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DISSERTATION

Antibiofilm strategies to combat prosthetic joint infections caused by Gram-negative bacteria

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List of abbreviations

Prosthetic joint infections	PJIs
Gram-negative	GN
Gram-positive	GP
Extracellular polymeric substances	EPS
Multidrug-resistant	MDR
Isothermal microcalorimetry	IMC
Methicillin-resistant *Staphylococcus aureus*	MRSA
Cation-adjusted Mueller-Hinton	CAMH
Luria-Bertani	LB
Brain Heart Infusion	BHI
Minimum biofilm bactericidal concentration	MBBC
Minimum biofilm eradicating concentration	MBEC
Fractional biofilm eradication concentration index	FBECI
Fosfomycin	FOS
Gentamicin	GEN
Ciprofloxacin	CIP
Meropenem	MER
Ceftriaxone	CEF
Growth control	GC
Negative control	NC
Synergism	S
No synergism	NS
No effect	NE
Antagonism	A
Single antibiotics	SIN
Simultaneous exposure	SIM
Staggered exposure	STA

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Zusammenfassung

Abstrakt

Biofilme die auf der Oberfläche von Gelenkprothesen wachsen, sind hochgradig refraktär gegenüber der alleinigen konventionellen antimikrobiellen Therapie. *Escherichia coli* und *Pseudomonas aeruginosa* sind hauptverantwortlich für gramnegative Prothesengelenksinfektionen (prosthetic joint infections: PJI). Die erfolgreiche Eradikation der Biofilmzellen allein mit konventionellen antimikrobiellen Therapien stellt nach wie vor eine Herausforderung dar. Daher besteht dringender Bedarf hinsichtlich der Entwicklung alternativer therapeutischer Strategien, um gramnegative PJI zu eliminieren. So haben wir zwei vielversprechende Ansätze – die, konventionelle Antibiotika-Kombinationen und Phagen-Antibiotika-Kombinationen – gegen die gramnegativen bakteriellen Biofilme entwickelt.

Ziel dieser Arbeit war es, die synergistische Antibiotika-Aktivität konventioneller Antibiotika-Kombinationen oder Phagen-Antibiotika-Kombinationen zur Bekämpfung gramnegativer bakterieller Biofilme *in vitro* zu untersuchen, um das Ergebnis der Behandlung gramnegativer PJIs zu verbessern.

Basierend auf unseren Ergebnissen zeigten unter den getesteten konventionellen Antibiotika-Kombinationen 75 % der Fosfomycin/Gentamicin-Kombinationen synergistische Effekte und stellten einen vielversprechenden therapeutischen Ansatz für Antibiotika-Kombinationen gegen *E. coli*-Biofilme dar, während 71,4 % einen Gentamicin/Ciprofloxacin-Kombinationen Synergismus aufwiesen und die optimale Behandlungsoption für *P. aeruginosa*-Biofilme darstellten. In Bezug auf Bakteriophagen-Antibiotika-Kombinationen beobachteten wir im Allgemeinen eine höhere Biofilm-Aktivität nach gestaffelter Exposition mit den Bakteriophagen, gefolgt von dem Antibiotikum, im Vergleich zu einer gleichzeitigen Exposition mit beiden antimikrobiell wirkenden Substanzen. Die zweite Studie zeigte, dass die synergistische Antibiofilm-Aktivität für die klinischen und Labor-*E. coli*-Biofilme bei gestaffelter Exposition von Phagen/Ciprofloxacin-, Phagen/Fosfomycin- und Phagen/Meropenem-Kombinationen erreicht werden konnte, während der Synergismus bei der gleichzeitigen Exposition der Phagen/Fosfomycin Kombination nur in den beiden *E. coli* Stämmen erreicht wurde. Überraschenderweise zeigte die gestaffelte Exposition der Phagen/Ciprofloxacin-Kombination nicht nur eine synergistische Wirkung gegen den klinischen *E. coli*-Biofilm, der gegen Ciprofloxacin resistent ist, sondern auch ein deutlich niedrigeres $MBEC_{phage}/MBEC_{alone}$-Verhältnis. In ähnlicher Weise ergab die gestaffelte Exposition von *S. aureus*/*P. aeruginosa*-Biofilmen zuerst mit zwei Verschiedenen Phagen PYO+Sb-1 und dann mit Ciprofloxacin eine signifikante Anti-Biofilm-Aktivität aus der dritten Studie, in der eine vollständige Eradikation des Biofilms bei einer Ciprofloxacin-Konzentration von 1 µg/mL erreicht werden konnte. Darüber hinaus zeigten unsere Ergebnisse auch die Zuverlässigkeit der neuartigen isothermen Mikrokalorimetrie (IMC)

zur Bewertung der Anti-Biofilm-Aktivitäten im Vergleich zu routinemäßigen Sonikations-/Koloniezählungstests, bei denen der Wärmefluss proportional zur mikrobiellen Lebensfähigkeit und Stoffwechselaktivität in Echtzeit gemessen wird.

Insgesamt ergab diese Studie, dass herkömmliche Antibiotikakombinationen in vitro eine effiziente Strategie gegen antibiotikaempfindliche Gram-negative Biofilme darstellen, und dass Phagen-Antibiotika-Kombinationen potentiell zur Bekämpfung multiresistender (MDR) gramnegativer bakterieller PJIs angewendet werden können. Für die klinische Anwendung der Phagen-Antibiotika-Kombinationen sind weitere vivo- und präklinische Studien erforderlich.

Abstract

Biofilms growing on the surface of prosthetic joints are highly refractory to the single conventional antimicrobial therapy, *Escherichia coli* and *Pseudomonas aeruginosa* present the mostly responsible for Gram-negative prosthetic joint infections (PJIs). Successful eradication of the biofilm cells with conventional antimicrobial therapies alone is still challenging. Therefore, there are urgent requirements to develop alternative therapeutic strategies in order to eliminate Gram-negative PJIs. Hence, we developed two promising approaches, conventional antibiotic combinations and phage-antibiotic combinations, against the Gram-negative bacterial biofilms.

The aim of this study was to investigate the synergistic antibiofilm activity of conventional antibiotic combinations or phage-antibiotic combinations to combat Gram-negative bacterial biofilms *in vitro*, in order to improve the outcome of treatment Gram-negative PJIs.

Based on our findings, among the tested conventional antibiotic combinations, 75% fosfomycin/gentamicin combinations had synergistic effects, presented a promising antibiotic combination therapeutic approach against *E. coli* biofilms, whereas 71.4% gentamicin/ciprofloxacin combination had synergism, exhibited the most optimal treatment option for *P. aeruginosa* biofilms. Regarding bacteriophage-antibiotic combinations, in general, we observed a higher antibiofilm activity after staggered exposure to the bacteriophage followed by the antibiotic compared with a simultaneous exposure of both antimicrobials. The second study showed the synergistic antibiofim activity for the clinical and laboratory *E. coli* biofilms could be achieved in staggered exposure of phage/ciprofloxacin, phage/fosfomycin and phage/meropenem combinations, whereas the synergism only achieved in the simultaneous exposure of phage/fosfomycin combination in both *E. coli* strains. Surprisingly, the staggered exposure of phage/ciprofloxacin combination not only showed synergistic effect against the clinical *E. coli* biofilm which has the ciprofloxacin resistant profile, but also presenting a considerably low $MBEC_{phage}/MBEC_{alone}$ ratio. Similarly, the staggered exposure of *S. aureus*/*P. aeruginosa* dual-species biofilms to phages PYO+Sb-1 first then to ciprofloxacin showed significant anti-biofilm activity from the third study, where a complete eradication of biofilm could be achieved at the concentration of ciprofloxacin 1 μg/mL. Moreover, our results also revealed the reliability of novel isothermal microcalorimetry (IMC) assay for evaluation of anti-biofilm activities compared to routine sonication/colony-counting assays, which measures the heat flow are proportional related to microbial viability and metabolic activity real-time.

Overall, this study revealed the new insights of the conventional antibiotic combinations are an efficient strategy against the susceptible Gram-negative biofilms *in vitro*, and phage-antibiotic combinations may have the potential clinical application to combat multidrug-resistant (MDR) Gram-negative bacterial PJIs, further vivo and pre-clinical trials are needed towards their clinical application.

1. Introduction

Prosthetic joint infections (PJIs) are devastating complications affecting 1% to 2% of patients undergoing primary total joint arthroplasty (1), which is related to significantly high morbidity and mortality. PJIs caused by Gram-negative (GN) bacteria account for 5% to 23% of the cases and are associated with a poor prognosis compared to those caused by Gram-positive (GP) bacteria (2-5). *Escherichia coli* is the most prevalent pathogen responsible for GN-PJIs followed by *Pseudomonas aeruginosa* (2). The pathogenesis of PJIs involves bacterial adhesion and the development of biofilm on the prosthetic implant (6). Biofilms are structured communities of sessile cells enclosed in hydrated extracellular polymeric substances (EPS) (7, 8). In the process of biofilm formation, the EPS mediates the adhesion of biofilm-embedded cells to biological or abiotic surfaces (9), acting also as the nutrient source and energy supply for the cells (10). Biofilms are considered one of the major factors in the failure of antimicrobial treatments due to their "phenotypic resistance" to numerous antibiotics and the immune system (11).

Extensive use of the first line of conventional antibiotics contributes to the occurrence of multidrug-resistant (MDR) GN-PJIs. Indeed, it has been reported that the number of MDR GN-PJIs has increased extremely from 2003 to 2012, for example, from 2 to 4.3% in the case of MDR *E. coli* or from 0.7 to 1.8% for MDR *P. aeruginosa* (12). Furthermore, there are limited new antibiotics for these difficult-to-treat infections, making urgent the search for novel alternatives to address GN-PJIs. Combination therapy involving current antibiotics with synergistic effects has been employed to treat PJIs efficiently (13), minimizing the rise of MDR strains and the requirement of antibiotic doses. Therefore, in our first *in vitro* study, we systematically evaluated the activity of first-line conventional antibiotics (fosfomycin, ciprofloxacin, and gentamicin), as monotherapy and in two-pair combinations, against biofilms of *E. coli* and *P. aeruginosa* strains, in order to find potential synergistic combinations that could be implemented for the treatment of GN-PJIs.

Since the discovery of bacteriophages (phages) in 1915, it has been applied to treat various bacterial infections, especially in several Eastern European countries. Phages are highly specific viruses, as they attack only a subset of bacterial species by binding to specific receptors on the bacterial cell wall, then injecting their DNA or RNA into cells (14). To date, even though regulatory authorities have not approved phage therapy in western countries yet, a growing number of preclinical studies about personalized phage therapy have been increasing for the treatment of chronic PJIs (15-17). The benefits of phage therapy are numerous compared to conventional antibiotic treatment alone (18). Phages exhibit an "auto-dosing" feature at the site of bacterial infection. Moreover, bacteriophages only infect the host bacterial cells without affecting the human eukaryotic cells due to their high specificity (19). Additionally, it has been reported that some phages encode depolymerase enzymes that can disrupt the EPS of biofilms (20), meanwhile, they lack cross-resistance with antibiotics, therefore, phages have been shown to kill MDR

cells more efficiently (21). Thus, the lytic bacteriophage therapy has been identified as a promising and effective treatment alternative against targeted pathogenic bacteria (22).

Ciprofloxacin is the recommended antibiotic for the treatment of GN-PJIs owing to its outstanding antibiofilm activity and bioavailability (23, 24), however, the increase of ciprofloxacin-resistant GN-PJIs leads to higher treatment failure rates. Recent studies have shown high efficacy of phage-antibiotic combinations against biofilms (20, 25). In our second study, we characterized a newly isolated bacteriophage vB_EcoM-WL-3 (ϕWL-3) active against *E. coli* and determined its combinatorial effect with different antibiotics, by either simultaneous or staggered application, against biofilms of a laboratory *E. coli* ATCC 25922 strain and a ciprofloxacin/ceftriaxone-resistant *E. coli* clinical isolate.

Furthermore, PJIs can be attributed to polymicrobial origins, *Staphylococcus aureus* and *P. aeruginosa* are frequently observed in polymicrobial biofilm infections, and it usually results in disastrous clinical outcomes than the monomicrobial infection. Phage cocktails are active against more than one bacterial species, which presented as a promising approach to treat the polymicrobial PJIs with the advantage of minimizing the occurrence of bacterial resistance and broadening the spectrum of antibacterial activity. The pyophage (PYO) cocktails and Staphylococcal bacteriophage are two commercial products manufactured by Eliava Biopreparations. PYO cocktails are composed of phages targeting *S. aureus*, *E. coli*, *P. aeruginosa*, *Streptococcus spp*., and *Proteus spp.*, whereas Staphylococcal bacteriophage contains the monophage Sb-1, a well-characterized and fully sequenced virus, which specifically targets *S. aureus* (26). In the third study, we investigated the effectiveness of both commercial phage preparations as the single therapy or in combination (simultaneous or staggered) with ciprofloxacin in eradicating *S. aureus*/*P. aeruginosa* dual-species biofilm. We hypothesized that PYO could target both bacterial species, the additional Sb-1 targeting not only *S. aureus* but also degrading the biofilm matrix, which results in completely eradicating the dual-species biofilm when combined with ciprofloxacin.

Overall, our aim was to bring *in vitro* evidence of promising treatment strategies for PJIs caused by the most frequent GN bacterial species.

2. Objectives of the work

The main aim of this study was to investigate the synergistic antibiofilm activity of different combinations of relevant antimicrobial agents (different classes of conventional antibiotic combinations or bacteriophage-antibiotic combinations) for the treatment of PJIs caused by GN bacteria. Therefore, three major objectives were set:

1. The investigation of synergistic antibiofilm activity of paired antibiotics (fosfomycin, ciprofloxacin, and gentamicin) against *E. coli* and *P. aeruginosa* biofilms. (**Study A:** Wang L et al., Synergistic activity of fosfomycin, ciprofloxacin and gentamicin

against *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. Frontiers in Microbiology (2019));

2. The investigation of synergistic antibiofilm activity of a novel bacteriophage ϕWL-3 conjuncted with antibiotics against the biofilms of a clinical ciprofloxacin/ceftriaxone-resistant *E. coli* and a laboratory reference strain *E. coli* ATCC 25922 by isothermal microcalorimetry (IMC). (**Study B**: Wang L et al., Bacteriophage–antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli in vitro* and in an experimental *Galleria mellonella* model. International Journal of Antimicrobial Agents (2020));

3. The investigation of the effectiveness of two commercial phage preparations to enhance ciprofloxacin activity in eradicating *S. aureus/P. aeruginosa* dual-species biofilm by IMC. (**Study C:** Tkhilaishvili T, Wang L, 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 medium

E. coli ATCC 25922, *P. aeruginosa* ATCC 27853 plus eight clinical *E. coli* and seven *P. aeruginosa* strains were used for the first study. A clinical ciprofloxacin/ceftriaxone-resistant *E. coli* and *E. coli* ATCC 25922 stains were used for the second study. The *P. aeruginosa* ATCC 27853 and MRSA ATCC 43300 strains were used for the third study. All of the bacterial strains were stored on a cryo-preservation system at -80 °C, and grown on blood agar plates at 37°C for 24 h. The antimicrobial assays were performed using cation-adjusted Mueller-Hinton (CAMH) broth for study A, Luria-Bertani (LB) broth for study B, and Brain Heart Infusion (BHI) broth for study C.

3.2. Antibiotics and bacteriophages

The powder of fosfomycin, meropenem, ceftriaxone and the injectable solutions of ciprofloxacin, gentamicin were provided by the respective manufacturers. The powder of antibiotics was dissolved in sterile 0.9% saline for testing. Fosfomycin was supplemented with glucose 6-phosphate (25 μg/mL) for antimicrobial testing.

A novel lytic bacteriophage vB_EcoM-WL-3 (ϕWL-3) infecting the *E. coli* ATCC 25922 and the clinical ciprofloxacin/ceftriaxone-resistant *E. coli* strains was isolated from wastewater from the Charité Campus Virchow-Klinikum. Commercially available PYO cocktails and Staphylococcal Bacteriophage were obtained as 10 mL liquid ampoules from Eliava Biopreparations.

