Chapter 1
Introduction

Introduction

Surgical infections

Dermal tissue injury destroys the most forceful strategy of human immunological defense: the barrier of the skin.[1] Therefore, all damaged dermal tissues can be infected. In surgical interventions, various precautions, such as sterilization of instruments and hygiene of the surgeon, are applied in order to prevent infection. Nevertheless, infection is still the most frequent surgical complication and infection rates range from 14% to 20% after general operative procedures.[2,3] Traumatic injuries are even more at risk for infectious complications because of preliminary bacterial contamination due to the accident. Approximately 30% of surgical wounds after trauma becomes infected.[4] Numerous patient-related and procedure-related factors influence the risk of infection and will be further discussed in the paragraph about *Biofilm formation*. Conventional therapy of infections includes antimicrobial therapy, disinfectants and/or surgery. However, in these times of increasing antibiotic resistance, persistent wound infection is a common problem in surgical practice.[5] Therefore, it is necessary to explore other treatment methods and investigate their mechanisms of action against infections.

Maggot Debridement Therapy

Maggot Debridement Therapy (MDT) may be one of the oldest effective wound treatment methods in the world. Despite clinical observations of beneficial effects of maggots in the healing process of infected wounds, the mechanism remains unclear.[6,7] This thesis investigates various possible modes of action and attempts to answer the main question: How do maggots operate?

The first evidence for the use of larvae to cure wounds originates from tribes of the Mayas in Peru and the aboriginals in Australia.[8] Unfortunately, there are no written documents of their experiences but images show their application (Fig. 1). In the 16th century, the first written report was found that described the effects of maggots infesting wounds. Ambroise Paré (1510-1590), a French military surgeon, documented their beneficial effects and noted the clean wounds after colonization.[8] The surgeon of Napoleon, Baron Dominique Larrey (1766-1842) also reported his observations of injured soldiers who had debrided and granulating wounds that healed quickly with maggots.[9]

Fig. 1 Maggots colonize a Mayan goda. Codex Dresdensis, ca. 11th century.

Many surgeons confirmed the observations of

Paré and Larrey, but John Zacharias (1837-1901), a confederate medical officer during the American Civil War was the first surgeon who introduced maggots for therapeutic use.[10] He noted: *'Maggots… in a single day would clean a wound much better than any agents we had at our command… I am sure I saved many lives by their use.'*

Until today, maggots still have the property to infestate wounds when hygiene is miserable, especially in times of (natural) disaster or war, e.g. after the atomic bomb explosion in Hiroshima in 1945 (Fig. 2). Individual cases are also frequently described, even in the 21st century.[11]

Fig. 2 A child sticks to his mother whose wounds were infestated by maggots after the atomic bomb explosion in Hiroshima. Japanese drawing by Ichida Yuji, August 17, 1945.

Although many observations and reports were noted about larval therapy, maggots became obsolete in the fourth decade of the last century. This change can be explained by medical history. In 1929, William Baer (1872-1931), orthopaedic surgeon at the John Hopkins Hospital in Baltimore introduced larvae of the *Lucilia sericata* for the treatment of children with osteomyelitis.[12] Baer described a fast debridement, the reduction of bacterial amounts, decreased odour and alkalinization of the wound surface. The descriptions of Baer were barely noticed, because the discovery of Penicillin by Alexander Fleming in the same time overwhelmed all these outcomes.[13] Maggots disappeared after the widespread production and use of this first antibiotic from 1944. However, only four years after the introduction of Penicillin, more than 50% of all *S. aureus* specimens produced β-lactamase, which rendered them resistant to the mould.[14] Antibiotic resistance to Penicillin and other types of antibiotics, increased in the time afterwards. The rising antibiotic resistance resulted in the failed healing of many infected wounds and due to this, maggots made their comeback in the late 1980's.[15] In the following years, MDT was reintroduced in hundreds of clinics in the US and Europe.[6,7,16] After the approval of MDT as a debridement therapy by the US Food and Drug Administration (510(k) #33391) in 2004,[17] larvae of *Lucilia sericata* are widely used to treat infected wounds.[18-20]

Evolutionary, the interaction between maggots and humans can be simply explained. Maggots are attracted to wound beds by nature, because they feed on debris, which is full of nutrients and allows them to grow fast and become adult flies. At the same time, the arrival of maggots in human wounds advances the healing process, resulting in tissue repair. There is mutualistic symbiosis between *Lucilia sericata* maggots and humans, which benefits the survival of both species.

