International Journal of Antimicrobial Agents 64 (2024) 107276

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Bacteriophages as potential antibiotic potentiators in cystic fibrosis: A new model to study the combination of antibiotics with a bacteriophage cocktail targeting dual species biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Zhifen Wang[a,#], Steven De Soir[a,b,#], Antoine Glorieux[a,b], Maya Merabishvili[b], Christiane Knoop[c], Daniel De Vos[b], Jean-Paul Pirnay[b], Françoise Van Bambeke[a,*]

[a] *Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium*
[b] *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Neder-over-Heembeek, Belgium*
[c] *Erasme Hospital, Université libre de Bruxelles, Brussels, Belgium*

ARTICLE INFO

Article history:
Received 27 February 2024
Accepted 10 July 2024

Editor: Dr Yan Zhu

Keywords:
Phages
Antibiotics
Cystic fibrosis
Dual-species biofilm
Staphylococcus aureus
Pseudomonas aeruginosa

ABSTRACT

Objectives: *Staphylococcus aureus* and *Pseudomonas aeruginosa* co-infections in patients with cystic fibrosis (CF) are associated with disease severity. Their treatment is complicated by biofilm formation in the sticky mucus obstructing the airways. We investigated the activity of phages-antibiotics combinations using a dual species biofilm (*P. aeruginosa/S. aureus*) formed in artificial sputum medium.

Methods: Biofilmswere incubated with broad-spectrum antibiotics (meropenem, ceftazidime, ciprofloxacin, tobramycin) combined with a cocktail of two (bacterio)phages (PSP3 and ISP) proven active via spot tests and double agar on *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923.

Results: At the highest tested concentrations (100 x MIC), antibiotics alone caused a 20–50% reduction in biomass and reduced *S. aureus* and *P. aeruginosa* CFU of 2.3 to 2.8 and 2.1 to 3.6 $\log_{10}$, respectively. Phages alone reduced biofilm biomass by 23% and reduced *P. aeruginosa* CFU of 2.1 $\log_{10}$, but did not affect *S. aureus* viability. Phages enhanced antibiotic effects on biomass and exhibited additive effects with antibiotics against *P. aeruginosa*, but not against *S. aureus.* Following inhibition of bacterial respiration by phages in planktonic cultures rationalised these observations by demonstrating that PSP3 was effective at multiplicities of infection (MOI) as low as 10^{-4} plaque forming units (PFU)/CFU on *P. aeruginosa*, but ISP, at higher MOI ($>$ 0.1) against *S. aureus*.

Conclusion: Pre-screening inhibition of bacterial respiration by phages may assist in selecting those showing activity at sufficiently low titers to showcase anti-biofilm activity in this complex but clinically-relevant in vitro model of biofilm.

© 2024 Elsevier Ltd and International Society of Antimicrobial Chemotherapy. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

1. Introduction

Cystic fibrosis (CF) affects over 150,000 people worldwide, mostly of Caucasian descent [1]. It is caused by genetic mutations in the *CFTR* gene. Altered CFTR channel leads to disruption in salt and water balance in mucosal epithelia, causing the thickening of mucus in the lungs.

This sticky mucus promotes persistent bacterial infections, which remain the main cause of morbidity and mortality in these patients [2]. Bacteria tend to form biofilms, that is, bacterial aggregates where cells become metabolically inactive and are protected by a self-produced extracellular matrix mainly consisting of polysaccharides, extracellular DNA and proteins, leading to antibiotic tolerance. The predominant pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, co-infect more than 25% of the patients [3]. *S. aureus,* highly prevalent in children and adolescents (50–80%), contributes to airway inflammation and reduced lung function, while *P. aeruginosa,* infecting 20–30% of children and up to 80% of adults, is a major cause of respiratory failures and death

* Corresponding author: Françoise Van Bambeke, Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium.
E-mail address: francoise.vanbambeke@uclouvain.be (F. Van Bambeke).
# Equal contribution as first authors

https://doi.org/10.1016/j.ijantimicag.2024.107276
0924-8579/© 2024 Elsevier Ltd and International Society of Antimicrobial Chemotherapy. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

Table 1
MIC of antibiotics[a] in MHB-ca and concentrations reached in the lungs or in the sputum.

Antibiotics	MIC (mg/L)		Lung (L) or sputum (S) concentration [route of administration]
	S. aureus ATCC 25923	*P. aeruginosa* PAO1	
Ciprofloxacin	8	0.25	3 mg/L (S) [oral] [25] 57-260 mg/L (S) [inhaled] [26]
Ceftazidime	0.5	2	2-7 mg/L (S) [IV] [27]
Meropenem	0.03	1	10-20 mg/L (L) [IV] [28]
Tobramycin	0.5	1	1000 µg/g (S) [inhaled] (SmPC)

[a] EUCAST resistance breakpoints (R): > 8 mg/L for meropenem and ceftazidime against *P. aeruginosa* (no breakpoint set for β-lactams against *S. aureus* but ATCC 25923 is an MSSA and therefore considered as susceptible), > 0.5 and 1 mg/L for ciprofloxacin against *S. aureus* and *P. aeruginosa*, respectively, and > 2 mg/L against both species for tobramycin.

