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RESEARCH ARTICLE

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Galleria mellonella as an alternative in vivo model to study implant-associated fungal infections

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Abstract

Fungal implant-associated bone infections are rare but difficult to treat and often associated with a poor outcome for patients. *Candida* species account for approximately 90% of all fungal infections. In vivo biofilm models play a major role to study biofilm development and potential new treatment options; however, there are only a very few in vivo models to study fungi-associated biofilms. Furthermore, mammalian infection models are replaced more and more due to ethical restrictions with other alternative models in basic research. Recently, we developed an insect infection model with *Galleria mellonella* larvae to study biofilm-associated infections with bacteria. Here, we further expanded the *G. mellonella* model to study in vivo fungal infections using *Candida albicans* and *Candida krusei*. We established a planktonic and biofilm-implant model to test different antifungal medication with amphotericin B, fluconazole, and voriconazole against the two species and assessed the fungal biofilm-load on the implant surface. Planktonic infection with *C. albicans* and *C. krusei* showed the killing of the *G. mellonella* larvae at 5×10^5 colony forming units (CFU). Treatment of larvae with antifungal compounds with amphotericin B and fluconazole showed significant survival improvement against planktonic *C. albicans* infection, but voriconazole had no effect. Titanium and stainless steel K-wires were preincubated with *C. albicans* and implanted inside the larvae to induce biofilm infection on the implant surface. The survival analysis revealed significantly reduced survival of the larvae with *Candida* spp. infection compared to noninfected implants. The treatment with antifungal amphotericin B and fluconazole resulted in a slight and nonsignificant improvement survival of the larvae. The treatment with the antifungal compounds in the biofilm-infection model was not as effective as in the planktonic infection model, which highlights the resistance of fungal biofilms to antifungal compounds like in bacterial biofilms. Scanning electron microscopy (SEM) analysis revealed the formation of a fungal biofilm with hyphae and spores associated with larvae tissue on the implant surface. Thus, our study highlights the

use of *G. mellonella* larvae as alternative in vivo model to study biofilm-associated implant fungal infections and that fungal biofilms exhibit high resistance profiles comparable to bacterial biofilms. The model can be used in the future to test antifungal treatment options for fungal biofilm infections.

KEYWORDS

Alternative in vivo model, biofilm, *Candida*, *Galleria mellonella*, implant-associated infection

1 | INTRODUCTION

Orthopedic implants, such as fracture fixation devices and total joint prostheses have proven their positive effect on patient quality of life. For both indications, metal implants are primarily used based on their biomechanical properties.[1] Despite their known functional benefits, all implants exhibit a certain risk of infection. However, colonization of medical devices with different pathogens, such as bacteria and fungi, that are forming biofilm on implant surfaces is a critical problem in clinical routine. After the formation of a biofilm, medical antibiotic or antimycotic treatment often fails due to manifold defense mechanisms.[2,3] Hence, biofilms established on medical devices usually require removal of the entire implant to achieve infect eradication. Fungal periprosthetic joint infection (PJI) represents a rare but often fatal etiology among all implant-associated bone infections. A Spanish retrospective multicenter study showed a fungal etiology of 1.3% of all culture-positive PJIs ($n = 2288$), where by *Candida* spp. were responsible for 90% of all fungal infections. *Candida albicans* was the most frequently isolated fungus (55%–65%), followed by *Candida parapsilosis* (13%–33%) and other species such as *Candida glabrata* (3%–7%) and *Candida tropicalis* (2%–4%).[4]

C. albicans is a commensal organism, which can be found at different sites of healthy individuals, for example, on the skin or as a part of the gastrointestinal and vaginal flora.[5] However, in hospitalized, and especially immunocompromised patients, it may cause a wide range of infections. In such individuals, the weakened immune system allows *Candida* cells to disseminate into the bloodstream and to invade deeper tissues causing life-threatening infections.[6,7] In addition, the presence of abiotic substrates such as catheters, artificial heart valves, and joint prosthesis may provide a niche for *Candida* attachment. Adhesion to such substrates is a prerequisite for further biofilm development, which represents a layer of yeast and hyphae embedded in extracellular polymeric material, mainly consisting of polysaccharides and proteins.[8,9] *C. albicans* catheter-associated infections are associated with high mortality rates. A general characteristic of biofilms is their decreased susceptibility to known antifungals, such as amphotericin B and azoles.[10]

Most of our current knowledge of *C. albicans* biofilm development is gained from in vitro studies on abiotic substrates such as polystyrene, or plastics of above-mentioned devices, that is, silicone, polyurethane. These models are quite advanced and mimic the situation in vivo as closely as possible. However, these systems do not involve the physiological conditions such as continuous blood flow and the immune system of the host.[11] Several in vivo models for *C. albicans* infections were developed such as central venous catheter models, the denture stomatitis model for oral candidiasis and a murine model for catheter-associated candiduria.[12,13] Kuracharikova et al. established a subcutaneous *C. albicans* biofilm model with implantation of infected catheter pieces on the back of rats and used to test susceptibility to fluconazole and echinocandin, as well as combinatorial therapy of diclofenac and caspofungin.[9,14]

In general, higher mammalian models are restricted to use due to animal welfare and ethical reasons. Especially infection experiments with inoculation of bacterial and fungal microorganisms often results in a high burden of the disease and pain in the animals.[15] Therefore, ethical approval is usually restricted. To provide best possible protection of research animals, each research project should follow the 3R (replacement, reduction, refinement) principles introduced for animal welfare by Russel and Burch in 1959.[16] Alternatively to higher mammalians, invertebrates such as *Drosophila melanogaster*, *Caenorhabditis elegans*, zebrafish, and *G. mellonella* have been widely used as infection models to study host–pathogen interactions as well as virulence of bacterial and fungal pathogens.[17–20] In addition, those models enabled testing of antimicrobial agents and drugs. These models are economical, ethically legitimate and easy to handle. Among those, the larva of the greater wax moth, *G. mellonella*, has been extensively used to test virulence of bacterial pathogens. Recently, our group established the *G. mellonella* larvae model to study implant-associated infection by implantation of metal Kirschner (K)-wires preincubated with bacteria into larvae, as well as studied the efficiency of antimicrobial compounds and bacteriophages against bacterial biofilm-associated infections.[21,22]

However, to the best of our knowledge, orthopedic metallic implants with fungal biofilm infections have not been tested yet in *G. mellonella*. Therefore, the aim of the current study was the establishment of this insect infection model for fungal infections associated with stainless steel and titanium implants. For this purpose, *Candida* spp. were used as model organisms. Larvae were implanted with *Candida* preincubated K-wires and the survival of the larvae was analyzed. Further, the efficiency of antifungal compounds against infection with *C. albicans* was tested in terms of fungal load and survival of *G. mellonella*. *C. albicans* biofilm was analyzed using scanning electron microscopy (SEM).

