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

Verheul, M.

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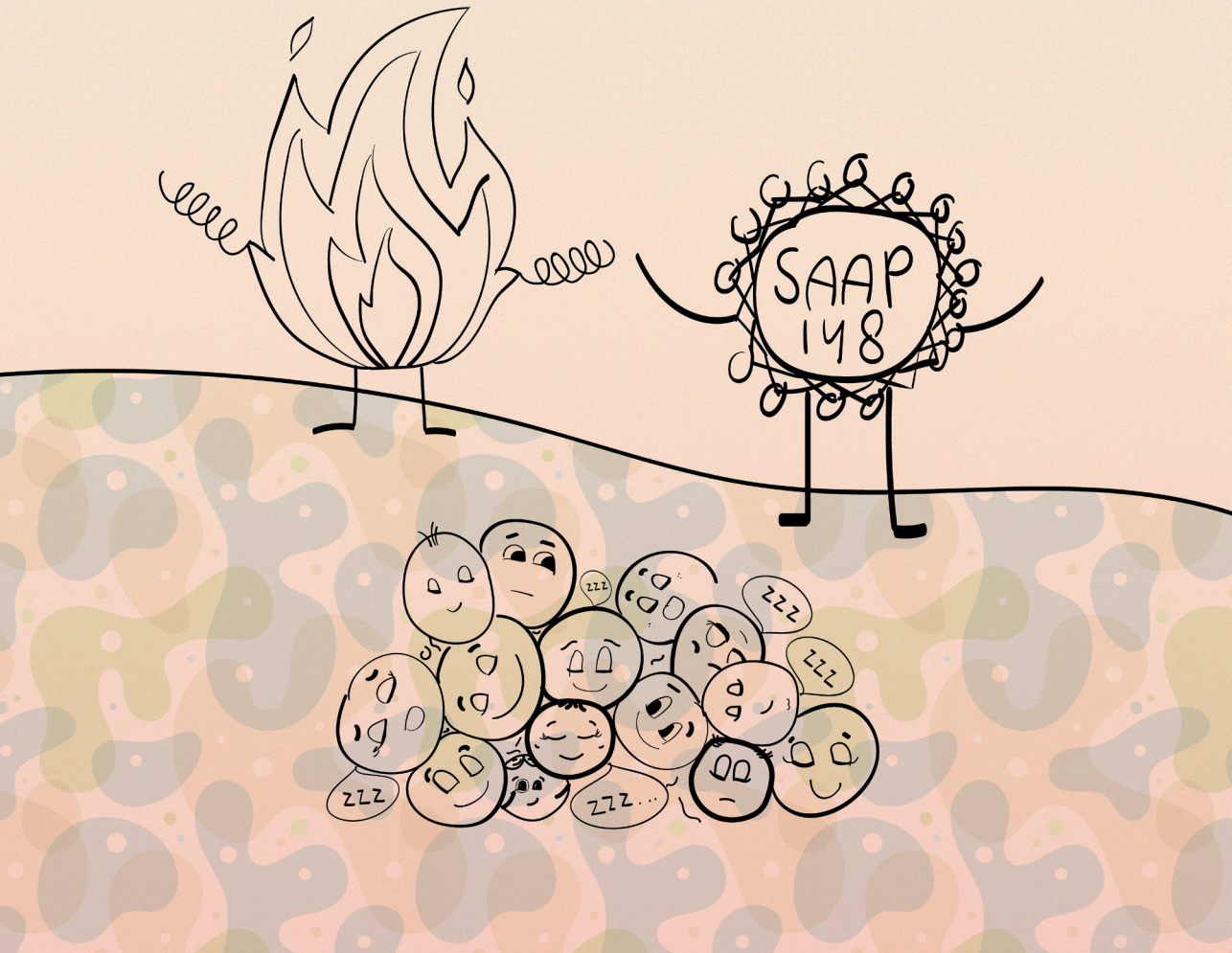

# Non-contact induction heating and SAAP-148 eliminate persisters within MRSA biofilms mimicking a metal implant infection

Mariëlle Verheul[1], Jan Wouter Drijfhout[2], Bart G. Pijls[3,†], Peter H. Nibbering[1,†]

[1] Leiden University Center for Infectious Diseases (LUCID), Leiden University Medical Center, Leiden, the Netherlands

[2] Department of Immunology, Leiden University Medical Center, Leiden, the Netherlands

[3] Department of Orthopedics, Leiden University Medical Center, Leiden, the Netherlands

