Article

Combination of bacteriophages and vancomycin in a co-delivery hydrogel for localized treatment of fracture-related infections

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Fracture-related infections (FRIs), particularly those caused by methicillin-resistant *Staphylococcus aureus* (MRSA), are challenging to treat. This study designed and evaluated a hydrogel loaded with a cocktail of bacteriophages and vancomycin (1.2 mg/mL). The co-delivery hydrogel showed 99.72% reduction in MRSA biofilm in vitro. The hydrogel released 54% of phages and 82% of vancomycin within 72 h and maintained activity for eight days, in vivo the co-delivery hydrogel with systemic antibiotic significantly reduced bacterial load by 0.99 log10 CFU compared to controls, with active phages detected in tissues at euthanasia (2×10^3 PFU/mL). No phage resistance was detected in the phage treatment groups, and serum neutralization resulted in only a 20% reduction in phage count. In this work, we show that a phage-antibiotic co-delivery system via CMC hydrogel is a promising adjunct to systemic antibiotic therapy for MRSA-induced FRI, highlighting its potential for localized, sustained delivery and improved treatment outcomes.

There are an estimated 178 million new bone fractures occurring globally and 36 million patients with fractures require surgery every year[1,2]. In approximately 5% of surgically operated patients, a fracture-related infection (FRI) occurs, resulting in prolonged antibiotic therapy, repeated surgical interventions, and recurrence rates of 6 to 9%[3,4]. The predominant causative pathogen is *Staphylococcus aureus*, with infections characterized by biofilm formation, antibiotic tolerance, and failure of the fracture to heal[5]. The challenges of treating biofilm-related FRI, and particularly those caused by antibiotic-resistant pathogens, have prompted the search for alternative or adjunct therapeutic approaches.

Bacteriophage (*phage*) therapy, which employs viruses to specifically target and kill invasive pathogens, has received renewed attention in recent years. Although the quality of clinical data is somewhat limited to date, the available evidence suggests that phage therapy has a 96% rate of clinical improvement in cases of musculoskeletal infections, with a good safety profile[6]. Phage therapy is generally administered as an adjunct to

conventional antibiotic therapy and in vitro studies show that the combination can yield improved outcomes[7,8], a phenomenon known as phage-antibiotic synergy (PAS). However, phage-antibiotic antagonism can also be observed in some cases, resulting in decreased treatment efficacy compared to phages or antibiotics alone[9].

In contrast to antibiotic therapy, where dosages and durations are precisely defined, the ideal mode of administration of phages is less clear[10]. Although local applications of phages (instillation) through a drain tube offer direct access to the infection site and have been routinely used in musculoskeletal infections[11], this approach has its own limitations including challenges in maintaining effective phage concentrations at the infection site[12] and the risk of superinfection with nosocomial pathogens that may colonize the drain tube. One alternative administration approach is the application of hydrogels loaded with phages[13]. This approach offers sustained and slow release of phages to the site of infection and reduces the need for drainage tubes[14,15]. Moreover, encapsulation of phages within such biomaterials can

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contribute towards attenuating antigenicity[16,17], thereby potentially decreasing the inactivation of phages by adaptive immune responses.

This study aims to develop an MRSA-targeting phage-loaded hydrogel simultaneously loaded with antibiotics. Specifically, a cocktail of two phages, both with enhanced anti-biofilm properties after an in vitro evolutionary process[18], is loaded within a carboxymethyl cellulose (CMC) hydrogel alongside vancomycin, hereafter called the co-delivery hydrogel. The stability and release kinetics of the co-delivery hydrogel were evaluated in vitro, followed by an in vivo efficacy assessment in a murine FRI model caused by MRSA. Outcome measures include antibacterial efficacy as well as the titer and distribution of phage in tissues, and an assessment of phage resistance and neutralization to enable a more regulated and effective delivery of phage.

Results

Antibiofilm activity of phage cocktail
GE-MRSA15-biofilm was formed onto porous glass beads (Fig. 1A & Supplementary Fig. 1, respectively). The antibiofilm effects of the phage cocktail (CUB_MRSA-COL_R23 and CUB_GE-MRSA15_R14) at a concentration of 10^7 PFU/mL, and vancomycin at 0.5, 1, 10, and 100 times the MIC, were evaluated alone and in combination using RT-qPCR. The phage cocktail achieved a 99.20% reduction of GE-MRSA15 CFUs compared to the growth control (Fig. 1B). The phage cocktail combined with vancomycin at a concentration of 0.5 times the MIC achieved the highest cell count reduction compared to the growth control (99.72%). Higher concentrations of antibiotics with phages demonstrated no additional antibiofilm activity compared to antibiotics alone at equivalent concentrations ($p > 0.05$) (Fig. 1B).

The stability and release kinetics of phages and vancomycin from co-delivery hydrogel
To identify the most effective hydrogel concentration (i.e. 2%, 3% or 4% w/v CMC) to deliver phages, the stability of the different agents in a hydrogel was evaluated. The outcomes indicate that CUB_GE-MRSA15_R14, and CUB_MRSA-COL_R23 exhibited approximately 1.9 $\log_{10}$ PFU/mL reduction after eight days of storage in all tested CMC hydrogel concentrations, compared to the initial titer observed in the freshly prepared hydrogel (Supplementary Fig. 2A & B, respectively). In addition, there was a 10% reduction in vancomycin under the same conditions (Supplementary Fig. 2A). The 3% CMC concentration was chosen for all subsequent testing because it provided a balance between sufficient phage and antibiotic delivery while maintaining optimal viscosity for local treatment applications. Subsequently, the stability of both the phage cocktail and vancomycin was assessed in the co-delivery hydrogel with 3% CMC. The co-delivery hydrogel exhibited approximately 1.9 $\log_{10}$ PFU/mL reduction over eight days (Fig. 2A). Vancomycin exhibited an 11.1% reduction in activity in the co-delivery hydrogel at day 8 (Fig. 2A).

The release profile of phages CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23 from the 3% CMC hydrogel exhibited a burst release within 24 h, reaching plateaus of approximately 57% and 74% after 72 h, respectively (Supplementary Fig. 3A & B, respectively). Meanwhile, vancomycin showed rapid release kinetics, with a near complete release observed after 72 h (Supplementary Fig. 3C). In the co-delivery hydrogel, the addition of vancomycin did not significantly affect the release kinetics of phage cocktail over time (Fig. 2B). However, the addition of phages led to a decrease in the amount of vancomycin (80% release within 72 h) released over time compared to conditions where vancomycin alone was delivered via the hydrogel (96% release within 72 h) (Fig. 2C). Statistical analyses were performed on the data for different hydrogel concentrations and buffer solution, but no significant differences were found. This demonstrates that the observed trends in phage and vancomycin release and stability are consistent across the conditions tested.

In vivo efficacy of co-delivery hydrogel against MRSA FRI
In total, 29 animals were included in this study. Five mice were excluded prematurely: two due to lack of weight bearing after revision surgery; one due to soft tissue/wound healing complications and lack of weight bearing after first surgery; one due to fracture of operated femur 1day post inoculation; and one due to fracture detected at euthanasia. A total of 24 mice were included in the final analysis, including Ctrl (no treatment, $n = 7$), Sys group (systemic vancomycin group, $n = 5$), cGel group (co-delivery hydrogel group, $n = 5$) and cGel+Sys group (co-delivery hydrogel combined systemic vancomycin group, $n = 7$). No significant difference ($P > 0.05$) was observed in the mean percentage weight change between the inoculation surgery and euthanasia between the different treatment groups or untreated control animals (Supplementary Fig. 4).