2 | MATERIALS AND METHODS

2.1 | *G. mellonella*, *Candida* spp. growth conditions and preparation of implant materials

G. mellonella larvae were ordered from Flotex Evergreen (Augsburg, Germany) and maintained on an artificial diet in an incubator at 30°C. For each experiment, 10 larvae weighing around ~200–250 mg and present in the last instar developmental stage were used. After infection, the *G. mellonella* larvae were maintained at 37°C.

In this study, *C. albicans* va49398 and *C. krusei* va50374, isolated from patients with PJI, were used. The resistant profile of these strains was determined by the automated VITEK 2 compact machine (bioMérieux). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimycotic agent that inhibits growth of the organism.

Brain–Heart-Infusion (BHI) broth was used to maintain the *Candida* spp. aerobically at 37°C by constant shaking at 180 rpm. An overnight culture was diluted 1:50 in fresh broth and grown to mid-exponential phase to an optical density (OD) of 1.0 at 600 nm. The *Candida* cultures were then washed twice with 0.9% NaCl. Thereafter, the *Candida* cultures were adjusted to the required numbers of colony forming units (CFU) based on the OD (OD of 1.0 equals to ~1.0×10^7 CFU/mL) and used for the experimental purpose.

Sterile stainless steel and titanium K-wires with a diameter of 0.8 mm (Synthes) were used as implant materials. Small pieces with a length of 4–5 mm were cut with of cable cutter and one edge of each K-wire was sharpened. The K-wires were sterilized in 70% ethanol for 30 min and subsequently washed two times with sterile phosphate-buffered saline (PBS).

2.2 | Survival analysis of *G. mellonella* with planktonic infection of *C. albicans* and *C. krusei*

To determine the virulence of the *Candida* isolates, these isolates were grown overnight in BHI broth, followed by subculturing into fresh broth (1:1). After 3 h, the OD was measured and the fungal inoculum suspension was adjusted to 1×10^9, 1×10^8, or 1×10^7 CFU/mL. Later, 10 µL of *Candida* inoculum suspension was injected into each larvae which is equivalent to that the larvae were injected in a gradient manner (10^7–10^5 CFU per larvae) and survival was observed for 5 days. To test the efficiency of antifungal compounds, amphotericin B (5 mg/kg), voriconazole (10 mg/kg) or fluconazole (15 mg/kg) were given against planktonic fungal infection in *G. mellonella* and survival of the larvae was analyzed.

2.3 | Establishment of in vivo *Candida* biofilm infection model using *G. mellonella*

The K-wires were sterilized in 70% ethanol for 30 min and subsequently washed two times with sterile PBS. To establish a fungal biofilm infection model, sterile stainless steel and titanium K-wires were incubated in fetal calf serum overnight at 37°C in an incubator. Later, these implants were preincubated in either *C. albicans* or *C. krusei* inoculum suspension at 1×10^6 CFU/mL for overnight 37°C. Later, these implants were washed in PBS and implanted into the larvae. The implantation process was performed as described in Mannala et al. Briefly, for the implantation of the K-wires inside the *G. mellonella*, the larvae were held by one person and another person performed the implantation with the help of a metal tweezer. The implants were placed in the rear part of the larvae through piercing the cuticle of the larvae with the sharp edge of implant material and was pushed inside the larvae simultaneously. For easy piercing and implantation, it is recommended to implant the K-wire at the segment region due to less cuticle thickness.[21] For the control group, the same process was applied but with sterile implants.

To determine the number of adherent *C. albicans* before implantation, implants were washed in PBS, followed by sonication and was plated out on the LB agar plates. The number of *C. albicans* or *C. krusei* CFU were counted after incubation of the plates at 37°C for 16 h.

After implantation, the larvae were maintained at 37°C for 5 days and their survival was observed each day yielding a survival curve for each experiment. We used 10 larvae for each group of the experimental setup and each experiment was repeated for a least three times.

2.4 | SEM

For the observation of biofilm on Day 3 after implantation, SEM was used. For the SEM analysis of the implants, the larvae were euthanized by exposing to −20°C for 15 min, dissected, implants were taken out and placed in Sørensen-Buffer. These implants were washed two times with Sørensen-Buffer to remove planktonic cells and then fixed with 2.5% Glutaraldehyde in 0.1 M Sørensen-Buffer pH 7.4 at room temperature for 1 h and dehydrated with lower to higher ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 96%) for 20 min, then finally two times with 100% ethanol for 20 min. Samples were dried in a critical point dryer (Balzers CPD 030, Bal-tec AG) and sputter coated with platinum (Sputter coater: Bal-tec SCD 005, with planetary rotation).

SEM analysis was performed (FEI Quanta 400 FEG [Thermo Fisher Scientific, FEI Deutschland GmbH) high vacuum mode at 3 kV). A similar procedure was followed to observe the in vitro analysis of *C. albicans* biofilm on stainless steel K-wire on Day 3 without inserting the K-wires in the larvae.

2.5 | Treatment of *C. albicans* biofilm infection with antifungal compounds

To test the efficiency of antifungal compounds against *Candida* biofilm infections, the larvae were implanted with stainless steel

K-wires preincubated with *C. albicans*. On Day 1 after implantation, we have tested the efficiency of amphotericin B (5 mg/kg), voriconazole (10 mg/kg), and fluconazole (15 mg/kg) against the *C. albicans* biofilms formed on the K-wires.

The effect of antifungal compounds was also measured in terms of *Candida* load on the implant surface and in the tissue of the larvae. For this, 2 days after start of the treatment, the implants were explanted from the larvae and both the implants and the larvae tissue was collected and processed for CFU analysis by sonication and homogenization, respectively. The sonicates and homogenates were serially diluted and plated on Souburad agar supplemented with antibiotics. The numbers of CFU were determined after overnight incubation at 37°C.

2.6 | Statistical analysis

Statistical analysis of the data was performed using SigmaPlot 10.0. For the CFU analysis Student *t*-test was applied and for the survival analysis logrank test was used.

3 | RESULTS

3.1 | Antifungal susceptibility analysis of *C. albicans* va49398 and *C. krusei* va50374 isolates and survival analysis in planktonic *G. mellonella* model

The susceptibility of the *C. albicans* va49398 and *C. krusei va50374* isolates from implant-associated infections against antifungal compounds was assessed using the VITEK 2 system. The results revealed that *C. albicans* va49398 is sensitive to amphotericin B (MIC of 0.75 mg/mL), fluconazole (0.38 mg/mL), and voriconazole (0.14 mg/mL), whereas, *C. krusei* va50374 showed resistance to fluconazole (36 mg/mL) and susceptibility to amphotericin B (0.36 mg/mL) and voriconazole (0.19 mg/mL) (Table 1). The virulence of the *Candida* spp. was determined by injecting *Candida* in a gradient manner (10^7–10^5 CFU per larvae). The results showed that both species were able to cause planktonic infection in *G. mellonella* based on the injected inoculum concentration (Figure 1). *C. albicans* va49398 (Figure 1A) killed all larvae within 4 days, whereas, *C. krusei* killed all larvae within 5 days at a concentration of 1×10^7 CFU.