3.3. Assessment of antibiofilm activity by isothermal microcalorimetry

The evaluation of antibiofilm activity of antibiotic combinations or phage-antibiotic combinations was performed by isothermal microcalorimetry (TA Instruments, New Castle, DE, USA). IMC measures the heat flow of biological processes in real-time, which is proportionally related to microbial viability and metabolic activity (27), allowing a reliable investigation of the susceptibility of biofilm-forming strains to different antimicrobial agents without the necessity of biofilm staining procedures. Mono- and dual-species biofilms were reproducibly grown in porous glass beads (ROBU®, Hattert, Germany) inoculated with the corresponding bacteria in the above mentioned medium and incubated 24 h at 37°C. After the incubation time, the beads were rinsed (3x) with sterile 0.9% NaCl to remove bacteria in suspensions, then exposed to serial dilutions of antimicrobial agents (phages or antibiotics alone or in combinations) and further incubated for 24 h at 37°C (unless otherwise specified). Furthermore, beads were washed (3x) again with sterile 0.9% NaCl and transferred into microcalorimetry ampoules containing 3 mL of fresh medium. The untreated biofilm served as the growth control, and the sterile bead with medium served as the negative control. Microcalorimetry ampoules were inserted into the respective channels and lowered to an equilibrium position for 15 min to reach an exact temperature of 37°C. Then, the heat flow (µW) was measured over time (h). The minimum biofilm bactericidal concentration (MBBC) was defined as the lowest concentration of antimicrobial agents that led to the absence of heat production after 48 h of incubation at 37 °C. Microcalorimetry figures were plotted by GraphPad Prism 6.01.

For the evaluation of the synergistic antibiofilm activity of phage-antibiotic combinations, two different approaches were conducted: (a) simultaneous exposure of biofilms to a fixed titer of phage (10^8 PFU/mL of ϕWL-3 or 10^6 PFU/mL of PYO/Sb-1) and sub-MBBC concentrations of antibiotics for 24h; (b) staggered exposure of biofilms to the fixed titer of phages during an optimized period (4 h for ϕWL-3 or 12 h for PYO/Sb-1) followed by a 24 hour-exposure to sub-MBBC concentrations of antibiotics.

3.4. Assessment of antibiofilm activity by sonication/colony-counting assay

After IMC experimentation, the viability of sessile cells on the glass beads was further evaluated by sonication/colony-counting assay. The beads of samples showing no heat production by IMC were selected for sonication, and following rinsed (3x) with sterile 0.9% NaCl and introduced in Eppendorf tubes containing 1 mL of Phosphate-buffered saline (PBS). Moreover, they were vortexed for 30 s, sonicated for 60 s in a sonication bath (BactoSonic, BANDELIN electronic, Berlin, Germany), and vortexed for another 30 s to dislodge adherent cells. Aliquots of 50 µL of the sonication fluids were plated on agar media for quantification of viable bacterial counts. The absence of any growth was considered as the minimum biofilm eradicating concentration (MBEC) (detection limit: <20 CFU/mL).

3.5. Evaluation of synergistic antibiofilm activity of antimicrobial combinations

The synergistic antibiofilm activity of antimicrobial combinations was evaluated by the sonication/colony-counting assay as described above, and it was calculated according to the fractional biofilm eradication concentration index (FBECI) (28) following the formula:

$$\mathrm{FBECI} = \mathrm{FBECI(a)} + \mathrm{FBECI(b)} = \frac{\mathrm{MBEC(a)\ combination}}{\mathrm{MBEC(a)\ alone}} + \frac{\mathrm{MBEC(b)\ combination}}{\mathrm{MBEC(b)\ alone}}$$

where "MBEC(a) combination" and "MBEC(b) combination" are the MBEC of antibiotic (a) in the presence of (b) and antibiotic (b) in the presence of (a), respectively; "MBEC(a) alone" and "MBEC(b) alone" are the MBEC of antibiotic (a) and antibiotic (b) as monotherapy, respectively. A FBECI ≤ 0.5 indicates synergism and a FBECI > 0.5 indicates no synergism.

The synergistic effect of phage-antibiotic combinations against biofilms was assessed based on a previous study (29), calculating the $MBEC_{phage}$/ $MBEC_{alone}$ ratio, with some modification. The $MBEC_{phage}$ corresponds to the obtained MBEC value of an antibiotic tested in combination with a fixed titer of phage and the $MBEC_{alone}$ represents the obtained MBEC value of the same antibiotic when tested alone. A ratio ≤ 0.25 indicates synergism, whereas a ratio between 0.25 and 1 indicates no effect, and a ratio > 1 indicates antagonism.

4. Results

This chapter contains key findings published in Frontiers in Microbiology (2019, 2020) (Study A and C) and International Journal of Antimicrobial Agents (2020) (Study B).

4.1. Evaluation of the anti-biofilm activity of combined antibiotics against laboratory strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 by IMC and sonication/colony-counting assays

Figure 1 and Table 1 summarized the antimicrobial effects (MBBC and MBEC) of conventional antibiotics as monotherapy and in combinations against biofilms of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 evaluated by IMC and sonication/colony-counting assay. We observed that the measured MBBC values by IMC were consistent with the MBEC values obtained after sonication/colony-counting.

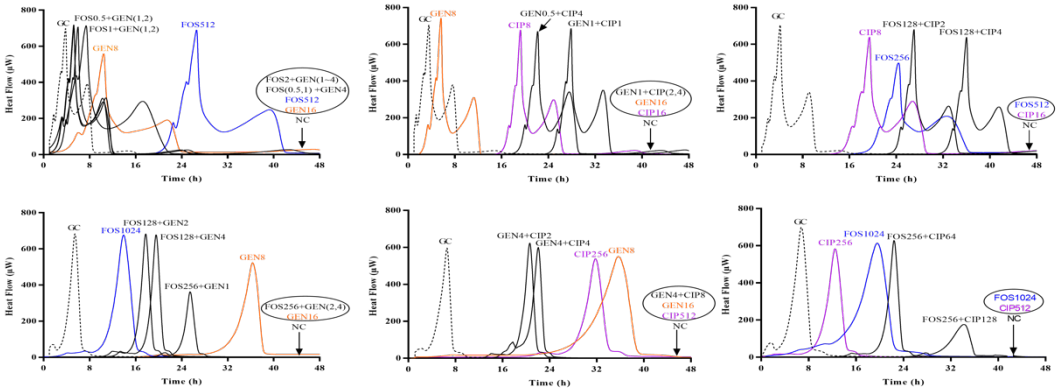

Figure 1. Evaluation of antimicrobial activity of paired antibiotics by microcalorimetry against *E. coli* ATCC 25922 (upper row) and *P. aeruginosa* ATCC 27853 (lower row) biofilms. Numbers represent antibiotic concentrations (in μg/mL). Circled values represent the MBBC. GC, growth control; NC, negative control; FOS, fosfomycin; GEN, gentamicin; CIP, ciprofloxacin. (Figure adapted from Figure 2 and Figure 3 in Study A: Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against *Escherichia coli* and *Pseudomonas aeruginosa* Biofilms. *Frontiers in Microbiology* 2019; 10: 2522. DOI: 10.3389/fmicb.2019.02522)

Gentamicin was the most active antibiotic as monotherapy against the biofilms of both ATCC strains presenting the same MBBC value (16 μg/mL), whereas ciprofloxacin showed a notable higher antibiofilm activity against *E. coli* (16 μg/mL) than *P. aeruginosa* (512 μg/mL). Fosfomycin exhibited poor antibiofilm activity against both tested strains (≥ 512 μg/mL). The strongest synergistic effect was observed by gentamicin/fosfomycin combination against *E. coli* biofilm (FBECI=0.06), followed by the gentamicin/ciprofloxacin combination (FBECI=0.19). Synergism by gentamicin/ciprofloxacin and fosfomycin/gentamicin combinations was also observed against *P. aeruginosa* biofilm, whereas fosfomycin/ciprofloxacin combination did not exhibit a synergistic effect against either strain (FBECI > 0.5).

Table 1. MBEC for fosfomycin (FOS), ciprofloxacin (CIP), gentamicin (GEN), and their combinations against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 biofilms

Strain	**MBEC (FBECI, interpretation)**					
	FOS	CIP	GEN	FOS+CIP	FOS+GEN	GEN+CIP
E. coli	512	16	16	128+8 (0.75, NS)	2+1 (0.06, S)	1+2 (0.19, S)
P. aeruginosa	>1024	512	16	256+256 (0.75*, NS)	256+2 (0.38*, S)	4+8 (0.26, S)

MBEC, minimal biofilm eradication concentration (values are expressed in μg/mL). FBECI, fractional biofilm eradication concentration index; S, synergism; NS, no synergism. *MBEC was considered equal to 1024 μg/mL for FBECI calculations. (Table adapted from Table 1 and Table 2 in Study A: Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against *Escherichia coli* and *Pseudomonas aeruginosa* Biofilms. *Frontiers in Microbiology* 2019; 10: 2522. DOI: 10.3389/fmicb.2019.02522)

4.2. Synergistic effect of antibiotic combinations against *E. coli* and *P. aeruginosa* clinical isolates by sonication/colony-counting assay

The same antibiotic combinations tested against the laboratory strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were also evaluated against biofilms formed by clinical isolates, and the eradicable activity was assessed by sonication/colony-counting assay. Tables 2 and 3 summarized the results of the MBEC for single and combined antibiotics against clinical *E. coli* and *P. aeruginosa* strains.

Table 2. MBEC for fosfomycin (FOS), ciprofloxacin (CIP), gentamicin (GEN), and their combinations against *E. coli* clinical strains.

Strain	MBEC (FBECI, interpretation)					
	FOS	CIP	GEN	FOS+CIP	FOS+GEN	GEN+CIP
Ec1	16	4	16	4+2 (0.75, NS)	1+1 (0.125, S)	0.5+0.5 (0.16, S)
Ec2	4	64	8	0.5+2 (0.16, S)	0.5+1 (0.25, S)	1+2 (0.16, S)
Ec3	16	0.032	8	4+0.016 (0.75, NS)	2+1 (0.25, S)	2+0.016 (0.75, NS)
Ec4	8	0.032	8	2+0.016 (0.75, NS)	2+0.5 (0.31, S)	2+0.016 (0.75, NS)
Ec5	8	64	16	2+1 (0.27, S)	1+1 (0.19, S)	4+0.5 (0.26, S)
Ec6	16	>1024	>1024	>4+256*(>0.5, NS)	2+16* (0.14, S)	>256*+256*(>0.5, NS)
Ec7	>1024	>1024	4	>256*+256*(>0.5, NS)	>256*+1 (>0.5, NS)	>1+256* (>0.5, NS)
Ec8	>1024	8	>1024	>256*+2 (>0.5, NS)	>256*+256* (>0.5, NS)	>256*+2 (>0.5, NS)

MBEC, minimal biofilm eradication concentration (values are expressed in µg/mL). FBECI, fractional biofilm eradication concentration index; S, synergism; NS, no synergism. *MBEC was considered equal to 1024 µg/ml for FBECI calculations. (Table adapted from Table 5 in Study A: Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against *Escherichia coli* and *Pseudomonas aeruginosa* Biofilms. *Frontiers in Microbiology* 2019; 10: 2522. DOI: 10.3389/fmicb.2019.02522)

The antibiofilm effect of each antibiotic varied widely among clinical isolates. From all of the tested antibiotic combinations against 8 *E. coli* isolates, a synergistic effect was observed in 2 isolates (25%) with fosfomycin/ciprofloxacin combination, in 6 isolates (75%) with fosfomycin/gentamicin combination and in 3 isolates (37.5%) with gentamicin/ciprofloxacin combination. On the other hand, gentamicin/ciprofloxacin combination exerted a synergistic effect in 5 *P. aeruginosa* isolates (71.4%), while fosfomycin/ciprofloxacin or fosfomycin/gentamicin combinations exhibited synergism in four isolates (57.1%), respectively.

Table 3. MBEC for fosfomycin (FOS), ciprofloxacin (CIP), gentamicin (GEN), and their combinations against *P. aeruginosa* clinical strains.

Strain	MBEC (FBECI, interpretation)					
	FOS	CIP	GEN	FOS+CIP	FOS+GEN	GEN+CIP
Pa1	>1024	4	8	128*+1 (0.38, S)	128*+2 (0.375, S)	2+1 (0.5, S)
Pa2	>1024	32	32	256*+16 (0.75, NS)	64*+4 (0.19, S)	4+2 (0.19, S)
Pa3	>1024	16	16	128*+2 (0.25, S)	128*+1 (0.19, S)	1+1 (0.13, S)
Pa4	>1024	8	16	32*+2 (0.28, S)	64*+1 (0.13, S)	4+1 (0.38, S)
Pa5	>1024	256	128	256*+128 (0.75, NS)	256*+64 (0.75, NS)	16+32 (0.25, S)
Pa6	>1024	16	>1024	64*+4 (0.31, S)	>256*+256*(>0.5, NS)	>256*+4 (>0.5, NS)
Pa7	>1024	>1024	16	>256*+256*(>0.5, NS)	>256*+4 (>0.5, NS)	>4+256*(>0.5, NS)

MBEC, minimal biofilm eradication concentration (values are expressed in µg/mL). FBECI, fractional biofilm eradication concentration index; S, synergism; NS, no synergism. *MBEC was considered equal to 1024 µg/mL for FBECI calculations. (Table adapted from Table 6 in Study A: Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against *Escherichia coli* and *Pseudomonas aeruginosa* Biofilms. *Frontiers in Microbiology* 2019; 10: 2522. DOI: 10.3389/fmicb.2019.02522)

4.3. Phage-antibiotic combinations against *E. coli* biofilms

The antibiofilm activity of ϕWL-3 phage or antibiotics alone or phage-antibiotic combinations against a clinical ciprofloxacin/ceftriaxone-resistant *E. coli* strain and the *E. coli* ATCC 25922 strain was evaluated by IMC. Thereafter, the presence of viable bacteria on the glass beads showing no heat-flow production was determined by sonication/colony-counting assay as described previously.

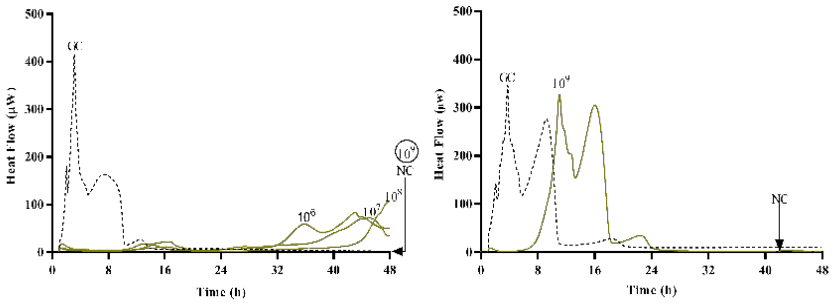

Figure 2. Microcalorimetry analysis of ciprofloxacin/ceftriaxone-resistant *E. coli* (left plot) and *E. coli* ATCC 25922 (right plot) biofilms treated with ϕWL-3 phage at different titers. Each curve shows the heat produced by viable bacteria presented in the biofilm after 24h of phage treatment or no treatment (GC). Numbers represent titers of phage (in PFU/mL). Circled values represent the MBBC, defined as the lowest antimicrobial concentration leading to absence of bacterial regrowth after 48 h. GC, growth control; NC, negative control. Data of a representative experiment are reported. (Figure adapted from Figure S4 and Figure S5 in Study B: Wang L, Tkhilaishvili T, Bernal Andres B, Trampuz A, Gonzalez Moreno M. Bacteriophage–antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli in vitro* and in an experimental *Galleria mellonella* model. *International Journal of Antimicrobial Agents* 2020; 56: 106200. DOI: 10.1016/j.ijantimicag.2020.106200)

Figure 2 presents that the phage alone as monotherapy could not inhibit both *E. coli* biofilms growth at titer 10^8 PFU/mL. Regarding antibiotic monotherapy, gentamicin and meropenem showed the highest antibiofilm activity against the clinical strain with a MBEC of 32 µg/mL (Table 4). The rest of the tested antibiotics showed no inhibitory activity against biofilm of the clinical strain when tested up to 1024 µg/mL. On the other hand, ciprofloxacin and gentamicin were the most effective antibiotics against biofilm of the ATCC strain with a MBEC of 16 µg/mL. Fosfomycin, meropenem and ceftriaxone exhibited poor antibiofilm activity, presenting MBEC values over 128 µg/mL.

