# Evaluating the potential of novel antimicrobial strategies in a multimodal treatment model for implant-related infections: antibiotics, antimicrobial peptides, bacteriophages, induction heating

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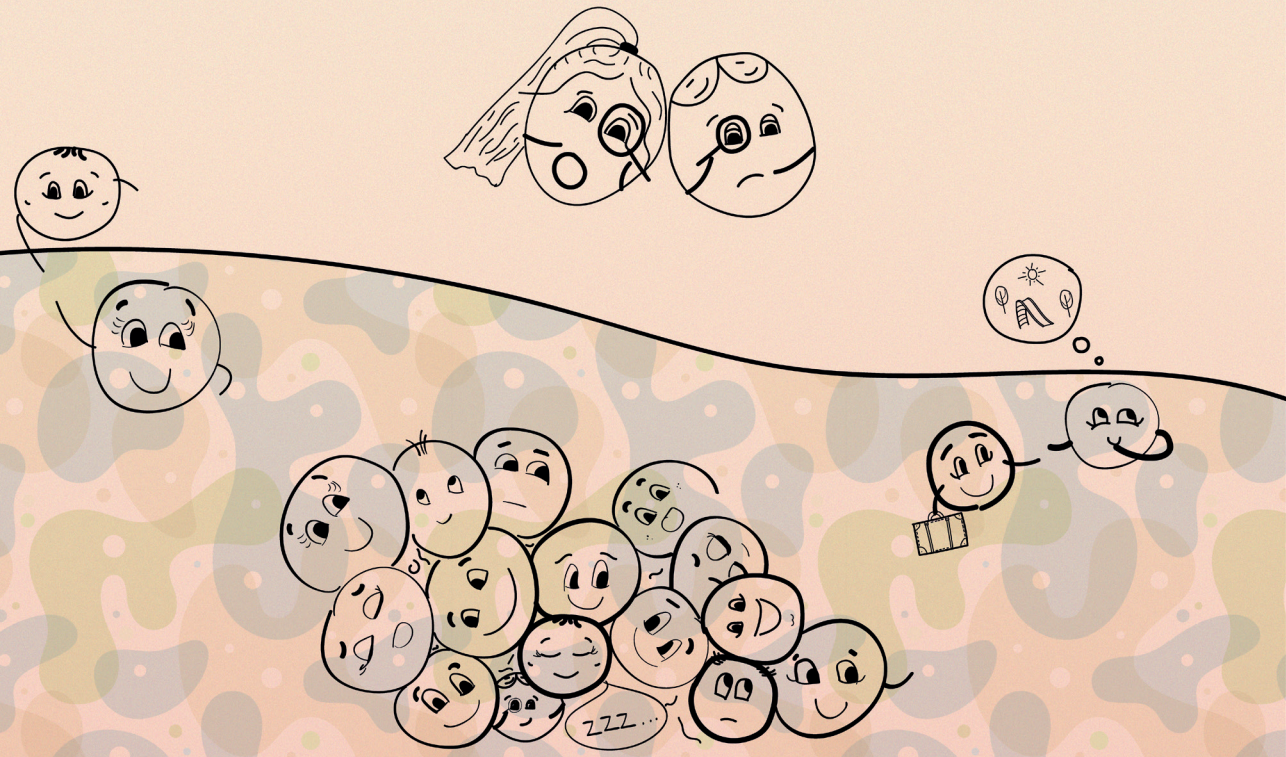

# General introduction

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## Clinical problem: metal-implant-associated infections

Metal biomaterials are widely used in medical applications, including joint arthroplasty and bone fixation in trauma surgery. Joint replacement surgery for the hips and knees is among the most common surgeries in orthopedics, with about 80.000 procedures annually in the Netherlands alone (1, 2). The volume of these surgeries has increased over the past decades, and this trend is expected to continue due to our ageing society, increased obesity, and the availability of these procedures in low and middle-income countries (3). In trauma surgery, metal plates, screws, and nails are crucial for enabling early mobilization of patients after trauma. Fixating fractured bones either internally (e.g., nails) or externally (e.g., plates and screws) ensures proper alignment and stability, and promotes healing (4).

