General discussion and Summary
Non-healing, chronic wounds often result in a reduced quality of life for patients, due to decreased physical, emotional and social function and are a major economic cost for both the patients, their families and Society as a whole. These wounds are particularly prone to occur in patients suffering from acute, extended trauma as well as in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus. Although there are numerous reasons for the development of a non-healing chronic wound, one of the major mechanisms underlying failure of healing is a prolonged and unregulated inflammatory response (Chapter 1). Whereas many therapies have been developed to address the problematic healing of these wounds, maggot therapy may be the most successful one, having a success rate around 7 out of every 10 wounds unresponsive to conventional therapies. Some characteristics (e.g. obesity, smoking, diabetes mellitus, wound duration and size) were not contra-indicative with respect to eligibility for maggot therapy, whereas others (chronic limb ischaemia, wound depth, and age) negatively influenced the outcome. Clearly, the modes of action of maggot therapy likely involve multiple wound healing processes. The studies reported in this thesis focussed on the effects of maggot excretions/secrections on processes related to the inflammatory phase of wound healing. The findings are summarised in Figure 1.

Figure 1 Effects of maggot secretions on cells, cellular products and processes associated with chronic wounds. ROS, reactive oxygen species; TIMPs, tissue inhibitor of metalloproteinases.
Maggot excretions/secretions combat bacterial infections

A major complication of wound healing is the occurrence of bacterial infections\(^3\)\(^-\)\(^5\), especially when the bacteria reside in biofilms\(^6\) which protect them from the actions of cells and molecules of the immune system\(^5\)\(^-\)\(^7\) and antibiotics\(^8\)\(^-\)\(^9\). Moreover, we found some antibiotics (vancomycin, daptomycin, and fluclouxacinil) enhance \(S.\) \(aureus\) biofilms, whereas other antimicrobial drugs (clindamycin and linezolid) were unable to totally eradicate the biofilms \((\text{Chapter 3})\). Consequently, bacterial re-growth may arise from the remaining biofilms and could be an explanation for the persistence of infections often encountered in chronic wounds. One of the beneficial effects of maggot excretions/secretions (ES) is the ability to inhibit the formation and breakdown of biofilms of \(S.\) \(aureus\) \((\text{Chapter 2})\). This biofilm breakdown occurred irrespective the presence of antibiotics \((\text{Chapter 3})\). In addition, ES broke down biofilms of \(P.\) \(aeruginosa\), when using 10-fold higher doses of ES than the effective concentrations used against \(S.\) \(aureus\) biofilms, whereas low concentrations of ES enhanced biofilm formation by these pathogens \((\text{Chapter 2})\). Others have shown that, \textit{in vitro}, \(P.\) \(aeruginosa\) but not \(S.\) \(aureus\), impairs maggot survival\(^10\). Based on these findings and as suggested by clinical experience\(^11\), we conclude that more maggots should be applied to make treatment successful for wounds colonized and infected with \(P.\) \(aeruginosa\), as compared to those by \(S.\) \(aureus\). As secretions interfered in a similar fashion with the TLR-2 and TLR-4 triggered intracellular pathways of monocytes \((\text{Chapter 5})\) and pro-inflammatory macrophages \((\text{Chapter 6})\), it is unlikely that a differential modulation of cell responses by Gram-positive and Gram-negative bacteria is the cause of the observed differences in effects of maggots between patients with wounds infected with \(S.\) \(aureus\) and \(P.\) \(aeruginosa\).