In vivo antibacterial efficacy
Bacterial load was quantified from the soft tissues, femur, and implants at euthanasia on day 13. In the untreated group, a high bacterial density was observed in all locations. The cGel+Sys treatment had the greatest antibacterial activity in soft tissue with 1.22 $\log_{10}$ CFU ($p < 0.05$) reduction in CFU compared with untreated animals; by 1.11 $\log_{10}$ CFU ($p < 0.05$) compared with the Sys group; and by 1.13 $\log_{10}$ CFU ($p < 0.05$) compared with the cGel group (Fig. 3A). The cGel+Sys group also significantly reduced the bacterial load in bone by 1.39 $\log_{10}$ CFU ($p < 0.01$) and in the implant by 0.91 $\log_{10}$ CFU ($p < 0.01$), respectively, compared with untreated animals (Fig. 3B, C, respectively). Overall, the cGel+Sys group showed superior antibacterial effect, achieving reductions of 0.99 $\log_{10}$ CFU in bacterial load compared to untreated controls, 0.81 $\log_{10}$ CFU versus Sys group, and 0.80 $\log_{10}$ CFU versus the gel group (all $p < 0.05$) at the infection site (Fig. 3D).

Phage titer in tissue at euthanasia
Phage titers in the cGel+Sys group (which had lowest CFU counts) were detected in soft tissue of only 1/7 animals (mean: 0.40 $\log_{10}$ PFU/mL), in the

Fig. 1 | SEM image and bacterial count determined for GE-MRSA15. Twenty-four-hour biofilm was formed on porous glass beads (PGB) by incubation in 1 mL medium inoculated with 1:100 dilution from a one-time use bacterial glycerol stock. **A** SEM image of bead in GE-MRSA15, magnifications × 2000; **B** Bacterial counts were determined by RT-qPCR after 24 h of GE-MRSA15 biofilm exposure to either phage cocktail (1:1 CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23, 10^7 PFU/mL), vancomycin (VAN at 0.5, 1, 10 and 100 times the MIC), or the combination of both. Growth control (GC) refers to GE-MRSA15 biofilm not exposed to antimicrobials. $MIC_{VAN} = 1$ µg/mL. The dots correspond to biological replicates ($n = 3$ per tested condition) and the bars represent the mean and standard error. VAN vancomycin.
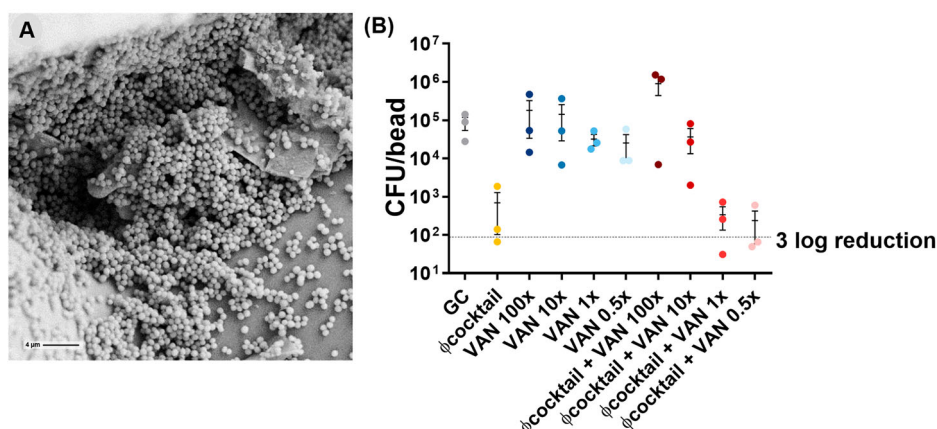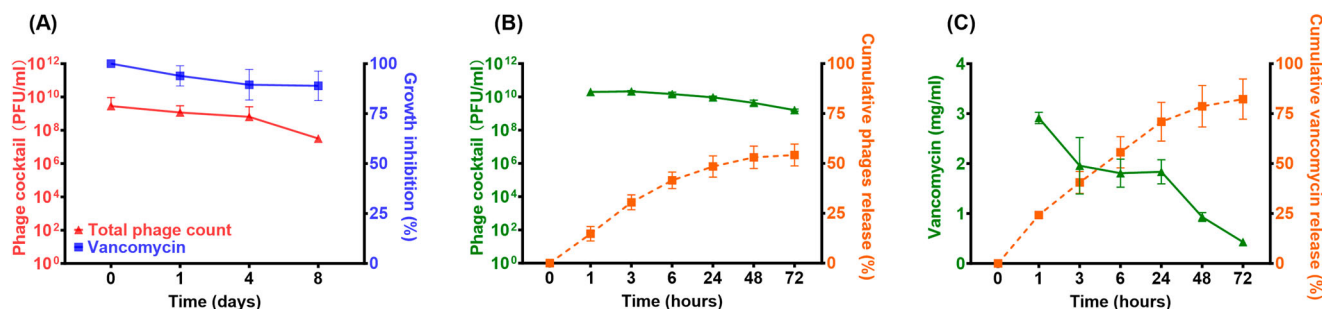
Fig. 2 | Stability and release profiles of phages and vancomycin in co-delivery hydrogel. A Stability of phages/vancomycin in co-delivery hydrogel. The red solid line represented the titer of phages in co-delivery hydrogel and should be read on the left Y-axis. The blue dashed line illustrated the stability of vancomycin in co-delivery hydrogel and should be read on the right Y-axis. The measurements were taken at different time points for 8 days. **B, C** The release profile of phage cocktail (CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23) and vancomycin in co-delivery hydrogel at 37 °C. The green solid line represents the drug concentration observed in the supernatant at specific timepoints and should be read on the left Y-axis. The orange dashed line illustrates the cumulative release profile up to the specific timepoints indicated and should be read on the right Y-axis. The measurements were taken at different time points for 72 h. The data are presented as means ± SD of results and error bars represent standard deviation. Experiments were performed with biological triplicates ($n = 3$ per tested condition).

