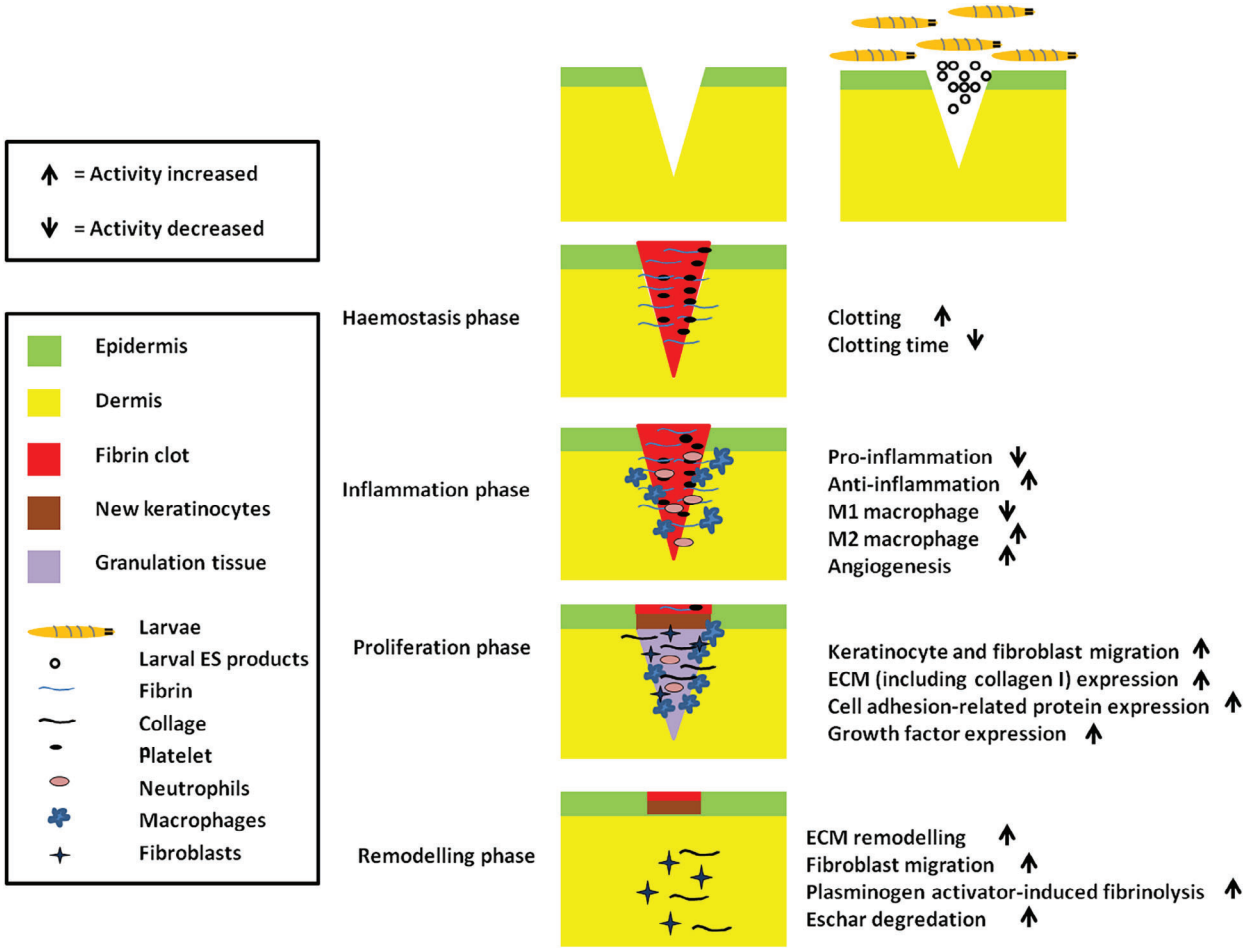


**Fig. 1.** The effect of larval ES products on wound healing stages.

During treatment, the larvae must also be protected from physical pressure, which could be difficult to achieve especially if the wound is under the sole of the foot or when it is applied on animals. The other practical disadvantage of MT is the requirement for the exchange of larvae and dressing every 1–3 days, that may be repeated for multiple times depending on the wound size and depth (Gilead *et al.*, 2012).

