Aus dem Institut Berlin-Brandenburger Centrum für Regenerative Therapien
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DISSERTATION

**BACTERIOPHAGES AS AN ALTERNATIVE STRATEGY IN
THE TREATMENT AND PREVENTION OF IMPLANT-
ASSOCIATED INFECTIONS**

zur Erlangung des akademischen Grades
Medical Doctor – Doctor of Philosophy (MD/PhD)

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Zusammenfassung
Abstrakt

Bakterielle Biofilme, die auf Oberflächen wachsen, sind gegenüber der konventionellen antimikrobiellen Therapie sehr refraktär. Die vollständige Eradikation des Biofilms besteht aus einem invasiven chirurgischen Eingriff in Kombination mit einer längeren systemischen antimikrobiellen Verabreichung. Daher gewann die Entwicklung neuer und alternativer Strategien zur Bekämpfung chronischer implantatassoziierter Infektionen zunehmend an Interesse. Aufgrund ihrer antimikrobiellen Eigenschaften wurden Bakteriophagen als vielversprechende Agent zur Behandlung bakterieller implantat-assoziierter Infektionen, insbesondere im Zusammenhang mit multiresistenten Bakterien, neu bewertet.

Ziel dieser Arbeit war es, die antimikrobielle Aktivität verschiedener Bakteriophagen, allein oder in Kombination mit konventionellen Antibiotika als alternativen Ansatz, zu untersuchen, um die Behandlung von Mono- und Dual-Spezies-Implantat-assoziierten Infektionen zu verbessern.

Die Ergebnisse zeigten das breite Potenzial der isothermen Mikrokalorimetrie (IMK) für das Screening und die Bewertung der Aktivität von Bakteriophagen zur Vorhersage eines Behandlungserfolgs bei Biofilm-Infektionen auf Nachfrage. Darüber hinaus wurden verschiedene Methoden wie Sonikation, konfokales Laserscanning und Rasterelektronenmikroskopie kombiniert, um die Anti-Biofilm-Aktivitäten von Bakteriophagen zu bewerten. Höhere Phagentiter und eine längere Exposition der Bakteriophagen waren erforderlich, um Biofilm- Bakterien im Vergleich zu planktonischen Pendants abzutöten. In den Experimenten zur Biofilm-Prävention zeigten unsere IMK-Ergebnisse jedoch, dass die Wärmeproduktion in Gegenwart von subinhibitorischen Titern von Phagen innerhalb von 24 Stunden aufgehoben wurde. Darüber hinaus erhöhten getestete Phagenformulierungen die Überlebensrate einer Infektion mit *Galleria mellonella* Larven *Staphylococcus aureus* im Vergleich zur unbehandelten Kontrolle. Wir stellten auch fest, dass konventionelle Antibiotika hohe minimale Biofilm eradizierende Konzentration (MBEK) Werte (zwischen 128 und >4096 µg/mL) aufwiesen, wenn sie allein gegen Biofilme untersucht wurden. Die Verabreichung von Phagen mit Antibiotika verbesserte die Antibiotika-Wirksamkeit gegen Mono- und Doppelspezies-Biofilm erheblich, insbesondere nach gestaffelter Exposition, wodurch die MBEK-Werte stark reduziert wurden. Interessanterweise zeigte der Staphylokokken Bakteriophage Sb-1 eine dosisabhängige Reduktion der Exopolysaccharidmatrix, während PYO Bakteriophage keine Wirkung zeigte, was darauf hindeutet, dass einige Phagen das Eindringen von Antibiotika in die tieferen Schichten von Biofilmen verbessern könnten. Diese Arbeit zeigte auch, dass sogar noch niedrigere Sb-1- Phagen-Titer phagenbehandelte *Staphylococcus aureus* -persistente Zellen abtöten können, wenn metabolisch inaktive Zellen in frischem Medium beimpft wurden und zu einem normal wachsenden Phänotyp zurückkehren. Insgesamt generiert diese Arbeit neue Erkenntnisse zur Verhinderung der Besiedlung von Implantaten und zur Abtötung von auf einer Oberfläche anhaftenden Biofilmbakterien. Zu ihrer klinischen Anwendung sind weitere präklinische und klinische Studien erforderlich.

Abstract

Bacterial biofilms growing on surfaces are highly refractory to the conventional antimicrobial therapy. Complete eradication of the biofilm consists of invasive surgical intervention combined with prolonged systemic antimicrobial administration. Hence, the development of new and alternative strategies to fight chronic implant-associated infections gained increasing interest. Due to their antimicrobial properties, bacteriophages have been revalued as a promising agents to treat bacterial implant-associated infections, especially related to multidrug resistant bacteria.

The aim of this work was to investigate the antimicrobial activity of different bacteriophages, alone or in combination with conventional antibiotics as alternative approach, in order to improve the treatment of mono and dual-species implant-associated infections.

Results revealed the wide potential of isothermal microcalorimetry (IMC) for screening and evaluating bacteriophage activity to predict a treatment success on demand for biofilm infections. Moreover, different methods such as sonication, confocal laser scanning and scanning electron microscopy were combined for the evaluation of anti-biofilm activities of bacteriophages. Higher phage titers and longer exposure of bacteriophages were required to kill biofilm bacteria in comparison with planktonic counterparts. However, in the experiments of biofilm prevention, our IMC results presented that the heat production was abolished in the presence of subinhibitory titers of phages within 24 hours. Additionally, tested phage formulations increased the survival of

Galleria mellonella larvae *Staphylococcus aureus* infection compared to untreated control. We also determined that conventional antibiotics had high minimum biofilm eradicating concentration (MBEC) values (ranging from 128 to >4096 µg/mL) when investigated alone against biofilms. The co-administration of phages with antibiotics improved considerably the antibiotic efficacy against mono and dual-species biofilm, especially after staggered exposure, strongly reducing the MBEC values. Interestingly, staphylococcal bacteriophage Sb-1 demonstrated a dose-dependent reduction of the exopolysaccharide matrix, whereas PYO bacteriophage had no effect, suggesting that some phages could enhance the penetration of antibiotics to the deeper layers of biofilms. This work also showed that even lower Sb-1 phage titers can kill phage-treated *Staphylococcus aureus*-persistent cells, when metabolically inactive cells were inoculated in fresh medium and returned to a normally growing phenotype.

Overall, this thesis generates new insights for preventing implant colonization and killing biofilm bacteria attached on a surface. Further pre-clinical and clinical trials are needed towards their clinical application.

1.   Introduction

The implantation of medical devices, such as orthopedic implants, cardiac valves, artificial hearts or deep brain stimulators has revolutionized modern medicine and strongly improved life quality and longevity of patients (1). However, indwelling devices are associated to the development of difficult to treat infections due to the structured communities known as biofilms, resulting into a significant socioeconomic and clinical burden (2, 3).

Biofilms formation is central to the pathogenesis of implant-associated infection, causing the persistence of chronic infection (4). The colonization of the foreign body can take place at the time of implantation either by a direct inoculation or due to an airborne contamination of the wound or microorganisms colonize the implant through hematogenous seeding or spreading from a contiguous infection after the operation (5). A biofilm is defined as a sessile microbial community in which microorganisms live attached to a surface embedded within a self-produced matrix of extracellular polymeric substances (EPSs), composed of host factors, lipopolysaccharides, proteins, lipids, glycolipids, and nucleic acids. Biofilm cells show complex community presenting functional and structural heterogeneity which includes a high level of persister cells, an isogenic subpopulation of bacteria characterized by a slow- or non-growing state (6, 7). These microbial population might be responsible for the recalcitrance of infections once the antibiotic selective pressure is reduced (8). In fact, persistence, as non-heritable phenotype, differs from resistance and remains the ability of bacteria to grow and replicate in the presence of antimicrobials. Indeed, levels of persisters frequently arise due to a state of dormancy and in biofilms (9). Hence, biofilm-embedded cells acquire extreme tolerability to the host immunity and antimicrobial killing and can be up to 1000-fold more resistant to antimicrobials than their free-floating counter parts (10).

Implant-associated infections can be initiated by a broad spectrum single pathogen or virulence factor, or can be attributed to a polymicrobial origin (11). The most important pathogens involved in implant-associated infections are staphylococci, including methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci (CoNS), such as *Staphylococcus epidermidis*, which account for approximately 50% of infections overall (12). Extensive research has targeted to find therapeutic solutions with the focus on staphylococcal biofilm infections. However, the emergence of spreading of staphylococcal strains resistant to different antimicrobial agents reduces treatment options (13). In addition, usually, polymicrobial implant-associated infections result in worse clinical outcomes than the single infections caused by either species (14).

The presence of the extracellular matrix and the heterogeneity of the cellular metabolic status presents key challenges for the treatment failures (15). Therefore, removal of the infected device is the standard clinical practice for implant-associated infection. Nevertheless, surgical debridement and implant retention with prolong, combined and high doses of systemic antimicrobial therapy may be attempted in some cases (16, 17). However, the constant presence of antibiotics at subtherapeutic concentrations can select for resistant bacteria in biofilm. This scenario is also complicated by a paucity of new antimicrobials currently under development (18) and has re-emerged interest in various novel and alternative therapies (19).

Among the some alternative therapeutic approaches currently under evaluation, bacteriophage therapy (phage therapy), based on the application of virulent (strictly lytic) viruses able to specifically kill bacteria, have been considered among the most promising options in both clinical and scientific fields, particularly when bacteria are resistant or tolerant to conventional antibiotic treatments (20, 21). After the discovery of bacteriophages by d'Herelle in 1917, phages as a therapeutic tool have been used for almost century mainly in the Eastern European countries (22). However, with the launching of conventional antibiotics, the application of phage therapy disappeared out of the focus of western medicine in Europe and the USA (23). The rapid increase of multidrug resistant bacterial strains recently has been renewed interest in phage therapy (24). Even though regulatory authorities in Europe and USA have not approved it yet, an ever-growing number of successfully applications of personalized phage therapy have been described on their own or in combination with antibiotics for the treatment of chronic infections (25-27).

Phage therapy offers a broad spectrum of advantages over conventional antimicrobial strategies (28). Due to their high specificity, bacteriophages attack only host bacterial cells without affecting the normal microflora and the risk to induce superinfections such as *Candida albicans* yeast infections or antibiotic-associated *Clostridium difficile* colitis, is low (29). Unlike antibiotics, phages exhibit a feature referred to as "auto dosing", regulating themselves at the site of infection. This behavior results in a localized increase in viral particle numbers with a low initial dose and thereby contribute to establish the optimal phage dose themselves or in a decrease when bacteria have been killed (30). Moreover, phages lacks of cross-resistance with antibiotics and have been demonstrated to kill multidrug resistant bacterial cells (31).

Recently, different studies indicated, that different phages alone or in combination with conventional antibiotics *in vitro* demonstrated ability to reduce viable sessile bacterial cells (Figure 1), suggesting a potential use for the treatment of biofilm-associated infections (32-34).
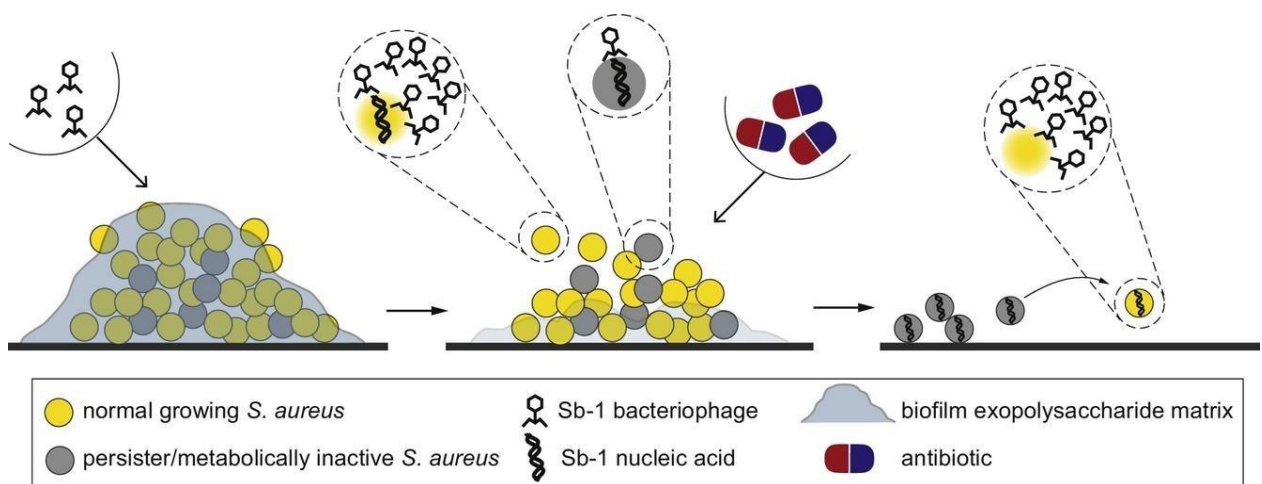

Figure 1 Schematic representation of the activity of staphylococcal bacteriophage (Sb-1) and antibiotics on a mature biofilm. Biofilms are aggregates of microbial cells with different metabolic status, including susceptible and persister cells, embedded within a self-produced matrix of extracellular polymeric substances (EPS). Unlike antibiotics, phages can infect all bacterial cells of the biofilm, multiply, and penetrate into the deeper layers. (Study C: Graphical abstract in Tkhilaishvili et al., *International Journal of Antimicrobial Agents* (2018))

Since clinical and scientific research on bacteriophages as an alternative antimicrobial strategy has been abandoned over the focus on conventional antibiotics for a long time, their therapeutic potential needs to be further investigated before application. Overall, our aim was to provide the insights for the alternative antimicrobial treatment strategy focuses on the activity of different lytic bacteriophages (alone or together with antibiotics) against bacteria that are associated with the occurrence of implant-associated infections due to biofilm formation.

2.   Objectives of the work

The main aim of this study was to investigate the effect of bacteriophages alone or in combination with different classes of antibiotics with a special focus on the antimicrobial activity towards prevention and treatment of biofilm associated infections. Therefore, the main objectives are:

1.   To establish a reliable, non-destructive and highly sensitive *in vitro* analytical tool for investigating the susceptibility to bacteriophages by monitoring bacterial metabolism and cell viability over the time both in planktonic and biofilm bacteria. (Study A: Tkhilaishvili et al., Real-time assessment of bacteriophage T3-derived antimicrobial activity against planktonic and biofilm-embedded Escherichia coli by isothermal microcalorimetry. *Research in microbiology* (2018));

2.   To determine *in vitro* the anti-biofilm preventive and eradication capability of mono- phage and phage cocktail in a *Staphylococcus aureus* biofilm using isothermal microcalorimetry (IMC) and confocal laser scanning microscopy. In addition, the *in vivo* efficacy of both phage formulations was evaluated in a *Galleria mellonella* model of *Staphylococcus aureus* systemic infection. (Study B: Tkhilaishvili et al., Antibacterial efficacy of two commercially available bacteriophage formulations, staphylococcal bacteriophage and PYO bacteriophage, against methicillin-resistant *Staphylococcus aureus*: Prevention and eradication of biofilm formation and control of a systemic infection of *Galleria mellonella* larvae. *Frontiers in Microbiology* (2020));

3.   To investigate anti-biofilm activity of staphylococcal phage in combination of different group of antibiotics in a *Staphylococcus aureus* biofilm in order to define the time of synergistic activity using IMC. The effect of phage treatment on biofilm and matrix was evaluated by confocal laser scanning microscopy. Phage activity against both less-metabolically active cells and persister cells was also tested. In addition, the lytic spectrum of staphylococcal phage on a collection of *Staphylococcus aureus* clinical isolates from implant-associated infections was evaluated. (Study C: Tkhilaishvili et al., Bacteriophage Sb-1 enhances antibiotic activity against biofilm, degrades exopolysaccharide matrix and targets persisters of *Staphylococcus aureus*. *International Journal of Antimicrobial Agents* (2018));

4.   To study the effectiveness of mono-phage and phage cocktail (targeting both bacterial species) to enhance antibiotic activity in eradicating *Staphylococcus aureus* /*Pseudomonas aeruginosa* dual-species biofilm using IMC. The morphological changes of biofilms were analyzed by scanning electron microscopy. (Study D: Tkhilaishvili et al., Using bacteriophages as a trojan horse to the killing of dual-species biofilm formed by *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus*. *Frontiers in Microbiology* (2020)).

3. Materials and methods

3.1. Bacterial strains and culture media

Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, E. coli TG1 and 57 *Staphylococcus aureus* clinical strains, including 28 MRSA and 29 methicillin-susceptible *Staphylococcus aureus* (MSSA), isolated from patients with orthopedic implant-associated infections, were used in this work. Bacteria were stored on a cryovial bead preservation system at -80 °C. Bacterial strains were grown on blood agar plate at 37°C for 24 h. Inoculum was prepared according to a McFarland turbidity of 0.5 ($\approx$1–5 $\times$ 10^8 CFU/ml of the tested strain). All antimicrobial assays for Study A and D were performed in Luria-Bertani (LB) broth, whereas Brain Heart Infusion (BHI) broth was used to investigate Study B and C. The growth medium for testing daptomycin and fosfomycin was supplemented with calcium chloride (50 mg/L) and glucose 6-phosphate (25 mg/L), respectively.

3.2. Bacteriophages and antibiotics

Commercially available staphylococcal bacteriophage Sb-1 and PYO bacteriophage was obtained as 10 ml liquid ampoules from the G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia and maintained at 4°C. Phage T3 was provided from the Institute of Virology, Helmut-Ruska-Haus, Berlin, Germany and were replicated in E. coli TG1 grown into LB broth and the lysate was stored at 4 °C. The susceptibility of the entire bacterial collection to phages was evaluated by spot test. The number of phages was determined by a quantitative plaque assay and expressed as plaque-forming unit (PFU)/mL, as reported previously. In addition, the susceptible strains were then tested by plaque assay to obtain the efficiency of plating (EOP). The EOP value was calculated as the ratio between the PFU on target clinical isolate and the PFU on ATCC 43300, which was used as the reference strain (Study C).

Fosfomycin, Rifampicin and Vancomycin were obtained in powder form and reconstituted using sterile pyrogen-free water. Daptomycin was provided as a powder and dissolved in sterile pyrogen-free 0.9% saline. Ciprofloxacin as injectable solution (2 mg/ml) were supplied and stock solutions of appropriate concentrations were prepared in sterile 0.9% saline.

3.3. Microcalorimetry set up and antimicrobial assay by heat measurement in real-time

Microcalorimetric measurements for the evaluation of antimicrobial activity of either phages or antibiotics vs. either planktonic or mono- and dual-species biofilm- embedded cells of bacterial strains were performed using an isothermal calorimeter TAM III (TA Instruments, New Castle, DE, USA) with a detection limit of heat production of 0.2 µW and equipped with 48 minicalorimeter channels. Airtight sealed sterile glass ampoules (4 ml-volume) were sequentially introduced used into the microcalorimetry channels and lowered to an equilibrium position for 15 min to reach a temperature of 37°C. Heat flow (µW) and total heat (J) were measured continuously against time (h) as measure of the instantaneous heat produced at any time point and as cumulative amount of heat produced during the experiment, respectively.

IMC measures the heat flow of biological processes, allowing the real-time monitoring of microbial viability in terms of heat produced due to the microbial metabolic activity. Moreover, IMC allows fast and reliable investigation of biofilm-forming strains and their susceptibility to different antimicrobials without the need for biofilm staining or physical harsh manipulation. Porous glass beads having a diameter of 4 mm, porosity 0.2 m2/g and pore size 60 µm was used for biofilm formation during static incubation in medium for 24 h at 37 °C. After incubation, rinsed beads were exposed to different titers/concentrations of phages/antibiotic for different time point (according to experiments) in glass ampoules. A growth control consisting in untreated biofilm was included, as well as a sterile bead as negative control. Microcalorimetry data was evaluated using the manufacturer's software and figures were plotted using GraphPad Prism 6.01.

3.4. Sonication and colony counting

After IMC biofilm experiments, to evaluate the reduction/eradication of biofilm cells, the beads showing no heat production together with untreated biofilms (growth controls) were subjected to sonication. Beads were vortexed for 30s with maximum power, sonicated at 40 kHz for 60s in a sonication bath, and vortexed for 30s again to dislodge biofilm bacteria. For conventional culture, sonication fluids were serially diluted in Eppendorf tubes and aliquots of 50 μL were quantified by viable count of the colony forming unit (CFU)/mL. The minimum biofilm eradicating concentration (MBEC) was defined as the lowest concentration/titre of antimicrobials required to kill all sessile cells resulting in the appearance of no colony after plating the sonication fluid (detection limit: < 20 CFU/mL).

3.5. Confocal Laser Scanning Microscopy (CLSM)

In Study B and C, the lytic effect of phage treatment on the prevention of ATCC 43300 biofilm formation and its eradication was evaluated by confocal laser scanning microscopy (CLSM). An overnight culture was diluted 1:100 and dispensed into an 8-well μ-Slide (Ibidi) to let the biofilm form. For prevention experiments, bacteria were simultaneously incubated (at 37°C for 24 h) in the presence of different titres of phages. For eradication experiments, bacteria were first let form biofilm into an 8-well μ-Slide (Ibidi) for 24h at 37°C, and then treated with different phage titers. Bacteria viability and biofilm thickness after phage co-incubation/treatment was determined by CLSM after staining cells with Syto9 (488nm/500–540 nm) and propidium iodide (PI) (561nm/600–650 nm) as recommended by the manufacturer. Samples were analysed by the microscope TCS SP5 using a $63 \times$ objective and a pinhole aperture of 1.0 Airy. For each image, the mean of fluorescent intensity was calculated as previously described (35).

3.6. Phage treatment in a *Galleria mellonella* model of *Staphylococcus aureus* infection

In Study B, phages were investigated for their ability to rescue MRSA-infected larvae from death. Larvae of *Galleria mellonella,* obtained from BioSystems Technology Ltd. (Exeter, Devon, UK), were stored at room temperature and were used within 3 days. Larvae were inoculated with 10 μL of bacterial suspension (containing $\approx 2.5\text{-}5 \times 10^6 - 2.5\text{-}5 \times 10^7$ CFU) in the last left proleg. Phages (1×10^5 PFU) or vancomycin (10 mg/kg) were delivered behind the last proleg on the opposite side to the bacterial injection site either 1 h post-infection (for treatment experiments) or 1 h pre-infection (for prevention experiments). Ten larvae per treatment were issued in all experiments. Larvae treated with PBS solution served as positive control group. Three negative control groups were also included in the experimental design including injected with phage suspension only, assessing phage toxicity. Larvae were stored in Petri dishes in the dark at 37°C for 168 h, were inspected every 24 h and were considered dead if they did not move when stimulated.

3.7. Evaluation of phage activity against persister cells

In Study C, persister status was induced in *Staphylococcus aureus* ATCC 43300 bacteria following two different methods. Firstly, persister cells were induced using carbonyl cyanide m-chlorophenylhydrazone (CCCP) as previously reported (36). Alternatively, a 24-h-old biofilm was treated with 512 μg/mL ciprofloxacin (or phosphate-buffered saline [PBS] as control) for 24 h at 37 °C. At the end of the treatment with either CCCP or ciprofloxacin, bacterial cells were washed (after scraping when biofilm-embedded), diluted to final concentration $\approx 5 \times 10^5$ CFU/mL and treated with 0, 10^4, or 10^7 PFU/mL (multiplicity of infection [MOI] 0, 0.02 and 20, respectively) for 3 h at 37 °C, in duplicate. For both conditions, one set of samples was then plated on BHI agar for CFU count, and the other was inoculated in fresh BHI broth and incubated for 24 h at 37 °C before being visually assessed for growth and plated for CFU counting.

3.8. Scanning Electron Microscopy (SEM)

In Study D, biofilm formed on glass beads, as previously described were washed in ddH2O (dipping) to remove unbound bacteria and chemically fixed. Subsequently, the samples were dehydrated in ethanol percent series and then dried at the critical point. Samples were mounted on aluminum stubs, coated with 20 nm layer of gold-palladium, and then observed in the microscope.

Further and more detailed information on the evaluation of methods as well as on the materials and equipment and their manufacturers can be found in the respective publications.

4. Results

This chapter contains key findings published in *Research in microbiology* (2018) (Study A), *Frontiers in Microbiology* (2020) (Study B and D) and *International Journal of Antimicrobial Agents* (2018) (Study C).