In 2008, Steenvoorde published his thesis about clinical aspects of MDT,[21] whereas this thesis focuses on the underlying mechanisms of action of MDT, in current times also known as biosurgery. These mechanisms are unclear, but could provide information for new treatment methods against infections. Investigation of the possible modes of action requires knowledge about basic microbiological and immunological processes in humans.

The process of physiological wound healing

Tissue damage immediately initiates the physiological process of wound repair, which consists of various phases: inflammation, tissue proliferation and tissue remodeling (Fig. 3).[22]

Once the skin is injured and blood vessels are ruptured, trombocytes and inflammatory mediators start migrating to the wound surface to stop the hemorrhage by respectively forming fibrin clots and causing vasoconstriction. Shortly after vasoconstriction, the release of histamine results in vasodilatation which causes edema of the tissue, due to leakage of proteins and water into the extravascular space. During the inflammatory phase that normally lasts five days after injury, cytokines, extracellular matrix proteins and other pro-inflammatory factors, including polymorphonuclear neutrophils, arrive at the injury site.[23] During the first two days after injury, recruited neutrophils debride devitalized tissue and phagocytose infectious agents. Thereafter, they are replaced by monocytes that mature at the wound site into macrophages and continue the debridement of the wound by phagocytosis and secretion of proteases. Their production of chemotactic mediators also stimulates angiogenesis and attracts cells that are necessary for the next phase of wound healing: the tissue formation or proliferative phase starting at day two post-injury and lasting for three weeks.

New stromal tissue is formed, called granulation tissue. This involves the migration of endothelial cells to the injury site and the accumulation of fibroblasts. At the same time neovascularization is continued, while part of the fibrin clots and extracellular matrix are degraded. The fibroblasts lay down initial collagen on the wound bed, creating the principal components of the extracellular matrix, such as fibronectin and hyaluron. Besides endothelial cells and fibroblasts, granulation tissue consists of blood vessels, inflammatory cells and myofibroblasts. It covers the wound bed during the inflammatory and proliferative phase. Myofibroblasts cause wound contraction and reduce the size of the defect. At the end of the second phase, the granulation tissue is fully replaced by collagen and epithelial cells and keratinocytes start to migrate into the wound area. The epithelial cells degrade the remaining fibrin clots and extracellular matrix and once the wound surface is covered by keratinocytes, they begin to secrete proteins that form a new basement membrane.
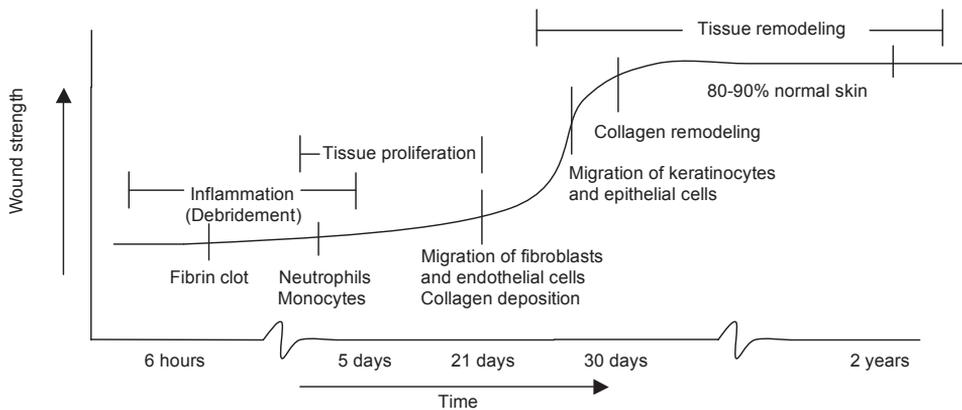
Figure 3 Overview of the process of physiological wound healing.