3.2 | Effect of antifungal compounds on the *C. albicans* va49398 planktonic infection in *G. mellonella*

To test the antifungal compounds against a planktonic infection, the larvae were injected with 1×10^5 CFU *C. albicans* per larvae and after 1 h the larvae were treated with antifungal compounds amphotericin B (5 mg/kg), fluconazole (15 mg/kg), or voriconazole (10 mg/kg). The results showed significant improvement of larvae survival with the treatment of amphotericin B (70%) (Figure 2A) and fluconazole (75%) (Figure 2B), whereas, no significant effect was observed in case of voriconazole (Figure 2C). The lack of effect by voriconazole might be due to the low concentrations used under in vivo conditions.

3.3 | *G. mellonella* as implant-associated biofilm infection model with *C. albicans* and *C. krusei*

To establish *G. mellonella* as a fungal biofilm implant infection model, we tested *C. albicans* and *C. krusei* as reference species. The results evidence a significant reduction of survival rates after the implantation of *C. albicans* preincubated stainless steel (10%) and titanium implants (10%) compared to controls (100%) (***$p \leq 0.001$) (Figure 3A). Similar to *C. albicans*, *C. krusei* also showed a significant reduction on the survival with stainless steel (15%) and titanium K-wires (25%) (***$p \leq 0.001$) (Figure 3B).

Determination of the total adherent *C. albicans* on the implant before implantation into the larvae showed comparable fungal adherence between stainless steel (7.200 ± 540 CFU) and titanium (6.700 ± 350 CFU) implants. Similarly, *C. krusei* showed adherence on stainless steel (4.800 ± 440 CFU) and titanium (5.030 ± 720 CFU).

3.4 | Biofilm visualization on implants with SEM analysis

Figure 4A–C shows the clear formation of a biofilm on stainless steel K-wires after 3 days of incubation in the *C. albicans* suspension confirming the biofilm forming capacity of *C. albicans*, and can therefore be used for the in vivo biofilm implant infection model. The *C. albicans* biofilm shows hyphae nets, spores, and pseudo spores as well as budding of the new spores (Figure 4A–C). The SEM images of sterile K-wires show a smooth surface (Figure 4D–F). Further, to visualize the biofilm maturation over time in *G. mellonella*, SEM analysis was performed on stainless steel implants that were

TABLE 1 Antifungal sensitivity assay of *Candida albicans* and *Candida krusei* isolates.

Candida isolate	Amphotericin B	Fluconazole	Voriconazole
***Candida albicans* va49398**	0.75 mg/mL (S)	0.38 mg/mL (S)	0.14 mg/mL (S)
***Candida krusei* va50374**	0.36 mg/mL (S)	32 mg/mL (R)	0.19 mg/mL (S)

Abbreviations: R, resistance; S, sensitive.

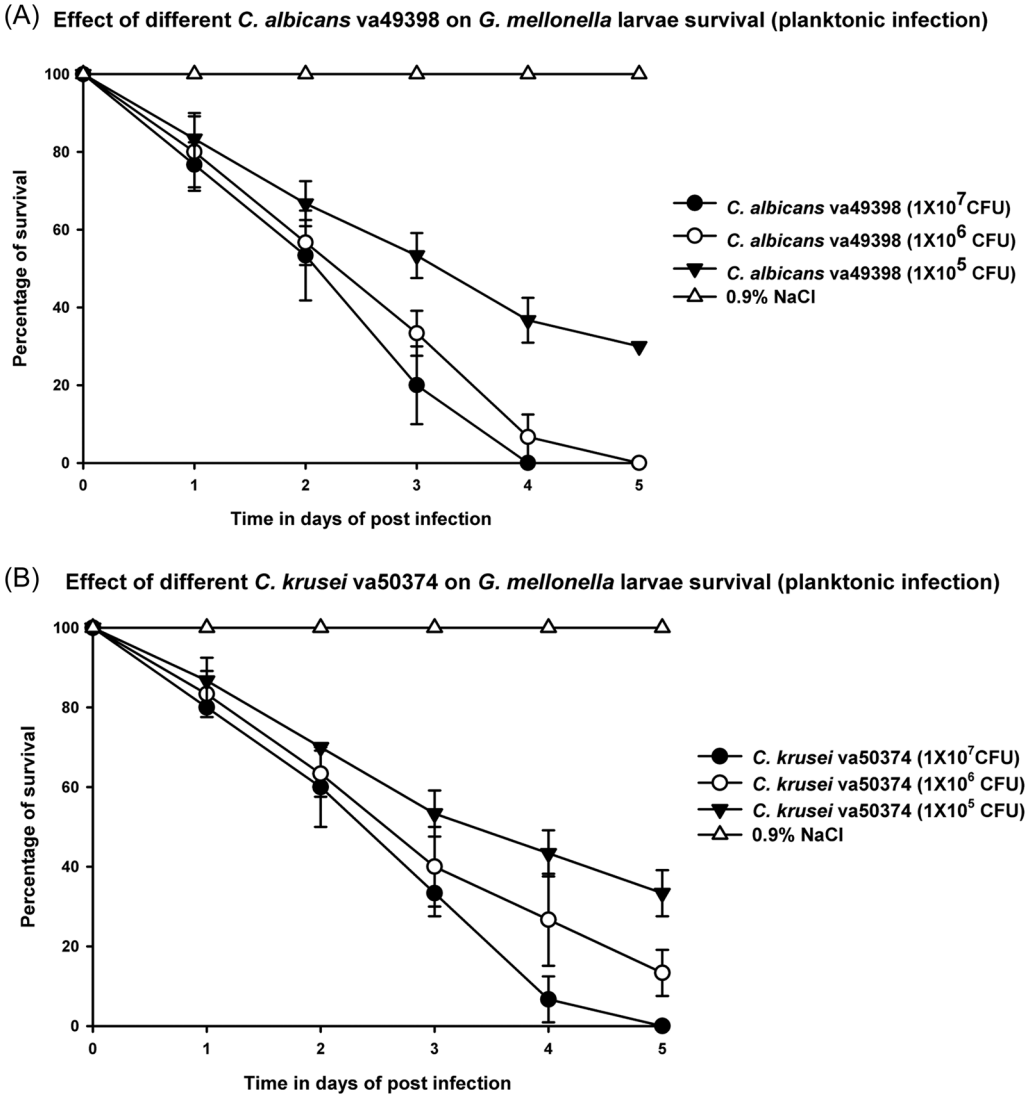

FIGURE 1 Survival curves of larvae after planktonic infection with *Candida albicans* and *Candida krusei*. To determine the virulence of the *Candida* isolates, the larvae were injected with 10 µl of *Candida* inoculum suspension, which is equivalent to that the larvae were infected in a gradient manner (10^7–10^5 CFU per larvae) and their survival was monitored for 5 days. Both *C. albicans* va49398 (A) and *C. krusei* va50374 (B) showed a dose-depending death of the larvae.

explanted from the larvae on Day 3. The results revealed that *C. albicans* biofilm on the K-wire surface with hyphae and spores was associated with the tissue of the larvae (Figure 5A–F). F igure 5G-I shows the surface of sterile K-wires after implantation into the *G. mellonella* larvae, covered with larvae tissue and absence of fungal hyphae and spores.