[2]. Given the scarcity of effective antibiotics against biofilms, there is an urgent need for new treatment strategies.

In this context, bacteriophages have emerged as potential antibiotic potentiators and biofilm disruptors [4]. Over the last two decades, bacteriophages garnered renewed interest and proved consistently safe. They have demonstrated lytic activity in the sputum from infected patients with CF [5], and synergistic interactions in combination with antibiotics, including in in-vitro models with CF isolates [6–8] or in a few patients with CF [9].

In this study, we aim to assess a combination of antibiotics with a bacteriophage cocktail consisting of two phages targeting *S. aureus* (phage ISP) and *P. aeruginosa* (phage PSP3) on a unique dual species biofilm model, growing in artificial sputum medium (ASM+ [10]), reflecting a clinically relevant environment. Selected for their broad host range [11,12] and demonstrated activity against single-species biofilms [12,13], these phages were combined with broad-spectrum antibiotics routinely used in patients with CF.

2. Materials and methods

2.1. Bacteria, phages, and antibiotics

The reference strains *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1 were used. Phage isolation and propagation techniques were described elsewhere [12]. Phage PSP3, active on *P. aeruginosa* (de novo isolated at the Queen Astrid Military Hospital [12]), and Phage ISP, active on *S. aureus* (Eliava Institute, Tbilisi, Georgia [11]) were selected for their activity on these strains. Ciprofloxacin, ceftazidime, meropenem and tobramycin (potency: 89%, 72%, 92% and 100%, respectively) were obtained from Bayer (Leverkusen, Germany), Panpharma (Luitré-Dompierre, France), Fresenius Kabi (Schelle, Belgium) and Galephar (Marche-en-Famenne, Belgium), respectively, and selected as representative broad-spectrum antibiotics active on both species.

2.2. Dual-species biofilms

Dual species biofilms were formed in artificial sputum medium (ASM+; see [10] for composition) based on a previously published protocol [14]. Briefly, *S. aureus* colonies from an overnight culture were suspended in cation-adjusted Mueller Hinton Broth (MHB-ca; Sigma-Aldrich), adjusted to a turbidity of 2.6 McFarland units, and diluted to approximately 2.1 10^7 CFU/mL. Two hundred µL of this suspension were inoculated in 96-well plates, which were incubated at 37°C for 2 h to allow bacterial adhesion. MHB-ca was then replaced with ASM+ and plates were further incubated at 37°C for 24 h. The medium was removed and replaced with 20 µL of *P. aeruginosa* suspension (from an overnight culture diluted in MHB-ca to approx. 1.35 10^7 CFU/mL) and 180 µL ASM+. Biofilms were then grown at 37°C for 48 h with refreshment of the medium after 24 h, in order to obtain a mature, stable dual species biofilm.

2.3. Antibiotic and phage activity on planktonic bacteria

MICs were determined in MHB-ca according to CLSI guidelines. Phage activity was determined by spot assay and double agar assay [12], and their effect on bacterial growth was determined in liquid culture in a 96-well plate reader OmniLog system (Biolog, Hayward, CA), by following the change in colour intensity upon reduction of a tetrazolium dye as a proxy for bacterial respiration. A bacterial suspension in PBS was adjusted to an OD_{600nm} of 0.5 and diluted 10-fold in lysogeny broth (LB) or in ASM+ containing a 100-fold diluted tetrazolium dye (average inoculum, 10^7 CFU/mL). Individual phages were added at different Multiplicities of Infection (MOI; PFU/CFU ratio) and colour intensity was read every 15 min at 37°C and converted to relative respiration units, with data analysis performed using the Omnilog Data Analysis Software 1.7.

2.4. Antibiotic and phage activity on dual species biofilms

ASM+ medium from mature biofilms was removed and replaced with ASM+ containing individual antibiotics alone or combined with a cocktail containing PSP3 and ISP, each at 10^9 PFU/mL. After 24 h of incubation at 37°C, the medium was removed, and biofilms were washed once with PBS. To measure residual CFU, biofilms were disrupted by mechanical scratching, resuspended in 200 µL of PBS, and sonicated (30 s at 60% amplitude; Q700; QSonica, Newton, CT) to release cells and disaggregate the biofilm structure. Aliquots were serially diluted, and plated onto Mannitol Salt Agar (MSA; peptone 5 g/L, NaCl 75 g/L, D-mannitol 10 g/L, agar 15 g/L) or *Pseudomonas* Isolation Agar (PIA; Sigma-Aldrich) to allow the selective counting of *S. aureus* and *P. aeruginosa* CFU, respectively. Residual biomass (which comprises cellular and noncellular fractions of biotic origin, as well as inorganic matter in the biofilm [15]) was measured after crystal violet staining [10].

2.5. Statistical analysis

Statistical analyses (2-way ANOVA with Tukey or Sidak post-hoc tests) were performed with GraphPad Software (version 10.1.2; GraphPad Software, San Diego, CA).

3. Results

3.1. Antibiotic susceptibility testing

Table 1 shows the MICs of antibiotics against *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1. In subsequent experiments, antibiotics were used at 1, 10, and 100 times the MIC of the least susceptible isolate in this pair.