4.1 IMC validates of the *in vitro* real-time assessment of the antimicrobial activity of bacteriophages as testing model

Our results in Study A, present IMC as fast and highly sensitive technique for the precise measurements in real time of the both planktonic and biofilm bacterial metabolic status and replication activity related to the heat production of phage-host interaction. The antimicrobial activity could be investigated in terms of either metabolism/growth inhibition, when the phages were co-incubated with the tested strain, or as bactericidal activity, when treated samples were evaluated for the presence of residual bacterial cells attached on glass beads after removal of the phages. In addition, as outcome from our study, we were able to calculate thermogenic parameters of biofilm treated with phage and compare to those obtained for the planktonic cells under the same conditions. The co-incubation of a 24 h *Escherichia coli* TG1 biofilm with increasing titers of phages (ranging from 10^2 to 10^7 PFU/mL) inhibited metabolism in dose-dependent manner. The first peak revealed in the presence of all phage titers and in the growth control (GC) within approximately 2.2 h. However, the first peak of the highest phage titer (10^7 PFU/ml) was almost undetectable and moreover, a reduction of more than 90% of the total heat produced be *Escherichia coli* biofilm-embedded cells was observed with the same highest phage titer (Study A: Figure 3 in Tkhilaishvili et al., *Research in microbiology* (2018)). As soon as the phage treatment was suspended (Figure 2), the re-inoculation of beads in fresh medium detected the presence of residual biofilm cells, which re-started replicating and metabolic reactivation was detected, although it was delayed in time. Even higher T3 phage titers were not able to completely eradicate biofilm. In fact, as reported in Figure 2, increasing phage titers correlated to strongly reduce viable cells embedded in the biofilm and therefore to more delayed onsets of biofilm exponential metabolic activity, as compared to the untreated control.
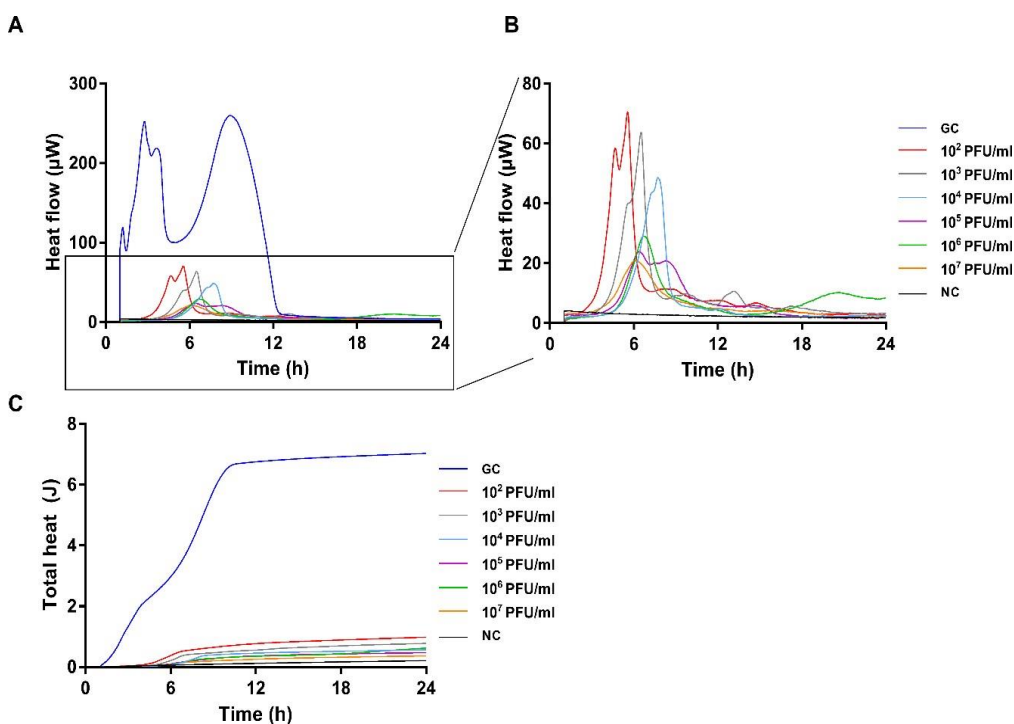

Figure 2 Microcalorimetry evaluation of recovering E. coli TG1 biofilm after treatment with T3 phage. Each curve shows the heat flow (A and B) and total heat (C) produced by E. coli TG1 biofilm on glass beads after

24 h of phage treatment or no treatment. GC, growth control; NC, negative control. (Study A: Figure 5 in Tkhilaishvili et al., *Research in microbiology* (2018)).

4.2 A longer exposure of bacteriophages exhibit more killing activity versus sessile cells of

Staphylococcus aureus in vitro

As reported in Study B, the evaluation of viable bacteria (by IMC in real-time and colony counting after bead sonication with plating of the sonication fluids) attached to the beads treated with Sb-1 and PYO phages during 48h-incubation revealed dose-dependent trend of reduction of MRSA CFUs/mL, as compared to the untreated growth control (Figure 3). However, a reduction of more than 90% of the total heat produced by MRSA biofilm-embedded cells was observed at 10^7 PFU/ml titers for both phage formulations.
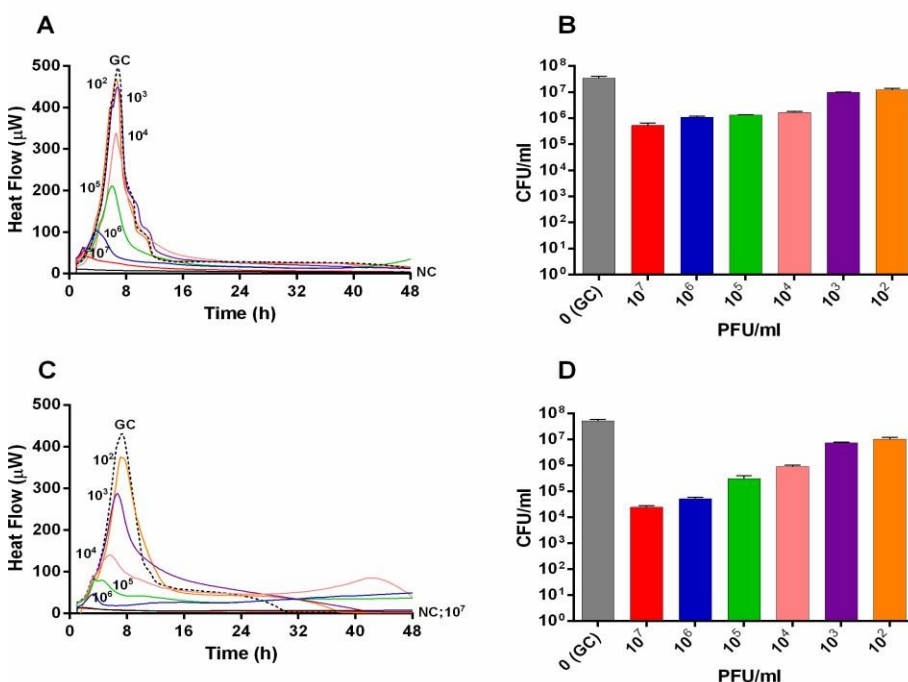
Figure 3 Evaluation of MRSA ATCC43300 biofilm susceptibility to either Sb-1 (A and B) or PYO (C and D) exposure, by isothermal microcalorimetry (A and C) and colony counting (B and D). Each curve shows the heat produced by viable bacteria attached on beads during 48 h treatment with different titers (ranging from 10^2 to 10^7 PFU/mL) of Sb-1 (A) and PYO (C), respectively. Histogram represents the mean of CFU number $\pm$ SEM of biofilm dislodged MRSA treated/untreated with Sb-1 (B) and PYO (D). Numbers above curves represent Sb-1 titers (in PFU/ml). GC, growth control (dashed line); NC, negative control. (Study B: Figure 2 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020))

The lack of an eradication even at higher titers after 24 h of both phage treatment was also confirmed by CLSM (Study B: Figure 3 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020)). By contrast, the analysis of sonication fluids of bead biofilms pre-treated with phages showed the eradication only following a co-incubation with the highest titer of Sb-1 and PYO, for either 7 or 5 days, respectively. The analysis of MRSA viability by dead/live staining by CLSM clearly revealed that no biofilms were detectable after the treatment of MRSA biofilm with phages (Figure 4). By definition, 10^7 PFU/mL and 10^6 PFU/mL were the MBEC of both phages after 7 and 5 days, respectively.
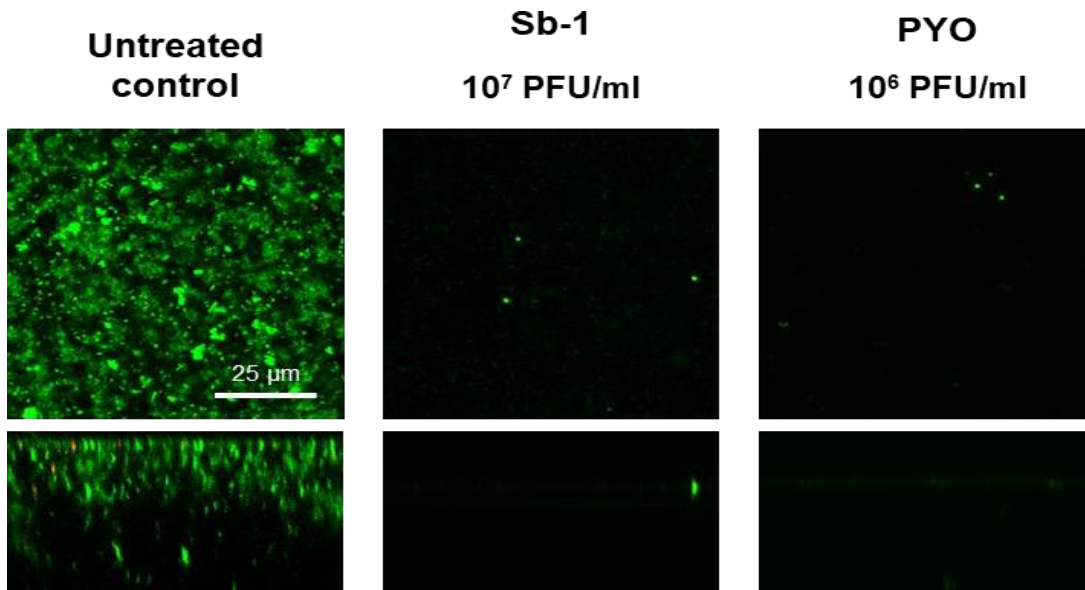
Figure 4 CLSM images of MRSA ATCC 43300 biofilm untreated and treated with Sb-1 and PYO. MRSA biofilm (24 h-old) was exposed for 7 and 5 days to 10^7 PFU/mL Sb-1 and 10^6 PFU/mL PYO, respectively. The viability of the cells was evaluated staining with green fluorescent labeled SYTO9 (488/500–540 nm) for alive bacteria and with red fluorescent propidium iodide (PI) (561/600–650 nm) for dead bacteria. Images are merged from the two channels. Upper and lower panels represent xy- and z- plans, respectively. Scale bar: 25 µm. (Study B: Figure 7 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020)).

4.3   Bacteriophages prevent MRSA biofilm formation *in vitro*

Study B also shows bacteriophages ability in successful preventing biofilm formation on glass beads evaluated by IMC, by colony counting of sonicated fluids and CLSM. A strong reduction of MRSA heat production was already observed at a lower titer (10^2 PFU/mL) of both bacteriophage formulations and a reduction of more than 90% of heat production was already revealed at 10^4 PFU/mL. The lack of heat production for 48h correlated with no sessile cells attached on glass beads (Figure 5). These results were confirmed by CLSM analysis as well (Study B: Figure 9 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020)).
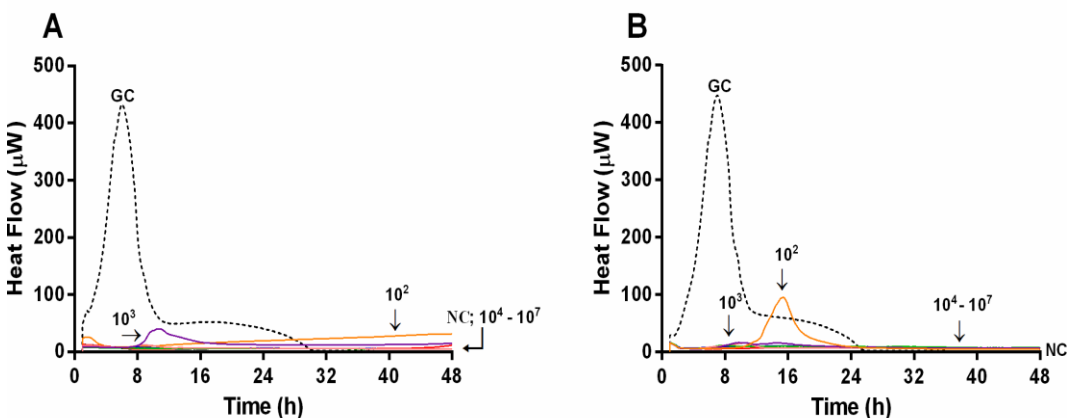

Figure 5 Evaluation of the ability of Sb-1 (A) and PYO (B) phages to prevent biofilm formation on porous glass beads by IMC. Each curve shows the heat produced by viable bacteria potentially attached on the glass beads after 24 h co-incubation with increasing titers of phages (ranging from 10^2 to 10^7 PFU/mL) in the presence of the abiotic surface. Numbers above curves represent Sb-1 titers (in PFU/ml). GC, growth control (dashed line); NC, negative control. (Study B: Figure 8 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020))

Then, to deepen the investigation for the better explanation the absence of heat production detected by microcalorimetric experiments, the viable bacteria (attached on the beads or free- floating bacteria in the supernatant) was evaluated by colony counting of either sonication fluids or supernatants. Sub-inhibitory titers (10^4 PFU/mL) of both phages formulations within 6 hours of co-incubation, showed a reduction of more than 3 log10 of free-swimming bacteria in the liquid medium, besides $\approx 10^2$ CFU/mL of bacteria was attached on the beads). However, after 24 h of co-incubation within phages, neither in the liquid medium nor in the sonication fluid no colonies were observed. A dose-dependent correlation was showed during colony counting of bacteria with increasing the titer of phages (Study B: Figure 10 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020)).

4.4 Bacteriophages have high *in vivo* efficacy for MRSA systemic infection in *Galleria mellonella* larvae

For the evaluation of the effectiveness of phages of MRSA systemic infection in *Galleria mellonella* larvae (Study B) two approaches were carried out. The first was a treatment whereby an acute 1h infection was allowed to establish prior to administration of phage or vancomycin; in the second model a phage formulation or vancomycin was applied to larvae 1h prior to bacterial exposure for preventing infection. In uninfected larvae injected with PBS, phage lysate (10^5 PFU/mL) or vancomycin 10 mg/kg, used as controls, a 100% survivability were obtained, showing that the antimicrobials caused no toxicity and the injections caused no trauma. Single therapy with phages or antibiotic administered before or after inoculation with MRSA improved larval survival. However, a dose dependent lethality was observed. The groups with a lower inoculum (2.5 x 10^6 CFU/mL) had a greater survival percentage. In addition, the data showed a higher survivability in larvae treated with the phage cocktail, as compared to single phage application. The survival rates of each experimental group are depicted in Study B: Figure 11 and Figure 12 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020).

4.5 Bacteriophages degrade the extracellular polysaccharide matrix

Study B and C reports on the evaluation of the effect of Sb-1 and PYO on extracellular matrix by confocal microscopy. Exposure of the 24h MRSA biofilm with increasing sub-eradicating titers of Sb-1 revealed the progressive degradation of the polysaccharide component (Figure 6) with statistically significant reduction of fluorescence mean intensity and no impact on cell viability (Study C: Figure 7 and Figure 8 in Tkhilaishvili et al., *International Journal of Antimicrobial Agents* (2018)). By contrast, application of PYO titers did not result into a gradual reduction of the blue staining in comparison to the untreated control, indicating that no visible degradation of the polysaccharide component occurs in the presence of PYO phages (Study B: Figure 4 in Tkhilaishvili et al., *Frontiers in Microbiology* (2020)).
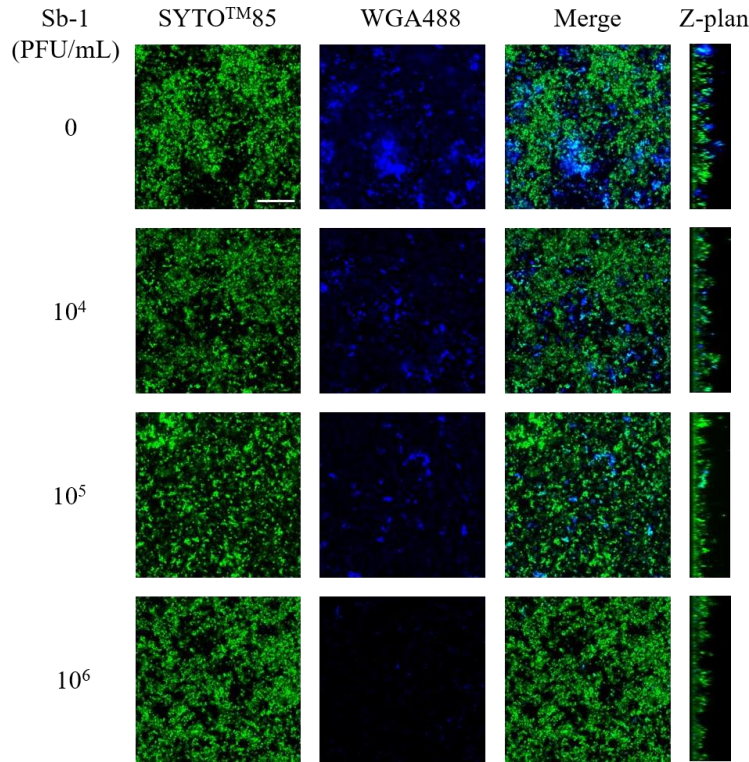
Figure 6 CLSM images of MRSA ATCC43300 biofilm untreated and treated with Sb-1. MRSA biofilm (24h-old) was exposed for 24 hours to different Sb-1 titers (ranging from 10^4 to 10^6 PFU/mL) and then stained with green fluorescent labeled WGA488 (488/500–600 nm) for exopolysaccharides and SYTO™85 (561/600–700 nm) for bacterial cells. An untreated control was also added. Scale bar: 25 μm. (Study C: Figure 6 in Tkhilaishvili et al., *International Journal of Antimicrobial Agents* (2018)).

4.6 Sb-1 bacteriophage exerts an anti-persister activity *in vitro*

As observed with Study C, the different free-floating cells dislodged from the biofilm were treated with either 10^4 PFU/mL or 10^7 PFU/mL Sb-1 phage and cell viability after phage treatment was detected by CFU counting. No reduction was demonstrated following treatment with 10^4 PFU/mL in any of the conditions tested. Interestingly, the 10^7 PFU/mL titer reduced CFU/mL (≈2-5 log 10 CFU), which indicates lytic activity of the phage regardless of the metabolic state of the cells. Of note, although a complete killing of biofilm cells was not achieved, when the treated biofilm cells from each condition were incubated into fresh media, no growth was revealed over 24 h (Figure 7). Indeed, the reversion of *Staphylococcus aureus* cells pretreated with sub-eradicating titers of Sb-1 to a metabolically active state resulted in the complete killing of the of biofilm viable cells (0 colonies on plate counts).
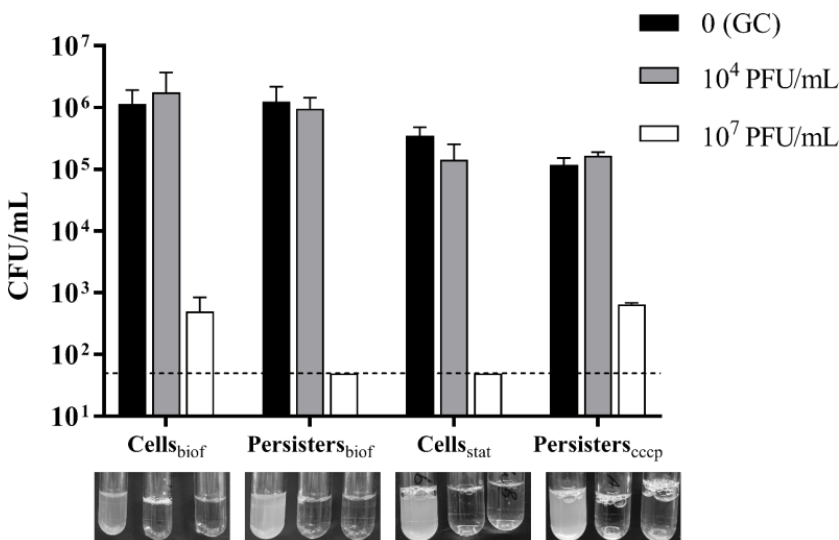
Figure 7 Lytic effect of Sb-1 against free-floating bacterial cells of MRSA ATCC 43300 in different metabolic conditions. Cells detached from biofilm (Cells$_{biof}$), persister cells isolated from biofilm after treatment with ciprofloxacin (Persisters$_{biof}$), stationary phase cells (Cells stat) and CCCP-*induced persisters (Persisters$_{cccp}$)* were exposed to either 10^4 (MOI 0.02) and 10^7 (MOI 20) PFU/mL Sb-1 in PBS + 1% BHI for 3 h at 37 °C. Untreated controls were also added. After phage treatment, cells were plated to enumerate CFUs. Data are means with standard deviation, and the dotted line represents the detection threshold. A set of samples treated as above-mentioned and washed to remove unadsorbed phages was also inoculated in fresh BHI for 24 h and then the turbidity was visually evaluated (on the lower panel). (Study C: Figure 9 in Tkhilaishvili et al., *International Journal of Antimicrobial Agents* (2018)).

4.7 Staggered phage and antibiotic treatment is the most effective for mono and dual-species biofilm eradication *in vitro*

As reported in Study C and D, we evaluated the synergistic effect of simultaneous and staggered phage-antibiotic combinations to eradicate the mono and dual-species biofilm. Overall, results indicated an increased anti-biofilm activity of the staggered exposure over the simultaneous exposure to antimicrobials. Specifically, when sub-eradicating concentrations of antibiotics were exposed in simultaneous treatment with sub-eradicating titers of phages to treat mono and dual-species biofilm-coated beads, a remarkable delay and/or reduction of heat flow produced by bacteria was revealed for all the antibiotics tested. However, a synergistic effect for the complete eradication of the mono-species MRSA biofilm was observed only when sub-eradicating titers of Sb-1 was combined with either 64 μg/mL rifampin or 32 μg/mL daptomycin, shown by the absence of growth on plate after sonication of the beads. Of note, no complete dual-species biofilm eradication effects were detected in the case of ciprofloxacin exposed in simultaneous treatment with bacteriophages.

