

***Lucilia sericata* medicinal maggots: a new source of antimicrobial compounds**

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Maggot debridement therapy (MDT) is increasingly being used as a fast and effective treatment of non-healing wounds. It has been demonstrated that the application of sterile larvae to an infected non-healing wound results in the removal of necrotic tissue (debridement), disinfection, rapid elimination of infecting microorganisms and enhancement of the healing process. Many studies have provided the evidence that the antimicrobial action results from both larval ingestion of wound bacteria, which appears to kill the bacteria as they pass through the larval digestive tracts, and by antimicrobial activity of larval components, including salivary gland secretions and faecal waste products. Therefore, identification and characterisation of antibacterial compound(s) and mechanisms involved in maggot therapy represents an interesting area of research. New biologically active molecules from maggots could be therapeutically used and medicinal maggots could be replaced by these active molecules in either their native or recombinant form in future.

A number of studies have aimed to determine the nature of the antibacterial activity exhibited by maggot excretion/secretion (ES) products. Several reports have described the presence of three categories of antibacterial factors in the ES of maggots, one category with a molecular mass <0.5 kDa and the other two with molecular masses of 0.5–10 and >10 kDa, respectively. Recently lucifensin, a novel larval defensin, has been identified as one of the antibacterial agents of medicinal maggots involved in MDT. Lucifensin was shown to be constitutively produced in the salivary glands of all larval stages during feeding. An infectious environment could induce its production in the fat body from where lucifensin is secreted solely into the haemolymph. Lucifensin possesses antibacterial activity against Gram-positive bacteria, most notably *Streptococcus* spp. and *Staphylococcus* spp., but it fails to inhibit the growth of *Escherichia coli* and *Pseudomonas aeruginosa*. It also exhibited antibacterial activity against clinical isolates of methicillin-resistant *S. aureus* (MRSA) – however, the minimal inhibitory concentration of lucifensin against MRSA was significantly higher than for methicillin-sensitive *S. aureus*. Nevertheless, MDT has been successfully used in the treatment of chronic wounds associated with antibiotic-resistant bacteria, including MRSA.

Bacteria within chronic wounds often reside in biofilms that protect them from antibiotics and the immune system. Chronic wounds are commonly associated with biofilms formed by bacteria such as *S. aureus* and *P. aeruginosa*, the most clinically relevant species. Maggot ES are differentially effective against biofilms of *S. aureus* and *P. aeruginosa* indicating that different molecules within ES are responsible for the biofilm disruption. As a result of biofilm breakdown, the bacteria become susceptible to actions of antibiotics and the immune system, as well as to the actions of maggots. Combinations of maggot ES and antibiotics could ensure complete breakdown of the biofilms, thereby preventing bacterial re-growth from the remaining matrix, and promote antibiotic action against the bacteria released from the biofilms. Molecules responsible for the anti-biofilm properties of ES remain unknown.

This review summarises the latest results in the identification and characterisation of antibacterial compound(s) and mechanisms involved in MDT. Some potential candidate biologically and therapeutically active molecules are discussed.

Keywords *Lucilia sericata*; medicinal maggots; wound bacteria; wound healing; antimicrobial peptides.