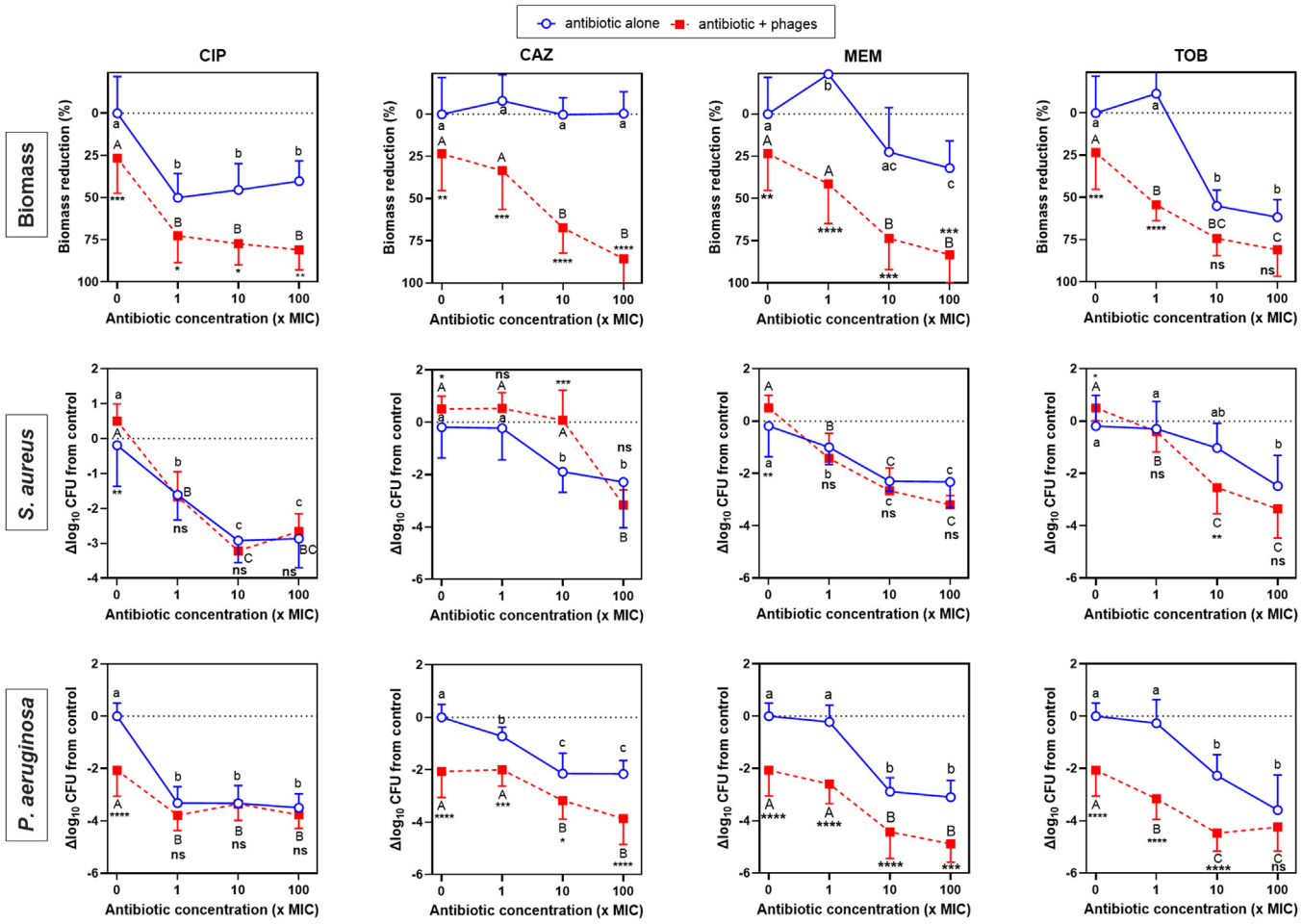

Fig. 1. Activity of antibiotics and phages against 48 h dual species biofilms formed by reference strains *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1. Biofilms were incubated during 24 h in control conditions or with antibiotics at 1, 10, or 100 x MIC of the less susceptible strain in the pair (ciprofloxacin [CIP], ceftazidime [CAZ], meropenem [MEM], or tobramycin [TOB], a cocktail of phages ISP and PSP3 each at 10^9 PFU/mL or the combination of one antibiotic with the phage cocktail. The successive rows show the reduction in biomass expressed in percentage of the crystal violet OD_{570nm} measured for non-treated biofilms (top), the reduction in viable counts of *S. aureus* (middle) or *P. aeruginosa* (bottom), both expressed in $\Delta\log_{10}$ CFU from control. Control values were 1.4 ± 0.35 (biomass), 5.8 ± 0.7 $\log_{10}$ CFU/mL for *S. aureus* and 8.7 ± 0.3 $\log_{10}$ CFU/mL for *P. aeruginosa.* Statistics: Two-way ANOVA analysis with Tukey post-test. Comparison between antibiotic concentrations: data points with different letters are statistically different from one another ($p < 0.05$; small letters: antibiotic alone; caps letters: antibiotic + phages). Comparison between antibiotic alone and antibiotic + phage at a given antibiotic concentration: ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data are mean ± SD (N≥3; n = 8).