For these reasons, development of easy-to-use, patient-friendly, and low-risk strategies are required for the treatment of chronic wounds, including those for which MT is not recommended. One approach includes the use of larval excretion/secretion (ES) products which are known to exert direct effects on the healing reactions since both 'free-range' and 'contained' larvae are used in treatment of chronic wounds. The aim of this review is to summarize the current literature on the effect of larvae ES products on cellular responses facilitating wound healing (Fig. 1).

## Literature search methodology

The National Library of Medicine ('PubMed') and the Google Scholar databases were searched to identify articles on larval ES product-mediated responses using the terms 'maggot' or '*Lucilia sericata*', or '*Lucilia cuprina*', or 'larva', or 'therapy', or 'secretions', or 'excretions', or 'extracts', or 'wound'. Articles on larvae on species other than *L sericata* and *L. cuprina*, along with those published before 2000 were excluded from the list of articles referred.

## The effect of ES products on the haemostasis phase of wound healing

The haemostasis phase that is initiated by the exposure of collagen upon wound formation is composed of two sub-phases. During the primary sub-phase, platelets become activated by the coagulation cascade and bind to the exposed collagen at the injury site. This is then followed by the spreading and adhesion of platelets to each other to form the platelet plug. The secondary sub-phase involves the activation of blood coagulation cascade and fibrin clot formation that occur alongside primary haemostasis. However, in contrast to the primary sub-phase, the secondary phase eventually leads to the formation of insoluble fibrin rather than platelet aggregation. The fibrin mesh and platelet aggregate from a complete clot that prevents further blood loss, provides a

barrier against invading microbes, serves as matrix for invading cells and functions as a reservoir of growth factors important for the healing process (Koh & DiPietro, 2011; Parker *et al.*, 2013; Sinno & Prakash, 2013; Kumar *et al.*, 2015; Thiruvoth *et al.*, 2015; Landén *et al.*, 2016; Nandi & Brown, 2016; Young *et al.*, 2016; Werner & Grose, 2017; Larouche *et al.*, 2018).

Larval ES products were shown to be influential in both phases of haemostasis. In a study conducted by Kahl *et al.* (2015) serine proteases in larval ES were shown to induce clotting of human plasma and whole blood, without influencing platelet activation or fibrinolysis. In a second study by Pöppel *et al.* (2016) a chymotrypsin-like serine protease isolated from *L. sericata* ES was reported to possess similarities with Jonah proteases from *Drosophila melanogaster* and a chymotrypsin from *L. cuprina*. In the same study, a recombinant form of the *L. sericata* Jonah chymotrypsin reduced the clotting time of human plasma and digested the ECM components such as fibronectin, laminin, and collagen IV.

## The effect of ES products on the inflammation phase of wound healing

The haemostasis phase is followed by the inflammation phase, which involves recruitment of inflammatory cells (e.g. neutrophils and monocytes) from the circulation with the help of ingredients such as chemoattractants and vasoconstrictors released by platelets in the clot (Thiruvoth *et al.*, 2015). Although this phase is crucial in removing invading pathogens, dead cells, and debris, the released mediators during this phase are also important for proliferation of keratinocytes, fibroblasts, and epithelial cells. As new tissues develop, the environment becomes less pro-inflammatory and more anti-inflammatory which skews the monocyte differentiation away from the pro-inflammatory (M1) towards the anti-inflammatory (M2) macrophage phenotype that promotes angiogenesis (Koh & DiPietro, 2011; Parker *et al.*, 2013; Sinno & Prakash, 2013; Kumar *et al.*, 2015; Thiruvoth *et al.*, 2015; Landén *et al.*, 2016; Young *et al.*, 2016; Werner & Grose, 2017; Larouche *et al.*, 2018).

Another important immune system component involved in inflammation and wound healing is the complement system, which can be activated by three major (classical, alternative, and lectin) and two minor (properdin and thrombin) pathways (Cazander *et al.*, 2012a). Among the complement fragments that are generated as a result of complement system activation, C3a and C5a can attract neutrophils to the site of their activation, and mediate the release of inflammatory agents into the tissues through degranulation of mast cells as well as basophils. Accordingly, inappropriate activation of complement system was reported in chronic wounds and both mast cells and basophils were demonstrated to augment wound healing (Cazander *et al.*, 2012a; Sinno & Prakash, 2013).

