Journal of Microbiological Methods 243 (2026) 107441

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Comparison of agar-based and checkerboard methods to assess phage–antimicrobial synergy in *Pseudomonas aeruginosa*

Umut Yılmaz *, Mehmet Ünlü, Gülhan Vardar Ünlü

Department of Medical Microbiology, Faculty of Medicine, Balikesir University, Balikesir 10145, Türkiye

ARTICLE INFO

Keywords:
Bacteriophage
Antibiotic
Peptide
Synergy
Pseudomonas aeruginosa

ABSTRACT

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* requires innovative therapeutic approaches. In this study, two lytic bacteriophages (BAUNPA1, BAUNPA3) were isolated from wastewater in Türkiye, and the combined efficacy of these phages with antibiotics (ciprofloxacin, colistin, meropenem, tobramycin) or antimicrobial peptides (murepavadin, pexiganan) was evaluated *in vitro*.Phages were characterized morphologically by electron microscopy and genotypically by restriction fragment length polymorphism, and their different identities were confirmed. Biological evaluation showed rapid replication (latent period: 30 min; burst sizes: 189 and 43 PFU/cell), lytic activity against clinical MDR isolates, and stability under physiological conditions (pH 6–8; temperature $\leq$ 50 °C). Synergy was assessed using modified double-layer agar and checkerboard assays under simultaneous and sequential applications. The checkerboard assay showed that synergy was significantly enhanced under phage-first application. BAUNPA1 showed strong synergy with meropenem (FICI $\leq$0.5) and colistin, while BAUNPA3 showed synergy with murepavadin and pexiganan. Agar-based methods have shown variable results, as plaque morphology does not always correlate quantitatively synergy. This study provides a comparative *in vitro* framework for assessing phage-antimicrobial interactions and, for the first time, reports reproducible synergy between intact lytic phages and murepavadin or pexiganan, highlighting the importance of methodological approach and application timing in synergy assessments.

1. Introduction

Pseudomonas aeruginosa is a leading cause of nosocomial infections, particularly in immunocompromised patients, characterized by its intrinsic antibiotic resistance, biofilm formation capacity, and rapid acquisition of resistance mechanisms (Qin et al., 2022; Juan et al., 2017). The escalating prevalence of MDR *P. aeruginosa* strains has substantially limited therapeutic options, with surveillance data indicating resistance rates exceeding 30% to key antibiotic classes including fluoroquinolones, carbapenems, and extended-spectrum penicillins (Cosentino et al., 2023; Horcajada et al., 2019). This resistance crisis necessitates the exploration of alternative therapeutic strategies, including phage-based interventions.

First introduced by Félix d'Hérelle in 1917 and widely used in the pre-antibiotic era, phages were used to treat various bacterial infections until their use declined with the advent of antibiotics (Summers, 2001). Bacteriophage therapy has re-emerged as a promising adjunct therapy to conventional antibiotics, particularly in infections where standard treatments have been ineffective (Hatfull et al., 2022; Suh et al., 2022). *Pseudomonas*-specific bacteriophages have been isolated since the early 20th century, with wastewater sources (especially hospital and municipal sewers) being the most productive reservoirs (Holloway et al., 1960). Today, most known *Pseudomonas* phages belong to the Caudovirales order. The emergence and global spread of MDR *P. aeruginosa* has revived interest in the antibacterial potential of lytic phages in planktonic cultures, leading to numerous *in vitro* studies demonstrating their rapid and strain-specific lytic capabilities (Holger et al., 2022). Lytic phages with broad host ranges are known to induce rapid bacterial reduction and intermittent lysis patterns; however, transient host adaptations and the emergence of phage-resistant subpopulations highlight the need for phage cocktails with broad host coverage (Knezevic et al., 2013). Consequently, combining multiple lytic phages has become a widely adopted strategy to enhance therapeutic resistance against genetically diverse *P. aeruginosa* strains. In parallel, the co-administration of phages with antibiotics has garnered significant interest. Studies evaluating the combination of *P. aeruginosa*-specific phages with aminoglycosides, β-lactams, quinolones, and polymyxins have shown that some phage-antibiotic pairings can produce synergistic

* Corresponding author.
E-mail address: umut.mikrobiyoloji0@gmail.com (U. Yılmaz).

https://doi.org/10.1016/j.mimet.2026.107441
Received 19 September 2025; Received in revised form 13 February 2026; Accepted 21 February 2026
Available online 25 February 2026
0167-7012/© 2026 Elsevier B.V. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

antibacterial effects (Hall et al., 2012; Nicholls et al., 2023).

Phage-antibiotic synergy (PAS) represents a promising therapeutic strategy with proven efficacy in numerous phage-antibiotic pairings (Dedrick et al., 2023). The growing clinical interest in PAS is rooted in decades of compassionate use experience, particularly in Georgia, Poland, and Israel, where structured programs have informed regulatory and research frameworks for phage therapy (Kutateladze, 2015; Nir-Paz et al., 2019; Yerushalmy et al., 2020; Uyttebroek et al., 2022). Despite this, the absence of a standardized methodological framework results in substantial methodological variations and inconsistent findings across studies assessing PAS (Glonti and Pirnay, 2022). Commonly used techniques include broth microdilution method, checkboard assay, agar dilution methods, and disk diffusion tests (Holger et al., 2022; Fatima and Hynes, 2025). Fundamental research on bacteriophage-antibiotic co-administration was conducted by Neter and Clark (1944), using a lytic *Staphylococcus aureus* phage in combination with penicillin. After a long period of limited research, Huff et al. (2004) revived *in vivo* research by demonstrating therapeutic synergy between an *Escherichia coli*-specific lytic phage and enrofloxacin in an avian model. Subsequently, Comeau et al. (2007) a significant conceptual advance by reported that sub-MIC concentrations of some antibiotics can significantly increase the production of lytic phages infecting *E. coli*, formally terming this phenomenon PAS. To quantify antimicrobial interactions, the time-kill curve assay is widely regarded as the standard approach. Its first application to validate PAS was demonstrated by Knezevic et al. (2013). Although it provides robust experimental validation, this method is labor-intensive and time-consuming, making it less practical for rapid and high-throughput screening of potential synergistic combinations. This situation has led to efforts to develop more accessible screening tools. In particular, Nikolic et al. (2022) conducted an extensive study to optimize the checkerboard method for evaluating phage-antimicrobial interactions. The researchers validated their optimized experiments against the time-kill curve standard and further extended the analysis to investigate the synergy dynamics of agents under both simultaneous and sequential applications.

This study characterized two lytic *P. aeruginosa* bacteriophages and evaluated their synergistic potential with four conventional antibiotics [ciprofloxacin (CIP), colistin (COL), meropenem (MEM), tobramycin (TOB)] and two peptides [murepavadin (MUR), pexiganan (PEX)]. Using complementary *in vitro* methods, including modified double-layer agar (MDLA) and checkerboard assays, our findings demonstrate that these approaches provide practical, reproducible tools for the rapid prescreening of promising phage-antibiotic/peptide combinations for therapeutic applications.

2. Materials and methods

2.1. Bacterial isolates, media, and routine culturing

A total of 75 clinical isolates were included: 50 *P. aeruginosa*, and 5 each of *E. coli, Stenotrophomonas maltophilia, Klebsiella pneumoniae, Acinetobacter baumannii, and Staphylococcus aureus*. Isolates were recovered from clinical specimens at the Microbiology Laboratory of Balıkesir University Faculty of Medicine, Türkiye. Species identification was performed using the Phoenix M50 system (BD, USA). *P. aeruginosa* ATCC 27853 was used for quality control. Antimicrobial susceptibility testing was conducted on Müeller-Hinton agar (Oxoid, UK) at 37 °C. For phage assays, Luria-Bertani (LB) agar (1.5% *w/v*) and LB soft agar (0.5% w/v) (Merck, Germany) were used following standard protocols. All bacterial strains were stored long-term at −80 °C in LB broth supplemented with 20% (*v*/v) glycerol. For routine use, strains were revived by streaking onto LB agar plates to obtain single, isolated colonies. Several colonies were then inoculated into 10 mL of LB broth and cultured overnight at 37 °C with shaking at 200 rpm.