These medical devices improve the patient's quality of life and functional capabilities in daily life (5, 6). However, approximately 2% of patients develop a peri-prosthetic joint infection (PJI) or a fracture-related infection (FRI) (7). Even more, the infection rates range between 3% and 31% in patients who are treated with mega-prosthetic implants after tumor resection (8-10). Notably, early postoperative metal implant-associated infections are frequently caused by *Staphylococci* such as *Staphylococcus aureus* (*S. aureus*).

Treatment strategies for PJI include revision surgery, initially focused on reducing the bacterial load and debridement of infected tissue, antibiotics, and implant retention (DAIR), and a minimum of 6 weeks of antibiotic therapy. If unsuccessful, the infected implant has to be removed during a one- or two-stage revision (extensive revision) surgery, followed by several weeks of antibiotic treatment. In one-stage revision surgery, the infected implant is removed and replaced within the same surgical procedure. Alternatively, in two-stage revision surgery, the infected implant and infected tissue are removed in the first phase, followed by antibiotic treatment for several weeks to months and insertion of the new implant in the second phase (11). DAIR involves mechanical cleaning of the infected implant, surgical removal of the infected peri-implant tissue, and high-dose antibiotic treatment (12). As the infected implant remains within the body, DAIR is considerably less invasive than extensive revision surgeries, in which the implant is removed (13).

The choice of treatment depends on various factors, such as the type of infection, the infecting microorganism, loosening of the prosthesis, and the physical condition of the patient (12). Metal implant-associated infections can be categorized as acute or chronic. Chronic infections are associated with reduced antibiotic efficacy, usually due to delayed or missed initial diagnosis, suboptimal antibiotic selection, and intermittent antibiotic treatment without addressing the infected tissue, resulting in osteomyelitis and antimicrobial-resistant (AMR) bacteria (14). Hence, extensive revision surgery (i.e., implant removal) is necessary along with antibiotics to treat chronic infections, provided the patient is fit enough for surgery. Patients with, for instance, severe comorbidity and high risk for revision surgery are treated with suppressive antibiotic therapy. Acute infections can be successfully treated by a DAIR procedure, in which the implant remains fixed within the bone. This procedure allows the removal of infected tissue and management of the biofilm, resulting in success rates up to 70% (15, 16).

Bacterial biofilm formation on the metal implant surface significantly impairs treatment effectiveness, leading to high rates of treatment failure and mortality (17). That is, the risk of mortality after PJI of the hip increased with an odds ratio of 3.58, and mortality rates 5 years after hip PJI accounted for 21% (18). Treatment failure after one-stage and two-stage revision

surgeries has been observed in about 13% and 15%, respectively (19). Further, single DAIR procedures fail in 2% to 40% of cases, requiring additional DAIR procedures or extensive revision surgery to treat these patients (20). Particularly, *Staphylococci* are associated with higher treatment failure rates than other causative pathogens in these infections (21, 22).

Prolonged antibiotic courses are often required to treat these patients with PJI, which often induce various adverse effects (23). Moreover, the duration of antibiotic suppression therapy can be indefinite (24). Extensive re-revision surgeries and sometimes even amputation of the infected limb are necessary to treat these patients (25). These surgical approaches are highly invasive for the patient (e.g., blood loss, 3 to 4 hours of surgery) and are associated with weeks to months of impaired mobility, pain, and psychological distress (26). Altogether, effectively managing metal implant-associated infections remains a complex multidisciplinary challenge, primarily due to bacterial biofilm formation on the metal implant surface and, if chronic, the long-term presence of infected bone and soft tissue.

