European Cells and Materials Vol.51 2025 (pages 136–144) DOI: 10.22203/eCM.v051a08 ISSN 1473-2262

Original Article

NISIN-ENRICHED COATINGS ON TITANIUM IMPLANTS PREVENT *STAPHYLOCOCCUS AUREUS* BIOFILM FORMATION: THE *GALLERIA MELLONELLA* MODEL AS A TESTING PLATFORM

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Abstract

Background: Bone and joint infections pose significant clinical challenges, often leading to severe complications and substantial healthcare costs. Traditional antibiotic therapies are becoming increasingly ineffective due to rising antibiotic resistance and the biofilm-forming ability of bacteria such as *Staphylococcus aureus* (*S. aureus*). Antimicrobial coatings offer a promising approach for the prevention and treatment of implant-associated and bone infections. Following the demonstrated *in vitro* efficacy of nisin, a naturally occurring antimicrobial peptide, in preventing *S. aureus* biofilm formation, this study investigates the *in vivo* potential of a nisin-enriched coating to prevent biofilm-related infections using the *Galleria mellonella* larva haematogenous implant infection model. **Methods**: Methicillin-sensitive *S. aureus* (MSSA) EDCC 5055 was used to infect larvae implanted with nisin-coated titanium Kirschner wires (K-wires). Survival rates and bacterial loads on both the K-wires and in larval tissue were analysed. Biofilm formation on K-wires was further analysed using scanning electron microscopy. **Results**: The results showed that nisin-coated K-wires significantly improved larval survival and reduced bacterial burden compared to control groups. Scanning electron microscopy confirmed the absence of biofilm formation on nisin-coated K-wires. **Conclusions**: These findings suggest that nisin-enriched coatings could be a viable strategy for preventing bone and joint infections. Additionally, this study demonstrates the feasibility of testing implant coatings in a cost-effective and ethically sound alternative *in vivo* model. Further evaluation and testing of the nisin-enhanced coating in vertebrate animal implant infection models is warranted.

Keywords: Nisin, titanium implant, *Staphylococcus aureus*, *Galleria mellonella*.

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Introduction

Bone and joint infections present significant challenges in clinical settings, often leading to severe complications, prolonged treatment durations, and substantial healthcare costs [1,2]. Addressing these infections is crucial, particularly in light of the increasing prevalence of antibiotic resistance, which compromises the effectiveness of conventional antibiotic therapies [3].

Biofilms, which are structured bacterial communities encased in a self-produced polymeric matrix, present a significant treatment challenge due to their enhanced resistance to antibiotics and the host immune response [4]. Although traditional antibiotic coatings may initially inhibit bacterial attachment to implant surfaces, they frequently lose efficacy over time, either due to insufficient long-time release or the development of bacterial resistance mechanisms [5]. This reduced efficacy not only compromises treatment but also contributes to the broader public health crisis of infections caused by antibiotic-resistant bacteria.

In search of alternative strategies, antimicrobial peptides (AMPs) have emerged as promising candidates due to their broad-spectrum activity and lower likelihood of inducing resistance [6]. Among these, nisin, a naturally occurring AMP produced by *Lactococcus lactis*, stands out [7]. Nisin has been widely used in the food industry as a preservative, recognized for its potent antimicrobial properties and established safety profile [8]. This history of safe use highlights its potential for medical applications, particularly in combating biofilm-related infections.

A recent study has demonstrated the promising application of nisin in medical settings. Specifically, the enrichment of an ultrathin hydrated Layer-by-Layer (LbL) coating, composed of biocompatible polyelectrolytes such as chondroitin sulfate A (CSA) and poly-L-lysine (PLL), crosslinked with genipin (GnP), has shown potential in inhibiting biofilm formation by *Staphylococcus aureus* (*S. aureus*) *in vitro* [9]. In this pioneering work, we used CSA, a polysaccharide found in the bone extracellular matrix, as a promoter of bone cell differentiation at the bone-implant interface. Additionally, we incorporated the PLL polypeptide as a promoter of cell adhesion. By crosslinking amino groups of PLL, GnP enhanced the mechanical properties of the coatings, optimizing their performance in relation to cell behaviour. When enriched with nisin, these films demonstrated a viable strategy for preventing bone and joint infections. However, it remains imperative to conduct *in vivo* investigations to further evaluate the therapeutic potential and validate these promising *in vitro* results.

Our group has recently developed a cost-effective and ethically sound *in vivo* alternative, the *Galleria mellonella* (*G. mellonella*) larvae implant-infection model, for evaluating promising implant materials [10]. Therefore, the present study aims to assess the antimicrobial efficacy of nisin-coated implants in direct infection scenarios, mimicking conditions observed in open fractures or postoperative settings, and establish foundational data using the *G. mellonella* larvae haematogenous implant-infection model. This investigation represents the first instance of testing an antimicrobial coating in this innovative and purposeful screening model, which will serve to prove the concept and lay the groundwork for further preclinical studies.