The antibiotics (CIP, COL, MEM, TOB) and the peptide MUR were obtained as pure compounds (active pharmaceutical ingredients) from MedChemExpress. The synthetic magainin-II analog PEX was obtained from Magainin Pharmaceuticals Inc. Commercially prepared antibiotic susceptibility test disks for disk diffusion tests [CIP (5 μg), COL (10 μg), MEM (10 μg), TOB (10 μg)] were obtained from Oxoid Ltd.

2.2. Bacteriophage isolation and enrichment

Bacteriophages BAUNPA1 and BAUNPA3 were isolated from wastewater samples collected in Balıkesir, Türkiye, under sterile conditions. For initial processing, 10 mL of each sample was centrifuged at 5000 ×*g* for 10 min at 4 °C, and the supernatants were filtered through 0.22 μm pore-size membranes (Sartorius Stedim Biotech, Germany) to eliminate bacterial and particulate contaminants. Enrichment was performed as previously described with minor modifications, by mixing 1 mL of the filtrate with 7 mL of sterile LB broth and 2 mL of overnight cultures of *P. aeruginosa* clinical isolates, selected as indicator hosts (Van Twest and Kropinski, 2009). The mixtures were incubated at 37 °C with gentle shaking for 18–24 h. Following incubation, cultures were centrifuged at 10,000 ×*g* for 10 min at 4 °C and re-filtered through 0.22 μm filters to obtain crude phage lysates. These lysates were stored at 4 °C and used in subsequent plaque assays and host range testing.

2.3. Purification and titration of isolated bacteriophages

Purification of BAUNPA1 and BAUNPA3 was performed using the DLA method through repeated single-plaque isolation as described by Yang et al., 2010. Serial 10-fold dilutions of crude lysates were prepared in sterile SM buffer (100 mM NaCl, 8 mM $MgSO_4{\cdot}7H_2O$, 50 mM Tris-HCl, pH 7.5). For each dilution, 100 μL of phage suspension was mixed with 100 μL of overnight *P. aeruginosa* host culture and 4 mL of 0.5% LB soft agar. The mixture was overlaid onto LB agar (1.5% agar, 5 mM $CaCl_2$) and incubated overnight at 37 °C. Well-isolated plaques with consistent morphology were picked and propagated in fresh LB broth with host bacteria. After overnight incubation, cultures were centrifuged (5000 ×*g*, 10 min, 4 °C) and filtered (0.22 μm). This cycle was repeated five times to ensure clonal purity. For phage titration, purified lysates were serially diluted, processed with DLA, and incubated overnight. Titers were calculated as PFU/mL based on plaque counts and dilution factors.

2.4. Phage DNA extraction and RFLP-based characterization

Phage DNA extraction and RFLP analysis were performed as described by Salih et al., 2022. High-titer phage suspensions (~10^{10} PFU/mL) were concentrated by centrifugation at 15,000 ×*g* for 120 min at 4 °C, and pellets were resuspended in SM buffer. DNase I (Thermo Fisher Scientific, USA) was added at 1 μg/mL to eliminate bacterial DNA, followed by incubation at 37 °C for 45 min. Enzymatic activity was inactivated at 70 °C for 15 min. Subsequently, Proteinase K (Triogen, Türkiye) was added at 20 μg/mL to lyse phage capsids, and samples were incubated at 56 °C for 45 min. DNA was extracted using the phenol: chloroform:isoamyl alcohol (25,24,1) method and precipitated with ethanol. The purified DNA was dissolved in nuclease-free water and stored at −20 °C. For RFLP, 1 μg of phage DNA was digested with *Hin*dIII (Thermo Fisher Scientific, USA), and fragments were separated on a 1% agarose gel in TAE buffer at 100 V for 30 min.

2.5. Phages morphological characterization by electron microscopy

Morphological analysis of BAUNPA1 and BAUNPA3 phages was performed using transmission electron microscopy (TEM). High-titer phage lysates (~10^{10} PFU/mL) were concentrated by ultracentrifugation at 25,000 ×*g* for 120 min at 4 °C using a fixed-angle rotor (Type 70 Ti, Beckman Coulter, USA). The supernatant was carefully discarded, and the pellet was gently resuspended in 100 μL of sterile SM buffer. For TEM grid preparation, carbon-coated, 300-mesh copper grids (Ted Pella, Inc., USA) were used. A 10 μL portion of the concentrated phage

suspension was applied to the grid and allowed to adsorb for 10 min at room temperature in a dust-free environment. Excess liquid was carefully removed by dabbing the edge of the grid with a piece of ashless filter paper (Whatman No. 1). The grids were then negatively stained with 10 μL of a 2% (*w/v*) uranyl acetate solution. The stain was applied for a full 30 s, after which the excess was immediately absorbed using filter paper. The grids were air-dried for 5 min before imaging. Imaging was performed using a JEOL JEM-1400 Flash transmission electron microscope (JEOL Ltd., Japan). Micrographs were acquired in bright-field mode at an acceleration voltage of 120 kV.

2.6. Host range determination and lytic activity of bacteriophages

The host range and lytic activity of BAUNPA1 and BAUNPA3 were assessed using the agar spot assay, as described by Melo et al., 2016 with minor modifications. A total of 75 clinical isolates were tested, including 50 *P. aeruginosa* strains and 5 isolates each of *E. coli, S. maltophilia, K. pneumoniae, A. baumannii*, and *S. aureus*.

For each assay, 100 μL of overnight bacterial culture was mixed with 5 mL of LB soft agar (0.5%) and overlaid onto LB agar plates (1.5%). Once solidified, 10 μL of purified phage lysate (~10^9 PFU/mL) was spotted onto the bacterial lawn and allowed to absorb for 10–15 min at room temperature. Plates were incubated at 37 °C for 18–24 h. Lytic activity was categorized as strong (clear zones), moderate (turbid zones), or weak (partial clearing). Each phage was tested in duplicate against all isolates, and reproducible lytic profiles were used to define the host specificity and potential therapeutic range.

2.7. Determination of optimal multiplicity of infection and one-step growth parameters

The optimal MOI for BAUNPA1 and BAUNPA3 phages was determined by infecting *P. aeruginosa* host strains (PA1 and PA3) at MOIs of 0, 1, 1, and 10, respectively. After 10 min adsorption at 37 °C, unbound phages were removed (12,000 ×g, 5 min, 4 °C), and bacterial pellets were resuspended in fresh LB and incubated for 4 h. Supernatants were filtered (0.22 μm) and titrated using the double-layer agar method (DLA). The MOI yielding the highest phage titer was defined as optimal (Karaynir et al., 2022).

One-step growth assays were performed to briefly assess latent period and burst size. Host cultures in mid-log phase were infected at an MOI of ~0.1, adsorbed (5 min, 37 °C), centrifuged (15,000 ×*g*, 5 min), washed, and transferred into fresh LB. Samples were taken every 10 min for 90 min, treated with 10% chloroform, and titrated. The latent period was the interval before detectable phage release; burst size was calculated by dividing the final phage titer by the initial number of infected cells.

2.8. Thermal and pH stability of bacteriophages

The thermal and pH stability of BAUNPA1 and BAUNPA3 were evaluated to assess environmental robustness, following modified protocols (Ali et al., 2023; Yazdi et al., 2018). For thermal stability testing, phage lysates (~10^9 PFU/mL) were incubated at 37, 50, 60, 70, 80, and 90 °C, with 4 °C serving as the non-stress control condition, representing a standard storage temperature for bacteriophages. At predetermined intervals (30–180 min), aliquots were collected, serially diluted, and titrated using the DLA method. Viability was expressed as PFU/mL.