Two approaches of phage-antibiotic combinations were conducted for this study: (1) The simultaneous exposure of *E. coli* biofilms to ϕWL-3 and sub-MBBC concentrations of an antibiotic for 24 h; (2) The staggered exposure of *E. coli* biofilms first to ϕWL-3 phage during 4 h followed by a 24 h exposure to sub-MBBC concentrations of an antibiotic.

Both approaches were evaluated by IMC (Figure 3 and Figure 4) and by sonication/colony-counting (Table 4).

As depicted in Figures 3 and 4, the staggered application of phage followed by antibiotics resulted in a higher antibiofilm effect with the necessity of lower concentration of antibiotics to completely inhibit heat flow production compared to simultaneous exposure. The simultaneous exposure of the clinical *E. coli* biofilm to ϕWL-3/antibiotic combinations revealed a reduction of heat-flow production compared to single antibiotics (Figures 3, left column), except for the ϕWL-3/ciprofloxacin and ϕWL-3/ceftriaxone combination, which showed up to 1024 μg/mL was unable to inhibit the growth of biofilm because of the resistant profile. As shown in Table 4, the synergism for complete eradication of biofilm was observed in the same combination. In contrast, it only presented synergistic activity with the simultaneous exposure of ϕWL-3/fosfomycin combination in the ATCC biofilm (Figures 4, left column) with more than a 2-log reduction of fosfomycin concentration. Unexpectedly, we observed an antagonistic effect in the simultaneous exposure of ϕWL-3/ceftriaxone combination with over 1024 μg/mL, however, the eradication concentration of ceftriaxone alone was 128 μg/mL. Moreover, the other three ϕWL-3/antibiotic combinations exhibited no effect for *E. coli* ATCC strain.

On the other hand, the synergistic activity exhibited in most of the staggered exposure, except the ϕWL-3/ceftriaxone and ϕWL-3/gentamicin combinations presenting no antimicrobial improvement for the clinical and ATCC strains, respectively (Figures 3 and 4, right column, and Table 4). The complete appreciable inhibition/eradication of both *E. coli* biofilms could be achieved in the staggered ϕWL-3/ciprofloxacin, ϕWL-3/fosfomycin and ϕWL-3/meropenem exposure. Surprisedly, the staggered exposure of ϕWL-3/ciprofloxacin not only showed synergism against the clinical *E. coli* biofilm, which has ciprofloxacin resistant profile, but also presenting a considerably low $MBEC_{phage}/MBEC_{alone}$ ratio. In addition, the antagonism observed in simultaneous exposure of ϕWL-3/ceftriaxone combination seems to be avoidable by the staggered exposure for *E. coli* ATCC strain.

Table 4. MBEC and $MBEC_{phage}/MBEC_{alone}$ ratio of phage–antibiotic combination against *E. coli* biofilms as a result of either simultaneous or staggered application. In brackets are shown the ratio value followed by the ratio interpretation.

Antibiotics	Single application		Simultaneous application		Staggered application	
	Clinical isolate	ATCC	Clinical isolate	ATCC	Clinical isolate	ATCC
CIP (μg/mL)	>1024	16	>1024* (1, NE)	8(0.5, NE)	4(0.004, S)	0.25(0.016, S)
FOS (μg/mL)	>1024	512	4(0.004, S)	64(0.125, S)	2(0.002, S)	32(0.06, S)
GEN (μg/mL)	32	16	8(0.25, S)	8(0.5, NE)	8(0.25, S)	8(0.25, NE)
MER (μg/mL)	32	128	4(0.125, S)	64(0.5, NE)	1(0.03, S)	16(0.128, S)
CEF (μg/mL)	>1024	128	>1024* (1, NE)	>1024* (8, A)	>1024* (1, NE)	32(0.25, S)

CIP: ciprofloxacin, FOS: fosfomycin, GEN: gentamicin, MER: meropenem, CEF: ceftriaxone. MBEC, minimal biofilm eradication concentration (values are expressed in μg/mL); S: Synergism; NE: No effect; A: Antagonism. *MBEC of the single antibiotic was considered equal to 1024 μg/mL for $MBEC_{phage}/MBEC_{alone}$ ratio calculations. (Table adapted from Table 1 and Table 2 in Study B: Wang L, Tkhilaishvili T, Bernal Andres B, Trampuz

A, Gonzalez Moreno M. Bacteriophage–antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli in vitro* and in an experimental *Galleria mellonella* model. *International Journal of Antimicrobial Agents* 2020; 56: 106200. DOI: 10.1016/j.ijantimicag.2020.106200)

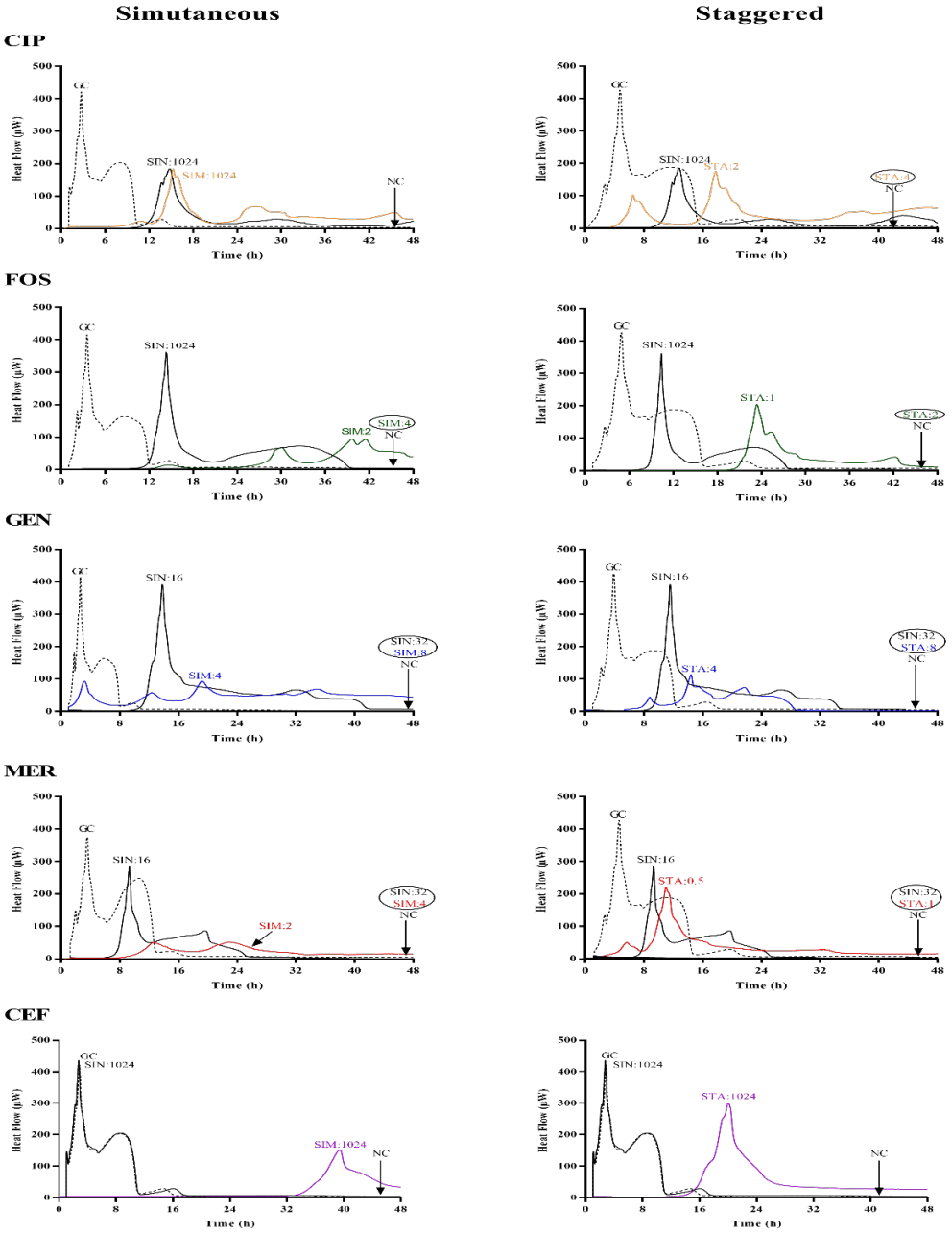

Figure 3. Microcalorimetry analysis of the clinical ciprofloxacin/ceftriaxone-resistant *E. coli* biofilm treated with single antibiotics or phage and sub-MBBC concentrations of antibiotic in a simultaneous (left column) or staggered (right column) manner. Each curve shows the heat produced by the recovering viable bacteria presented in the biofilm after treatment. Numbers represent concentrations (in µg/mL) of ciprofloxacin (CIP), fosfomycin (FOS), gentamicin (GEN), meropenem (MER) and ceftriaxone (CEF). Circled values represent the MBBC, defined as the lowest antimicrobial concentration leading to absence of bacterial regrowth after 48 h. GC, growth control; NC, negative control. SIN: Single antibiotics; SIM: simultaneous exposure; STA: staggered exposure. Data of a representative experiment are reported. (Figure adapted from Figure 4 and Figure S4 in Study B: Wang L, Tkhilaishvili T, Bernal Andres B, Trampuz A, Gonzalez Moreno M. Bacteriophage–antibiotic combinations against ciprofloxacin/ceftriaxone-resistant

Escherichia coli in vitro and in an experimental *Galleria mellonella* model. *International Journal of Antimicrobial Agents* 2020; 56: 106200. DOI: 10.1016/j.ijantimicag.2020.106200)

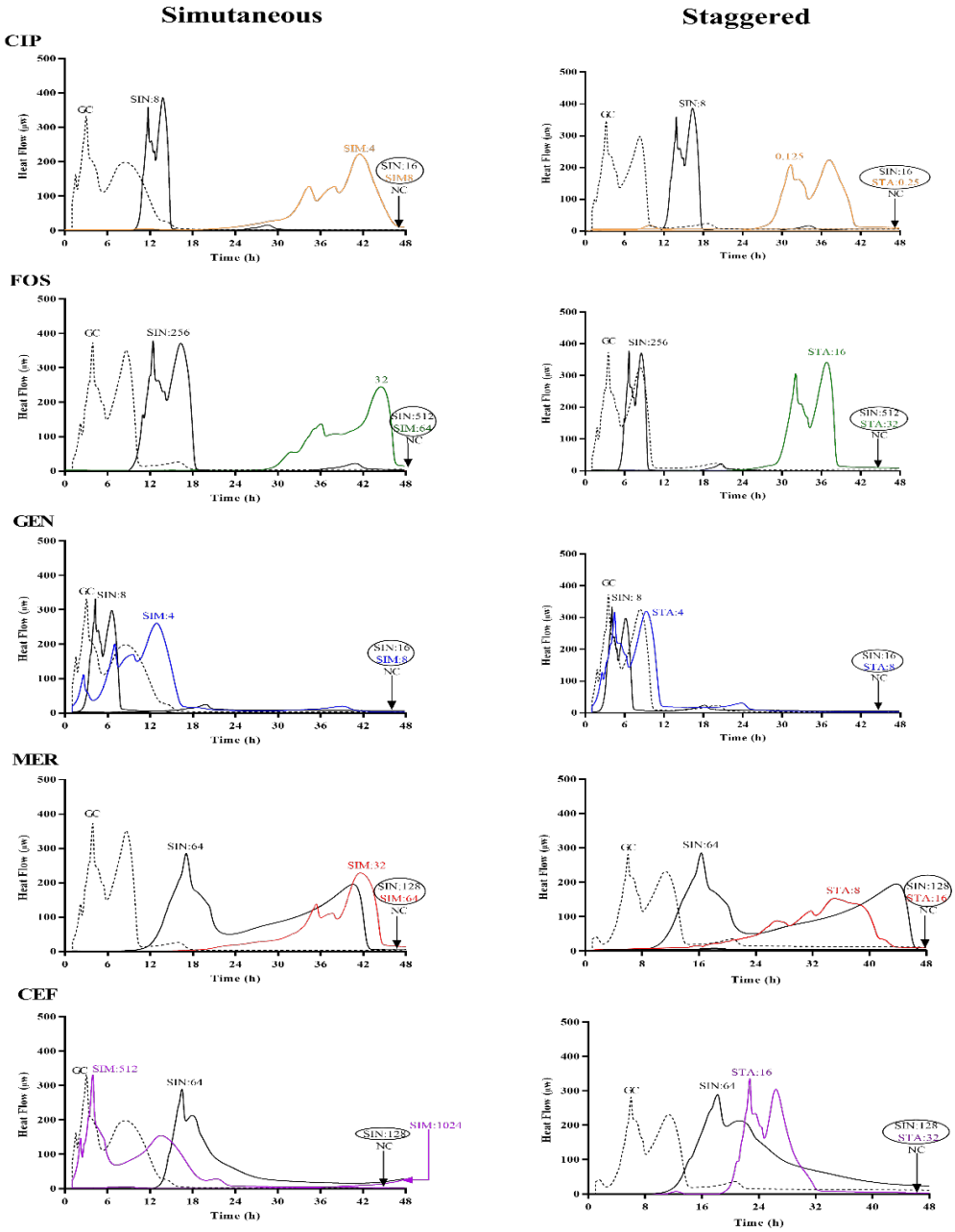

Figure 4. Microcalorimetry analysis of *E. coli* ATCC 25922 biofilm treated with single antibiotics or phage and sub-MBBC concentrations of antibiotic in a simultaneous (left column) or staggered (right column) manner. Each curve shows the heat produced by the recovering viable bacteria presented in the biofilm after treatment. Numbers represent concentrations (in µg/ml) of ciprofloxacin (CIP), fosfomycin (FOS), gentamicin (GEN), meropenem (MER) and ceftriaxone (CEF). Circled values represent the MBBC, defined as the lowest antimicrobial concentration leading to absence of bacterial regrowth after 48 h. GC, growth control; NC, negative control. SIN: Single antibiotics; SIM: simultaneous exposure; STA: staggered exposure. Data of a representative experiment are reported. (Figure adapted from Figure S5 and Figure S6 in Study B: Wang L, Tkhilaishvili T, Bernal Andres B, Trampuz A, Gonzalez Moreno M. Bacteriophage–antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli in vitro* and in an experimental *Galleria mellonella* model. *International Journal of Antimicrobial Agents* 2020; 56: 106200. DOI: 10.1016/j.ijantimicag.2020.106200)

4.4. Phage-antibiotic combination against *S. aureus*/*P. aeruginosa* dual-species biofilm

As reported in study C, we evaluated the efficacy of two commercial phage preparations to enhance ciprofloxacin activity in eradicating *S. aureus*/*P. aeruginosa* dual-species biofilm.

Overall, results indicated an increased antibiofilm activity by staggered exposure over the simultaneous exposure to the tested antimicrobials. The exposure of dual-species biofilm to ciprofloxacin monotherapy was not enough to completely inhibit the heat flow production when tested up to 512 μg/mL, whereas the exposure to phages PYO+Sb1 showed a noteworthy delay on the heat flow production without complete inhibition, indicating a moderate antibiofilm activity.

The simultaneous exposure of sub-MBBC concentrations of ciprofloxacin combined with PYO+Sb-1 revealed a remarkable delay and reduction of heat flow production compared to the heat flow produced by the growth control. Specifically, it was able to reduce over a 90% of the heat flow production when used at concentrations ranging from 4 to 64 μg/mL, but showing no complete eradication of the biofilm. On the contrary, the staggered exposure of dual-species biofilms first to phages PYO+Sb-1 during 12 h followed by exposure to ciprofloxacin, complete eradication of the biofilm could be achieved at a MBEC of 1 μg/mL.

5. Discussion

The management of GN-PJIs is difficult due to the lack of a "gold standard" treatment strategy (5). Ciprofloxacin is considered the first line of antibiofilm antibiotics for the treatment of GN-PJIs (30). However, the rapid emergence and spread of resistance to ciprofloxacin result in limited treatment options for GN-PJIs when used frequently and indiscriminately as monotherapy. In order to avoid monotherapy failure, several studies have demonstrated the benefits of applying antibiotic combinatorial therapies, particularly for the treatment of GN biofilm infections, due to the potential synergy between drugs (31, 32).

Previous studies have shown the suitability of IMC as a nonconventional technique to assess the susceptibility of planktonic cells and biofilms to antibiotics (33, 34). In our studies, we also found a correlation between the MBBC values obtained by IMC and the MBEC values obtained by the classic sonication assay.