## The biofilm cycle

Bacterial adhesion is the initial stage of biofilm formation, its cycle depicted in **Figure 1**, by which bacteria protect themselves against exogenous stressors. Bacteria can form biofilms on biotic and abiotic surfaces, and within fluids (27). Following adhesion, bacteria form microcolonies, large aggregates, and a self-produced matrix of proteins, exopolysaccharides, lipids, and extracellular DNA (eDNA). This matrix hinders the access and efficacy of immune effector cells and antimicrobial agents (28). The formation of water channels and pores allows the transport and storage of nutrients, waste products, and other small molecules throughout the biofilm layers (29).

*S. aureus* biofilm formation and regulation are influenced by quorum sensing, a chemical communication process utilized by bacteria, primarily through the accessory gene regulator (*agr*) system (30). The *agr* system regulates the expression of toxins and virulence factors in biofilm-embedded bacteria and is involved in their resistance to antibiotics (31). Additionally, biofilms facilitate the exchange of resistance genes by horizontal gene transfer, leading to rapid AMR development and reduced antimicrobial treatment efficacy (32, 33).

As the biofilm matures, heterogeneous subpopulations arise due to varying gradients of pH, nutrients, and oxygen (34). Biofilm-embedded bacteria can become tolerant to antimicrobial therapy, which can occur spontaneously or can be induced by an external stressor (35). Tolerant bacteria are difficult to eradicate with antimicrobials due to their reduced metabolic activity and antimicrobial target activity, resulting in an increased duration for killing compared to sensitive bacteria (35). Persisters are a subset of tolerant bacteria in a sensitive bacterial population. Hence, the duration for killing persisters with antimicrobial agents is increased compared to the bulk of bacteria, resulting in the biphasic killing curve characteristic of persisters (36). These bacteria notoriously induce infection relapse after discontinuing antimicrobial treatment (37).

Bacterial tolerance and persistence are phenotypic phenomena that differ from antimicrobial resistance. While the minimal inhibitory concentration (MIC) of resistant bacteria increases, the MIC of tolerant bacteria (and thus persisters) remains similar to that of a susceptible bacterial population (36, 38). Importantly, bacterial tolerance and persistence seem to precede and enhance the development of AMR (39).

In the highly dynamic biofilm, single bacteria or bacterial aggregates can disperse into

