MICROBIOLOGICAL AND ULTRASTRUCTURAL EVALUATION OF BACTERIOPHAGE 191219 AGAINST PLANKTONIC, INTRACELLULAR AND BIOFILM INFECTION WITH *STAPHYLOCOCCUS AUREUS*

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Abstract

Infections of orthopaedic implants, such as fracture fixation devices and total-joint prostheses, are devastating complications. *Staphylococcus aureus* (*S. aureus*) is a predominant pathogen causing orthopaedic-implant biofilm infections that can also internalise and persist in osteoblasts, thus resisting antibiotic therapy. Bacteriophages are a promising alternative treatment approach. However, data on the activity of bacteriophages against *S. aureus*, especially during intracellular growth, and against *in vivo* biofilm formation on metals are scarce. Therefore, the present study evaluated the *in vitro* efficacy of *S. aureus* bacteriophage 191219, alone as well as in combination with gentamicin and rifampicin, to eradicate *S. aureus* strains in their planktonic stage, during biofilm formation and after internalisation into osteoblasts. Further, the invertebrate model organism *Galleria mellonella* was used to assess the activity of the bacteriophage against *S. aureus* biofilm on metal implants with and without antibiotics. Results demonstrated the *in vitro* efficacy of bacteriophage 191219 against planktonic *S. aureus*. The phage was also effective against *in vitro S. aureus* biofilm formation in a dose-dependent manner and against *S. aureus* internalised in an osteoblastic cell line. Transmission electron microscopy (TEM) analysis showed bacteriophages on *S. aureus* inside the osteoblasts, with the destruction of the intracellular bacteria and formation of new bacteriophages. For the *Galleria mellonella* infection model, single administration of phage 191219 failed to show an improvement in survival rate but appeared to show a not statistically significant enhanced effect with gentamicin or rifampicin. In summary, bacteriophages could be a potential adjuvant treatment strategy for patients with implant-associated biofilm infections.

Keywords: *Staphylococcus aureus*, bacteriophage, biofilm, implant-associated infection, *Galleria mellonella*.

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List of Abbreviations	
BHI	brain heart infusion
CFU	colony-forming unit
EPS	extracellular polymeric substances
G. mellonella	*Galleria mellonella*
MOI	multiplicity of infection
MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-susceptible *S. aureus*
OD	optical density
PBS	phosphate buffer saline
PFU	plaque-forming unit
S. aureus	*Staphylococcus aureus*
TEM	transmission electron microscopy
TSB	tryptic soy broth

Introduction

Orthopaedic implants, such as fracture fixation devices and total joint prostheses, have proven their positive effect on patients' quality of life.

Metal implants are primarily used based on their biomechanical properties. Despite their known functional benefits, all implants exhibit a certain risk of deep infection (Moriarty *et al.*, 2016; Riebeiro *et al.*, 2012). Earlier studies have identified the so-called "race for the surface" phenomenon between bacteria and host proteins and subsequent biofilm formation by bacteria as a key element for the pathophysiology of implant-associated bone infections (Davidson *et al.*, 2019; Gristina and Costerton, 1985; Romanò *et al.*, 2015).

S. aureus is a clinically significant pathogen that is predominantly involved in implant-associated bone infections, colonising necrotic tissue and abiotic surfaces, thus forming biofilms. *S. aureus* biofilms are embedded in an extracellular matrix composed of either self-produced extracellular polysaccharides, DNA and proteins or host-derived matrices such as fibrin. Further, *S. aureus* can invade osteoblasts and enter a "persister" state in which exposure to high levels of antibiotics can be survived due to a lack of metabolic activity (Gimza and Cassat, 2021). Antimicrobial drug diffusion into biofilms is limited by several factors such as the physical barrier of EPS, including polysaccharides, proteins, DNA and RNA. The efficiency of antimicrobial drugs against bacteria embedded in biofilm is reduced with enhanced exchange of antibiotic resistance genes and slower growth rates (Akanda *et al.*, 2018). Hence, biofilm microorganisms are up to 1,000 times more resistant to growth-dependent antimicrobial agents than their planktonic equivalent (Arciola *et al.*, 2015; Gebreyohannes *et al.*, 2019). Therefore, once a biofilm is formed, eradication of bacteria and of the infection is challenging and reinfections can often not be avoided. Thus, treatment concepts for implant-associated bone infections mainly depend on the duration of the infection since maturation of the biofilm is a key factor for the therapeutic opportunity of implant retention (Zimmerli and Sendi, 2017). The remarkable resistance of biofilms to conventional antibiotic therapy has sparked a fair amount of research such as on alternative combination agents, synthetic surfaces and antimicrobial coatings (Alt, 2017; Izakovicova *et al.*, 2019; Ribeiro *et al.*, 2012). One promising approach involves bacteriophages, ubiquitously present in the environment as the most abundant biological agent on Earth (Baltinovic *et al.*, 2019). These viruses infect bacteria, replicate inside them and finally are released through lysis, killing the host. Hence, bacteriophages are potentially potent antimicrobial agents, which were already being used to treat bacterial infections when discovered in 1917 by Felix d'Herelle (d'Hérelle, 1917). Nowadays, especially in light of the increasing multidrug-resistant infections, bacteriophages are being re-considered as a possible therapy option.

Whereas first preclinical studies show promising results, *in vitro* data on the efficacy of bacteriophages in the treatment of bone and joint infection is scarce and further research is required to translate bacteriophage therapy into clinical practice (Ferry *et al.*, 2020, Ferry *et al.*, 2021; Gibb and Hadjiargyrou, 2021). Specifically, evidence on the effectiveness of bacteriophages against intracellular growing *S. aureus* is missing. Further, infection models are required to evaluate the activity of bacteriophages *in vivo*. Replacing models from higher mammals with the *G. mellonella* insect model would seem to be an appropriate alternative for studying implant-associated *S. aureus* biofilm infections (Mannala *et al.*, 2020a; Tkhilaishvili *et al.*, 2020; Wang *et al.*, 2020). However, the efficiency of bacteriophages on *S. aureus* biofilm infection in *G. mellonella* with metallic implants mimicking orthopaedic infections has not yet been investigated. Therefore, the present study aimed to evaluate the *in vitro* and *in vivo* effectivity of phage 191219 (D&D Pharma GmbH, Pyrmont, Germany) against *S. aureus* during its planktonic, biofilm and intracellular growth phases as well as in an implant-associated *G. mellonella* biofilm infection model.