1. Introduction

Chronic, non-healing wounds are a major healthcare problem worldwide. Recent estimates indicate that 1 to 2% of the population in developing countries will experience chronic skin wounds during their lifetime [1]. Treatment of chronic wounds is in general time-consuming and often unsuccessful, resulting in anatomical and functional damage. It is now accepted that the tissue of all chronic wounds is colonised by polymicrobial flora, which is a major contributing factor to delayed wound healing [2]. At present, there are two main strategies used to prevent and treat clinical infections in non-healing wounds: 1) systemic and topical antibiotics or 2) antiseptics. However, according to a recent study [3], antibiotics do not promote wound healing. This fact, together with the increasing problem of bacterial resistance to antibiotics, questions their general use for treating bacterial colonisation. There is an urgent need for introducing novel or re-emerging effective strategies, like maggot debridement therapy (MDT), in treating chronic wounds unresponsive to antibiotic therapy. Several clinical studies have shown that the application of sterile larvae of *Lucilia sericata* results in the removal of necrotic tissue (debridement), disinfection, rapid elimination of infecting microorganisms and enhancement of the healing process [4–6]. The mechanism of action probably results from both larval ingestion of wound bacteria and by antimicrobial activity of larval excretion/secretion (ES) products [7–9]. MDT has attracted great attention due to its successful application and efficacy in elimination of multi-drug resistant wound pathogens [10]. Therefore, identification and characterisation of antibacterial compound(s) and mechanisms involved in MDT represents an interesting area of research. New biologically active molecules from maggots could be therapeutically

used and, in future, medicinal maggots could be replaced by these active molecules in either their native or recombinant/synthetic form.

2. The biology of *Lucilia sericata*

L. sericata is a common blowfly found in most areas of the world, and the most well-known of the numerous green bottle fly species (Fig.1). This fly belongs to the family Caliphoridae within the order Diptera. It is coastal in its distribution and prefers warm and moist climates. The female lays her eggs in meat, fish, animal corpses, infected wounds of humans or animals, and excrement. The larvae of this insect feed on most decomposing tissue. Larvae of *L. sericata* are facultative parasites, unable to ingest the vital tissue [11]. Thanks to a well-defined life cycle, behaviour and facultative parasitism, maggots of *L. sericata* have been used widely in several fields, for example: 1) Forensic needs - due to the well-known life cycle, the stage of the insect's development on a corpse is used to calculate a minimum period of colonisation, so that it can be used to aid in determining the time of death of the victim [12]; 2) Clinical needs - the larval applications for maggot therapy [13-15]; 3) Research needs - the whole maggots' body and excretions and secretions of *L. sericata* maggots contain substances with antimicrobial and immunomodulating character that have potential as drugs for the treatment of chronic wounds [10, 16-22].



Fig. 1 Imago (left) and larva (right) of *Lucilia sericata*

L. sericata larvae are typical white pale maggots with a tapering anterior and a truncated posterior end. Anatomy of the maggot's digestive system and characterisation of the digestive process requires special attention, because digestion plays a crucial role for maggot therapy [7]. One pair of salivary glands continuously produces digestive and proteolytic enzymes that are secreted into the surroundings (Fig. 2A). In particular, there were found proteases such as collagenases, amylases, peptidases and antibacterial factor called lucifensin [20]. The mid-gut in *L. sericata* larvae consists of three parts – anterior, middle and posterior segments – with different characters (Fig. 2B). The maggots feed by dipping their front end into the liquid nutritive substrate, while breathing through their posterior respiratory apertures. During the feeding they form feeding communities and secrete digestive enzymes together. They produce a cocktail of proteolytic and antimicrobial substances called ES products of the gut as well as salivary glands origin. *In vitro* examination of ES products revealed substances including serine proteases, chymotrypsin, chymotrypsin-like and trypsin-like proteases, and the antimicrobial peptide lucifensin [19-22].