For pH stability, SM buffer was adjusted to pH 2, 4, 6, 8, 10, and 12 using hydrochloric acid (0.001–0.1 M HCl) or sodium hydroxide (0.001–0.2 M NaOH), and verified using a digital pH meter (Orion 420 A+, Thermo Scientific, USA). Phage lysates (100 μL) were added to 900 μL of each pH-adjusted buffer and incubated at 37 °C for 3 h. Phage viability was determined by DLA following serial dilution. A pH 7 buffer was used as the neutral control. All tests were performed in triplicate, and results were reported as mean PFU/mL.

2.9. Broth microdilution method

The minimum inhibitory concentrations (MICs) of antibiotics and peptides against 50 clinical *P. aeruginosa* isolates were determined using the broth microdilution method, as previously described (Yilmaz et al., 2024). The assay was conducted in U-bottom 96-well microtiter plates using Cation-Adjusted Mueller-Hinton Broth (CAMHB; Oxoid, UK) as the test medium. Each well initially received 50 μL of CAMHB, and serial two-fold dilutions were prepared to obtain final concentration ranges of 0.0312–64 μg/mL for antibiotics, 0.0155–32 μg/mL for MUR, and 0.125–256 μg/mL for PEX. Bacterial suspensions were adjusted to a 0.5 McFarland standard (approximately 10^8 CFU/mL), then diluted 1:100 in CAMHB to yield 10^6 CFU/mL, and 50 μL of this inoculum was added to each well, resulting in a final bacterial density of 5×10^5 CFU/mL. Each assay included appropriate controls: growth control (CAMHB with bacteria), solvent control (CAMHB with diluent), sterility control (CAMHB with antimicrobial agent only), and medium control (CAMHB alone). The microplates were incubated at 37 °C for 18–24 h, and the MIC was defined as the lowest concentration of the tested agent that completely inhibited visible bacterial growth. All tests were conducted in triplicate, and *P. aeruginosa* ATCC 27853 was included as a quality control strain in accordance with EUCAST 2025 guidelines.

2.10. Determination of phage-antimicrobial synergy

To assess potential synergistic interactions between bacteriophages and antimicrobial agents, two *in vitro* methods were employed: the MDLA assay and the checkerboard microdilution assay. Synergy studies were conducted using four MDR *P. aeruginosa* clinical isolates (PA29, PA34, PA49, and PA50). These strains were specifically chosen because they are susceptible to phage lysis, allowing for the phenotypic assessment of combinatory effects through quantitative changes in plaque diameter and number, while their MDR profile represents a clinically relevant challenge.

2.11. Disk diffusion assay

As previously described, 100 μL of bacteriophage lysate and 100 μL of host bacterial suspension were mixed with 5 mL of molten soft agar (0.5%) and poured onto pre-solidified LB agar plates (Comeau et al., 2007; Kaur et al., 2012; Loganathan et al., 2024). After solidification at room temperature (~15 min), sterile antibiotic- or peptide-impregnated filter paper disks were placed on the soft agar surface. Plates were incubated at 37 °C for 18–24 h. Control conditions included: bacteria only, bacteria + phage, bacteria + antimicrobial disk, and bacteria + phage + disk. After incubation, inhibition zone diameters were measured. Additionally, the diameters of 10 randomly selected phage plaques located nearest to the inhibition zones were measured to assess plaque diameter alterations.

2.12. Addition of sub-MIC antimicrobials to the bottom agar

Based on MIC data, sub-MIC of antibiotics or peptides were aseptically added to molten LB bottom agar (1.5%) after cooling to 40–50 °C and sterile filtration (0.22 μm). The prepared agar was poured into sterile Petri dishes and allowed to solidify. Then, 5 mL of soft agar containing 100 μL of phage lysate and 100 μL of host bacterial suspension was overlaid. Control plates included: bottom agar with antimicrobial + bacteria (no phage), and bottom agar without antimicrobial + phage + bacteria. Following incubation at 37 °C for 18–24 h, plates showing homogeneous plaque distribution were selected. The diameters of 10 randomly selected central plaques were measured, and changes in phage titer were evaluated (Santos et al., 2009).

2.13. Addition of sub-MIC antimicrobials to the soft agar

Sub-MIC of antimicrobial agents were added directly to molten soft agar (0.5%) after cooling to 40–50 °C and sterile filtration (0.22 μm). Then, 100 μL of phage lysate and 100 μL of host bacterial suspension were added and mixed prior to being overlaid onto the bottom agar. Control conditions included: soft agar with antimicrobial + bacteria, and soft agar without antimicrobial + phage + bacteria. After solidification (~15 min at room temperature), plates were incubated at 37 °C for 18–24 h. Plates with evenly distributed plaques were selected for analysis. The diameters of 10 randomly selected plaques in the central region were measured, and phage titer changes were assessed (Stachurska et al., 2021; Kaur et al., 2012).

2.14. Checkerboard assay

The checkerboard method was used to evaluate potential synergistic interactions between phages and antimicrobial agents (antibiotics or peptides), following previously described protocols (Al-Anany et al., 2021; Al-Anany et al., 2024) with modifications in the MOI range, informed by expert consultation (personal communication, Dr. Alexander Hynes). *P. aeruginosa* strains were cultured to mid-log phase (OD_{600} ~ 0.2). Phages were diluted across a broad MOI range (1000 to 0.0001), and antimicrobials were two-fold diluted starting from 4 × MIC (Fig. S1). In 96-well U-bottom microplates, phages and antimicrobials were arranged vertically and horizontally, respectively. After combining agents and adding bacterial suspension, plates were incubated at 37 °C for 18–24 h. Sequential applications (phage- or antimicrobial-first with a 2 h delay) were also tested to evaluate timing-dependent effects. Synergy was evaluated using the fractional inhibitory concentration index (FICI), calculated as (Odds, 2003):

FICI = (MOI of phage in combination / MOI of phage alone) + (Antimicrobial concentration in combination / MIC of antimicrobial alone).

Results were interpreted as synergistic (FICI ≤0.5), no interaction (0.5 < FICI ≤4), or antagonistic (FICI >4), in accordance with the criteria proposed by Odds, 2009.

2.15. Cytotoxic activity of peptides

The cytotoxicity of the peptides PEX and MUR was evaluated using the L929 mouse fibroblast cell line (ATCC, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 1% gentamicin, and incubated at 37 °C in a humidified 5% CO_2 atmosphere. Viable cell counts were determined *via* trypan blue exclusion using a Thoma chamber. For the assay, 2×10^4 cells/well were seeded into 96-well flat-bottom microplates in 100 μL of medium. Serial dilutions of PEX (512–0.25 μg/mL) and MUR (64–0.01 μg/mL) were added (100 μL/well), and plates were incubated for 48 h. Cell viability was assessed using the MTT assay as described by Ferrari et al., 1990, and absorbance was measured at 570 nm with a BioTek 800TS spectrophotometer. All experiments were performed in triplicate on three independent days.