Materials and Methods

Bacteriophages
S. aureus phage 191219 was provided by D&D Pharma GmbH, Pyrmont, Germany and propagated on the *S. aureus* EDCC 5055 strain. Briefly, overnight grown *S. aureus* bacteria were sub-cultured into fresh BHI medium and incubated on a shaker at 37 °C until the OD at 600 nm (OD_{600}) reached 1. After that, 5 mL of bacteriophage solution (5×10^8 PFU/mL) was added to 25 mL of bacterial solution and further incubated at 37 °C for 4 h. Phages present in bacterial suspension were obtained by centrifugation at 5086 ×*g* for 10 min, followed by filtering the supernatant through 0.45 μm and 0.2 μm filters. The concentration of phage solution was determined by plaque assay using bacteriophage serial dilutions (Bonilla *et al.*, 2016).

Bacteria and *G. mellonella*
S. aureus EDCC 5055 (MSSA) and *S. aureus* DSM 21979 (MRSA) strains were used. *S. aureus* EDCC 5055, which was isolated from a human wound infection, is known for its high biofilm formation capacity and its whole genome sequence is available (Mannala *et al.*, 2017; 2018). *S. aureus* DSM 21979 was isolated from the nasal swab of an infected patient and showed methicillin resistance. BHI broth was used to maintain *S. aureus* strains aerobically at 37 °C by constant shaking at 180 rpm.

G. mellonella larvae were purchased from EVERGREEN GmbH (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 stage were used. After infection, *G. mellonella* larvae were maintained at 37 °C.

Effect of phage 191219 on planktonic growth of bacterial pathogens

The efficiency of the bacteriophages on *S. aureus* planktonic growth was tested using the double layer agar method. Both *S. aureus* EDCC 5055 and DSM 21979 strains were used for the planktonic assay. Briefly, logarithmic growth phase *S. aureus* cultures were diluted to 10^7 CFU/mL. Later, this solution was mixed with bacteriophage dilutions (10^{-1}-10^{-8}) in 5 mL total volume of semi solid BHI medium (0.7 % agar), poured onto the solid agar medium (BHI with 1.5 % agar) and incubated at 37 °C for plaque formation. The growth inhibition of *S. aureus* by bacteriophages in liquid medium was assessed by culturing the bacteria for 24 h in a 96-well plate at a concentration of 10^7 CFU/mL with bacteriophages at MOI 10, 1, 0.1 and 0.01. The bacterial growth at 37 °C was monitored after 24 h incubation by measuring the OD_{600}. These bacterial cultures were washed 3 times with PBS to remove the bacteriophages in the supernatant, the pellet was suspended in PBS and finally plated onto the BHI agar plates to determine the bacterial concentration. For each group, 3 replicates were performed.

Effect of phage 191219 on bacterial biofilms formed in 96-well plate

To check the effect of phage 191219 on biofilms, the biofilm assay for *S. aureus* EDCC 5055 was performed in a 96-well plate, as described by Mannala *et al.* (2018). Briefly, bacteria were grown in TSB overnight at 37 °C with constant shaking at 180 rpm. The bacterial cultures were diluted into fresh medium (1:50) and the diluted bacterial cultures were transferred into U-bottom 96-well plate under static conditions and incubated for 1 d at 37 °C.

After 1 d, the 96-well plate was washed once with PBS. Bacteriophages (10^6-10^9 PFU) in TSB medium were added to well-biofilms and incubated for 1 d at 37 °C. Afterwards, the biomass in the wells was washed twice with PBS and stained with 0.01 % crystal violet solution for 10 min. Then, the wells were washed twice with PBS to remove unbound

crystal violet. The cell-wall-bound crystal violet was dissolved by using 100 % ethanol and absorbance was measured at 595 nm using a UV spectrophotometer (Bio-Rad GmbH). Further, to count the bacteria before and after treatment with bacteriophage, the wells were washed twice followed by dissolving the biofilm in 200 µL of PBS solution by stirring using a a sterile inoculation loop. The use of the inoculation loop might leave some of the biofilm clumps on the well wall, which is a limitation of the method. This recovered biofilm solution was serially diluted and plated on agar plates. The CFUs were counted and results were analysed between control (t0) and treated groups after 24 h, as previously described (Thieme *et al.*, 2019). 8 replicates were performed for each group.

Phage 191219 effect on intracellular replicating bacteria inside osteoblasts

Infection of osteoblasts was performed according to Mohammed *et al.* (2014). The SAOS-2 osteoblast-like cell line was grown in McCoy's 5A medium. For the infection assay, SAOS-2 cells were cultured to a semi-confluent layer in 24-well plates. *S. aureus* EDCC 5055 bacteria were incubated overnight. Bacteria were added at a MOI of 10 per well. The number of osteoblasts per well was determined using a Neubauer chamber before starting the infection experiment. After 30 min incubation, McCoy's 5A medium was replaced by medium supplemented with 30 µg/mL gentamicin to kill only the remaining extracellular bacteria without affecting the intracellular bacteria. After gentamicin treatment for 30 min, the medium was removed, the cells were washed with PBS once and McCoy's 5A medium with the bacteriophages (10^9 PFU/well) was added to the wells. Then, cells were incubated at 37 °C for 2, 4 and 24 h, respectively. Next, the supernatants were discarded and the cells were lysed using 0.2 % Triton X-100 in sterile, cold, distilled water for 10 min. The lysates were diluted 10 times in 1× PBS and plated onto BHI agar. After 24 h of incubation at 37 °C, the number of bacterial CFU was determined. These results were compared with the effects of rifampicin (8 µg/

Table 1. Activity of bacteriophages against the planktonic growth phase of *S. aureus* strains. To assess the activity of bacteriophages against the planktonic growth phase, bacteriophages and *S. aureus* EDCC 5055 (MSSA) (1×10^7 CFU/mL) or *S. aureus* DSM 21979 (MRSA) (1×10^7 CFU/mL) were co-cultured for 24 h at 37 °C in BHI medium at MOI 10, 1, 0.1 and 0.01. After incubation, ODs of the cultures were measured, cultures were washed 3 times with sterile PBS, pelleted down and finally plated onto BHI agar plates to estimate the bacterial concentration.