3.5 | Effect of the antifungal compounds on *C. albicans* biofilm infection in *G. mellonella*

The effect of amphotericin B and fluconazole on biofilm infection improved slightly the survival of the larvae compared to the control group. However, the difference to the treatment-free group was not significantly different, which highlights the typical biofilm infection feature with increased resistance toward antimicrobial compounds (Figure 6A,B). Voriconazole showed no effect on planktonic and biofilm *C. albicans* infections in *G. mellonella* (Figure 6C).

However, the bioburden analysis showed an antifungal effect of amphotericin B and fluconazole with significant reduction of the number of CFU of *C. albicans* on the surface of the K-wire (Figure 7A) and in the tissue of the larvae (Figure 7B).

4 | DISCUSSION

In vivo infection experiments are associated with significant pain and discomfort for the animals and should therefore be used in a very strictly manner. Russel and Burch proposed specific

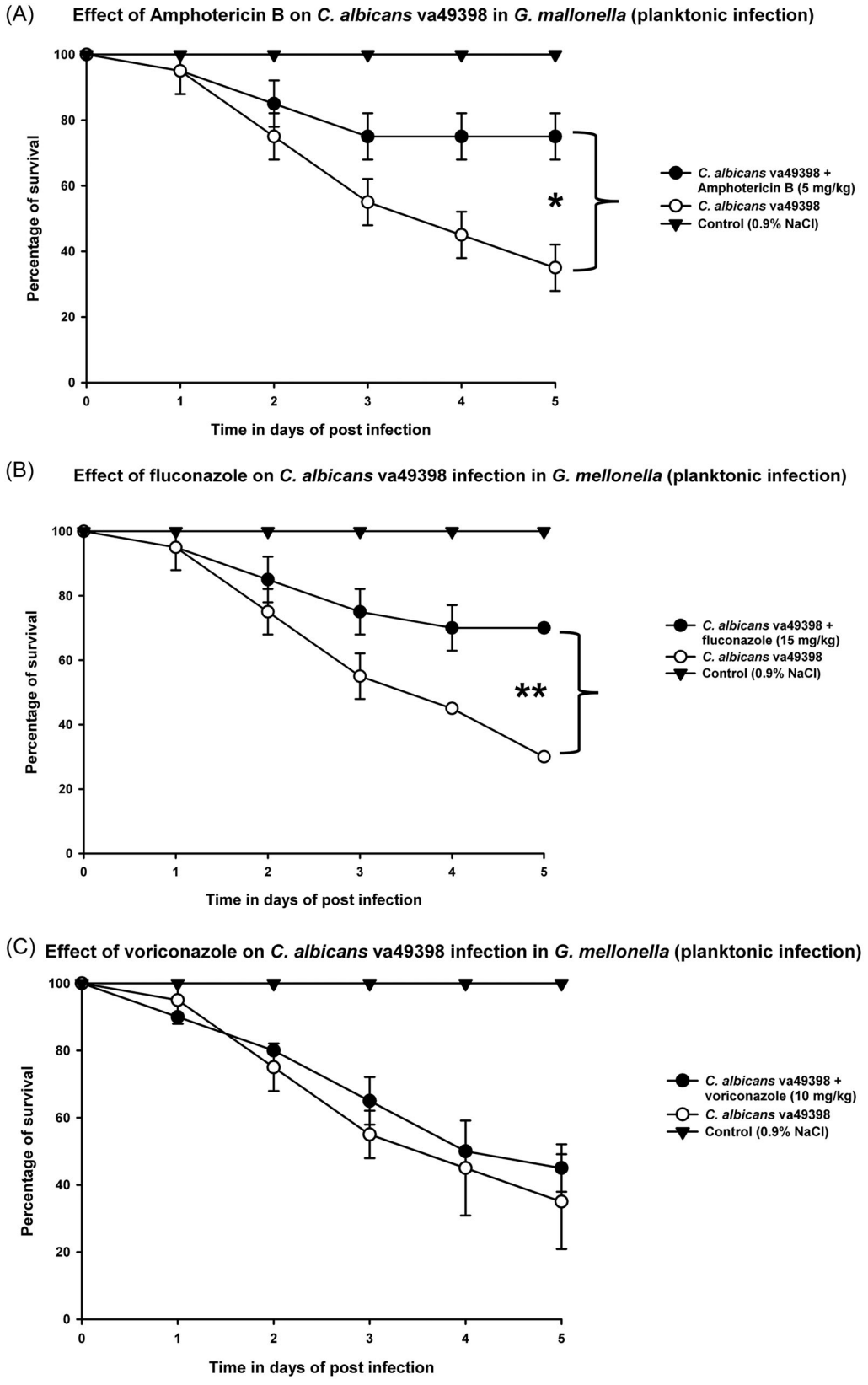

FIGURE 2 Effect of antifungal compounds on *Candida albicans* planktonic infection in *Galleria mellonella* larvae. To test the efficiency of antifungal compounds, amphotericin B (5 mg/kg), fluconazole (15 mg/kg), and voriconazole (10 mg/kg) were given against *C. albicans* infection in *G. mellonella* and survival of the larvae was analyzed. The amphotericin B (A) and fluconazole (B) showed significant reduction whereas voriconazole showed no effect on the survival of the larvae (C). The data was analyzed from three independent experiments and statistical analysis was performed using logrank test. (*$p \leq 0.05$; **$p \leq 0.001$).