By contrast, the staggered exposure of mono and dual-species biofilms to phages first and then, after different incubation times, to conventional antibiotics was associated with the most pronounced inhibition of heat flow production for all the combinations tested. Specifically, pretreatment with phages against MRSA biofilm resulted in a synergistic eradicating effect with all the antibiotics tested. Moreover, the synergistic eradicating concentrations of rifampin and daptomycin were 2 and 3 dilutions below the ones in the simultaneous exposure, respectively. However, among all staggered tested antibiotic–phage combinations against MRSA biofilm, only the pretreatment with sub-inhibitory concentrations of rifampicin, vancomycin and daptomycin followed by incubation with Sb-1 revealed a synergistic effect at higher antibiotic concentrations (Figure 8).
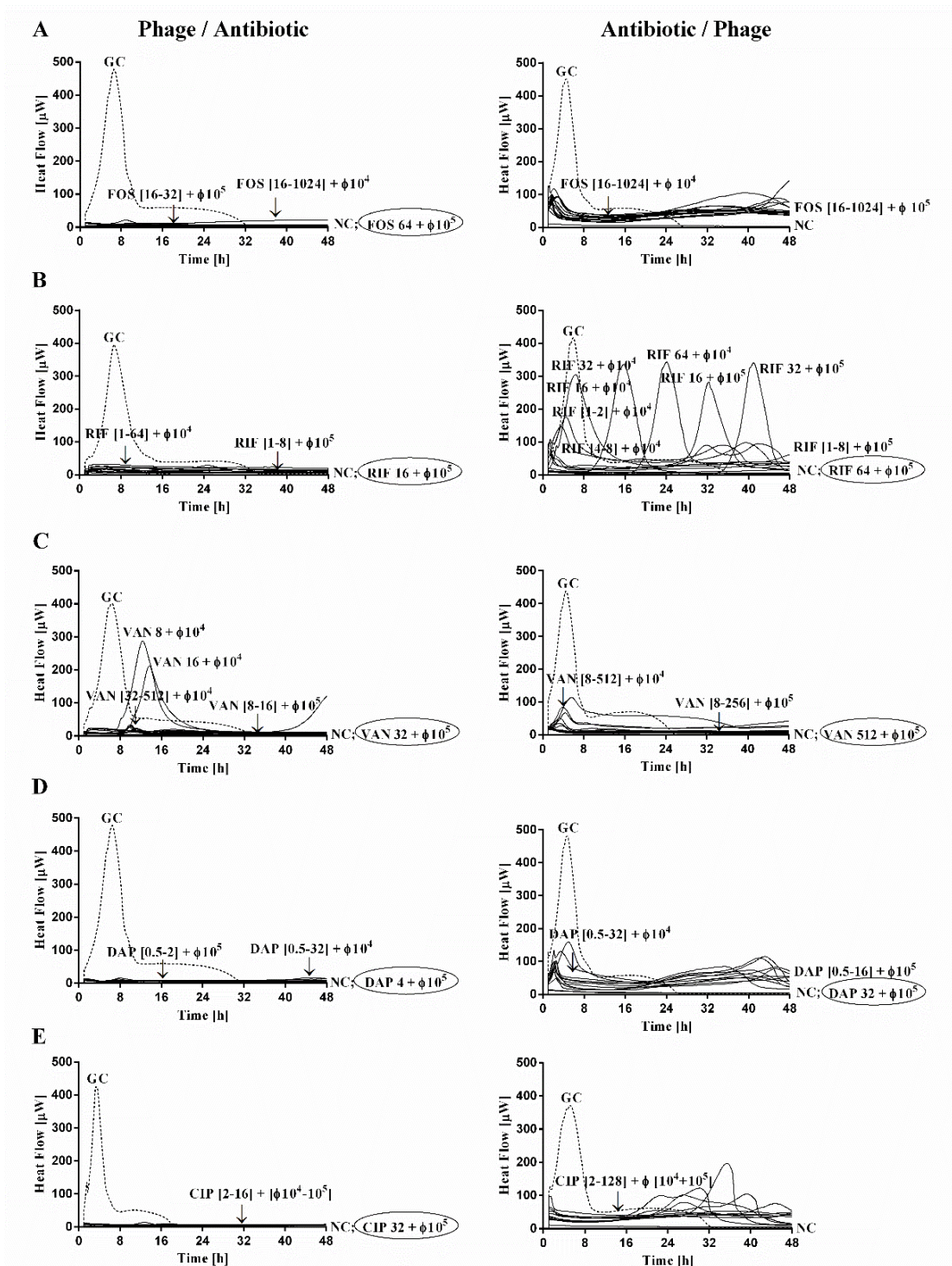
Figure 8 Evaluation of MRSA ATCC 43300 biofilm to staggered exposure of Sb-1 followed (graphs on the left) or preceded (graphs on the right) by fosfomycin (A), rifampin (B), vancomycin, (C) daptomycin (D) and ciprofloxacin (E) by microcalorimetry. Each curve shows the heat produced by viable bacteria present in the biofilm after the staggered treatment. Untreated controls were also added. The combinations were tested with fixed concentrations of antibiotics (1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 ×the MBEC biofilm) and either 10^4 or 10^5 PFU/mL (subinhibitory titers) of phage Sb-1. Numbers above curves represent antibiotic concentrations (in µg/mL) and titers of Sb-1 (in PFU/mL). Circled values represent the MBEC, defined as the lowest antimicrobial concentration leading to absence of bacterial regrowth after 48 h and no colonies after sonication and plating. GC, growth control (dashed line); NC, negative control. (Study C: Figure 5 in Tkhilaishvili et al., *International Journal of Antimicrobial Agents* (2018)).

In addition, with pretreatment with phages against *S. aureus/P. aeruginosa* dual-species biofilm demonstrated the highest anti-biofilm activity when the antibiotic was added after 12h of pre-exposure to either PYO or PYO+Sb-1 bacteriophages, where a complete eradication of the biofilm could be achieved at MBEC of ciprofloxacin of 2 mg/L and 1 mg/L, respectively (Figure 9).
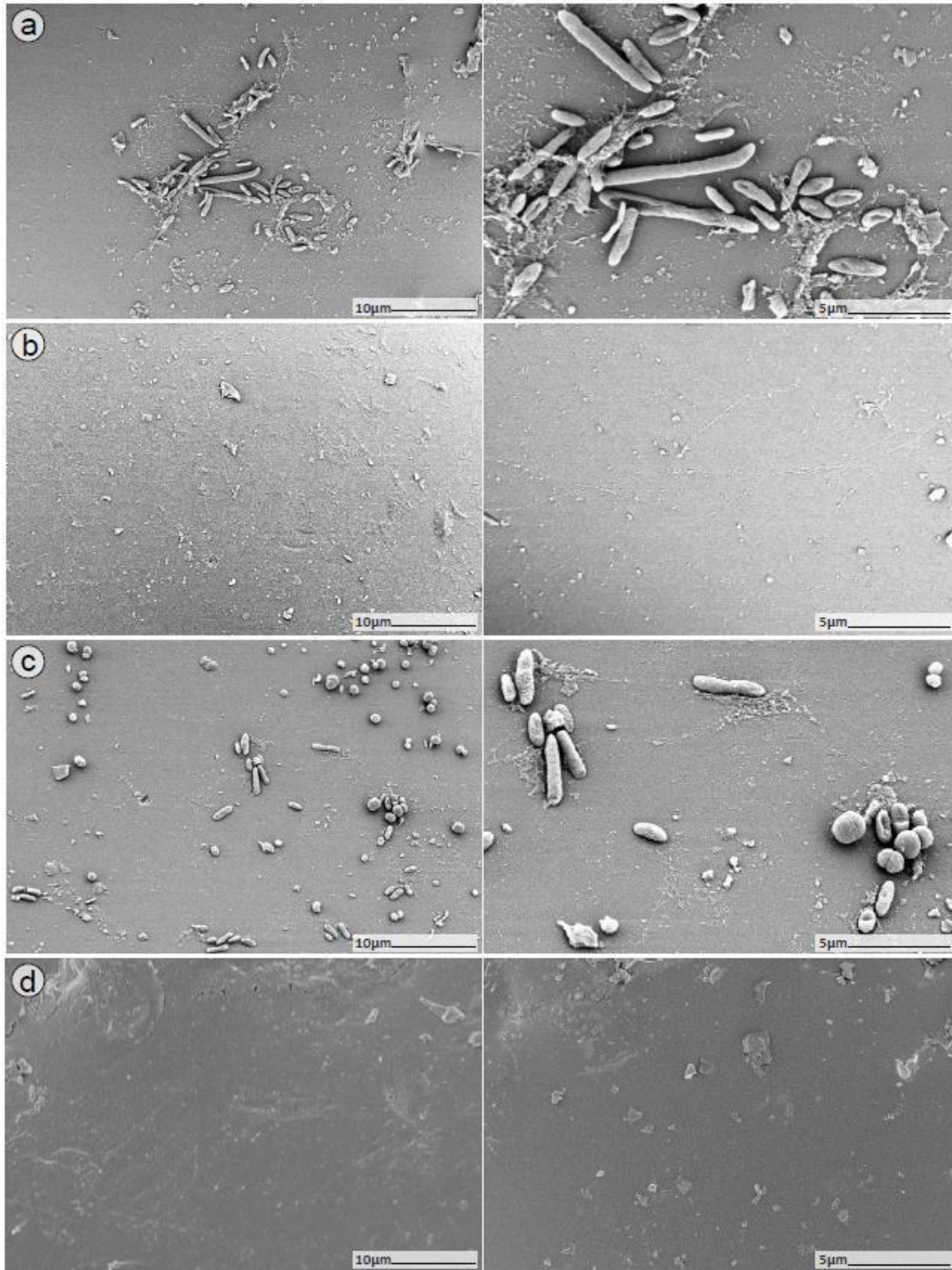

Figure 9 *S. aureus/P. aeruginosa* dual-species biofilm grown on porous glass beads for 24h and treated with a combinatorial therapy of (a) simultaneous exposure to PYO and ciprofloxacin (1 mg/L, 24h); (b) staggered exposure to PYO (12h) followed by ciprofloxacin (1 mg/L, 24h); (c) simultaneous exposure to PYO+Sb-1 and ciprofloxacin (1 mg/L, 24h); or (d) staggered exposure to PYO+Sb-1 (12h) followed by ciprofloxacin (1 mg/L, 24h).

5. DISCUSSION

The biofilm formation on the surface plays a pivotal role in implant-associated infection, representing complex communities of microorganisms in which various metabolic activities and interactions occur (37). Implant-associated infections are extremely challenging to eradicate due to their tolerance and refractivity to the activity of host immunity and conventional antibiotics, causing a limited therapeutic options (38). Therefore, bacteriophages have been regaining interest as potential alternative strategy for the treatment of biofilm-associated infections (39).

In the thesis, we stablished an optimized *in vitro* biofilm model of the application of IMC, demonstrating the real-time monitoring of the lytic activity of phages and their interaction with planktonic and biofilm bacteria in terms of heat production related to the cellular metabolic activity. 48 channels IMC can determine in parallel the lytic activity of different phages with different phage titers and therefore it is possible to identify and characterize the best lytic phage and the best phage dose active specifically against the various clinical strains isolated from the patient. As proof of concept, we showed that IMC can be an important analytical tool especially for a fast and reliable investigation of biofilm-forming strains and their susceptibility to bacteriophages without the need for biofilm staining or physical harsh manipulation comparing to other standardized biofilm studies (40) for rapidly setting a personalized phage therapy for complicated implant-associated infections.

In order to observe the phage activity different parameters acquired from microcalorimetry experiments were evaluated. The minimum virus titer that inhibits the heat production and that exhibits a bactericidal effect were calculated versus planktonic and biofilm cells. The possibility to sonicate and plate each sample after the calorimetric analysis, allowed us to evaluate also the eradication activity of bacteriophages. Specifically, in Study A, we determined, that T3 phage was able to strongly inhibit the heat produced by biofilm-embedded E. coli cells and reduce their regrowth, however none of the tested phage titers could completely eradicate biofilm after 24 h-treatment, inferring the establishment of an equilibrium between virus and host as described earlier (41). A similar trend was observed in Study B and D, where microcalorimetric analysis in real-time revealed that the incubation with higher titer of either Sb-1 or PYO exhibited a killing activity versus mono or dual-species sessile cells of *Staphylococcus aureus* or *Staphylococcus aureus /Pseudomonas aeruginosa*, but it did not result in an eradication of the biofilm within 24h, as tested even by colony counting and CLSM or SEM analysis.

Bacteriophages, infect their host cells to replicate themselves and release the viral progeny, meaning an exponential increase of the number of virus particles over time (42, 43). Therefore, longer exposure time in order to increase the number of infecting particles and improve the penetration of phages into the deepest biofilm layers might be necessary to obtain complete eradication of biofilm cells. Given this hypothesis in Study A, we evaluated if a longer exposure could eradicate all sessile bacteria. As a result, in Study B we observed that the *in vitro* killing of all *S. aureus* biofilm-embedded cells was possible when bacteria were incubated over 5 and 7 days with either PYO (10^6 PFU/mL) or Sb-1 (10^7 PFU/mL), respectively. Interestingly, our results determined that PYO had no effect on the exopolysaccharide component of the matrix whereas Sb-1 could degrade the matrix, proposing a distinct enzymatic activity between the phage formulations examined. Thus, we also speculated that delayed lytic activity of phages against biofilms can be plainly more due to the physiological condition of biofilm community, and not to diffusion challenges (44). However, the degradation of some of matrix components may facilitate the penetration of antimicrobials to the bottom of the sessile cells and could enhance antibiotic activity against biofilms.

The thesis points towards the possible use of phages for the prevention of the infection as the best strategy to reduce the cases of implant-associated infections. Thus, with the study B we also aimed to

investigate bacteriophages ability to prevent *S. aureus* biofilm formation. By contrast to *S. aureus* biofilm eradication, either Sb-1 or PYO could prevent *S. aureus* biofilm formation within 24h at lower titer (10^4 PFU/mL) than that required even for the elimination of all planktonic cells of the primary inoculum. This disparity might be explained by the fact that in the existence of the foreign material part of the free-floating bacteria attaches on the beads and results into diminishing number of the replicating planktonic bacteria and therefore can be easily lysate by phages. The attachment of *S. aureus* comes about 3h from bacterial inoculum, as previously reported (45). CFUs determining after 3 h-incubation of *S. aureus* treated with 10^4 PFU/mL of Sb-1 and PYO displayed that phages did not interfere with bacterial adhesion process. Although we could not obviously distinguish between biofilm prevention and disruption, here *S. aureus* biofilm disruption could be excepted due to the fact that 10^4 PFU/mL phage titer had only a poor activity in the biofilm treatment test.

The infection environment can extremely impact on phage efficacy and therefore various *in vivo* studies, using *Galleria mellonella* infection model, have been evaluated for the therapeutic and/or prophylactic activity of phages (46, 47). However, to the best of our knowledge, Study B for the first time provided the investigation of phages against MRSA-infected *Galleria mellonella* larvae. Our data indicated an improved survival of larvae comparing to the activity revealed by vancomycin used as antibiotic control or to the untreated sample. Moreover, our results showed that a single phage dose was sufficient to decrease the mortality of larvae and the time of application of phage had an essential role. In addition, Study B suggests that phage cocktail compared to single phage has an advanced effect on *Galleria mellonella* larvae, however different timings and dosage of the phage exposure, needs to be evaluated.

Another crucial aspect from this thesis was to evaluate the effect of combined phage-antibiotic treatment against mono and dual-species biofilm-embedded cells. Phage-antibiotic synergy is the result of combinatorial effect of sub-inhibitory concentrations of antibiotics and phages to enhance antimicrobial activity resulting in bacterial decline (48). Previous studies have reported the benefit of the staggered exposure over a simultaneous application when combining phages and antibiotics, possibly due to antagonistic modes of action (49-51). However, our results showed simultaneous exposure with phage Sb-1 and either rifampin or daptomycin also resulted in a synergic eradication of *S. aureus* biofilm. Although, strong synergistic effect of a phage-rifampin combination on *S. aureus* biofilm was described by Rahman et al (52), to the best of our knowledge, with Study C we reported for the first time the synergism between phages and daptomycin against biofilm. Differently, when we evaluated in Study D the application of dual-species biofilms to ciprofloxacin and phages simultaneously, we observed a paradoxical effect, showing a higher delay/decrease in heat flow production with lower antibiotic concentrations in combination with phages. An increased effect of combinatory therapy appears to rely also on the host strain and on mechanism of action of the administrated antibiotic. Thus, this ineffective combinatorial activity could be avoided by the use of antibiotics with modes of action that do not compete with the viral amplification or by exposing bacteria in a staggered rather than a simultaneous manner to phages and antibiotics.

Indeed, the best outcome against mono and dual-species biofilms under combinatorial treatment was found to be the staggered administration of phage with all antibiotics tested. In the matter of rifampin and daptomycin, in which the simultaneous exposure of the Sb-1 already revealed a synergistic effect, the pre-exposure to Sb-1 followed by antibiotic treatment eliminated the *S.aureus* biofilm with even rather lower antibiotic concentrations. Interestingly, staggered application to fosfomycin, vancomycin, and ciprofloxacin strongly enhanced the bactericidal activity of the antibiotic that was not determined with simultaneous treatment. Therefore, antibiotic concentrations still reachable in clinical practice eradicated biofilm. Recently was reported that synergistic interaction between phages and antibiotics

strongly depends also on time points of the administration of antimicrobials (48). Thus, with Study D in addition we determined the optimal time for the staggered exposure of phages and antibiotic against dual-species biofilm. Here, our results revealed that the strongest anti-biofilm effect could be reached when ciprofloxacin was added after 6 or 12 h of pre-incubation to phages.

In addition, to the best of our knowledge our study for the first time investigated whether phages could target persister cells of *S. aureus* biofilm, which remain viable over the course of antibiotic exposure and contributes to the relapse of the biofilm infections (9). Our results revealed that higher titer of Sb-1 could direct kill persisters, however even sub-inhibitory titers of Sb-1 had lytic activity on persister cells after their reversion to a normal growing phenotype ("Trojan horse" effect) resulted in bacterial cell lysis. Thus, we speculated that phages in combination with conventional antibiotics may minimize the relapse of infection related to the recalcitrance of persister cells.

With this thesis, the reported findings strongly emphasizes valuable *in vitro* and *in vivo* data on the use of bacteriophages alone or in combination with conventional antibiotics against mono and dual-species biofilm infections, highlighting new insights and possible explanations as potential approach for preventing device colonization and killing sessile bacteria attached on a surface. Moreover, by the use of commercially available phage preparation, we demonstrated the therapeutic effectiveness of phages against bacterial strains that has not been used specifically for their isolation. Further preclinical and clinical studies are needed to support the development of phage therapy as alternative or complementary strategy for implant-associated infections.

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Statutory Declaration

"I, Tamta Tkhilaishvili, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Bacteriophages as an alternative strategy in the treatment and prevention of implant-associated infections", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines.

The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

_____   _____

Date   Signature

Declaration of contribution to the listed publications

Tamta Tkhilaishvili contributed the following to the below listed publications:

Publication 1: **Tkhilaishvili T[1]**, Di Luca M[1], Abbandonato G, Maiolo EM, Klatt AB, Reuter M, Möncke-Buchner E, Trampuz A. Real-time assessment of bacteriophage T3-derived antimicrobial activity against planktonic and biofilm-embedded Escherichia coli by isothermal microcalorimetry. Research in microbiology (2018); 169(9):515-521.doi: 10.1016/j.resmic.2018.05.010.

[1]authors equally contributed as first authors

Contribution in detail:

- Planning and organization of experiments in agreement with PD Dr. Trampuz and Dr. Mariagrazia Di Luca.

- Performing of all experiments, in detail: Evaluation of the antimicrobial assays against planktonic and biofilm bacteria by real-time microcalorimetry assays and assessment of biofilm eradication by sonication of beads and colony counting.

- Processing and evaluation of all data, interpretation of results, preparation of all tables and figures.

- Writing the manuscript and working on the final version of the paper.

Publication 2: **Tkhilaishvili T**, Wang L, Tavanti A, Trampuz A, Di Luca M. Antibacterial efficacy of two commercially available bacteriophage formulations, staphylococcal bacteriophage and PYO bacteriophage, against methicillin-resistant *Staphylococcus aureus*: Prevention and eradication of biofilm formation and control of a systemic infection of *Galleria mellonella* larvae. Frontiers in Microbiology (2020); 7;11:110. doi: 10.3389/fmicb.2020.00110.

Contribution in detail:

- Planning and organization of experiments in agreement with PD Dr. Trampuz and Dr. Mariagrazia Di Luca.

- Performing of all *in vitro* and *in vivo* experiments, in detail: Evaluation of the antimicrobial assays against planktonic and biofilm bacteria by real-time microcalorimetry assays and assessment of biofilm eradication by sonication of beads and colony counting. Testing of phage formulations in a *Galleria mellonella* model of *S. aureus* systemic infection.

- Processing and evaluation of all data, interpretation of results, preparation of all tables and figures.

- Writing the manuscript and working on the final version of the paper.

Publication 3: Tkhilaishvili T, Lombardi L, Klatt AB, Trampuz A, Di Luca M. Bacteriophage Sb-1 enhances antibiotic activity against biofilm, degrades exopolysaccharide matrix and targets persisters of *Staphylococcus aureus.* International Journal of Antimicrobial Agents (2018). 52(6):842-853. doi: 10.1016/j.ijantimicag.2018.09.006.

Contribution in detail:

- Planning and organization of experiments in agreement with PD Dr. Trampuz and Dr. Mariagrazia Di Luca.

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- Processing and evaluation of all data, interpretation of results, preparation of all tables and figures.

- Writing the manuscript and working on the final version of the paper.

Publication 4: Tkhilaishvili T, Wang L, Perka C, Trampuz A, Gonzalez Moreno M. Using bacteriophages as a trojan horse to the killing of dual-species biofilm formed by *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus*. Frontiers in Microbiology (2020); 11:695. doi: 10.3389/fmicb.2020.00695

Contribution in detail:

- Planning and organization of experiments in agreement with PD Dr. Trampuz.
- Performing of all experiments, in detail: Evaluation of the antimicrobial assays against planktonic and biofilm bacteria by real-time microcalorimetry assays and assessment of biofilm eradication by sonication of beads and colony counting.
- Processing and evaluation of all data, interpretation of results, preparation of all tables and figures.
- Writing the manuscript and working on the final version of the paper.

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Signature, date and stamp of supervising university professor / lecturer

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Signature of the doctoral candidate

Printed copies of selected publications

Publication 1 (Study A): Real-time assessment of bacteriophage T3-derived antimicrobial activity against planktonic and biofilm-embedded Escherichia coli by isothermal microcalorimetry.
Tkhilaishvili T[1], Di Luca M[1], Abbandonato G, Maiolo EM, Klatt AB, Reuter M, Möncke- Buchner E, Trampuz A.
[1]authors equally contributed as first authors
Research in microbiology (2018).
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Publication 2 (Study B): Antibacterial efficacy of two commercially available bacteriophage formulations, staphylococcal bacteriophage and PYO bacteriophage, against methicillin-resistant *Staphylococcus aureus*: Prevention and eradication of biofilm formation and control of a systemic infection of *Galleria mellonella* larvae.
Tkhilaishvili T, Wang L, Tavanti A, Trampuz A, Di Luca M
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Tkhilaishvili T, Lombardi L, Klatt AB, Trampuz A, Di Luca M.
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Publication 4 (Study D): Using bacteriophages as a trojan horse to the killing of dual-species biofilm formed by *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus.*
Tkhilaishvili T, Wang L, Perka C, Trampuz A, Gonzalez Moreno M.
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Publication 1 (Study A): Real-time assessment of bacteriophage T3-derived antimicrobial activity against planktonic and biofilm-embedded Escherichia coli by isothermal microcalorimetry.

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Antibacterial Efficacy of Two Commercially Available Bacteriophage Formulations, Staphylococcal Bacteriophage and PYO Bacteriophage, Against Methicillin-Resistant *Staphylococcus aureus*: Prevention and Eradication of Biofilm Formation and Control of a Systemic Infection of *Galleria mellonella* Larvae

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Sessile bacteria growing on surfaces are more resistant to standard antibiotics than their planktonic counterpart. Due to their antimicrobial properties, bacteriophages have re-emerged as a promising approach to treat bacterial biofilm-associated infections. Here, we evaluated the ability of two commercially available phage formulations, Staphylococcal bacteriophage (containing the monophage Sb-1) and PYO bacteriophage (a polyphage), in preventing and eradicating an *in vitro* biofilm of methicillin-resistant *Staphylococcus aureus* (MRSA) by isothermal microcalorimetry and high-resolution confocal laser scanning microscopy (CLSM). Moreover, to assess the potential *in vivo* efficacy of both phage preparations, a *Galleria mellonella* model of MRSA systemic infection was used. Microcalorimetry measurement showed that 10^7 PFU/ml (the highest tested titer) of both phage formulations were able to inhibit planktonic growth in a concentration-dependent manner. However, MRSA biofilm was eradicated only by co-incubation of 5–7 days with the highest phage titers, respectively. In the experiments of biofilm prevention, isothermal microcalorimetry revealed that the heat production was completely abolished in the presence of sub-inhibitory titers (10^4 PFU/ml) of phages. These data were also confirmed by confocal laser scanning microscopy. Both phage formulations increased the survival of *G. mellonella* larvae preventing or treating MRSA infection compared to untreated control. In conclusion, tested phage formulations are promising for preventing device

colonization and killing biofilm bacteria attached on a surface. Novel strategies for direct coating and release of phages from material should be investigated.

INTRODUCTION

Staphylococcus aureus is causing a variety of community-acquired and healthcare-associated infections (Magill et al., 2014; Tong et al., 2015). In addition, *S. aureus* exhibits the ability to form biofilm on either native tissues or implanted medical devices resulting in tolerance to high concentrations of antimicrobials (Zimmerli et al., 2004; Arciola et al., 2012). Infections caused by biofilm-embedded bacteria are difficult to eradicate due to an extracellular polymeric matrix, which protects them from antimicrobials and host immune cells (de la Fuente-Nunez et al., 2013; Paharik and Horswill, 2016). Indeed, the heterogeneity of the biofilm cell populations, including antibiotic-tolerant persister cells, characterized by a slow- or non-growing state, makes biofilm-embedded bacteria significantly less susceptible to antimicrobials than their free-floating counterparts (Van Acker et al., 2014; Flemming et al., 2016). Moreover, the emergence of spreading of staphylococcal strains resistant to different antimicrobial agents, including methicillin, vancomycin, daptomycin and/or rifampicin (O'Neill et al., 2006; Kos et al., 2012; Hassoun et al., 2017; Ma et al., 2018) represents a serious threat to global health (Sugden et al., 2016; Monaco et al., 2017).

This scenario is further complicated by the fact that production pipelines for the development of novel antibiotics has been running dry over the past few decades, resulting in an crucial requirement to identify novel therapeutic strategies to control bacterial infections mainly due to multi-drug resistant bacteria embedded in a biofilm (Ribeiro et al., 2016).