MOI with bacteriophage	*S. aureus* EDCC 5055 (MSSA) OD value	*S. aureus* EDCC 5055 (MSSA) number of colonies/mL	*S. aureus* DSM 21979 (MRSA) OD value	*S. aureus* DSM 21979 (MRSA) number of colonies/mL
Control	1.25	1.94×10^8	1.39	2.06×10^8
10	0.009	$\leq 1.0 \times 10^1$	0.007	$\leq 1.0 \times 10^1$
1	0.073	3.8×10^2	0.082	5.5×10^2
0.1	0.205	5.6×10^6	0.243	9.4×10^6
0.01	0.435	4.9×10^7	0.492	6.8×10^7

mL) and gentamicin (30 μg/mL) on the intracellular survival of bacteria inside osteoblasts. In addition, synergistic effects of bacteriophages with gentamicin or rifampicin antibiotics to clear intracellular *S. aureus* were analysed. For each group, 3 replicates were performed. Further, the stability and activity of the bacteriophages were investigated performing a plaque assay on osteoblast lysates that were treated with bacteriophages following the *S. aureus* infection. For this purpose, the *S. aureus*-infected osteoblasts were treated with two concentrations of bacteriophages (5×10^8 PFU and 1×10^9 PFU) and incubated for 24 h. Subsequently, the wells were washed 3 times with PBS and the cells treated with citrate buffer (pH 4) for 5 min to inactivate extracellular phages. Next, the wells were washed twice with PBS and the cells trypsinised, centrifuged and lysed using 0.2 % Triton X-100. This lysate was serially diluted (1:10) in sterile PBS and mixed with bacterial suspension in semisolid-agar medium. Next, the mixed solution was spread onto agar plates. The plates were observed for plaque formation after overnight incubation at 37 °C. A control of osteoblasts treated with only bacteriophages (1×10^9 PFU/well) was also included. As a positive control, phage 191219 solution with 4×10^9 PFU was used.

TEM analysis

The effect of bacteriophages on bacteria was visualised using TEM imaging, after normal culture conditions as well as following the osteoblast infection. Under normal culture conditions, *S. aureus* bacterial suspension (OD_{600}: 0.5) was treated with bacteriophages at MOI 50 at 37 °C and samples were collected by centrifugation (457 ×*g*) for 10 min at the 10 min and 12 h time points. In case of osteoblast infection, SAOS-2 cells were seeded on cell culture Petri dishes (1×10^6 cells/dish) and infected with *S. aureus* EDCC 5055 at MOI 30, as previously described. After gentamicin treatment, cells were treated with bacteriophages (10^9 PFU/mL), trypsinised and collected by centrifugation (5086 ×*g*) for 10 min at the 6 h time point for TEM imaging. MOI 30 and 6 h time point were used for the detection of the bacteriophage effect on the bacteria inside the osteoblasts.

These pellets were first fixed in Karnovsky fixative (for 15 min at room temperature; Heim *et al.,* 2015) and, after that, they were enclosed with agarose. For the embedding process, cells were fixed again with 1 % osmium tetroxide at pH 7.3 and dehydrated through a graded series of ethanol. Next, the samples were embedded in EMbed-812 epoxy resin (Science Services, Munich, Germany) and polymerised at 60 °C for 48 h. Then, the LYNX microscopy tissue processor (Reichert-Jung, Wetzlar, Germany) was used. Semi-thin-sections (75 μm), for the selection of relevant areas, and ultra-thin sections (80 nm) were cut using the Reichert Ultracut S Microtome (Leica-Reichert, Wetzlar, Germany). Then, the ultra-thin-sections were contrasted using aqueous 2 % uranyl-acetate and 2 % lead-citrate solution for 10 min each.

Electron-microscope imaging was performed using a Zeiss LEO 912AB electron-microscope (Lüke *et al.,* 2020).

Testing of phage 191219 effect on implant-associated infections in the *G. mellonella* model

Stainless steel K-wires with a diameter of 0.8 mm, were purchased from Synthes, Zuchwil, Switzerland. 4-5 mm long pieces were sterilised in 70 % ethanol before performing the experiment. For the infection process, implants were pre-incubated in bacterial growth culture medium with 1×10^6 CFU/mL *S. aureus* EDCC 5055 for 30 min while shaking at 150 rpm. Later, the implants were washed with 10 mL PBS and implanted on the rear side in the abdomen of the larvae by piercing the cuticle of the larvae using the sharp edge of the K-wire (Mannala *et al.,* 2020a). For the control group, the same process was applied but without bacterial contamination of the implants. Before implantation, the number of adhered bacteria was estimated by sonicating the samples and plating them on agar plates. For each testing group, 10 larvae were used.

To determine the effect of bacteriophages on bacterial survival in the early-stage stainless steel implant biofilm infection model, 50 μL of bacteriophages (10^9 PFU/larva) were injected at day 1 into the abdomen of a larva that had already been implanted with an *S. aureus*-preincubated implant, as previously described (Mannala *et al.,* 2020a). To determine the synergistic effect of antibiotics with bacteriophages against *S. aureus* biofilm infection, the larvae were injected with 10 μL of gentamicin (120 mg/kg) and rifampicin (70 mg/kg) at their rear part and survival of the larvae was observed for 5 d. All experiments were performed 3 times.

Statistical analysis

Statistical analysis was performed using sigmaP lot 10.0 (SYSSTAT software Inc.,Chicago, IL, USA). For the analysis of bacterial numbers, Student's *t*-test was applied. For the survival analysis, log-rank test was performed. Data were represented as means ± standard deviation from 3 independent experiments, with at least 3 technical replicates in each experiment. Data were considered to be statistically significant for $p < 0.05$.

Results

Phage 191219 exhibited *in vitro* activity against planktonic *S. aureus* with proliferation of bacteriophages within *S. aureus*

First, the activity of phage 191219 was assessed against *S. aureus* EDCC 5055 and *S. aureus* DSM 2197 using the double agar overlay method. The results showed clear plaque formation with both strains. Next, the efficiency of phage 191219 against planktonic growth was measured. Phage 191219 and bacteria were cultured at MOI 10, 1, 0.1 and 0.01 and

bacterial number was measured by CFU analysis. The CFU analysis showed that a significant antimicrobial activity of the bacteriophages was measured at MOI 10 and 1. There was no significant reduction in planktonic growth at MOI 0.1 and 0.01 (Table 1).

TEM investigation revealed that numerous phage 191219 particles had attached to the *S. aureus* after 12 h, with a large number of newly formed bacteriophages within the bacteria (Fig. 1a). Furthermore, the infected bacteria showed disrupted cell walls and even bacterial degradation, with detectable remnants of dead bacteria after 12 h of

treatment (Fig. 1b). At the early time point of 10 min after treatment, no significant attachment of phages (191219) to *S. aureus* bacteria was detected.