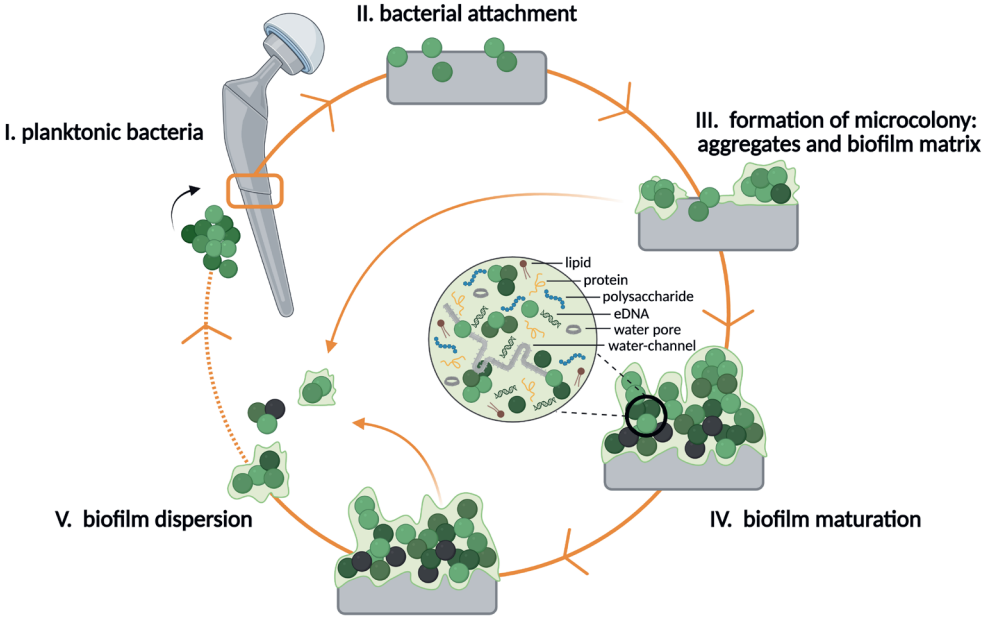


**Figure 1. The biofilm cycle.** An overview of the different stages of bacterial biofilm formation on metal implants, such as prosthetic joints. First, planktonic bacteria infect (I) the tissue surrounding the metal implant and thereafter attach (II) to the metal implant surface. After irreversible adhesion, the bacteria proliferate and form a microcolony (III) by their aggregation and their embedment within a self-produced matrix (consisting of lipids, proteins, polysaccharides, extracellular DNA (eDNA), water channels and pores). Over time, the bacterial biofilm matures (IV): the bacterial population expands, various subpopulations arise (indicated by the different shades of green bacteria), and the matrix becomes more complex and dense. Bacteria, and bacterial aggregates, can disperse (V) from the biofilm to the surrounding tissue and potentially initiate an (biofilm-associated) infection elsewhere. Image created using BioRender.com.

the surroundings and potentially form a (biofilm-associated) infection elsewhere (40). When not induced by exogenous stressors, biofilm dispersion is a controlled process often mediated by the *agr* system in *S. aureus* (41). Furthermore, the biofilm-dispersed fraction is remarkably heterogeneous and associated with high metabolic activity, virulence, and antibiotic resistance (42). Even more, biofilm dispersal enables the dissemination of AMR genes that bacteria acquired within the biofilm.

## Interplay between the immune system, the metal implant, and *S. aureus*

The host's innate immune response to the implanted biomaterial, known as the foreign body reaction (FBR), plays a crucial role in the ability of bacteria to colonize metal implants (43). First, the adherence of serum proteins to the implant activates the immune system, leading to neutrophil attachment and the induction of an inflammatory environment (44). Recruited monocytes differentiate into pro-inflammatory macrophages, aimed at engulfing the foreign body. However, when these phagocytes fail to accomplish this, their immune effector functions become exhausted. This phenomenon, known as "frustrated phagocytosis", causes pro-inflammatory macrophages to shift towards an anti-inflammatory state, which results in impaired bacterial eradication (43). Additionally, insufficient integration of the implant surface by tissue cells can further increase the risk of bacterial colonization, a theory known

as the "race for the surface" (45). Essentially, the more tissue cells that attach to the implant, the lower the risk of bacterial adhesion, and vice versa.

Upon infection, *S. aureus* further modulates the host's immune response towards tolerance by polarizing macrophages towards an anti-inflammatory state. In addition, *S. aureus*-mediated recruitment of myeloid-derived suppressor cells (MDSCs) dampens the immune response by impairing phagocyte recruitment and T-cell maturation (46). This anti-inflammatory milieu, induced by both the FBR and the bacteria, creates a window of opportunity for bacterial biofilm formation on the implant surface.

Although neutrophils can infiltrate and phagocytose biofilm-embedded bacteria, their efficacy reduces as the biofilm matures and becomes less susceptible to eradication (47). Mature *S. aureus* biofilms express high levels of toxins (e.g., α-toxin, leukocidin AB) and evasion molecules (e.g., extracellular adherence protein (Eap)), both impairing the bacterial-eradicating functions of phagocytes and their ability to infiltrate the biofilm (48, 49). Macrophages that could invade the biofilm showed limited phagocytic capacity, an anti-inflammatory phenotype, and high levels of cell death (50). Taken together, the *S. aureus* biofilm redirects the host immune response to tolerate the infection, contributing to its persistence.

## Alternative treatment strategies

Metal implant-associated infections are a significant complication in orthopedic and trauma surgery due to their resilience against the effects of host immune cells and current treatments. These infections significantly affect the patient's quality of life, the healthcare system, and society (7). In addition, inadequate antimicrobial therapy is associated with the emergence of antimicrobial resistance (AMR) (51). The global emergence of AMR bacterial strains further hampers the treatment efficacy of antibiotics, highlighting the urgency of developing alternative approaches to combat these infections and limit AMR (52).