Bacteriophage (phage) therapy, based on the employment of viruses specifically killing bacterial cells, is considered an encouraging option for treating staphylococcal infections which result refractory to conventional antibiotics (Gordillo Altamirano and Barr, 2019). After the discovery of bacteriophages by d'Herelle in 1917, phages as therapeutic agents have been used for almost century mainly in the Eastern European countries in humans (Kutateladze and Adamia, 2010). However, with the launching of conventional antibiotics, the application of phage therapy in Western countries promptly decreased (Kortright et al., 2019). The rapid increase of multi-drug-resistant bacterial strains recently has been renewed interest in phage therapy and even though regulatory authorities in Europe and U.S. have not approved it yet, several examples of successfully applications of personalized phage therapy have been reported (Jennes et al., 2017; Chan et al., 2018; Exarchos et al., 2019; Nir-Paz et al., 2019; Tkhilaishvili et al., 2019) as compassionate use under the umbrella of Article 37 of the Helsinki Declaration.

Phage therapy offers some advantages over conventional antimicrobial strategies. Due to their high specificity, bacteriophages attack only host bacterial cells without affecting the normal microflora (Ly-Chatain, 2014). Unlike antibiotics, bacteriophages are self-propagating and self-limiting viruses, regulating themselves at the site of infection. This behavior results in a localized increase in viral particle numbers with a low initial dose as long as the targeted bacteria are present and in a decrease when bacteria have been killed (Dabrowska, 2019). Moreover, since the resistance mechanisms arose for all class of antibiotics do not alter phage infection, phages have been demonstrated to kill multidrug-resistant bacterial cells.

In addition, for different phages, the ability to reduce *in vitro* viable sessile bacterial cells was also proved (Khalifa et al., 2015; Liu et al., 2016; Kumaran et al., 2018), suggesting a potential use for the treatment of biofilm-associated infections.

Among commercially available phage formulations for therapeutic use in human, Staphylococcal bacteriophage (Sb) and PYO bacteriophage (PYO) were developed and employed as anti-infective treatments at Eliava Institute in Georgia (Fish et al., 2018; Ujmajuridze et al., 2018). Sb is a mono phage preparation that contains a well characterized and fully sequenced virus Sb-1 (Kvachadze et al., 2011), whereas PYO is a complex preparation targeting different bacterial species including *S. aureus*, *Streptococcus* spp., *E. coli*, *Pseudomonas aeruginosa*, and *Proteus* spp. with batch to batch variations (Kvachadze et al., 2011; Villarroel et al., 2017).

Sb has been successfully employed to treat *S. aureus* infections in different patients suffering from digital osteomyelitis and foot ulcers (Fish et al., 2016). Analogously, PYO have been used to cure staphylococcal wound infections by either washing the wound or applying a dressing impregnated with the phage cocktail (Pokrovskaya et al., 1942; Markoishvili et al., 2002).

Recently, we have showed that Sb is able to degrade the components of extracellular matrix and be effective against persister cells of *S. aureus* (Tkhilaishvili et al., 2018b). In addition, this phage formulation exhibited a rapid synergistic activity in eradicating *S. aureus* biofilm after 24 h-treatment in combination with different classes of antibiotics (Tkhilaishvili et al., 2018b).

Although both phage formulations were widely used in Former Soviet Union (Sulakvelidze et al., 2001; Myelnikov, 2018), their lytic effect against sessile bacteria has not been investigated in a preclinical setting yet.

Here, we evaluated the ability of Sb and PYO to prevent and eradicate an *in vitro* biofilm of methicillin-resistant *S. aureus* (MRSA) by isothermal microcalorimetry (IMC) and high-resolution microscopy. In addition, the *in vivo* efficacy of both phage formulations was also evaluated in a *Galleria mellonella* model of *S. aureus* systemic infection.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophages

MRSA ATCC 43300 was used for all experiments. Bacteria were stored in a cryovial bead preservation system (Roth, Karlsruhe, Germany) at −80°C. Bacterial strains were grown on blood agar plate (VWR Chemicals, Leuven, Belgium) at 37°C for 24 h. Inoculum was prepared according to a McFarland (BioMerieux Marcy l'Etoile, France) turbidity of 0.5 ($\approx$1–5 × 10^8 CFU/ml of the tested strain). Commercially available formulations of staphylococcal bacteriophage and PYO bacteriophage were obtained as 10 ml liquid ampoules from the Eliava Biopreparations, a company associated with the G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia. PYO formulation Phage stocks were maintained at 4°C. Vancomycin was supplied by Teva Pharma AG (Aesch, Switzerland) as 10 mg of powder in ampoules. The stock solution of 50 mg/ml was prepared in sterile saline.

Titration of Bacteriophage Suspensions

Phages titers were determined by a quantitative plaque assay as previously described (Tkhilaishvili et al., 2018a). Phage formulations were diluted using 10-fold serial dilutions in phosphate buffer to an estimated concentration yielding plaque numbers that could easily be counted. From appropriate dilutions, 0.5 ml bacteriophage lysate and 0.3 ml host bacteria from overnight culture were added to 3 ml top agar (ca. 50°C) and immediately poured onto Brain-Heart Infusion agar plates. Two plates for each dilution were used. After overnight incubation of plates at 37°C, plaques were counted and the titer was calculated in PFU/ml.

Microcalorimetry Assay

An isothermal calorimetry instrument (Thermal Activity Monitor, Model 3,102 TAM III, TA Instruments, New Castle, DE, USA) equipped with 48 channels was used to determine the antimicrobial activity of bacteriophages, as previously reported (Tkhilaishvili et al., 2018a,b). Airtight sealed ampoules were sequentially introduced into the microcalorimetry channels and lowered to an equilibrium position for 15 min to reach a temperature of 37°C. The heat generated in real-time by planktonic and biofilm-embedded cells treated with phages and by recovering bacteria after treatment were continuously measured. Heat flow (μW) was measured at 120 s-intervals and recorded for either 24 or 48 h.

Phage Lytic Activity Against Planktonic Methicillin-Resistant *Staphylococcus aureus* by Isothermal Microcalorimetry and CFU Counting

Free-floating bacteria were added to microcalorimetric ampoules containing 3 ml of BHIB (final inoculum 1–5 × 10^6 CFU/ml) and 10-fold serial dilutions of phages (ranging from 10^2 to 10^7 PFU/ml). A growth control containing bacteria without phages, as well as a negative control with phages only was also included.

Bacterial heat production was monitored for 24 h at 37°C and data were plotted as heat flow (in μW) and total heat (J) versus time. The minimum heat inhibiting concentration of phages (MHICP) was defined as the lowest titer inoculated with bacteria at the experiment starting point that inhibited growth-related heat production during 24 h-incubation in the microcalorimeter by more than 90% in comparison to the untreated control (growth control). After calorimetric analysis, 50 μl of the culture and related 10-fold serial dilutions were plated onto BHI agar for colony counting. MBC was defined as the minimum bactericidal titer of phages which determined a reduction of more than 3 log$_{10}$ CFU/ml comparing to the CFU/ml of inoculum size. Experiments were performed in triplicate.

Phage Lytic Activity Against Biofilm Methicillin-Resistant *Staphylococcus aureus* by Isothermal Microcalorimetry Testing and Sonication/Colony Counting

Real-Time Isothermal Microcalorimetry

MRSA biofilms were formed on porous glass beads having a diameter 4 mm, pore size 60 μm, and surface area approximately 60 cm^2 (VitraPor; ROBU, Hattert, Germany). Briefly, 10 beads were statically incubated with 2–3 colonies of MRSA into 10 ml BHI broth at 37°C. After 24 h incubation, beads were carefully washed three times using sterile PBS and incubated with 10-fold serial dilution phage titers (ranging from 10^2 to 10^7 PFU/ml) into the microcalorimetry glass ampoules filled with a final volume of 3 ml fresh BHI broth. Sterile beads and beads with untreated biofilm were also included as a negative (sterility) and positive (growth) control. IMC analyses were recorded for 48 h at 37°C. The minimal heat inhibitory concentration of phages for biofilm (MHICP$_{biofilm}$) bacteria was defined as "the lowest phage titer inhibiting growth-related heat production related to the viability of biofilm cells during 48 h-incubation in the microcalorimeter more than 90% (corresponding more than 2 log$_{10}$-reduction of CFU) compared to the growth control. Experiments were performed in triplicate."

Evaluation of the Eradication of Biofilm Methicillin-Resistant *Staphylococcus aureus* by Isothermal Microcalorimetry

Twenty-four-hour-old biofilms on the beads prepared as described above were co-incubated to the 10-fold serial dilution bacteriophage titers (ranging from 10^2 to 10^7 PFU/ml) in plastic FAC tubes (Corning Science, Reynosa, Mexico) for 24–48–72–120–168 h. Then, beads were carefully rinsed (3×) using sterile PBS to remove planktonic bacteria and phages, incubated in ampoules with fresh medium and inserted into the calorimeter for the eradication analysis. Growth medium with untreated beads was used as the positive (growth) control, and growth medium with sterile beads served as the negative (sterility) control. The minimum biofilm bactericidal concentration of phages (MBBCP) was defined as the lowest antimicrobial concentration that strongly reduced the number of viable bacterial cells within the biofilm, and therefore leading to

undetectable heat values for 24–48 h. Experiments were performed in triplicates.

Biofilm Prevention Assay by Isothermal Microcalorimetry

An inoculum, prepared according to a McFarland standard turbidity of 0.5 was diluted to a final concentration of $1-5 \times 10^6$ CFUs/ml, as reported above. Ten beads were incubated with $1-5 \times 10^6$ CFU/ml of MRSA together with 10-fold serial dilution phage titers (ranging from 10^2 to 10^7 PFU/ml) into 10 ml BHI broth for 24 h at 37°C in static condition. After 24 h-incubation, beads were carefully rinsed (3×) with sterile PBS and incubated in sterile glass ampoules with 3 ml BHI broth. Sterile beads and beads with untreated biofilm were also here included as a negative (sterility) and positive (growth) control. The IMC analysis was performed at 37°C for 48 h, defining the minimum biofilm preventing concentration (MBPCP) of phages as the lowest phage titer that prevented the formation of biofilm on the glass beads, thus leading to an undetectable heat flow signal during 48 h-incubation in the calorimetry.

Sonication/Colony Counting

After IMC biofilm experiments, to evaluate the reduction/eradication of biofilm cells, the beads showing no heat production together with untreated biofilms (growth controls) were washed (3×) using sterile PBS to remove the rest of phages and planktonic bacteria and transferred to individual Eppendorf tubes with 1 ml saline. Beads were vortexed for 30 s with maximum power, sonicated at 40 kHz for 60 s in a sonication bath (BactoSonic; Bandelin Electronic, Germany), and vortexed for 30 s again to dislodge biofilm bacteria. For conventional culture, sonication fluids were serially diluted in Eppendorf tubes and aliquots of 50 µl were quantified by viable count of CFU/ml. In unpublished control experiments, in which *S. aureus* cultures were sonicated up to 30 min (with the same above mentioned conditions), no statistically significant difference in CFU values was observed in comparison to non-sonicated cultures, indicating that under these conditions sonication does not kill bacteria. The minimum biofilm eradicating concentration (MBECP) of phage titers was defined as MBBCP titers, but resulting in 0 CFU/ml on plates after CFU counting of the sonicated beads, as previously described (Tkhilaishvili et al., 2018b).

Confocal Laser Scanning Microscopy

The lytic effect of phage on the prevention of biofilm formation and its eradication was evaluated by CLSM. Brieflyan overnight bacterial culture (diluted 1:100) was distributed into an 8-well µ-Slide (Ibidi) to form biofilm. For prevention experiments, bacteria were simultaneously incubated (at 37°C for 24 h) in the presence of different titres of phages. For eradication experiments, bacteria were first let form biofilm into an 8-well µ-Slide (Ibidi) for 24 h at 37°C, and then treated with different phage titers. Bacteria viability and biofilm thickness after phage co-incubation/treatment was determined by CLSM after staining cells with Syto9 (488 nm/500–540 nm) and propidium iodide (PI) (561 nm/600–650 nm) as recommended by the manufacturer (Live/dead BacLight Bacterial Viability Kit Molecular Probes, Life technologies). Samples were analyzed by the microscope TCS SP5 (Leica, Heidelberg, Germany) using a 63× objective and a pinhole aperture of 1.0 Airy. For each image, the mean of fluorescent intensity was calculated as previously described.

Phage Treatment in a *Galleria mellonella* Model of Methicillin-Resistant *Staphylococcus aureus* Infection

Larvae of *G. mellonella* were obtained from BioSystems Technology Ltd. (Exeter, Devon, UK). Larvae were stored at room temperature and were used within 3 days. Phages were tested for their ability to rescue MRSA-infected larvae from death. Bacteria were prepared for injection as previously described (Gibreel and Upton, 2013). Larvae were inoculated with 10 µl of bacterial suspension (containing $\approx 2.5-5 \times 10^6 - 2.5-5 \times 10^7$ CFU) in the last left proleg. For the treatment, phages (1×10^5 PFU) or vancomycin (10 mg/kg) were delivered behind the last proleg on the opposite side to the bacterial injection site either 1 h post-infection (for treatment experiments) or 1 h pre-infection (for prevention experiments). Ten larvae per treatments were issued in all experiments. Larvae infected and treated with PBS solution served as positive control group. Three negative control groups were also included in the experimental design: one group that underwent no manipulation, one group injected with PBS only, which controlled for the impact of any negative effect from the injection process, and one group injected with phage suspension only, assessing phage toxicity. Larvae were stored in Petri dishes in the dark at 37°C for 168 h. Larvae were inspected every 24 h and were considered dead if they did not move when stimulated.

Data Analysis

Microcalorimetry data analysis was accomplished using the manufacturer's software (TAM Assistant; TA Instruments, New Castle, DE). Figures were plotted using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA), and resulted data were expressed as heat flow (µW) and total heat (J) versus time (h). In the *G. mellonella* model of infection, survival data were plotted using the Kaplan-Meier method.

RESULTS

Antibacterial Activity of Sb and PYO *versus* Planktonic Methicillin-Resistant *Staphylococcus aureus*

The viability of planktonic MRSA was investigated in real-time over 24 h by IMC measuring the heat produced by MRSA in the presence of phages and by CFUs counting after phage treatment (**Figure 1**). An untreated growth control was also added. As shown in **Figures 1A,C**, either Sb or PYO rapidly inhibited the planktonic growth of MRSA in a titer-dependent manner compared to the untreated growth control. Indeed, no heat production was observed in the presence of 10^7 PFU/ml of both phage formulations within 24 h-incubation, indicating that 10^7 PFU/ml titer corresponds to MHICP.
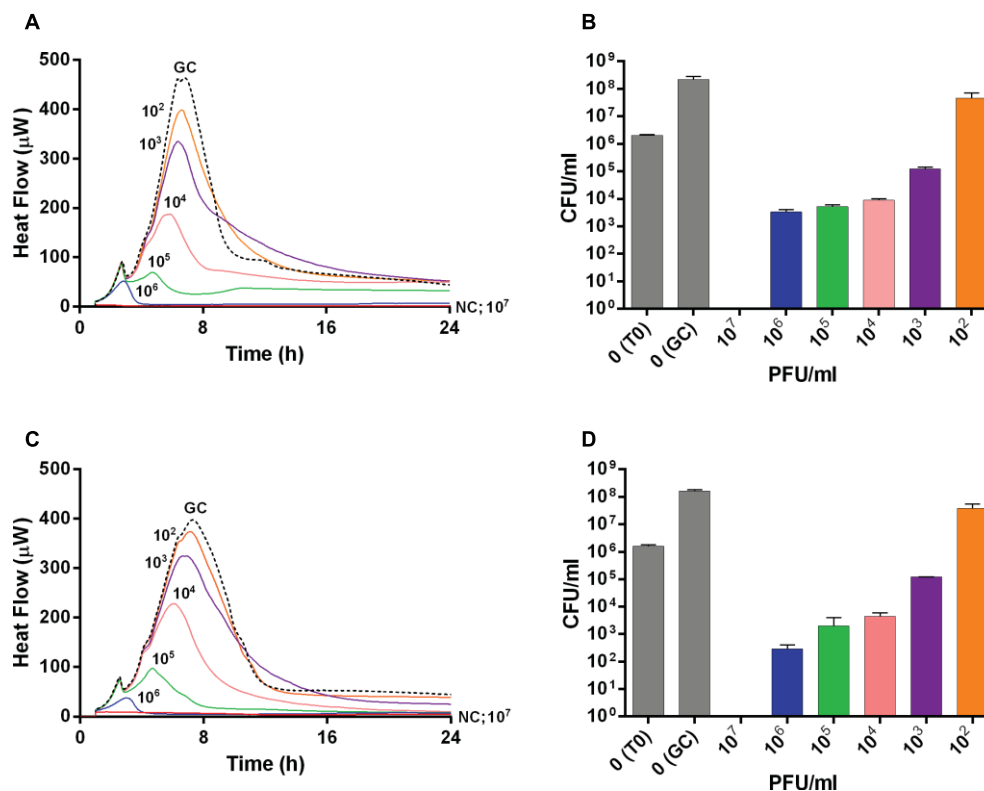
FIGURE 1 | Evaluation of MRSA ATCC43300 susceptibility to Sb **(A,B)** and PYO **(C,D)** exposure, by isothermal microcalorimetry **(A,C)** and colony counting **(B,D)**. Each curve shows the effect of different titers of phages on the heat produced by viable bacteria during 24 h of treatment with Sb **(A)** and PYO **(C)**. Histogram represents the mean of CFU number ± SEM of planktonic MRSA treated/untreated with Sb **(B)** and PYO **(D)**. Numbers above curves represent Sb titers (PFU/ml). GC, growth control (dashed line); NC, negative control; T0, initial inoculum.

A similar dose-dependent trend was observed by colony counting of bacteria after 24 h incubation with different phage titers. As shown in **Figures 1B,D**, an increase of ≈2 $\log_{10}$ CFU/ml was observed in the GC samples, as compared to the inoculum size (T0). A reduction of more than 2 $\log_{10}$ was already obtained with 10^4 PFU/ml of Sb and PYO phages, respectively, compared to the CFU/ml number of MRSA initial inoculum (1–5 × 10^6 CFU/ml). In the presence of 10^7 PFU/ml titers of both phage formulations, no CFUs were observed (plating detection limit = 20 CFU/ml), suggesting that such titer is MBCP.

Antibacterial Activity of Sb and PYO Against Biofilm-Embedded Methicillin-Resistant *Staphylococcus aureus*

The interaction between phages and 24 h-old *S. aureus* biofilm was also analyzed in real-time by microcalorimetric measurements. The thermogenic curves of biofilm-embedded cells treated and untreated with phages are shown in **Figure 2**. Either Sb and PYO inhibited the replication of sessile bacteria in a titer-dependent manner compared to the growth control, resulting in a suppression of the heat production over 48 h-incubation (**Figures 2A,C**). However, a reduction of more than 90% of the total heat produced by MRSA biofilm-embedded cells was

observed at 10^7 PFU/ml titers for both phage formulations and therefore was defined as MHICP$_{biofilm}$.

Then, the evaluation of viable bacteria attached to the beads was performed by colony counting after bead sonication and plating of the sonication fluids. A similar dose-dependent trend of reduction of MRSA CFUs/ml was observed for all samples treated with both phages, as compared to the untreated growth control (**Figures 2B,D**). Here, the colony counting showed a reduction of more than 2 $\log_{10}$ when bacteria were treated with 10^7 PFU/ml of Sb and more than 3 $\log_{10}$ with 10^7 PFU/ml of PYO phage compared to the growth control (plating detection limit = 20 CFUs/ml). The lack of an eradication at higher titers after 24 h of phage treatment was also confirmed by CLSM (**Figure 3**).

In our previous work, we have shown that Sb is able to degrade the extracellular polysaccharide matrix of *S. aureus* biofilm (Tkhilaishvili et al., 2018b). By using CLSM, we also assessed the effect of PYO on extracellular matrix (**Figure 4**). Twenty-four-hour-old biofilm of MRSA was stained with both a dye specific for the poly-N-acetylglucosamine residues (blue) of the extracellular polysaccharides and with syto 85 specific for the cellular DNA (green). In contrast to what observed for Sb (Tkhilaishvili et al., 2018b), none of PYO titers determined a progressive reduction of the blue staining in comparison to
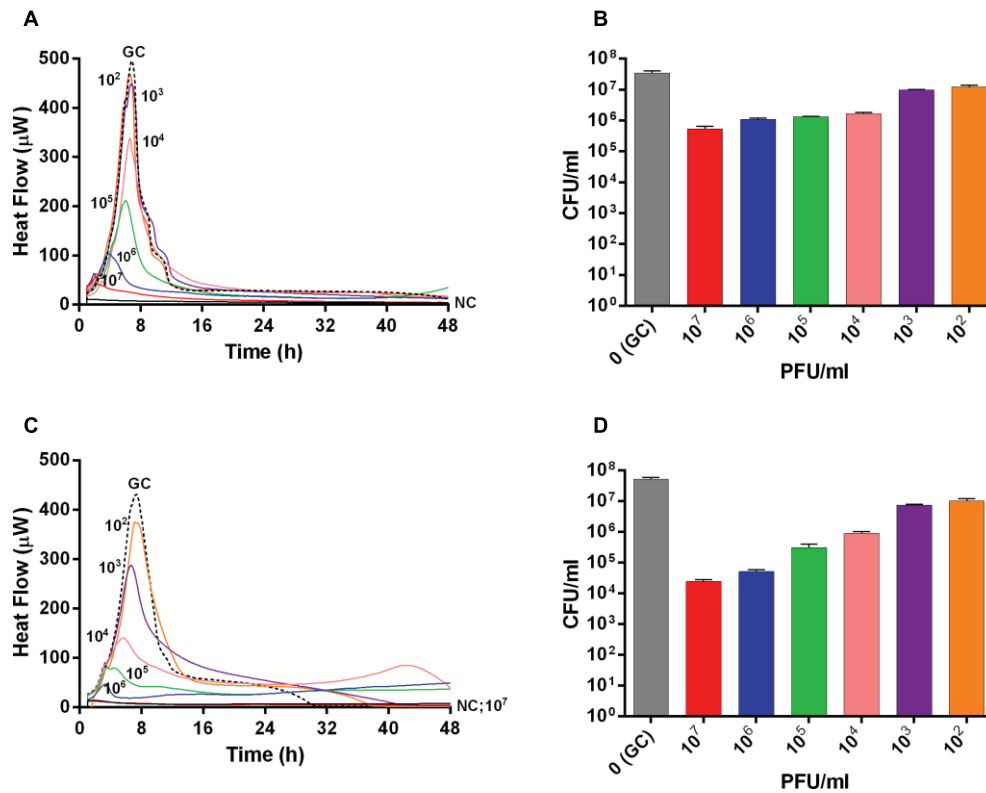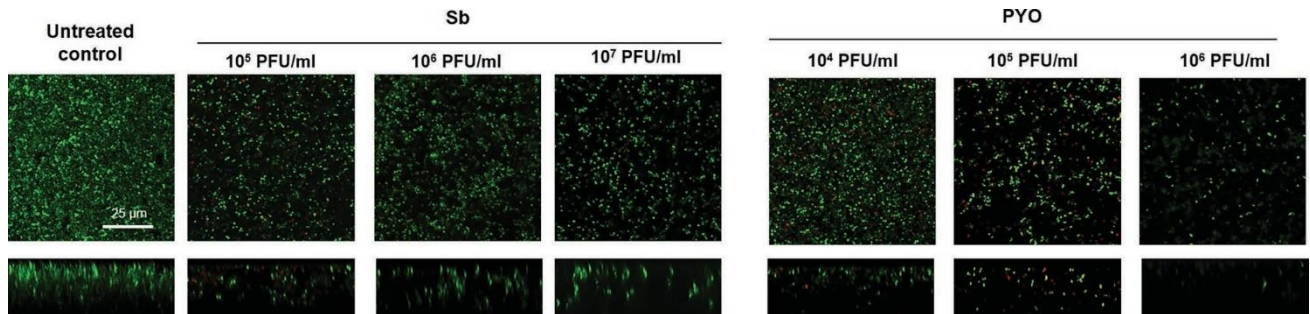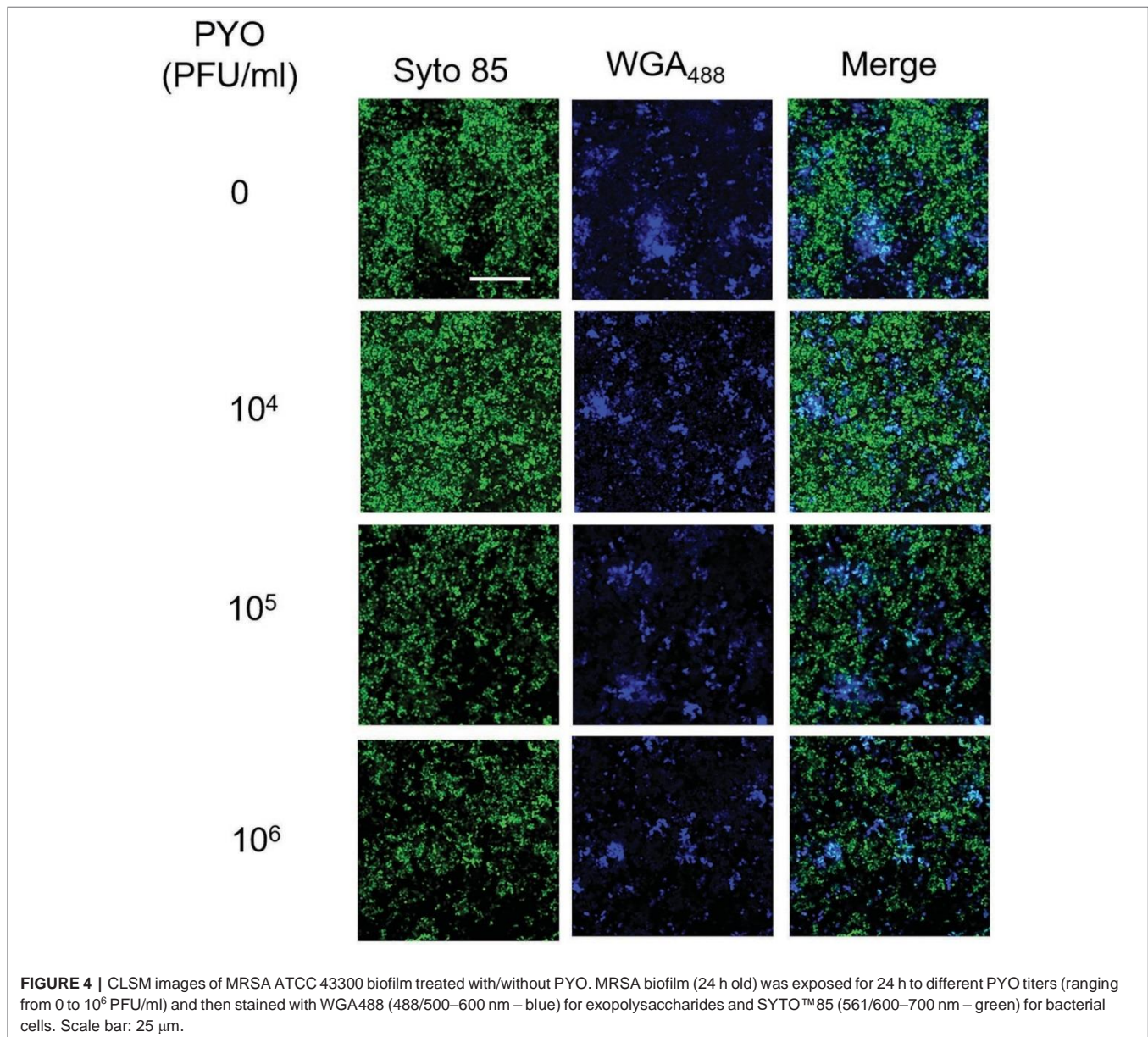
FIGURE 2 | Evaluation of MRSA ATCC43300 biofilm susceptibility to either Sb **(A,B)** or PYO **(C,D)** exposure, by isothermal microcalorimetry **(A,C)** and colony counting **(B,D)**. Each curve shows the heat produced by viable bacteria attached on beads during 48 h treatment with different titers (ranging from 10^2 to 10^7 PFU/ml) of Sb **(A)** and PYO **(C)**, respectively. Histogram represents the mean of CFU number ± SEM of biofilm dislodged MRSA treated/untreated with Sb **(B)** and PYO **(D)**. Numbers above curves represent Sb titers (in PFU/ml). GC, growth control (dashed line); NC, negative control.