[†] These authors contributed equally to this work

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*Minor adaptations*

## Graphical abstract

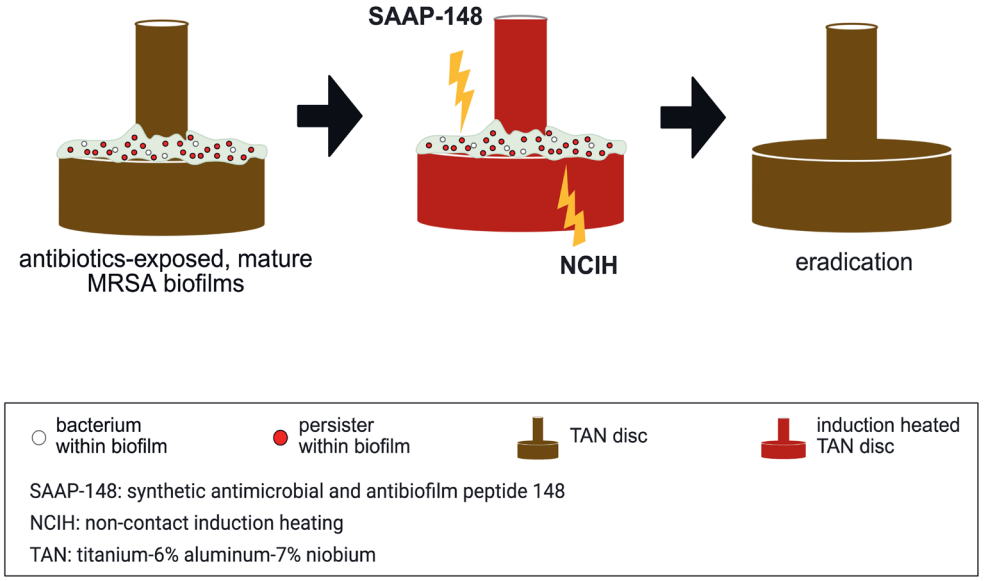


*Figure created using BioRender.com*


## Abstract

Implant-associated infections are the primary cause of complications following orthopedic surgery. Due to biofilm and persister formation, current treatments, i.e., surgical debridement followed by antibiotics, often fail. There is an urgent need for alternative strategies to combat such infections. Therefore, the present study investigated the effects of non-contact induction heating (NCIH), the antimicrobial peptide SAAP-148, and combinations thereof on bacterial counts in seven-day mature biofilms and in persister-enriched biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) on titanium-aluminum-niobium (TAN) discs. Enrichment of persisters was achieved by daily exposure of mature biofilms to high doses of rifampicin and ciprofloxacin for three consecutive days. To heat up the TAN discs, a miniaturized induction heater was built and successfully validated. Using this apparatus, NCIH resulting in surface temperatures up to 85 °C eradicated all the bacteria in immature biofilms but not in mature biofilms, whereas persisters were already eliminated at surface temperatures ≥ 70 °C. SAAP-148 at concentrations > 25.6 µM reduced the persister counts in antibiotics-exposed mature biofilms. As surface temperatures > 60 °C can have detrimental effects on the surrounding tissues, the maximum temperature of NCIH used in combination with SAAP-148 on persisters was set to 60 °C. Results revealed that this combination was slightly more effective than the peptide or NCIH alone in eliminating biofilm-embedded persisters. NCIH and SAAP-148 can be applied both invasively and non-invasively in various treatment scenarios. Together, combinations of NCIH and SAAP-148 might be a promising treatment strategy to combat metal-implant associated infections.

## Introduction

An implant-associated infection is a major complication of orthopedic surgery and is responsible for implant failure. Current treatments often fail to combat these persistent infections, which results in high morbidity and mortality rates, and a major economic burden (1, 2). Infections of (prosthetic) implants are predominantly caused by *Staphylococci,* such as methicillin-resistant *Staphylococcus aureus* (MRSA) that form a biofilm on the implant surface (3, 4). The extracellular polymeric matrix of the biofilm protects the biofilm-embedded bacteria from the actions of antibiotics, as well as effector cells and molecules of the immune system (5). Also, the various bacterial subpopulations within the biofilm show different metabolic activities, which alter their response to antibiotics, i.e., subpopulations with low metabolic activity tolerate antibiotics and will therefore persist within the biofilm (6). Upon discontinuation of the antibiotic treatment, these persisting bacteria can cause infection relapse (7), thus explaining the failure of the antibiotic treatment (8). Other treatments that are used to remove implant-associated infections include debridement, antibiotics and implant retention (DAIR) and revision surgery (9, 10). Unfortunately, these treatments are invasive, often not successful and associated with additional complications.

Novel non-invasive strategies, such as heating up metal implants by using high-frequency alternating magnetic fields (AMF; also known as induction heating), have been proposed to reduce or even eradicate 24 h bacterial biofilms (11, 12). The principle of AMF to generate heat in metal objects has been used on several biomedical technologies as it specifically heats up metals and does not directly harm the surrounding tissue (13). Recently, the effect of heat generation by AMF has been tested in an *in vitro* model of *Pseudomonas aeruginosa* and MRSA biofilms on metal implant mimics (11). Exposure for longer than 3 min to ~ 80 °C results in elimination of bacteria within biofilms and increased sensitivity of bacteria to antibiotics. Similarly, heating up titanium alloys by non-contact induction heating (NCIH) results in a temperature of 80 °C after 3.5 min and almost total eradication of the bacterial load of MRSA 24 h biofilms (14). Moreover, a synergistic effect could be observed upon the combination of 60 °C NCIH and antibiotics, resulting in a significant elimination of viable bacteria within biofilms (14). Another alternative strategy for implant-associated infections is the use of antimicrobial peptides such as the synthetic antimicrobial and anti-biofilm peptides (SAAP; (15)). SAAPs were developed based on the blueprint of human antimicrobial peptide LL-37 and exert a lower rate of resistance development due to their multifunctional mechanisms of action (16, 17). Notably, SAAP-148 has shown promising anti-biofilm activity by totally eradicating MRSA biofilms from polystyrene surfaces, wounded *ex vivo* human skin, and superficially wounded murine skin (18). Moreover, SAAP-148 is more effective than several other antimicrobial peptides in eliminating bacteria, including persisters, in mature MRSA biofilms on various surfaces including titanium-aluminum-niobium (TAN) discs (19).