Interestingly, disruption of bacterial biofilms by ES does not involve the killing of bacteria as the micro-organisms released from the biofilm remained viable \((\text{Chapter 2})\). However, several reports describe bactericidal properties of ES against planktonic bacteria\(^12\)\(^-\)\(^14\). The reason for this apparent discrepancy is that the concentration of ES effective against biofilms is considerably lower than those needed to kill the bacteria. Moreover, this level of ES is not within the range achieved in wounds during maggot therapy. Therefore, maggots cannot be considered as a replacement for antibiotics, but should be used only as a supplementary treatment. Of note, it has been reported that antibiotics do not affect the viability of maggots\(^15\). The consequence of biofilm breakdown is that bacteria will become subject to the actions of antibiotics and the immune system as well as to ingestion and subsequent degradation by maggots\(^16\)\(^,\)^\(^17\). Unexpectedly, we initially observed that antibiotics were inactive or less effective against the bacteria that were released from \(S.\) \(aureus\) biofilms. We argued that such bacteria are initially resistant to antibiotics due to their metabolic state; once the bacteria started to multiply, they became more susceptible to antimicrobial action \((\text{Chapter 3})\). In agreement with this, daptomycin, which acts on dormant (static) and exponentially growing bacteria,
was the most active antibiotic in this respect. Moreover, ES increased the activity of daptomycin against bacteria derived from biofilms. Taken together, it can be stated that maggot secretions breakdown bacterial biofilms, thereby subjecting the released bacteria to the action of antibiotics and the immune system. As a result, the unopposed stimulation of the inflammatory response by the bacterial products released from bacteria within biofilms may come to a halt.

**Maggot secretions regulate inflammatory responses**

Maggot secretions did not affect the ability of neutrophils and monocytes to phagocytose and intracellularly kill bacteria (Chapter 5 and 6). The two main mechanisms involved in bacterial killing by neutrophils are the production of reactive oxygen species (ROS) and degranulation, i.e. the release of enzymes, antimicrobial peptides etc into the phagolysosome containing the micro-organisms. H₂O₂ is the most stable ROS and as elastase is a very destructive enzyme, we therefore focussed on the effects of maggot secretions on the production and release of these molecules by neutrophils. Our results show that secretions dose-dependently inhibit these activities in response to the chemotactic peptide fMLP and the protein kinase C activator PMA (Chapter 5).

Besides clearing infections, monocytes and especially macrophages, play a major role in regulating cellular behaviour and other processes in the wounds. We therefore investigated the effect of secretions on the production of pro-inflammatory cytokines by these cells. Maggot secretions reduced the LPS-induced production of several pro-inflammatory cytokines by monocytes as well as that by cells in whole blood (Chapter 6). Similar findings apply to pro-inflammatory macrophages that differentiated from monocytes (i.e. as induced by growth factors) in the presence of the secretions (Chapter 7). Taken together, maggot secretions reduce the production and/or release of pro-inflammatory mediators by phagocytes, thereby contributing to the inhibition of pro-inflammatory activity in chronic wounds. The observed effects of secretions on cell functions are unlikely to be based in altered expression profiles as maggot secretions induced different and/or contrasting effects on the expression of cell surface receptors on neutrophils (Chapter 5), monocytes (Chapter 6) and macrophages (Chapter 7).

Pro-inflammatory macrophages are also responsible for the recruitment and activation of Th1 lymphocytes, through cytokine production, via expression of co-stimulatory molecules and by antigen processing and presentation. These T-cells in turn induce activation of pro-inflammatory macrophages, thereby enhancing their pro-inflammatory responses. Since maggot secretions inhibit the production of pro-inflammatory cytokines and reduce the expression of the T-cell co-receptor CD86 on macrophages (Chapter 7), Th1 cell proliferation and function may be reduced. Furthermore, preliminary experiments showed secretions to decrease the IFN-γ production by T-cells in whole blood stimulated with monoclonal antibodies directed against CD3 and CD28 for 24 h (unpublished observations).
By contrast, using a different experimental set-up, we observed an increased production of IFN-γ when stimulating the cells with mAbs against CD2 and CD28. Clearly, the effects of secretions on T-cells should be investigated further as no conclusions can be obtained from the present data.