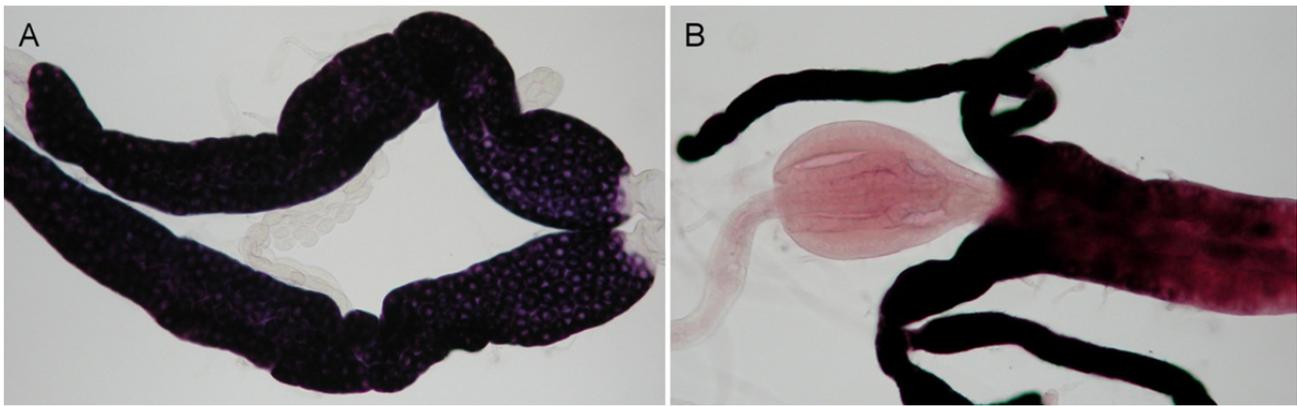


Fig. 2 Salivary gland secretions and faecal waste products form larval excretion/secretions – a cocktail of proteolytic and antimicrobial substances.

One pair of salivary glands continuously produces digestive, proteolytic enzymes and antibacterial lucifensin, which are secreted into the surroundings (A, salivary glands from third larval instar of *L. sericata* after in situ hybridisation with cDNA probe specific to lucifensin). The midgut is the source of substances like serine proteases, chymotrypsin, chymotrypsin-like and trypsin-like proteases that act inside the gut and are also excreted (B, first part of the midgut from third larval instar of *L. sericata* after in situ hybridisation with cDNA probe specific to chymotrypsin).

3. Wound-healing process

The primary function of the skin is to serve as a protective barrier against the environment. Loss of the integrity of a large area of the skin as a result of injury or illness may lead to major disability or even death [23]. Normal wound healing is a dynamic and complex process involving a series of coordinated events, including bleeding, coagulation, initiation of an acute inflammatory response to the initial injury, regeneration, migration and proliferation of connective tissue and parenchyma cells, as well as synthesis of extracellular matrix (ECM) proteins, remodelling of new parenchyma and connective tissue and collagen deposition regulated by cytokines and growth factors [24]. Four well-defined phases can be recognised during a healing process - haemostasis (coagulation), inflammation, repair (cell migration, proliferation, matrix repair, and epithelization), remodelling and maturation of the scar tissue [25]. Clinically, wounds can be categorised as either acute or chronic non-healing wounds depending upon the timescale of healing or the tendency to relapse [26]. One of the underlying mechanisms responsible for the failure of chronic wounds to heal is an out-of-control inflammatory response that is self-sustaining [27]. The abnormal presence of bacteria in wounds prolongs the wound-healing inflammatory phase. The majority of wounds are polymicrobial, involving both aerobes and anaerobes. The aerobic pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiellae*, *Proteus spp.* and β -haemolytic *Streptococci* have been most frequently cited as the cause of delayed wound healing and infection [28]. The most common anaerobes present in the chronic wounds are *Peptostreptococcus spp.*, *Serratia spp.*, *Bacteroides*, *Peptoniphilus*, *Fingoldia*, *Anaerococcus*, and *Peptostreptococcus spp.* isolates [29].

4. MDT in wound care

Maggots of the blowfly *L. sericata* have been successfully used as a debridement agent for chronic and infected wounds throughout history. There are records that indicate that larvae were allowed to infest the wounds of many ancient cultures and tribes [30-32]. Later, MDT was introduced into Western medicine following World War I by Baer and extensively used to treat osteomyelitis and gas gangrenous wounds [33]. Following the discovery of penicillin, the use of surgical maggots was abandoned [13]. However, due to the appearance of antibiotic resistance, the resurgence in the use of maggots in wounds began again recently in the late 1980s and early 1990s. Nowadays, maggots are produced aseptically and delivered by commercial companies to wound-care centres and hospitals worldwide. It is now a universally acknowledged fact that MDT can be successfully used to treat chronic, long-standing, infected wounds, which have previously failed to respond to conventional treatment. Its beneficial influence can be seen in a short period of time and the result of maggot treatment is clear (Fig.3).