The aim of the present study was to assess the effect of NCIH and NCIH in combination with SAAP-148 on bacteria in biofilms on metal surfaces mimicking a metal implant-associated infection. For this purpose, persisters in seven-day mature biofilms were obtained by high dose antibiotic treatment and subsequently exposed to NCIH, SAAP-148 and combinations thereof. As the TAN discs were designed to fit in the wells of a 96-wells plate, a custom-built NCIH device was developed to heat up the TAN discs. The combination of NCIH and SAAP-148 was expected to combat the biofilm-embedded persisters with a high efficacy by attacking the biofilm from both the basal (implant) and top (peri-implant) side.

## Materials and Methods

**Antibiotics and SAAP-148 peptide**
Ciprofloxacin (Sigma-Aldrich, 86393-32-0) was diluted in MilliQ water (5.12 mg/mL) and rifampicin (Sigma-Aldrich, 13292-46-1) in DMSO (4 mg/mL) and stored at -20 °C until use. The SAAP-148 peptide (sequence LKRVWKRVFKLLKRYWRQLKKPVR) was synthesized by solid phase chemistry on an automated multiple peptide synthesizer (SyroII, MultiSyntech, Witten, Germany) as described elsewhere (18). The molecular mass of the N-terminal acetylated and C-terminal amidated peptide was confirmed by mass spectrometry and the purity of the peptide exceeded 95%, as determined by reverse phase high-performance liquid chromatography and detection at 214 nm. The lyophilized peptides were stored at -20 °C until use.

**Titanium-aluminum-niobium discs**
Medical grade titanium-7% aluminum-6% niobium (TAN; iso5832/11) discs (diameter 5 mm, height 1.5 mm) with a handle allowing gentle removal of the discs from the wells made specifically to fit in 96-wells plates were a kind gift of dr. T.F. Moriarty (AO Research Institute, Davos, Switzerland). For re-use, TAN discs were rinsed and submerged overnight in 70% EtOH after which they were air-dried and autoclaved.

**Non-contact induction heating (NCIH)**
TAN discs were exposed to a pulsed electro-magnetic field (PEMF) of 101 kHz at a maximum of 135 Watts from a custom-built induction heater (**Figure 1A**). The induction heater featured a solenoid type coil with 10 turns of 6 mm copper tubing and it was designed to accommodate a 50 mL plastic centrifuge tube. This tube was equipped with a silicon mold in the center to fit a 2 mL micro-tube with the TAN disc inside. This set-up was designed to consistently place the TAN disc in the center of the PEMF generated by the solenoid coil, therefore allowing temperature control by adjusting the heating time when the association between time and temperature was known. The relationship between time and temperature was determined in triplicate with two fiber-optic temperature sensors connected to a fiber-optic signal conditioner (Neoptix, ReFlex-4; Neoptix Inc, Quebec City Canada; **Figure 1B**). Fiber-optic temperature sensors were not affected nor heated by the PEMF, therefore ensuring reliable temperature measurements in the presence of a PEMF.

**Culture of methicillin-resistant *Staphylococcus aureus* (MRSA)**
Frozen stocks of MRSA LUH14616 (sequence type 247, NCCB 100829, kindly provided by dr. S. Croes, Department of Medical Microbiology, Maastricht University Medical Center, Maastricht, the Netherlands) in 20% glycerol were thawed to spread on trypticase soy agar with 5% sheep blood plates (Biomerieux, Marcy-l'Étoile, France, 43009) and cultured overnight at 37 °C. Subsequently, several colonies were taken from these plates and cultured in tryptic soy broth medium (TSB, Oxoid, Basingstoke, Hampshire, UK, CM0129) for 2.5 h at 200 rpm and 37 °C. The mid-logarithmic phase bacteria were harvested by centrifugation (1,000x *g* for 10 min), washed with phosphate-buffered saline (PBS; pH 7.4) and then, based on the optical density at 600 nm, diluted to 1 x $10^7$ colony-forming units (CFU)/mL in the desired medium.

**MRSA biofilm and persister formation**
MRSA was diluted in brain heart infusion broth (BHI, Oxoid, CM1135) for seven-day biofilms and in BM2 medium (62 mM potassium phosphate buffer (pH 7) with 7 mM $(NH_4)2SO_4$, 200