During the remodeling phase of the wound, redundant cells die by apoptosis and collagen is remodeled and realigned. The remodeling phase can last up to two years. The cicatrix that is formed slowly loses its redness because of apoptosis of the redundant blood vessels, and finally the skin retrieves its normal color.

Chronic wounds

Successful repair of tissue injury requires resolution of the inflammatory response.[22] A prolonged inflammatory phase can lead to the formation of non-healing, chronic wounds. Most chronic wounds are associated with a number of clinical diseases, such as vascular compromise, diabetes mellitus and pressure necrosis.[22] Other risk factors for impaired wound healing are immune-related diseases and/or the use of immunosuppressants, connective tissue disorders, malignancies, edema, radiation therapy, poor nutrition, alcohol abuse and smoking.[24]

Chronic wounds are often characterized by debris on the surface, which results from the impaired healing process. Persistent chemotaxis of neutrophils to the wound bed, unrestrained proteolytic activity and disturbed oxidant/antioxidant balance cause damage of the surrounding tissue rather than repair.[22] Effective debridement is therefore essential to facilitate and continue the process of wound healing.[25]

Debridement is defined as the removal of necrotic and infected tissue and foreign material from and around a wound surface.[26] Maggots are especially indicated for debridement of wound beds.[17] They distinguish the vital and necrotic tissues, which is very remarkable. How do larvae remove this debris so precisely? One of the hypotheses is that excretions and secretions which they produce, interfere with the debris and dissolve it. Though debris can consist of several

components, in this thesis, the effect of maggot excretions and secretions was investigated on an essential part of debris, present in many chronic, infected wounds; the biofilm.

Biofilm formation

Bacteria that grow on surfaces become embedded in an extracellular polysaccharide matrix, which is called biofilm. This biofilm protects these bacteria from antibiotics and host immune defenses.[27] Until 30 years ago, scientists thought that the bacterial pathogens that could cause infection were always free-living (planktonic) bacteria.[28] However, further research has led to the insight that bacteria can also cause biofilm-associated infections, that are more difficult to treat than infections caused by free-living bacteria. In nature, 99.9% of all bacteria live in well-developed biofilms.[28] Biofilm formation is an essential survival strategy of bacteria. In daily clinical practice, bacteria in biofilms on prostheses, other foreign bodies or in bone tissue constitute a major problem because of their difficult treatment.[29,30]

Biofilm formation is a dynamic process that starts with the attachment of bacteria to a surface and ends with the development of a mature, hydrated polymeric matrix that can neither be destroyed by disinfectants, antimicrobials nor by host defenses.[31,32] The maturation process can take twelve hours up to weeks (Fig. 4). Bacteria in biofilms are resistant to antimicrobial concentrations that are thousand times higher than the concentrations that kill planktonic cells.[33]
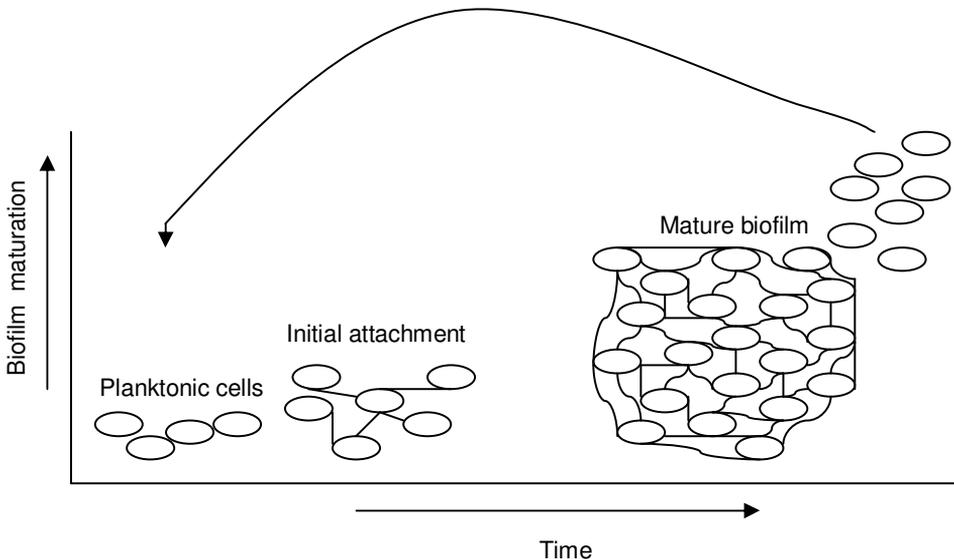