Fig. 3 | Quantitative bacteriological evaluation of tissues after treatment. Soft tissue (**A**), implant (**B**), bone (**C**) and summary (**D**). They were shown for the Ctrl ($n = 7$), Sys group ($n = 5$), cGel group ($n = 5$) and cGel+Sys group ($n = 7$). All animals received treatment for five days after revision surgery except Ctrl that receive NaCl. Each symbol represents data from a single mouse. The data were presented as mean ± standard deviation of results and error bars represent standard deviation, and statistical significance was determined using a Kruskal-Wallis test followed by Tukey posttest (*$p < 0.05$ and **$p < 0.01$). CFU colony forming units.
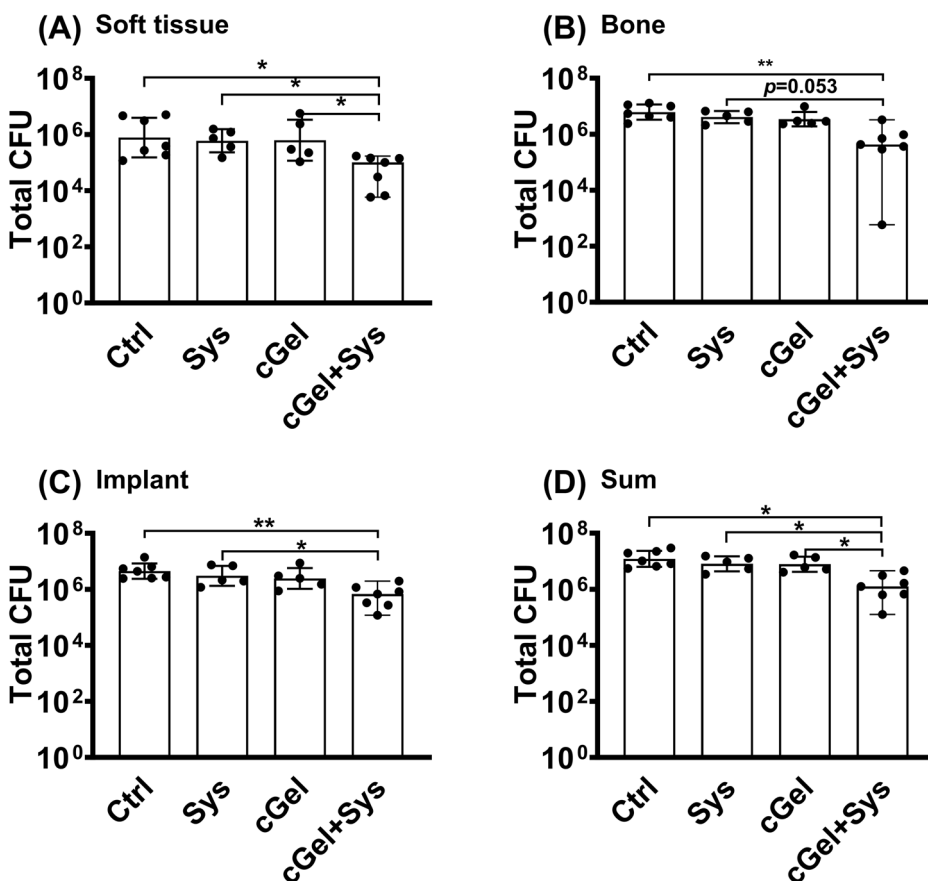
bone of 2/7 animals (mean: 0.75 $\log_{10}$ PFU/mL), and in the implant of 1/7 animals (mean: 0.41 $\log_{10}$ PFU/mL, Fig. 4A). In contrast, in the cGel group (which had the same total phage dose, but higher CFU counts) phages were detected in the soft tissue, bone, and implant in 4/5 animals, indicating a widespread distribution of phages across the tested locations (Fig. 4A). Moreover, in the bone, the cGel group exhibited significantly higher phage titers compared to the cGel+Sys group ($p < 0.05$). When evaluating all sampled locations collectively, a similar significant difference in phage presence was also observed ($p < 0.05$) (Fig. 5A). The bacterial load is positively associated with phage titer (r = 0.77 and $p = 0.003$) (Supplementary Fig. 5).

Bacterial resistance to phage at euthanasia

In groups that received phage therapy, isolated strains that were retrieved from the soft tissue, bone, and implant at euthanasia were used to determine whether they had developed resistance to the phages. When isolated strains were tested with phage CUB_GE-MRSA15_R14, a higher EOP was observed in the Gel+Sys group than in the Gel group ($p < 0.05$, Supplementary Fig. 6A), suggesting an enhanced susceptibility to CUB_GE-MRSA15_R14 when combined with systemic vancomycin. In contrast, when isolated strains were tested with phage CUB_MRSA-COL_R23, a lower EOP was observed in the Gel+Sys group than in the Gel group

Fig. 4 | Evaluation of phage therapy against MRSA: distribution, resistance, and neutralization. A The presence of phages against GE-MRSA15 in soft tissue, implant, bone and their summary at euthanasia. The total titer of the two phages was reported due to the indistinguishability between CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23 by plaque morphology. Samples for which no phage was recovered are plotted as 1 on the x-axis. **B** The efficiency of plating (EOP) for the isolates retrieved after euthanasia of the animals against phage cocktail. The isolates from soft tissue, bone, and implant were harvested for phage susceptibility tests. Each symbol represents data from a single colony collected from mice. **C** The percentage of neutralized phage particles. Each symbol represents data the mean for triplicate samples from one mouse. The data were presented as mean ± standard deviation of results and error bars represent standard deviation, and statistical significance was determined using a student's t-test (*$p < 0.05$). PFU, plaque-forming units.
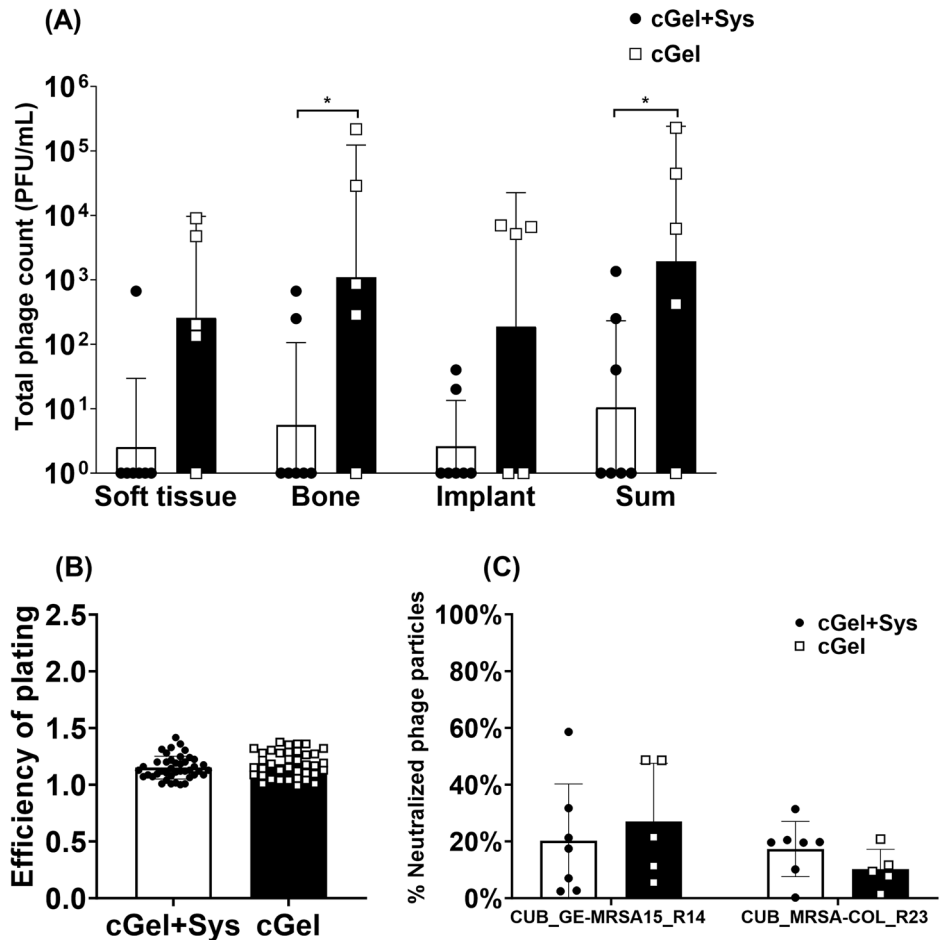
($p < 0.05$, Supplementary Fig. 6B), suggesting that systemic vancomycin might inadvertently promote a selective environment that encourages the survival of strains with higher resistance to CUB_MRSA-COL_R23. However, no significant difference in EOP between groups was observed using a phage cocktail for testing and the EOP of all isolated strains was greater than one (Fig. 4B).