FIGURE 3 | CLSM images of MRSA ATCC 43300 biofilm treated with/without phages. MRSA biofilm (24 h-old) was exposed for further 24 h to different phage titers (10^4–10^7 PFU/ml). The viability of the cells was evaluated staining biofilm with SYTO9 (488/500–540 nm – green) for alive bacteria and with propidium iodide (PI) (561/600–650 nm – red) for dead bacteria. Images are merged from the two channels. Upper and lower panels represent xy- and z-plans, respectively. Scale bar: 25 μm.

the untreated control, suggesting that no visible degradation of the polysaccharide component seems to occur in the presence of PYO phages.

Moreover, isothermal microcalorimetry was used to investigate the presence of residual biofilm cells attached on glass beads in terms of metabolic heat production. Beads were re-inoculated in fresh medium, after incubation with different phage titers and at different time points (ranging from 24 h up to 168 h). Indeed, co-incubation with increasing titers of either Sb and

PYO formulations over the time led to a minor heat production compared to the heat released by untreated controls, suggesting a strong decrease in the number of alive bacteria attached on the beads when treated with phages (**Figures 5, 6**, respectively). The analysis of sonication fluids of bead biofilms pre-treated with phages and incubated in the calorimeter at 37°C for 24 h in fresh medium revealed that MRSA biofilm was eradicated only following a co-incubation with the highest titer of Sb and PYO, for either 7 or 5 days, respectively (**Figures 5E, 6D**).

FIGURE 4 | CLSM images of MRSA ATCC 43300 biofilm treated with/without PYO. MRSA biofilm (24 h old) was exposed for 24 h to different PYO titers (ranging from 0 to 10^6 PFU/ml) and then stained with WGA488 (488/500–600 nm – blue) for exopolysaccharides and SYTO™85 (561/600–700 nm – green) for bacterial cells. Scale bar: 25 μm.

These results were confirmed by CLSM imaging of MRSA biofilm treated for 7 and 5 days with Sb and PYO, respectively. The analysis of MRSA viability by dead/live staining clearly indicating that no biofilms were detectable after the treatment of MRSA biofilm with phages (**Figure 7**). By definition, 10^7 PFU/ml and 10^6 PFU/ml were the MBEC of Sb and PYO after 7 and 5 days, respectively.

Ability of Sb and PYO to Prevent Methicillin-Resistant *Staphylococcus aureus* Biofilm Formation

Microcalorimetric measurements were also performed to evaluate the ability of bacteriophages in preventing biofilm formation on glass beads. In this experiment, by using microcalorimetry,

we aimed at evaluating if any biofilm or attached bacteria were present on the beads which were previously co-incubated with phages and bacteria (simultaneously), for 24 h. After co-incubation, beads were washed to remove free floating phages and bacteria and then inoculated in fresh medium in the calorimeter. If phages prevented bacterial attachment and biofilm formation, beads had no bacteria on top, so no heat was produced within 48 h of measuring. **Figure 8** shows the heat flow detected during 48 h monitoring of heat produced by MRSA, generated by viable bacteria attached on the beads, previously co-incubated with either Sb (**Figure 8A**) or PYO phage (**Figure 8B**).

A strong reduction of MRSA heat production was already achieved at a lower titer (10^2 PFU/ml) of both bacteriophages, and a reduction of more than 90% of heat production was already observed at 10^4 PFU/ml of both phage formulations.
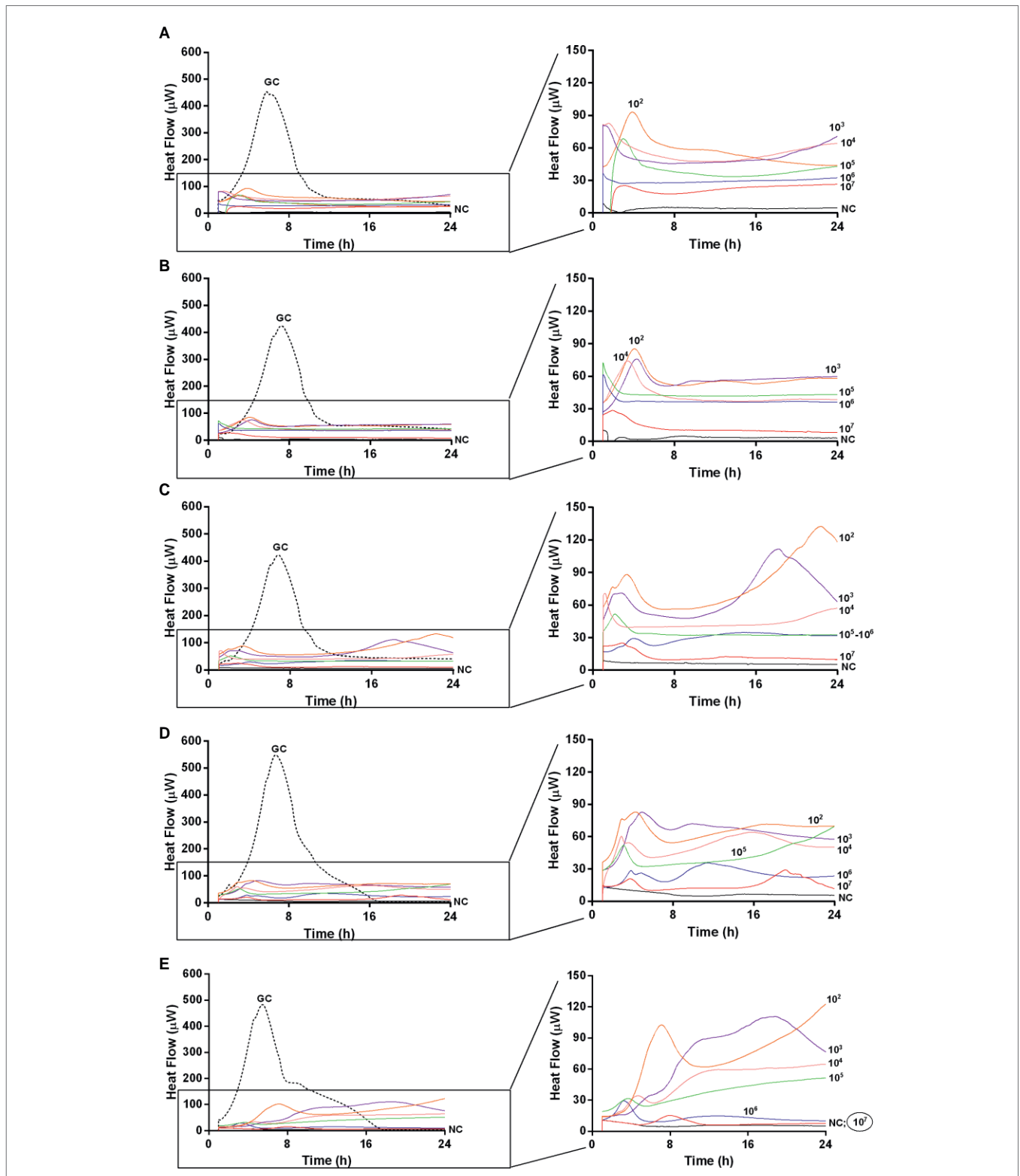
FIGURE 5 | Evaluation of MRSA ATCC43300 biofilm susceptibility to Sb phage at different time exposures by IMC. Each curve shows the heat produced by viable bacteria present in the biofilm after 24 h **(A)**, 48 h **(B)**, 72 h **(C)**, 120 h **(D)**, 168 h **(E)**, treatment with different phage titers (ranging from 10^2 to 10^7 PFU/ml). Graphs on the right represent the magnification of the graphs on the left. Numbers above curves represent Sb titers (in PFU/ml). Circled values represent the MBEC. GC, growth control (dashed line); NC, negative control.
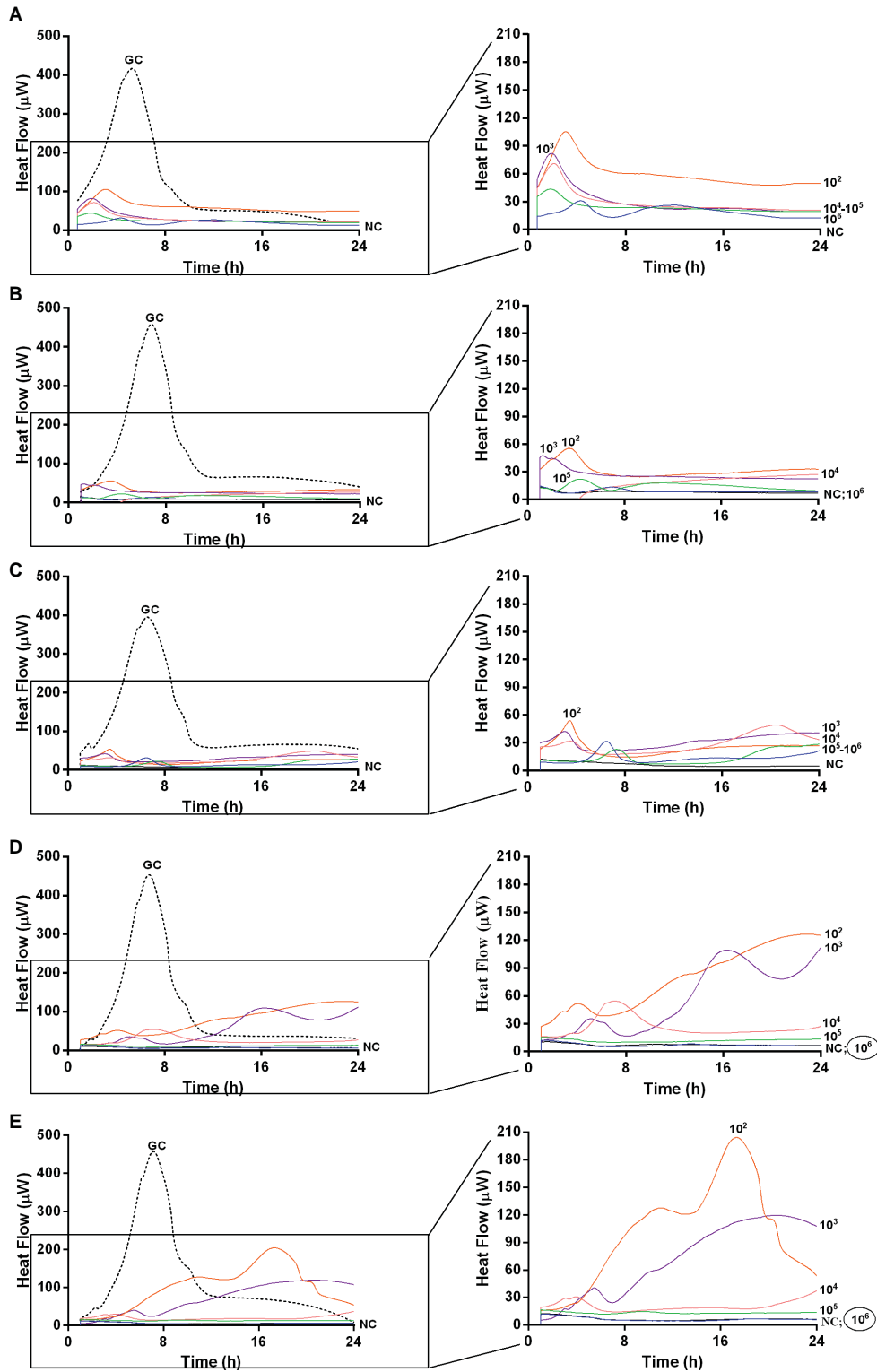
FIGURE 6 | Evaluation of MRSA ATCC43300 biofilm susceptibility to PYO at different time exposures by IMC. Each curve shows the heat produced by viable bacteria present in the biofilm after 24 h **(A)**, 48 h **(B)**, 72 h **(C)**, 120 h **(D)**, 168 h **(E)** treatment with different phage titers (ranging from 10^2 to 10^7 PFU/ml). Graphs on the right represent the magnification of the graphs on the left. Numbers above curves represent PYO titers (in PFU/ml). Circled values represent the MBECP. GC, growth control (dashed line); NC, negative control.
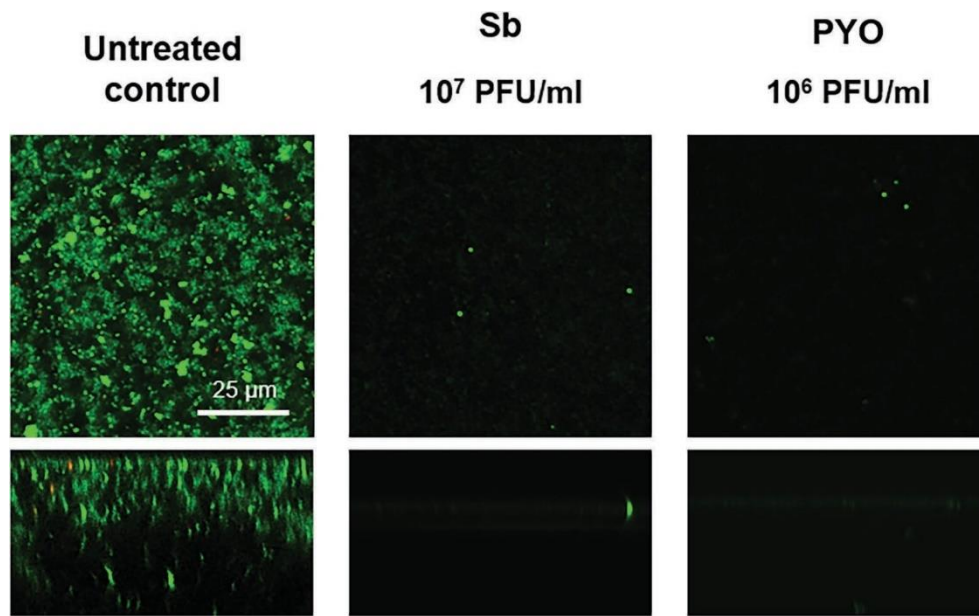
FIGURE 7 | CLSM images of MRSA ATCC 43300 biofilm untreated and treated with Sb and PYO. MRSA biofilm (24 h-old) was exposed for 7 and 5 days to 10^7 PFU/ml Sb and 10^6 PFU/ml PYO, respectively. The viability of the cells was evaluated staining with green fluorescent labeled SYTO9 (488/500–540 nm) for alive bacteria and with red fluorescent propidium iodide (PI) (561/600–650 nm) for dead bacteria. Images are merged from the two channels. Upper and lower panels represent xy- and z-plans, respectively. Scale bar: 25 μm.
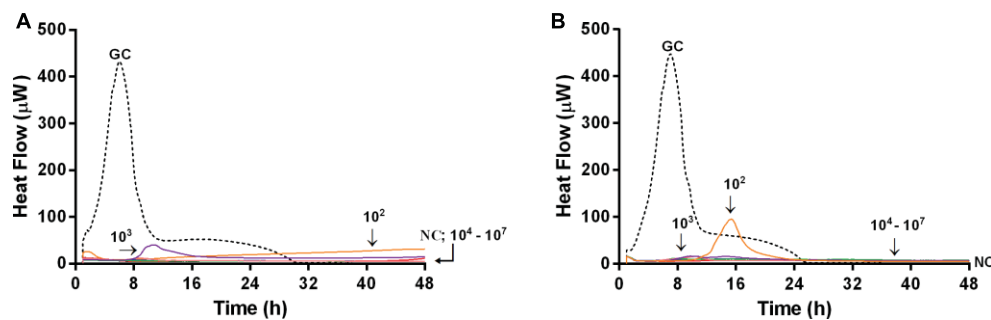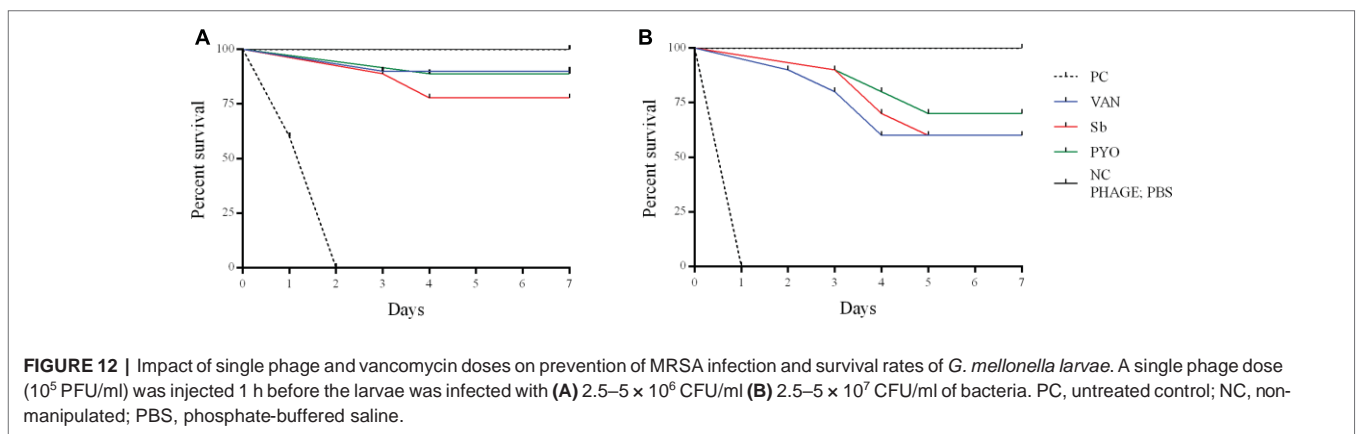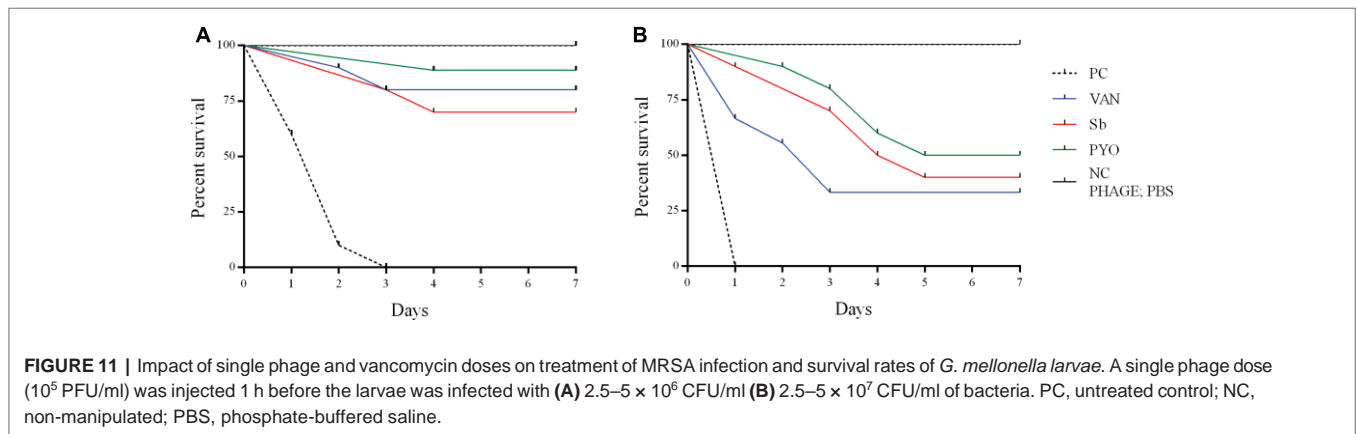

FIGURE 8 | Evaluation of the ability of Sb **(A)** and PYO **(B)** phages to prevent biofilm formation on porous glass beads by IMC. Each curve shows the heat produced by viable bacteria potentially attached on the glass beads after 24 h co-incubation with increasing titers of phages (ranging from 10^2 to 10^7 PFU/ml) in the presence of the abiotic surface. Numbers above curves represent Sb titers (in PFU/ml). GC, growth control (dashed line); NC, negative control.

The lack of heat production for 48 h correlated with no biofilm-embedded cells attached on porous surface of glass beads.

These results were confirmed by CLSM analysis of the presence of biofilm MRSA, whose planktonic cells were previously incubated with phages and then labeled by dead/live staining. As shown in **Figure 9**, a strong reduction or the absence of the alive bacterial cells attached on the wilco surface after co-incubation with 10^3 PFU/ml and 10^4 PFU/ml, of both phages compared to the untreated control was observed.

In order to better understand the absence of heat production detected by microcalorimetric experiments, the evaluation of viable bacteria attached on the beads or free-swimming bacteria in the supernatant was performed by colony counting of either sonication fluids or supernatants. As shown in **Figure 10**, sub-inhibitory titers (10^4 PFU/ml) of both phages formulations within 6 h of co-incubation showed a reduction of more than $3 \log_{10}$ of free-floating bacteria in the liquid medium, besides $\approx 10^2$ CFU/ml of bacteria was attached on the beads. However, after 24 h of co-incubation within phages, no colonies were detected, neither in the liquid medium nor in the sonication fluid. A dose-dependent correlation was observed during colony counting of bacteria with increasing the titer of phages.