3.2. Activity of phages and antibiotics against dual species biofilms

The kinetics of growth of the dual species biofilm illustrated in Fig. S1 suggests a predominance of *P. aeruginosa* with respect to both CFU counts and biomass. Fig. 1 shows the activity of individual antibiotics alone or combined with the phage cocktail against 48 h dual species biofilms.

Considering first the treatment effects on biomass, ceftazidime was ineffective, while meropenem reduced it by approx. 20% (at concentrations ≥ 10 times MIC), and tobramycin and ciprofloxacin, of approx. 50% (at concentrations ≥ 10 and 1 time the MIC, respectively), with no improvement when their concentration was increased. Phages alone caused a 23% reduction in biomass. Phage-antibiotic combinations were vastly more active than antibiotics or phages alone, with a drop of biomass of at least 70% observed when phages were combined with ciprofloxacin at 1 time its MIC and with other antibiotics at 10 times their MIC.

Considering then the treatment effects on bacterial counts, all antibiotics alone caused a concentration-dependent decrease in CFU, which was significant for concentrations ≥ 1 time the MIC for ciprofloxacin but ≥ 10 times the MIC for the other antibiotics, against both strains. At the highest concentration tested, reductions of *S. aureus* and *P. aeruginosa* CFU reached -2.8 and -3.5 $\log_{10}$ for ciprofloxacin, -2.5 and -3.6 $\log_{10}$ for tobramycin, -2.3 and -3.1 $\log_{10}$ for meropenem, and -2.3 and -2.1 $\log_{10}$ for ceftazidime, respectively. Phages alone were inactive on *S. aureus* but reduced *P. aeruginosa* CFU by 2.1 $\log_{10}$. In combination with antibiotics, no improvement was observed against *S. aureus* (except with tobramycin at 10 times the MIC), but a globally additive effect was observed against *P. aeruginosa* except with ciprofloxacin (at all concentrations) and tobramycin (at 100 times the MIC), i.e., in conditions for which the antibiotic alone was already highly effective (decrease of 3.5 $\log_{10}$).

3.3. Inhibition by phages of bacterial respiration in planktonic cultures

As the phage cocktail improved antibiotic activity against *P. aeruginosa* but not *S. aureus* despite an efficiency of plating close to 1, we further characterised individual phage activity against planktonic bacteria. To this effect, we followed the respiration rate of each bacterium over time in the presence of individual phages in both LB and ASM+. Both phages remained stable over time in both