Percentage of phage neutralization

Serum from the mice exposed to phages was analyzed to evaluate the capacity of serum to inactivate phages after 13 days of exposure in vivo. The mean percentage of neutralized phage particles at euthanasia compared with pre-exposure serum is shown in Fig. 4C, with no statistical differences being observed between the groups. This suggests that adaptive immune responses had not impacted phage treatment at this time point.

Discussion

The treatment of FRI, particularly those caused by MRSA, often requires long-term antibiotic use and multiple revision surgeries[19]. A potential approach to support the effective treatment of MRSA-induced FRI is the combination of phages and antibiotics[20]. In this study, the efficacy of a co-delivery hydrogel loaded with a phage cocktail and vancomycin was evaluated. This approach allows the application of phages in a single dose directly at the site of infection, thus overcoming the requirement for repeated doses when given by instillation or intravenous infusion. The in vitro results demonstrated that a phage cocktail combined with subinhibitory concentrations of vancomycin achieved the highest CFU reduction within an MRSA biofilm compared with the growth control. The co-delivery hydrogel maintained the lytic efficacy of phages for at least eight days in vitro and released approximately 55% of its incorporated phages, which

corresponds to 1.6×10^9 PFU/mL, within 72 h. In vivo, the co-delivery hydrogel combined with systemic vancomycin significantly reduced the bacterial load compared to systemic vancomycin and untreated controls. Active phages were still detected at euthanasia in the tissues (2×10^3 PFU/mL) in co-delivery hydrogel group. Phage therapy combined with systemic vancomycin increased susceptibility to phage CUB_GE-MRSA15_R14 but potentially promoted resistance to phage CUB_MRSA-COL_R23, with no significant difference observed using a phage cocktail. Taken together, these outcomes confirm the value of a co-delivery CMC hydrogel system to decrease the bacterial load and increase phage persistence in vivo.

The antibiofilm capacity of natural phages can be enhanced through an in vitro-directed evolution strategy, involving the application of selection pressures such as biofilm-challenged environments, which facilitates the training of phages to enhance their efficacy in killing target pathogens[18,21]. In our study, we used the evolved phage cocktail reported in Ponce et al.[18], which was the result of a directed evolution experiment to specifically increase biofilm activity of the included phages. The concentration of phages in the cocktail was also based on findings of Ponce et al., in which phage concentrations of 10^7 PFU/mL demonstrated an enhanced capability to suppress biofilm of GE-MRSA15[18]. Moreover, the concentration of 10^7 PFU/mL aligns with typical therapeutic doses in human patients[22]. The evolved phage cocktail in our study showed an enhanced antibiofilm performance relative to natural ancestor phages, as determined by RT-qPCR. To further enhance antibiofilm activity, we sought to simultaneously expose biofilms to antibiotics, as it was described previously that the antibacterial effect of phage therapy was enhanced with the addition of antibiotics[23], although not for all phage/antibiotic combinations[24]. In this work, we show that exposure of mature-grown MRSA biofilm to the phage cocktail in combination with subinhibitory concentration (0.5x MIC) of vancomycin

Fig. 5 | Scheme representing the formulation of co-delivery hydrogel and mouse study design. A The 3% w/v co-delivery hydrogel was formulated by mixing CUB_GE-MRSA15_R14, CUB_MRSA-COL_R23, and vancomycin with CMC powder at room temperature. **B** The mice were randomly assigned to one of four treatment groups. cGel group: a single dose of locally administered co-delivery hydrogel; cGel+Sys group: a single dose of locally administered co-delivery hydrogel and systemic subcutaneous vancomycin; Sys group: subcutaneous vancomycin (100 mg/kg) administration for five days; and Ctrl group: only received 0.3 mL of 0.9% NaCl solution. **C** The timeline of the animal experiment. After osteotomy, plate, and screw osteosynthesis, mice were inoculated with GE-MRSA15 and observed for five days and subsequently underwent revision surgery and treatment on day 5. From day 10 to day 13, mice did not receive any treatment. All mice were euthanized on day 13, and tissues were collected for analysis. VAN: vancomycin, NS: normal saline (0.9% NaCl). This figure was created with Biorender (biorender.com).
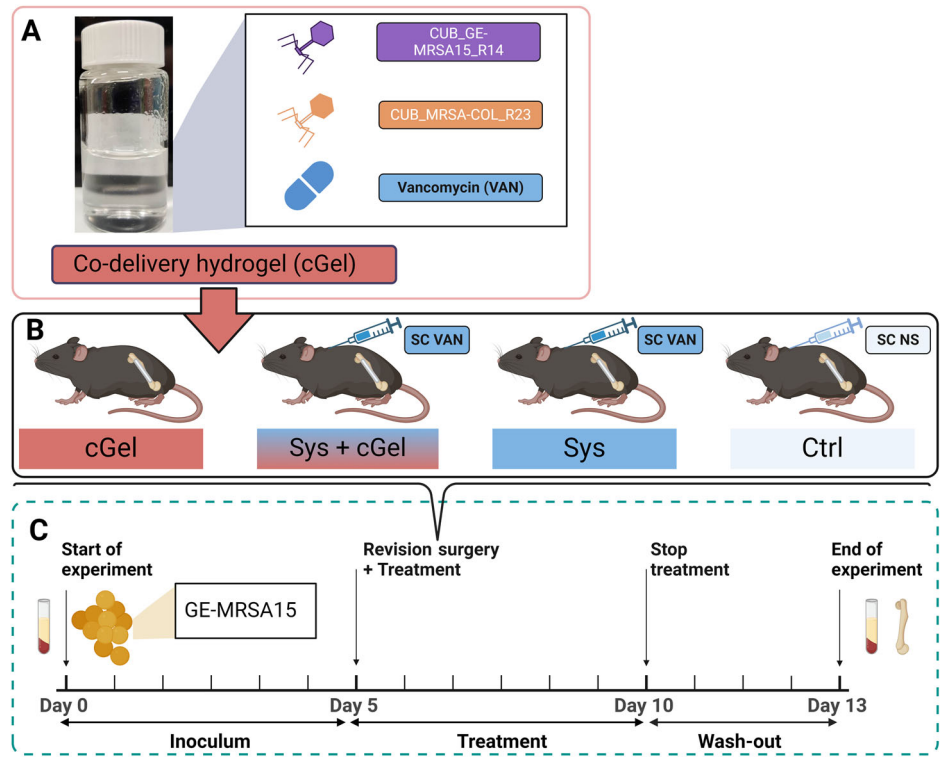
resulted in the highest reduction (99.20%) in bacterial load. At higher antibiotic concentrations, however, the phage cocktail demonstrated no additional antibiofilm activity compared to antibiotics alone at equivalent concentrations in this study. Wang et al. showed no synergism between simultaneous exposure of phage Sb-1 and vancomycin at high concentrations against GE-MRSA15 (the same bacterial strain used in our study)[25]. They hypothesized Sb-1 infection (for which the teichoic acid of the bacterial cell wall is presumably used as a receptor) might be negatively affected by the impact of vancomycin on the bacterial cell wall. The exposure of the biofilm to low concentrations of antibiotic together with phages, as seen in our study, may perhaps be enough to enhance bacterial killing without antagonizing the innate activity of phages. In some cases, combining phages with antibiotics may be necessary to comply with established treatment protocols or regulatory guidelines, particularly in clinical trials or in practices where antibiotic use is standard. The combination can help integrate phage therapy into existing treatment paradigms more smoothly. Furthermore, using both phages and antibiotics can be a strategy to prevent or delay the development of resistance. If bacteria develop resistance to the phage, the antibiotic could still be effective, and vice versa[26].