### *The effect of ES products on immune response*

Chronic wounds are associated with excessive inflammatory reactions that exert inhibitory effects on wound healing processes and are characterized by abundant neutrophil infiltration, reactive oxygen species (ROS) production and destructive enzyme generation (Zhao *et al.*, 2016; Yan *et al.*, 2018). The pro-inflammatory environment skews the monocyte differentiation into pro-inflammatory M1 macrophages which, together with neutrophils, are associated with reduced tissue inhibitors of matrix metalloprotease (TIMPs) expression and increased levels of pro-inflammatory cytokine, and matrix metalloprotease (MMP) production. This leads to further inflammation, ECM breakdown, and preclusion of proliferation during wound healing (Zhao *et al.*, 2016).

In a study using transcriptome analysis of wound-associated keratinocytes, endothelial cells, fibroblasts, and monocytes, ES products were shown to predominantly modulate immune response pathways while also influencing other wound-healing processes such as cell migration and angiogenesis indirectly through cytokine release (Dauros Singorenko *et al.*, 2017). In further support for the immunomodulatory role of ES products, larval secretions were demonstrated to have a negative impact on the pro-inflammatory cytokines such as tumour necrosis factor [TNF]-α and interleukin [IL]-12p40 production, while increasing the expression levels of anti-inflammatory cytokine IL-10 (van der Plas *et al.*, 2009a; Téllez *et al.*, 2018). A similar anti-inflammatory effect was also observed in a study in which ES products were able to reduce the levels of lipopolysaccharide (LPS)-inducedIL-8 release by fibroblasts (Dauros Singorenko *et al.*, 2017). Reduction of pro-inflammatory cytokine levels by ES products was also reported in acute traumatic rats (Li *et al.*, 2013), Furthermore, a protein from *L. cuprina* larval secretions, named blowfly larval immunosuppressive protein, was reported to reduce mitogen-induced lymphocyte proliferation, as well as interferon (IFN)-γ, IL-4, IL-10, and IL-13 mRNA expression levels while upregulating TNF-α and tumour growth factor (TGF)-β gene expression levels (Elkington *et al.*, 2009).

ES products can also influence the intracellular killing mechanisms utilized by immune cells since crude *L. sericata* salivary gland extract was reported to suppress opsonized zymosan-stimulated blood superoxide generation and myeloperoxidase release (Pečivová *et al.*, 2008). Nevertheless, the relevant literature is inconsistent, and in a previous study by van der Plas *et al.* (2009b) secretions did not exert any influence on phagocytosis and intracellular killing of *Staphylococcus aureus* by human monocytes. In another study by the same group, ES products inhibited N-formylmethionyl-leucyl-phenylalanine (fMLP)-mediated elastase release and hydrogen peroxide production by neutrophils while not affecting phagocytosis and killing of *Candida albicans* by neutrophils (van der Plas *et al.*, 2007).

Larval secretions were able to reduce nuclear factor-kappa β (NF-κB) (p65) activity, and divert the monocyte differentiation into pro-angiogenic M2 macrophages away from the pro-inflammatory M1 type (van der Plas *et al.*, 2009a; Tombulturk *et al.*, 2018). MMP-2 and MMP-9 levels were also diminished in *L. sericata* ES product-treated diabetic wounds which was suggested to be mediated by inhibition of AP-1 and p53 expression (Tombulturk *et al.*, 2019). Larval secretions were also shown to act through modulation of NF-E2-related factor-2 to ameliorate inflammation and oxidative stress in acute experimental colitis (Wang *et al.*, 2019).

The complement system also seems to be affected since complement proteins such as C3 and C4 were shown to be broken down by larval secretions (Cazander *et al.*, 2012b). In addition, heat-sensitive serine proteases in larval ES were demonstrated to target and degrade multiple target proteins, including C3a and C5a, in all major complement activation pathways, and reduce neutrophil activation levels generated in response to C3a/C5a fragments (Tamura *et al.*, 2017). In the same study, heat and pre-treatment with serine protease inhibitor did not affect the ability of ES products to reduce C5b-9 complex formation which suggests a second complement-inhibiting molecule in ES.

Additionally, ES products can reduce the level of pro-inflammatory response indirectly by acting through their anti-microbial activities. Larval secretions have been reported to include components such as ammonium carbonate, calcium, allantoin, and urea that prevent microbial growth by increasing the pH of the environment (Yan *et al.*, 2018). The secretions were also shown to possess anti-microbial peptide activity against pathogens- including bacteria such as *Pseudomonas aeruginosa*, and *Staphylococcus aureus* and fungi such as *Candida albicans*- commonly found on wounds, as well as against biofilm formation. Interestingly, the activity was reported to be less effective against Gram-negative than Gram-positive bacteria in some studies (Yan *et al.*, 2018). Nevertheless, pre-surgical maggot debridement of wounds was shown to reduce postoperative infection rates (Sherman & Shimoda, 2004).