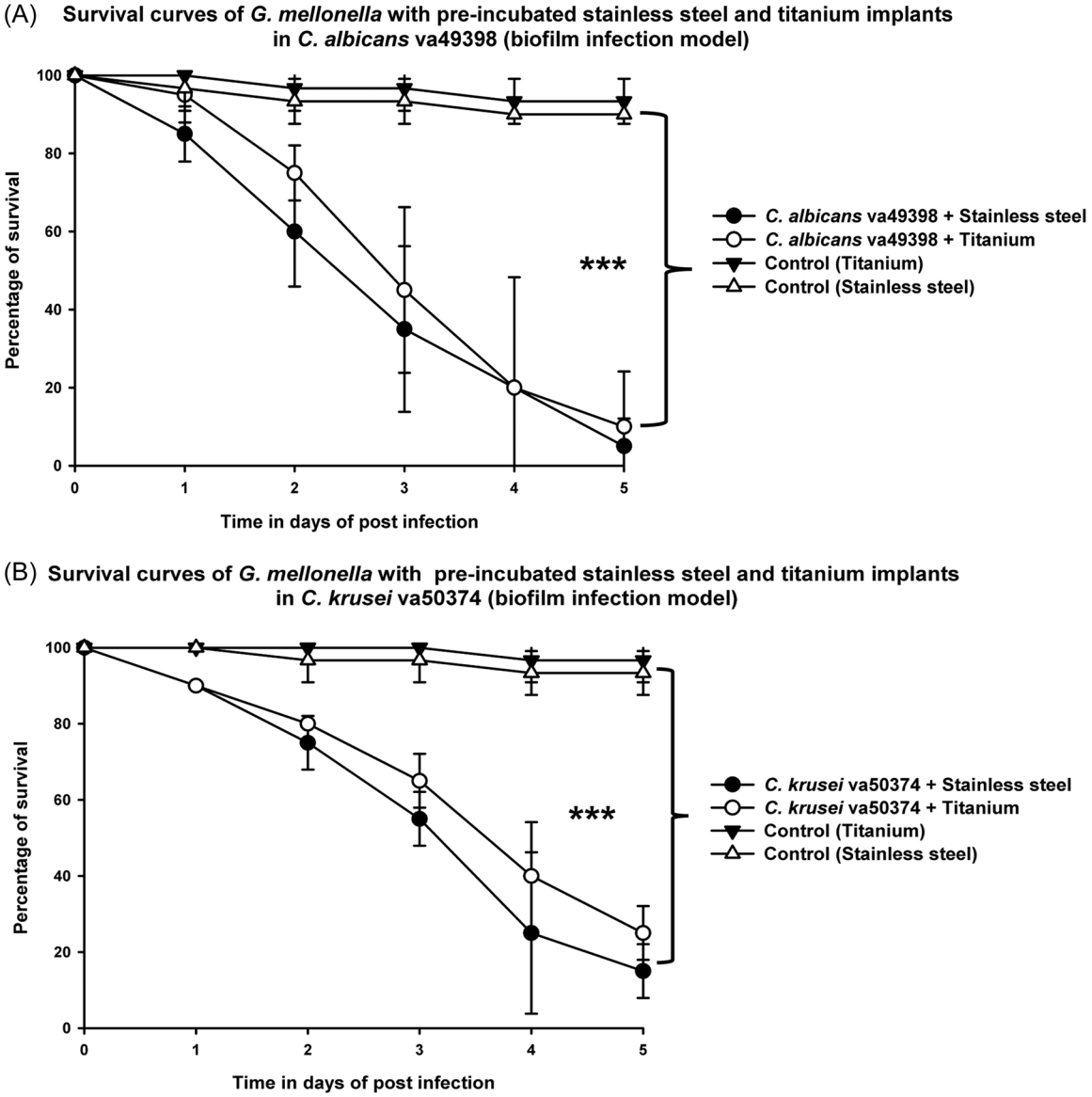

FIGURE 3 Establishment of *Galleria mellonella* as fungal biofilm implant infection model. To establish *G. mellonella* as fungal implant infection model, we have tested stainless steel and titanium K-wires preincubated with *Candida albicans* and *Candida krusei*. The implants were incubated in the fungal suspensions for 1 hour followed by wash steps and implanted in the larvae. Next, the larvae were incubated and monitored for their survival for 5 days. The survival analysis revealed the development of *Candida*-associated implant infection by both *C. albicans* (A) and *C. krusei* (B). The data was analyzed from three independent experiments and statistical analysis was performed using logrank test. (***$p \leq 0.001$).

recommendations for the use and alternatives of animal research with their 3R principles for animal welfare, which are still being used nowadays and paved the way to a more ethical and restricted use of animal experiments. Further advancements, such as the ARRIVE (Animals in Research: Reporting In Vivo Experiments)[23] or the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence)[24] guidelines are implemented for quality and decision making in animal research.

As aforementioned, the invertebrate larvae model *G. mellonella* has been extensively used to test virulence of bacterial and fungal pathogens, toxicity of chemicals and antibacterial, and antimycotic activity as they are easy to handle and able to survive at 37°C, which is the relevant human physiological temperature.[19,20,25] Recently, our group established *G. mellonella* as an implant infection model by implanting metal K-wires into the larvae body. The larvae with *S. aureus* preincubated K-wires developed an infection and SEM analysis showed biofilm formation on the surface. This model was further used to test antibiotic and bacteriophage therapies against implant-associated infections that mimic PJI patients.[21,22] Our study enabled further expanding this larvae model to study biofilm infections and thus, reducing the usage of higher mammals in animal experimentation. In fungi research, very limited number of in vivo models are developed, which also face similar ethical problems.