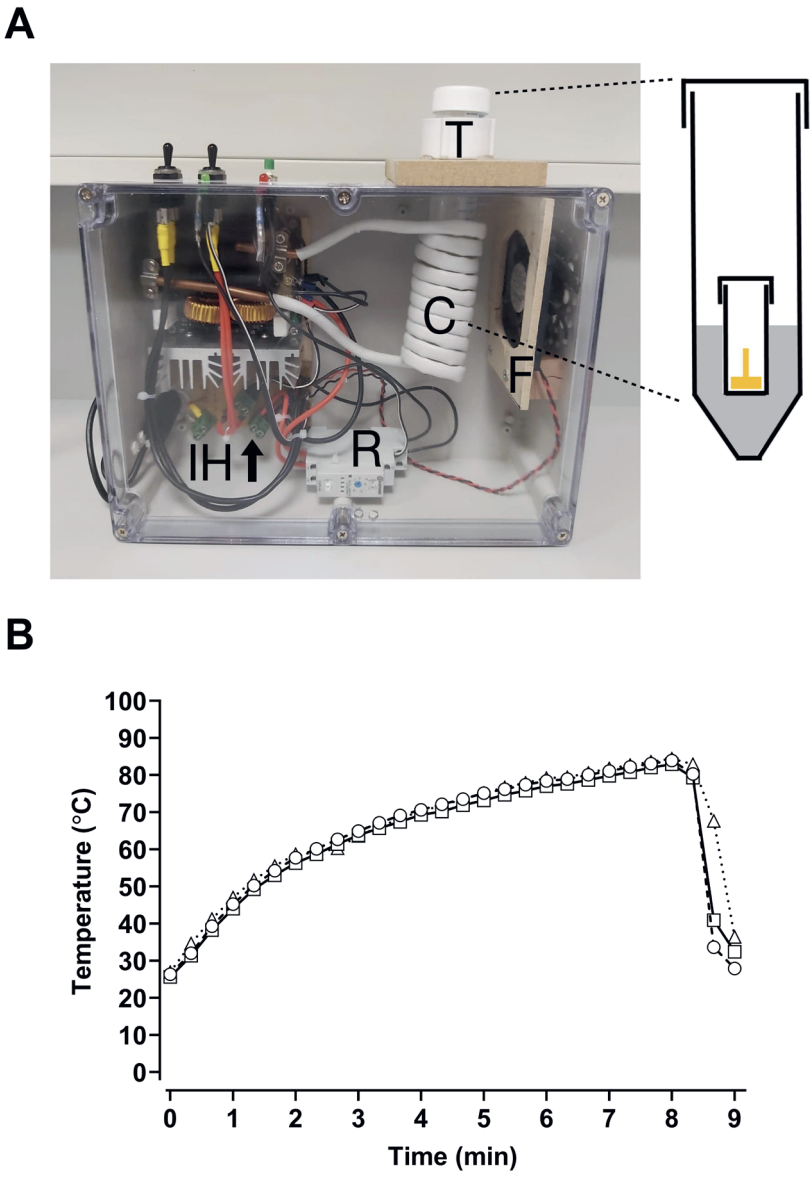


**Figure 1. The relationship between duration of induction heating by the custom-built non-contact induction heating (NCIH) equipment and surface temperature of titanium-aluminum-niobium (TAN) discs.** (**A**) The custom-built induction heater and a schematic drawing of the orange TAN disc in the 2 mL micro-tube, placed in a silicon mold (gray) in the center of the 50 mL plastic centrifuge tube (IH = induction heater circuit; C = coil to generate a pulsed electromagnetic field; T = docking station for 50 mL tube; F = fan for convection cooling of coil and induction heater circuit; R = time release to control heating time). (**B**) Fiber-optic measurements were performed to determine the relationship between the duration of NCIH and the surface temperature of TAN disc in phosphate buffered saline. Results are means of duplicates from three independent runs plotted per 20 sec for the duration of 9 min.

mM $MgSO_4$, 10 mM $FeSO_4$, 40% glucose with 50% casamino acids) for 24 h biofilms. Briefly, 100 µL of bacterial suspension was pipetted into 96-wells plates containing TAN discs. After 24 h or seven days, the wells were washed and the discs with biofilms were gently transferred to polystyrene 96-wells plates or polypropylene 96-wells plates for exposure to antibiotics or SAAP-148, respectively. Plates with 24 h biofilms were sealed with non-breathable plastic sealing film (Amplistar Adhesive Plate Sealers, Westburg, Leusden, the Netherlands, WB 2-3830). Mature biofilms on plates were sealed with breathable rayon sealing film (VWR, 391-1262). For enrichment in persisters, mature biofilms were washed daily with PBS and then 100 µL of BHI suspension with high-dose ciprofloxacin (1280 µg/mL) and rifampicin (10 µg/mL) was added. After 24 h, the antibiotics were refreshed daily for up to five days. Controls received similar concentrations of BHI suspension in MilliQ water. Biofilms were allowed to form in a plastic box at 37 °C to maintain a humidified environment. Medium controls were used to monitor any possible contamination.

**Effect of treatments on bacterial counts in biofilms**

Biofilms were exposed to NCIH at various target temperatures (60 to 85 °C), to SAAP-148 peptide or to combinations of NCIH and SAAP-148 at various dilutions in PBS for 2 h. For these combinations, biofilms were first exposed to the peptide and subsequently to NCIH. Prior to treatment, planktonic bacteria were removed from the wells by two washes with PBS. After treatment, TAN discs with biofilms were transferred to 96-wells plates containing 100 µL of PBS and sonicated (10 min at 40 kHz). Serial dilutions of bacterial suspensions were plated on Mueller Hinton agar plates (Oxoid, CM0337) and CFU was counted after incubation overnight (37 °C).