2.16. Statistical analysis

In this study, plaque diameter and count data obtained from the MDLA method were statistically analyzed. Combinations were created by incorporating sub-MIC concentrations of antibiotics or peptides into either the bottom or soft agar, and results were compared to phage-only controls using paired samples *t*-tests. Separate analyses were performed for BAUNPA1 and BAUNPA3, with statistical significance set at $p < 0.05$. For FICI evaluations, phage–antimicrobial combinations were tested under three application models: simultaneous, phage-first, and antimicrobial-first. Differences in FICI values across application types were assessed using one-way ANOVA, and pairwise comparisons were performed with independent samples *t*-tests. All analyses were conducted using SPSS version 25.0 (IBM-SPSS Inc., Chicago, IL, USA). Cytotoxicity was evaluated using an MTT assay, and data are presented as mean ± SD of three independent experiments. Dose–response curves were fitted using a four-parameter logistic (4PL) regression model to calculate IC_{50} values. Statistical comparisons between treatment concentrations and the untreated control were performed using one-way ANOVA followed by Dunnett's post-hoc test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Isolation and characterization of bacteriophages

Two lytic bacteriophages, BAUNPA1 and BAUNPA3, specific for *P. aeruginosa* were isolated from wastewater samples in Balıkesir, Türkiye. Plaque morphology was assessed using the DLA method (Fig. S2). Genomic DNA extraction yielded distinct bands, and *Hin*dIII digestion revealed distinct restriction patterns indicative of genomic diversity (Fig. S3). TEM analysis showed that BAUNPA1 has an isometric head and tail, while BAUNPA3 has an isometric head and no visible tail (Fig. S4).

3.2. Replication dynamics and host range

Phage replication at MOI 0.1, 1, and 10 indicated optimal propagation at MOI 10. One-step growth revealed a 30-min latent period and burst sizes of 189 PFU (BAUNPA1) and 43 PFU (BAUNPA3) (Fig. S5). Agar spot assay showed BAUNPA1 completely lysed 20% and partially lysed 14% of the 50 *P. aeruginosa* isolates. BAUNPA3 exhibited broader activity, with complete lysis in 36% and partial lysis in 24% of strains. When tested against non-*Pseudomonas* species (extended host range screening), only a single *A. baumannii* isolate showed partial lysis by BAUNPA3 (Table S1).

Stability of Bacteriophages Under Different Temperature and pH Conditions.

Both phages remained stable at 37–50 °C over 180 min. Titers decreased at 60 °C and complete inactivation occurred at 80–90 °C within 30 min. No reduction was seen at +4 °C (Fig. S6a, 6b). At pH 7–8, activity was retained, while extreme pH values (2,10−12) caused significant inactivation. BAUNPA1 was more stable between pH 4–6 than BAUNPA3 (Fig. S6c).

3.3. MIC distributions of antibiotics and peptides

In vitro activity of antibiotics and peptides against 50 *P. aeruginosa* isolates isolated from clinical samples was determined using the broth microdilution method (Table S2). COL showed the lowest MIC values ($MIC_{50/90}$: 0.5/1 μg/mL) and MUR showed the lowest MIC values ($MIC_{50/90}$: 0.25/0.5 μg/mL), exhibiting consistent activity and the highest *in vitro* potential (narrow MIC distribution) among all 50 isolates. For synergy studies, the MIC values (μg/mL) of the tested antibiotics and antimicrobial peptides for the selected strains are presented in Table S3.

In contrast, MEM showed the lowest $MIC_{50/90}$ values (0.5/16 μg/mL), CIP showed the lowest $MIC_{50/90}$ values (0.25/16 μg/mL), and TOB showed the lowest MIC values (0.5/8 μg/mL). Low MIC_{50} values indicate intrinsic activity against the vast majority of the population, while significantly high MIC_{90} values highlight a significant subset of isolates with reduced susceptibility or acquired resistance, reflecting the well-documented heterogeneity in clinical *P. aeruginosa* collections. PEX showed the highest MIC values ($MIC_{50/90}$: 16/32 μg/mL), indicating relatively lower intrinsic *in vitro* activity against this Gram-negative pathogen.

3.4. Synergistic interactions based on the modified double-layer agar method

In MDLA disk diffusion tests, phage-antimicrobial combinations resulted in very limited, isolate-specific increases in both inhibition zone diameters and phage plaque sizes ($p < 0.05$). BAUNPA1, when used in combination with MEM and PEX, showed expansions in the inhibition zone diameter (up to 4–5 mm) on only one isolate (Table S4). BAUNPA3 demonstrated relatively weaker activity in the selected antibiotic combinations, resulting in low-level inhibition zone expansions (up to 2–3 mm) and limited plaque diameter increases (≤0.5 mm) Table S6. Quantitative data are presented in Supplementary Tables S4-S7 and Figs. 1 and 2.

Adding antibiotic/peptide at sub-MIC concentrations to the bottom agar medium resulted in strain- and treatment-dependent increases in plaque number and/or plaque diameter compared to phage-only controls; the most pronounced effects were observed in conditions containing MEM, CIP, and COL, while some combinations showed no statistically significant change (Figs. 3 and 4). BAUNPA1 showed measurable increases in plaque diameter (MEM, CIP and TOB) and significant increases ($p > 0.05$) in plaque number (MEM, CIP, COL and MUR) across multiple isolates. BAUNPA3 caused minimal changes in plaque diameter but led to increases in plaque number (MEM, CIP and COL) depending on the isolate. Detailed quantitative data are given in Tables S8-S11.

Adding antibiotic/peptide at sub-MIC concentrations to soft agar medium resulted in selective, strain-dependent changes in plaque diameter and plaque number. For BAUNPA1, significant increases in plaque number were observed, particularly under MEM-containing conditions; however, strain-dependent increases were also detected for the other tested CIP, COL, and TOB (Table S13). In contrast, BAUNPA3 showed no significant change in plaque diameter in combinations, and only limited increases in plaque number were seen with MEM (Figs. 5 and 6). BAUNPA1 caused an increase in measurable plaque diameter in a single isolate (MEM only) and in plaque number in multiple isolates (except MUR). In contrast, BAUNPA3 did not affect plaque diameter, but sub-MIC antimicrobial addition resulted in an increase in plaque number in two isolates (MEM only). Detailed quantitative data are given in Tables S12–S15.

3.5. Synergistic interactions determined by the checkerboard assay

Application sequence impacted synergy. Simultaneous application of BAUNPA1 with MEM, CIP, and COL yielded the highest synergy (FICI range 0.13 to 0.51 for MEM), while TOB, MUR, and PEX showed no interactions effects (0.5 < FICI ≤4) (Table S16-S17). When BAUNPA1 was applied before antimicrobials, synergy improved and was observed across all agents (Table 1). Reverse application reduced effects, especially with peptides. For BAUNPA3, simultaneous use with MEM and CIP showed strong synergy, and phage-first sequential application extended synergy to all agents (Table 2 and Table S18-S22). Only COL maintained consistent synergy regardless of sequence.

3.6. MTT-based cytotoxicity analysis

PEX demonstrated low cytotoxicity, with a calculated IC_{50} of 170.43 μg/mL. One-way ANOVA confirmed a significant concentration-dependent effect on cell viability ($p < 0.0001$). Cell viability remained statistically indistinguishable from the control at concentrations ≤8 μg/mL (96.7 ± 0.6%, $p > 0.05$). A significant reduction was first observed at 16 μg/mL (80.0%, $p < 0.05$), followed by a progressive decline at higher concentrations (*e.g.*, 69.7 ± 0.6% at 128 μg/mL). In contrast, MUR exhibited markedly higher cytotoxicity, with a substantially lower IC_{50} of 45.23 μg/mL (approximately 3.8-fold lower than that of PEX). ANOVA similarly revealed a highly significant effect of concentration ($p < 0.0001$). Cell viability was not significantly affected at concentrations ≤16 μg/mL (≥97.0%, $p > 0.05$), whereas a sharp and statistically significant decrease was observed at 32 μg/mL (72.3 ± 0.6%, $p < 0.001$) and 64 μg/mL (18.3 ± 0.6%, $p < 0.001$).