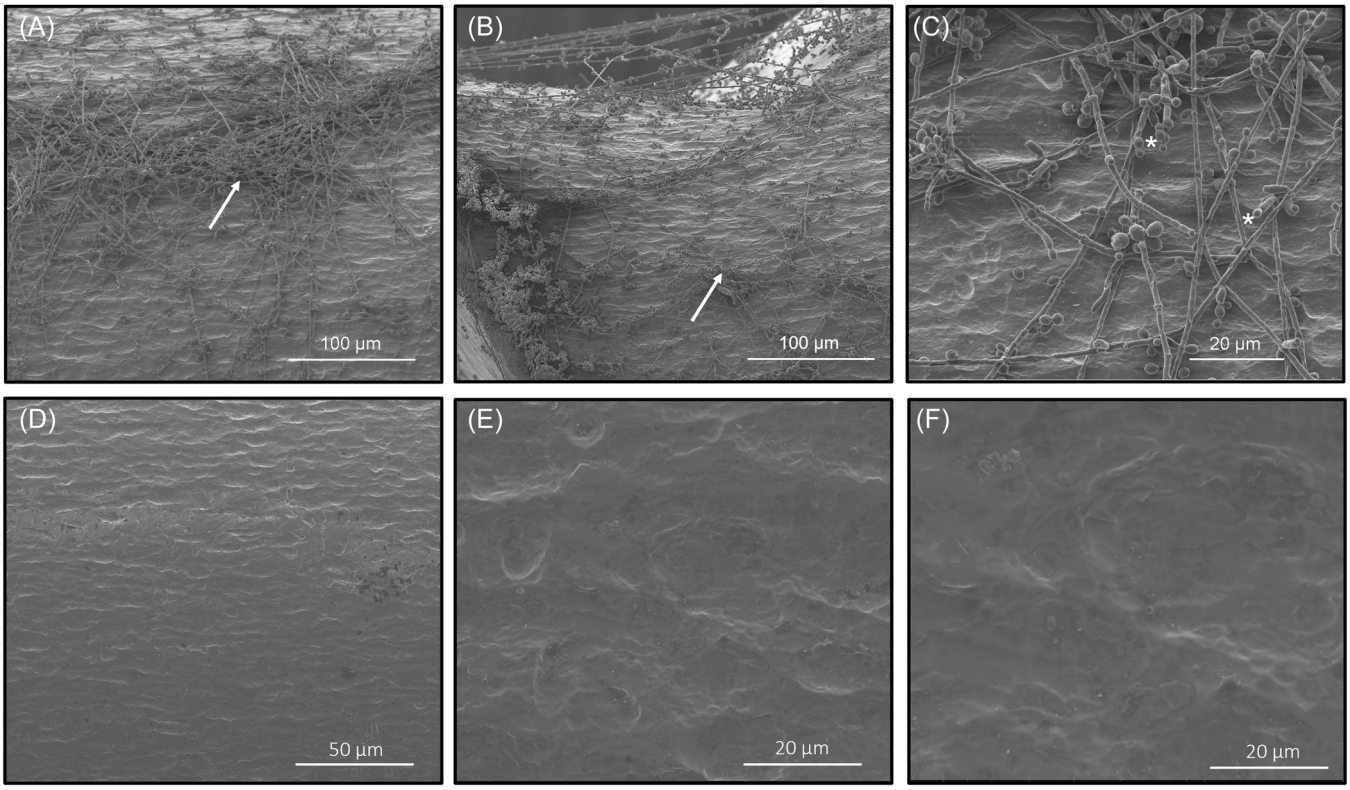

FIGURE 4 Scanning electron microscopy (SEM) analysis of in vitro biofilm formation on the K-wires. To visualize the in vitro *Candida* biofilm formation on the K-wires, the implant materials were incubated in a *Candida* suspension in vitro. The results showed hyphae nets (arrow symbol), spores, and budding of the new spores (star symbol) (A–C) on the implant surface. SEM images (D–F) show the clear surface of sterile K-wires.

Therefore, the aim of the current work was to establish and to evaluate this alternative insect infection model with implantation of stainless steel and titanium implants to mimic fungal biofilm infections for orthopedic purposes.

We first established a planktonic infection model with the different fungal species, which showed both planktonic infection of the larvae by the two different fungal species and the effectiveness of antifungal treatment by amphotericin B and fluconazole.

The biofilm infection part of the study with implants pre-incubated with *C. albicans* and *C. krusei* revealed a significant reduction of survival rates of the infected larvae compared to uninfected controls. The adhesion assay showed adherence of *Candida* during the preincubation to the K-wire that resulted in the colonization, development of biofilm and systemic infection and finally, death of the larvae. The treatment options against *Candida* implant infection with antifungal compounds such as amphotericin B and fluconazole exhibited a slightly nonsignificant improvement of the larvae survival. However, these treatment options were effective against planktonic infection. This highlights the increased resistance to the antifungal compounds due to biofilm formation, which is a typical feature in all biofilm-associated infections.[26] Further SEM analysis revealed the clear biofilm formation by *Candida* on the surface of the K-wires both in the in vitro and in the in vivo settings. The in vitro biofilm showed hyphae nets, yeast spores, and budding yeast structures. In case of in vivo, the biofilm is visualized along with larvae tissue with hyphae and yeast spores. In an in vivo mouse model, *C. albicans* was able to form a biofilm on the mucosal layer at 48 h after vaginal inoculation.[27] Further, Kernien et al. showed *C. albicans* biofilm formation on the luminal surface of a rat venous catheter at 24 h postinoculation.[28] In summary, the presented *G. mellonella* biofilm K-wire infection model mimics all typical features of a implant-associated fungal infection and can therefore be considered as a clinically relevant in vivo fungal biofilm infection model.

The bioburden analysis showed a significant reduction of *C. albicans* load on the implant surface as well as in the tissue of the larvae, which is not in line with the survival curve analysis.

The host response of *G. mellonella* toward fungal pathogens is based on its innate immune system solely, due to the lack of an adoptive immune system, which is present in higher mammals. This adoptive immune system involves specific B-cell and T-cell responses toward microorganisms, including fungal pathogens.[29] Despite the evolutionary distance to mammals, the *G. mellonella* immune system is broadly similar to the innate immune system of mammals and consists of pattern recognition receptors, a complement-like system and hemocytes as phagocytic cells to eliminate pathogens. In contrast to mammals, *G. mellonella* has phenol oxidase that produces melanin from tyrosine as antimicrobial strategy to kill pathogens.[30] C-type lectin receptors (CLRs) play a major role in the antifungal defense by