The dual delivery of antibiotic and phage was approached using a relatively simple hydrogel. The phage cocktail and vancomycin remained stable within the CMC hydrogel for eight days, providing a prolonged release for a minimum of three days, suggesting that the CMC hydrogel is suitable for delivering both phages and vancomycin. This performance contrasts with buffer solutions, where similar stability trends were observed but lacked the additional functional benefits provided by hydrogels. Hydrogel networks that have loose crosslinking are prone to releasing phages more rapidly than those with dense crosslinking[27]. The non-covalent nature of CMC gelation, which occurs spontaneously in water without the addition of reactive chemicals as crosslinkers, is an advantage to preserve phage and vancomycin stability. CMC is a hydrophilic polymer widely used in hydrogel components for delivering phages[28,29]. Its hydrophilic properties and gel-like nature enable it to retain water and provide a moist environment for the phages, facilitating sustained release over a prolonged period and improving treatment efficacy[30].

In vivo, the co-delivery hydrogel combined with systemic vancomycin significantly reduced the bacterial load compared to systemic vancomycin

and untreated controls. This is contrasting with prior findings where such an effect was not observed. For example, Cobb et al.[31] reported no additive effect when combining phage and an extremely high dose of fosfomycin against *S. aureus*-related osteomyelitis compared to either agent alone in the hydrogel. Onsea et al.[14] employed a topical hydrogel with phage ISP (10^9 PFU/ml) for FRI in rabbits and combined it with subcutaneous nafcillin administration and oral rifampicin for a duration of seven days. However, despite the notable reduction in bacterial load observed in the soft tissue, the bacterial load on the bone and implant remained high across all groups and no significant differences were found. The high vascularity within soft tissues facilitates the systemic circulation and delivery of antibiotics. In contrast, the reduced blood supply and distinctive structure of bone tissue during injury present challenges to effective antibiotic penetration, providing a plausible explanation for the limited impact on bacterial load in this region. Combined with our findings, the incorporation of sub-inhibitory concentrations of antibiotics into hydrogels may be important to enhance the antibacterial effect of phages. Furthermore, our study's findings should be considered within the broader context of bacterial burden reduction thresholds. Typically, a 2- to 3-log reduction or thresholds below $10^3 – 10^4$ CFU is needed to define successful treatment in animal models[32], but our results achieved a one-log reduction, from 10^7 to 10^6 CFU. While this falls short of the typical reduction, it's important to note that this reduction is in addition to the effects of vancomycin, a potent antibiotic against MRSA. This combination represents a significant improvement, especially in cases with prevalent antibiotic resistance. Although greater reductions would be desirable, our results demonstrate the effectiveness of combining phages with vancomycin and support the potential of this strategy to enhance treatment outcomes. These findings encourage further optimization and clinical trials to explore more effective combinations and achieve clinically relevant reductions.

Furthermore, we observed higher phage titers in tissues treated solely with co-delivery hydrogel versus those receiving combined therapy. This may be due to systemic antibiotics disrupting the bacterial hosts needed for phage replication. A direct correlation between phage titer and bacterial load was observed. Lower bacterial loads were associated with decreased phage titers, which might initially seem counterintuitive. However, this relationship underscores the phages' dependency on bacterial hosts for replication.

Consequently, effective antibacterial action, while beneficial for infection control, naturally leads to a reduction in available hosts for phage replication, thus explaining the lower phage titers. Understanding this balance is crucial for designing effective phage-based biomaterials, especially in environments where bacterial densities can fluctuate significantly.

Clinical studies indicate varying resistance levels to phages among bacteria, with lower resistance observed for *staphylococcal* phages (17%) compared to *P. aeruginosa* (36%) and *E. faecalis* (43%) phages[33]. This variance is attributed to the polyvalence of *staphylococcal* phages, supporting the use of phage cocktails to expand host range and target diversity. In our in vivo study, differences were found between the Gel and Sys+Gel groups when isolated strains were tested with CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23, respectively. Conversely, when the isolated strains were tested with a phage cocktail, no differences in sensitivity were observed between the treatment groups. The advantage of a phage cocktail lies in its ability to maintain effectiveness even if bacteria develop resistance to one of its components, as other phages within the cocktail can still lyse the bacterial cells. Moreover, while higher phage doses can effectively reduce bacterial populations, they also increase selection pressure, potentially accelerating resistance development[34]. Our approach mitigates this issue using a hydrogel with a slow-release mechanism, thereby moderating selective pressure and reducing the risk of resistance emergence.

The use of phages as therapeutic agents in vivo is promising; however, it raises concerns about the host's immune response potentially neutralizing the phages. Data in the scientific literature is rather limited, although in one published case report, a patient with FRI developed neutralizing antibodies 8-18 days after phage therapy[35]. Another study found that these antibodies can persist for over a year in some individuals with osteomyelitis[36]. These findings suggest that phage therapy may not be repeatedly administered, although there is little to no information on potential for repeat administration with different phages and how they may also be neutralized. Addressing these challenges, the deployment of innovative delivery systems has shown promise. A study focusing on *Klebsiella pneumoniae*-induced respiratory tract infections in mice demonstrated that encapsulation of liposome provided 100% protection for phage from neutralizing antibody[37]. In our study, phages were encapsulated within the hydrogel, and we observed a low level of phage neutralization, approximately 20%. This is in stark contrast to parallel research where phages delivered in a buffer solution induced a significantly higher rate of antibody production ranging from 50% to 80% by day 8, as compared to the 20%-30% neutralization observed for phage-loaded hydrogel by day 8[15]. Moreover, the FRI study by Onsea et al. demonstrated, on average, 45% of phage neutralization occurred in rabbits that received phage ISP in saline by day 14, whereas in rabbits that received phage ISP loaded in hydrogel, no neutralization was observed by day 14[14]. Although differences in phage, bacteria and hydrogel types across studies prevent a direct comparison, these findings highlight that the encapsulation within hydrogels provided a protective barrier that reduces immune system exposure, resulting in significantly lower neutralization levels. The longer study duration (14 days) in Onsea et al. shows that hydrogel-encapsulated phages effectively provide sustained protection, as evidenced by the absence of neutralization by day 14.