Various alternatives have been researched to combat *S. aureus* biofilms, including repurposed drugs (i.e., halicin (53), penfluridol (54)), quorum-sensing inhibitors (i.e., 2-(2-methyl-2-nitrovinyl)furan (55), quercetin (56)), photodynamic therapy (57), radio-immunotherapy (58), bioactive glass (59), and enzybiotics (60). Here, the focus lies on bacteriophages, non-contact induction heating of metal implants, and antimicrobial peptides as alternative treatment strategies for metal implant-associated infections.

### Bacteriophages

In the early 1900s, bacteriophage (phage) therapy was a common method for treating bacterial infections (61). Although its use was discontinued in the Western World with the discovery of antibiotics, difficult-to-treat infections and the global spread of multidrug-resistant (MDR) bacteria have reignited interest in phage therapy as a promising alternative treatment strategy.

Phages are the natural predators of bacteria and are highly diverse, ubiquitous, and abundant on Earth (62). These viruses can infect and kill a specific bacterial species or bacterial strain(s) within a species. Phages recognize a specific surface receptor or multiple receptors on the bacterial cell wall, e.g., polysaccharides and peptide sequences, which can be highly diverse across bacterial strains (63). After attaching to a susceptible bacterium, lytic phages insert their genome into the bacterial cytoplasm and hijack the bacterial replication and translation machinery to ensure viral replication (64). This results in bacterial lysis, cell

death, and the release of the phage progeny to infect neighboring bacteria.

Due to their high specificity, phages must be selected for therapeutic applications based on their activity against the infecting bacterial strain. While selecting suitable phages can be time-consuming and complex, the advantage of their high specificity is that phage therapy minimally disrupts the patient's microbiota compared to antibiotic treatment (65). Further, phages can be pre-adapted through co-evolution with the respective bacteria to enhance bacterial eradication and reduce the development of phage resistance (66, 67).

Besides their direct bactericidal function, phages can modulate the host's immune response by directly interacting with phagocytes and influencing phagocytosis, ROS production, and cytokine production (68). Both pro- and anti-inflammatory responses can be induced by phages, with a higher tendency towards anti-inflammatory effects (69). Further, phages can influence antibody production and effector cell polarization (70). Importantly, phage therapy can induce phage-neutralizing antibodies that can reduce the efficacy of phage therapy (71). Despite the presence of such antibodies, successful treatment outcomes were observed (72). Synergy between phages and the host immune system has been reported, resulting in enhanced clearance of infections caused by MDR bacteria (73). An improved understanding of the interactions between phages, bacteria, and the immune system is crucial for successful phage therapy.

Currently, phage therapy is still considered experimental in Western Europe and restricted to administration as a compassionate use program, allowing investigational medicine in serious and life-threatening diseases (74). Various case reports and clinical trials have been published using phage therapy to control difficult-to-treat infections, including bone and joint infections (75). Generally, the incidence of adverse events due to phage therapy was low, and adverse events that have been reported were considered mild and resolvable (76). Patients with PJI who received phage therapy during surgical debridement did not show infection recurrence. Importantly, suppressive antibiotic therapy was required for the satisfactory outcomes of these patients (77).

Clinical phage trials, however, could not reproduce the positive treatment outcomes observed in case reports (78). These clinical trials used predefined phage cocktails that were not effective against all bacterial strains in the study, which explains the absence of statistically significant positive treatment outcomes (79). Personalized approaches to phage therapy would be more suitable for clinical phage trials (79). Recently, a retrospective observational study reported positive outcomes of personalized phage therapy combined with standard-of-care antibiotics (80).