Fig. 3 The successful outcome of a patient following treatment with *Lucilia sericata* larvae.

Over the last decade, MDT has been recognised by many clinicians as a potential adjunct to conventional therapy, and many patients with non-healing, chronic ulcers have been treated. Numerous case reports and case series have described the successful use of MDT in a variety of ulcers, including necrotising fasciitis, perineal gangrene, post-surgical wound infections and burns. However, comparative clinical trials and in particular randomised controlled trials investigating the efficacy of MDT are sparse, which highlights the need for more and better-designed investigations [6].

5. Antimicrobial potency of medicinal maggots

One of the confirmed beneficial effects of MDT is the removal of pathogenic bacteria. Several studies provided the evidence about antibacterial activity in ES of *L. sericata* maggots but there were some discrepancies depending on the choice of the bioassay used to examine the activity. According to a turbidimetric assay of bacterial growth, native ES showed significant antibacterial activity against both Gram-positive and Gram-negative species such as *S. aureus*, *Bacillus thuringiensis*, *E. coli*, *Enterobacter cloacae* and *P. aeruginosa* [16]. Using the standard agar diffusion assay, the successful growth inhibition of Gram-positive *S. aureus* and *Streptococcus pyogenes* isolates has been reported after treatment with ES that had been freeze dried before use, but no activity has been shown against Gram-negative *P. aeruginosa* [4]. Employing a colony-forming units bioassay, antibacterial activity against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria has been documented but the antibacterial activity of the maggot ES against *E. coli* was significantly lower compared to activity against *S. aureus* [10]. Similarly, antibacterial activity against both Gram-positive and Gram-negative bacteria including *S. aureus* and *E. coli* has further been documented but whole-body extracts and the haemolymph were used instead of ES [34]. Although it is still not clear if ES are significantly active against Gram-negative bacteria, MDT has been successfully used in the treatment of chronic wounds associated with such bacteria species [10].

Many studies have focused on the determination of antimicrobial compounds from maggots of the blowfly *L. sericata* to elucidate the positive effects of MDT in humans [4, 9, 16-18, 20, 35, 36, 46]. Several reports have described the presence of three categories of antibacterial factors in the ES products of maggots, one category with a molecular mass <0.5 kDa and the other two with molecular masses of 0.5–10 and >10 kDa, respectively [9, 16, 17, 35].

It has been demonstrated that ES are able to lyse the cells of *Micrococcus lysodeikticus*. This lytic activity was different from lysozyme-like activity and was retained in a greater than 10 kDa fraction [16]. It has been also suggested that maggot ES exhibit a bacteriostatic effect. Attention has been also paid on the morphology changes of bacteria treated with <500 Da fraction of ES. Both *B. cereus* and *E. coli* showed morphological changes such as forming filamentation, which causes an increase in the length, and other morphological changes coming to the lemon and the racket shape formation indicating a swelling of the cell [9].