4. Discussion

The main objective of this study was to systematically evaluate the *in vitro* synergistic potential of combinations of two novel *P. aeruginosa* phages (BAUNPA1 and BAUNPA3) with antibiotics and peptides, using agar-based and broth-based experimental platforms in parallel. The findings show that PAS detection and interpretation depend largely on the methodological platform used, and that there are differences between qualitative methods based on solid media and quantitative

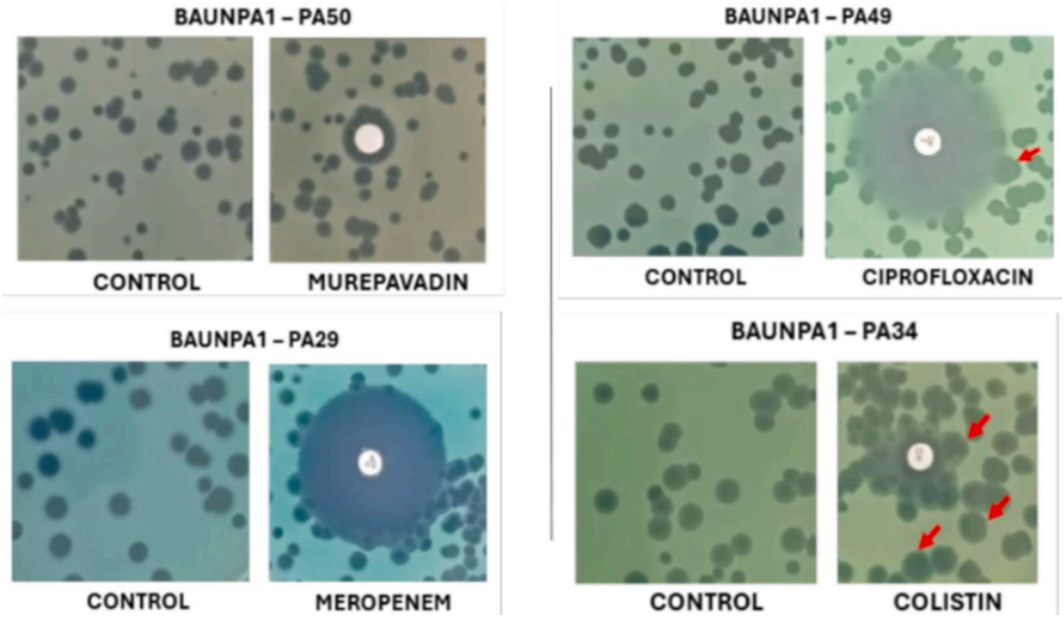


Fig. 1. Disk diffusion synergy images showing BAUNPA1 phage plaques with and without antibiotic/peptide combination against *P. aeruginosa*. Red arrows indicate plaque growth compared to phage-only controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

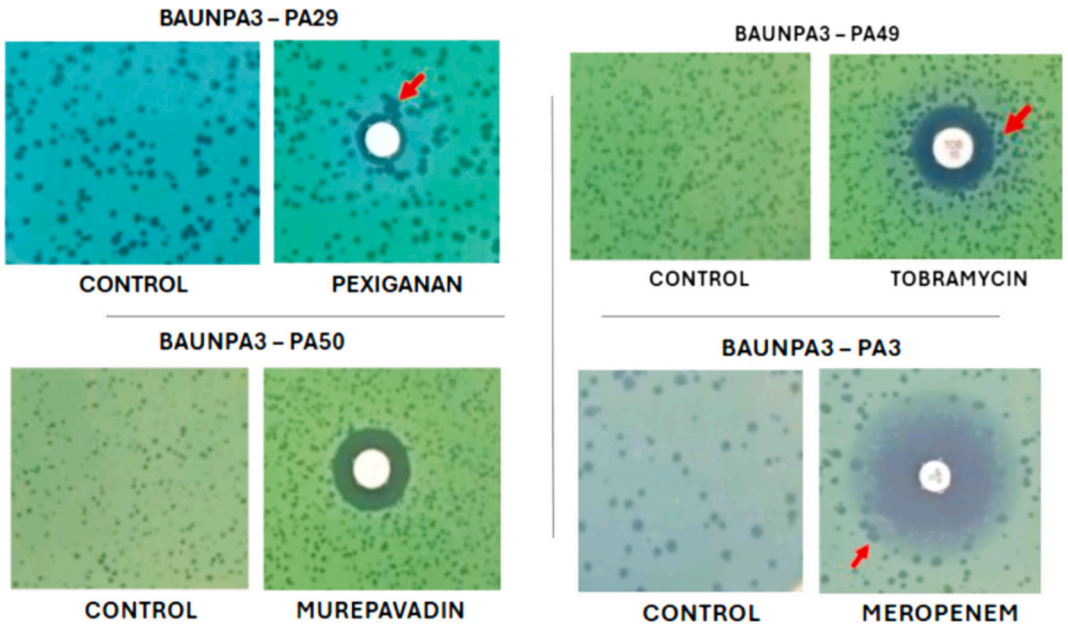


Fig. 2. Disk diffusion synergy images showing BAUNPA3 phage plaques with and without antibiotic/peptide combination against *P. aeruginosa*. Red arrows indicate plaque growth compared to phage-only controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

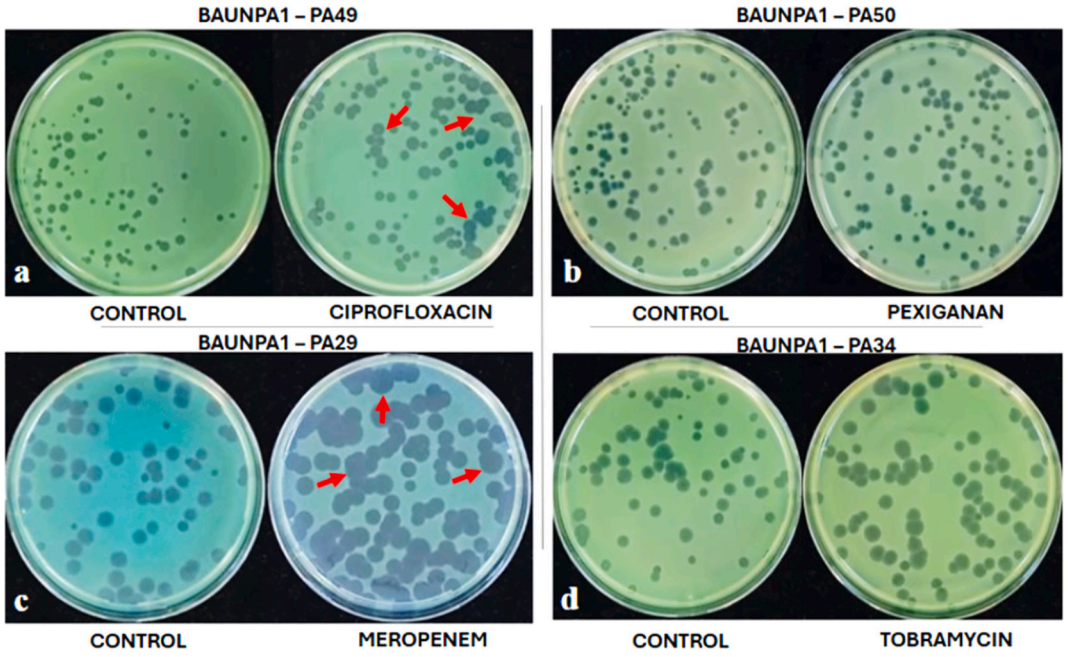


Fig. 3. Effects of sub-MIC concentrations of antibiotics addition into the bottom agar on BAUNPA1 phage plaque formation against *P. aeruginosa* strains. Red arrows indicate plaque enlargement compared with phage-only controls. Increases in plaque numbers are also apparent relative to the controls. In petri dishes without the red arrow, no difference in phage plaque size was observed compared to the control groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

systems based on broth cultures.