Phage 191219 was active against *in vitro S. aureus* biofilm

To evaluate the role of bacteriophages against *in vitro S. aureus* biofilm, bacteriophages at various concentrations were added to the biofilm formed on the plastic surface of the 96-well plate. After 24 h of bacteriophage treatment, the biofilm was analysed using both crystal violet and CFU analysis methods.
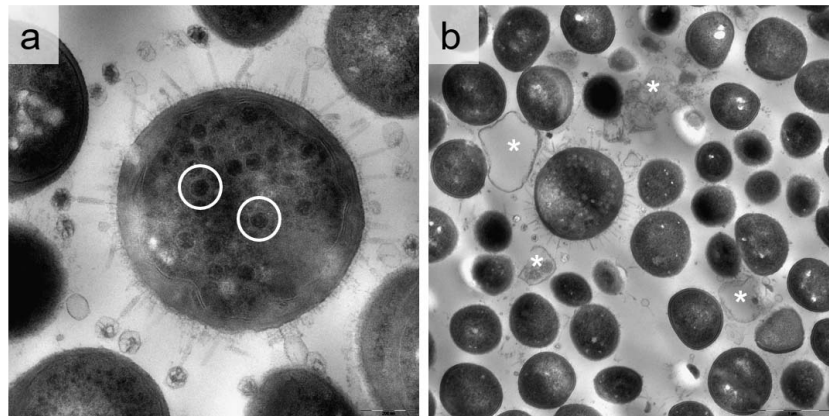

Fig 1. Visualisation of the effect of phage 191219 by TEM. *S. aureus* EDCC 5055 bacteria were treated with bacteriophages at MOI 50 and incubated at 37 °C. The bacteria were collected at 10 min and 12 h, fixed and analysed using TEM. (**a**) A bacterium with numerous bacteriophages binding at its surface and newly formed phages within the bacterium (examples marked by white circles) after 12 h. (**b**) Furthermore, after 12 h, damaged bacteria (white spots) and debris as well as remnants of dead bacteria were detectable (*). At the early time point of 10 min, significant attachment of phages to *S. aureus* was not detected.
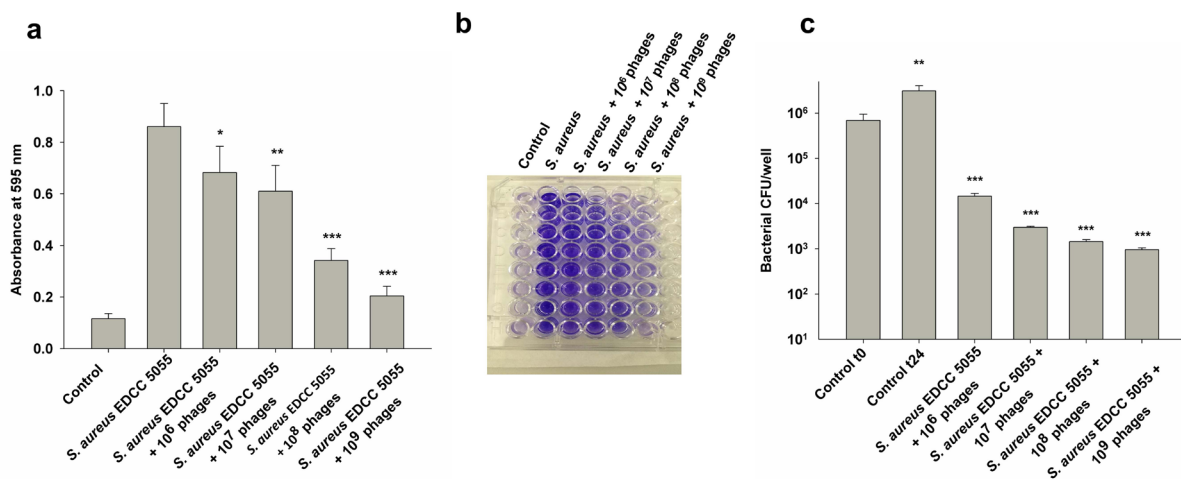

Fig 2. Antimicrobial activity of phage 191219 on *S. aureus* biofilms measured using the crystal violet method and CFU analysis. The antimicrobial activity of the bacteriophages on biofilms *in vitro* was measured by incubating for 24 h the several fold dilutions of bacteriophages in TSB medium with biofilms formed on 96-well U-bottom plate. After 24 h, the wells were washed twice with PBS, stained with 0.1 % crystal violet solution and incubated at room temperature for 10 min. Later, unbound colour was removed using PBS washes, dissolved in ethanol and absorbance was measured at 595 nm. A concentration of 10^6 PFU/well already resulted in a significant reduction in the biofilm biomass on the 96-well plate and the reduction in biomass was profound at increasing phage concentrations. (**a**,**b**) Visualisation of the outcome of the crystal violet assay. For the bacterial count determination, after 24 h treatment, wells were washed twice with PBS and bacterial cell number was determined by CFU analysis. (**c**) Representation of the obtained CFU/well for each tested condition. Bacteriophage concentrations of $10^7, 10^8$ and 10^9 PFU showed 2.6, 2.8 and 3-log reduction in biofilm-embedded bacteria compared to control (t0), respectively (*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; *t*-test). Data are represented as mean ± standard deviation from 3 independent experiments.

Results showed that the bacteriophage dose of 10^9 PFU was effective in eradicating the biofilm as evidenced by a decrease in crystal violet absorbance as well as a significant reduction in CFUs for the treated group (9.59×10^2 CFUs/well) when compared to control at time point t0 (1.29×10^6 CFUs/well) (*** $p \leq 0.001$) (Fig. 2). Treatment of biofilm with bacteriophages at a concentration of 10^8 and 10^7 PFU/ well showed 2.8 and 2.6 log reduction in *S. aureus* in biofilms, respectively.

Phage 191219 improved the low effect of gentamicin on intracellular growing *S. aureus* inside osteoblasts but not the high effect of rifampicin

SAOS-2 osteoblasts were infected with *S. aureus* and treated with bacteriophages at 10^9 PFU/well. The bacterial number was determined at 2, 4 and 24 h using cell lysis and CFU analysis (Fig. 3). Results showed that bacteriophages significantly affected the intracellular growing bacteria compared to control after 4 and 24 h (*** $p \leq 0.001$). The additional use of bacteriophages with gentamicin showed further inhibition of *S. aureus* intracellular growth at 4 and 24 h compared to solely gentamicin treatment (*** $p \leq 0.001$). The combination of bacteriophages with rifampicin could not further improve the good intracellular effect of rifampicin alone.

The plaque assay with osteoblast lysates showed the presence of bacteriophages inside the osteoblasts after 24 h as well as phage propagation inside the *S. aureus*-infected osteoblasts. The plaque assay with serial dilution of osteoblast lysates showed that there was no difference in the number of bacteriophages between the 1×10^9 PFU/well- and 5×10^8 PFU/well-

treated groups (data not shown). The number of bacteriophages inside *S. aureus*-infected osteoblasts was many folds increased compared to the control non-infected osteoblasts treated with bacteriophages (1×10^9 PFU/well). The cell lysate of the control group non-infected osteoblasts treated with bacteriophages revealed the internalisation of phages (5×10^2 PFU/well) into osteoblasts (data not shown).