Further studies have focused on the lower molecular weight (MW) fractions supposed to be present in the samples less than 1 kDa or even less than 500 Da. The whole-body extracts and haemolymph fractions from non-sterile maggots lysed Gram-positive and Gram-negative bacteria including *P. aeruginosa*, *Klebsiella pneumoniae* and MRSA isolated from wounds. The fraction with a MW of less than 1 kDa lysed over 90% of the bacteria within 15 minutes by causing an influx of K^+ and changing the membrane potential of bacteria [34]. Low MW compounds from the maggots whole-body extract or haemolymph extracts of *L. sericata* were identified as p-hydroxybenzoic acid (138 Da), p-hydroxyphenylacetic acid (152 Da) and octahydro-dipyrrolo[1,2-a;1',2'-d] pyrazine-5,10-dione (194 Da), also known as the cyclic dimer of proline (or proline diketopiperazine or cyclo[Pro,Pro]) [17]. From the biotechnological and clinical points of view, the molecules with low MW are more interesting due to their easier production, thermal and chemical stability and penetration properties. Novel antibacterial molecules with low MW have been identified in sterile maggot ES [36]. To fractionate less than 500 Da size fractions of sterile ES, C30 HPLC columns have been used. The fractions were assayed against clinically relevant species including MRSA, *Staphylococcus spp.*, *Bacillus spp.*, *E. coli*,

Pseudomonas spp., *Proteus spp.*, *Enterococcus spp.*, *Serratia spp.*, *Candida spp.* and *Enterobacter spp.* The active fractions were pooled and concentrated. Accurate mass analysis indicated that the active antibacterial agent has an empirical formula of $C_{10}H_{16}N_6O_9$. This formula has been registered as the antibiotic named Seraticin [36].

Later, it was reported that lucifensin, a novel larval defensin found in ES products, gut, salivary glands, fat body and haemolymph, is the larger MW antibacterial agent of medicinal maggots involved in MDT [20]. Lucifensin (411 Da) consists of 40 amino-acid residues and three intramolecular disulphide bridges and is very similar to dipteran defensins. Insect defensins are small (~5 kDa), basic, cysteine-rich antibacterial peptides with efficacy against Gram-positive bacteria whereas Gram-negative bacteria, yeast and filamentous fungi are less sensitive to insect defensins [37-39]. Most defensins almost immediately kill bacterial cells by permeabilisation of their cytoplasmic membrane [40-42]. It has been documented that the recombinant form of lucifensin exhibits antibacterial activity against *Streptococcus spp.*, *S. epidermidis* and *En. faecalis*, but it failed to inhibit the growth of *E. coli* and *P. aeruginosa* at a concentration below 128 mg/l [18]. Using *in situ* hybridisation, expression of lucifensin was confirmed in the salivary glands of all larval stages. Expression was occasionally detected in a few cells of the fat body and in the grease coupler of the salivary glands. Surprisingly, no expression of lucifensin was detected in the gut although lucifensin was originally purified from this tissue. This could mean that, after secretion from salivary glands into the environment, lucifensin is ingested by maggots along with food and passes through the digestive tract [43].

The antibacterial activity often results from a constitutive expression of antibacterial factors (produced at a constant level) or from an inductive expression of antibacterial factors upon bacterial stimulation [36]. Nearly a century ago, it was reported that non-sterile maggots produced more antibacterial active ES than sterile maggots [44, 45]. This finding was later confirmed by comparing bioactivity of sterile maggots and maggots that were removed from chronic wounds. The whole-body extract and haemolymph extract of chronic wound maggots were three- to six-fold more active than sterile ones. The mechanically induced antimicrobial activity in maggots was also investigated. Injuring the sterile maggots with a needle containing bacteria resulted in an increase of antimicrobial haemolymph-related activity [34]. Similarly, septic-injured larvae were used to study genes that are differentially expressed in response to the infection. Suppression subtractive hybridisation methodology resulted in the identification of 65 novel *L. sericata* genes including potential signalling proteins (e.g. inhibitor of apoptosis 2 protein) and a number of digestive enzymes including lipases and proteinases. Additionally, numerous putative antimicrobial peptides (AMPs), such as a potential *Lucilia* defensin, dipterin and three novel proline-rich AMPs have been found [46]. Since lucifensin was identified as one of the antibacterial peptides in ES products, it has been suggested that wound pathogens could induce its expression and secretion. However, it has been shown that an infectious environment could increase the production of lucifensin only in the fat body of *L. sericata* larvae. Lucifensin is secreted solely from this tissue into the haemolymph (similar to other insect defensins) and not into ES products. The production and antimicrobial activity of lucifensin in ES products is not affected by pathogens from the environment during wound healing [43]. These conclusions are supported by a recent report where neither the sterility of the larvae nor the larval environment influenced the antibacterial potency of ES products [47]. It is vital to distinguish between pure ES product and different whole-body or haemolymph extracts containing also fatbody products. Synthesis of antimicrobial peptides in the fat body (a functional equivalent of the mammalian liver) and their rapid release into the haemolymph is an important – and the best characterised – aspect of the insect immune response. In *Drosophila*, seven antimicrobial peptides with different activity spectra are synthesised by the fat body during the immune response [48]. These peptides serve as part of the immune system of the animal and are active within the body and not released into the outer environment.