**Data analysis**

Kruskal-Wallis tests followed by Mann-Whitney U tests (GraphPad Software 9) were performed to determine the significance of the difference in CFUs between treated and control samples. $p < 0.05$ was considered statistically significant.

## Results

**Calibration of the heat induction equipment**

The temperature at the surface of TAN discs was measured every 1 sec during a period of 8 min of NCIH using fiber-optic sensors to establish the relationship between the duration of NCIH and the surface temperature of TAN discs in PBS (**Figure 1B**). Results revealed that within the first 2 min the surface temperature rapidly increased from 27 °C to 60 °C. Thereafter, the surface temperature linearly increased by 10 °C per 2 min until 80 °C and, subsequently, increased up to 85 °C during the 2 min interval. After transfer of the TAN discs to wells containing PBS at 37 °C, the temperature of the discs dropped to ambient temperature within 1 min. Based on these data, a time relay was used to control the heating time. The times for induction heating of TAN discs in presence of 100 µL of PBS were set at 2 min for 60 °C, 4 min for 70 °C, 6 min for 80 °C and 8 min for 85 °C.

**Effect of heat-treatment duration on the bacterial counts in seven-day mature biofilms**

To determine if reaching the temperature rather than the duration of exposure to that temperature killed the bacteria within biofilms, the number of viable bacteria within seven-day mature biofilms on TAN discs was determined upon exposure to a range of temperatures (60, 70 or 80 °C) using a heat-block for various durations. Results revealed a heat-dependent reduction in biofilm-embedded bacteria, with a maximal reduction down to 500 CFU/mL

after exposure to 80 °C. Longer exposure of biofilms (4 min compared to 2 min) to the heat-block at 60 °C did not show a further reduction in bacterial load. Further, subjecting the biofilms for 4 min at 70 and 80 °C exerted a maximal, negligible 10-fold reduction in CFU/mL compared to 2 min exposure to these temperatures. Data indicated that longer durations at a selected temperature resulted in a steady-state antibacterial effect rather than a further reduction in bacterial counts in the biofilms.

**Development of the *in vitro* model for persisters in biofilms**

To develop an *in vitro* model that partially mimicked metal implant-associated infections, the effects of daily exposure to high doses of rifampicin and ciprofloxacin on bacteria residing within seven-day mature MRSA biofilms on TAN discs for a period of five days were determined. Results revealed that the number of viable bacteria in the biofilms rapidly dropped ($p < 0.05$) from approximately 1 x $10^8$ CFU/mL to about 1 x $10^5$ CFU/mL during the first two days of antibiotic exposure and thereafter the bacteria tolerated the antibiotics for at least an additional three days (**Figure 2A**). The numbers of viable bacteria in control biofilms did not change ($p > 0.05$) during these five days (**Figure 2B**). Based on these data, three days of antibiotic treatment has been used in further experiments to study the effects of innovative treatments on persisters in biofilms.

**Effect of heat induction on bacteria in biofilms and persisters**

To allow for a comparison with previously published studies on NCIH with 24 h biofilms, the effects of NCIH on bacteria in 24 h MRSA biofilms on TAN discs were first determined after various durations of induction heating. Results revealed a surface temperature-dependent reduction ($p < 0.05$) in bacterial counts, with complete eradication of the bacteria seen at a surface temperature of 85 °C (**Figure 3A**). However, a maximum 5-log reduction in bacterial



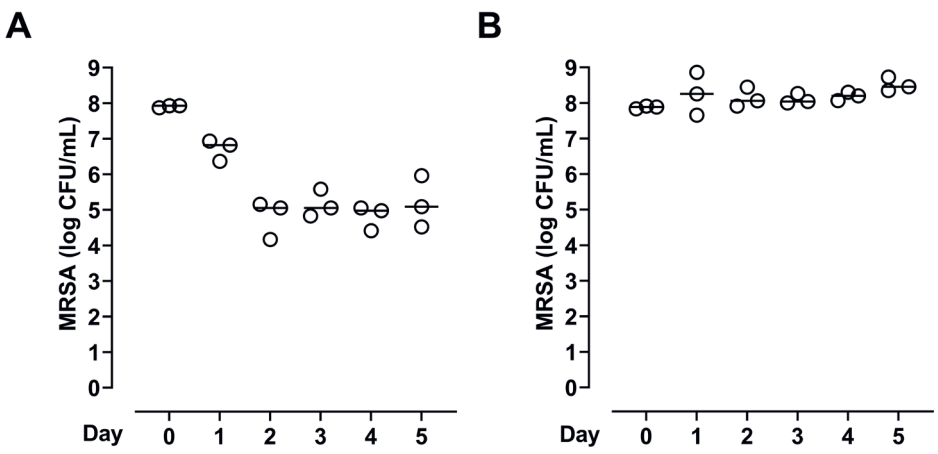


**Figure 2. Effect of high-dose antibiotics on bacteria in mature biofilms.** Seven-day mature *Staphylococcus aureus* LUH14616 (MRSA) biofilms on titanium-aluminum-niobium (TAN) discs were exposed daily for up to five consecutive days to a combination of (**A**) high doses of rifampicin and ciprofloxacin or (**B**) the diluent of the antibiotics as control. After each day, TAN discs with biofilms were transferred to wells containing phosphate buffered saline, sonicated, and thereafter the number of viable bacteria was assessed microbiologically by counting the colony forming units (CFU). Results are from three independent experiments each performed in triplicate; the solid line indicates the median log CFU/mL. Significance of the differences between the days of exposure was calculated using Kruskal-Wallis test *(***A***: $p < 0.05$,* **B***: $p > 0.05$).*

counts was seen for MRSA in seven-day mature biofilms on TAN discs after heating the surface to ≥ 70 °C (**Figure 3B**), indicating that NCIH was not sufficient to eradicate all biofilm-encased bacteria. Regarding the persister model, NCIH of the TAN discs to 60 °C led to a 10-fold reduction of the number of persisters (**Figure 3C**), whereas heating to surface temperatures ≥ 70 °C resulted in total eradication of persisters within the biofilms ($p < 0.001$).