Materials and Methods

Chemicals

Nisin Z was sourced from Anhui Minmetals Development (Hefei, China). Tris(hydroxymethyl)aminomethane (Tris) or Trizma® base, saline, phosphate-buffered saline (PBS), poly(ethyleneimine) (PEI; branched, 750 kDa, 50 wt % solution in water), chondroitin sulfate A (CSA; 20–30 kDa) and poly-L-lysine (PLL; alpha, linear, 40–60 kDa) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris-NaCl buffer (10 mM Tris, 150 mM NaCl, pH 7.4 adjusted with HCl) was prepared using pure water (resistivity 18.2 MΩ cm). Polyelectrolytes and nisin Z solutions were prepared in Tris-NaCl buffer at concentrations of 5 mg/mL for PEI and 1 mg/mL for CSA, PLL, and nisin. Genipin (GnP), used as a crosslinker, was obtained from Fujifilm (Tokyo, Japan) and dissolved in PBS at a concentration of 0.25 % (w/v).

Bacteria

In the current study, the methicillin-sensitive *S. aureus* (MSSA) EDCC 5055 strain was used. This strain was originally isolated from a human wound infection at the University Hospital Giessen (Giessen, Hesse, Germany) [11]. The *S. aureus* EDCC 5055 strain is well-known for its strong biofilm-forming ability, and its whole genome sequence is publicly available [12]. Brain-Heart-Infusion (BHI) broth (Merck, Darmstadt, Hesse, Germany; 1.10493.0500) was used to culture the bacteria aerobically at 37 °C with constant shaking at 180 rpm. An overnight bacterial culture was diluted 1:100 in fresh BHI broth and incubated at 37 °C under shaking until it reached the logarithmic growth phase. The bacterial cells were then pelleted by centrifugation, washed once with PBS, and resuspended in PBS. The suspension was adjusted to 5×10^7 colony forming unit (CFU)/mL based on optical density measurements at 600 nm. To confirm the inoculum concentration, tenfold serial dilutions were performed, and the samples were plated on lysogeny broth (LB) agar plates (Carl Roth, Karlsruhe, Baden-Württemberg, Germany; Yeast extract-2363.4, Trypton-8952.4, Agar-5210.5, NaCl-3957.2).

Antimicrobial Nisin-Enriched Layer-by-Layer Coating

Titanium Kirschner (K)-wires (designated as "T"; 0.8 mm diameter, 5–6 mm in length; Depuy Synthes, Zuchwil, Solothurn, Switzerland; 492.090) were first rinsed with ethanol, dried, and then cleaned with UV-ozone for 60 min. Afterwards, they were immersed in ethanol overnight and dried again. The K-wires were then placed on a sample holder and immersed for 30 min in a 5 mg/mL PEI Tris-NaCl buffer solution, followed by a 10 min rinse in Tris-NaCl buffer (Fig. 1). PEI was used as a precursor layer before the LbL film buildup, as it is widely accepted that this uniform anchoring layer provides a reproducible surface for LbL substrates [13].

Next, the substrates were immersed for 10 min into a 1 mg/mL CSA Tris-NaCl buffer solution, followed by another 10-min rinse in Tris-NaCl buffer. This process was then repeated with PLL. The alternating process between CSA and PLL was continued until six CSA/PLL layer pairs were assembled. The coating was subsequently rinsed with PBS to remove any aminated molecules of Tris, and crosslinked with GnP in darkness overnight.

After crosslinking, the coated K-wires were rinsed with both PBS and Tris-NaCl buffer. An outer layer of CSA was then adsorbed. Half of the K-wires were immersed in a nisin solution for 48 hours (designated as "T + C + Nisin"), while the remaining K-wires were immersed in Tris-NaCl