In our first study (Study A), the selected antibiotics present different cellular pathways to target bacteria. Fosfomycin has a broad antibacterial activity with a unique mechanism, which irreversibly inhibits an early stage of bacterial cell wall biosynthesis by impeding phosphoenolpyruvate transferase (35). Moreover, it does not bind with negatively charged bacterial glycocalyx, resulting in an easier penetration through multilayered

biofilms. Thus, fosfomycin is usually used to treat complicated infections with good efficacy and tolerability. Another antibiotic, ciprofloxacin, is known for the inhibition of DNA gyrase, and it also has good penetrative properties and a longtime effect on the biofilm (36). Gentamicin primarily acts by binding to the 30S ribosomal subunit, resulting in disruption of bacterial protein synthesis. Therefore, the combination of two antibiotics could be possible to make the cross bactericidal action against GN bacterial infections. It has been reported that fosfomycin combined with fluoroquinolones has a synergistic effect against biofilm in *P. aeruginosa* infections (37), and Corvec et al. also found that fosfomycin plus gentamicin exhibited significant antibiofilm activity against extended-spectrum-β-lactamase-producing *E. coli* (31). However, no other study has systematically investigated the synergistic effect of fosfomycin, ciprofloxacin and gentamicin combinations against *E. coli* and *P. aeruginosa* biofilms under the same experimental settings *in vitro* before our study.

Our results showed that the combination of fosfomycin and gentamicin seems to be a promising strategy against *E. coli* biofilms. Nevertheless, gentamicin combined with ciprofloxacin represents an attractive application against both, *E. coli* and *P. aeruginosa,* GN species. Recent studies have revealed that fosfomycin could alter the permeability of bacteria by affecting cell wall synthesis (38), which contributes to the uptake of the fluoroquinolone into the cytoplasm and triggers inhibition of protein synthesis (39). On the other hand, Yamada et al. observed that the role of ciprofloxacin was considered to be related to damage of the outer membrane and to facilitate fosfomycin penetration (40). Regarding permeability in biofilms, ciprofloxacin exhibited a higher penetration rate (>75%) than gentamicin (73%) in *P. aeruginosa* biofilms (36), whereas it presented a similar kinetic of penetration than fosfomycin in *E. coli* and *P. aeruginosa* biofilms (41). Overall, it can be assumed that fosfomycin, ciprofloxacin and gentamicin have a good penetration into biofilms in our first study. Thereby, the hypothesis that the different efficiency in the ability of each antibiotic combination to exert a synergistic effect may be caused by other reasons, such as *P. aeruginosa* possesses the express specific channel porins for the uptake of different nutrients which results in low outer membrane permeability, however, *E. coli* possess general diffusion channel porins in their outer membrane (42).

Over the last years, lytic bacteriophages have reemerged as therapeutic agents alone or in conjunction with antibiotics for the treatment of MDR infections. Lytic phages can hijack the bacterial metabolism, replicate intracellularly and lyse the host, releasing their progenies for the infection of other cells (43). Besides, they potentially help diminish antibiotic resistance and provide another line of defense against MDR bacteria. In this sense, our second (Study B) and third (Study C) studies revalued the promising utility of self-isolated and commercial lytic phages to combine with antibiotics for eliminating ciprofloxacin-resistant *E. coli* biofilm or dual-species biofilms associated with PJIs, respectively.

Phage-antibiotic combinations have been shown in several studies to facilitate infection clearance due to less cross-resistance against MDR bacterial infection compared to single antibiotic treatment (44, 45). Moreover, this combinatorial therapy can not only significantly reduce the emergence of bacteriophage and antibiotic-resistant bacterial cells in biofilm, but also decrease bacterial virulence (46-48). Our studies revealed that the staggered administration of the first bacteriophage followed by antibiotics resulted in complete biofilm eradication at lower antibiotic concentrations compared to the simultaneous application, which is consistent with previous studies (49-51). The reason might be that some antibiotics can interfere with aspects of bacterial physiology that are essential for phage replication when applied simultaneously (52).

Indeed, in our Study B, a different outcome was observed by simultaneous administration of phage and two antibiotics with different mechanisms of action, namely fosfomycin and ciprofloxacin, against *E. coli* biofilms. This may be explained by the action of ciprofloxacin killing the bacteria by the inhibition of DNA gyrase as we described before, then interfering with the propagation of phage inside of the bacterial cells. Fosfomycin, as the smallest-molecule antibiotic by inhibiting the cell wall synthesis, is easy to diffuse in the biofilm (53), thus, it has a potential cross synergistic effect with phages. However, the synergistic activity against both *E. coli* strains was observed by the staggered administration of phage with ciprofloxacin, fosfomycin and meropenem combination, especially for the clinical *E. coli* with ciprofloxacin-resistant profile, we hypothesized that lytic bacteriophage could target receptors belong to the multidrug efflux systems, which actively removes ciprofloxacin from the bacterial cell (54-56), this may be the reason why the staggered application of ϕWL-3 and ciprofloxacin formulation could restore the susceptibility of *E. coli* to ciprofloxacin in the biofilm treatment. On the contrary, the synergistic anti-biofilm activity was not observed with phage/ceftriaxone combination in the clinical *E. coli* strain, probably due to high-level resistance to ceftriaxone, which is consistent with the previous study that the level of bacterial resistance can influence the phage-antibiotic efficacy (57). Additionally, the simultaneous exposure of phage/ceftriaxone combination exhibited the antagonistic effects in *E. coli* ATCC strain, perhaps the phage and ceftriaxone interact with each other effects, but the mechanism is still not clear (52, 58). Furthermore, our study also revealed that the improved effect of combining phages and antibiotics seems to depend not only on the mechanism of antibiotic action and on the chronological order of administration, but also on the host strain, where different antibiofilm effects were observed between both tested *E. coli* strains exposed to analogous phage-antibiotic combinations. Therefore, additional studies are required to clarify the underlying mechanism behind each synergistic and antagonistic activity of phage-antibiotic combinations against biofilms.

In Study C, we showed the greater challenge of treating *S. aureus*/*P. aeruginosa* dual-species biofilms compared with mono-species biofilms, where monotherapy with ciprofloxacin revealed drug concentrations to eradicate biofilm much higher than the ones reachable in clinical practice. Our results showed the simultaneous exposure of the phage/ciprofloxacin combination could not completely inhibit the growth of the dual-

species biofilm, whereas the successful eradication of dual-species biofilm could be achieved in a staggering exposure with a lower concentration of ciprofloxacin. A similar hypothesis as our second study is that the ciprofloxacin may inhibit bacterial DNA replication, as a result, it may impair the phage amplification. Therefore, we were able to strongly reduce the concentration of ciprofloxacin needed for dual-species biofilm eradication to a dose achievable in intravenous or oral antibiotic administration by using staggered exposure of phages (59, 60).

In conclusion, PJIs remain a serious concern during implanting a prosthetic joint in clinical settings, and the successful eradication of the biofilm cells with the single conventional antibiotic therapies seems to be a major challenge in this field. With this thesis, the reported findings present two effective and promising strategies (conventional antibiotic combinations or phage-antibiotic combinations) against the gram-negative biofilms *in vitro*, we highlight the conventional antibiotic combinations is an efficient strategy against the susceptible gram-negative biofilms *in vitro*, and phage-antibiotic combinations bring new insights into the potential clinical application associated with MDR gram-negative bacterial PJIs. Further complimentary vivo and preclinical studies are needed to support these findings for PJIs.

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Statutory Declaration

"I, Lei Wang, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Antibiofilm strategies to combat prosthetic joint infections caused by Gram-negative bacteria", 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.

I have conducted my doctoral research project completely. Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

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 shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

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."

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Date Signature

Declaration of contribution to the listed publications

Lei Wang contributed the following to the below listed publications:

Publication 1: **Wang L,** Luca MD, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic activity of fosfomycin, ciprofloxacin and gentamicin against *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. Frontiers in microbiology 2019; 10.
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Contribution in detail:

- Planning and organization of experiments in agreement with PD Dr. Trampuz and Dr. Gonzalez Moreno.
- 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: **Wang L,** Tkhilaishvili T, Andres BB, Trampuz A, Gonzalez Moreno M. Bacteriophage-antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli in vitro* and in an experimental *Galleria mellonella* model. International Journal of Antimicrobial Agents 2020: 106200.
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- Planning and organization of experiments in agreement with PD Dr. Trampuz and Dr. Gonzalez Moreno.
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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 3: Tkhilaishvili T, **Wang L**, Perka C, Trampuz A, Moreno MG. 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.
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Contribution in detail:

- Performing of some *in vitro* experiments, in detail: Assessment of biofilm eradication by sonication of beads and colony counting.
- Interpretation of some results.
- Writing some part of the manuscript.

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Signature of the doctoral candidate

Printed copies of selected publications

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Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against *Escherichia coli* and *Pseudomonas aeruginosa* Biofilms

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Gram-negative (GN) rods cause about 10% periprosthetic joint infection (PJI) and represent an increasing challenge due to emergence of antimicrobial resistance. *Escherichia coli* and *Pseudomonas aeruginosa* are among the most common cause of GN-PJI and ciprofloxacin is the first-line antibiotic. Due to emergence of fluoroquinolone resistance, we evaluated *in vitro* the activity of fosfomycin, ciprofloxacin, and gentamicin, alone and in combinations, against *E. coli* and *P. aeruginosa* biofilms. Conventional microbiological tests and isothermal microcalorimetry were applied to investigate the anti-biofilm activity of the selected antibiotics against standard laboratory strains as well as clinical strains isolated from patients with prosthetic joint associated infections. The biofilm susceptibility to each antibiotic varied widely among strains, while fosfomycin presented a poor anti-biofilm activity against *P. aeruginosa*. Synergism of two-pair antibiotic combinations was observed against different clinical strains from both species. Highest synergism was found for the fosfomycin/gentamicin combination against the biofilm of *E. coli* strains (75%), including a gentamicin-resistant but fosfomycin-susceptible strain, whereas the gentamicin/ciprofloxacin combination presented synergism with higher frequency against the biofilm of *P. aeruginosa* strains (71.4%). A hypothetical bacteriolysis effect of gentamicin could explain why combinations with this antibiotic seem to be particularly effective. Still, the underlying mechanism of the synergistic effect on biofilms is unknown. In conclusion, combinatorial antibiotic application has shown to be more effective against biofilms compared to monotherapy. Further *in vivo* and clinical studies are essential to define the potential treatment regimen based on our results.

Keywords: ***Escherichia coli*, *Pseudomonas aeruginosa*, biofilm-associated infection, antibiotic activity, synergism, clinical isolates, antibiotic resistance, isothermal microcalorimetry**

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INTRODUCTION

Gram-negative (GN) rods cause about 10% of periprosthetic joint infection (PJI) and represent an increasing treatment challenge due to the emergence of resistance worldwide (Shah et al., 2016; Thompson et al., 2018). Enterobacteriaceae are most frequently isolated in GN-PJI, followed by *Pseudomonas aeruginosa* (Fantoni et al., 2019). GN-PJI can occur after hematogenous

seeding from a distant infectious focus (i.e. urinary or intestinal tract) or can be introduced during arthroplasty and manifest in the early postoperative period (Sendi et al., 2010). Over a period of a few years (2003–2012), the occurrence of PJIs due to multidrug-resistant GN bacteria has increased significantly, in the case of *E. coli*, from 2 to 4.3%, and for *P. aeruginosa*, from 0.7 to 1.8% (Benito et al., 2016). Antimicrobial resistance in GN rods is increasing at both, community and hospital levels, and is often associated with treatment failure (Virginio et al., 2019). The worldwide rise of carbapenem-resistant GN bacilli is of major concern for the public health. While formally this problem was mostly related to *Pseudomonas* and *Acinetobacter* species, the rising trend in *E. coli* may lead to almost untreatable community-acquired infections (Tangden and Giske, 2015).

In spite of its vast impact on patients and the health-care system (Haddad et al., 2017), the management of GN-PJI is difficult due to the lack of a "gold standard" treatment strategy (Zmistowski et al., 2011; Goel et al., 2017). In fluoroquinolone-susceptible GN rods, ciprofloxacin is recommended for PJI (Sousa and Abreu, 2018). However, the growing quinolone-resistance in GN bacteria makes the treatment of GN-PJI more challenging complicating the clinical outcome (Rodriguez-Pardo et al., 2014; Benito et al., 2016). Due to unmet medical needs of currently available antibiotics, combination therapy has been investigated as an alternative strategy for GN-PJI treatment (Taha et al., 2018). Especially, revival of older antibiotics such as fosfomycin gained attention for treatment of multi-drug resistant GN rods (Walsh et al., 2016).

Beside antimicrobial resistance, treatment of PJI is challenged by the microbial persistence on the surface of implants forming biofilms (Taha et al., 2018). In biofilms, microbes exhibit "phenotypical resistance" to standard antibiotics (Donlan and Costerton, 2002). Therefore, it is essential to look at possible anti-biofilm activities of single or combined antibiotics. Several pre-clinical investigations of fosfomycin combination therapy have shown synergistic activity against biofilms of GN bacteria (Falagas et al., 2016), particularly in combination with fluoroquinolones or aminoglycosides (Michalopoulos et al., 2011; Corvec et al., 2013). Nevertheless, no systematic studies investigated these combinations against *P. aeruginosa* and *Escherichia coli* biofilms in the same experimental settings. Thus, we focused on combinations involving fosfomycin, ciprofloxacin, and gentamicin as representatives of the above-mentioned antibiotic classes. These three antibiotics present a bactericidal effect against bacteria showing different mechanisms of action. Fosfomycin has a unique mode of action inhibiting irreversibly an early stage of bacterial cell wall biosynthesis (Dijkmans et al., 2017), whereas ciprofloxacin inhibits bacterial DNA replication (Thai and Zito, 2019) and gentamicin inhibits the bacterial protein synthesis (Kumar et al., 2008).

Accurate experimental data from the investigation of combinatorial therapy with paired antibiotics might bring new evidences on their potential in the treatment of GN-PJI. Hence, we evaluated the *in vitro* activity of single and combinations of fosfomycin, ciprofloxacin, and gentamicin against planktonic and biofilms of *P. aeruginosa* and *E. coli* strains, including resistant clinical isolates obtained from patients with prosthetic joint associated infections, by using conventional microbiological tests and isothermal microcalorimetry.

MATERIALS AND METHODS

Bacterial Strains

E. coli (ATCC 25922) and *P. aeruginosa* (ATCC 27853) laboratory standard strains were used in this study. Moreover, eight *E. coli* and seven *P. aeruginosa* clinical isolates obtained from consecutive patients diagnosed with PJI between 2015 and 2017 were used for this study. For the diagnosis of PJI, the PRO-IMPLANT diagnostic criteria were used (Li et al., 2018; Izakovicova et al., 2019). The clinical isolates were used from the biobank collection, which is part of the prospective institutional PJI cohort. The study was approved by the institutional ethical committee (EA1/040/14) and was conducted in accordance with the most recent iteration of the Declaration of Helsinki. According to the ethical approval, participants' informed consent was waived and all data were pseudonymized. Bacteria were stored at −80°C using a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Canada).

Antimicrobial Agents

Fosfomycin (5 g; InfectoPharm, Heppenheim, Germany) was provided as purified powder by the manufacturer. Ciprofloxacin injectable solution (2 mg/ml; Fresenius Kabi GmbH, Bad Homburg, Germany) and gentamicin injectable solution (40 mg/ml; Ratiopharm GmbH, Ulm, Germany) were purchased from the respective manufacturers. Stock solutions of appropriate concentrations were prepared in sterile 0.9% saline.

Etest

Etest (bioMerieux, Marcy-l'Étoile, France) was performed in Mueller-Hinton agar (MHA) (Becton, Dickinson and Company, Germany) following the manufacturer's instructions. The minimum inhibitory concentration (MIC) was determined as the concentration at which the inhibition ellipse intersected the scale of the strip after incubation at 37°C for 24 h. To evaluate the susceptibility, the antimicrobial susceptibility breakpoints from the CLSI (CLSI, 2015) were used. All experiments were performed in triplicates.