**Maggot secretions inhibit migration of phagocytes**
Apart from components and products released by bacteria, chemokines released at the site of inflammation can attract large numbers of inflammatory cells. Therefore, we investigated whether maggot secretions influenced migration and chemokine production by phagocytes. The results revealed that secretions altered the production of several chemokines by monocytes. Similar effects were observed using macrophages differentiated from monocytes in the presence of secretions. Using supernatants of monocyte-cultures incubated with secretions, we observed reduced monocyte chemotaxis, as compared to supernatants of control cultures. However, secretions dose-dependently inhibited the migration of both neutrophils and monocytes towards fMLP directly, making the changes in chemokines of an overall lesser importance regarding the outcome. Thus, inhibition of leucocyte migration by maggot secretions may contribute to reduced pro-inflammatory responses in chronic wounds.

**Wound matrix and debridement**
Although maggot excretions/secretions break down bacterial biofilms and suppress pro-inflammatory responses of phagocytes, these effects may be insufficient to reverse an impaired wound healing. When the wound has been infected for a considerable time, the actions of the pathogenic bacteria and/or the immune cells combined likely have led to the destruction of the provisional matrix (and also the surrounding healthy tissue), which then no longer supports re-epithelialisation and granulation tissue formation. This means that the corrupted tissue has to be removed. This cannot be accomplished by the wound components alone as the lysis of fibrin clots (fibrinolysis) may be impaired in chronic wounds, due to enhanced levels of the fibrinolysis inhibitor PAI. We found enhanced plasminogen activator-induced fibrinolysis by a serine protease present in the secretions of maggots. Consequently, suboptimal levels of plasminogen activators may be sufficient for effective fibrinolysis in chronic wounds. Interestingly, secretions were unable to dissolve plasma clots. These findings are in contrast to those of another report that described the use of a ‘clot’ composed of fibrin only. We therefore concluded that the clot composition may be an important factor for the activity of the enzymes within maggot secretions. Together, wound debridement by maggots may involve a combined action of fibrinolytic activity of the wound components and enzyme activity within the secretions.
Granulation tissue formation
Clinical observations indicate that maggots induce the formation of granulation tissue\textsuperscript{27,28}. This could result from debridement in combination with suppression of pro-inflammation and clearance of the bacteria. However, we found that maggot secretions enhanced the production of the growth factors VEGF and bFGF by monocytes (unpublished observations) and pro-angiogenic macrophages (Chapter 7). In agreement with this earlier reports observed secretions-induced enhanced levels of bFGF in ulcer tissues\textsuperscript{29}. Growth factors, IL-8 and low levels of TNF-\(\alpha\) are involved in endothelial cell migration and proliferation which are essential for angiogenesis\textsuperscript{30,31}. Moreover, our preliminary data showed elevated IL-8 levels in fluid samples from wounds treated with maggots for 3 or 4 days, as compared to fluids obtained from these wounds just before the start of the therapy (unpublished observations). Thus, the increased pro-angiogenic activity may induce neovascularisation and the concurrent formation of granulation tissue.

Active components within maggot excretions/secretions
Maggot excretions/secretions contain a wide variety of components that may induce various effects on human cells and the processes involved in wound healing. The results in Chapters 4, 6 and 7 were obtained with maggot secretions whereas the less ‘pure’ mixtures of excretions/secretions were used in the experiments described in Chapters 2, 3 and 5. Additional experiments showed that maggot secretions breakdown biofilms as well (unpublished observations). Although we did not test the effect of secretions on neutrophils, we assume that the active component is identical to the one responsible for the actions of maggot secretions on monocytes and macrophages.