Figure 4 The maturation process of biofilm formation

The most important differences between bacteria in biofilm and planktonic microorganisms that can explain the difference in antibiotic resistance are the reduced diffusion of antimicrobial agents in the biofilm matrix as a result of chemical reactions, the change of local environment, e.g. pH and limited oxygen concentration.

Finally, an essential difference is the metabolic state of biofilm bacteria; the genotype and phenotype of bacteria in biofilms have been changed, as well as the protein expression and this results in metabolic quiescence.[33] Bacteria in biofilms are not only resistant to conventional antimicrobial therapies, they even contribute to the spread of antibiotic resistance.[34] The bacterial population in a biofilm has a high density, which results in a very effective intra-cellular transfer of resistance and virulence genes.

Although biofilm bacteria live in a stationary phase, the biofilm continues to grow because of an intercellular communication process within the matrix that is called *quorum-sensing*.[31] This bacterial signaling system is a complex process, in which the regulation of gene expression and metal ions play an essential role. However, the exact mechanism of quorum-sensing is not well understood; it is the subject of intensive investigation by many research groups in the entire world.

The main problem of biofilms in clinical practice is that they are associated with infections that are very difficult to treat, e.g. osteomyelitis and prosthesis-associated infections.[32] If antibiotics do not destroy biofilm bacteria, are there other effective anti-biofilm treatments? Up to date, the treatment possibilities are limited.[35] With the current knowledge of biofilm formation, we could think of methods to interfere with the biofilm bacteria themselves, e.g. by providing alternative electron acceptors or fermentable substrates to change the metabolic quiescence; with the extracellular matrix, e.g. the local environment; and with the maturation process including the quorum-sensing phenomenon. Preventive methods for biofilm-associated infections of prostheses could be the use of biomaterials with an anti-adhesive surface, e.g. a surface impregnated with an anti-biofilm substance, to avoid colonization and subsequent infection.[36]

In current clinical practice, biofilm-associated infections of prostheses require in almost all cases removal of the implant, or months of antibiotic treatment; this results in delay of recovery and complications for the patient.[35] The risk for developing a deep prosthesis-related infection after surgery varies, according to the literature from 1-2% in joint replacement and between 5% and 10% after fracture fixation.[32] Biofilm-associated infections of medical devices are often diagnosed with a delay. Patients present with an acute infection that is temporarily suppressed by antibiotics and appears to recover, but when planktonic cells, that are periodically released from the mature biofilm, come into the bloodstream, systemic infection returns.[32,33] Especially in a patient with (any) implant device (arthroplasty, catheter, pacemaker etc.) and recurrent, unexplained fever and

sickness, the presence of biofilm-associated infection must be considered (Table 1).

Table 1

> ***How to recognize a biofilm-associated infection in clinical practice?*** [31]
>   1. *Clinical signs of infection with (repeatedly) negative cultures*
>   2. *Chronic infection with periodically acute exacerbation of symptoms*
>   3. *(Partial) failure of response to conventional antimicrobial therapy*

Knowledge about biofilm formation on wound surfaces is limited. The studies that were performed, show that biofilm-associated infections of wounds inhibit the normal wound healing process and cannot be treated sufficiently with conventional antimicrobial or surgical methods alone.[38] It has been reported that biofilm is part of the wound debris and that debridement is an essential tool for maintaining wound biofilms in a weakened state.[25,38] This important information refers to one of the hypotheses that is investigated in this study: if maggots debride wounds, do they also reduce biofilm formation?