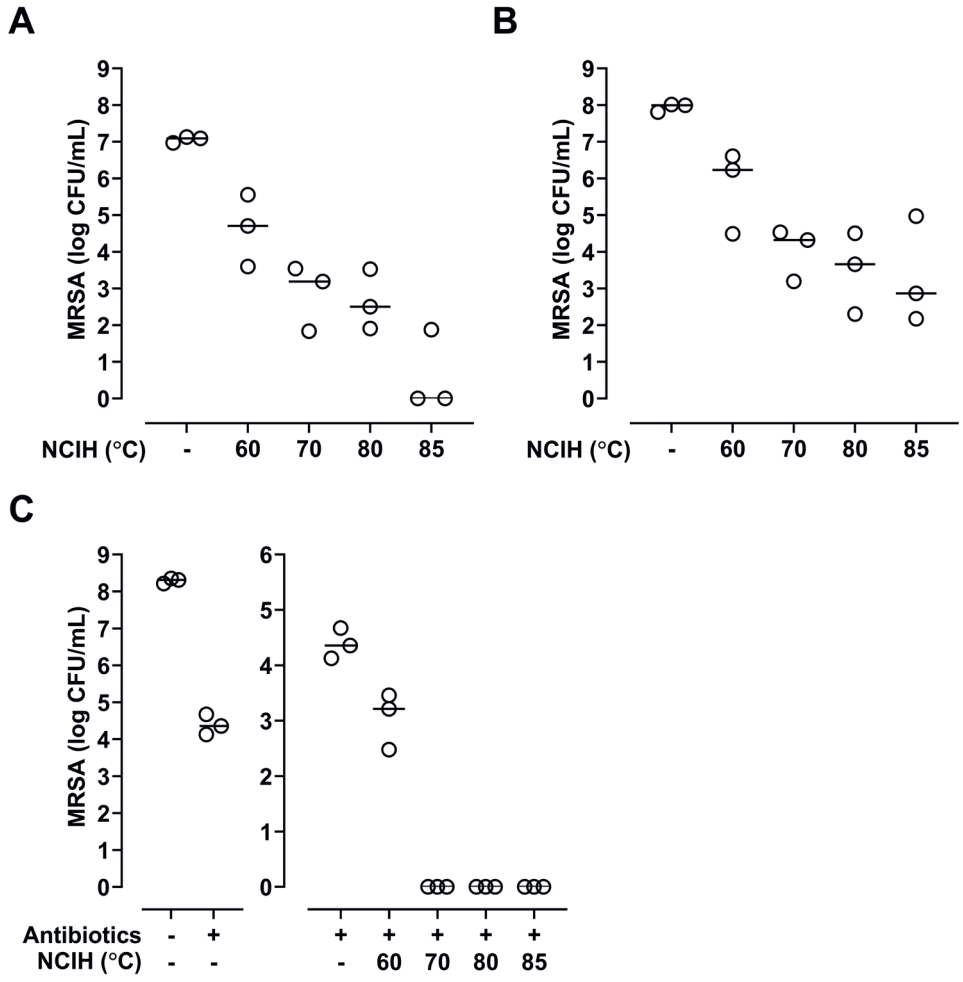


**Figure 3. Effect of surface temperature on bacteria, including persisters, residing in biofilms.** (**A**) 24 h methicillin-resistant *Staphylococcus aureus* LUH14616 (MRSA) biofilms, (**B**) seven-day mature MRSA biofilms, and (**C**) seven-day mature, three day antibiotics (high-dose rifampicin and ciprofloxacin)-exposed MRSA biofilms on titanium-aluminum-niobium (TAN) discs were transferred to vials containing PBS for surface heating by non-contact induction heating (NCIH) for various intervals. Thereafter, the TAN discs with biofilms were transferred to wells containing PBS and subjected to sonication for microbiological determination of the number of viable bacteria. Results are from three independent experiments each performed in quadruplicate; the solid line indicates the median log colony forming units (CFU)/mL. Significance of the differences between the various surface temperatures was calculated using Kruskal-Wallis test (**A**, **C**: $p < 0.0001$, **B**: $p < 0.05$).