Table 1 summarises all identified or predicted antimicrobial agents in *L. sericata* maggots that are/could be involved in MDT.

Table 1 A list of previously identified antimicrobial agents in *L. sericata* maggots.

Name	MW	Source	Active against	Reference
p-hydroxybenzoic acid	138 Da	whole body extract	<i>M. luteus</i> , <i>P. aeruginosa</i>	[17]
p-hydroxyphenylacetic acid	152 Da	whole body extract	<i>M. luteus</i> , <i>P. aeruginosa</i>	[17]
octahydro-dipyrrolo[1,2-a;1',2'-d] pyrazine-5,10-dione	194 Da	whole body extract	<i>M. luteus</i> , <i>P. aeruginosa</i>	[17]
$C_{10}H_{16}N_6O_9$ - Seraticin	<500 Da	ES	MRSA, <i>Staphylococcus spp.</i> , <i>Bacillus spp.</i> , <i>E. coli</i> , <i>Pseudomonas spp.</i> , <i>Proteus spp.</i> , <i>Enterococcus spp.</i> , <i>Serratia spp.</i> , <i>Candida spp.</i> , <i>Enterobacter spp.</i>	[36]
Lucifensin	411 Da	gut, salivary glands, fat body, haemolymph, ES	<i>Streptococcus spp.</i> , <i>Staphylococcus spp.</i> , <i>En. faecalis</i> , MRSA	[20]
Putative AMP (Ls-AMP1) FG360477	279 Da	subtracted cDNA library of whole body maggots	not tested	[46]

Putative AMP (Ls-AMP2) FG360493	394 Da	subtracted cDNA library of whole body maggots	not tested	[46]
Putative AMP (Ls-AMP3) FG360534	432 Da	subtracted cDNA library of whole body maggots	not tested	[46]
Lys-rich lysozyme 2 FG360533	1389 Da	subtracted cDNA library of whole body maggots	not tested	[46]
Putative lysozyme 1 ZY200249	1374 Da	salivary gland/crop extract	not tested	[18]
Putative lysozyme 1 ZY200358	1384 Da	salivary gland/crop extract	not tested	[18]

6. Anti-biofilm potency of medicinal maggots

Bacteria within chronic wounds often reside in biofilms that protect them from antibiotics and the immune system, which slows the healing process. The bacterial biofilm is an aggregated community of stable microbial cells, which adhere to each other and/or to a surface, and are embedded in a complex self-produced mixture of extracellular polymers. Chronic wounds are commonly associated with biofilms formed by bacteria such as *S. aureus* and *P. aeruginosa*, the most clinically relevant species [49]. Application of larval therapy leads to the successful disruption of already established biofilms as well as to the inhibition of their formation. There are a number of recent studies that attempt to elucidate the effect of maggot ES on the ability of bacteria to form biofilm communities, and the potential use of ES as an agent to disrupt existing bacterial biofilms [49-53].