Broth Macrodilution Assays

The MIC and the minimum bactericidal concentration (MBC) phase were determined for fosfomycin, ciprofloxacin, and gentamicin by the broth macrodilution assay (BMD) in cation-adjusted Mueller-Hinton broth (CAMHB) (BD, Le Pont de Claix, France), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 1999). An inoculum of approximately 5×10^5 CFU/ml were used. Two-fold serial dilutions of each antibiotic were prepared in 1 ml medium in plastic tubes and incubated for 24 h at 37°C. The MIC

was defined as the lowest concentration of antibiotic that completely inhibited visible growth.

After the incubation, all tubes without visible growth were vigorously vortexed, aliquots of 100 µl were plated on Tryptic Soy Agar (TSA) (Oxoid, Basingstoke, UK) plates, and the numbers of bacteria were determined. The MBC was defined as the lowest antimicrobial concentration that killed ≥99.9% of the initial bacterial inoculum after 24 h. The medium was supplemented with 25 mg/L glucose-6-phosphate for testing of fosfomycin. Glucose-6-phosphate induces the transport system *via* which fosfomycin is actively absorbed into the bacteria (Dijkmans et al., 2017). All experiments were performed in triplicates.

Assessment of Antimicrobial Activity by Isothermal Microcalorimetry and Sonication/Colony-Counting

The antimicrobial activity of fosfomycin, ciprofloxacin, and gentamicin against either *E. coli* or *P. aeruginosa* ATCC strains was determined by isothermal microcalorimetry (IMC) as described previously (Butini et al., 2018). Briefly, planktonic bacteria (5×10^5 CFU/ml) were treated with serial dilutions of antibiotics in CAMHB, and production of heat was measured for 24 h. The minimum heat inhibitory concentration (MHIC) was defined as the lowest concentration of antibiotic able to suppress the metabolic heat production of planktonic bacteria.

E. coli and *P. aeruginosa* biofilm formation was assessed by incubating porous glass beads (ROBU®, Hattert, Germany) in inoculated CAMHB with 2–3 colonies of the corresponding bacteria at 37°C. The ratio between beads and diluted bacterial suspension was 1 bead:1 ml, with a maximum of 10 beads per 50 ml Falcon tube. After 24 h incubation, beads were washed three times with sterile 0.9% saline to remove planktonic bacteria and exposed to serial dilutions of antibiotic in 1 ml of CAMHB and incubated for a further 24 h at 37°C. The media were supplemented with 25 mg/L glucose-6-phosphate for fosfomycin testing. After exposure to antibiotics, beads were washed three times with 0.9% saline, placed in glass ampoules containing 3 ml of CAMHB and introduced into the calorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, New Castle, USA). Sterile beads were used as negative control. Production of heat was recorded for 48 h to detect bacterial activity. The minimum biofilm bactericidal concentration (MBBC) was defined as the lowest concentration of antibiotic that strongly reduced biofilm cells viability and led to the absence of heat flow production after 48 h of incubation at 37°C.

Moreover, the biofilm-eradicating activity of these three antibiotics on clinical isolates from both species was evaluated by sonication and colony-counting as in a previous study (Gonzalez Moreno et al., 2019). 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).

The synergistic effect of antibiotic combinations was evaluated against both ATCC species following the IMC assay as described above and through CFU counting of the sonicated beads. The synergistic activity was evaluated by calculation of the fractional biofilm eradication concentration index (FBECI) as described in a previous study (Dall et al., 2018), where a FBECI of ≤0.5 indicates a synergistic effect. The FBECI was calculated following the equation: FBECI = FBECI A + FBECI B = $MBEC_A$ combination/ $MBEC_A$ alone + $MBEC_B$ combination/$MBEC_B$ alone, where $MBEC_A$ combination and $MBEC_B$ combination are the MBEC of compound A in the presence of B and compound B in the presence of A, respectively; $MBEC_A$ alone and $MBEC_B$ alone are the FBECI of compound A and compound B, respectively.

Data from IMC were analyzed by the manufacturer's software (TAM Assistant; TA Instruments) and Prism 7.0 (GraphPad Software, La Jolla, CA). All experiments were performed in triplicates.

RESULTS

Activity of Antibiotics Against *E. coli* and *P. aeruginosa* ATCC Strains

The antimicrobial activity of fosfomycin, ciprofloxacin, and gentamicin against planktonic and sessile *E. coli* and *P. aeruginosa* ATCC strains was assessed using BMC, Etest, IMC (**Figures 1, 2**) and by plating of sonication fluid. **Table 1** summarizes the susceptibilities of both species.

The observed MIC values evaluated by Etest and BMD were comparable to those obtained by IMC. Both ATCC strains were susceptible to all three tested antibiotics. Ciprofloxacin was the most active antibiotic against planktonic bacteria from both strains, followed by gentamicin. Fosfomycin showed a remarkable lower bactericidal activity against *P. aeruginosa* with MIC and MBC values 16 and 64 times higher respectively compared to *E. coli*.

Gentamicin was the most active antibiotic against the biofilm of both strains presenting a MBBC of 16 µg/ml (**Figures 2E,F**), whereas ciprofloxacin showed a notable higher anti-biofilm activity against *E. coli* (MBBC = 16 µg/ml) compared to *P. aeruginosa* (MBBC = 512 µg/ml) (**Figures 2C,D**). Fosfomycin exhibited a poor anti-biofilm activity against both tested ATCC strains (**Figures 2A,B**).

Results showed that the concentrations of antibiotics necessary to completely eradicate the biofilm (MBEC) of both ATCC strains correlated with the bactericidal concentrations observed by calorimetry (MBBC) for all the tested antibiotics.

Anti-biofilm Activity of Combined Antibiotics Against *E. coli* and *P. aeruginosa* ATCC Strains

The synergistic effect of two-pair antibiotics against biofilm of both ATCC strains was investigated by IMC combining fosfomycin/ciprofloxacin, fosfomycin/gentamicin, and gentamicin/ciprofloxacin. Results are summarized in **Table 2**. Calorimetric curves are depicted in **Figure 3**.

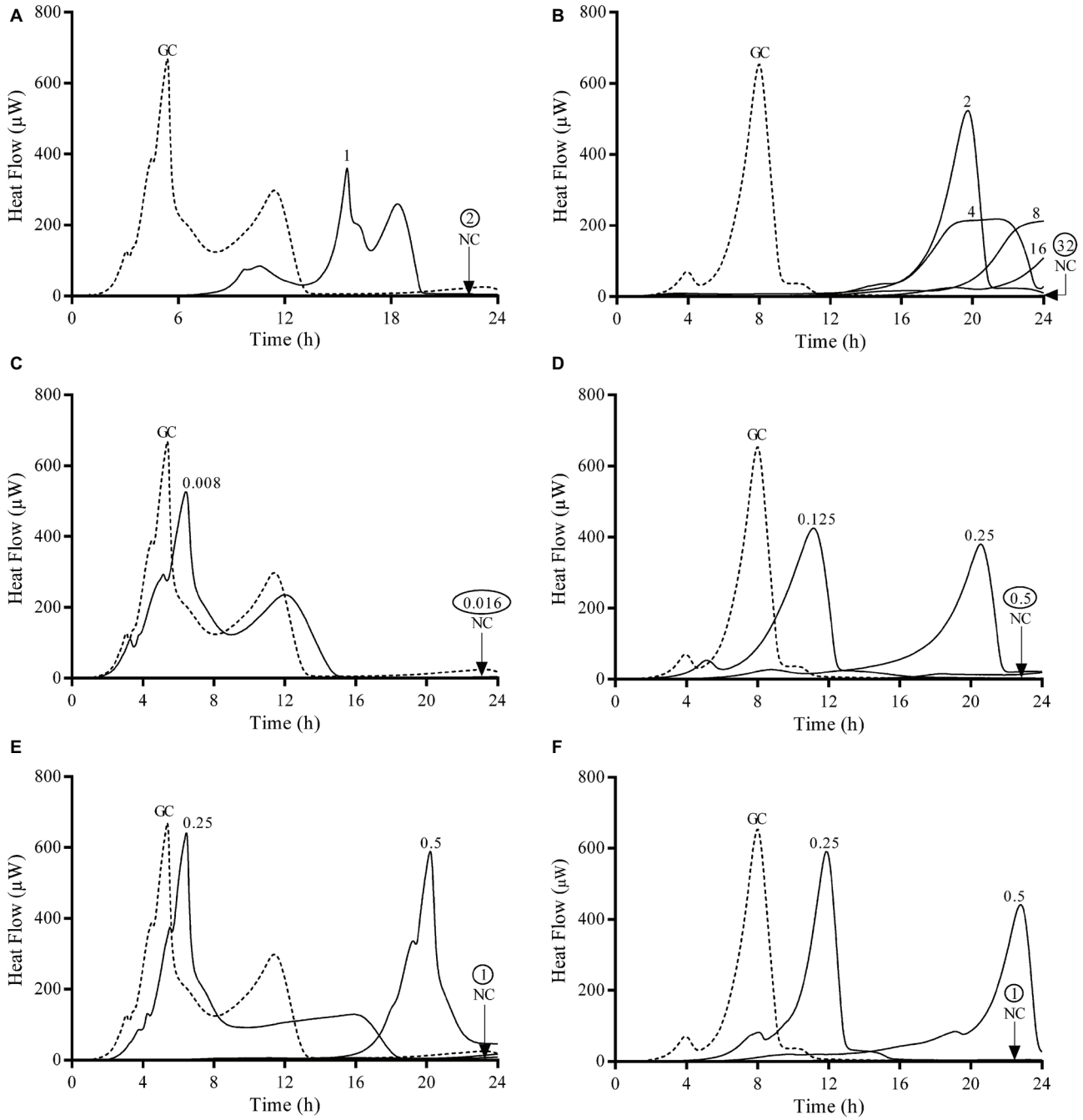

FIGURE 1 | Microcalorimetry analysis of planktonic bacteria exposed to serial antibiotic concentrations for 24 h. Numbers represent concentrations (in μg/ml) of fosfomycin **(A,B)**, ciprofloxacin **(C,D)**, and gentamicin **(E,F)** against *E. coli* ATCC 25922 (left column) and *P. aeruginosa* ATCC 27853 (right column). Circled values represent the MHIC. GC, growth control; NC, negative control. Data of a representative experiment are reported.

The strongest synergistic effect was observed when gentamicin was combined with fosfomycin (FBEC = 0.06) against *E. coli* biofilm followed by the combination of gentamicin with ciprofloxacin, whereas these two antibiotic combinations showed similar synergistic effect against *P. aeruginosa* biofilm. Fosfomycin/ciprofloxacin combination did not show synergism against the biofilm of both strains.

Antibiotic Susceptibility of *E. coli* and *P. aeruginosa* Clinical Strains

The MIC of *E. coli* and *P. aeruginosa* clinical strains to fosfomycin, ciprofloxacin, and gentamicin was determined by Etest. The results are summarized in **Tables 3** and **4**.

E. coli and *P. aeruginosa* strains were considered susceptible to fosfomycin when MIC ≤64 μg/ml, to ciprofloxacin when

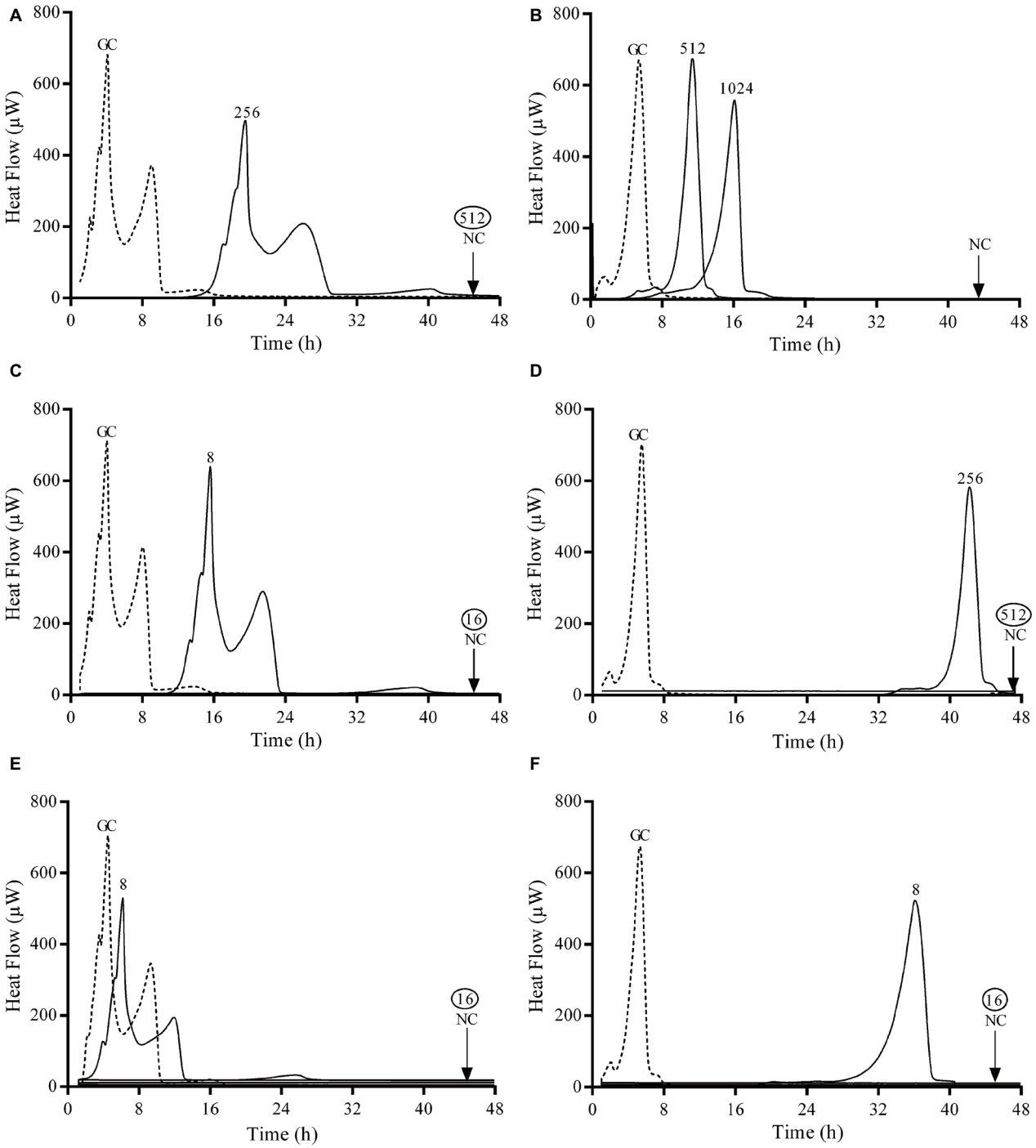

FIGURE 2 | Microcalorimetry analysis of biofilm bacteria after exposure to serial antibiotic concentrations for 24 h. Numbers represent concentrations (in μg/ml) of fosfomycin **(A,B)**, ciprofloxacin **(C,D)**, and gentamicin **(E,F)** against *E. coli* ATCC 25922 (left column) and *P. aeruginosa* ATCC 27853 (right column). Circled values represent the MBBC. GC, growth control; NC, negative control. Data of a representative experiment are reported.

MIC ≤1 μg/ml and to gentamicin when MIC ≤4 μg/ml according to CLSI (CLSI, 2015).

Most strains were susceptible to the tested antibiotics, except Ec6 (resistant to ciprofloxacin and gentamicin), Ec7 (resistant to fosfomycin and ciprofloxacin), Ec8 (resistant to fosfomycin and gentamicin), Pa 6 (resistant to gentamicin), and Pa 7 (resistant to ciprofloxacin).

Ciprofloxacin exhibited the lowest MIC in sensitive strains for both bacterial species (MIC range 0.008–0.25 μg/ml), whereas fosfomycin showed higher activity on susceptible strains of

TABLE 1 | Antimicrobial susceptibility of planktonic and adherent *E. coli* and *P. aeruginosa* determined by conventional broth macrodilution (BMD), Etest, isothermal microcalorimetry (IMC), and sonication/colony-counting.