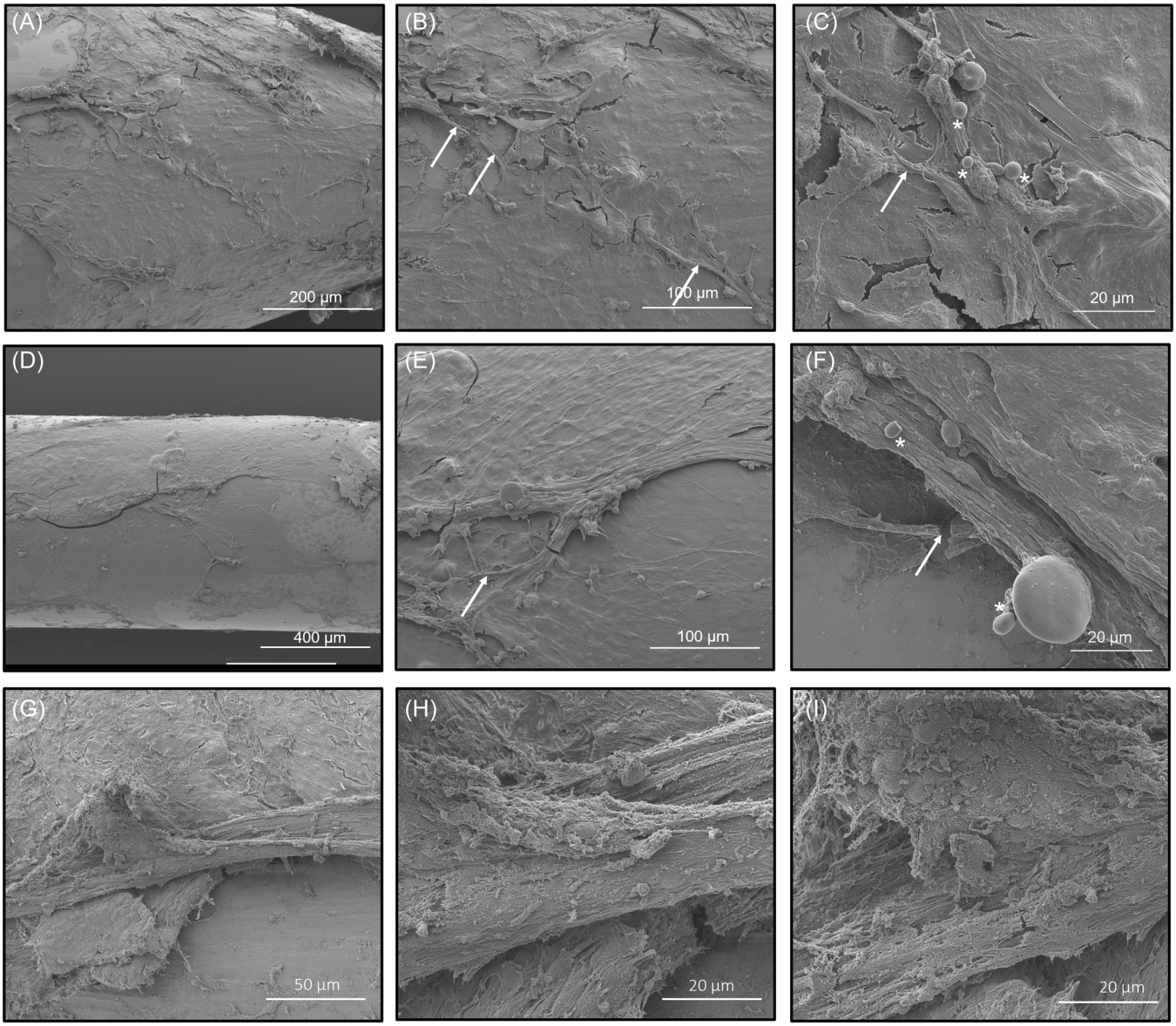

FIGURE 5 Scanning electron microscopy (SEM) analysis of in vivo biofilm formation of implanted K-wires. To visualize the *Candida* biofilm formation on the K-wires in vivo, the implants were explanted from the larvae on Day 3. Two samples were analyzed (A–C; D–F) with different magnifications. Both samples showed the biofilm is merged with larvae tissue on the surface of the K-wires. The images show the hyphae (arrow symbol) and spores (star symbol) along with the tissue. SEM images (G–I) show the surface of sterile K-wires after implantation in the larvae, with the surface covered with larvae tissue and absence of spores and hyphae.

attaching to fungal cell wall carbohydrates, such as mannans and glucans. After binding of the CLRs to the fungal surface, it leads to activation of inflammatory responses, release of cytotoxic molecules and phagocytosis of fungal cells.[31] In *G. mellonella*, CLRs have been also identified that attach to the carbohydrates and thereby promote fungal clearance. These CLRs are specific to the fungal cell wall carbohydrates N-acetyl D-galactosamine and β-(1,3)glucan L-fucose.[32,33]

The model is further of relevance due to its low costs and its potential for rapid and high throughput analysis, for example, for screening of antimycotic materials, such as coatings or other anti-infective treatment strategies. Application and decision processes with animal welfare committees, which are sometimes complicated and time-consuming for vertebrae infection experiments, can be avoided by the use of the presented model.

Despite these positive aspects, there are several limitations to the model and this study. The major draw backs of te *G. mellonella* model are lack of an adaptive immune system and its short life cycle that makes it impossible to study chronic infections. Furthermore, the absence of a skeletal system hinders typical bone-associated reactions and limits the conclusion of these results for orthopedic implant-related infections.