**Effect of SAAP-148 monotherapy and in combination with induction heating on persisters within biofilms**

Since persisters are the main cause of therapy failure in implant-associated infections, the effect of SAAP-148 on persisters in antibiotics-exposed mature biofilms on TAN discs was first confirmed. Results revealed a dose-dependent reduction ($p < 0.05$) in bacterial counts for persisters in a biofilm by peptide concentrations > 12.8 µM, with almost all persisters eradicated by the highest peptide concentration (51.2 µM) (**Figure 4**). Next, the effect of SAAP-148 followed by induction heating to a surface temperature of 60 °C on persisters in biofilms was determined. Results showed that this combination was slightly more effective in reducing persisters in antibiotics-exposed mature biofilms than the peptide or NCIH alone. Mann-Whitney U test indicated that the combination of NCIH with 12.8 µM SAAP-148 was significantly ($p < 0.05$) more effective than the peptide, but not induction heating, alone (**Figure 4**).

## Discussion

Two innovative, non-antibiotic strategies to combat metal implant-associated infections with MRSA were investigated using a novel *in vitro* model (19). For this model, seven-day mature biofilms on TAN discs were developed and exposed for three days to high doses of rifampicin and ciprofloxacin, an antibiotic combination that is commonly used to treat implant-associated infections caused by *Staphylococcus aureus*/MRSA. Control experiments

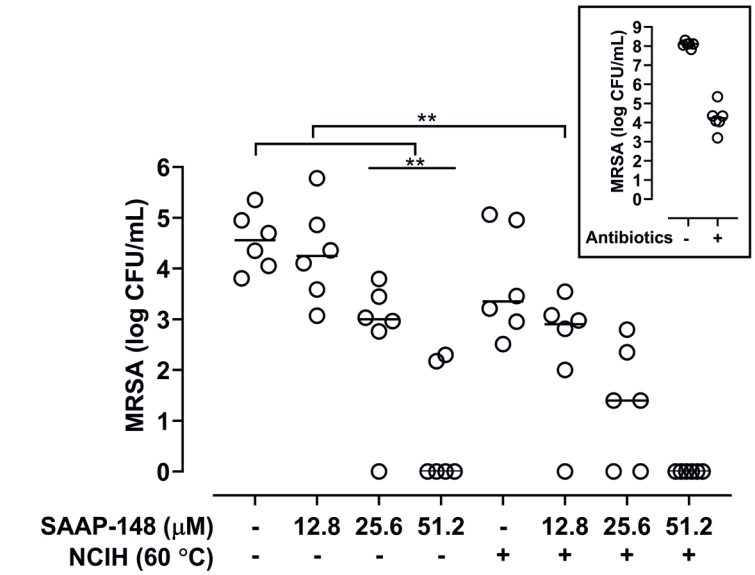


**Figure 4. Effect of SAAP-148 in combination with non-contact induction heating (NCIH) on persisters within MRSA biofilms.** Seven-day mature methicillin-resistant *Staphylococcus aureus* LUH14616 (MRSA) biofilms on TAN discs were exposed daily to high-dose antibiotics (rifampicin and ciprofloxacin) and subjected for 2 h to increasing concentrations of SAAP-148 with or without subsequent NCIH to 60 °C. The insert indicates the effect of antibiotics on bacteria in the mature biofilm. All biofilms were subjected to sonication for microbiological determination of the number of viable bacteria. Results are from six independent experiments each performed in quadruplicate with a solid line indicating the median log colony forming units (CFU)/mL. Significance of the differences between the various conditions was calculated using Kruskal-Wallis test ($p < 0.0001$) and subsequently Mann-Whitney U tests. ** $p < 0.01$.

indicated that the surviving bacteria indeed developed tolerance to the subsequent antibiotic treatment. First, the effects of NCIH and of the novel antimicrobial peptide SAAP-148 on bacterial subpopulations including persisters in biofilms were determined. To circumvent undesirable side-effects of these treatments on peri-implant tissues, the second strategy focused on establishing the possible additive effects of combining NCIH with SAAP-148 on the counts of persisters in biofilms.

The results showed a temperature-dependent reduction in bacterial counts in immature biofilms with complete bacterial eradication seen at a surface temperature of 85 °C, whereas bacterial counts in mature biofilms decreased in a temperature-dependent fashion up to 70 °C and thereafter remained unchanged. The observed effect of NCIH on immature biofilms is in line with our earlier observation that almost all bacteria within 24 h biofilms are eliminated by a heat shock of 85 °C (14). The difference in susceptibility of bacteria in mature and immature biofilms to induction heating could be related to the amount of protective extracellular matrix, which is considerably larger in mature biofilms. Further, the present study focused predominantly on (persister-enriched) mature biofilms since others have shown that 24 h biofilms may not be optimal to study the effects of novel strategies and drugs on bacteria in mature biofilms (20). In agreement, Cazander et al. reported that at least five to eight days are required to develop the mature biofilm profile that is observed in clinical implant-associated infections (21). Since the persister subpopulation is the major cause of treatment failure in metal implant-associated infections, the finding that NCIH resulting in temperatures ≥ 70 °C completely eradicated all bacteria in persister-enriched mature biofilms are of particular importance. The difference in the efficacy of NCIH on bacteria in mature biofilms and persisters within biofilms may be related to the bacterial numbers in these models; the number of persisters being 10,000-fold lower than the number of bacteria in mature biofilms. As reported earlier (19), the SAAP-148 peptide at the highest non-hemolytic concentration (18) effectively reduced the counts for persisters in antibiotics-exposed mature biofilms, but does not eradicate all persisters.