Positive treatment outcomes of (personalized) phage therapy can be attributed to phage-antibiotic synergy. This synergy depends on several factors, including the host environment and the phage-antibiotic combination (54). Mechanisms underlying phage-antibiotic synergy that have been described include enhanced phage adsorption due to antibiotic-induced bacterial filamentation (81), improved phage production resulting from antibiotic-induced delayed lysis (82), and attenuated antibiotic sensitivity due to phage infection (54). In the latter, phages reduce the MIC of antibiotics to which bacteria were initially resistant.

**Non-contact induction heating**

Another strategy to eradicate bacterial biofilms is heating metal implants to high temperatures. Non-sporulating bacteria, such as *S. aureus*, are already inactivated at

temperatures ≥ 50 °C. Hence, high temperatures damage important physiological processes and the structural integrity of bacteria (83). Since the effectiveness of heat does not depend on the metabolic activity of bacteria, heat may also affect antibiotic-tolerant bacteria within biofilms. In addition, this heating approach could significantly affect the bacteria attached to the metal implant, which are often difficult to eradicate with antibiotics. The specific heating of metal implants requires pulsed electromagnetic fields (PEMF), which are used by non-contact induction heating (NCIH). PEMF induces eddy currents in the metal, resulting in localized heating of the metal due to electrical resistance. This heating can be controlled by adjusting the frequency and strength of the magnetic field.

Importantly, NCIH induced structural damage to biofilms *in vitro* and effectively reduced the bacterial load within (84). The combination of NCIH at temperatures above 60 °C with antibiotics synergistically reduced biofilm-embedded bacteria of various species (85-87). The mechanism of action of NCIH most likely resembles that of conventional heat treatment, which induces defects in the bacterial cell wall integrity, hampers bacterial reproduction, and weakens the biofilm integrity (88). Further, previous research has shown that NCIH can induce bacterial membrane disruption and thereby can improve the efficacy of antimicrobials against MDR bacteria when the resistance mechanism involves the bacterial cell membrane (85).

NCIH can be applied invasively during surgical debridement procedures (i.e., DAIR) in patients with metal-implant-associated infections. This application also allows the eradication of bacteria in areas that are difficult to reach by mechanical cleaning of the metal implant (e.g., stem-inserting screw holes at the neck of femoral stems (89)), while keeping the soft tissues away to limit tissue damage. In cases of multiple surgical debridement procedures, NCIH could be performed several times.

Alternatively, NCIH could be applied non-invasively (i.e., closed wound). Homogenous heating and temperature control of the implant, considering its shape and type, are important factors in limiting tissue damage (90). Mathematical models indicated that thermal damage to the surrounding tissues is restricted to approximately 3 mm around the implant when implant-related biofilms are exposed to temperatures above 70 °C (91, 92). This suggests that lower temperatures at the implant-bone interface should be considered to minimize potential tissue damage.

**Antimicrobial peptides**

Antimicrobial peptides (AMPs), or host defense peptides, are small, naturally occurring molecules with broad-spectrum activity against various pathogens, including bacteria (93). AMPs play an important role in the innate immune systems of humans and many other organisms by exerting direct killing and/or immune-modulatory activities (94). Most AMPs in mammals belong to the group of cathelicidins and defensins, which can be produced by, for instance, epithelial cells and phagocytes in response to structures from pathogens, e.g., pathogen-associated molecular patterns (PAMPs) and cytokines. Moreover, AMPs are stored as inactive precursors in (neutrophil) granules at very high concentrations for local release at infection sites (95).

AMPs are typically positively charged, with cationic and hydrophobic sequences, crucial features for their interaction with the negatively charged bacterial surface, membrane insertion, and discrimination between target and host cells (96). While most AMPs induce direct killing by damaging the bacterial membrane, AMPs can also have intracellular

targets that are reached after translocation across the bacterial membrane (94). Due to the membrane-targeting activity of AMPs, the emergence of resistance is relatively low (97). Moreover, AMPs have been reported to be effective against bacteria with significantly reduced metabolic activity (98).