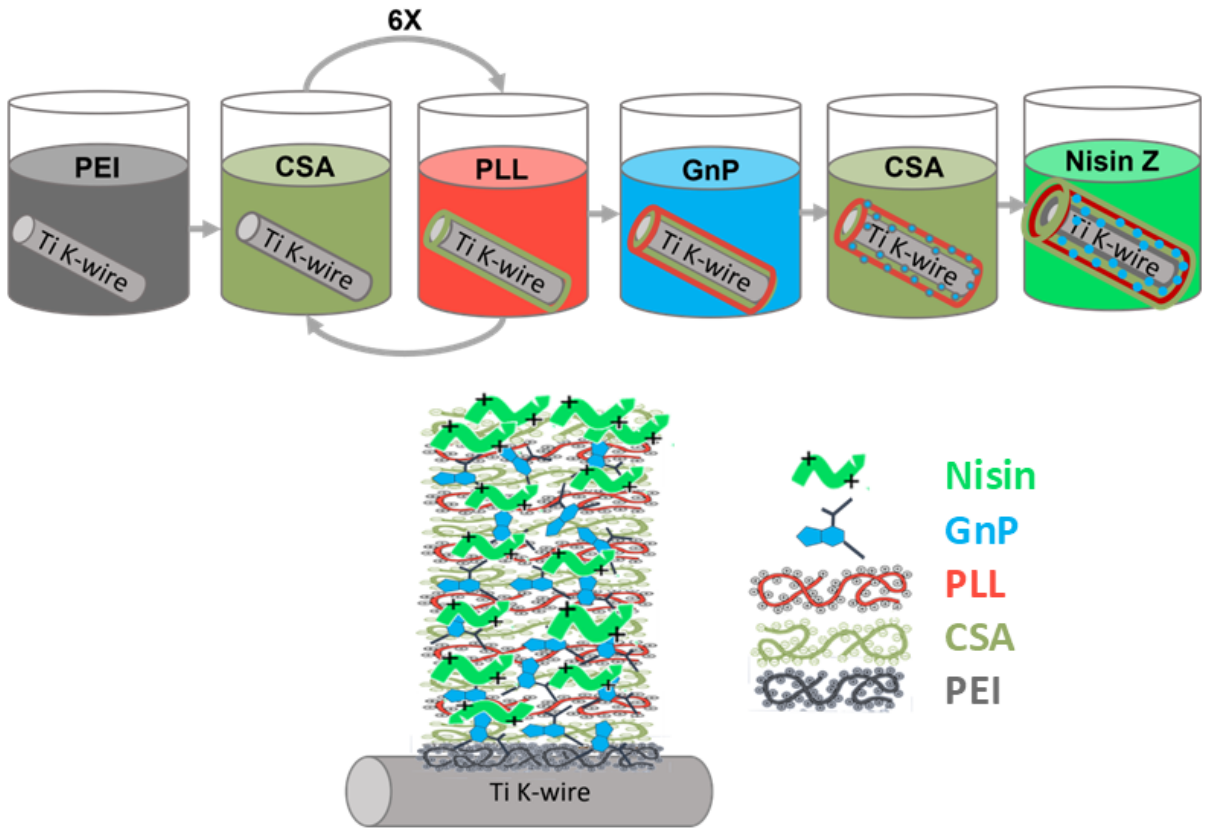

Fig. 1. Schematic representation of the Layer-by-Layer coating process for titanium K-wires. This figure illustrates the step-by-step preparation and coating of titanium K-wires using the Layer-by-Layer (LbL) technique. It depicts the precursor layer application with poly(ethyleneimine) (PEI), alternating deposition of chondroitin sulfate A (CSA) and poly-L-lysine (PLL) layers (6 times), genipin (GnP) crosslinking, and final functionalization with nisin Z to create an antimicrobial coating. The figure was created with Microsoft PowerPoint. K-wires, Kirschner wires.

buffer as controls ("T + C"). Finally, all K-wires were rinsed with Tris-NaCl buffer, followed by PBS, and dried under gentle nitrogen flow.

Galleria mellonella

G. mellonella larvae were obtained from Evergreen GmbH (Augsburg, Bavaria, Germany; 2013) and maintained on a wheat germ diet (Tropic Shop GmbH, Nordhorn, Lower Saxony, Germany; 20008) at room temperature. Throughout the experiment, the larvae were incubated at 37 °C. For survival experiments, ten larvae in the final instar stage, weighing approximately 400–450 mg, were used per group, and each experiment was repeated three times (n = 30 per group). To determine the bacterial load on the K-wire surface and within larval tissue, an additional six larvae per group were utilized.

Antimicrobial Activity of Nisin-Coated K-Wires in the Galleria mellonella Larvae Haematogenous Implant-Infection Model

To evaluate the antimicrobial activity of the nisin coating, nisin-coated K-wires (T + C + Nisin) were implanted into the rear end of the *G. mellonella* larvae by piercing the cuticle with the sharp end of the K-wire (Fig. 2). The larvae were incubated at 37 °C for one hour, after which each larva was injected with 10 μL of an *S. aureus* suspension in PBS (5×10^5 CFU/larva). Larval survival was monitored over a 5-day period. Three control groups were included: titanium K-wires with an injection of 10 μL PBS ("T (non-infected control)"), titanium K-wires with coating process but without nisin ("T + C"), and uncoated titanium K-wires ("T"), both with an injection of 10 μL of *S. aureus* suspension.