Maggot ES are differentially effective against biofilms of *S. aureus* and *P. aeruginosa* indicating that different molecules within ES are responsible for the observed effects [49]. Mature biofilms of *S. aureus* and *P. aeruginosa* were treated with ES from sterile maggots. It was shown that the potential anti-biofilm effect largely depended on the bacterial species present in the biofilm. A sufficient amount of ES material to prevent the formation of biofilm in *S. aureus* was 0.2 mg, while the active concentration of sterile maggot ES able to disrupt established biofilms in *S. aureus* was 2 mg. In contrast, sterile maggot ES initially promoted *P. aeruginosa* biofilm formation, but after 10 h the biofilms collapsed. Degradation of *P. aeruginosa* biofilms started after 10 h and required 20 mg of ES. Boiling of ES abrogated their effects on *S. aureus*, but not on *P. aeruginosa* biofilms, indicating that different molecules within ES are responsible for the observed effects. Modulation of biofilms by ES did not involve bacterial killing or effects on quorum sensing systems [49].

The anti-biofilm activity of ES products against *P. aeruginosa* of sterile maggots was examined in the first and in the third instar. It was found that both kinds of ES were able to prevent biofilm formation and degrade existing biofilms of *P. aeruginosa*, with more effective secretions being produced by third instar maggots than first instar maggots. In addition, the physiochemical properties of the anti-biofilm components of maggot ES have been characterised and ES bioactivity were found to be stable at room temperature [50]. In contrast, another study has shown that ES activity against *S. epidermidis* biofilm formation was temperature and time dependent, inactivated by heat treatment, and disruption depended on the mechanism of intercellular adhesion. The molecule(s) responsible for this effect was >10 kDa in size and appeared to have protease or glucosaminidase activity [51].

Later, the effects of sterile maggot ES on biofilm production upon the surfaces that are generally used in a medical practice was investigated. This study examined biofilms of various bacterial species like *S. aureus*, *S. epidermidis*, *Klebsiella oxytoca*, *E. faecalis* and *E. cloacae* on polyethylene, titanium and stainless steel. The quantities of biofilm formation between the bacterial species on the various biomaterials were compared. Maggot ES were added to established biofilms. The strongest biofilms were formed by *S. aureus* and *S. epidermidis* on polyethylene and the weakest on titanium. The presence of ES reduced biofilm formation on all biomaterials by up to 92% [52].

As a result of biofilm breakdown, the bacteria become susceptible to actions of antibiotics and the immune system as well as to actions of maggots. Combinations of maggot ES and antibiotics could ensure complete breakdown of the biofilms, thereby preventing bacterial re-growth from the remaining matrix, and promote antibiotic action against the bacteria released from the biofilm [53]. Despite several studies, the exact structure of molecules responsible for the anti-biofilm properties of ES remains unknown.

7. Protease activity of medicinal maggots

An important part of larval therapy is removal of necrotic tissue from the wound [54, 55]. This helps reduce the number of microbes, toxins and other substances that increase the risk of infection and prolong wound healing [56]. MDT provides a removal of debris with faster healing rates compared with other conventional debridement treatments [57, 58]. Exudates, wound odour and pain scores were all significantly decreased after MDT and necrotic tissue was reduced by 33% in 4 weeks compared to dressings alone [59]. Various mechanisms by which maggots function in wounds have

been suggested, including liquefaction of necrotic tissues by proteases, digestion of necrotic tissue, change in wound pH from acid to a beneficial alkaline pH by the excretion of ammonia and the irrigation of bacteria from the wound [7].

The success of MDT has been divided in two beneficial effects: the disturbance of the wound and the secretion of proteases by the larvae [60]. Debridement is believed to be provided by wound disturbance caused as the maggots crawl around the wound bed using their chitinised mouth hooks [54]. The mouth hooks disrupt the membranes and allow the penetration of enzymes present in maggot ES [61]. Larvae in wounds secrete a mixture of proteolytic enzymes that cause disruption of necrotic tissue into a slurry form, which serves as larval food source [19]. The proteolytic digestion of wound ECM components is the initial act of tissue repair, leading to haemostasis, thrombosis, inflammatory cell activation and finally tissue reconstruction [62].