Although the findings in this work are promising, it is important to acknowledge a few limitations in our study. First, the in vivo portion of the study evaluated the system's performance over a relatively short duration. A longer-term assessment would provide a more comprehensive understanding of its effectiveness. Duration of therapy is a key factor in antibiotic therapy outcome, and a longer duration of antibiotic therapy in this model may increase efficacy with the impact of phage therapy waning after a single application. How phage titer changes over a longer period after a single application in the co-delivery hydrogel is an important future research target. Second, a phage cocktail was used. Therefore, distinguishing the activity of individual phages from the in vivo samples was challenging due to the similarity in plaque morphology for the two types of phages. As a counter-argument, phage cocktails are often the clinical routine, and potentially offer better antibacterial efficacy. The trade-off between the

single phage versus cocktail is thus less than clear. Third, the study did not directly compare the efficacy of co-delivery hydrogel with delivering single agents through encapsulation in vivo, such as phage in buffer solution, phage-only hydrogel, vancomycin-only hydrogel and systemic vancomycin combined with vancomycin loaded hydrogel. Comparative studies would help assess the relative efficacy and advantages of the proposed approach in comparison to other delivery system. Based on our findings, and these limitations, future research and development in this area should focus on better understanding of the relationship between tissue phage titer and antibacterial efficacy. Similarly, the relative antibacterial efficacy of different phages, alone or in phages, remains incompletely understood and finally, higher phage titers in the hydrogel and sequential release of phage followed by antibiotic could help to achieve even greater reductions in bacterial load. The use of cocktails, comprising of evolved phages to either increase half-life in vivo or further increase biofilm activity may also enable greater efficacy in the future.

Our study revealed the potential of simultaneously loading a cocktail of phages and vancomycin into a co-delivery hydrogel to combat MRSA-induced FRI. This co-delivery hydrogel provides a stable and slow release of phages, simplifying treatment with a single application, which also effectively reduced bacterial load in vivo. Moreover, the co-delivery hydrogel extends the lifespan of phage at the surgical site, and encouragingly, phages delivered via this system do not appear to induce the development of phage resistance and fewer neutralized phage particles were induced. This approach holds promise in addressing the challenges posed by biofilm-related infections, offering a new avenue for more effective and patient-friendly treatment strategies in the realm of FRI management. Further research and clinical trials are warranted to validate and refine these encouraging results for broader clinical applications.

Methods
Bacterial strains and antimicrobials
The methicillin-resistant *S. aureus* strain (GE-MRSA15) was sourced from the biobank collection associated with the prospective institutional cohort on periprosthetic joint infections at Charité–Universitätsmedizin Berlin in Berlin, Germany[25]. GE-MRSA15 was used as the test microorganism for all in vitro and in vivo experiments in this study. The antibiotic susceptibility of the isolate was tested by Labor Berlin – Charité Vivantes GmbH, Berlin, Germany. Results showed that GE-MRSA15 is rifampicin-resistant (MIC 4 μg/mL), gentamicin-resistant (MIC 16 μg/mL) and vancomycin susceptible (MIC 1 μg/mL). Bacteria were kept at -20 °C in a 25% glycerol solution for long-term storage. Fresh cultures were grown in tryptic Soy Broth (TSB, Sigma-Aldrich, Steinheim, Germany) or Müller-Hinton Broth (MHB, Sigma-Aldrich, Steinheim, Germany) as appropriate. Bacteria were grown on solid media when required using tryptic soy agar (TSA, Sigma-Aldrich, Steinheim, Germany).

CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23, two evolved phages with increased antibiofilm activity, were used in this work as monophages or as a 1:1 cocktail containing both phages[18]. GE-MRSA15 was used as the host bacteria for both phages. Due to similar plaque morphology, phage count was calculated as total plaque-forming units (PFU) count, and not as separate counts for each phage.

Vancomycin powder (Hexal, Holzkirchen, Germany) was reconstituted in sterile phosphate-buffered saline to desired concentration (PBS, OmniPur PBS Tablettes; Sigma-Aldrich, Steinheim, Germany).

Bacterial biofilm formation and imaging
Bacterial biofilms were formed on sterile 4 mm sintered porous glass beads (ROBU, Hattert, Germany) incubated in a sterile 24-well plate (Corning Inc., Corning, USA) (one bead per well) containing 1 mL TSB inoculated with a 1:100 dilution of a single-use glycerol stock of the GE-MRSA15 strain. Plates were kept humidified at 37 °C and 150 rpm for 24 h. For scanning electron microscopy (SEM), biofilm-containing beads were thoroughly washed in PBS to eliminate unattached bacteria prior to being fixed in a solution of 4% paraformaldehyde and 2.5% glutaraldehyde in 50 mM

HEPES for 24 h at 4 °C. Next, the samples were dehydrated in an increasing ethanol series and dried at the critical point, mounted on aluminum stubs, sputter coated with a 20 nm layer of gold-palladium and examined in the GeminiSEM 300 (Carl Zeiss Microscopy GmbH, Germany) operating at 3 kV with the SE2 electron detector.

In vitro analysis of phage-antibiotic combinations against biofilm
The antibacterial efficacy of the phage cocktail, vancomycin or their combination against GE-MRSA15 biofilm was assessed by real-time quantitative polymerase chain reaction (RT-qPCR) based on a previously described method[38].

First, 24 h-old-biofilms were formed on porous glass beads as described above, thoroughly washed with PBS, and exposed to either phage cocktail (10^7 PFU/mL, the concentration of phage cocktail was selected based on prior in vitro study using the same phage cocktail and MRSA[18]), vancomycin at concentrations of 0.5, 1, 10 and 100 times the MIC, or the combination of phage cocktail and vancomycin (same concentrations mentioned above) in MHB at a final volume of 1 mL and incubated at 37 °C for 24 h. For vancomycin, the concentrations tested (0.5, 1, 10, and 100 times the MIC) were chosen to cover a broad range that includes sub-inhibitory to significantly supra-inhibitory levels, which allowed us to assess the synergistic effects of phages and vancomycin across a spectrum of antibiotic pressures.

After 24 h of antimicrobial exposure, beads were washed in PBS and transferred to an Eppendorf tube containing 1 mL PBS and subsequently sonicated in a BactoSonic ultrasound bath at 40 kHz and 0.2 W/cm² (BANDELIN electronic, Berlin, Germany) for 20 mins. Then, the sonicated solution was used for DNA extraction from the dislodged biofilm-bacteria-cells, using the DNeasy UltraClean Microbial Kit (QIAGEN, Hilden, Germany). Subsequently, the NZYTech *Staphylococcus aureus* Real-time PCR Kit was used according to the manufacturer's instructions (NZYTech, Lisboa, Portugal). The extracted DNA was amplified and quantified in the Mastercycler RealPlex2 (Eppendorf, Hamburg, Germany). For each experiment, as part of the PCR kit, a positive control, negative control, and internal extraction control were included. Experiments were performed in biological triplicates.