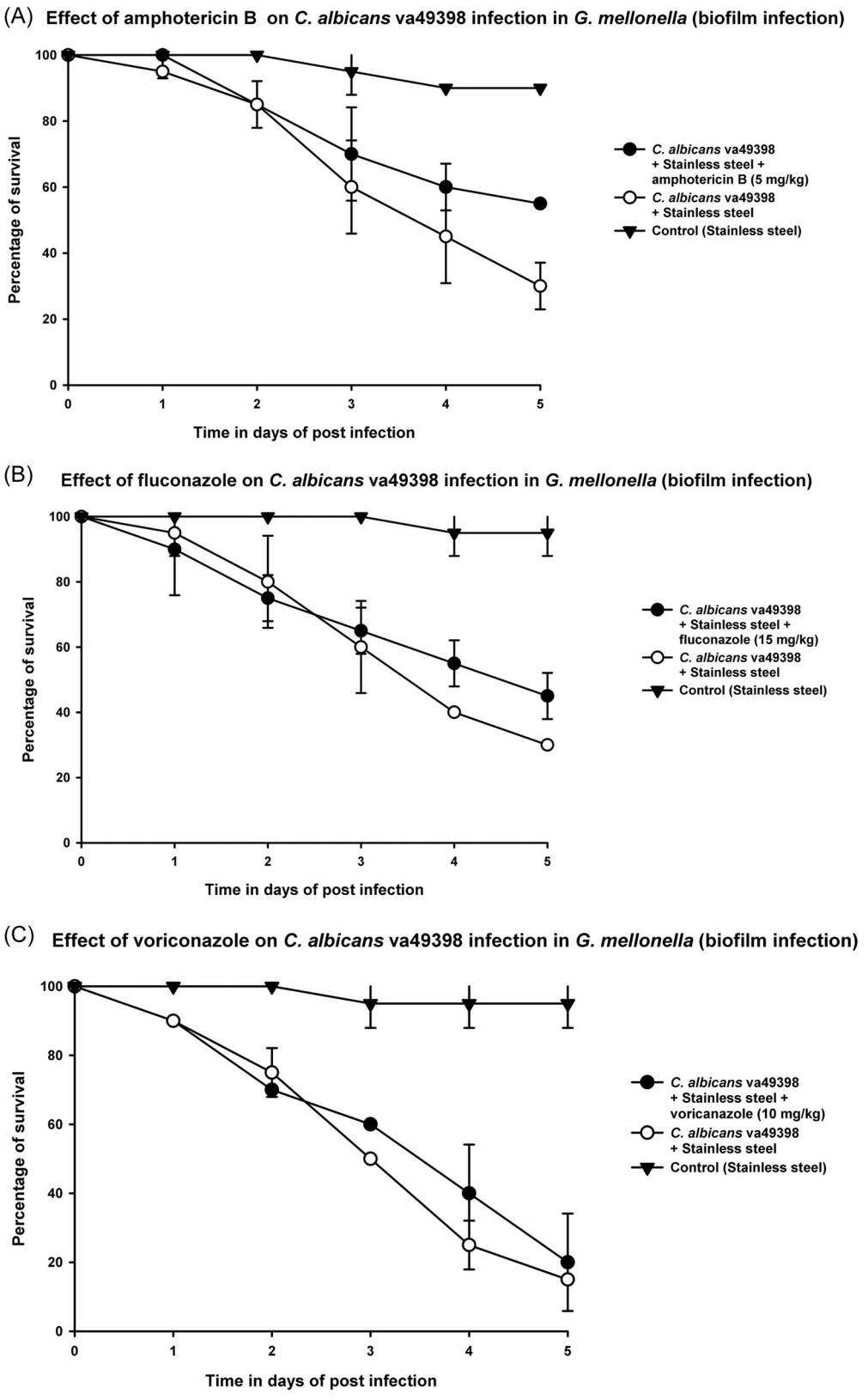

FIGURE 6 Effect of antifungal compounds against *Candida albicans* biofilm implant infection in *Galleria mellonella*. We tested amphotericin B, fluconazole, and voriconazole compounds against *C. albicans* infection. We have implanted the larvae with stainless steel K-wires with preincubated *C. albicans*. After 1 day, the antifungal compounds amphotericin B (5 mg/kg), fluconazole (15 mg/kg), and voriconazole (10 mg/kg) were added. The survival of the larvae was monitored for 5 days. The survival analysis revealed a slight, nonsignificant increase in larvae survival with treatment of amphotericin B (A) and fluconazole (B), but there was no effect of voriconazole (C). The data was analyzed from three independent experiments and statistical analysis was performed using logrank test.

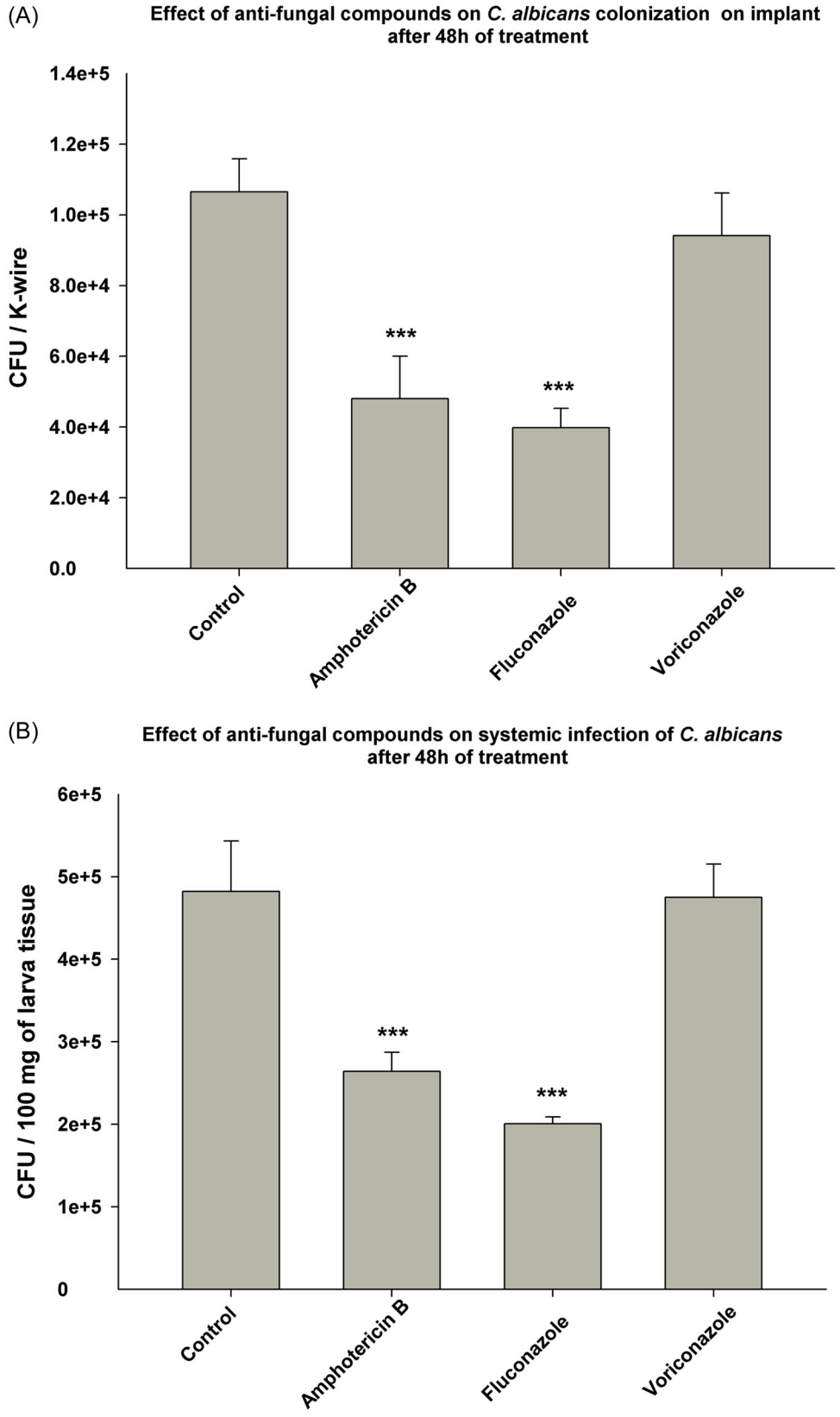

FIGURE 7 Effect of antifungal compounds on *Candida albicans* load on implant and tissue of *Galleria mellonella*. We have implanted the larvae with stainless steel K-wires preincubated with *C. albicans*. After 1 day, the antifungal compounds amphotericin B (5 mg/kg), fluconazole (15 mg/kg), and voriconazole (10 mg/kg) were added. At 2 days posttreatment, both the implants and the tissue of the larvae were collected, and processed for CFU analysis. The *Candida* burden was significantly reduced with the treatment of amphotericin and fluconazole on the implant surface (A) as well as in the tissue of the larvae (B), whereas there was no effect of voriconazole. The data was analyzed from three independent experiments and statistical analysis was performed using student *t*-test. (*$p \leq 0.05$; **$p \leq 0.001$).

5 | CONCLUSION

In conclusion, our results further showed that *G. mellonella* can be used as an alternative in vivo model to study fungal biofilm-associated implant infections for metallic orthopedic devices. Despite the lack of an adoptive immune system and a musculoskeletal system, the *G. mellonella* larvae could be used as preclinical model to screen and evaluate antifungal compounds and other new treatment strategies against fungal biofilm infections. Thus, the model has the potential to reduce animal infection experiments for fungal biofilm-infection with vertebrates in the future.

AUTHOR CONTRIBUTIONS

Gopala Krishna Mannala, Markus Rupp, and Volker Alt developed the approach and designed the experiments. Gopala Krishna Mannala, Nike Walter, Konstantin Johannes Scholz, Michaela Simon, and Martijn Riool performed the experiments and analyzed the results. Gopala Krishna Mannala, Markus Rupp, and Volker Alt interpreted the data and wrote manuscript with editing support from Nike Walter, Konstantin Johannes Scholz, Michaela Simon, and Martijn Riool. All authors have read the manuscript and approved the submitted manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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