Besides direct killing, AMPs have various immunomodulatory functions that enhance bacterial elimination by phagocytosis, immune cell recruitment at inflammatory sites, modulation of immune cell differentiation, and initiation of adaptive immunity (99). Furthermore, AMPs can promote angiogenesis and wound repair, neutralize LPS, and dampen excessive pro-inflammatory immune responses (100).

Based on the blueprints of natural AMPs, synthetic analogues have been produced and optimized to enhance their antimicrobial activity using machine-learning technology. This resulted in the synthetic antimicrobial and antibiofilm peptides (SAAP), among other libraries. SAAP-148 offers an interesting alternative to antibiotics by killing MDR bacteria without inducing resistance and eliminating *S. aureus* persisters within mature biofilms (98, 101).

AMPs can prevent bacterial attachment to surfaces, hamper biofilm formation and maturation, and eliminate established biofilms by degrading the biofilm matrix and lysing bacteria within (102, 103). However, the application of AMPs is limited by various challenges. For example, AMPs can induce significant cytotoxicity at concentrations required for antimicrobial activity (104, 105). Under physiological conditions, AMPs exhibit low stability and short half-lives due to their susceptibility to proteolytic degradation (106, 107). Further, AMPs have a limited bioavailability and activity due to their binding to serum and plasma proteins, and are rapidly cleared from the circulation (108, 109).

Despite their antimicrobial and immune-modulating activities, the limitations listed above make AMPs less suitable alternatives to antibiotic treatment compared to phage therapy and NCIH. Nevertheless, the combination of AMPs and antibiotics synergistically reduced biofilm-embedded bacteria, indicating that AMPs could be interesting additives for the treatment of biofilm-associated infections (110).

## Multimodality approach

Eradicating *S. aureus* biofilms with a single treatment approach is unlikely to be the way forward, given the heterogeneity and resilience of biofilm-embedded bacteria. Treatment of metal-implant-associated infections frequently involves surgical debridement and antibiotic combinations to increase the treatment efficacy and reduce the emergence of bacterial resistance (111). Following this rationale, the effects of the aforementioned alternative treatment strategies should be investigated in a multimodality approach to enhance their biofilm-eradicating capacity and to curb AMR.

## Thesis outline

This thesis investigates the effects of phages, NCIH, SAAP-148, and their combinations on bacteria within biofilms on metal discs mimicking metal implants *in vitro*. **Chapter 1** describes the infections associated with metal implants and the sequelae, the basic treatment concepts, and various alternatives in treating these implant-associated infections.

Although phage therapy is increasingly under investigation, the effects of phages on bacteria in the various stages of the biofilm cycle are poorly understood. **Chapter 2** investigates the effects of phage ISP against *S. aureus* in planktonic phase, attached to the metal implant mimic, within immature and (persister-enriched) mature biofilms, and dispersed from mature biofilms. The mechanisms involved in the phage-tolerant phenotype of biofilm-dispersed bacteria, as observed in Chapter 2, are investigated in **Chapter 3** using a proteomic workflow supported by microbiological and biochemical assays. In addition, this chapter determines whether this tolerance is restricted to phage ISP. Since the occurrence of phage-antibiotic synergy depends on the phage and antibiotic combination, **Chapter 4** describes whether pre-screening the effects of phage-antibiotic combinations on planktonic phase bacteria can predict their efficacy on mature biofilm-embedded bacteria. The effect of NCIH on immature biofilm models *in vitro* has previously been investigated, yet its effect on clinically relevant biofilm models remains undetermined. **Chapter 5** investigates the effects of NCIH and its combination with SAAP-148 on persister-enriched *S. aureus* biofilms. **Chapter 6** examines whether sequential combinations of antibiotics, SAAP-148, or phage ISP with NCIH can enhance NCIH's efficacy in eradicating biofilm-embedded bacteria, thereby allowing lower temperatures to be used to limit potential tissue damage.

**Chapter 7** summarizes and discusses the findings of this thesis, evaluates the advantages and limitations of the various alternatives to antibiotics and their combinations, and provides recommendations for future studies. Finally, a Dutch summary is provided in the **Appendix.**

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