In agar-based assays, including the MDLA method and formats adapted for disk diffusion, phage-antimicrobial interactions can be rapidly assessed with high visual accessibility. MDLA has become increasingly popular for PAS assessment; however, caution is required in its interpretation due to methodological limitations (Table 3). In this study, the MDLA-disk diffusion configuration did not show statistically significant differences in antibiotic or peptide inhibition site diameters in the presence of phages. This finding is consistent with previous agar-based studies showing that inhibition site expansion is an insensitive and often unreliable marker for PAS, especially in systems where antimicrobial diffusion kinetics rather than bactericidal activity is dominant (Loganathan et al., 2024; Morris et al., 2025). In contrast, changes in plaque morphology—specifically plaque diameter and plaque number—emerged as the most sensitive agar-based indicators of phage-antimicrobial interactions. Adding sub-MIC levels of antibiotics or peptides to bottom agar or soft agar resulted in modest but statistically significant increases in plaque diameter and/or plaque number. Among

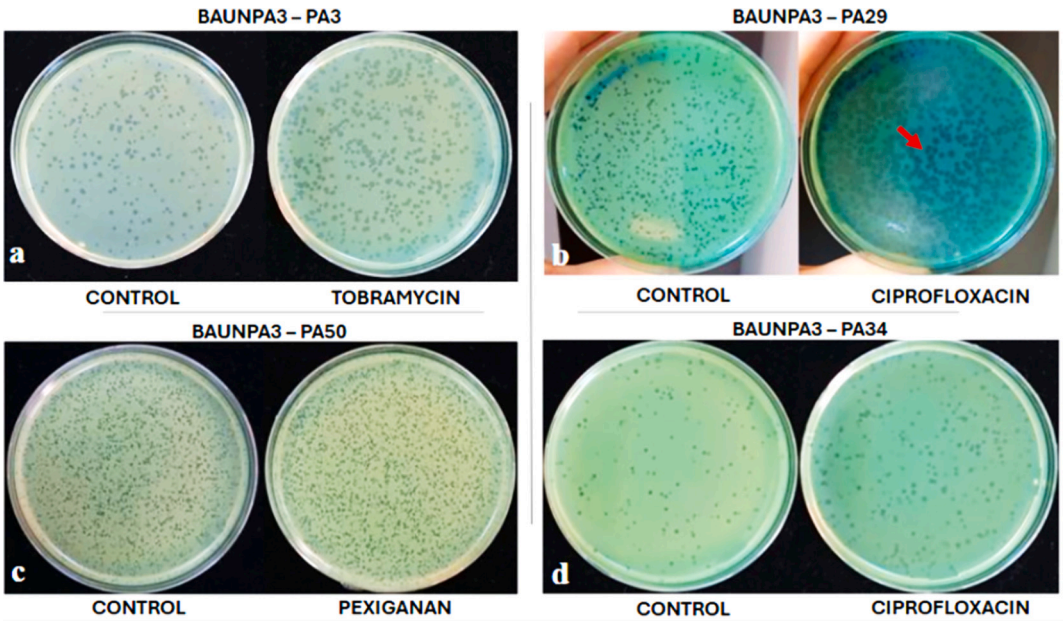


Fig. 4. Effects of sub-MIC concentrations of antibiotics addition into the bottom agar on BAUNPA3 phage plaque formation against *P. aeruginosa* strains. Red arrows indicate plaque enlargement compared with phage-only controls. Increases in plaque numbers are also apparent relative to the controls. In petri dishes without the red arrow, no difference in phage plaque size was observed compared to the control groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

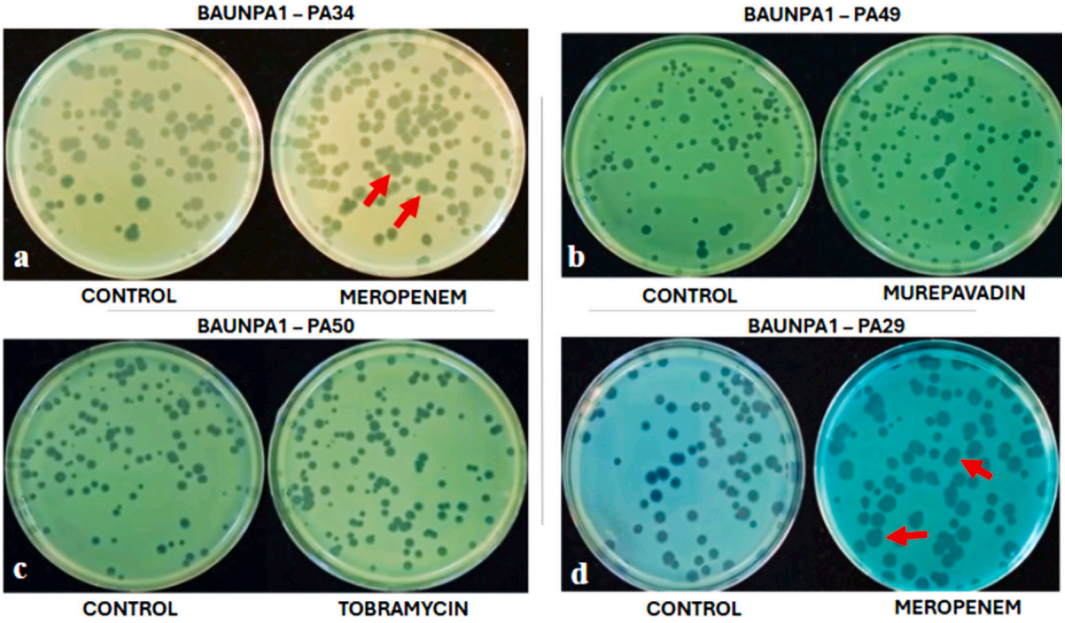


Fig. 5. Effects of sub-MIC concentrations of antibiotics addition into the soft agar on BAUNPA1 phage plaque formation against *P. aeruginosa* strains. Red arrows indicate plaque enlargement compared with phage-only controls. Increases in plaque numbers are also apparent relative to the controls. In petri dishes without the red arrow, no difference in phage plaque size was observed compared to the control groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the agents tested, MEM caused the most pronounced increase in plaque diameter, while MEM, COL, and CIP were associated with increased plaque number. These effects were largely dependent on the agar configuration: addition to bottom agar primarily increased plaque diameter, while addition to soft agar more consistently increased plaque number. Importantly, both approaches reduced inter-plaque heterogeneity and improved plaque contrast and countability, resulting in a more homogeneous plaque appearance.

These observations are consistent with previous agar-based studies showing that sub-MIC antibiotic/peptide exposure can modulate bacterial physiology to promote phage replication, including filamentation induction, changes in cell division dynamics, and expansion of phage adsorption surface area, but does not necessarily increase bacterial killing (Santos et al., 2009; Stachurska et al., 2021). However, consistent with methodological analyses reported in the literature, agar-based assays have not reliably distinguished between true antibacterial synergy and purely permissive interactions that facilitate phage replication (Loganathan et al., 2024). Therefore, increases in plaque diameter or