Analysis of TEM images showed the effect of the bacteriophages in *S. aureus* inside the osteoblasts (Fig. 4). Phages were detected in the osteoblasts in all stages of the lytic cycle, such as binding to the bacterial cell wall, proliferation inside the bacteria and lysis of the bacteria (Fig. 5). Remnants of lysed bacteria with deteriorated cell walls were also found with newly formed bacteriophages in close vicinity (Fig. 4,5).

***In vivo* testing of phage 191219 effect on *S. aureus* biofilm in the *G. mellonella* infection model**

The effect of phage 191219 was assessed against early-stage *S. aureus* biofilm formation on the implanted K-wire inside the larvae. The adhered bacteria on pre-incubated K-wires were $5,300 \pm 480$ CFUs per K-wire, as determined by sonication. In the *G. mellonella* early-stage biofilm infection model, the singular bacteriophage application was not effective against *S. aureus* infection, as survival rates were comparable to those of the untreated control group (Fig. 6). Further, the treatment with rifampicin against *S. aureus* biofilm infection significantly improved the larval survival (** $p \leq 0.01$), whereas gentamicin did not. Simultaneous treatment with bacteriophages and either gentamicin or rifampicin appeared to slightly
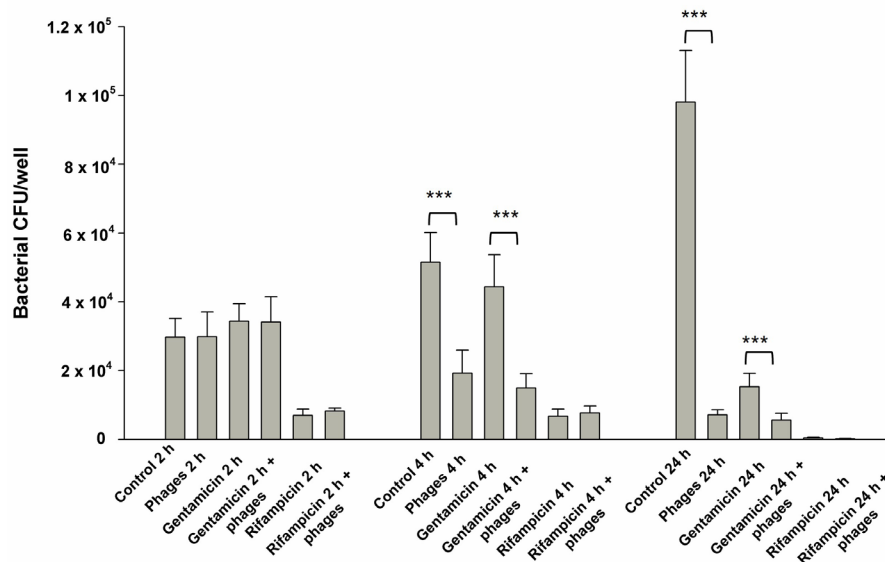
Fig 3. Effect of phage 191219 on intracellular growth of *S. aureus* inside the osteoblasts. The effect of the bacteriophages on the intracellular growth of *S. aureus* inside the osteoblasts was tested by treating the *S. aureus*-infected osteoblasts with 10^9 PFU/well, followed by CFU analysis at 2, 4 and 24 h time points. In addition, the synergistic effect of gentamicin and rifampicin to clear intracellular *S. aureus* was analysed. The results showed a significant antimicrobial effect on the intracellular survival of *S. aureus* compared to control after 4 h. Similarly, bacteriophages showed an improved effect in combination with gentamicin at 4 and 24 h time points, with significant inhibition of intracellular bacterial growth, but not in combination with rifampicin (*** $p \leq 0.001$; *t*-test). Data are represented as mean ± standard deviation from 3 independent experiments.

enhance the effect of the respective antibiotic, despite the results not being statistically significant.

Discussion

In the present study, the *in vitro* efficacy of phage 191219 to eradicate planktonic stage *S. aureus* strains – following biofilm formation as well as after internalisation into osteoblasts – alone as well as in combination with gentamicin and rifampicin was tested. Furthermore, to the best of the authors' knowledge, the present was the first work to study the effects of bacteriophages in a *G. mellonella* biofilm model with an already incorporated metal implant mimicking orthopaedic infections, as previous studies with phages in *Galleria* models have only looked at planktonic bacterial infections.

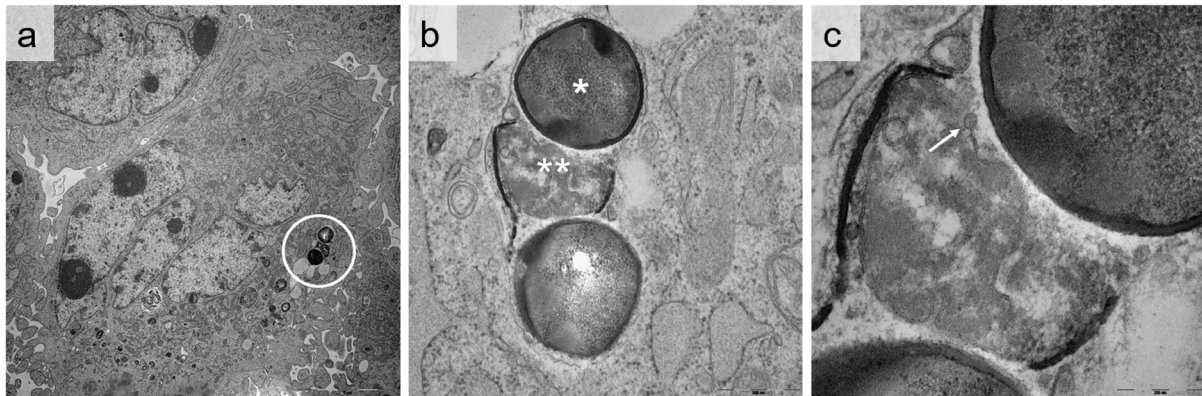Two *S. aureus* strains (MSSA and MRSA) were treated during their planktonic stage with distinct