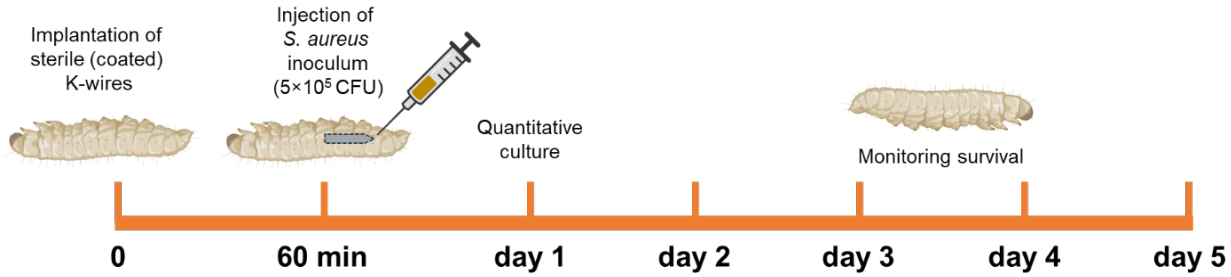

Fig. 2. Schematic representation of the *G. mellonella* haematogenous implant-infection model used in this study. K-wires without coating ("T"), K-wires with the coating process but without nisin ("T + C"), and nisin-coated K-wires ("T + C + Nisin") were implanted into the larvae, and 10 μL *S. aureus* inoculum (5×10^5 CFU/larva) was injected after one hour. As a non-infected control, non-coated K-wires were implanted, followed by an injection of 10 μL after one hour ("T (non-infected control)"). The survival of the larvae was monitored for 5 days. Each experimental group contained 30 larvae. At one day after infection, the number of CFU at the implant surface and in the tissue of the larvae was quantitatively determined (additional larvae, n = 6 per group). The figure was made with Microsoft PowerPoint, with parts being created using https://www.biorender.com. *S. aureus*, *Staphylococcus aureus*; *G. mellonella*, *Galleria mellonella*; CFU, colony forming unit.

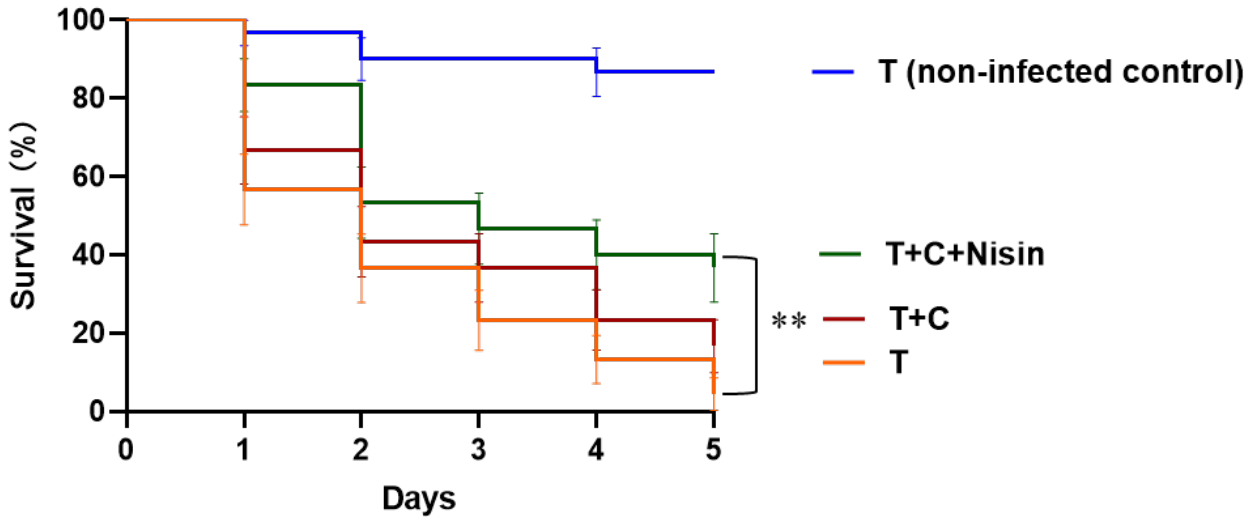

Fig. 3. Prevention of *Staphylococcus aureus* haematogenous implant infection by nisin-coated K-wires in *Galleria mellonella* larvae. Titanium Kirschner wires (K-wires) were implanted into the larvae, followed by an injection of 10 μL *S. aureus* inoculum (5×10^5 CFU/larva) after 1 hour, or an equivalent volume of phosphate-buffered saline (PBS) for the non-infected control group. The graph displays the percentage survival over time (in days) for larvae implanted with uncoated K-wires ("T"), K-wires subjected to the coating process but without nisin ("T + C"), and nisin-coated K-wires ("T + C + Nisin"). Non-infected larvae served as additional controls ("T (non-infected control)"). Survival data were derived from three independent experiments (n = 10 larvae per experiment), and statistical analysis was performed using log-rank test. $^{**}p \leq 0.01$.

Quantitative Culture

The antimicrobial efficacy of the nisin coating was evaluated by determining the bacterial load on the K-wire surface and within the larval tissue. On day 1, K-wires from all experimental groups (n = 6) were explanted from the larvae. Both the K-wires and the larval tissue were collected and processed to quantify the bacterial burden.