Both NCIH and SAAP-148 should not result in adverse effects, such as cytotoxicity. In this context, temperatures > 60 °C were considered undesirable due to the risk of transmission, to some extent, of heat to the peri-implant tissue. This is based on the observation that induction heating of a titanium memory rod up to 60 °C in rats does not cause necrosis (22). Chopra et al. reported that *in vivo* alternating magnetic field exposure reaching temperatures up to 100 °C results in (irreversible) damage to tissues closely surrounding the metal implant (11). The present study revealed that exposure of antibiotics-exposed mature biofilms to SAAP-148 for 2 h followed by induction heating to 60 °C was slightly more effective than the heat shock or the peptide alone, with the highest concentration of the peptide in combination with induction heating completely eradicating all persisters in biofilms. Of note, the effect of the combination did not depend on the order in which the peptide and induction heating were applied (results not shown). A possible explanation for this enhanced effect of NCIH in combination with SAAP-148 on persisters in mature biofilms could be that the treatments attack the bacteria in the biofilms from different sides, i.e., NCIH from the basal side (implant) and the peptide from the top (peri-implant). In addition, the modes of action of these treatments also differ completely. Based on these findings, it was concluded that combinations of NCIH and SAAP-148 could become a promising strategy for the treatment of metal implant-associated infections.

To heat up the TAN discs in a controlled fashion, a miniaturized version of the NCIH

apparatus was built and successfully validated. This equipment was designed to reach a target temperature rather than to maintain the temperature for a prolonged duration, as was the case in previous NCIH studies (14). The rationale for this approach was twofold. First, a closed feedback temperature control system for non-invasive heating at 60 °C may be difficult to achieve clinically (23), indicating that the NCIH model featured here represents a practical approach. Secondly, the temperature rather than duration of that temperature may be more effective against bacteria. In agreement, experiments investigating the effects of duration of exposure to a temperature using a heat block revealed that longer durations at a selected temperature resulted in a steady-state antibacterial effect rather than a further reduction in bacterial counts in biofilms. In line with this, recent studies on thermal stability of proteins among different species (e.g., human, bacterial, and fungi) have suggested that stability of key proteins of these pathogens is affected by lower temperatures than that of human proteins (24, 25).

The future medical application of NCIH could be divided into non-invasive and invasive use. During the non-invasive use, the metal implant is heated non-invasively through the skin, while great care is taken to avoid excessive heating to areas where the implant is fixed to the bone or areas that are in close contact with crucial anatomical structures such as nerves and major blood vessels. This may be performed in a segmental way with tailored heating protocols for each segment: segmental induction heating (26). In the future, the non-invasive application of induction heating may first help patients for whom surgical treatment is not possible and who, for instance, receive suppression antibiotic therapy. NCIH will most likely be part of a multi-modality treatment that also involves antibiotics and adjuvants (non-antibiotic chemical agents). Alternatively, NCIH can be used invasively, e.g., during surgery, to increase the effectiveness of the surgical procedure such as in DAIR: NCIH kills micro-organisms by heat and it could be used to heat the implant and parts of the implant that cannot be reached and mechanically cleaned. The soft tissue can be kept away from the heated part of the implant in order to protect it from thermal damage. Furthermore, surgery allows for direct temperature control by direct temperature measurement (e.g., thermocouple) or infrared thermal imaging.



However, it should be mentioned that the current *in vitro* model for metal implant-associated infections suffers from some limitations. First, all experiments were performed with only a single strain (although a clinical isolate) of MRSA (18). Another major limitation is the absence of peri-implant tissue and local factors, such as exudate or wound fluid. These limitations may be circumvented in future experiments by using TAN discs in 12-well plates containing cultures of relevant cell types, such as myeloid-derived suppressive cells (27), inflammatory macrophages and wound fluid collected from patients undergoing prosthetic joint or hip replacement surgery. While the current experiments have focused on the effects of combined treatment on persisters in mature biofilms, the effects of this combination on bacteria in mature biofilms remain unexplored. Lastly, it is unknown whether bacteria in mature biofilms can become tolerant to induction heating in the unlikely event of receiving multiple heat shocks as treatment.

## Conclusion

Neither NCIH nor the SAAP-148 peptide at clinically desirable conditions could eradicate all MRSA subpopulations residing within antibiotics-exposed mature biofilms on TAN discs. Combinations of SAAP-148 and induction heating to 60 °C were slightly more effective

than these approaches alone. Of note, the combination of the highest non-hemolytic concentration of SAAP-148 and induction heating to 60 °C resulted in complete eradication of all bacterial subpopulations in MRSA biofilms. Furthermore, NCIH and the peptide can be applied both invasively and non-invasively. Therefore, combinations of NCIH and SAAP-148 may be useful as a treatment for patients with implant-associated infections in whom the current treatment has failed. Moreover, such combinations could also be applied in an earlier stage of the treatment, e.g., during debridement of the implant, or even when replacing the current treatment involving debridement and antibiotics.

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## Conflict of interest

One of the authors (BGP) is listed as a co-inventor on a provisional patent application from the Leiden University Medical Center (WO2020/067898). The Leiden University Medical Center is also holder of patent no. WO2015088344 (co-inventors JWD and PHN) relating to the peptide described in this paper.

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