Prophylaxis and Treatment of Methicillin-Resistant *Staphylococcus aureus* Systemic Infection in *Galleria mellonella* Larvae by Either Sb or PYO Phages

To evaluate the application of the phages as prophylactic or remedial treatment for MRSA infection, we determined their
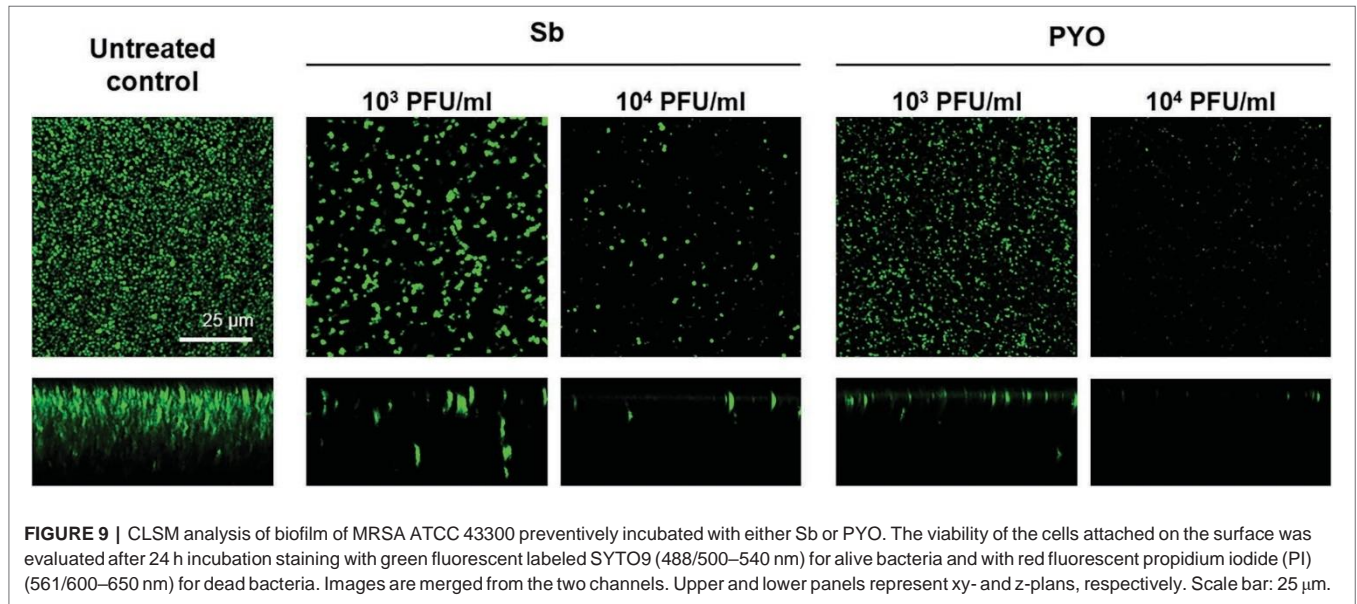
FIGURE 9 | CLSM analysis of biofilm of MRSA ATCC 43300 preventively incubated with either Sb or PYO. The viability of the cells attached on the surface was evaluated after 24 h incubation staining with green fluorescent labeled SYTO9 (488/500–540 nm) for alive bacteria and with red fluorescent propidium iodide (PI) (561/600–650 nm) for dead bacteria. Images are merged from the two channels. Upper and lower panels represent xy- and z-plans, respectively. Scale bar: 25 μm.
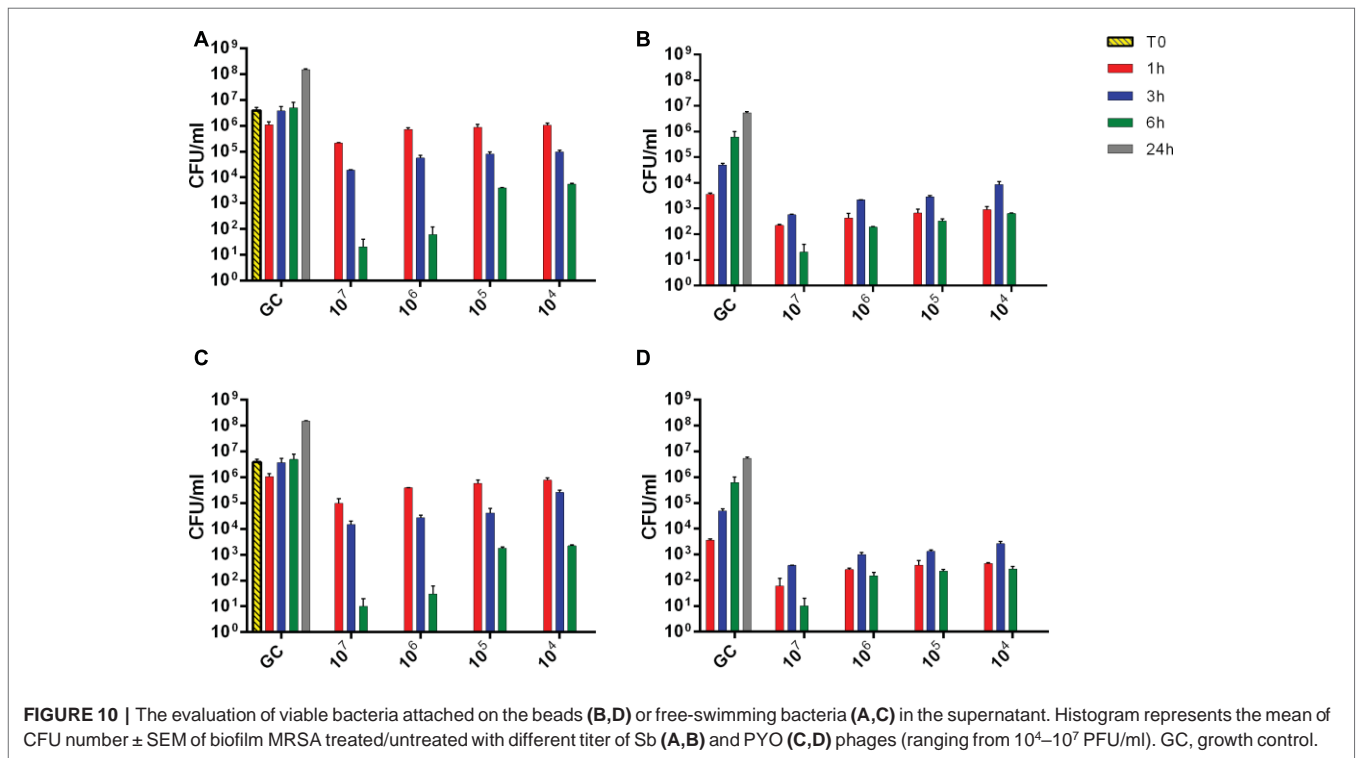
FIGURE 10 | The evaluation of viable bacteria attached on the beads **(B,D)** or free-swimming bacteria **(A,C)** in the supernatant. Histogram represents the mean of CFU number ± SEM of biofilm MRSA treated/untreated with different titer of Sb **(A,B)** and PYO **(C,D)** phages (ranging from 10^4–10^7 PFU/ml). GC, growth control.

efficacy in the *G. mellonella* larvae using the same bacterial strain and phages combination used for the *in vitro* assays (**Figures 11, 12**). Two models of phage therapy were examined. The first was a treatment whereby an acute 1 h infection was allowed to establish prior to administration of phage or vancomycin (**Figure 11**); in the second model, a phage formulation or vancomycin was administered to larvae 1 h prior to bacterial challenge to prevent infection (**Figure 12**). No mortality was recorded in the negative controls. Moreover, phages (10^5 PFU/

ml) and vancomycin 10 mg/kg alone had no effect on larval survival, as compared to the PBS-treated controls and were therefore considered to be non-toxic at these doses. Larval survival was affected by the inoculum dose, with higher doses of bacteria (2.5×10^7 CFU/ml) reducing the time of larval survival (**Figures 11B, 12B**). Single treatment with different doses of phages or antibiotic administered before or after inoculation with MRSA improved larval survival. However, the groups with a lower inoculum (2.5×10^6 CFU/ml) had a greater survival

FIGURE 11 | Impact of single phage and vancomycin doses on treatment of MRSA infection and survival rates of *G. mellonella larvae*. A single phage dose (10^5 PFU/ml) was injected 1 h before the larvae was infected with **(A)** $2.5–5 \times 10^6$ CFU/ml **(B)** $2.5–5 \times 10^7$ CFU/ml of bacteria. PC, untreated control; NC, non-manipulated; PBS, phosphate-buffered saline.

FIGURE 12 | Impact of single phage and vancomycin doses on prevention of MRSA infection and survival rates of *G. mellonella larvae*. A single phage dose (10^5 PFU/ml) was injected 1 h before the larvae was infected with **(A)** $2.5–5 \times 10^6$ CFU/ml **(B)** $2.5–5 \times 10^7$ CFU/ml of bacteria. PC, untreated control; NC, non-manipulated; PBS, phosphate-buffered saline.

percentage (**Figures 11A, 12A**). In addition, the results showed that increased survival rate was observed with the usage of a phage cocktail, as compared to single phage administration.

DISCUSSION

The emergence of antibiotic resistance has reduced the available treatment options for bacterial infections (O'Connell et al., 2013; Li and Webster, 2018). Moreover, the ability of *S. aureus* to form biofilm in the presence of a medical indwelling device represents an additional challenge, since only few antibiotics, such as vancomycin, daptomycin, fosfomycin, and rifampicin, result effective for the treatment of staphylococcal biofilm-associated infections (Singh et al., 2010; Mihailescu et al., 2014). In most cases, biofilm bacteria are tolerant to high concentrations of antibiotics due to the presence of persisters, a subpopulation of bacterial cells phenotypically tolerant to antibiotics, determined the need of prolonged therapies (Lebeaux et al., 2014; Butini et al., 2019). As a result, bacteriophages have been re-emerged as potential alternative strategy for the treatment of biofilm-associated infections (Wu et al., 2015; Pires et al., 2017).

Among different commercially available phage formulation, Sb is constituted of Sb-1 a fully sequenced single phage

preparation, while PYO is a cocktail of different phages and the one used in this study contained the completely sequenced Staphylococcal phage ISP (Vandersteegen et al., 2011).

In our previous work, by using IMC, we observed that T3 phage exerted killing activity against planktonic and biofilm-embedded *E. coli* in a titer dependent manner and the highest titer tested (10^7 PFU/ml) was able to kill all planktonic cells, but it was not able to eradicate sessile bacteria after 24 h incubation (Tkhilaishvili et al., 2018a). A similar trend was observed here. Microcalorimetric analysis in real-time showed that the incubation with 10^7 PFU/ml titer of either Sb or PYO exhibited a killing activity versus sessile cells of *S. aureus* ATCC43300, but it did not result in an eradication of the biofilm within 24 h, as attested even by colony counting and CLSM imaging. When we compared the biofilm untreated control (growth control) to the biofilm samples treated with the highest titers of phages tested for 24 h, we observed that 90% of heat reduction of the curves in calorimetric graphs corresponds to a moderate reduction of *S. aureus* viability ($\approx 2 \log_{10}$ and $\approx 3.5 \log_{10}$ CFU/ml) for Sb and PYO treatments, respectively. This is consistent with the fact that the detection limit of the calorimeter is $\approx 10^5$ CFU/ml (Butini et al., 2019), therefore in order to better quantify the remaining bacterial cells on glass beads the colony counting is needed.

Bacteriophages, as all viruses, infect their host cells to replicate themselves and release the viral progeny, meaning an exponential increase of the number of virus particles over time (Yin and Redovich, 2018). In addition, in our previous work, we showed that phages can also kill persister cells after their resumption to a normal growing phenotype (Tkhilaishvili et al., 2018b). Therefore, we evaluated if a longer exposure could eradicate all sessile bacteria. We observed that the *in vitro* killing of all *S. aureus* biofilm-embedded cells was possible when bacteria were incubated over 5 and 7 days with either PYO (10^6 PFU/ml) or Sb (10^7 PFU/ml), respectively. A prolonged exposure characterized by a rise of phage number during bacterial lysis might facilitate the interaction by phages with bacterial cells, including those localized into the deepest biofilm layers, which determine consequently the death of all the adherent cells.

In a previous work, we also showed that Sb can degrade the exopolysaccharide component of the matrix. Here, the CLSM analysis showed that the treatment of biofilm based on PYO had no effect on the extracellular components of the matrix suggesting a different enzymatic activity between the phage formulation tested. However, the slower lytic activity of phages against biofilms can be apparently more due to the physiological state of sessile community and not to diffusion barriers (Abedon, 2017).

As an established biofilm is difficult to be eradicated, the prevention of the infection is still considered the best strategy to reduce the cases of infections (Chen et al., 2013). Either Sb or PYO showed an *in vitro* ability to prevent *S. aureus* biofilm formation within 24 h at titer (10^4 PFU/ml) lower than that needed for the killing of all planktonic cells of the initial inoculum as shown in **Figure 1**. In such experiment, we observed only a 2 $\log_{10}$ CFU reduction of bacteria treated with 10^4 PFU/ml comparing to the untreated control. This discrepancy might be due to the fact that in the presence of the beads part of the planktonic bacteria attaches on the material, resulting less metabolically active. This effect reduces the number of the replicating free-floating bacteria and be more easily lysate by phages. This effect was observed for both phages formulation tested independently. As reported by *in vitro* observations, the adhesion of *S. aureus* seems to occur within 3 h from bacterial inoculum (Moormeier et al., 2014). CFUs counting after 3 h-incubation of *S. aureus* treated with 10^4 PFU/ml of either Sb or PYO showed that there is no difference in the CFU number of attached cells from treated and untreated samples, suggesting that Sb and PYO did not interfere with bacterial adhesion process. Although we cannot clearly discriminate between biofilm prevention and biofilm disruption, here mature biofilm disruption could be excluded by the fact that the phage titer 10^4 PFU/ml (determining bacteria eradication on the beads) had only a poor effect in disrupting the biofilms in the "antibiofilm activity test" (**Figure 2**).

In vivo experiments, performed in a *G. mellonella* model of *S. aureus* systemic infection, showed that both phage formulations at higher titer tested improved the survival of larvae comparing to the untreated sample and analogously to the effect exhibited by vancomycin used as antibiotic control. Previous *in vivo* studies of the therapeutic potential of phages against different bacterial pathogens have used in a *G. mellonella* infection model (Desbois and Coote, 2011; Johnston et al., 2016; Silva et al., 2017). However, to the best of our knowledge, this is the first study to test phages for their ability to rescue MRSA-infected *G. mellonella* larvae from death. In our study, the phage was applied at 1 h before or after MRSA infection in order to determine if these phage formulations had a prophylactic and/or treatment effect. Results suggest that the time of administration of phage plays an important role in therapy of MRSA infection in larvae. When phages were applied 1 h before infection, the survival rates were higher than in the groups administered phage after 1 h infection. In addition, our study showed that the effectiveness of phage therapy increased with usage of phage cocktail compared to single phage and a single phage dose was enough to reduce the mortality of larvae infected with MRSA *in vivo*. The low MOI (0.1 and 0.01) used in this study allowed for determination of the efficacy of the phages while avoiding the phenomenon of "lysis from without." Interestingly, a MOI 0.1 determined the survival of more larvae comparing to the larval survival with a MOI 0.01, suggesting that the ratio between phages and bacteria is relevant for the success of the therapy. However, the relevance of the *G. mellonella* model to predict the phage efficacy with higher MOIs and different timings of phage administration remains to be determined.

CONCLUSION

In this study, our research strongly suggests that phage PYO bacteriophage and Sb phages are promising for preventing device colonization and killing biofilm bacteria attached on a surface. Novel strategies for addressing coating and release of phages from material should be further investigated. In addition, both phage formulations increased the survival of *G. mellonella* larvae preventing or treating MRSA infection compared to untreated control. Future work should be considered using the phages against clinical strains and efficiency of treatment for systemic infections must be evaluated in more complex animal models.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

TT, MD, and ATr conceived and designed the experiments. TT and MD performed the experiments, analyzed the data, and drafted the manuscript, with the contribution of ATr, LW, and ATa.

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Using Bacteriophages as a Trojan Horse to the Killing of Dual-Species Biofilm Formed by *Pseudomonas aeruginosa* and Methicillin Resistant *Staphylococcus aureus*

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Pseudomonas aeruginosa and *Staphylococcus aureus* are pathogens able to colonize surfaces and form together a mixed biofilm. Dual-species biofilms are significantly more resistant to antimicrobials than a monomicrobial community, leading to treatment failure. Due to their rapid bactericidal activity, the self-amplification ability and the biofilm degrading properties, bacteriophages represent a promising therapeutic option in fighting biofilm-related infections. In this study, we investigated the effect of either the simultaneous or staggered application of commercially available phages and ciprofloxacin versus *S. aureus/P. aeruginosa* dual-species biofilms *in vitro*. Biofilms were grown on porous glass beads and analyzed over time. Different techniques such as microcalorimetry, sonication and scanning electron microscopy were combined for the evaluation of anti-biofilm activities. Both bacterial species were susceptible to ciprofloxacin and to phages in their planktonic form of growth. Ciprofloxacin tested alone against biofilms required high concentration ranging from 256 to >512 mg/L to show an inhibitory effect, whereas phages alone showed good and moderate activity against MRSA biofilms and dual-species biofilms, respectively, but low activity against *P. aeruginosa* biofilms. The combination of ciprofloxacin with phages showed a remarkable improvement in the anti-biofilm activity of both antimicrobials with complete eradication of dual-species biofilms after staggered exposure to Pyophage or

Pyophage + Staphylococcal phage for 12 h followed by 1 mg/L of ciprofloxacin, a dose achievable by intravenous or oral antibiotic administration. Our study provides also valuable data regarding not only dosage but also an optimal time of antimicrobial exposure, which is crucial in the implementation of combined therapies.

INTRODUCTION

Although many common infectious diseases can be initiated by a single pathogen or virulence factor, others can be attributed to a polymicrobial origin (Peters et al., 2012). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are commonly found in mixed biofilm infections including chronically infected wounds, indwelling medical devices, cystic fibrosis lung infection or diabetic foot ulcers among others (Tande and Patel, 2014; Chew et al., 2018). Usually, polymicrobial biofilm infections result in worse clinical outcomes than the single infections caused by either species (Serra et al., 2015; Limoli et al., 2016). Treatment is often complicated due to the synergies of polymicrobial biofilms on limiting the effectiveness of antibiotics (Wolcott et al., 2013). Radlinski found that the interaction of *S. aureus* with *P. aeruginosa* within a biofilm can alter *S. aureus'* susceptibility to different antibiotics (Radlinski et al., 2017), whereas other authors also suggested a phenotypic change of *S. aureus* to a small colony variant (SCV) in the presence of *P. aeruginosa* (Chew et al., 2018), increasing its tolerance toward antibiotics (Garcia et al., 2013).

The lack of effective therapies against polymicrobial biofilm infections is a pressing need for the development of new antimicrobial strategies. Bacteriophages (phages) have regained interest as promising therapeutic option in fighting biofilm-related infections due to their rapid bactericidal activity, the self-amplification ability and potential biofilm degradative properties (Harper et al., 2014). However, there are only limited studies investigating the activity of phages against polymicrobial biofilms (Sillankorva et al., 2010; Kay et al., 2011; Chhibber et al., 2015; Oliveira et al., 2018; Melo et al., 2019) and just recently Akturk et al. (2019) evaluated the simultaneous and staggered administration of a *P. aeruginosa*-targeting monophage and conventional antibiotics on *S. aureus/P. aeruginosa* dual- species biofilms.

Pyophage (PYO) and Staphylococcal bacteriophage (Sb-1) are two commercially available phage preparations manufactured by Eliava Biopreparations, a company associated with the G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia. Sb-1 is a *Staphylococcus*-targeting phage preparation containing the well characterized and fully sequenced Sb1 phage (Kvachadze et al., 2011), whereas PYO is composed by a cocktail of phages targeting *S. aureus, Streptococcus spp., Escherichia coli, P. aeruginosa*, and *Proteus species*. An advantage of using phages cocktails lies in a more broad antibacterial spectrum of activity while minimizing the emergency of bacterial resistance (Chan et al., 2013). Furthermore, phages

may encode extracellular polysaccharides (EPS) depolymerases to facilitate their penetration within biofilms (Fernandes and Sao-Jose, 2018). Indeed, in a previous study, we observed the ability of Sb-1 to degrade the extracellular polysaccharide component of *S. aureus* biofilm, which could have improved synergism with antibiotics (Tkhilaishvili et al., 2018b). Thus, in the present study, we investigated the effectiveness of both phage preparations to enhance antibiotic activity in eradicating *S. aureus/P. aeruginosa* dual-species biofilm. We hypothesize that while PYO can target both bacterial species, the addition of Sb-1 targeting not only *S. aureus* but also the biofilm matrix, could help in completely eradicating the dual-species biofilm when combined with an antibiotic. Mono- and dual-species biofilms of *S. aureus* and *P. aeruginosa* were reproducibly grown in porous glass beads and exposed to phages, ciprofloxacin and their simultaneous or staggered combinations. Furthermore, the morphological changes of biofilms induced by each treatment condition were analyzed with scanning electron microscopy (SEM).

MATERIALS AND METHODS

Bacterial Strains and Bacteriophages

Methicillin-resistant *S. aureus* (MRSA) ATCC 43300 and *P. aeruginosa* ATCC 27853 strains were used in this study. Bacteria were stored on a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, ON, Canada) at 80 °C.

Phages Sb-1 and PYO were provided as 10 mL liquid ampoules by the Eliava Institute for Bacteriophages, Microbiology and Virology (Tbilisi, Georgia) and maintained at 4°C. The phage titer was determined by titration and expressed as PFU/mL. A fixed titer of PYO corresponding to 10^5 PFU/mL for MRSA and 10^4 PFU/mL for *P. aeruginosa* and of Sb-1 corresponding to 10^6 PFU/mL for MRSA were used for all tests.

Biofilm Formation Assay

In this study, we applied an optimized *in vitro* assay for biofilm formation using porous sintered glass beads (diameter, 4 mm; pore size, 60 µm; porosity, 0.2 m^2/g; ROBUVR, Hattert, Germany) following the assay described by Zimmerli et al. (1994) with some modifications.

Considering the findings from previous studies predominantly showing an out-competition of *S. aureus* growth by *P. aeruginosa* growth (Filkins et al., 2015; Woods et al., 2019) bacterial inoculums in our study were prepared at a ratio of 1 *P. aeruginosa* to 1000 MRSA bacterial cells.

In order to allow mono- and dual-species biofilm formation on the glass beads, a bacterial suspension of MRSA corresponding to 5×10^6 CFU/mL and *P. aeruginosa* corresponding to 5×10^3 CFU/mL were incubated – alone or combined – in Luria-Bertani broth (LB, Sigma-Aldrich, Steinheim, Germany) in the presence of porous glass beads at $37°C$ under static conditions. After 3, 6, 12, or 24 h of incubation, beads were washed three times in sterile 0.9% saline to remove non-adherent bacteria suspended in the incubation medium. The number of MRSA and *P. aeruginosa* bacteria adhering on the glass beads was determined by sonication and colony counting (see section "Sonication of Biofilms Formed on Porous Glass Beads and Plating for Colony Counting") in Mannitol salt agar (VWR Chemicals, Leuven, Belgium) and Cetrimide selective agar media (Sigma-Aldrich, Steinheim, Germany) respectively. The dual-species biofilm formed in the beads was also visualized by SEM (see section "SEM of Biofilms on Porous Glass Beads").

Twenty-four hours old dual-species biofilms with a 1:1 ratio of MRSA and *P. aeruginosa* bacterial cells on the beads were used for anti-biofilm activity tests.

Sonication of Biofilms Formed on Porous Glass Beads and Plating for Colony Counting

The presence of attached cells to the glass beads was evaluated by CFUs counting of sonicated beads as previously described (Gonzalez Moreno et al., 2019). After biofilm formation, glass beads were washed three times with 0.9% saline and introduced in Eppendorf tubes containing 1 mL of sodium-phosphate buffer solution (PBS). Samples were vortexed for 30 s and then subjected to sonication in an ultrasound bath at 40 kHz and 0.2 W/cm^2 (BactoSonic, BANDELIN electronic GmbH & Co., KG, Berlin, Germany) for 1 minute, followed by additional 30 s vortexing. 10-fold serial dilutions of the sonication fluid were plated onto the appropriate media and colonies were counted after 18–24 h incubation at $37°C$ and expressed as CFUs/mL.