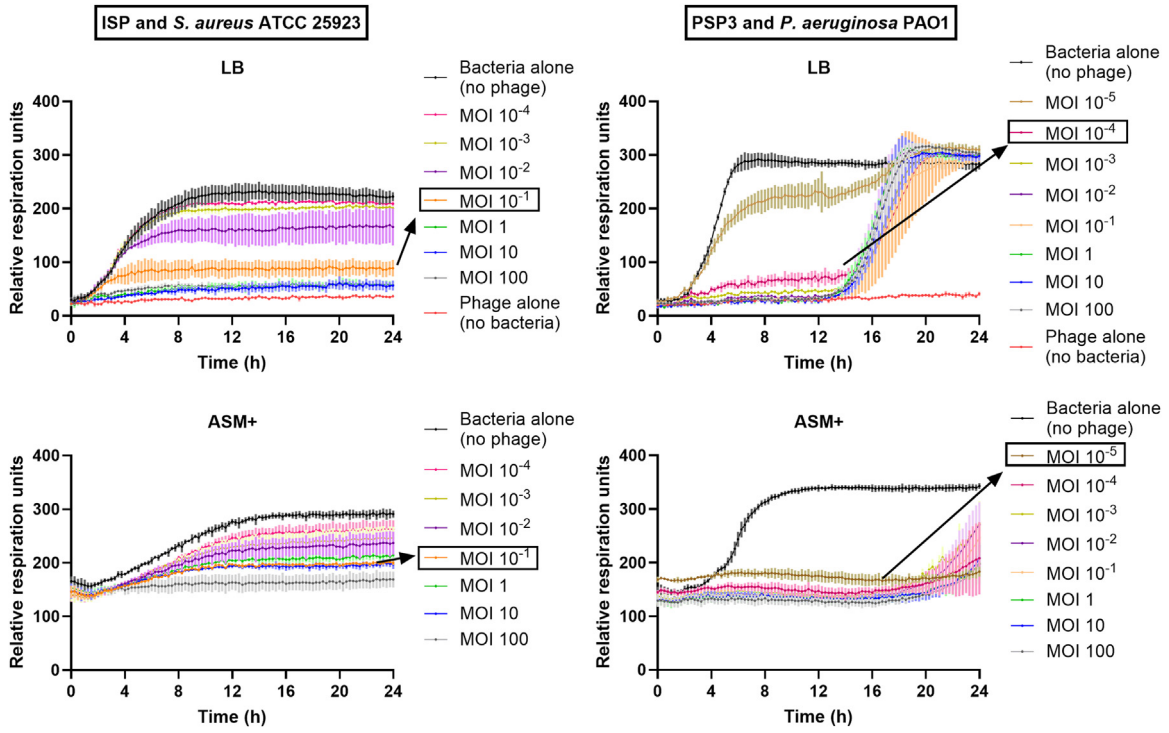

Fig. 2. Activity of phages ISP and PSP3 against planktonic cultures of *S. aureus* ATCC 25923 (left) or *P. aeruginosa* PAO1 (right), respectively. Bacteria (initial inoculum: 10^7 CFU/mL) were incubated during 24 h in control conditions or with phages at different MOI in LB (top) or ASM+ (bottom) media. A negative control (phages in the absence of bacteria) and a positive control (bacteria in the absence of phages) were also included. Colour intensity was measured every 15 minutes to follow the reduction of a tetrazolium dye by metabolic active bacteria. Data are mean ± SD (n = 3). The arrow and box in each panel point to the lowest MOI for which an almost complete inhibition of bacterial respiration is observed for at least 12 h.

media, and bacterial growth was slightly lower in ASM+ *vs.* LB (Fig. S2). In both media, phage ISP completely inhibited the respiration of *S. aureus* ATCC 25923 at a MOI > 0.1. Its activity decreased progressively with dilution and vanished at MOI of 10^{-5}. Phage PSP3 totally prevented PAO1 respiration during 14 h at MOI ≥ 10^{-4} in LB, but inhibition remained stable over 20 h in ASM+ for MOI 10^{-5} (Fig. 2).

4. Discussion

Using a dual species biofilm cultivated in a highly viscous medium reminiscent of the conditions prevailing in the lungs of patients with CF [10], we found that antibiotics exhibit some degree of activity, particularly when used at elevated concentrations, not clinically achievable for drugs given by parenteral administration (Table 1). Interestingly, this activity is higher than that observed in the corresponding mono-species biofilms [10,16], as previously described for broad-spectrum antibiotics in a much simpler dual species biofilm model [17]. When combined with a cocktail comprising two phages respectively active on *S. aureus* and *P. aeruginosa,* antibiotic activity was improved against *P. aeruginosa* counts, and biomass was further reduced. The lack of effect against *S. aureus* was unexpected, considering that the phage ISP was active against *S. aureus* ATCC 25923 in double agar assay as well as on planktonic cultures grown in ASM+, and showed activity against single species biofilms grown in much less complex medium [13].

One potential reason for decreased phage activity against biofilms may be attributed to the barrier effect of the mucoid environment in the artificial sputum medium. To the best of our knowledge, phage activity against dual species biofilms in this medium has not been previously evaluated, and the influence of mucus on phage activity is still debated [9]. While one study indicates that a phage cocktail remains active against *P. aeruginosa* in CF sputum samples [5], there are no data for *S. aureus.* The presence of mucus is suggested to enhance phage proximity to bacteria and persistence at the infection site [18], while intestinal mucus has been reported to impair phage killing capacity [19]. We show here that phages remain stable and as active in ASM+ as in conventional LB medium against planktonic bacteria. Interestingly, phage activity against *S. aureus* is hindered in human plasma that promotes bacterial aggregation, masking phage surface receptors [20], a phenomenon that may also occur in biofilms due to self-aggregation of bacteria.

Another factor contributing to reduced phage activity may therefore arise from the biofilm lifestyle, which confers antibiotic tolerance due to decreased bacterial metabolism and the protective matrix barrier. Phage activity against dual species biofilm is not well-characterised, particularly in conditions resembling those found in patients with CF. Many studies focus on *P. aeruginosa* using CF isolates often cultivated in conventional culture media. Martin *et al.* observed enhanced reduction in biomass and bacterial counts with a combination of antibiotics and a four-phage cocktail [7]. In contrast, Fiscarelli et al. [8] rather found stimulation of pseudomonal biofilms exposed to either sub-inhibitory concentrations of antibiotics or several phages but significant reduction of biomass in biofilms treated with phages and tobramycin. Chang et al. [6] reported synergistic interactions between phage PEV20 and ciprofloxacin against *P. aeruginosa* biofilms whereas phages alone were not active. Regarding staphylococci, studies mainly relate to implant-related biofilm infections, where the matrix effect impedes phage activity, particularly against *S. epidermidis* [21].

Studies on dual species biofilms indicate that synergy between antibiotics and phages can occur, though not consistently, and is notably influenced by factors such as the concentrations of phages

and antibiotics, their simultaneous or sequential administration, and the frequency of phage applications over time [22]. Higher synergy is observed when both active agents are present at suboptimal concentrations. In this context, it is worth mentioning that, in combination with phages, antibiotics at 1 time the MIC (i.e., easily clinically achievable concentrations; see Table 1) reduce *P. aeruginosa* counts and biomass to an extent similar to that observed for antibiotics alone at 100 times the MIC (i.e. supratherapeutic concentrations for drugs given IV or orally). This suggests the clinical potential of a combined treatment modality.

Our phage cocktail achieved a 2 $\log_{10}$ CFU reduction for PAO1 only, along with a 20–25% reduction in biofilm biomass, and these effects were markedly amplified when combined with antibiotics. Of note, neither of these two phages produces depolymerases [11,12], that is, enzymes hydrolysing biofilm matrix polymers. A recent review compiling all existing data on phage activity against biofilms [23] suggests that an increased burst size, phage inoculum, and infection time lead to more significant biomass reduction, which aligns with our findings. Conversely, CFU reduction is typically observed with phages possessing smaller genomes that can replicate more rapidly, a characteristic not met by our phages with genome sizes of 138,715 bp for ISP and 66,308 bp for PSP3 [11,12].