Hydrogel preparation
The hydrogel used as a phase vehicle in this study was based on aqueous dispersions of sodium carboxymethyl cellulose (CMC, Sigma-Aldrich, USA). To determine whether phage or antibiotic stability was affected by the hydrogel (Section 2.5), suspensions of 2%, 3%, and 4% (w/v, CMC powder: suspension) hydrogels were prepared at room temperature and loaded with phage or vancomycin. The buffer used to suspend phages was Dulbecco phosphate buffer saline (DPBS, [2.7 mM potassium chloride (KCl), 1.5 mM potassium phosphate monobasic (KH_2PO_4), 137.9 mM sodium chloride (NaCl), 8.1 mM sodium phosphate dibasic ($Na_2HPO_4.12H_2O$)]) and for vancomycin, this was 0.9% NaCl (Thermofisher, USA). Briefly, CMC hydrogel was prepared by slowly incorporating CMC powder into the suspension while continuously stirring to ensure the formation of a homogeneous hydrogel[39]. The final concentration of each selected phage was 9×10^9 PFU/mL, and that of vancomycin, 1.2 mg/mL.

After initial experiments to identify the optimal CMC preparation, the final co-delivery hydrogel was formulated by integrating CUB_GE-MRSA15_R14, CUB_MRSA-COL_R23, and vancomycin into CMC powder at room temperature, resulting in a 3% w/v concentration (Fig. 1A). The final concentration of the phage cocktail, CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23, was 3×10^9 PFU/mL, and that of vancomycin, 0.4 mg/mL.

Stability of vancomycin and phage cocktail in hydrogel
The stability of phages/vancomycin in buffer and CMC hydrogel, as well as the stability of their combination in co-delivery hydrogel, was evaluated over an eight-day period. To assess this, 1 mL of each hydrogel and buffer was placed in glass vials and incubated at 37 °C. The zone of inhibition (ZOI) was assessed to identify the antibacterial activity of vancomycin within the hydrogel. GE-MRSA15 was cultured in TSB for 16 h, and the suspension was adjusted to an optical density at 600 nm (OD_{600}) of 0.1 using a Multiskan GO Spectrophotometer (Thermo Scientific). Subsequently, 100 µL of the adjusted inoculum was dispensed onto a Mueller–Hinton agar (MHA, Oxoid, Basingstoke, UK) plate and evenly distributed using sterile cotton swabs. Blank paper disks (SensiDisk, USA) were placed on the plates, followed by the addition of 20 µL of vancomycin in buffer, vancomycin-loaded CMC hydrogel, or co-delivery hydrogel onto the disks, obtained at different time points during an eight-day period. The plates were then incubated at 37 °C for 24 h. After the incubation period, the ZOI was measured on a millimeter (mm) scale using a Scan® 1200 (Interscience, Saint Nom, France) to quantify the antibacterial effect. The percentage of growth inhibition was calculated using the following formula: Percentage of growth inhibition = (Treatment ZOI/Control ZOI) × 100%. The Control ZOI refers to the ZOI observed on day 0, while the Treatment ZOI represents the ZOI observed on the testing day.

The stability of phage was assessed using the plaque assay method. The evaluation encompassed CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23 separately in buffer, CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23 separately in CMC hydrogel, and the phage cocktail in the co-delivery hydrogel. One mL of each hydrogel and buffer was placed in glass vials and incubated at 37 °C. Briefly, 100 µL of the diluted sample was taken and mixed with 100 µL of bacterial culture. The mixture was then added to 4 mL of soft agar containing 3% TSB and 0.6% agar. After gentle mixing, the solution was transferred to a TSA plate which was incubated overnight in a 37 °C incubator. Following incubation, the PFU were counted to determine the phage titer. The assessment was conducted on Day 0, Day 1, Day 4, and Day 8. All measurements were in biological triplicate from three independent samples.

Vancomycin and phage release from hydrogel
The release of vancomycin and phages from the hydrogel was evaluated over 72 h. One mL of CUB_GE-MRSA15_R14 (9×10^9 PFU/mL) in CMC hydrogel, CUB_MRSA-COL_R23 (9×10^9 PFU/mL) in CMC hydrogel, vancomycin (1.2 mg/mL) in CMC hydrogel, and co-delivery hydrogel was prepared in separate glass vials. Subsequently, 1 mL of PBS was added on top of the hydrogel, and the vials were stored at 37 °C. At each timepoint (1 h, 3 h, 6 h, 24 h, 48 h, 72 h), 1 mL of PBS was collected and replaced with the same quantity of PBS. First, released phages were tested by the double-agar method at the mentioned time points. The cumulative amount of released phages was plotted against time. Second, the release of vancomycin was tested using a colorimetric method[40]. A standard calibration curve relating absorption intensity to concentration was constructed for vancomycin using the Multiskan GO Spectrophotometer at a wavelength of 280 nm. Subsequently, the concentration of vancomycin released was determined by measuring absorbance and interpolating the corresponding value on the calibration curve.

Study design and animal welfare
The in vivo study was approved by the Ethical Committee of the Canton of Grisons in Switzerland (approval numbers GR/13/2021 and GR/08E/2022) and conducted at an Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited research institute. Twenty-nine female C57bl/6 mice specific pathogens-free (SPF), purchased from Charles River (Germany), aged 20-28 weeks, were included in the study after being given a two-week acclimatization period and declared in good health by a veterinarian examination. They were kept on a 12-hour dark/12-hour light cycle and fed a standard mouse diet. The animal grouping, animal model and timeline were shown in Fig. 5B, C, respectively. It involved creating a femoral osteotomy and inoculating it with GE-MRSA15 during index surgery, followed by a revision surgery involving debridement and irrigation on Day 5 to remove infected tissue. At time of revision surgery, mice were randomly divided into treatment groups or negative controls (receiving sterile saline instead of treatment). After five days of treatment following revision surgery,

there was a three-day wash-out to avoid false negative cultures, before the mice were euthanized on the 13th day after inoculation surgery (Fig. 5B, C, respectively). Blood samples were taken before the first operation and before euthanasia, and serum was separated and frozen for later neutralization experiments. Radiographs were taken after each surgery and at the end of the study to check for proper alignment and healing.