***E. coli* ATCC 25922**						
Antibiotic	**Etest**	**BMD**		**IMC**		**Sonication**
	MIC	**MIC**	**MBC**	**MHIC**	**MBBC**	**MBEC**
Fosfomycin	1	2	4	2	512	512
Ciprofloxacin	0.012	0.008	0.016	0.016	16	16
Gentamicin	0.5	0.5	4	1	16	16
***P. aeruginosa* ATCC 27853**						
Antibiotic	**Etest**	**BMD**		**IMC**		**Sonication**
	MIC	**MIC**	**MBC**	**MHIC**	**MBBC**	**MBEC**
Fosfomycin	16	32	256	32	>1,024	>1,024
Ciprofloxacin	0.25	0.25	1	0.5	512	512
Gentamicin	1	0.5	4	1	16	16

Concentration values are expressed in μg/ml.

TABLE 2 | MBBC and FBEC values for fosfomycin (FOS), ciprofloxacin (CIP), and gentamicin (GEN) in combination against *E. coli* and *P. aeruginosa*.

Antibiotic	***E. coli* (ATCC 25922)**		***P. aeruginosa* (ATCC 27853)**	
	MBBC (μg/ml)	**FBEC (interpretation)**	**MBBC (μg/ml)**	**FBEC (interpretation)**
FOS + CIP	128 + 8	0.75 (NS)	256 + 256	0.75* (NS)
FOS + GEN	2 + 1	0.06 (S)	256 + 2	0.38* (S)
GEN + CIP	1 + 2	0.19 (S)	4 + 8	0.26 (S)

*MBBC, minimal biofilm bactericidal concentration; FBEC, fractional biofilm eradication concentration; S, synergism, NS, no synergism; *$MBBC_{FOS}$ of P. aeruginosa was considered equal to 1,024 μg/ml for FBEC calculations.*

E. coli (MIC range 0.064–1 μg/ml) than *P.* aeruginosa (8–48 μg/ml). Gentamicin-susceptible strains from both species presented similar susceptible profile with a MIC range of 0.5–3 μg/ml.

Synergistic Effect of Antibiotic Combinations Against *E. coli* and *P. aeruginosa* Clinical Strains

The same two-pair antibiotic combinations tested against the ATCC strains were used to evaluate their ability to eradicate biofilms from clinical strains by sonication/colony counting. **Tables 5** and **6** summarize the results of the MBEC for single and combined antibiotics against *E. coli* and *P. aeruginosa* clinical strains.

The biofilm susceptibility to each antibiotic varied widely among clinical isolates. Among the eight tested *E. coli* isolates, synergism based on fosfomycin/ciprofloxacin combinations was observed in two isolates (25%), while the combinations fosfomycin/gentamicin and gentamicin/ciprofloxacin resulted synergistic in six (75%) and three isolates (37.5%), respectively.

Moreover, the fosfomycin/gentamicin combination showed a synergistic effect against Ec6, a gentamicin-resistant but fosfomycin-susceptible *E. coli* strain, whereas the synergistic effect was not observed when the fosfomycin/gentamicin combination was tested against Ec7, a fosfomycin-resistant but gentamicin-susceptible *E. coli* strain.

On the other hand, the synergism of gentamicin/ciprofloxacin was observed in five *P. aeruginosa* isolates (71.4%), while four isolates (57.1%) were susceptible to the combination of either fosfomycin/ciprofloxacin or fosfomycin/gentamicin.

DISCUSSION

Fluoroquinolones are the first choice as anti-biofilm antibiotics for the treatment of GN-PJI (Boyle et al., 2019). However, emergence and spread of resistance to fluoroquinolones and aminoglycosides has decreased the existing treatment options for GN infections (Tucaliuc et al., 2015). Combination therapy with fosfomycin has been recommended, particularly against fluoroquinolone resistant organisms (Boyle et al., 2019). Nonetheless, there is a lack of systematic studies investigating antibiotic combinations under the same experimental settings on GN biofilms. In this study, we have generated original data showing synergistic activity of two-pair antibiotic combinations against either *E. coli* or *P. aeruginosa* biofilms *in vitro*.

Conventional (Etest, BMD and colony counting) and nonconventional (IMC) laboratory tests were applied to evaluate the susceptibility to antibiotics of either planktonic or biofilm bacteria. As seen also in previous studies (Gonzalez Moreno et al., 2017, 2019), the MHIC values obtained by IMC showed consistency to the MIC values obtained by BMD and Etest, proving the reliability of IMC for antimicrobial testing on large

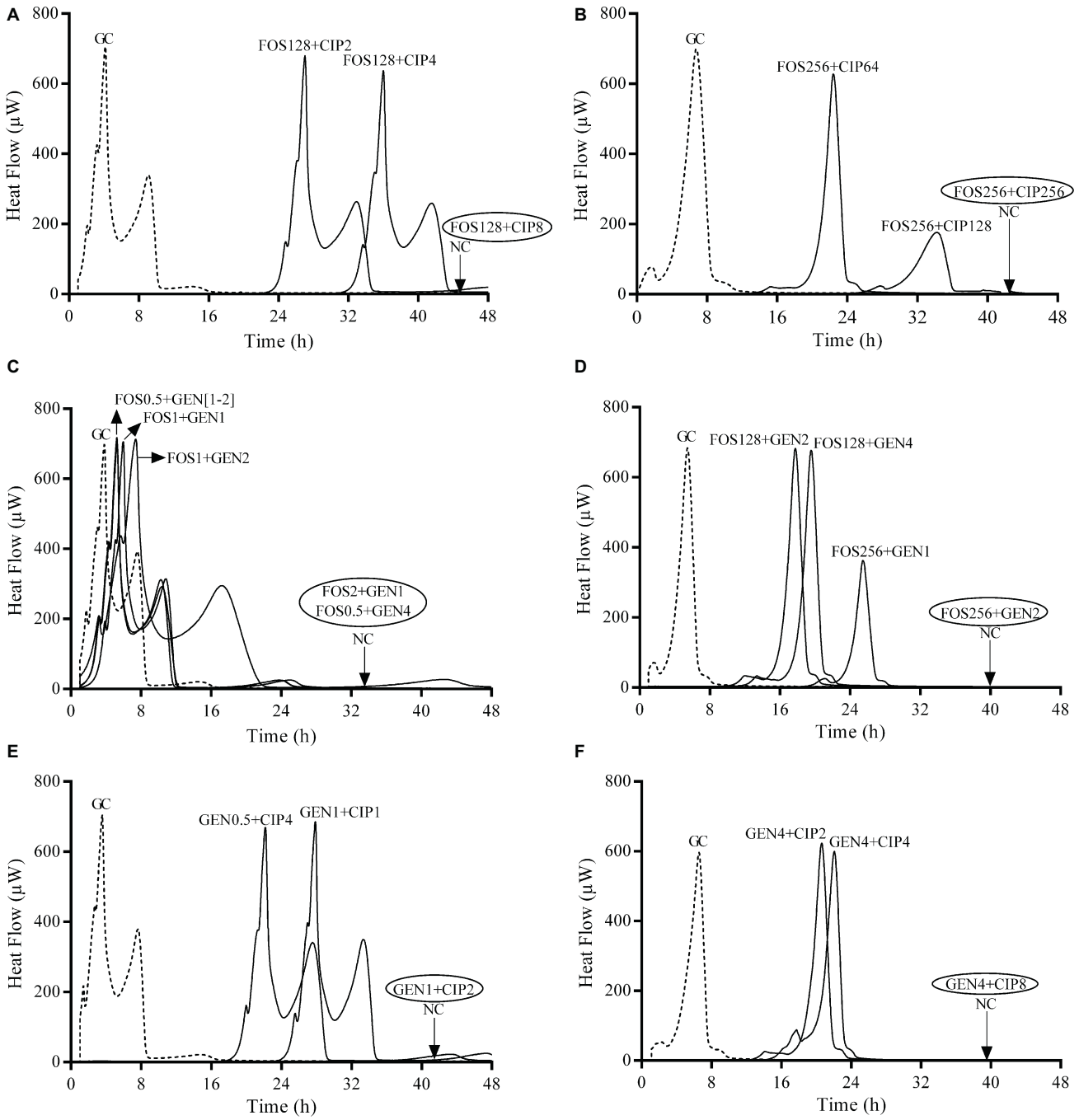

FIGURE 3 | Evaluation of synergistic activity of paired antibiotics by IMC against *E. coli* ATCC 25922 **(A,C,E)** and *P. aeruginosa* ATCC 27853 **(B,D,F)** biofilms. Numbers represent concentrations (in μg/ml). Circled values represent the MBBC. GC, growth control; NC, negative control; FOS, fosfomycin; GEN, gentamicin; CIP, ciprofloxacin. Data of a representative experiment are reported.

scale. Moreover, the concentrations of antibiotics needed to eradicate biofilms were analogous to those showing biofilm bactericidal activity by IMC.

As already reported for many microorganisms (Stewart, 2015), in our study, the eradication of GN biofilms required considerably higher concentrations of all three tested antibiotics (4 to 2,723-fold higher) compared with the killing of their planktonic bacteria. Fosfomycin had no anti-biofilm activity against *P. aeruginosa* strains despite the use of high concentrations of antibiotic (up to 1,024 μg/ml) (**Table 6**). These results suggest that outcomes obtained on planktonic cells cannot be transferred to biofilms, underling the importance of developing standardize methods to evaluate antimicrobial activity on biofilms (Macia et al., 2014). In our work, we employed IMC in

combination with sonication as reliable methods for the *in vitro* analysis of bacterial biofilms (Butini et al., 2018).

In biofilm infections, where antimicrobial monotherapies are not effective or not applicable due to the rapid development of antibiotic resistance (Wu et al., 2015; Greimel et al., 2017; Xu et al., 2018), the use of antibiotic combinatorial therapies has been shown to be particularly relevant due to the potential synergy between drugs (Wu et al., 2015). Therefore, we also investigated the *in vitro* synergistic activity of paired antibiotics against *E. coli* and *P. aeruginosa* ATCC strains and clinical isolates.

A study limitation is the lack of a full chequerboard analysis for antibiotic combinations, which could bring more insights on the synergistic/antagonistic activity. We evaluated only concentrations of antibiotics that could reveal a synergistic effect based on the MBEC values of the single antibiotics to be combined. For MBEC >1,024 μg/ml, a fixed value of 1,024 μg/ml was considered for the calculation of the FBEC index, thus some combinations which were interpreted as not synergistic could turn out to have a synergistic effect considering higher MBEC values. However, with this approach, the observed positive synergistic effects of antibiotic combinations are certain and usually presenting considerably lower MBEC values compared to the MBEC values of single antibiotics, which are difficult to reach in clinical practice.

The three tested antibiotic combinations showed synergistic activity, to varying degrees, against different clinical strains from both species partially differing from the results observed with the ATCC strains. The considerable reduction of MBEC values with antibiotic combinations ranging from 2-fold to 16-fold in case of *P. aeruginosa* strains or 2-fold to 128-fold in case of *E. coli* strains compared to single drug was predominantly in the range, which is achievable by intravenous or oral antibiotic administration (Dijkmans et al., 2017; Thabit et al., 2019).

Even though several studies have reported that fosfomycin showed an estimable synergistic effect, among others, with

TABLE 3 | MIC of *E. coli* by Etest.

Antimicrobial	Ec1	Ec2	Ec3	Ec4	Ec5	Ec6	Ec7	Ec8
Fosfomycin	0.19	0.094	0.125	0.064	0.75	0.25	128(R)	64(R)
Ciprofloxacin	0.016	0.016	0.008	0.008	0.008	8(R)	8(R)	0.008
Gentamicin	1	0.5	1	2	1	96(R)	1	96(R)

MIC values are expressed in μg/ml; R, resistant (according to CLSI).

TABLE 4 | MIC of *P. aeruginosa* by Etest.

Antimicrobial	Pa1	Pa2	Pa3	Pa4	Pa5	Pa6	Pa7
Fosfomycin	32	48	8	16	32	24	48
Ciprofloxacin	0.19	0.064	0.125	0.125	0.094	0.19	12(R)
Gentamicin	1.5	1.5	2	3	2	128(R)	1.5

MIC values are expressed in μg/ml; R, resistant (according to CLSI).

TABLE 5 | MBEC for fosfomycin (FOS), ciprofloxacin (CIP), gentamicin (GEN), and their combinations against *E. coli* clinical strains.

Strain	MBEC (FBEC, interpretation)					
	FOS	CIP	GEN	FOS + CIP	FOS + GEN	GEN + CIP
Ec1	16	4	16	4 + 2 (0.75, NS)	1 + 1 (0.125, S)	0.5 + 0.5 (0.16, S)
Ec2	4	64	8	0.5 + 2 (0.16, S)	0.5 + 1 (0.25, S)	1 + 2 (0.16, S)
Ec3	16	0.032	8	4 + 0.016 (0.75, NS)	2 + 1 (0.25, S)	2 + 0.016 (0.75, NS)
Ec4	8	0.032	8	2 + 0.016 (0.75, NS)	2 + 0.5 (0.31, S)	2 + 0.016 (0.75, NS)
Ec5	8	64	16	2 + 1 (0.27, S)	1 + 1 (0.19, S)	4 + 0.5 (0.26, S)
Ec6	16	>1,024	>1,024	>4 + 256* (>0.5, NS)	2 + 16* (0.14, S)	>256* + 256* (>0.5, NS)
Ec7	>1,024	>1,024	4	>256* + 256*(>0.5, NS)	>256* + 1 (>0.5, NS)	>1 + 256* (>0.5, NS)
Ec8	>1,024	8	>1,024	>256* + 2 (>0.5, NS)	>256* + 256*(>0.5, NS)	>256* + 2 (>0.5, NS)

*MBEC, minimal biofilm eradication concentration (values are expressed in μg/ml). FBEC, fractional biofilm eradication concentration; S, synergism; NS, no synergism; *MBBC was considered equal to 1,024 μg/ml for FBEC calculations.*

TABLE 6 | MBEC for fosfomycin (FOS), ciprofloxacin (CIP), gentamicin (GEN), and their combinations against *P. aeruginosa* clinical strains.

Strain	MBEC (FBEC, interpretation)					
	FOS	CIP	GEN	FOS + CIP	FOS + GEN	GEN + CIP
Pa1	>1,024	4	8	128* + 1 (0.38, S)	128* + 2 (0.375, S)	2 + 1 (0.5, S)
Pa2	>1,024	32	32	256* + 16 (0.75, NS)	64* + 4 (0.19, S)	4 + 2 (0.19, S)
Pa3	>1,024	16	16	128* + 2 (0.25, S)	128* + 1 (0.19, S)	1 + 1 (0.13, S)
Pa4	>1,024	8	16	32* + 2 (0.28, S)	64* + 1 (0.13, S)	4 + 1 (0.38, S)
Pa5	>1,024	256	128	256* + 128 (0.75, NS)	256* + 64 (0.75, NS)	16 + 32 (0.25, S)
Pa6	>1,024	16	>1,024	64* + 4 (0.31, S)	>256* + 256*(>0.5, NS)	>256* + 4 (>0.5, NS)
Pa7	>1,024	>1,024	16	>256* + 256*(>0.5, NS)	>256* + 4 (>0.5, NS)	>4 + 256*(>0.5, NS)

*MBEC, minimal biofilm eradication concentration (values are expressed in μg/ml). FBEC, fractional biofilm eradication concentration; S, synergism; NS, no synergism; *MBBC was considered equal to 1,024 μg/ml for FBEC calculations.*

gentamicin and ciprofloxacin against *P. aeruginosa* planktonic cells (Kastoris et al., 2010), there is limited evidence for these combinations against biofilms. The fosfomycin/ciprofloxacin combination has being shown to be effective against *P. aeruginosa* biofilms in other experimental set-ups (Xiong et al., 1995; Mikuniya et al., 2005), whereas we could not find studies investigating the fosfomycin/gentamicin combination. Nonetheless, previous studies have shown synergistic effect when fosfomycin was combined with other aminoglycosides (Cai et al., 2009; Anderson et al., 2013) as well as other fluoroquinolones (Kumon et al., 1995; Monden et al., 2002; Mikuniya et al., 2005, 2007) against *P. aeruginosa* biofilms.