Three groups of proteolytic enzymes were identified in the larval ES products. Serine proteases with two different subgroups (trypsin-like and chymotrypsin-like proteases) and aspartyl proteases and metalloproteinases [19]. Maggot ES was found to be able to solubilise fibrin clots and degrade the ECM components like fibronectin, laminin, and acid-solubilised collagen types I and III by the activity of the chymotrypsin-like serine protease. This is thought to be involved in the remodelling of the ECM components by the expression of protease-activated receptors, which become active in the presence of serine proteases [19, 36].

Recently a recombinant chymotrypsin I isolated from *L. sericata* larvae has been prepared. The recombinant chymotrypsin cleaved the majority of proteins from slough/eschar from venous leg ulcers. It represents the first protease in the larvae of *L. sericata* to be identified and characterised [21]. The current studies show that more proteases whose precise functions are still unknown are present in ES products.

8. Conclusions

The mechanisms underlying the healing properties of maggot therapy have been examined since the initiation of maggot therapy as a medical treatment option. The molecular and cellular mechanisms of the maggot healing effect are still not entirely elucidated, as well as substances produced by maggots that are responsible for the stimulation of the healing process of chronic wounds. The mechanism of action probably results from both larval ingestion of wound bacteria and from antimicrobial activity of larval ES products [7-9]. However, there is also question of whether any bioactivity identified in maggots originates from the maggots themselves rather than from any infective or symbiotic bacteria present in the maggot body [36]. The eradication of pathogens in chronic wounds during larval therapy may be also associated with the activity of skin cells itself, particularly keratinocytes, which, when activated, secrete antimicrobial peptides into the environment. Several mechanisms, including induction of fibroblast migration by ES of maggots, have been proposed [64]. A recent report described the increase of hepatocyte growth factor (HGF) in blood samples taken from the femoral vein of the affected limb during one session of MDT. HGF is known as an important factor that is involved in cutaneous wound healing [65]. It was considered that formation of healthy granulation tissue observed during MDT results from increased HGF [66]. The wound-healing capacity of maggot secretions has many aspects, and further research will reveal more interactions with human cells [36].

One of the promising ways to better understand the mechanisms and survival strategies of maggots in such an inhospitable environment is to know where, in which tissue and when, during which developmental stage, different substances are produced. To identify substances responsible for the positive effects of larval therapy on the healing of chronic wounds, purification of the active compounds from different larval extracts and secretions was used in previous studies. This approach seems to be sub-optimal and could cause some misleading conclusions as whole-body extracts are often used. For instance, lucifensin was originally isolated from the gut of *L. sericata* [20], but a closer study of its expression has shown that lucifensin is produced exclusively in the salivary glands and fat body [43].

Most previous studies have tested various larval extracts and secretions that represent a mixture of several substances. The subsequent purification of active fractions often resulted in the loss of activity, and a large amount of extracts and secretions was necessary to use for the successful isolation. The final yield of active substance was always very low and sometimes ended only in a partial characterisation. Employing the techniques of recombinant DNA seems to be a more promising strategy. Nowadays, two cDNA libraries of *L. sericata* are available [18, 46] and also a recently published transcriptome [63]. These data could facilitate scientific discovery and give us the possibility of using different techniques. Using *in silico* analysis, it will be possible to predict certain candidates for antibacterial peptides and proteases, which could participate in the positive effects of larval therapy. Further, it will be possible to prepare such peptides and proteins using recombinant techniques and to test their activities using *in vitro* assays separately and not as a part of a mixture. This approach will allow us to better understand the mechanism of how larvae are able to remove necrotic tissue from the wound, eliminate various pathogens and generally enhance the healing process. Active recombinant peptides and proteins could be used in the future as new biotherapeutics.

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