During our attempts to isolate and characterise the active components within maggot excretions/secretions we gained some knowledge on this topic. First of all, the breakdown of \textit{S. aureus} biofilms was facilitated by heat-sensitive molecules (enzymes) within ES (Chapter 2). By contrast, heat-resistant molecules affected \textit{P. aeruginosa} biofilms. Maggots produce a wide variety of molecules with antimicrobial activity. Using gel-filtration and RP-HPLC, we isolated a small number of peptides/proteins from the haemolymph of maggots that potentially exert antimicrobial activity (unpublished observations). These molecules were also present in the excretions/secretions of the maggots. Chromatographic techniques and mass spectrometry, together with functional assays, revealed that the active component of maggot secretions enhancing fibrinolysis was a trypsine-like serine protease. The component in the secretions that affects phagocytes remains to be elucidated. As also observed for \textit{P. aeruginosa} biofilms, the active molecule did not bind to a C18-RP-HPLC column indicating that it is not a peptide/protein (unpublished observations). By contrast, we were able to reveal aspects of the mechanism by which secretions may affect phagocytes. Within 15 sec after the addition to the cells, maggot secretions maximally increased the intracellular concentration of cAMP both in neutrophils (Chapter 5) and monocytes (Chapter
The cAMP signalling cascade can lead to a range of immunomodulatory effects on cells such as neutrophils, monocytes, macrophages and T lymphocytes\textsuperscript{32}. It has been reported that activation of cAMP pathways is associated with a reduced production of pro-inflammatory cytokines while enhancing the production of IL-10 and VEGF\textsuperscript{32-34}. Furthermore, elsewhere it has been reported that a rise in cAMP is involved in a decreased migration\textsuperscript{35,36}, degranulation and respiratory burst\textsuperscript{37,38} while inhibiting apoptosis in several cell types\textsuperscript{39-41}. Overall it can be concluded that the effects of maggot secretions on phagocytes is explainable on the basis of elevated intracellular cAMP levels.

**Therapeutic considerations**

Maggots are applied to wounds using either a ‘free-range’ or a ‘contained’ (biobag) technique. It has been reported that debridement by free-ranging maggots but not maggots in biobags, can lead to bleeding of wounds\textsuperscript{42}. The most widely accepted explanation is that crawling of the maggots can cause bleeding\textsuperscript{43} although there is a lack of scientific evidence to support this view. It is also possible that enzyme activity of maggots and enhanced fibrinolysis result in breakdown of the provisional matrix before the underlying tissue is healed. In this context, it is likely that a large part of the secretions stick to the biobags, thereby lowering the level of active molecules in the wounds compared to that obtained with free-ranging maggots. In agreement with this, it is reported that free-ranging maggots are more successful than maggots in biobags\textsuperscript{44}.

We found that maggot secretions affect a broad range of processes related to chronic wounds. Based on these results, it may be possible to replace maggots by their secretions thereby eliminating the so-called ‘yuk-factor’ which plays a negative role in the acceptance of maggot therapy.

**Summary and Conclusion**

Maggot excretions/secretions breakdown biofilms of both Gram-positive and Gram-negative bacteria, exposing them to the immune system, antibiotics, and ingestion and subsequent degradation by the maggots. Furthermore, proteases in maggot secretions enhance debridement by increasing the fibrinolytic activity of wound components and by degrading matrix components directly. Additionally, maggot secretions inhibit the pro-inflammatory responses of phagocytes but do not affect their ability to ingest and intracellularly kill microorganisms. Finally, secretions induce the production of growth factors essential for angiogenesis. Clearly, all these effects may be beneficial for the recovery of chronic wounds. The reason why maggots exert these effects may well be a simple matter of survival. Similar to other multi-cellular organisms, maggots have evolved ways to deal with detrimental bacteria, using antimicrobial molecules and, in case of biofilms, with enzymes and other additional mechanisms. Furthermore, maggots obtain their nutrients from dead tissue. To digest the tissue remnants they produce numerous enzymes. In addition, common
mechanisms for parasitic organisms to successfully invade the host are by inducing fibrinolysis and/or suppressing the host immune system, since inflammatory responses of the cells from the host may be detrimental to survival of the parasite. Clearly, maggots do not harm their human hosts. However, uncontrolled myasis, as observed in some cattle, can become lethal and should therefore be avoided.

In conclusion, the results described in this thesis provide new insights into the modes of action of maggot therapy in chronic wounds. The success of maggot therapy may be explained by the broad spectrum of processes that are modulated by maggot secretions. These results contribute to further acceptance of this efficient and successful therapy for the treatment of chronic wounds.
Chapter 8

References