In this context, assessing phage activity against planktonic cultures by continuous monitoring of bacterial respiration provides valuable insights. Previous research indicates that a disparity might be observed between phage activity measured by plaque assay and their efficacy on biofilms [7]. We noticed that ISP requires a higher MOI to prevent *S. aureus* ATCC 25923 growth in planktonic cultures compared to PSP3 against *P. aeruginosa* PAO1. Given that MOI ranged from 1000 to 10 at the time of phage addition to biofilms (10^9 PFU added to biofilms containing approx. 10^6 and 10^8 CFU/mL of *S. aureus* and *P. aeruginosa,* respectively), our findings suggest that effective action on biofilms in ASM+ requires phages to exhibit efficacy at considerably lower MOI against planktonic bacteria, emphasising the challenge of their low bioavailability in this complex biofilm model. This also suggests that the concept of tolerance in biofilms may extend to phages, not just antibiotics. Additionally, it is worth noting that the use of phage PSP3 at low titers and without simultaneous antibiotic was effective only for 14 h in LB, possibly indicating resistance development when the phage is used alone.

Our study has some limitations. Firstly, we only used two strains and two phages, due to the complexity and labor-intensive nature of our biofilm model, which makes high-throughput screening impractical. This also explains why we did not test various treatment application conditions, despite their potential impact on the results. Nevertheless, our data suggests that a future experimental strategy could involve pre-screening phage effect on bacterial growth in planktonic cultures before biofilm testing. Lastly, we did not extensively examine the biofilm matrix composition or the phage's ability to penetrate biofilms, as these complex analyses would represent an independent undertaking beyond the scope of the present study.

In a recent position paper [24], the taskforce of the Antibacterial Resistance Leadership Group (ARLG) underscored a series of knowledge gaps in the field of phage therapy. These include the lack of consensus on optimal evaluation methodologies to evaluate phages or phage-antibiotic combinations to predict their clinical efficacy, the need to characterise their activity against biofilms, or the necessity of considering how the environment at the site of infection may influence their activity. These considerations are crucial as we await data from well-conducted clinical trials. In this context, our study pioneers the evaluation of phage-antibiotic combinations against relevant dual-species biofilms in an in-vitro model relevant of CF. The established model and evaluation process can now be applied to clinical isolates, and the identified pitfalls may guide further investigations to evaluate alternative phages or treatment protocols with optimised efficacy.

Declarations

Funding: This work was supported by the Belgian Fonds de la Recherche Scientifique (FNRS-FRS) grant J.0177.23 to F.V.B and by a grant from the King Baudouin Foundation (Fonds Forton) and the Belgian Mucovereniging/Association Muco to F.V.B. and J.P.P. Z.W. was recipient of a Chinese Scholarship Council (CSC) PhD grant and was further supported by the King Baudouin Foundation (Fonds Forton) and the Belgian Mucovereniging/Association Muco; S.D.S received a Doctiris doctoral fellowship from Innoviris (grant 2019-PHD-9). A. G. is supported by a grant from the King Baudouin Foundation (Fonds Forton) and the Belgian Mucovereniging/Association Muco. M.M. is supported by the Royal Higher Institute for Defence. F.V.B. is Research Director from the Belgian FNRS-FRS.

Competing interest: The authors have no conflict of interest to declare.

Ethical approval: Not applicable.

Sequence Information: Not applicable.

CRediT authorship contribution statement

Zhifen Wang: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Steven De Soir:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Antoine Glorieux:** Investigation, Formal analysis, Writing – review & editing. **Maya Merabishvili:** Resources, Methodology, Writing – review & editing. **Christiane Knoop:** Writing – review & editing. **Daniel De Vos:** Methodology, Funding acquisition, Writing – review & editing. **Jean-Paul Pirnay:** Methodology, Funding acquisition, Writing – review & editing. **Françoise Van Bambeke:** Conceptualization, Formal analysis, Funding acquisition, Writing – original draft.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2024.107276.

References

[1] Guo J, Garratt A, Hill A. Worldwide rates of diagnosis and effective treatment for cystic fibrosis. J Cyst Fibros 2022;21(3):456–62. doi:10.1016/j.jcf.2022.01.009.
[2] Thornton CS, Parkins MD. Microbial epidemiology of the cystic fibrosis airways: past, present, and future. Semin Respir Crit Care Med 2023;44(2):269–86. doi:10.1055/s-0042-1758732.
[3] Cystic_Fibrosis_Foundation. Cystic Fibrosis Foundation Patient Registry Annual Data Report 2018. 2019.
[4] Lusiak-Szelachowska M, Miedzybrodzki R, Drulis-Kawa Z, Cater K, Knezevic P, Winogradow C, et al. Bacteriophages and antibiotic interactions in clinical practice: what we have learned so far. J Biomed Sci 2022;29(1):23. doi:10.1186/s12929-022-00806-1.
[5] Saussereau E, Vachier I, Chiron R, Godbert B, Sermet I, Dufour N, et al. Effectiveness of bacteriophages in the sputum of cystic fibrosis patients. Clin Microbiol Infect 2014;20(12):O983–90. doi:10.1111/1469-0691.12712.
[6] Chang RYK, Das T, Manos J, Kutter E, Morales S, Chan HK. Bacteriophage PEV20 and ciprofloxacin combination treatment enhances removal of pseudomonas aeruginosa biofilm isolated from cystic fibrosis and wound patients. AAPS J 2019;21(3):49. doi:10.1208/s12248-019-0315-0.
[7] Martin I, Morales S, Alton E, Davies JC. Lytic bacteriophage is a promising adjunct to common antibiotics across cystic fibrosis clinical strains and culture models of pseudomonas aeruginosa infection. Antibiotics (Basel) 2023;12(3):593. doi:10.3390/antibiotics12030593.