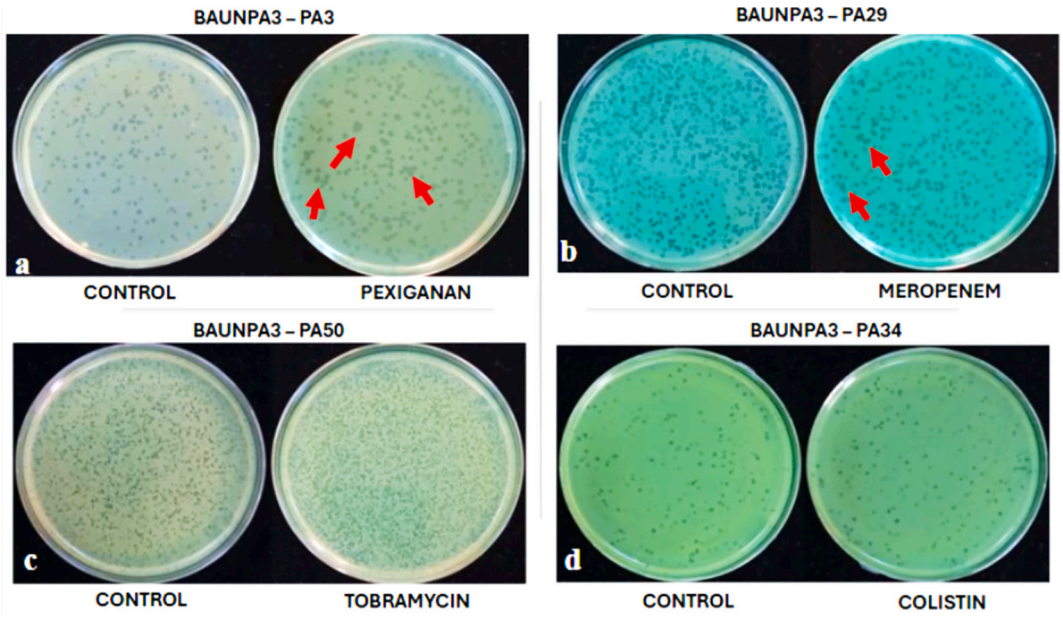


Fig. 6. Effects of sub-MIC concentrations of antibiotics addition into the soft agar on BAUNPA3 phage plaque formation against *P. aeruginosa* strains. Red arrows indicate plaque enlargement compared with phage-only controls. Increases in plaque numbers are also apparent relative to the controls. In petri dishes without the red arrow, no difference in phage plaque size was observed compared to the control groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Fractional inhibitory concentration index (FICI) values and interaction results of BAUNPA1–antibiotic combinations in sequential application (phage first).

Strain	CIP			MEM			COL			TOB		
	FIC_{CIP}	FIC_{p1}	FICI	FIC_{MEM}	FIC_{p1}	FICI	FIC_{COL}	FICp1	FICI	FIC_{TOB}	FICp1	FICI
1	0.24	0.10	**0.34**	0.25	0.10	**0.35**	0.25	0.10	**0.35**	0.24	0.10	**0.34**
29	0.12	0.10	**0.25**	0.25	0.10	**0.35**	0.06	0.10	**0.16**	0.125	0.10	**0.225**
34	0.06	0.10	**0.16**	0.06	0.10	**0.16**	0.03	0.10	**0.13**	0.25	0.10	**0.35**
49	0.25	0.10	**0.35**	0.06	0.10	**0.16**	0.125	0.10	**0.22**	0.25	0.10	**0.35**
50	0.50	0.10	0.60	0.125	0.10	**0.22**	0.06	0.10	**0.16**	0.50	0.10	0.60

p1: BAUNPA1, CIP: ciprofloxacin, COL: colistin, MEM: meropenem, TOB: tobramycin. Synergistic interactions (FICI ≤0.5) are highlighted in bold.

Table 2
Fractional inhibitory concentration index (FICI) values and interaction results of BAUNPA3–antibiotic combinations in sequential application (phage first).

Strain	CIP			MEM			COL			TOB		
	FIC_{CIP}	FICp3	FICI	FIC_{MEM}	FICp3	FICI	FIC_{COL}	FICp3	FICI	FIC_{TOB}	FICp3	FICI
3	0.25	0.10	**0.35**	0.25	0.10	**0.35**	0.5	0.01	0.51	0.24	0.10	**0.34**
29	0.24	0.10	**0.34**	0.06	0.10	**0.16**	0.06	0.10	**0.16**	0.25	0.10	**0.35**
34	0.24	0.01	**0.25**	0.12	0.10	**0.22**	0.25	0.01	**0.26**	0.12	0.10	**0.22**
49	0.12	0.01	**0.13**	0.06	0.10	**0.16**	0.12	0.10	**0.22**	0.25	0.10	**0.35**
50	0.25	0.10	**0.35**	0.06	0.10	**0.16**	0.25	0.01	**0.26**	0.50	0.10	0.60

p3: BAUNPA3, CIP: ciprofloxacin, COL: colistin, MEM: meropenem, TOB: tobramycin. Synergistic interactions (FICI ≤0.5) are highlighted in bold.

number reflect changes in phage replication kinetics rather than being definitive evidence of increased bactericidal efficacy (Santos et al., 2009; Kaur et al., 2012; Stachurska et al., 2021). Given that plaque morphology is influenced by numerous experimental variables, including agar composition, diffusion properties, bacterial inoculation density, and phage life cycle characteristics, it cannot be considered a sole indicator of PAS. Consequently, the MDLA findings in this study should be interpreted not as direct evidence of synergistic antimicrobial activity, but as methodological improvements that enhance plaque detectability and experimental standardization, particularly for phages producing small or difficult-to-detect plaques.

In contrast, quantitative assessment using a liquid-based checkerboard assay revealed the interaction more clearly. Synergy, defined as FICI ≤0.5, was largely dependent on the antimicrobial class, phage identity, and, most importantly, the order of application. Simultaneous and phage-first application of phages and antimicrobials yielded synergistic and interactive results. In particular, sequential application of MEM, COL, and CIP using a phage-first strategy consistently provided higher synergy frequencies. This pattern was reproducible for both BAUNPA1 and BAUNPA3; however, synergistic interactions were limited to a maximum of five isolates, underscoring the significant strain specificity of PAS.

Rather than demonstrating broad therapeutic applicability, these findings highlight the critical role of bacterial physiological state and experimental timing in shaping interaction outcomes. Mechanistically, early phage infection can primegate bacterial cells through membrane disruption or the induction of metabolic stress, thereby enhancing subsequent antibiotic susceptibility. Conversely, antibiotic pre-exposure,

Table 3
Agar-based studies on phage–antibiotic ınteractions.

Author (Year)	Bacteria/ Phage	Antibiotic (s)	Method	Findings
Comeau et al. (2007)	*E. coli*	CFT, CIP	MDLA-Disk Diffusion	A significant increase in phage plaque size was observed. Indicates that antibiotics may enhance phage replication.
Kaur et al. (2012)	*S. aureus*	LZD, TET CIP, AMP	MDLA-SA	Significant increase in plaque size reported. Latent period shortened and burst size increased.
Stachurska et al. (2021)	*E. coli*	43 different antibiotics including (CIP,COL, MEM)	MDLA–7 Different Modifications	Greatest increase in plaque diameter observed when antibiotic was added to the bottom agar. Addition to bottom and soft agar improved phage plaque detection and enumeration.
Loganathan et al. (2024)	*S. aureus*	FOX, VAN CIP, OXC	MDLA – Disk Diffusion and Checkerboard	Increase in plaque size observed with FOX. Synergy detected with others *via* checkerboard method. Synergy may not always correlate with plaque morphology.
Khong et al. (2024)	*E.faecium*, *E.faecalis*, *S. maltophilia*	VAN, AMP LZD, CFT, LEV	MDLA-(Strip Placement)	Pronounced synergy with VAN in enterococci. Variable responses in *S. maltophilia* strains depending on the antibiotic.
Moryl et al. (2024)	*E. faecalis*	IMP, AMC, AMP, NOR	MDLA-Disk Diffusion	Increased plaque size with three antibiotics; antagonistic effect with NOR. Latent period shortened and burst size increased.
Morris et al. (2025)	ESKAPE	CIP, LEV, AZT	MDLA-Disk Diffusion	Significant increase in inhibition zones with CIP and LEV. Disk diffusion may be useful for predicting synergy, but liquid-based methods are required for confirmation.
This study	*P. aeruginosa*	CIP, COL, MEM, TOB, MUR, PEX	MDLA–3 Different Modifications	MEM showed the greatest increase in plaque size, while MEM, CIP, and CIP led to an increase in plaque number.