The role of complement in wound healing

The complement system is part of the innate immune defense and complement activation plays an important role in the activation of the inflammatory response to injury.[39] Invading organisms or tissue injury can trigger the complement cascade, which can occur via three pathways: the classical pathway (CP), the alternative pathway (AP) and the mannose-binding lectin pathway (MBL-P).

      The CP can be activated by antibodies, when complement factor C1 interacts with antigen-IgM or aggregated antigen-IgG complexes or by other molecules, e.g. when polyanions, gram-negative bacteria or bound C-reactive protein directly interfere with C1. CP is regulated by C1 inhibitor.

      MBL-P activation is not antibody dependent. The serum protein mannose-binding lectin binds to mannose or fructose on the wall of a bacterium, yeast or virus and further proceeds on the same cascade as the CP.

      AP activation is initiated when components of microbial cell surfaces, mostly polysaccharides of Gram-negative bacteria, cleave small amounts of C3, the central complement factor. The AP is regulated by properdin, factor H, factor I, factor D and decay-accelerating factor (CD55).

      The result of activation of any of these pathways is cleavage of central factor C3 into C3a and C3b by C3 convertase. Finally the terminal pathway of
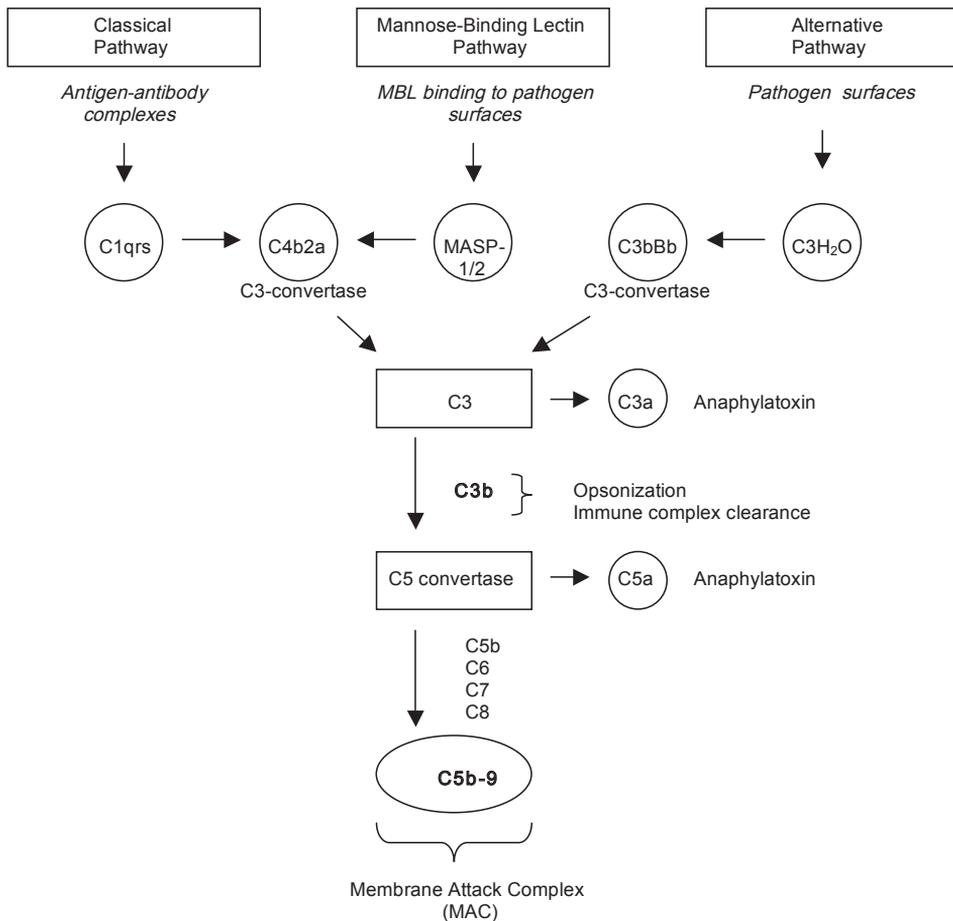
Classical Pathway	Mannose-Binding Lectin Pathway	Alternative Pathway