### *The effect of ES products on production of growth factors*

As new tissues are generated, macrophages switch to the alternatively activated M2 macrophage phenotype which is involved in the resolution of inflammation by releasing anti-inflammatory cytokines such as TGF-β. These cells are also important in the promotion of ECM synthesis, angiogenesis, and wound contraction (Larouche *et al.*, 2018).

Angiogenesis results in transition to a proliferative phase. It is mediated by proangiogenic factors such as vascular endothelial growth factor (VEGF) released by inflammatory cells and is necessary to provide oxygen and nutrients required for cellular processes involved in wound healing.

A study on the amino acid-like compounds in ES products of *L. sericata* larvae showed a proangiogenic effect mediated by three amino acids (histidine, valinol, and 3-guanitidinopropionic acid) on human endothelial cells, but not on fibroblasts (Bexfield *et al.*, 2010). Similar results were also reported by another study which demonstrated increased endothelial cell proliferation, and increased expression of VEGF receptor 2 upon ES application (Sun *et al.*, 2016). Stimulation with ES products was also demonstrated to elevate basic fibroblast growth factor and connective tissue growth factor expression in ulcers of diabetes mellitus rats (Wang *et al.*, 2008). Another growth factor influenced by the application of larval secretions is the hepatocyte growth factor (HGF) which is also an important contributor of angiogenesis (Xin *et al.*, 2001). In a study by Honda *et al.* (2011) HGF expression was induced by a positive feedback loop of HGF receptor/signal transducer and activator of transcription 3 (STAT3). Induction of STAT3 signalling was also reported by Li *et al.* (2015) which demonstrated increased levels of STAT3, TGF-β, and SMAD family member 3 upon treatment with ES products. Moreover, in another study, ES product-mediated endothelial cell migration was shown to be partially mediated by AKT, but not by ERK1/2 (Wang *et al.*, 2010).

ES products were also reported to act via induction of microRNAs (miRNAs) and increased expression levels of miR-18a/19a and miR-126, a well-known angiogenesis regulatory microRNA (miRNA), were observed in ES-stimulated human umbilical vein endothelial cells (Zhang *et al.*, 2017; Wang *et al.*, 2020). Additionally, in a previous study miR18a/19a – TSP-1 axis was held responsible for ES-mediated angiogenesis (Wang *et al.*, 2020).

*In vivo* studies also supported the role of ES-induced miRNAs expression in wound healing processes. In a study using an experimental rat model, elevated expression levels of rno-miR-99a* and rno-mir-877 were suggested as biomarkers for wound healing induced by ES products (Coskunpinar *et al.*, 2015).In another study using diabetic rats, larval secretions were suggested to increase miR146a expression to facilitate wound healing and reduce inflammatory response (Kilinc *et al.*, 2020).

## The effect of ES products on the proliferative phase of wound healing

Angiogenesis results in a transition to the proliferative phase, during which the granulation tissue made up of fibroblasts, granulocytes, macrophages, blood vessels, and collagen bundles, replaces the wound matrix formed in haemostasis. Fibroblasts have a vital role in granulation tissue formation, since apart from cytokines, chemokines, and growth factors they deposit ECM components (including collagen) and secrete proteinases involved in degradation of provisional matrix. Once sufficient matrix has been produced, fibroblasts differentiate into myofibroblasts that are involved in wound contraction (Landén *et al.*, 2016; Young *et al.*, 2016).

The proliferation phase is associated with wound re-epithelization which involves migration and proliferation of epithelia stem cells from hair follicles or sweat glands as well as keratinocytes from the basal layer of the wound area. While the cells are released from their original source by the activities of collagenases and elastases, the migration process is triggered by the loss of cell-contact inhibition leading to higher permeability for calcium, which facilitates cytoskeletal re-organization for cellular migration. Cellular migration would continue until it covers the whole wounded area and is stopped by the formation of new intercellular adhesion structures (Landén *et al.*, 2016).