Some authors propose that fosfomycin alters the membrane permeability of *P. aeruginosa* by affecting cell wall synthesis, which should lead to enhanced uptake of the fluoroquinolone ofloxacin (Monden et al., 2002). On the other hand, one study has suggested that the role of ciprofloxacin is thought to be related to damage of the outer membrane, enhancing fosfomycin penetration (Yamada et al., 2007). Regarding permeability in biofilms, it was reported that ciprofloxacin had a higher penetration rate (>75%) than gentamicin (73%) in *P. aeruginosa* biofilms (Abdi-Ali et al., 2006), but showed a similar kinetic of penetration than fosfomycin into the bacterial biofilm of both, *E. coli* and *P. aeruginosa* species (Rodríguez-Martínez et al., 2007). Based on these studies, it can be argued that all three antibiotics are able to penetrate well into biofilms. Thus, it could be hypothesized that the differences observed on the ability of each antibiotic combination to exert a synergistic effect might be attributed to other factors, such as killing of persister cells as proposed for streptococci in combinations including gentamicin (Gonzalez Moreno et al., 2017), rather than the enhancement of penetration. Further studies are required to clarify the underlying mechanism of their synergistic effect on biofilms.

The fosfomycin/gentamicin combination was the most active against *E. coli* strains. These results also correlate with the findings from Corvec et al., where fosfomycin plus gentamicin presented a significant high cure rate in an *in vivo* foreign-body infection model (Corvec et al., 2013). Moreover, the fosfomycin/gentamicin combination showed a synergistic effect toward a gentamicin-resistant strain, but the same combination was not synergistic toward a fosfomycin-resistant strain. Conventionally, the mechanism of action of gentamicin has been considered at the 30S ribosomal level. Nevertheless, some authors have suggested that gentamicin has two potentially lethal effects on Gram-negative cells, one being the inhibition of protein synthesis and the other one being the surface perturbation (Kadurugamuwa et al., 1993a,b). Thus, a bacteriolysis effect mediated through perturbation of the cell surface by gentamicin could explain the synergism observed by the fosfomycin/gentamicin combination toward a gentamicin-resistant strain. It could be speculated that, in the case of a gentamicin-resistant but fosfomycin-susceptible strain, while fosfomycin can act against susceptible bacterial cells, gentamicin could also actively target resistant bacterial cells through bacteriolysis, resulting in complete biofilm eradication. However, in the case of a gentamicin-susceptible but fosfomycin-resistant strain, no synergistic effect is observed because only gentamicin can act against bacteria, whereas fosfomycin becomes ineffective. The two antimicrobial effects of gentamicin might also explain why combinations with this antibiotic seem to be particularly effective. Still, elucidations for synergistic effect based on planktonic findings would need to be confirmed also for biofilms.

In conclusion, the use of fosfomycin in combination with gentamicin seems to be a promising therapeutic approach against *E. coli* biofilm related infections. Nevertheless, against both Gram-negative species, combination of gentamicin with ciprofloxacin represents the most optimal treatment option. Further *in vivo* and clinical studies are essential to define the potential treatment regimen based on the combination of these two antibiotics. Moreover, our study presents IMC as a sensitive technique to provide reliable data on as important field of clinical microbiology as it is the screening for biofilm-eradicating approaches.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The clinical isolates were used from the biobank collection, which is part of the prospective institutional PJI cohort. The study was approved by the Institutional Ethical Committee (EA1/040/14) and was conducted in accordance with the most recent iteration of the Declaration of Helsinki. According to the ethical approval, participants' informed consent was waived and all data were pseudonymized.

AUTHOR CONTRIBUTIONS

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

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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.

Keywords: *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, biofilm-associated infection, dual-species biofilm, antibiotic-bacteriophage combination, bacteriophages, isothermal microcalorimetry, scanning electron microscopy

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_2O (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×) 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×) 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 (**Figure1C**) and at 24 h of incubation

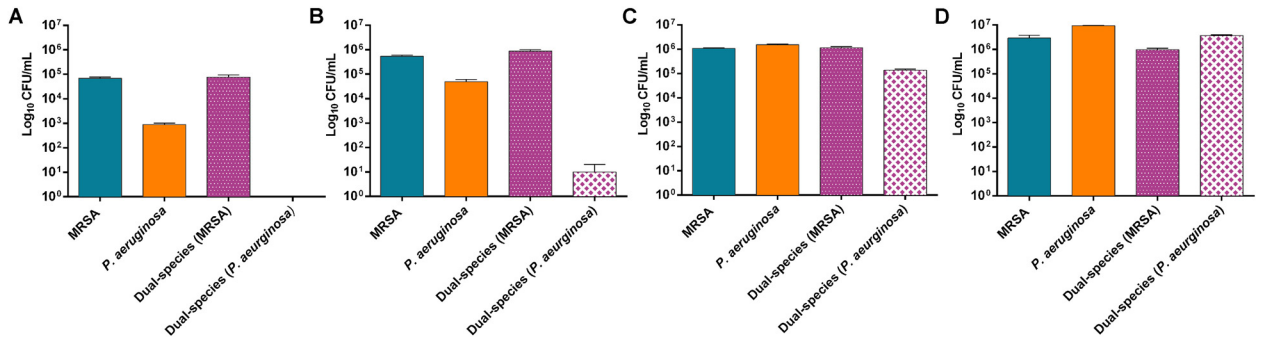

FIGURE 1 | Distribution pattern of bacterial populations over the time. Number of viable cells (in log10 CFU/mL) of *P. aeruginosa* and MRSA on mono- and dual-species biofilms formed after 3 h **(A)**, 6 h **(B)**, 12 h **(C)** and 24 h **(D)**. Data are reported as CFU/mL mean ± standard deviation of 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**).

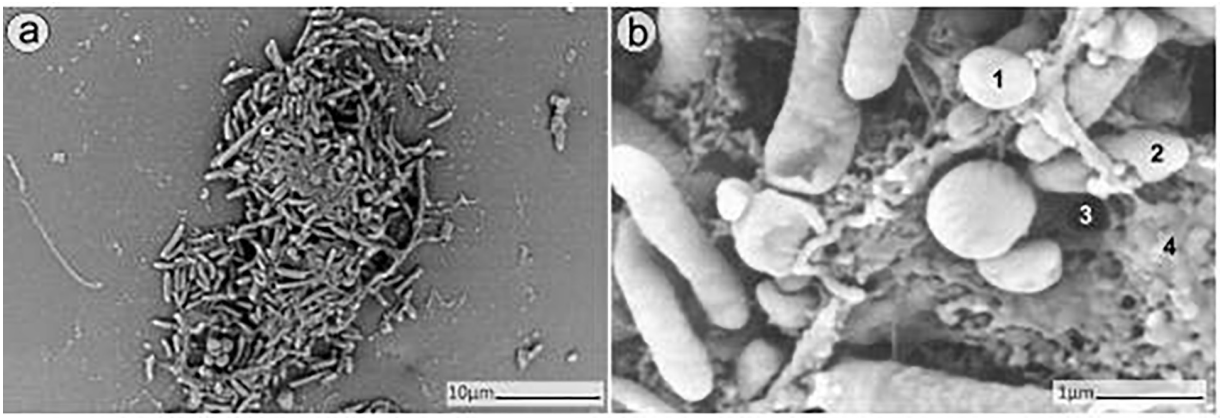

FIGURE 2 | SEM analysis of dual-species biofilm formed by MRSA (ATCC 43300) and *P. aeruginosa* (ATCC 27853) on porous glass beads after 24h of incubation. Image **(b)** is a close-up from **(a)**. Numbers 1 and 2 indicate a MRSA bacterium and a *P. aeruginosa* bacterium respectively, whereas 3 and 4 point out a water channel and the extracellular polymeric matrix of the biofilm correspondingly.

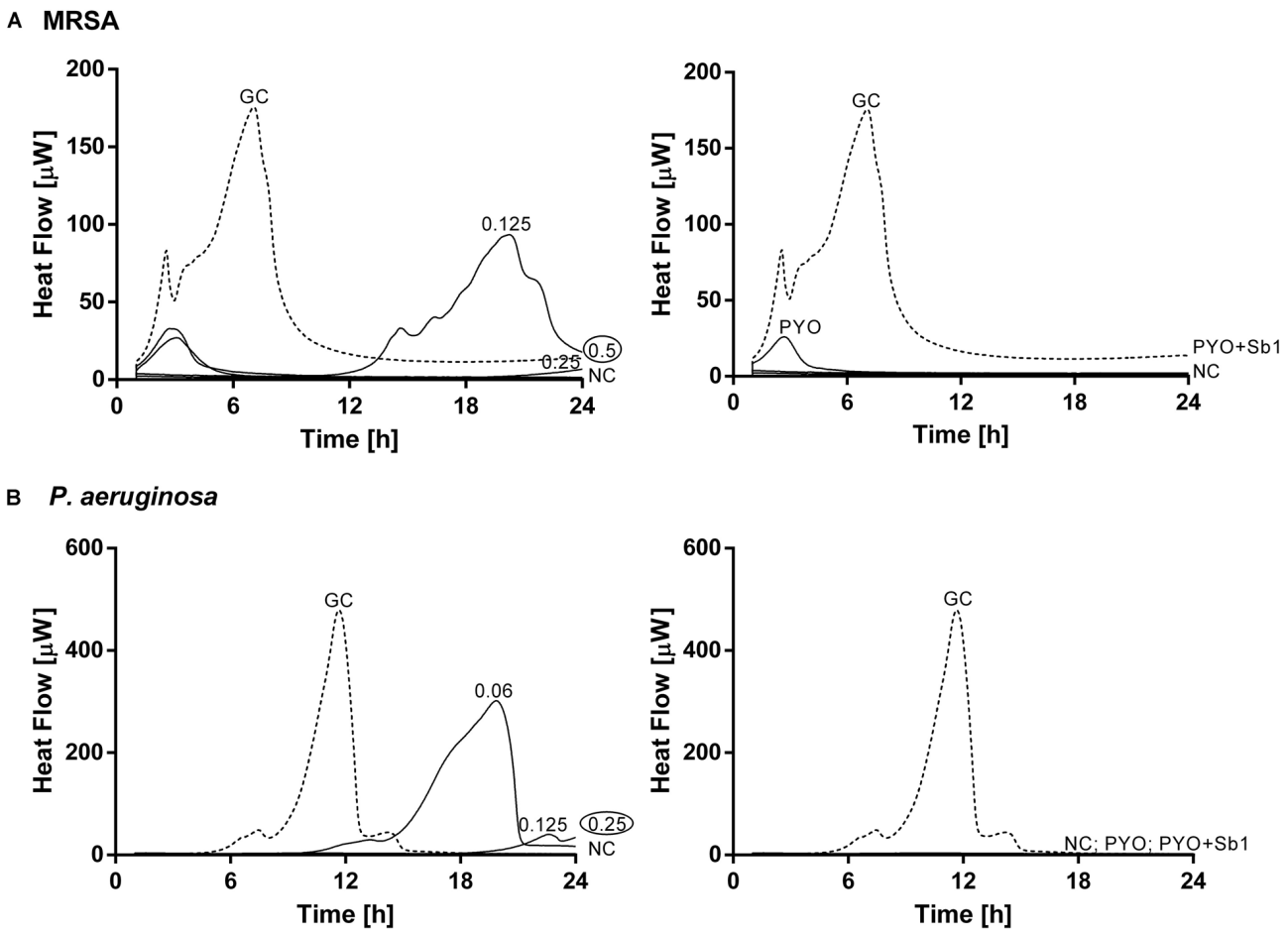

FIGURE 3 | Microcalorimetry analysis of planktonic MRSA ATCC 43300 **(A)** and *P. aeruginosa* ATCC 27853 **(B)** cells treated with two-fold increasing concentrations of ciprofloxacin (left column, numbers represent concentrations in mg/L of antibiotic) or with phages (right column). A circled value 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.

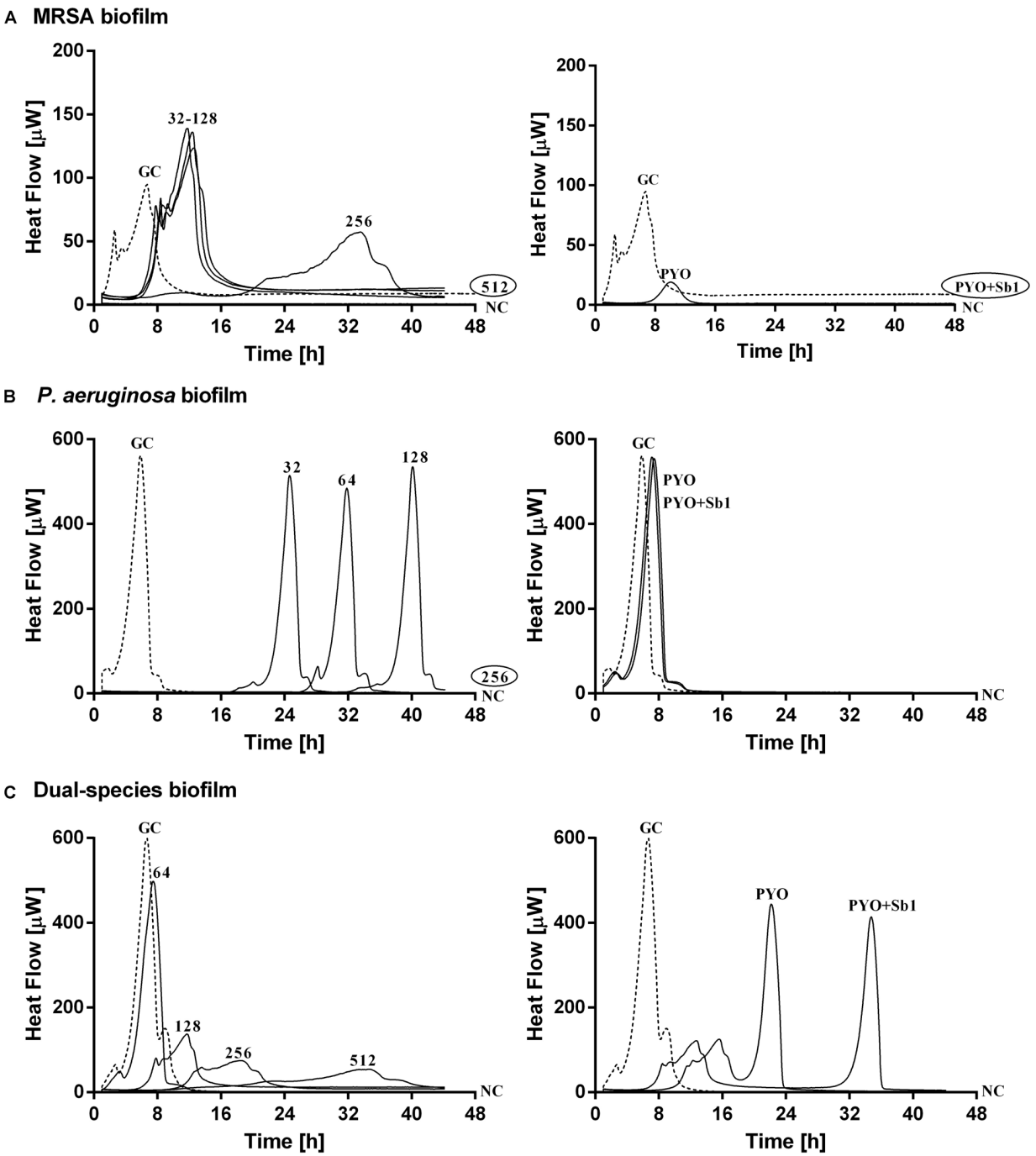

FIGURE 4 | Microcalorimetry analysis of **(A,B)** mono- and **(C)** dual-species MRSA (ATCC 43300) and *P. aeruginosa* (ATCC 27853) biofilms treated with two-fold increasing concentrations of ciprofloxacin (left column, numbers represent concentrations in mg/L of antibiotic) or with phages (right column). 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

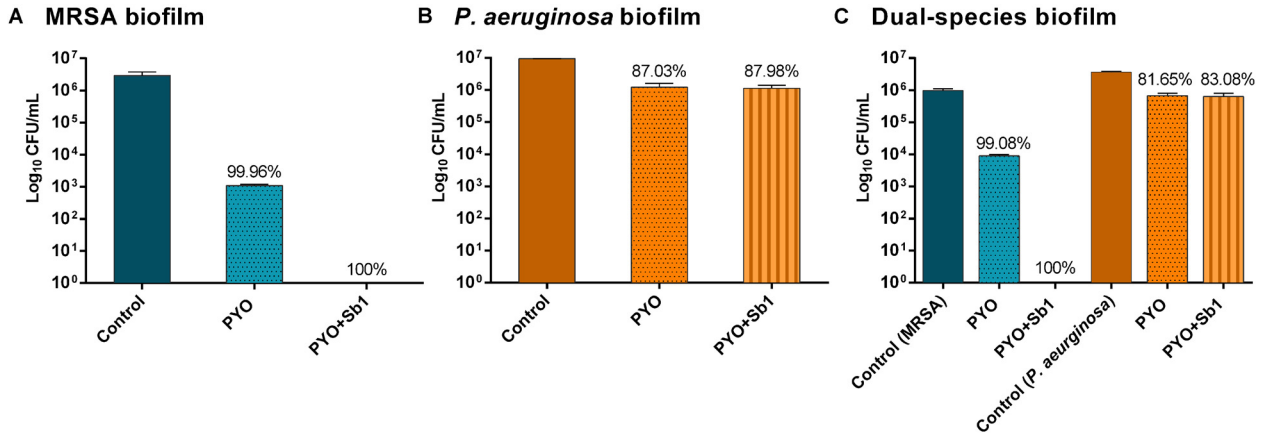

FIGURE 5 | Effect of PYO and Sb-1 phage preparations on viability of biofilm-embedded mono and mix bacteria populations. *S. aureus/P. aeruginosa* mono- **(A,B)** and dual-species **(C)** biofilms formed on porous glass beads were exposed to phages. Data are reported as log10 CFUs/mL mean ± standard 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]×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.