SEM of Biofilms on Porous Glass Beads

For SEM imaging, biofilm was formed on porous glass beads as described above. Afterward, all beads were washed in ddH$_2$O (dipping) to remove unbound bacteria and chemically fixed. Subsequently, the samples were dehydrated in ethanol percent series and then dried at the critical point. Samples were mounted on aluminum stubs, coated with 20 nm layer of gold-palladium, and then observed in the microscope (DSM 982 GEMINI, Zeiss Oberkochen).

Antimicrobial Assay by Microcalorimetry and Sonication/Colony Counting

An isothermal microcalorimeter (TAM III; TA Instruments, New Castle, DE, United States) equipped with 48 channels was used to determine the antimicrobial activity of either antibiotic and/or phages against planktonic, mono- and dual- species biofilms as previously reported (Butini et al., 2018; Tkhilaishvili et al., 2018a,b). Briefly, MRSA or *P. aeruginosa* planktonic cells (10^5 CFU/mL) were exposed to two-fold serial

dilutions of ciprofloxacin or to each phage preparation in LB, and heat production was measured for 24 h. The minimum heat inhibitory concentration (MHIC) was defined as the lowest concentration of antimicrobial able to suppress the metabolic heat production of planktonic bacteria.

Mono- and dual-species biofilms formed on porous glass beads as previously described were rinsed $(3 \times)$ with 0.9% saline and exposed to fresh LB containing ciprofloxacin or phages. After 24 h of incubation at $37°C$, beads were rinsed $(3 \times)$ with 0.9% saline and inserted in microcalorimetry ampoules containing 3 mL of fresh LB and introduced into the calorimeter. The viability of bacteria on the glass beads after the antibiotic treatment was detected by measuring their heat production at $37°C$ for 48 h. For samples where not heat production was detected, the complete biofilm eradication was determined by CFU counting of the sonicated beads after the microcalorimetric assay. The minimum biofilm bactericidal concentration (MBBC) was defined as the lowest concentration of antibiotic that strongly reduced the viability of biofilm cells and led to the absence of heat flow production from treated beads when incubated during 48 h in fresh medium. The minimum biofilm eradicating concentration (MBEC) was defined as the lowest concentration of antibiotic required to kill all sessile cells resulting in the appearance of no colony after plating sonication fluid (detection limit: 20 CFU/mL) (Gonzalez Moreno et al., 2019; Wang et al., 2019). All experiments were performed in triplicate and repeated three times.

To evaluate the antimicrobial effect of antibiotic/phage combinations, two different approaches were carried out: (i) simultaneous exposure of biofilms to PYO or PYO Sb-1 and sub-inhibitory concentrations of ciprofloxacin for 24 h; (ii) staggered exposure of biofilms to PYO or PYO Sb-1 phages for 3, 6, 12, or 24 h followed by a 24 h-exposure to sub- inhibitory concentrations of ciprofloxacin. The viability of bacteria on the glass beads after the antibiotic/phage treatment was determined by both, calorimetry and sonication/colony- counting as previously mentioned.

For each tested condition throughout all experiments, grown biofilms were rinsed with 0.9% saline prior exposure to fresh LB containing the respective antimicrobials.

Microcalorimetry data was evaluated using the manufacturer's software (TAM Assistant; TA Instruments, New Castle, DE, United States) and figures were plotted using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, United States).

RESULTS

Formation of Mono- and Dual-Species Biofilm

MRSA and *P. aeruginosa* were used to grow mono- and dual-species biofilms. The evaluation of the bacteria adhered to the beads over time showed a considerably higher concentration of MRSA cells at 3 and 6 h of incubation in dual-species biofilms compared to *P. aeruginosa* cells (**Figure 1A,B**), whereas at 12 h of incubation the concentration of *P. aeruginosa* increased substantially (**Figure 1C**) and at 24 h of incubation
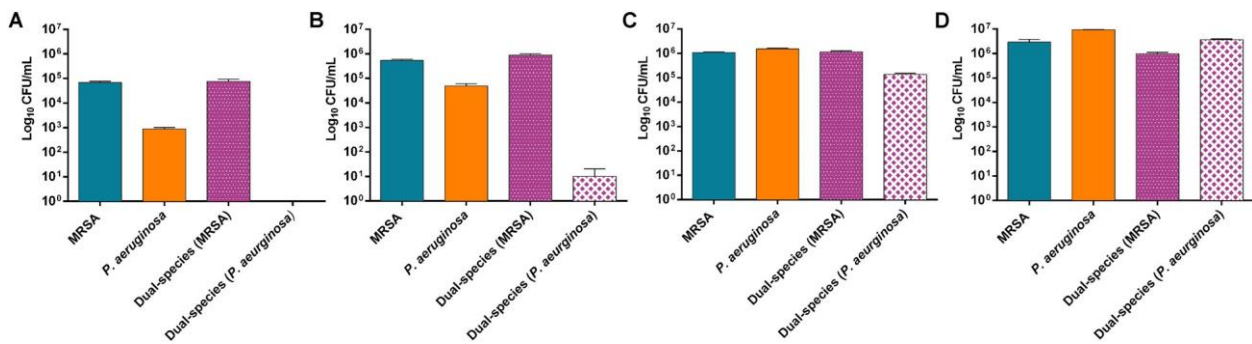
FIGURE 1 | Distribution pattern of bacterial populations over the time. Number of viable cells (in log10 CFU/mL) of *P. aeruginosa* and MRSA on mono- and dual-species biofilms formed after 3 h (A), 6 h (B), 12 h (C) and 24 h (D). Data are reported as CFU/mL mean ± standard deviation of at least three independent experiments. Figure 2 | Dual-species biofilm formed by MRSA (ATCC 43300) and *P. aeruginosa* (ATCC 27853) on porous glass beads after 24 h of incubation. Image (B) is a close-up from (A). Numbers 1 and 2 indicate a MRSA bacterium and a *P. aeruginosa* bacterium, respectively, whereas three and four point out a water channel and the extracellular polymeric matrix of the biofilm correspondingly.

the concentration of *P. aeruginosa* showed values comparable to those from MRSA (**Figure 1D**).

Results showed approximately a 1:1 ratio of MRSA (9.7 10^5 CFU/mL) and *P. aeruginosa* (3.7 10^6 CFU/mL) bacterial cells on the beads of dual-species biofilms after 24 h of incubation. MRSA and *P. aeruginosa* mono-species biofilms presented bacterial concentrations comparable to those observed on the dual-species biofilm after 24 h of incubation.

The SEM analysis of 24 h-old dual-species biofilms showed the capability of MRSA and *P. aeruginosa* to adhere and form an even mixed biofilm on the porous glass beads (**Figure 2**).

Antimicrobial Activity of Ciprofloxacin or Phages Against Planktonic, Mono- and Dual-Species Biofilms

The antimicrobial susceptibility of planktonic cells (**Figure 3**) or mono- and dual-species biofilms (**Figure 4**) to ciprofloxacin or to phages was determined by isothermal microcalorimetry.

The calorimetry analysis for planktonic bacteria showed that MRSA and *P. aeruginosa* were susceptible to ciprofloxacin with MHIC values of 0.5 and 0.25 mg/L, respectively. The exposure of MRSA to PYO revealed a high reduction of heat production compared to the growth control, a complete inhibition of the heat production could be observed in the case of *P. aeruginosa*. The combination of PYO+Sb-1 showed complete growth inhibition against both bacterial species.

Mono-species biofilms from both strains were susceptible to considerable high concentrations of ciprofloxacin (512 mg/L for MRSA and 256 mg/L for *P. aeruginosa*), whereas the antibiotic was not able to completely inhibit the heat flow production of the dual-species biofilm when tested up to 512 mg/L.

The exposure of MRSA biofilm to PYO revealed a drastic reduction of the heat production compared to the growth control, and with the addition of Sb-1, a complete inhibition of the biofilm could be achieved. On the contrary, neither PYO nor

PYO+Sb1 showed a noteworthy anti-biofilm activity against *P. aeruginosa* biofilm, whereas on dual-species biofilm, a delay on the heat production could be observed on treated samples with PYO, indicating a moderate anti-biofilm activity, which was seen improved by the addition of Sb1 but with no complete inhibition of the biofilm.

Biofilm-Eradicating Activity of Phage Preparations

In order to evaluate the biofilm-eradicating activity of the two phage preparations, mono- and dual-species biofilms were exposed to PYO or to PYO Sb-1 for 24 h and then, viable bacteria attached to the beads were detected by colony counting after bead sonication and plating of the sonication fluids.

A higher reduction of MRSA viable bacteria after exposure to PYO could be observed (**Figure 5A**) compared to *P. aeruginosa* biofilm, where no considerable bacterial reduction was determined (**Figure 5B**). Moreover, a complete eradication of MRSA biofilm was observed after exposure to PYO+Sb-1, although this phage combination did not improve the killing of *P. aeruginosa* biofilm compared to PYO alone.

Regarding the phage activity against dual-species biofilm (**Figure 5C**), a reduction of more than 2 log10 of MRSA and around 1 log10 of *P. aeruginosa* cells was observed after exposure to PYO in comparison to the growth control. The combination of PYO Sb-1 showed a complete eradication of MRSA cells on the dual-species biofilm, whereas no substantial reduction of *P. aeruginosa* cells was observed.

Anti-biofilm Activity of Ciprofloxacin in Combination With Phages Against Dual-Species Biofilm

Simultaneous exposure of sub-inhibitory concentrations of ciprofloxacin combined with PYO or PYO Sb-1 revealed a remarkable delay and reduction of heat flow production compared to the heat flow produced by the growth control (**Figure 6**).
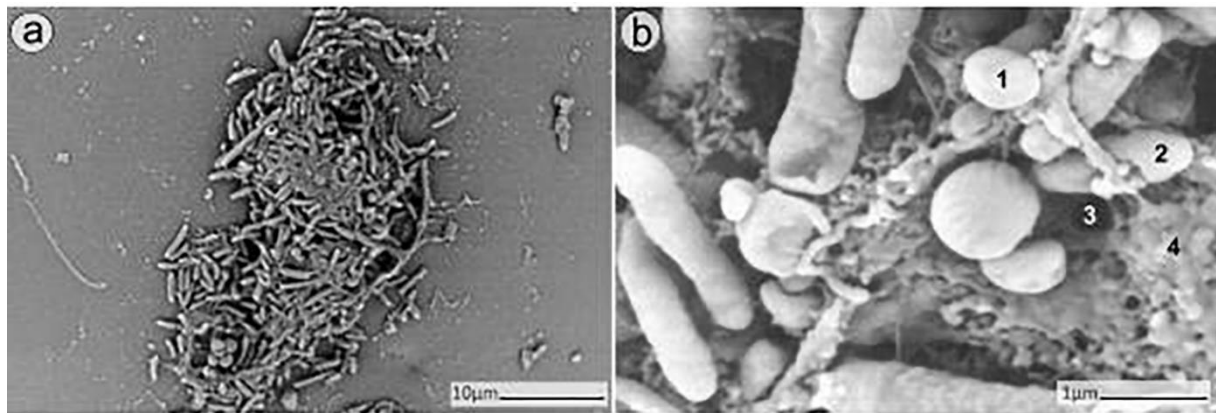
FIGURE 2 | SEM analysis of dual-species biofilm formed by MRSA (ATCC 43300) and *P. aeruginosa* (ATCC 27853) on porous glass beads after 24h of incubation. Image (b) is a close-up from (a). Numbers 1 and 2 indicate a MRSA bacterium and a *P. aeruginosa* bacterium respectively, whereas 3 and 4 point out a water channel and the extracellular polymeric matrix of the biofilm correspondingly.
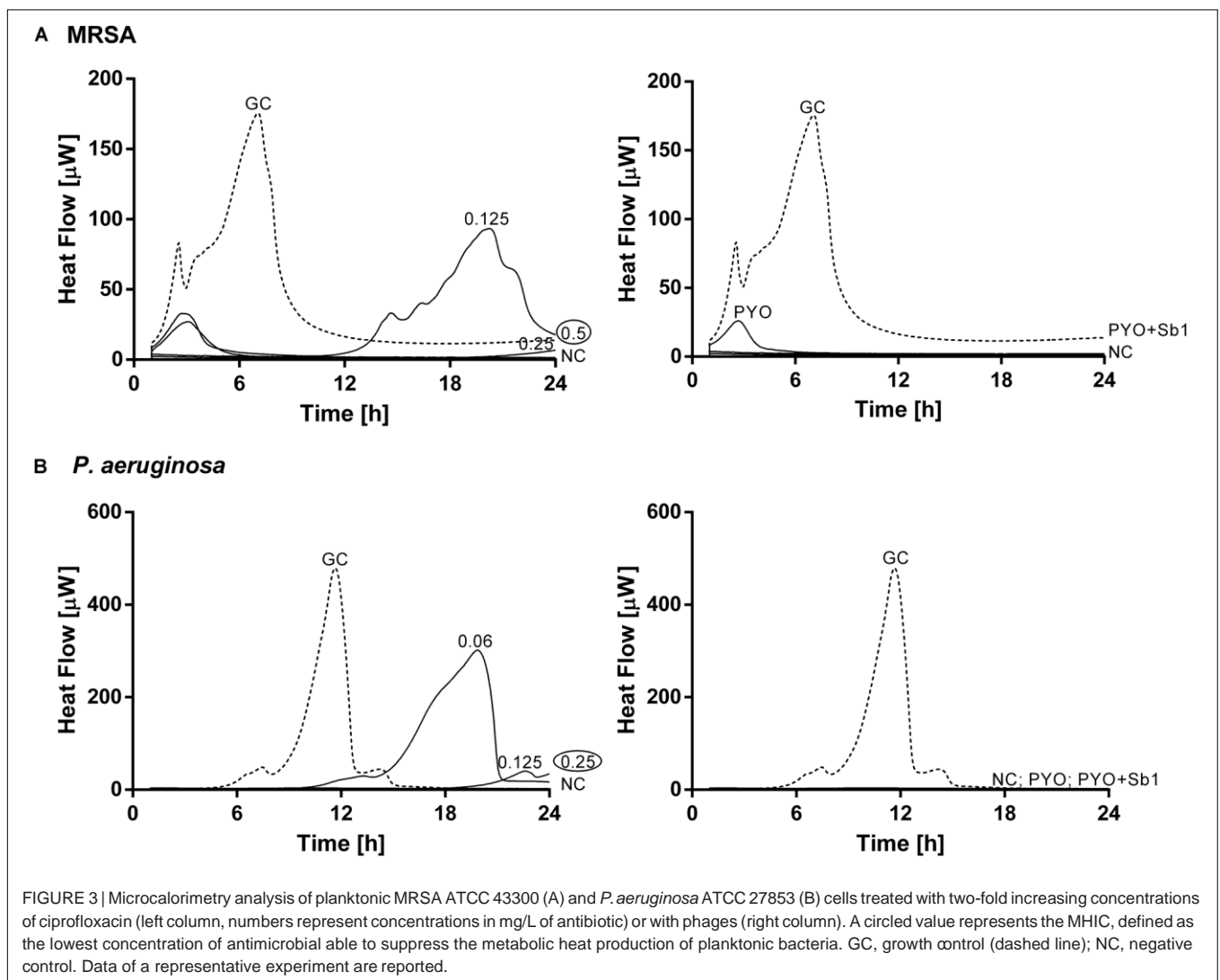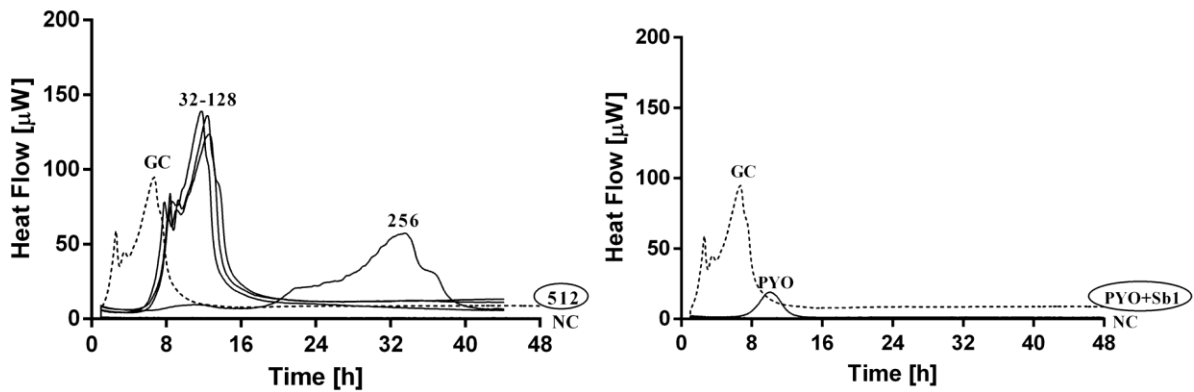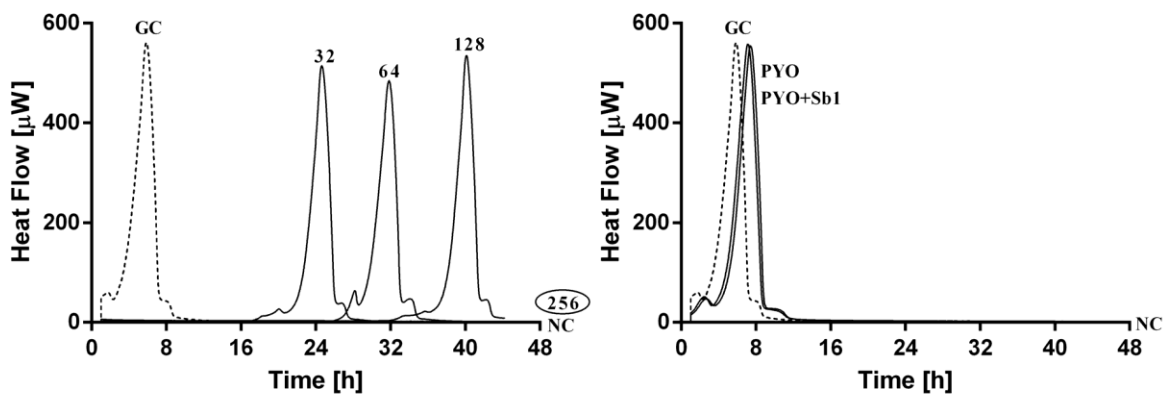

FIGURE 3 | Microcalorimetry analysis of planktonic MRSA ATCC 43300 (A) and *P. aeruginosa* ATCC 27853 (B) cells treated with two-fold increasing concentrations of ciprofloxacin (left column, numbers represent concentrations in mg/L of antibiotic) or with phages (right column). A circled value represents the MHIC, defined as the lowest concentration of antimicrobial able to suppress the metabolic heat production of planktonic bacteria. GC, growth control (dashed line); NC, negative control. Data of a representative experiment are reported.
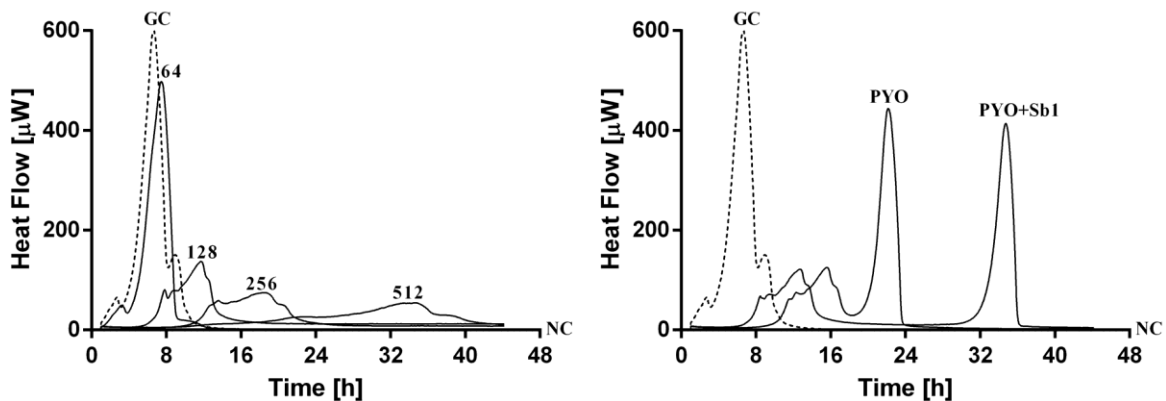
FIGURE 4 | Microcalorimetry analysis of (A,B) mono- and (C) dual-species MRSA (ATCC 43300) and *P. aeruginosa* (ATCC 27853) biofilms treated with two-fold increasing concentrations of ciprofloxacin (left column, numbers represent concentrations in mg/L of antibiotic) or with phages (right column). Each curve shows the heat produced by viable bacteria present in the biofilm after 24 h of antibiotic or phage treatment. A circled value represents the MBEC, defined as the lowest concentration of antibiotic that strongly reduced the viability of biofilm cells leading to the absence of heat flow production from treated beads when incubated during 48 h in fresh medium and no colonies after sonication and plating. GC, growth control (dashed line); NC, negative control. Data of a representative experiment are reported.

The combination of PYO and ciprofloxacin revealed a decrease of over 90% in heat flow production of samples treated with 16–64 mg/L of antibiotic compared to the growth control, whereas in the case of combining PYO Sb-1 and ciprofloxacin, a concentration of antibiotic as low as 4 mg/L was already able to reduce over a 90% of the heat flow production if compared
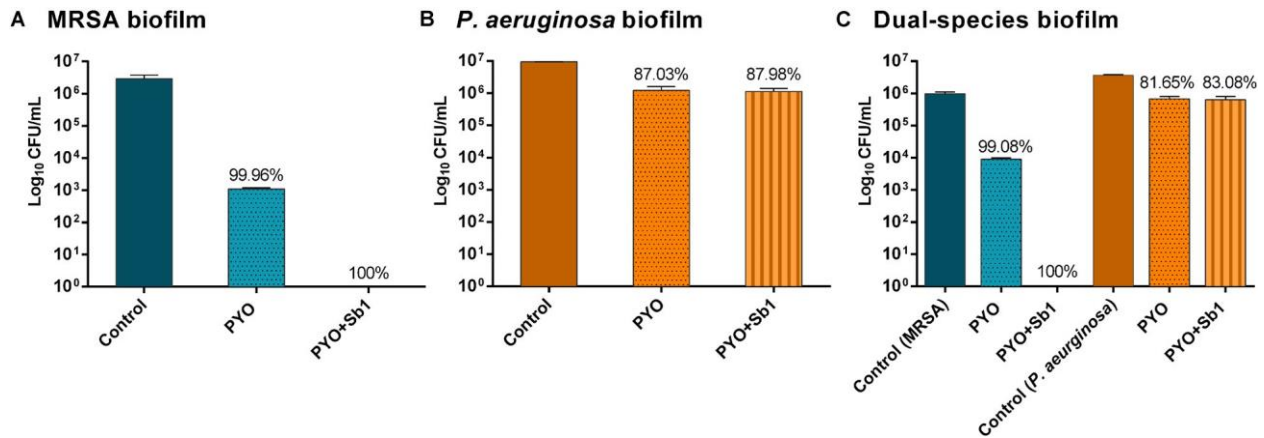
FIGURE 5 | Effect of PYO and Sb-1 phage preparations on viability of biofilm-embedded mono and mix bacteria populations. *S. aureus/P. aeruginosa* mono- (A,B) and dual-species (C) biofilms formed on porous glass beads were exposed to phages. Data are reported as log10 CFUs/mL mean ± standard deviation of at least three independent experiments. Percent of cell reduction of treated samples compared to untreated samples was calculated as: percent reduction = $[(A-B)/A] \times 100$, where A is the mean number of viable bacteria of the growth control and B is the mean number of viable bacteria after exposure to PYO or PYO+Sb-1.
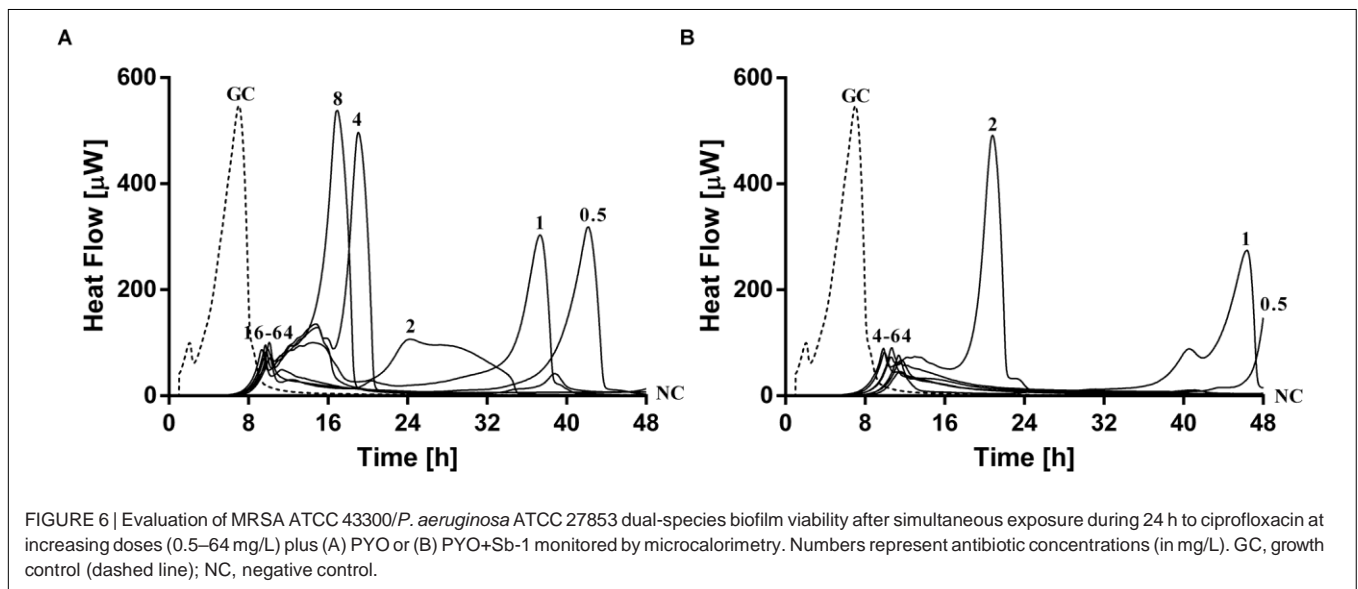
FIGURE 6 | Evaluation of MRSA ATCC 43300/*P. aeruginosa* ATCC 27853 dual-species biofilm viability after simultaneous exposure during 24 h to ciprofloxacin at increasing doses (0.5–64 mg/L) plus (A) PYO or (B) PYO+Sb-1 monitored by microcalorimetry. Numbers represent antibiotic concentrations (in mg/L). GC, growth control (dashed line); NC, negative control.

to that one measured for the growth control. Still, no complete biofilm eradication was observed with any of the both tested treatment combinations.