Previous *in vitro* studies demonstrated enhanced keratinocyte and fibroblast migration upon stimulation with ES products (Horobin *et al.*, 2005, 2006; Smith *et al.*, 2006; Téllez *et al.*, 2018). They were also shown to stimulate fibroblast growth and induce cellular metabolism and protein production which are important for both formation of the microfibrillar net used for migration of fibroblasts and proper production of ECM

components (Polakovičova *et al.*, 2015). Furthermore, Lucilin peptide was identified as an ES product that is able to induce cellular migration of human keratinocytes (Téllez *et al.*, 2018). In an *in vivo* study that compared gene expression profile between ES-treated and control samples at the transcriptional level, there were 38 differentially expressed mRNAs many of which were responsible for the expression of ECM, cell adhesion-related proteins, and growth factors (Polat *et al.*, 2014). In another *in* vivo study using a diabetic rat wound model, enhanced level of collagen I expression was detected upon treatment with ES products (Tombulturk *et al.*, 2018).

### The effect of ES products on the remodelling phase of wound healing

During the last phase, the remodelling phase, ECM components become increasingly well-organized and undergo further change as collagen III is replaced with collagen I, which is stronger but takes longer to deposit (Landén *et al.*, 2016; Young *et al.*, 2016). This phase is also associated with a reduction in cellular content due to the apoptosis of inflammatory cells and regression of vasculature, while scar formation is the final outcome of wound repair (Thiruvoth *et al.*, 2015).

Apart from ECM remodelling, larval ES products were also suggested to facilitate tissue formation via coordination of cellular responses and induction of fibroblast migration, that involve 'chymotrypsin-like' serine proteinase activity (Chambers *et al.*, 2003; Horobin *et al.*, 2006). Chymotrypsin from larvae was shown to be resistant to endogenous wound protease inhibitors (Telford *et al.*, 2011) and was able to degrade wound eschar (Telford *et al.*, 2010; Pritchard & Brown, 2015). The larval secretions were also demonstrated to display DNAse activity, which helps wound healing by digesting DNA associated with wound eschar and with *P. aeruginosa* biofilms (Brown *et al.*, 2012). In addition, glycosidases in larval secretions also altered susceptibility of eschar proteins to proteases involved in debridement of chronic wounds (Telford *et al.*, 2012). Furthermore, larval secretions were effective in increasing the generation of plasmin and fibrinolysis via plasminogen activation, without influencing the coagulation cascade. The agent responsible for the cleavage of plasminogen was later found to be a serine protease (named Sericase), which was also reported to utilize a non-proteolytic cofactor in secretions, for plasminogen activator-induced fibrinolysis (van der Plas *et al.*, 2014).

## Conclusions

Today, chronic wounds still pose a significant burden to healthcare systems and the quality of life for those affected. The TIME principle of wound bed preparation is a systemic approach to increase the wound potential to heal by removing molecular and cellular barriers. Regular tissue debridement is regarded as the main component, as it can facilitate wound healing by removing the necrotic tissues and reducing both the infection risk and inflammatory response level.

Even though MT was initially proposed as a biological debridement method due to the necrophagous feeding behaviour of *Lucilia* spp. larvae, today it is also known to act through other components of TIME. Besides keeping the wound hydrated, larval ES products were also suggested by various studies to facilitate regulation of inflammation and infection, as well as promotion of cellular proliferation. ES products were also demonstrated to affect all phases of wound healing. Therefore, development of novel ES-based approaches could replace MT in chronic wound treatment in situations where limitations associated with the latter cannot be overcome. Nevertheless, the disadvantage of not having the beneficial effects of flushing action of wound fluids and mechanical action of using live larvae must also be considered for the success of these applications.

On the other hand, studies demonstrating increased anti-bacterial and anti-biofilm activities of ES products due to prior exposure of larvae to bacteria imply the possible contribution of pre-treatment conditions on the larval ES-mediated bio- activities (Huberman *et al.*, 2007; Jiang *et al.*, 2012). Therefore, future studies on ES products with particular attention on the conditions in which larvae are reared would further contribute to the development of ES-based approaches for the treatment of chronic wounds. Development of easy-to-use, patient-friendly and low-risk approaches can also benefit from studies using larval body or fatty acid extracts which were also reported to display similar wound healing activities as ES products (Zhang *et al.*, 2010; Bian *et al.*, 2017; Dong *et al.*, 2018; Wang *et al.*, 2018; Zong *et al.*, 2020).

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## Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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