Fig 4. Visualisation of the effect of phage 191219 on osteoblasts by TEM. For the osteoblast infection, the SAOS-2 cells were infected with *S. aureus* at MOI 30 and treated with bacteriophages after the gentamicin treatment step. (**a**) Infected osteoblast. Scale bar: 5 μm. (**b**) Magnification of **a**. The infected osteoblast after treatment showed fewer (*) and totally damaged (**) bacteria within the cell. Scale bar: 500 nm. (**c**) Magnification of **b**. Dead bacterium showing a destroyed cell wall and newly formed phages (arrow). Scale bar: 200 nm.
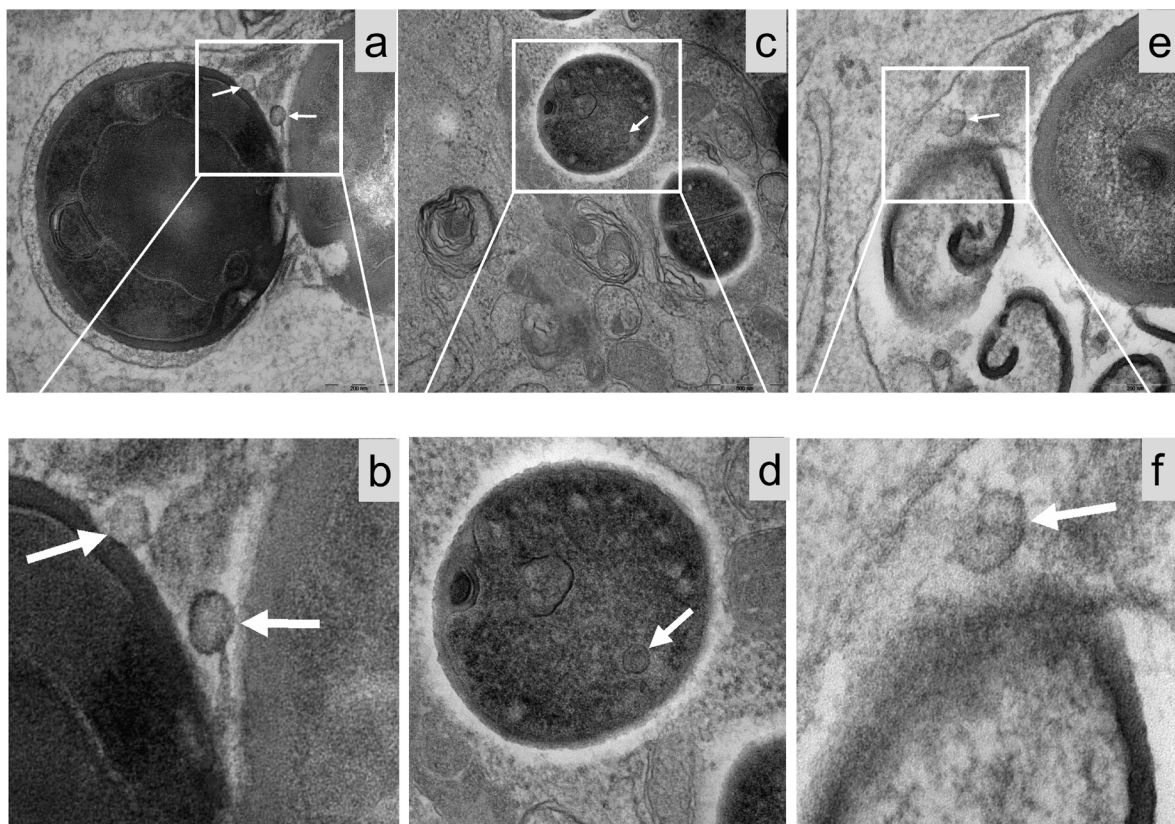

Fig 5. Visualisation of all lytic stages of phage 191219 in osteoblasts infected with *S. aureus* by TEM. TEM analysis of osteoblasts infected with *S. aureus* and treated with bacteriophages revealed the lytic stages of phage 191219 such as (**a,b**) attachment of phages to the bacteria, (**c,d**) proliferation inside the bacteria and (**e,f**) lysis of the bacteria. Scale bar: 500 nm. The lysis of the bacteria led to the release of phages and dead bacteria into the cytoplasm.

doses of 191219 *S. aureus* bacteriophages. Results showed that bacteriophages administered at a MOI 1 were sufficient to kill both MSSA and MRSA strains. Morris *et al.* (2019) tested a cocktail of distinct bacteriophage types against *S. aureus* strains in their planktonic stage, reporting a reduction in bacterial growth of 90-100 %. In another study, bacteriophages were modified using CRISPR-Cas9 to remove staphylococcal cytotoxin and enterotoxin genes. The bacteriophage killed 1×10^5 CFU *S. aureus* culture within 6 h of treatment, being more effective than the unmodified type (Park *et al.*, 2017). TEM investigations confirmed the attachment of numerous bacteriophages to the bacterial cell after 12 h and attacked bacteria showed disrupted cell walls and even bacterial degradation, confirming the effect of the phages on *S. aureus*.

From the biofilm assay, it became evident that increasing doses of phage 191219 led to decreased bacterial biofilm burden on plastic surfaces. Further, the treatment with 1×10^9 PFU bacteriophages resulted in a 3 $\log_{10}$ reduction in bacterial biofilm inoculum after 24 h. Morris *et al.* (2019) showed that treatment of established biofilm on titanium scaffolds with the bacteriophage cocktail could reduce 6.8 $\log_{10}$ to 6.2 $\log_{10}$ CFU compared to a control, however, not after exposure to cefazolin (50 mg/mL). Further research has shown the effectiveness of a temperate bacteriophage genetically modified with CRISPR/Cas9, removing major virulence genes and expanding the host specificity through modification of the tail fibre protein. The modified bacteriophage was effective at a dose of 1×10^8 and 2.5×10^8 PFU, with a total clearance of bacterial biofilm, whereas vancomycin (1,024 µg/mL) did not affect the biofilm (Cobb *et al.*, 2019). Similarly, several other *in vitro* studies (Alves *et al.*, 2014; Tkhilaishvili *et al.*, 2020)

have confirmed the efficacy of bacteriophages against *S. aureus* biofilm, which could be corroborated by the results of the present study. Alves *et al.* (2014) revealed a significant biofilm reduction after 48 h treatment using a novel phage DRA88. Tkhilaishvili *et al.* (2020) showed complete eradication of biofilm-embedded bacteria on glass beads after treatment with Sb-1 and PYO phages for 5-7 d. The work conducted by Kumaran *et al.* (2018) targeted the question whether treatment order matters, showing significantly more *S. aureus* reduction when treatment with the bacteriophage SATA-8505 precedes the antibiotic (cefazolin, vancomycin, dicloxacillin, tetracycline and linezolid). The apparent improved effect in the present study with simultaneously administered antibiotics enhancing bacteriophage activity in the *G. mellonella* assay endorsed previously published data (Chaudry *et al.*, 2017; Kolenda *et al.*, 2020; Tkhilaishvili *et al.*, 2018). Kolenda *et al.* (2020) showed a synergistic effect with simultaneous treatment of *S. aureus* biofilms with a cocktail of 3 phages along with either vancomycin or rifampicin antibiotics.