The K-wires were subjected to sonication at 45 kHz for 2 min in a water bath sonicator (Ultrasonic Cleaner USC-T; VWR, Ismaning, Bavaria, Germany; 142-0087), while the larval tissue was homogenized using a micro tissue homogenizer (Fisher Scientific, Schwerte, North Rhine-Westphalia, Germany; 15344182). The resulting sonicates from the K-wires and the homogenates from the larval tissue were serially diluted tenfold in PBS and plated onto LB agar plates supplemented with ampicillin to suppress the growth of skin-derived bacteria. The number of CFU per sample was determined after overnight incubation at 37 °C. Bacterial load was expressed as $\log_{10}$ CFU per 100 mg of larval tissue or $\log_{10}$ CFU per implant.

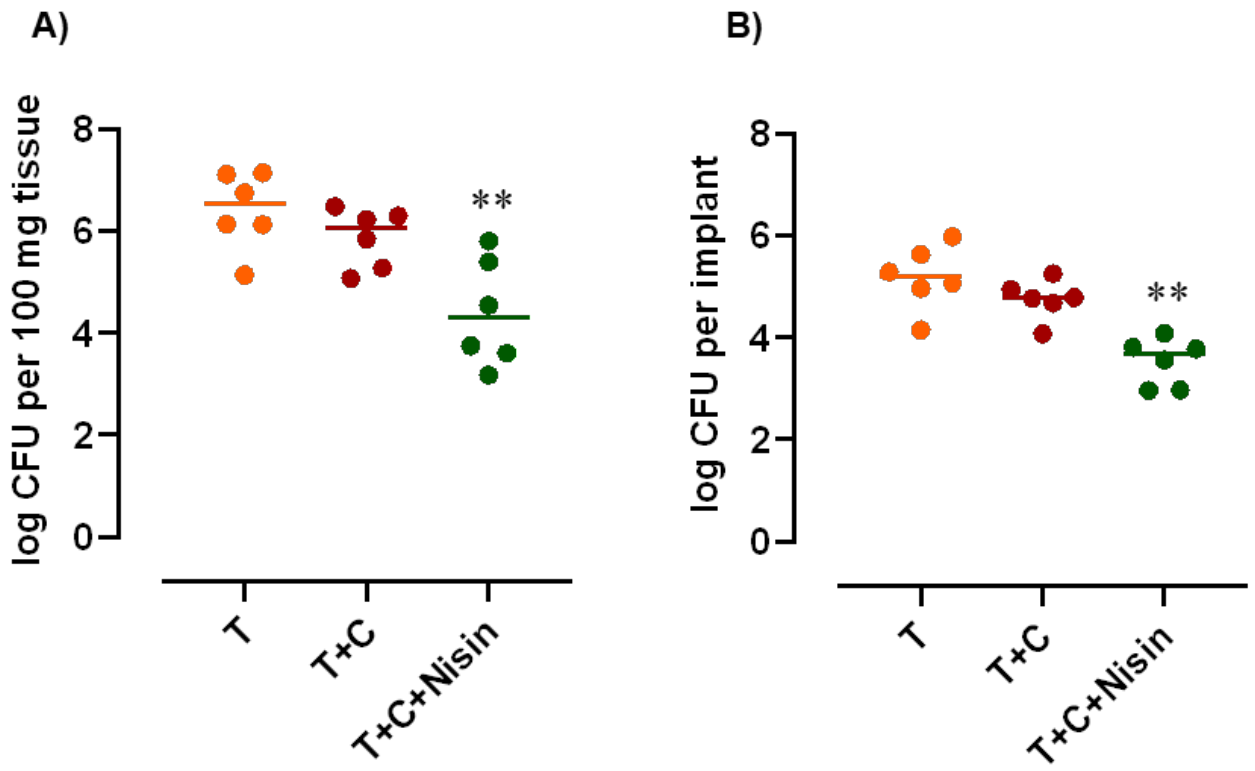

Fig. 4. Effect of nisin coating on bacterial burden in larval tissue and on the implant surface. Titanium Kirschner wires (K-wires) were implanted into the larvae, followed by an injection of 10 μL of *S. aureus* inoculum (5×10^5 CFU/larva) after 1 hour. The bacterial load is displayed as: (**A**) *S. aureus* in larval tissue (log CFU per 100 mg tissue) and (**B**) *S. aureus* on the K-wires (log CFU per implant), measured one day after implantation. Data are shown for uncoated K-wires ("T"), K-wires subjected to the coating process but without nisin ("T + C"), and nisin-coated K-wires ("T + C + Nisin"). Quantitative culture data were obtained from 6 larvae per group, and statistical analysis was performed using Mann-Whitney U test. $^{**}p \leq 0.01$.