Antigen-antibody complexes — *MBL binding to pathogen surfaces* — *Pathogen surfaces*

C1qrs → C4b2a ← MASP-1/2 — C3bBb ← $C3H_2O$

C4b2a: C3-convertase   C3bBb: C3-convertase

C3 → C3a Anaphylatoxin

C3b } Opsonization / Immune complex clearance

C5 convertase → C5a Anaphylatoxin

C5b C6 C7 C8

C5b-9

Membrane Attack Complex (MAC)

Figure 5 A simplified overview of the complement cascade

the complement system with factors C5b to C9 is reached. These factors form the membrane attack complexes (MAC), which form pores in the microbial wall resulting in cell lysis.

Not only immune complexes and pathogens can initiate the complement system, also aseptic tissue injury causes complement activation.[40] The inflammatory response as described in the process of wound healing is the result of complement activation and is necessary for successful tissue repair. However, inappropriate complement activation can also cause injury and contribute to severe tissue damage, caused by exactly the same mediators.[41] In the impaired wound healing process, the inflammatory phase is prolonged, which is the result of a disturbed complement activation.[42] Does this mean that in chronic wound

situations the complement system is excessively activated? Or is the complement activation not powerful enough to opsonize the debris and is stimulation of the complement system needed? In current science, these questions are still unanswered.

Maggots are successful in healing chronic wounds. Therefore, the hypothesis arose that larvae and/or their excretions and secretions interfere with the complement system. A possible influence of maggots on complement activation could provide information for new treatment methods against various (inflammatory) diseases, related with our innate immune system.

The scope of the thesis

The scope of this thesis is to obtain insight in the underlying microbiological and immunological mechanisms of action of Maggot Debridement Therapy. MDT is supposed to work in three areas: disinfection, debridement and stimulation of wound healing. *Chapter 2* and *Chapter 3* focus on the part of disinfection. In clinical practice, it is simply thought that maggots consume bacteria and therefore aid in disinfection of the wound surface. However, there is no evidence for this notion. *Chapter 2* investigates the susceptibility of strains of six bacterial species, which are regularly found in infected wounds, to live maggots and/or their excretions/secretions to find proof supporting direct antibacterial activity. During MDT, faster healing of wounds in combination with antibiotics has been observed. Based on these facts it was hypothesized that maggot excretions and secretions enhance the antibacterial effect of antimicrobial agents and the results of this study are described in *Chapter 3*. The role of maggot excretions and secretions in dissolving debris, especially in affecting biofilms on biomaterials in vitro, is investigated in *Chapter 4.1* and *4.2*. In current MDT, sterile maggots are captured in small permeable bags that allow free exchange of maggot excretions and secretions to the wound surface. Wounds, suspected for biofilm formation, successfully healed with larvae in these bags. This could mean that maggot excretions and secretions are of importance in debriding biofilms, which is tested for six bacterial species on three commonly used prosthetic materials. In *Chapter 5* the effect of maggot excretions and secretions on the three pathways of complement activation is studied. The complement system is essential to mediate a physiological wound healing process. In impaired wound healing, this physiological balance is disturbed. Perhaps maggot excretions and secretions stimulate wound healing by affecting inappropriate complement activation. The review in *Chapter 6* discusses the different modes of action of MDT, except for the results of the complement study. This review also shows an overview of clinical aspects of MDT, indications, contra-indications, modes of application and possible future prospects. Finally, the entire overview and the discussion about

the results of this thesis will be completed in *Chapter 7*, including the effects of maggot excretions and secretions on complement activation in human sera. *Chapter 7* explains how maggots operate on wounds.

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