Analyses of treated *S. aureus*-infected osteoblasts revealed that bacteriophages were more effective in comparison to gentamicin alone, whereas a combination of both agents further but slightly reduced bacterial CFU counts. Rifampicin alone seemed to be the most efficient in eradicating internalised *S. aureus*; a combination of bacteriophages with rifampicin cleared most of the bacteria after 24 h. The combination of gentamicin or rifampicin and phage 191219 failed to show significant effects on the intracellular growth of bacteria compared to the antibiotic alone. This might be due to the different time durations required for the antimicrobial activity of phage/antibiotic combinations and the intracellular
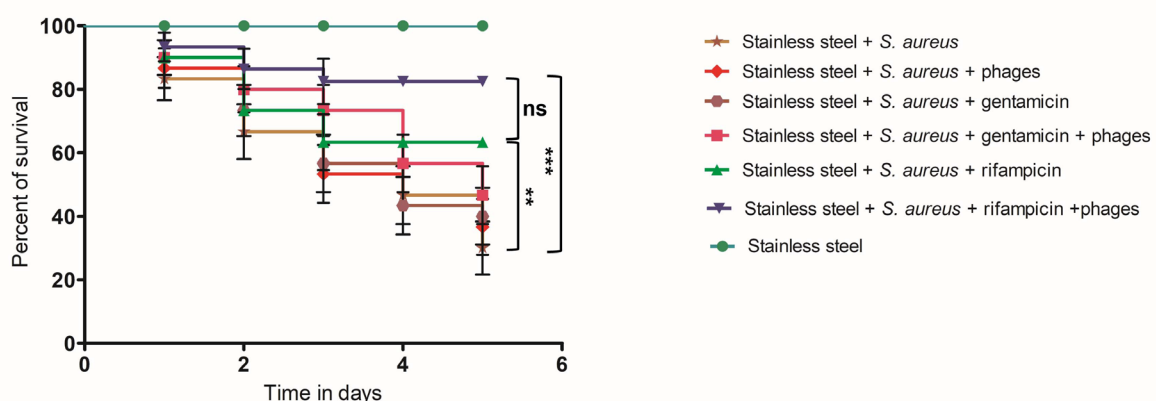

Fig 6. Effect of phage 191219 against *S. aureus* in a *G. mellonella* early-stage implant biofilm model. For the biofilm infection model, the larvae were implanted with pre-incubated K-wires, followed by treatment with bacteriophages alone and in combination with rifampicin and gentamicin 24 h post-implantation. The synergy between the phages and the antibiotics was compared with their respective controls. Results showed a significant improvement in survival rate for the larvae treated with rifampicin compared to the untreated control group. The combination of rifampicin and phages appeared to have slightly improved the larval survival, although not significantly. Neither phages, gentamicin nor the combination of both treatments significantly improved the larval survival compared to the untreated control group (ns, non-significant, *** $p \leq 0.001$; ** $p \leq 0.01$; log-rank test). In each experiment, 10 larvae were used for each group. Data are represented as mean ± standard deviation from 3 independent experiments.

conditions. In the present study, ultrastructural analysis of the phages in *S. aureus*-infected osteoblasts showed the activity of phages, with binding of the phages to the bacteria, proliferation of new phage particles and subsequent lysis of the bacterial cell and release of the phages into the cell cytoplasm. Phages are found ubiquitously in nature and the human body harbours a large number of bacteriophages together with their bacterial host. Moreover, they constitute the key component of the gut microbiome (Bichet *et al.*, 2021). Phages are detected in the circulatory systems, organs and tissue indicating that they are capable of translocating from the gut and penetrating throughout the body (Ghose *et al.*, 2019; Huh *et al.*, 2019). Recently, Nguyen *et al.* (2017) have shown that epithelial cells ingest phages through non-specific mechanisms, such as macropinocytosis, and transcytose them from the apical surface toward the basolateral side of the cell (Nguyen *et al.*, 2017). Thus, it is possible that the osteoblasts can internalise bacteriophages into macropinocytotic vesicles. Those vesicles might then fuse with phagolysosomes containing the bacteria and make it possible for the phages to attach to those bacteria. However, data on bacteriophage-mediated killing of *S. aureus* internalised in osteoblast are scarce. So far, only Kolenda *et al.* (2020) investigated three different bacteriophages (PP1493, PP1815 and PP1957), alone and in combination with vancomycin and rifampicin, against *S. aureus* biofilm as well as in an osteoblast infection model. The authors showed that the bacteriophages were able to internalise; however, the data suggest a lack of activity against intracellular bacteria in MG63 osteoblasts. The authors hypothesise that bacteriophages might be shuttled inside the osteoblasts by the bacteria, as it was previously indicated that bacteriophage MR-5 does not affect the natural intracellular killing of *S. aureus* by macrophages (Kaur *et al.*, 2014). This contrasts with the present study results, which showed a significant inhibition of *S. aureus* growth in osteoblasts already 4 h following bacteriophage treatment. The present study showed bacteriophages inside *S. aureus* within osteoblasts with destruction of bacterial walls and newly formed bacteriophages within the osteoblasts. The discrepancy compared to the results of Kaur *et al.* (2014) might be explained by i) the use of distinct *S. aureus* strains, which might differ in intracellular metabolic activity; ii) the use of distinct osteoblastic cells possibly influencing bacterial dormancy; iii) different activity of the phages used.