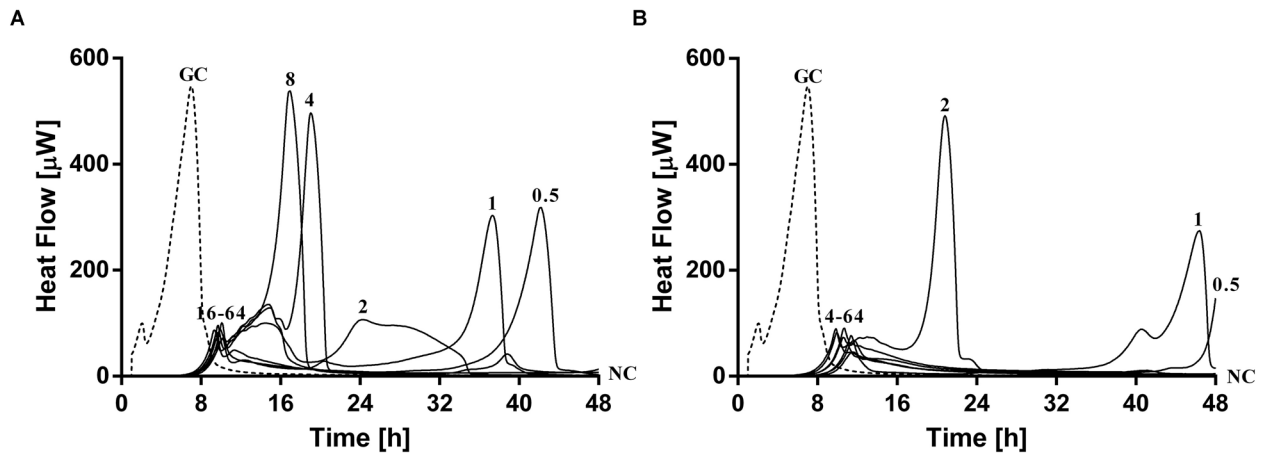

FIGURE 6 | Evaluation of MRSA ATCC 43300/*P. aeruginosa* ATCC 27853 dual-species biofilm viability after simultaneous exposure during 24 h to ciprofloxacin at increasing doses (0.5–64 mg/L) plus **(A)** PYO or **(B)** PYO+Sb-1 monitored by microcalorimetry. Numbers represent antibiotic concentrations (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

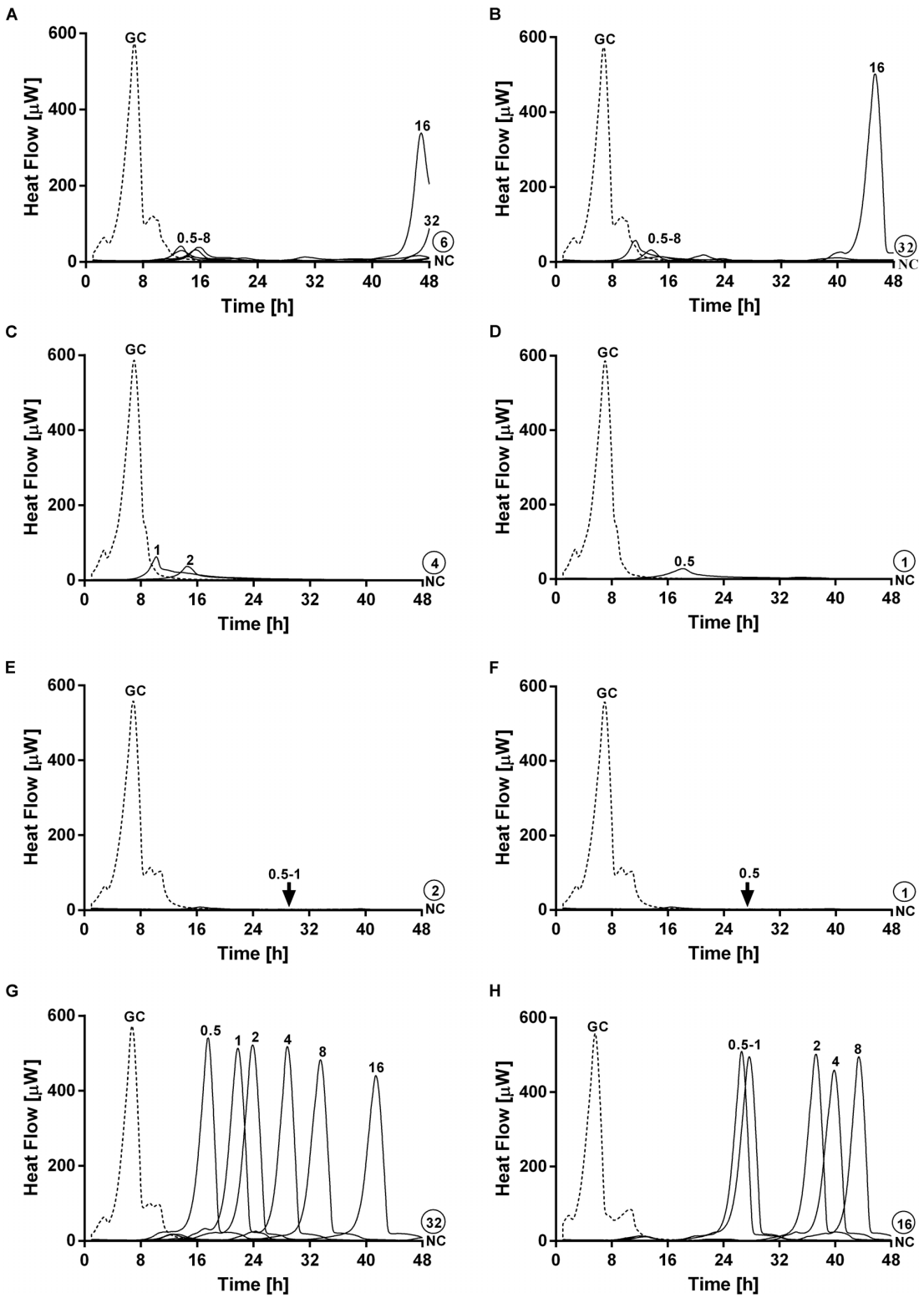

FIGURE 7 | Evaluation of MRSA ATCC 43300/*P. aeruginosa* ATCC 27853 dual-species biofilm viability after staggered exposure to phages and ciprofloxacin monitored by microcalorimetry. Each curve shows the heat produced by viable bacteria present in biofilms pretreated for 3 h **(A,B)**, 6 h **(C,D)**, 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.

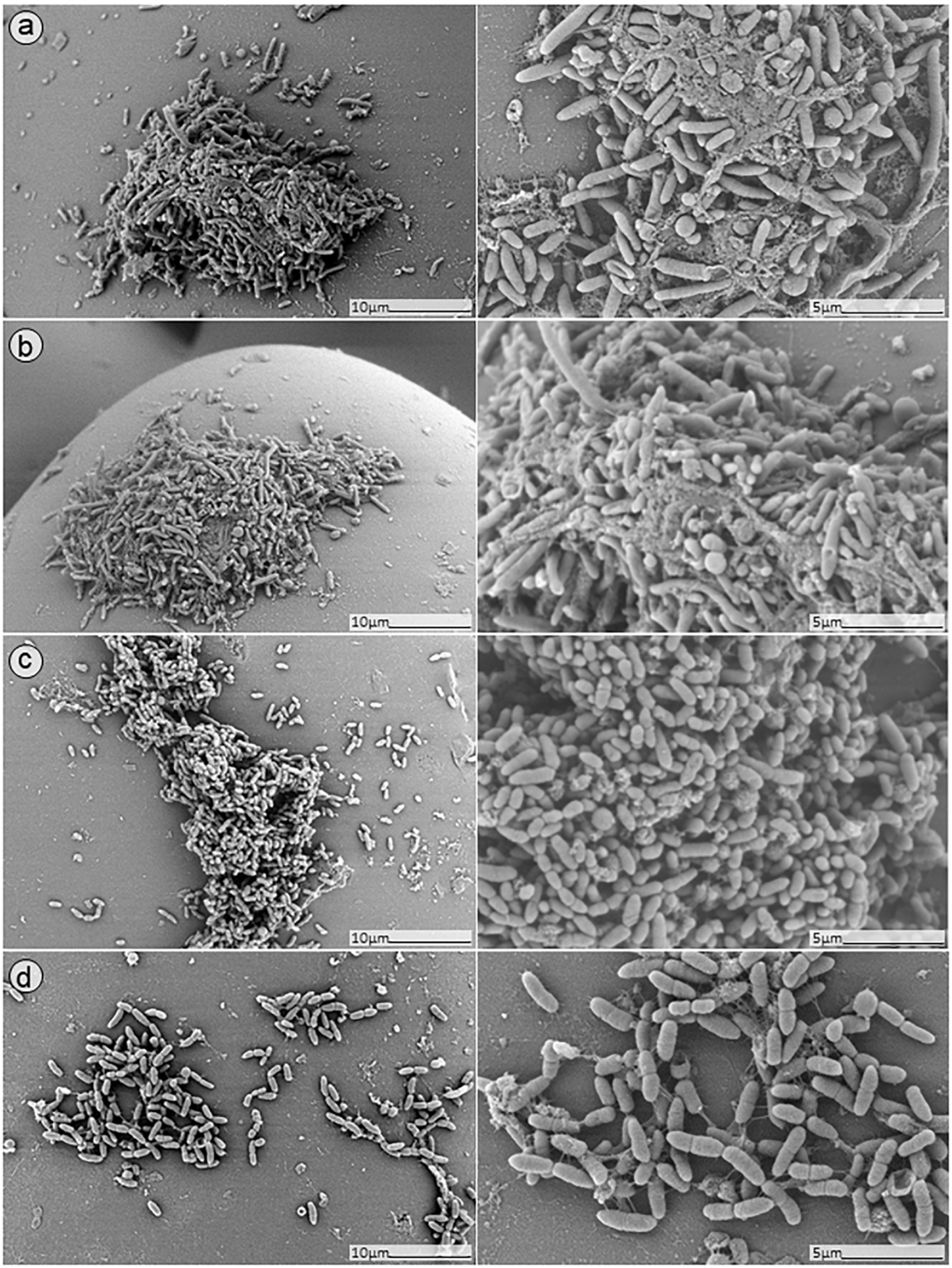

FIGURE 8 | SEM analysis of *S. aureus/P. aeruginosa* dual-species biofilms grown on porous glass beads for 24 h without treatment **(a)** and after exposure to 24 h monotherapy with **(b)** ciprofloxacin (1 mg/L); **(c)** PYO; or **(d)** pyo+sb-1.

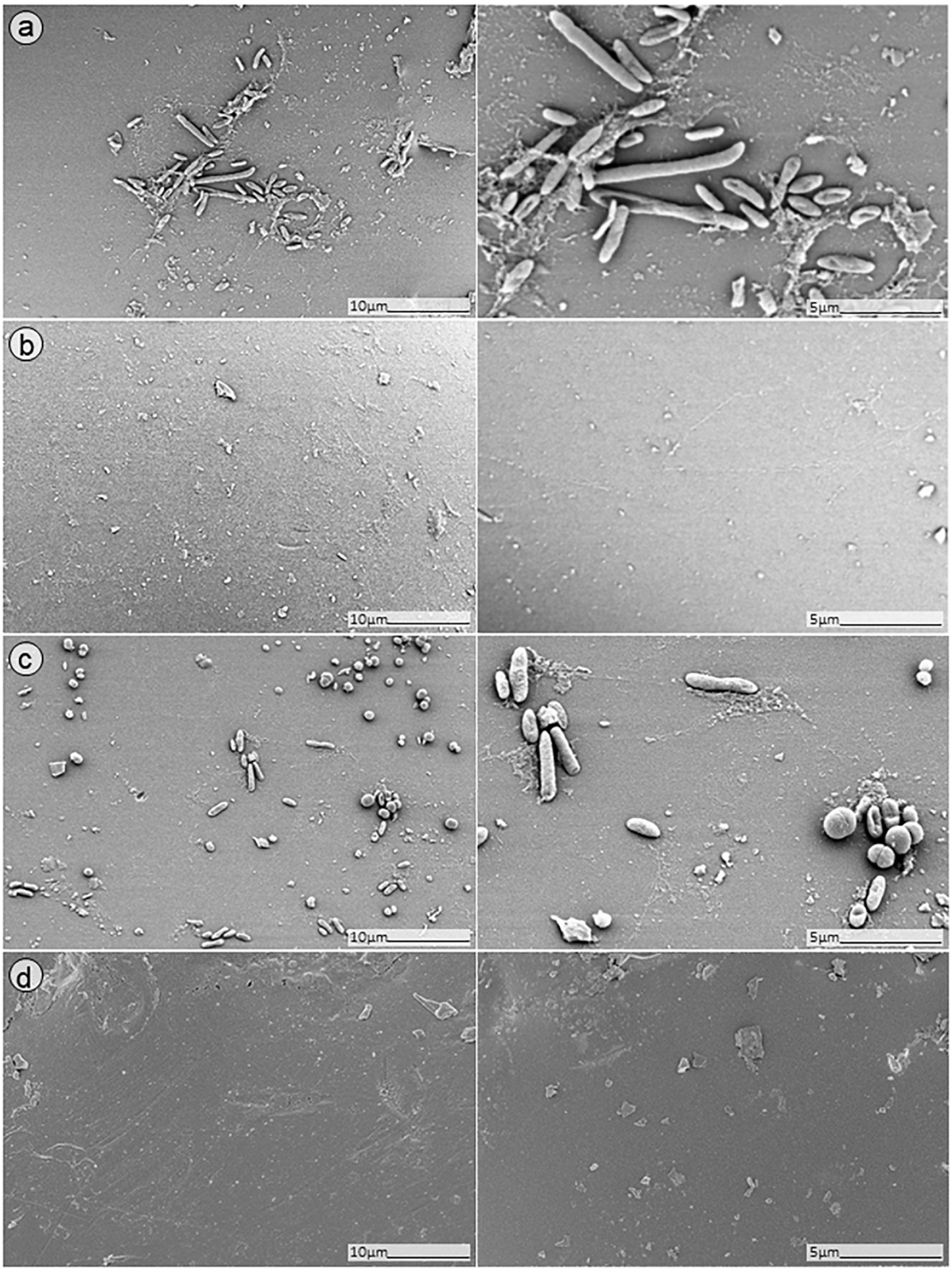

FIGURE 9 | SEM analysis of *S. aureus/P. aeruginosa* dual-species biofilm grown on porous glass beads for 24 h and treated with a combinatorial therapy of **(a)** simultaneous exposure to PYO and ciprofloxacin (1 mg/L, 24 h); **(b)** staggered exposure to PYO (12 h) followed by ciprofloxacin (1 mg/L, 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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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Curriculum Vitae

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

Complete list of publications

1. **Wang L**, Tkhilaishvili T, Andres BB, Trampuz A, Moreno MG. 2020. Bacteriophage-antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli* in vitro and in an experimental Galleria mellonella model.
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