Scanning Electron Microscopy (SEM)

One day after implantation, K-wires were removed from the larvae and immersed in PBS. The samples were washed twice with PBS to eliminate planktonic cells and then fixed with 2.5 % glutaraldehyde (NeoFroxx GmbH, Einhausen, Hesse, Germany; LC-10058) at 4 °C for 24 hours. After fixation, the glutaraldehyde was removed by washing the samples six times with PBS. The samples were then dehydrated through a graded ethanol series (30 %, 50 %, 70 %, 80 %, and 96 % ethanol), with each step lasting 15 min, followed by three washes with 100 % ethanol for 30 min. Once dehydrated, the samples were dried using a critical point dryer (Leica EM CPD300, Leica, Wetzlar, Hesse, Germany) and sputter-coated with gold and palladium (Polaron Sputter Coater SC760, Leica, Wetzlar, Hesse, Germany). SEM analysis was performed using a LEO1530 microscope operating at 15 kV (Ziess, Oberkochen, Baden-Württemberg, Germany).

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism (version 7, San Diego, CA, USA). The primary outcome, survival, was analysed using the Kaplan–Meier method. Survival curves were generated for each experimental group, and differences between the curves were assessed using the log-rank (Mantel-Cox) test. The Mann-Whitney U test was used to evaluate differences in bacterial load between groups. All statistical tests were two-sided, with a *p*-value of less than 0.05 considered statistically significant.

Results

Nisin-Coated K-Wires Show Improved Larval Survival, Indicating Antimicrobial Properties

Larvae implanted with nisin-coated K-wires (T + C + Nisin) exhibited a significant improvement in survival (36.6 %) compared to those receiving uncoated titanium K-wires (T; 3.3 %; $p < 0.01$). However, no significant improvement was observed when compared to larvae receiving K-wires with the control coating (T + C; 16.6 %; $p = 0.2$; Fig. 3). These results suggest that the coating process itself may exhibit antimicrobial effects, potentially aiding in bacterial eradication.