G. mellonella was recently shown to be a suitable non-vertebrate animal model to study biofilm infections, with the insertion of contaminated metal implants (stainless steel, titanium) mimicking orthopaedic biofilm infections (Mannala *et al.*, 2020a). To the best of the authors' knowledge, *G. mellonella* was previously only used for planktonic bacterial infection experiments with the addition of bacteriophages and multiple studies showed enhanced larval survivability due to bacteriophage treatment (Jeon and Yong, 2019; Manohar *et al.*, 2018; Thiry *et al.*, 2019; Tkhilaishvili *et al.*, 2020; Wang *et al.*, 2020). For instance, Tkhilaishvili *et al.* (2020) showed that the administration of the bacteriophages Sb and PYO results in reduced mortality, whereby survival rates are higher when the bacteriophages are applied before infection with *S. aureus* and further increases with a higher bacteriophage dose. Analogous effects are observed following treatment with vancomycin. Wang *et al.* (2020) characterised the activity of the new phage φWL-3, which was isolated from hospital sewage, against *E. coli* biofilms *in vitro* and in a planktonic infection *in vivo* model using *G. mellonella*. φWL-3 was effective against a planktonic *E. coli* infection in *G. mellonella* and the effectiveness was significantly enhanced by simultaneous treatment with phosphomycin (Wang *et al.*, 2020). The present study results of the *G. mellonella* biofilm model suggested that a single bacteriophage treatment alone did not result in a significantly increased survival rate of the larvae compared to untreated controls, whereas rifampicin significantly increased the survival rates. Phage treatment appeared to exhibit enhanced effects in combination with gentamicin or rifampicin, despite this result not being statistically significant. The bacteriophage treatment might have failed because only one treatment was applied. The coexistence of bacteriophages and bacteria could result in the rise of phage-resistant bacterial strains through various mechanisms, such as point mutations in receptor genes, CRISPR/Cas9 system and restriction-modification systems (Onsea *et al.*, 2020). However, phage resistance might not be the reason for the failure of bacteriophage therapy in *G. mellonella*, as the phage resistant bacterial strains face reduced fitness and loss of virulence with mutations in receptor genes. Capparelli *et al.* (2010) showed that the virulence of a phage-resistant *S. aureus* strain was decreased in a mouse infection model and used an attenuated strain to induce immunity against S. *aureus*.

There are several limitations to the present study. First, only one phage (191219) was used and results for this phage cannot be generalised for other phages. Further, only one single dose of the bacteriophages was used and multiple and/or repetitive treatment might enhance their activity, which was not investigated. Finally, the *G. mellonella* biofilm infection model also has its limitations due to the absence of a humoral immune system. Therefore, the results have to be interpreted with caution for any potential clinical translation.

Conclusions

While bacteriophages exhibited strong activity against planktonic and intracellular *S. aureus* in an osteoblastic cell line, only high concentrations of bacteriophages were effective against *S. aureus*

biofilms *in vitro*. Ultrastructural analysis highlighted the activity of phages on intracellular growing *S. aureus*, which is responsible for relapse and chronic implant-associated bone infections. For implant-associated biofilm infections tested in the *G. mellonella* infection model, single administration of bacteriophages failed to show improvement of larval survival rates. However, an improvement of the bacteriophage effect with the addition of gentamicin and rifampicin was suggested, albeit the result not being statistically significant. From the clinical perspective, the data suggested that the administration of bacteriophages might be a potential adjuvant treatment strategy for implant-associated biofilm infections, which has to be proven in further preclinical and clinical trials.

Acknowledgements

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Discussion with Reviewers

Reviewer 1: Bacterial internalisation may be equally, or more, significant within macrophages than within osteoblasts. How do you think a phage may enter macrophages and target the intracellular *S. aureus* within?

Authors: Similarly to phagocytosis of bacteria, the bacteriophages can be taken up by macrophage phagocytosis as well as by endocytosis and transcytosis by epithelial cells. Once the bacteriophage enters the cell, due to host specificity, it binds to the bacterial membrane and injects its DNA inside the bacteria, resulting in phage progeny formation and lysis of the bacteria.

Mercedes Gonzalez Moreno: The authors used K-wires as metallic implants for the *in vivo* model to mimic orthopaedic biofilm infections. So, why not also use the K-wire model for *in vitro* evaluation of anti-biofilm efficacy? Biofilm can be formed on K-wires and remaining adherent bacteria to the K-wire after treatment can be measured by sonication and CFUs quantification.

Authors: As the effect of bacteriophages on the biofilm was already tested using a U bottom 96-well plate, the K-wire was directly tested using the *in vivo* model *G. mellonella*. We will consider *in vitro* evaluation of anti-biofilm efficacy using the K-wire model in future projects.

Mercedes Gonzalez Moreno: The *G. mellonella* infection model using K-wire implants described by Mannala *et al.* (2020a), and referred to by the authors, shows initial adhesion of *S. aureus* to the surface of the K-wire after 30 min of incubation with bacteria

and maturation of the biofilm at day 3. Can the authors, therefore, claim the presence of a biofilm on the K-wire after 30 min of incubation with bacteria? The *in vivo* model used in the present study seems rather to address an early stage (bacterial adhesion) of biofilm formation.

Authors: We agree with the reviewer, that the biofilm is not mature after 30 min of incubation with bacteria. However, early stage of biofilm formation can be assumed after 24 h of implantation, when the bacteriophages were added.

Mercedes Gonzalez Moreno: Combinations of antimicrobial agents or combination of a phage with an antimicrobial agent are promising for the successful therapeutic management of biofilm infections. To reveal synergistic effects between phage-antibiotic combinations, it seems reasonable to test sublethal concentrations of certain antibiotics (Ryan *et al.*, 2012, additional reference). How do the authors intend to determine phage-antibiotic synergy?

Authors: Gentamicin (30 µg/mL) and rifampicin (8 µg/mL) in sublethal concentrations show an effect on the intracellular survival of bacteria (Mohammed *et al.*, 2014); therefore, these concentrations were chosen in the present study. A synergistic antimicrobial effect was defined by determining whether any combination of phage + antibiotic improved the antimicrobial activity compared to their effect individually.

Mercedes Gonzalez Moreno: Considering the mechanism of action of these two antibiotics, inhibiting DNA transcription (rifampicin) or bacterial protein synthesis (gentamicin), it raises the question of how the specific mechanism of action of an antibiotic may also affect its ability to synergise with phages. One could think that the inhibitory effects of rifampicin and gentamicin within the bacterial cell could be an impairment to phage propagation as well. Other antibiotics such as vancomycin or daptomycin, targeting the bacterial cell wall, appear to be good candidates for combination therapy with phages. In the treatment of infected orthopaedic implants, although rifampicin is the only antibiotic that is considered to possess anti-biofilm activity and it is recommended for the treatment of MRSA infections, it is never applied as a monotherapy due to the high risk of resistance development. Gentamicin, however, is not a recommended antibiotic for the treatment of MRSA infections. What was the authors' reasoning behind the choice of these two antibiotics for their study?

Authors: Rifampicin was chosen due to its very well-known activity against intracellular *S. aureus*. Indeed, rifampicin should never be applied as a monotherapy due to the high risk of infection development. Gentamicin, which is regularly used in orthopaedic surgery, was chosen as a kind of negative

control. The aim was to identify whether the phage treatment would make the bacteria more susceptible to gentamicin.

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Additional Reference

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Editor's note: The Scientific Editor responsible for this paper was Fintan Moriarty.