Surgical procedure, treatment and postoperative care
Surgical procedures were performed as previously described[15]. Anesthesia was induced and maintained with a mixture of sevoflurane solution (sevofluran Baxter®, Baxter AG, Switzerland) in oxygen (ca. 7 Vol%, 800-1000 ml/min O2 for induction and 2-3% sevoflurane, 600 ml/min O2 for maintenance). Analgesia was provided preoperatively by Buprenorphine (0.03 mg/mouse subcutaneously) and Carprofen (12.5 mg/kg subcutaneously) and continued postoperatively by administering Tramadol in drinking water (0.2 mg/ml) for 5 days after each surgical procedure. After aseptic preparation of the surgical site, the left femur and a 4-hole plate (RIS.401.100; RISystem AG, Switzerland) was applied to the bone and fixed with four angular stable screws (RIS.401.110; RISystem AG, Switzerland) following the implant manufacturer's protocols. Subsequently, a 0.44 mm osteotomy was created in the center of the plate by using the MouseFix Drill-&Saw guide and a Gigly hand saw. A swab sample was taken from the surgical site above the muscles for bacterial culture to check for contamination before inoculation, and the incision was closed with sutures after adding 2 μL of GE-MRSA15 (1×10^4 CFU) into the osteotomy. Treatment began five days after infection, with all mice receiving revision surgery under anesthesia and aseptic preparation of the surgery site as previously described. Infected tissue was removed, and the surgical site was flushed with 1 mL of sterile Ringer solution (Scharlau, Barcelona, Spain), which we collected for bacterial culture, before the mice received their assigned treatments. Before surgical closure, the mice allocated to groups receiving phage therapy (cGel, cGel+Sys) received a wash solution of 50 μL of phages (10^{10} PFU/mL) in buffer that was pipetted into the surgical wound and left in place for 5 min. Subsequently, an additional hydrogel treatment consisting of 50 μL of co-delivery hydrogel was pipetted into the surgical wound above the femur immediately prior to surgical closure. The Sys group received subcutaneous (SC) vancomycin (100 mg/kg) twice daily for 5 days after revision surgery. The mice from the systemic treatment group (Sys) and cGel+Sys group received vancomycin injections (100 mg/kg subcutaneously) twice daily for 5 days. The mice from the control group (Ctrl) received 0.3 mL of 0.9% NaCl solution twice daily for the same 5-day treatment period after revision surgery (Fig. 5C). The mice were observed and scored using a study-specific scoring system for welfare assessment twice a day for the first 5 postoperative days, followed by daily scoring until euthanasia. The mice were monitored for their general eating behavior and the load they placed on the operated leg. The surgical incision, respiration, eyes, fur, and feces are also monitored. The weight was also monitored at surgery, 3 days postoperatively, and at euthanasia. The results were noted on the score sheet and used to identify animals requiring early intervention or early euthanasia.

Inoculum preparation
A single colony of GE-MRSA15 was suspended in 20 mL of TSB and incubated at 37 °C with shaking overnight prior to surgery, to ensure fresh cultures in the logarithmic growth phase. One mL aliquot of the overnight culture was transferred to a 20 mL pre-incubated TSB flask and incubated with shaking at 100 rpm at 37 °C for a period of 2–2.5 h for sub-culturing. Approximately 30 min before the surgery, the sub-culture was subjected to centrifugation at $3220 \times g$ for 7 mins at room temperature. The supernatant was discarded, and the pellet was re-suspended using PBS. This process was repeated following centrifugation under same conditions. At the time of surgery, mice were inoculated with 10^4 CFU of strain (OD_{600} of 0.01) at the logarithmic growth phase confirmed through counting of colony-forming units (CFU) on TSA plates.

Bacterial quantification
The bacterial load was measured in soft tissue, bone, and implants of mice after euthanasia on day 13 to assess the outcome of infection and treatment. Femurs and soft tissue overlying the implant were homogenized (Omni TH, tissue homogenizer TH-02/TH21649) separately in 4 mL sterile PBS. The plate and screws were removed from the femurs, transferred to a glass test tube containing 4 mL PBS and sonicated in an ultrasonic water bath (Model RK 510 H, Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 3 mins. Each sample was serially diluted ten-fold before being cultured on 5% horse blood agar plates (BA, Oxoid Ltd., Hampshire, UK). Plates were incubated at 37 °C and bacterial quantification was performed after 24 h. Plates were incubated at room temperature for an additional 24 h to check for any signs of slow-growing colonies or contaminants.

Determination of phage concentration post-mortem
The double agar method was employed to determine the presence of phages in bone, soft tissue, and implant obtained from the phage-treated mice at euthanasia. An aliquot of each tissue sample was centrifuged at $3220 \times g$ for 10 min at 4 °C to separate cells and debris. The supernatant was collected and passed through a 0.45 μm filter and subsequently through a 0.22 μm filter (Millex, Merck Millipore, Ireland) to remove bacterial debris. Serial dilutions were performed in phage buffer, and the resulting dilutions were subjected to phage titer with host strain GE-MRSA15 using the double agar overlay method, as previously described.

Phage susceptibility tests
In order to identify any phage-resistant strains emerging in vivo, the lytic efficacy of the original GE-MRSA15 strain and bacterial colonies that were recovered after euthanasia were compared. Specifically, in the groups that received phage therapy (cGel+Sys and cGel), two bacterial colonies from the primary plate were randomly picked from soft tissue, bone, and implant, respectively (total of six colonies per animal). The activity of the CUB_GE-MRSA15_R14 and CUB_MRSA-COL_R23 and phage cocktail was assessed to determine whether any phage resistance emerged in vivo. Bacterial susceptibility to the phages was tested using the double agar method. The efficiency of plating (EOP) was determined as the ratio of the phage titer, and it was calculated as follows: EOP = phage titer on the phage-treated strain/ phage titer on the phage-naive strain. A reduced susceptibility was indicated by an EOP of less than 1.

Phage neutralization assay
The development of phage neutralization during phage therapy was assessed by conducting a phage neutralization assay using the serum obtained from animals that received phage treatment. In brief, 900 μL of diluted serum (1:100) was incubated with 100 μL of CUB_GE-MRSA15_R14 (10^7 PFU/mL) and 100 μL of CUB_MRSA-COL_R23 (10^7 PFU/mL), respectively, at 37 °C for 30 mins. After being diluted by a factor of 10,000 in cold 0.9% NaCl solution, the mixture underwent double agar overlay titrations. The percentage of neutralized phage particles was calculated using the formula: percentage of neutralized phage particles = $(P_0 - P_1)/ P_0 \times 100\%$, where P_1 represented the phage titer after incubation with serum collected at euthanasia and P_0 represented the phage titer after incubation with serum collected before initial surgery.

Data analysis
Data were analyzed using SPSS statistics Version 22 (IBM, Chicago, IL, USA). The normality of continuous data was tested with the Shapiro-Wilk test and the homogeneity of variances was tested using the Levene's test. In case of parametric data, a one-way ANOVA or Student's t-test (with either equal variance assumed or not) was used to compare differences between groups. In the case of non-parametric data, the Kruskal-Wallis or Mann-Whitney U test was used, as appropriate. Tukey posttest was performed to compare different groups. The Pearson correlation test was performed to test the correlation of bacterial burden (CFU) and phage titer (PFU). P values below 0.05 were considered significant. Data were expressed as

mean ± standard error and were plotted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

Data availability
All relevant data used to support the findings of this study are included within the article. Additional information and data are available from the authors upon reasonable request.

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Author contributions

Conceptualization, M.D., M.G.M., A.T., R.L., T.F.M., W.J.M.; methodology, B.C., L.P.B., M.C., V.P., P.N., M.G.M.; formal analysis, B.C., M.C. and T.F.M.; investigation, B.C., L.P.B., M.C., V.P,. C.C., S.Z. and W.F.; resources, A.T., R.G.R., R.L., T.F.M. and W.J.M.; writing–original draft preparation, B.C., M.C., L.P.B. and T.F.M.; writing–review and editing, B.C., L.P.B., M.C., V.P., M.D.E., C.C., S.Z., M.G.M., A.T., J.W., J.O., R.L., T.F.M. and W.J.M.; visualization, B.C., L.P.B., M.G.M. and T.F.M.; supervision, W.J.M., R.L. and T.F.M.; All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information