[8] Fiscarelli EV, Rossitto M, Rosati P, Essa N, Crocetta V, Di Giulio A, et al. In vitro newly isolated environmental phage activity against biofilms preformed by pseudomonas aeruginosa from patients with cystic fibrosis. Microorganisms. 2021;9(3):478. doi:10.3390/microorganisms9030478.
[9] Ling KM, Stick SM, Kicic A. Pulmonary bacteriophage and cystic fibrosis airway mucus: friends or foes? Front Med (Lausanne) 2023;10:1088494. doi:10.3389/fmed.2023.1088494.
[10] Diaz Iglesias Y, Van Bambeke F. Activity of antibiotics against pseudomonas aeruginosa in an in vitro model of biofilms in the context of cystic fibrosis: influence of the culture medium. Antimicrob Agents Chemother 2020;64(4):e02204–19. doi:10.1128/AAC.02204-19.
[11] Vandersteegen K, Mattheus W, Ceyssens PJ, Bilocq F, De Vos D, Pirnay JP, et al. Microbiological and molecular assessment of bacteriophage ISP for the control of Staphylococcus aureus. PLoS One 2011;6(9):e24418. doi:10.1371/journal.pone.0024418.
[12] De Soir S, Paree H, Kamarudin NHN, Wagemans J, Lavigne R, Braem A, et al. Exploiting phage-antibiotic synergies to disrupt Pseudomonas aeruginosa PAO1 biofilms in the context of orthopedic infections. Microbiol Spectr 2023:e0321923. doi:10.1128/spectrum.03219-23.
[13] Vandersteegen K, Kropinski AM, Nash JH, Noben JP, Hermans K, Lavigne R. Romulus and Remus, two phage isolates representing a distinct clade within the Twortlikevirus genus, display suitable properties for phage therapy applications. J Virol 2013;87(6):3237–47. doi:10.1128/JVI.02763-12.
[14] Woods PW, Haynes ZM, Mina EG, Marques CNH. Maintenance of S. aureus in co-culture with P. aeruginosa while growing as biofilms. Front Microbiol. 2018;9:3291. doi:10.3389/fmicb.2018.03291.
[15] Klopper KB, de Witt RN, Bester E, Dicks LMT, Wolfaardt GM. Biofilm dynamics: linking in situ biofilm biomass and metabolic activity measurements in real-time under continuous flow conditions. NPJ Biofilms Microbiomes 2020;6(1):42. doi:10.1038/s41522-020-00153-9.
[16] Diaz Iglesias Y, Wilms T, Vanbever R, Van Bambeke F. Activity of antibiotics against staphylococcus aureus in an in vitro model of biofilms in the context of cystic fibrosis: influence of the culture medium. Antimicrob Agents Chemother 2019;63(7):e00602–19. doi:10.1128/AAC.00602-19.
[17] Trizna EY, Yarullina MN, Baidamshina DR, Mironova AV, Akhatova FS, Rozhina EV, et al. Bidirectional alterations in antibiotics susceptibility in Staphylococcus aureus-Pseudomonas aeruginosa dual-species biofilm. Sci Rep 2020;10(1):14849. doi:10.1038/s41598-020-71834-w.
[18] Barr JJ, Auro R, Sam-Soon N, Kassegne S, Peters G, Bonilla N, et al. Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. Proc Natl Acad Sci U S A 2015;112(44):13675–80. doi:10.1073/pnas.1508355112.
[19] Green SI, Gu Liu C, Yu X, Gibson S, Salmen W, Rajan A, et al. Targeting of mammalian glycans enhances phage predation in the gastrointestinal tract. mBio 2021;12(1):e03474 -20. doi:10.1128/mBio.03474-20.
[20] Shinde P, Stamatos N, Doub JB. Human plasma significantly reduces bacteriophage infectivity against staphylococcus aureus clinical isolates. Cureus 2022;14(4):e23777. doi:10.7759/cureus.23777.
[21] Melo LDR, Pinto G, Oliveira F, Vilas-Boas D, Almeida C, Sillankorva S, et al. The Protective Effect of Staphylococcus epidermidis Biofilm Matrix against Phage Predation. Viruses 2020;12(10):1076. doi:10.3390/v12101076.
[22] Akturk E, Melo LDR, Oliveira H, Crabbe A, Coenye T, Azeredo J. Combining phages and antibiotic to enhance antibiofilm efficacy against an in vitro dual species wound biofilm. Biofilm 2023;6:100147. doi:10.1016/j.bioflm.2023.100147.
[23] Meneses L, Brandao AC, Coenye T, Braga AC, Pires DP, Azeredo J. A systematic review of the use of bacteriophages for in vitro biofilm control. Eur J Clin Microbiol Infect Dis 2023;42(8):919–28. doi:10.1007/s10096-023-04638-1.
[24] Suh GA, Lodise TP, Tamma PD, Knisely JM, Alexander J, Aslam S, et al. Considerations for the use of phage therapy in clinical practice. Antimicrob Agents Chemother 2022;66(3):e0207121. doi:10.1128/AAC.02071-21.
[25] Reed MD, Stern RC, Myers CM, Yamashita TS, Blumer JL. Lack of unique ciprofloxacin pharmacokinetic characteristics in patients with cystic fibrosis. J Clin Pharmacol 1988;28(8):691–9. doi:10.1002/j.1552-4604.1988.tb03202.x.
[26] Stass H, Weimann B, Nagelschmitz J, Rolinck-Werninghaus C, Staab D. Tolerability and pharmacokinetic properties of ciprofloxacin dry powder for inhalation in patients with cystic fibrosis: a phase I, randomized, dose-escalation study. Clin Ther 2013;35(10):1571–81. doi:10.1016/j.clinthera.2013.08.003.
[27] Turner A, Pedler SJ, Carswell F, Spencer GR, Speller DC. Serum and sputum concentrations of ceftazidime in patients with cystic fibrosis. J Antimicrob Chemother 1984;14(5):521–7. doi:10.1093/jac/14.5.521.
[28] Paal M, Scharf C, Denninger AK, Ilia L, Kloft C, Kneidinger N, et al. Target site pharmacokinetics of meropenem: measurement in human explanted lung tissue by bronchoalveolar lavage, microdialysis, and homogenized lung tissue. Antimicrob Agents Chemother 2021;65(12):e0156421. doi:10.1128/AAC.01564-21.