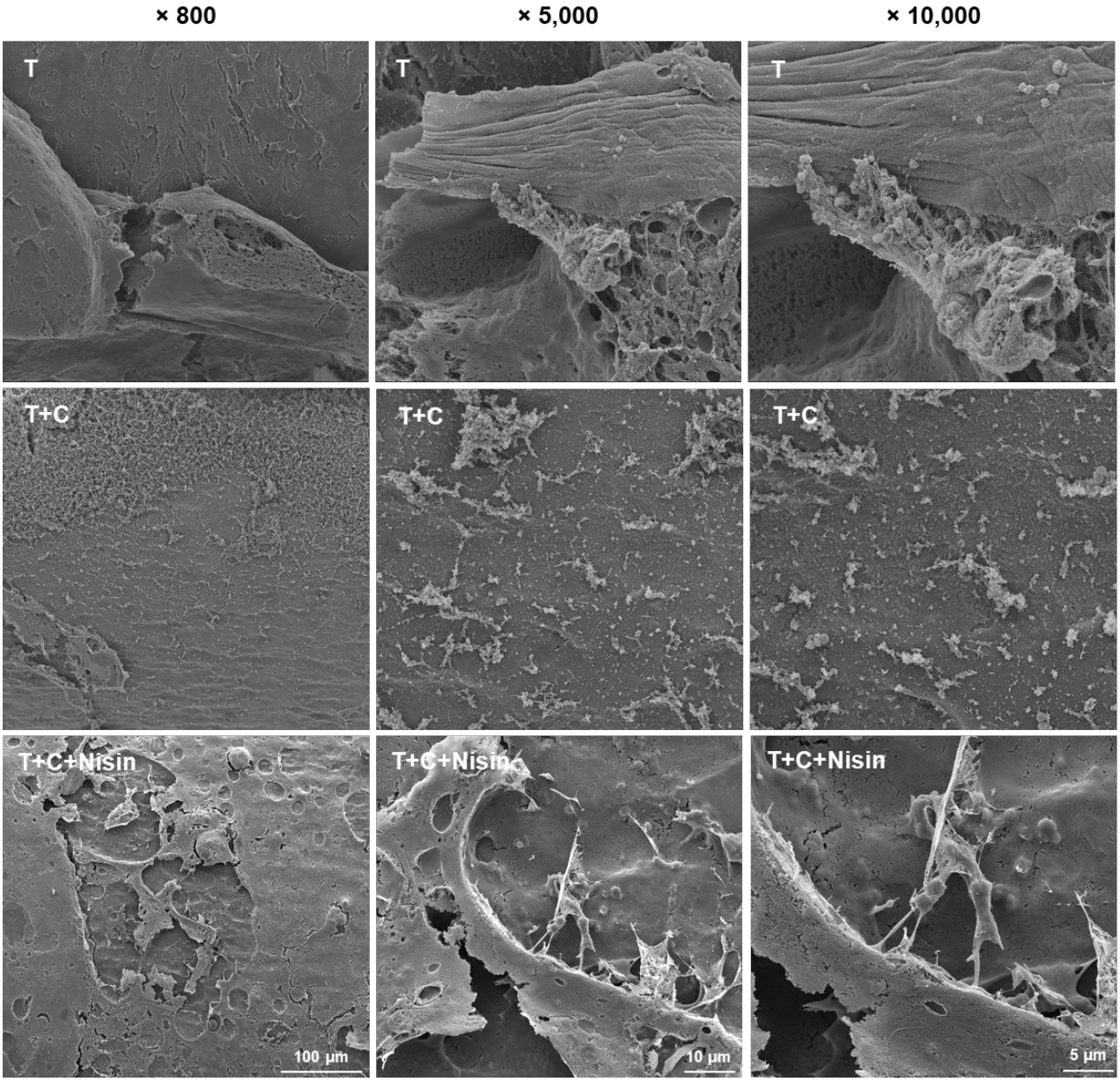

Fig. 5. Biofilm visualization using scanning electron microscopy (SEM). SEM analysis of *S. aureus* attachment on the different K-wires after 24 hours of incubation in the *G. mellonella* haematogenous implant-infection model. Hardly any biofilm formation is seen on the surface of the nisin-coated K-wires (T + C + Nisin), and strongly reduced biofilm formation on the surface of control coated K-wires (T + C), when compared to the uncoated control K-wires (T), which exhibited significant biofilm formation. Scale bars indicate 100 μm (800× magnification), 10 μm (5000× magnification), and 5 μm (10,000× magnification).

Effect of Nisin Coating on the Bacterial Burden on the Surface of the K-Wire and in Larval Tissue

To assess the effect of the nisin coating on bacterial colonization on both the K-wire surface and in the larval tissue, the larvae were dissected 24 hours post-infection, and tissue and implants samples were collected. The results showed a significant reduction in bacterial load in the tissue, with more than a 2-log difference in the nisin-coated K-wire group (T + C + Nisin; log 4.3 CFU/100 mg tissue) compared to the non-coated control group (T; log 6.4 CFU/100 mg tissue; $p < 0.01$; Fig. 4**A**). On the surface of the K-wires, the nisin-coated samples (T + C + Nisin; log 3.5 CFU/implant) showed a 1.5-log reduction ($p < 0.01$), while the control coated samples (T + C; log 4.7 CFU/implant) showed a 0.5-log reduction ($p = 0.093$) compared to the uncoated control group (T; log 5.2 CFU/implant; Fig. 4**B**). These findings demonstrate that the nisin coating contributes to reduced bacterial colonization on the surface of the K-wire and in the surrounding tissue.

SEM Analysis

The SEM analysis of nisin-coated K-wires (T + C + Nisin) revealed an absence of biofilm formation on their surfaces (Fig. 5). In contrast, the uncoated control K-wire samples (T) exhibited significant biofilm formation. Notably, K-wire samples with the control coating (T + C) showed a reduction in biofilm formation compared to the uncoated controls. These findings are consistent with the quantitative results from the survival and bacterial burden analyses, further supporting the efficacy of the nisin coating in preventing biofilm development.

Discussion

The present study demonstrates that a nisin-enriched LbL implant coating, which was successfully tested *in vitro*, can also reduce the risk of implant infection *in vivo*, though it cannot completely prevent infection in all cases. This study is the first to demonstrate that implant coatings can be evaluated using the alternative animal *Galleria mellonella* model in an acute infection setting for implant infection prophylaxis.

Improved Larval Survival and Reduced Biofilm Formation with Nisin-Coated Implants

In the present study, incorporating the antimicrobial peptide (AMP) nisin Z into the implant coating led to more than a 30 % increase in larval survival compared to the uncoated control group, highlighting its potential to prevent implant-related infections. The control coating without nisin exhibited weaker antimicrobial effects, which were not statistically significant when compared to the nisin-coated group. These results suggest that the nisin-enriched coating plays a crucial role in preventing implant infections in *G. mellonella* larvae. The antimicrobial action of nisin likely stems from its mechanism of disrupting bacterial cell membranes by forming pores, which inhibits bacterial adhesion to surfaces [14]. Additionally, nisin binds to lipid II, a critical molecule in bacterial cell wall synthesis, disrupting cell wall formation and compromising bacterial structural integrity [15]. By hindering cell wall synthesis, nisin not only prevents bacterial proliferation but also inhibits biofilm maturation.

Like other anti-infective strategies, the nisin-enriched coating demonstrated a measurable, though not complete, antimicrobial effect [16,17]. This is likely due to the established bacterial load in the model. An excessively high bacterial load would lead to the death of all larvae, while a very low load might not result in infection and could be managed by the innate immune system of *G. mellonella* larvae.

Antimicrobial Effect of LbL Coating

An antimicrobial effect was also observed from the LbL coating alone in terms of larval survival, bacterial load, and SEM analysis. This can be explained by physical and chemical changes to the implant surface [18]. However, the precise mechanism of this effect remains unclear, as no antibiotics, AMPs, or other active agents were applied to the LbL coating in the control group [19]. Moving forward, combining the observed antimicrobial properties of the LbL coating with additional AMPs could offer a synergistic approach for optimizing implant surface treatments.