Paradoxically, low concentrations of ciprofloxacin (0.5–1 mg/L) in combination with phages showed a higher delay/decrease in heat flow production, correlating with a lower loading of bacterial cells in the beads, if compared to the heat flow curves observed after exposure to concentrations of ciprofloxacin ranging from 2 to 8 mg/L, in combination with phages.

On the other hand, a staggered exposure of phage and antibiotic against dual-species biofilms was evaluated by microcalorimetry. The obtained results are depicted in **Figure 7**. Dual-species biofilms were first exposed to phages at different incubation times (3, 6, 12, and 24 h) and then to sub-inhibitory concentrations of ciprofloxacin for 24 h. After calorimetry, the

complete eradication of the biofilm was further investigated by sonication and colony counting of those samples showing no heat flow production after 48 h of incubation.

Results showed the highest anti-biofilm activity when the antibiotic was added after 12 h of pre-exposure to either PYO or PYO+Sb-1, where a complete eradication of the biofilm could be achieved at MBEC of ciprofloxacin of 2 mg/L (**Figure 7E**) and 1 mg/L (**Figure 7F**) respectively. Similarly, relatively low MBEC values were also obtained when ciprofloxacin was added after 6 h of biofilm pre-treatment with PYO (MBEC = 4 mg/L) (**Figure 7C**) or PYO Sb-1 (MBEC = 1 mg/L) (**Figure 7D**). On the contrary, when biofilms were incubated for 3 or 24 h with phages prior addition of ciprofloxacin, higher MBEC values ranging from 16 to 64 mg/L were observed. Generally, the PYO+Sb-1/ciprofloxacin combination exhibited MBEC values
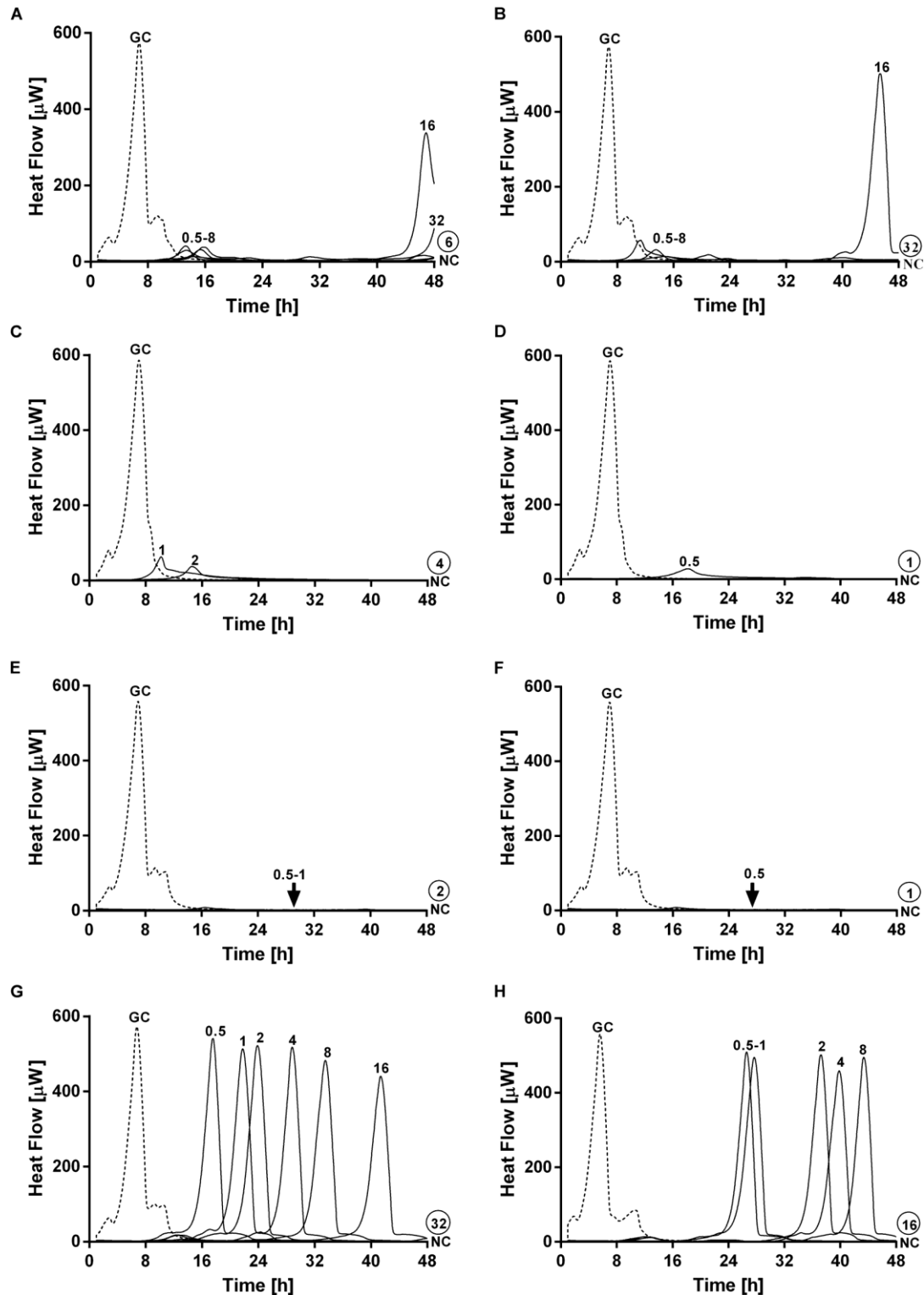
FIGURE 7 | Evaluation of MRSA ATCC 43300/*P. aeruginosa* ATCC 27853 dual-species biofilm viability after staggered exposure to phages and ciprofloxacin monitored by microcalorimetry. Each curve shows the heat produced by viable bacteria present in biofilms pretreated for 3 h (A,B), 6 h (C,D), 12 h (E,F) and 24 h (G,H) with PYO (graphs on the left) or PYO+Sb-1 (graphs on the right) followed by exposure to ciprofloxacin at increasing doses (0.5–64 mg/L) for 24 h. Numbers above curves represent antibiotic concentrations (in mg/L). Circled values represents the MBEC, defined as the lowest concentration of antibiotic that strongly reduced the viability of biofilm cells leading to the absence of heat flow production from treated beads when incubated during 48 h in fresh medium and no colonies after sonication and plating. GC, growth control (dashed line); NC, negative control.
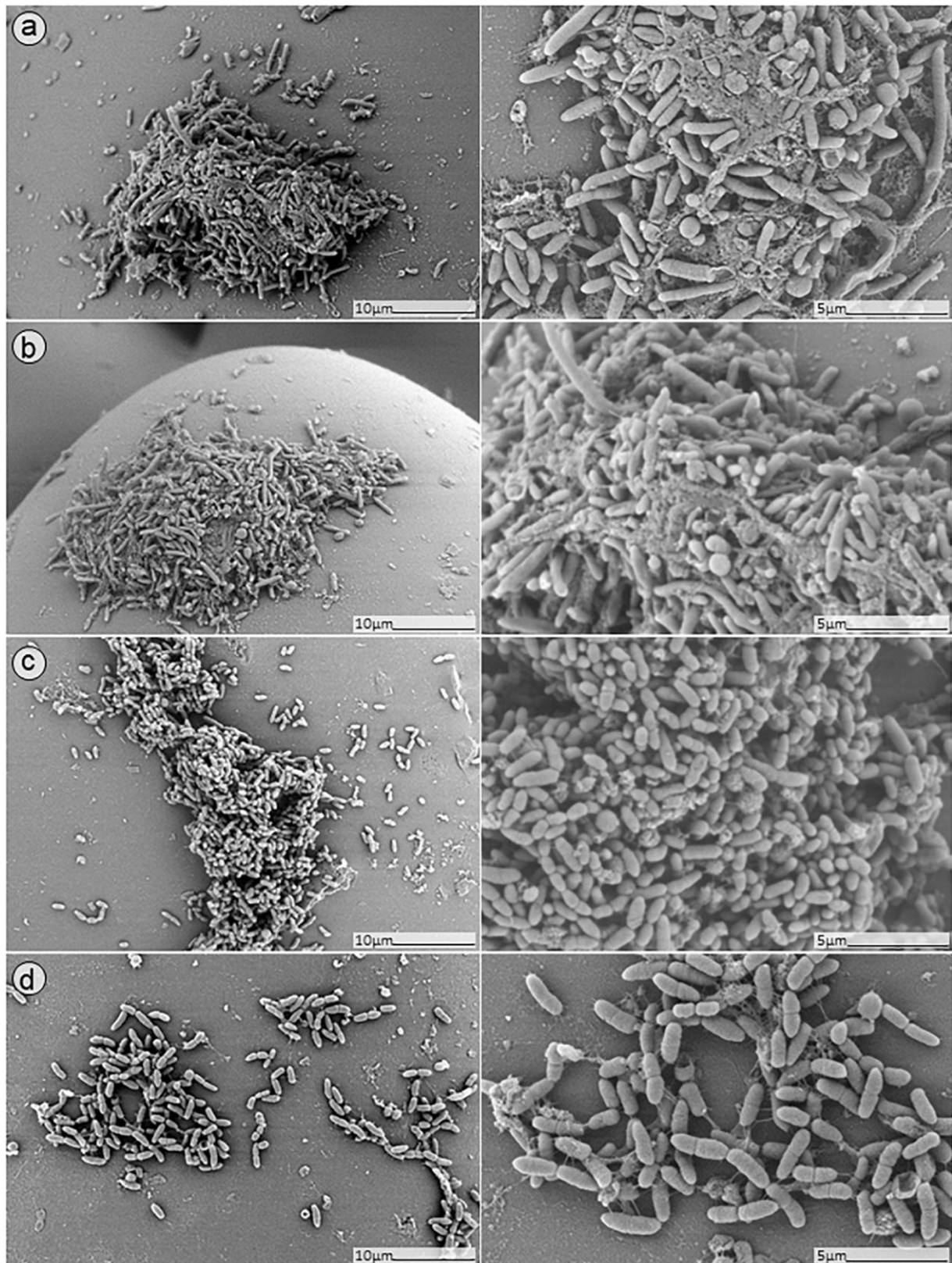
FIGURE 8 | SEM analysis of *S. aureus/P. aeruginosa* dual-species biofilms grown on porous glass beads for 24 h without treatment (a) and after exposure to 24 h monotherapy with (b) ciprofloxacin (1 mg/L); (c) PYO; or (d) pyo+sb-1.
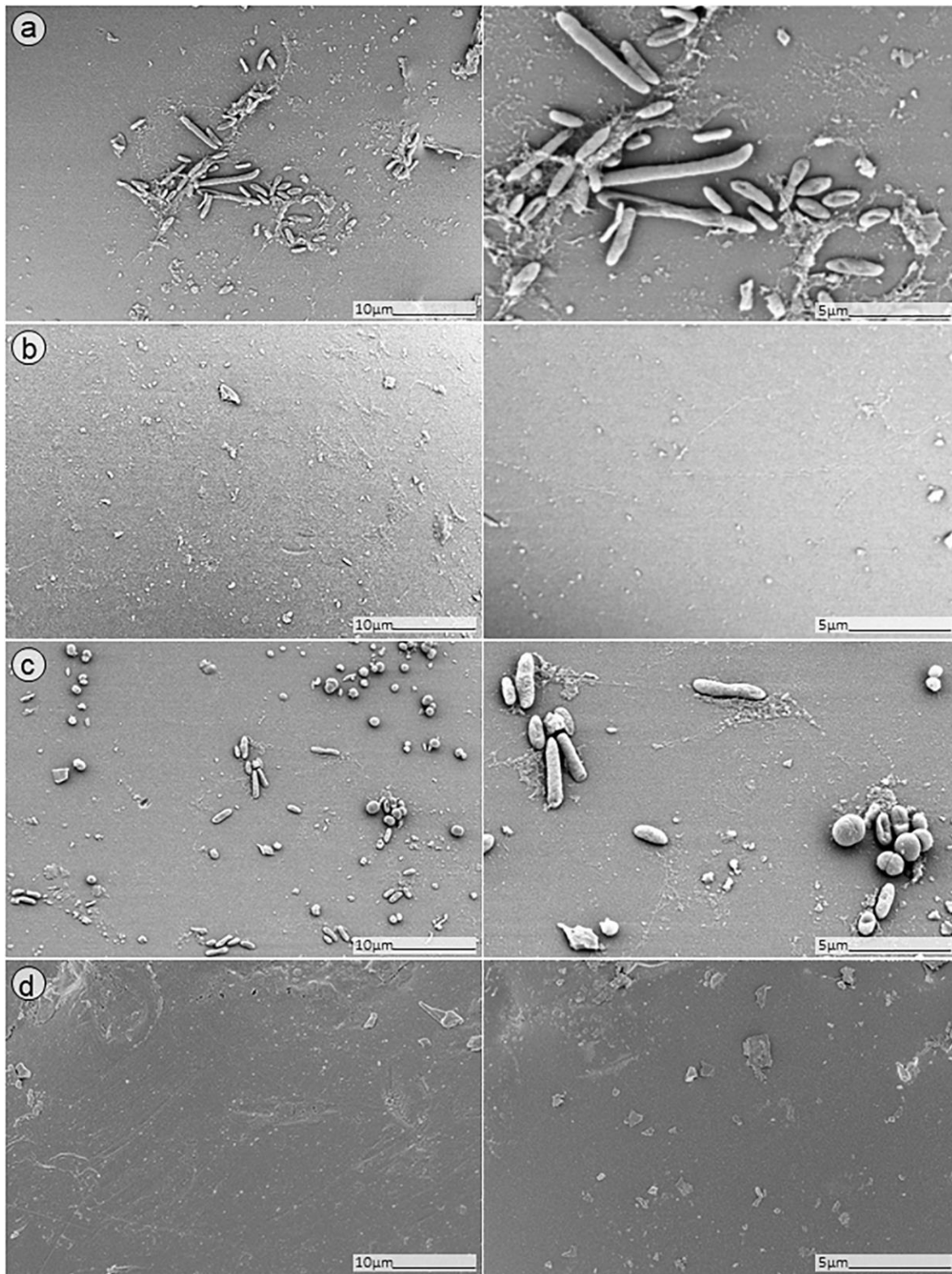
FIGURE 9 | SEM analysis of *S. aureus/P. aeruginosa* dual-species biofilm grown on porous glass beads for 24 h and treated with a combinatorial therapy of (a) simultaneous exposure to PYO and ciprofloxacin (1 mg/L, 24 h); (b) staggered exposure to PYO (12 h) followed by ciprofloxacin (1 mg/L, 24 h); (c) simultaneous exposure to PYO+Sb-1 and ciprofloxacin (1 mg/L, 24 h); or (d) staggered exposure to PYO+Sb-1 (12 h) followed by ciprofloxacin (1 mg/L, 24 h).

2–4 times lower than the PYO/ciprofloxacin combination at all the tested incubation times.

SEM Analysis

In order to further confirm our findings, dual-species biofilm after exposure to either antimicrobials alone or in combinations were visualized by SEM (**Figures 8**, **9**). The microscopy analysis revealed comparable outcomes to those obtained by microcalorimetry and sonication/colony-counting.

No presence of bacteria on the beads was observed after biofilm exposure to phages for 12 h followed by 24 h of exposure to 1 mg/L ciprofloxacin (**Figure 9**, images B,D). Ciprofloxacin alone at that same concentration (1 mg/L) showed abundant biofilm formation on the bead (**Figure 8**, image B), indicating no anti-biofilm activity at that concentration.

Different outcomes were observed when biofilms were exposed to PYO+Sb-1 (**Figure 8D**), were a relatively lower abundance of biofilm and especially of MRSA bacterium cells could be visualized, compared to PYO alone (**Figure 8C**). Finally, the beads exposed to simultaneous incubation with phages and ciprofloxacin (**Figure 9**, images A,C) showed a sharp decrease of biofilm without complete eradication.

DISCUSSION

S. aureus and *P. aeruginosa* are two bacterial pathogens commonly isolated in mixed-species biofilm infections (Hotterbeekx et al., 2017). A vast number of studies suggest that, when both bacterial species interact to form biofilm, *S. aureus* is predominantly outcompeted by *P. aeruginosa* (Machan et al., 1991; Mashburn et al., 2005; Filkins et al., 2015; Woods et al., 2019). However, other studies found that both species may benefit each other during the infection and keep a stable co-existence (Pastar et al., 2013; DeLeon et al., 2014; Woods et al., 2019). In our study, we observed a similar trend, where the growth of *S. aureus* biofilm was outcompeted by *P. aeruginosa* during a period of 24 h *in vitro*, despite the higher initial inoculum size of *S. aureus* in relation to *P. aeruginosa*, and both species could form an even mixed biofilm after 24 h of co-incubation, as shown by colony-counts and SEM.

It has been shown that *S. aureus/P. aeruginosa* coinfections result in enhanced virulence and resistance to antibiotics (DeLeon et al., 2014). Our results also revealed that a higher concentration of ciprofloxacin was necessary to inhibit the growth of dual-species biofilms when compared with mono- species biofilms. The MBEC values obtained in all cases are too high to be reached in the clinical practice (Kontou et al., 2011; Thabit et al., 2019). In this scenario, bacteriophages appear to be an alternative strategy to treat biofilm-forming infections. Over the past few years, numerous studies have been carried out investigating the effectiveness of phages against mono- and dual- species biofilm (Sillankorva et al., 2010; Chhibber et al., 2015; Gutierrez et al., 2015; Gonzalez et al., 2017; Melo et al., 2019). Many of these studies pointed to a notable dependency between the phages and the bacterial species involved on the biofilm for the efficacy of the phage treatment. Indeed, it is generally accepted

that the efficacy of phages against bacteria is influenced by several factors, among others, the host specificity, the treatment method, environmental conditions or accessibility to target bacteria (Ly-Chatain, 2014). The impact of host specificity for the therapeutic use of phages is also under debate (Ross et al., 2016; Hyman, 2019). Thus, in contrast to other studies where they make use of self-isolated phages targeting the bacterial strains under study, for our study we chose to investigate commercially available phage preparations with the potential to a more straightforward implementation in a clinical setting.

Our results showed that, even though planktonic cells from both tested species were susceptible to the PYO phage-cocktail, when tested against biofilms, only MRSA showed a substantial reduction on bacterial viability, especially as mono-species biofilm, whereas a lower efficacy was observed against dual-species biofilms. A possible explanation for this might be a limited phage penetration within the biofilm, what could be improved by the addition of the MRSA targeting and matrix-degrading Sb-1 phage. The combination of PYO Sb-1 showed a major eradication of MRSA cells on the dual-species biofilm, as seen also by SEM, however, no substantial reduction of *P. aeruginosa* cells was observed. A possible additional effect on the reduction of MRSA could be due to the natural competition between the two species as mentioned above.

To enhance the effect of phages, the combined exposure with an antibiotic was assessed. Phage- antibiotic synergy is the result of combining sub-inhibitory concentrations of antibiotics with phages to foster phage productivity and thus phage- mediated bacterial decline (Tagliaferri et al., 2019). Previous studies have shown the benefit of the staggered application when combining antibiotics and phages, while a simultaneous exposure could result in hindering their anti-biofilm efficacy, possibly due to antagonistic modes of action (Chaudhry et al., 2017; Kumaran et al., 2018; Akturk et al., 2019). Indeed, when we analyzed by calorimetry the exposure of dual-species biofilms to ciprofloxacin and PYO or PYO +Sb-1 simultaneously, we observed a paradoxical effect, where lower concentrations of ciprofloxacin in combination with phages showed a higher delay/decrease in heat flow production compared to higher antibiotic concentrations. We assume that the mode of action of ciprofloxacin inhibiting bacterial DNA replication might hamper the phage amplification (replication) (Constantinou et al., 1986). Therefore, lower doses of ciprofloxacin could have a minor interference with phage replication or could not reduce the concentration of bacteria to levels below which phages can replicate, if compared to higher antibiotic doses (Levin et al., 1977). This counterproductive effect could be perhaps prevented by the use of antibiotics with modes of action that do not compete with the viral amplification, or also, by exposing bacteria in a staggered rather than a simultaneous manner to phages and antibiotics. As seen in our study, a complete eradication of dual-species biofilm could be only achieved by staggered administration of phages followed by a sub-inhibitory concentration of ciprofloxacin.

As recently stated by Tagliaferri et al. (2019), synergistic interactions between antimicrobial agents may be strongly dependent on the treatment conditions such as dosage,

frequency, time points and order of administration. Hence, we were interested on determining the optimal time point for the staggered administration of phages and the antibiotic. Our results showed that the highest anti-biofilm activity could be reached when ciprofloxacin was added after 6 or 12 h of pre-exposure to PYO-Sb-1. SEM analysis also revealed the absence of adherent bacterial cells on the glass beads.

Differently, pre-incubation of dual-species biofilms with phages for 3 or 24 h prior addition of ciprofloxacin exhibited higher MBEC values, confirming that, not only dosage but also an optimal time of antimicrobial exposure is crucial in the implementation of the combined therapies.

In conclusion, this work provides valuable original data on the combinatorial use of phage and antibiotic against *S. aureus/P. aeruginosa* dual-species biofilm that might bring new insights into the potential application of such a treatment to combat polymicrobial infections. Monotherapy with ciprofloxacin revealed drug concentrations to eradicate biofilm (MBEC >512 mg/L) much superior to the ones reachable in clinical practice, whereas a combinatorial treatment by staggered administration of phages and ciprofloxacin strongly reduced the MBEC of ciprofloxacin to a dose (MBEC = 1 mg/L) achievable by intravenous or oral antibiotic administration (Kontou et al., 2011; Thabit et al., 2019). Moreover, by the use of commercially available phage preparation in this study, we were able to show the effectiveness of these preparations against bacterial strains that have not been used specifically for their isolation.

Over the last years, bacteriophages have been extensively studied as therapeutic agents alone or in conjunction with other therapeutics. *In vivo* models (Tagliaferri et al., 2019) and a few clinical trials (Merabishvili et al., 2017; Furfaro et al., 2018; Jault et al., 2019) have demonstrated effectiveness of phage treatment against *P. aeruginosa* and *S. aureus* infections, without any reported adverse effects. However, little has been published about polymicrobial biofilm infections. Although these infections are less common, their treatment presents a major challenge. Hence, further preclinical and clinical studies are essential to support the development of phage/antibiotic combination therapy for polymicrobial infections.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

TT and AT conceived and designed the experiments. TT performed the experiments. TT and MG analyzed the data and drafted the manuscript, with the contribution of LW, CP, and AT. All authors reviewed and revised the final drafts of this manuscript.

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CURRICULUM VITAE

My curriculum vitae does not appear in the electronic version of my thesis for reasons of data protection.

Complete list of publications

Published peer-reviewed articles

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