Bacteriophages as potential antibiotic potentiators in cystic fibrosis:

A new model to study the combination of antibiotics with a bacteriophage cocktail targeting dual species biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Zhifen Wang[1,$], Steven De Soir[1,2,$], Antoine Glorieux[1,2], Maia Merabishvili[2], Christiane Knoop[3], Daniel De Vos[2], Jean-Paul Pirnay[2], Françoise Van Bambeke[1,*]

[1] Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium
[2] Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Neder-over-Heembeek, Belgium
[3] Erasme Hospital, Université libre de Bruxelles, Brussels, Belgium

[$] equal contribution as first authors

SUPPLEMENTARY MATERIAL

Figure S1

Kinetics of formation of single species biofilms of *S. aureus* ATCC 25923 or *Pseudomonas aeruginosa* PAO1 or of dual species biofilms including both strains in ASM+. *S. aureus* was inoculated at approx. 2.1 10^7 CFU/mL in MHB-ca and allowed to adhere for 2 h at 37°C, in order to reach ≥ 10^6 CFU/mL of adhering bacteria (6.45 ± 0.5 10^6). MHB-ca was then replaced with ASM+ and plates were further incubated at 37°C with daily renewal of the medium. For dual species biofilms, *P. aeruginosa* suspension (at approx. 1.35 10^6 CFU/mL in ASM+) was added 24 h after *S. aureus.*

These conditions were optimized in order to reach stable bacterial counts and biomass in the dual species biofilms for both species over time with the strains used in this study.

a-b: CFU counts for *S. aureus* and *P. aeruginosa*, respectively; **c:** biomass, evaluated by the absorbance of crystal violet.

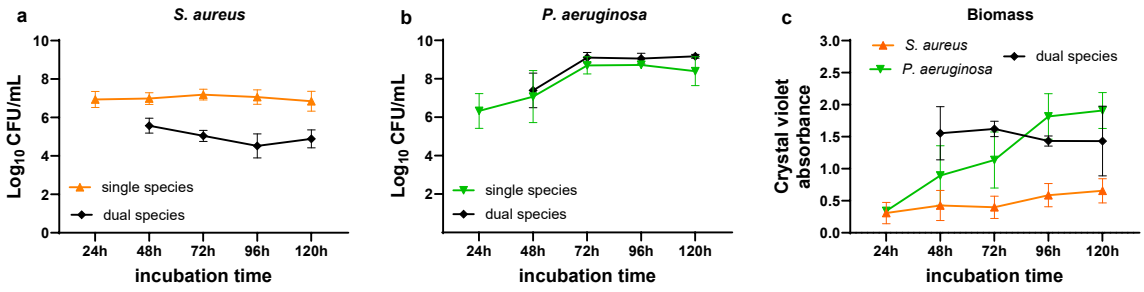

Figure S2

Comparison of CFU (bacteria) and (PFU (phages) over time of incubation in LB or ASM+.
Bacterial growth after 24 h is significantly lower in ASM+ as compared to LB (2-way ANOVA with Sidak post-hoc test; n=3); no statistical difference observed for PFU number between both media (n=2).