Direct Inoculation Allows for Efficient Screening of Antimicrobial Coatings

The direct inoculation of *G. mellonella* larvae with *S. aureus* EDCC 5055 (5×10^5 CFU/larva) post-implantation resulted in robust biofilm formation in the control group. While a pre-incubation step to allow biofilm formation on the implants before implantation could be considered, it is less practical for investigating antimicrobial coatings. Pre-incubation may mask the *in vivo* effect of the coating, as it could already act during the *in vitro* preparation stage. Thus, the results of this study are particularly significant, as they demonstrate for the first time that this type of coating can be effectively assessed in the *G. mellonella* implant infection model.

Strengths & Limitations

The use of the *G. mellonella* model offers several advantages: it does not require ethical permissions, is easy to handle, cost-effective, and allows for statistical robustness by enabling studies with a larger number of animals. Recently, we demonstrated the utility of this model in evaluating the efficacy of commercially available antibiotic-loaded bone cement (ALBC) against *S. aureus*. In this study, polymerized ALBC specimens were implanted into the larvae, followed by a bacterial challenge. The results showed improved larval survival and significant eradication of *S. aureus* infection. These findings align with clinical outcomes observed in patients undergoing interim implant replacement with ALBC spacers to treat infections [20]. Despite these strengths, this study has several limitations. Most notably, *G. mellonella* larvae lack an immune system comparable to humans. While humans have an adaptive immune system with specialized cells and antibodies that targeting specific pathogens, *G. mellonella* relies on an innate immune system with generalized responses to a wide range of pathogens. However, the larvae's cellular immune system is believed to resemble mammalian phagocytotic responses [21]. Another limitation is the absence of a skeletal system and bone-associated tissues in the larvae, which restricts the ability to assess the impact of biomaterial coatings on bone metabolism. As such, this model does not allow for a comprehensive study of bone-related processes, highlighting the need for vertebrate models in subsequent testing stages. From an infection perspective, the short observation period (limited to five days due to larval development) does not allow for the investigation of mature biofilms, which are crucial in chronic infections. Alternative animal models

with longer survival times would allow for more clinically relevant investigations.

Conclusions

The findings of this study suggest that nisin-enriched coatings could be a promising strategy for preventing bone and joint infections. Furthermore, this study demonstrates the feasibility of testing implant coatings using an ethically sound and cost-effective alternative *in vivo* model. Based on these findings, further evaluation and nisin-enhanced coatings in vertebrate animal models is warranted to fully assess their potential for preventing implant-associated infections.

List of Abbreviations

AMP, antimicrobial peptide; CFU, colony forming unit; CSA, chondroitin sulfate A; *G. mellonella*, *Galleria mellonella*; GnP, genipin; K-wire, Kirschner wire; LB, lysogeny broth; LbL, Layer-by-Layer; MSSA, methicillin-sensitive *Staphylococcus aureus*; PBS, phosphate-buffered saline; PEI, poly(ethyleneimine); PLL, poly-L-lysine; *S. aureus*, *Staphylococcus aureus*; SEM, scanning electron microscopy; T, uncoated titanium K-wire; T + C, control-coated K-wire; T + C + Nisin, nisin-coated K-wire; Tris, tris(hydroxymethyl)aminomethane; BHI, Brain-Heart-Infusion; ALBC, antibiotic-loaded bone cement.

Availability of Data and Materials

The data are available upon request from corresponding author.

Author Contributions

MRup, GKM, NW, DM, GL, RMYW, WHC, CB, VA, MRio and PT contributed to the design of work. GKM, CR, GL, BL and PT performed the experiments. MRup, GKM, CR, GL, BL, MRio and PT analysed the data. MRup, GKM, NW, DM, CR, GL, RMYW, WHC, BL, CB, VA, MRio and PT contributed to original draft of the manuscript, review and editing. All authors read and approved the final manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Ethical review and approval were not applicable for this study because it utilized the non-vertebrate *G. mellonella* larvae model. The study utilized the *S. aureus* strain EDCC 5055, which is isolated over a decade ago at the University Hospital Giessen (Giessen, Germany), and does not require any additional ethical approval under current legislation.

Acknowledgments

The authors would like to thank Ruth Schewior, Daniela Drenkard and Tobias Gsottberger for their excellent technical assistance, and Gerlinde Ferstl for the SEM analysis.

Funding

The authors did not receive any financial or material support for the research presented in this article.

Conflict of Interest

The authors declare no conflicts of interest.

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Editor's note: The Scientific Editor responsible for this paper was Kai Zheng.

Received: 14th November 2024; **Accepted**: 17th March 2025; **Published**: 26th June 2025