CFT: Cefotaxime, LZD: Linezolid, TET: Tetracycline, AMP: Ampicillin, FOX: Fosfomycin, VAN: Vancomycin, OXC: Oxacillin, LEV: Levofloxacin, IMP: Imipenem, AMC: Amoxicillin-clavulanic acid, NOR: Norfloxacin, AZT: Aztreonam, MUR: Murepavadin, PEX: Pexiganan, MDLA: Modified double-layer agar, SA: Soft agar.

particularly with protein synthesis-inhibiting agents, can suppress phage replication and reduce combinatorial efficacy (Chaudhry et al., 2017; Nikolic et al., 2022). Phage-peptide combinations were subject to similar limitations. While synergy with MUR and PEX was detectable under phage pre-exposure conditions, these effects were moderate and inconsistent among isolates. This observation aligns with limited and consistent literature on direct phage-peptide synergy, where strain dependence and experimental design significantly constrain detectable interactions (Kim et al., 2022; Wilkinson et al., 2025).

The MDLA method can be used as a predefined preliminary approach for determining phage-antimicrobial interactions because it is faster, more practical, and more reproducible than the checkerboard method. However, given the methodological limitations inherent in agar-based platforms, the liquid-based checkerboard method can also be applied simultaneously to validate these interactions. This comparative framework highlights that agar-based and liquid-based methods investigate fundamentally different biological events. Agar-based methods are particularly suitable for qualitative screening, optimization of plaque visualization, and methodological improvement during phage isolation and characterization (Santos et al., 2009). In contrast, liquid culture-based methods provide quantitative, strain-based information regarding interaction outcomes, including the critical effect of dosing order. Neither approach alone is sufficient for comprehensive characterization of PAS. In particular, it is a documented inconsistency in previous comparative studies that synergistic interactions detected in liquid culture do not manifest as plaque growth in solid medium, and *vice versa* (Loganathan et al., 2024; Khong et al., 2024). Accordingly, the combined and complementary application of both methods may provide a more accurate qualitative and quantitative assessment of phage-antimicrobial interaction results.

This study has several limitations. The number of isolates used in synergy studies was limited; time-death kinetics were not evaluated; and *in vivo* validation was not performed. Accordingly, the findings should not be extrapolated to clinical practice. The primary contribution of this work is methodological: it demonstrates that *in vitro* platform selection fundamentally influences both the detectability and the interpretation of phage–antimicrobial interactions. PAS is a method-dependent phenotype, and even when detected, it remains conditional, strain-specific, and temporally constrained. Agar-based modifications improve plaque visualization and experimental reproducibility but do not measure antibacterial synergy. Checkerboard-based assays provide greater quantitative resolution yet are sensitive to procedural variables. A structured, dual-platform workflow minimizes the risk of phenotypic interpretation and can provide a more reliable basis for prioritizing phage-antibiotic combinations for subsequent mechanistic and *in vivo* investigations.

5. Conclusion

This study compared agar-based and liquid-based methods that can be used to determine phage-antibiotic synergy *in vitro* in clinical phage microbiology laboratories using lytic bacteriophages specific to *P. aeruginosa*. In agar-based experiments, sub-MIC supplementation of antibiotics/peptides did not significantly alter inhibition sites, but improved bacteriophage plaque diameter, number, and homogeneity, particularly with MEM, COL and CIP. Checkerboard assays performed in liquid culture medium consistently demonstrated isolate- and phage-dependent synergistic interactions; concurrent and phage-first application, in particular, revealed synergy. Although limited to validated *in vitro* models, these findings provide a strong rationale for further studies exploring the potential of these combinations.

CRediT authorship contribution statement

Umut Yılmaz: Writing – review & editing, Software, Resources, Methodology. **Mehmet Ünlü:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Gülhan Vardar Ünlü:** Writing – review & editing, Software, Resources, Methodology.

Ethics approval

This study was approved by the Non-Interventional Research Ethics Committee of Balıkesir University, with the decision number 2025/35.

Funding

This study was supported by the Scientific Research Projects Unit of Balıkesir University under project number 2022-036.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to express our sincere gratitude to Prof. Dr. Bülent Bozdoğan and Dr. Abdülkerim Karaynır for their valuable contributions to the experimental procedures. We are also deeply thankful to Assoc. Prof. Bükay Yenice Gürsu for her expert support in the electron microscopy analyses, and to Dr. İbrahim Çavuş for his assistance with the cytotoxicity assays.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2026.107441.

Data availability

No datasets were generated or analyzed during the current study.

References

Al-Anany, A.M., Fatima, R., Hynes, A.P., 2021. Temperate phage-antibiotic synergy eradicates bacteria through depletion of lysogens. Cell Rep. 35, 109172. https://doi.org/10.1016/j.celrep.2021.109172.

Al-Anany, A.M., Fatima, R., Nair, G., Mayol, J.T., Hynes, A.P., 2024. Temperate phage-antibiotic synergy across antibiotic classes reveals new mechanism for preventing lysogeny. mBio 15, e00504-24. https://doi.org/10.1128/mbio.00504-24.

Ali, S., Karaynir, A., Salih, H., Öncü, S., Bozdoğan, B., 2023. Characterization, genome analysis and antibiofilm efficacy of lytic Proteus phages RP6 and RP7 isolated from university hospital sewage. Virus Res. 326, 199049. https://doi.org/10.1016/j.virusres.2023.199049.

Chaudhry, W.N., Concepción-Acevedo, J., Park, T., Andleeb, S., Bull, J.J., Levin, B.R., 2017. Synergy and order effects of antibiotics and phages in killing *Pseudomonas aeruginosa* biofilms. PLoS One 12, e0168615. https://doi.org/10.1371/journal.pone.0168615.

Comeau, A.M., Tétart, F., Trojet, S.N., Prère, M.F., Krisch, H.M., 2007. Phage-antibiotic synergy (PAS): Beta-lactam and quinolone antibiotics stimulate virulent phage growth. PLoS One 2, e799. https://doi.org/10.1371/journal.pone.0000799.

Cosentino, F., Viale, P., Giannella, M., 2023. MDR/XDR/PDR or DTR? Which definition best fits the resistance profile of *Pseudomonas aeruginosa*? Curr. Opin. Infect. Dis. 36, 564–571. https://doi.org/10.1097/QCO.0000000000000966.

Dedrick, R.M., Smith, B.E., Cristinziano, M., Freeman, K.G., Jacobs-Sera, D., Belessis, Y., Whitney Brown, A., Cohen, K.A., Davidson, R.M., van Duin, D., et al., 2023. Phage therapy of *Mycobacterium* infections: Compassionate use of phages in 20 patients with drug-resistant mycobacterial disease. Clin. Infect. Dis. 76, 103–112. https://doi.org/10.1093/cid/ciac453.

Fatima, R., Hynes, A.P., 2025. Phage-antibiotic combinations for *Pseudomonas*: Successes in the clinic and *in vitro* tenuously connected. Microb. Biotechnol. 18, e70193. https://doi.org/10.1111/1751-7915.70193.

Ferrari, M., Fornasiero, M.C., Isetta, A.M., 1990. MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. J. Immunol. Methods 131, 165–172. https://doi.org/10.1016/0022-1759(90)90187-z.

Glonti, T., Pirnay, J.P., 2022. *In vitro* techniques and measurements of phage characteristics that are important for phage therapy success. Viruses 14, 1490. https://doi.org/10.3390/v14071490.

Hall, A.R., De Vos, D., Friman, V.-P., Pirnay, J.-P., Buckling, A., 2012. Effects of sequential and simultaneous applications of bacteriophages on populations of *Pseudomonas aeruginosa in vitro* and in wax moth larvae. Appl. Environ. Microbiol. 78, 5646–5652. https://doi.org/10.1128/AEM.00757-12.

Hatfull, G.F., Dedrick, R.M., Schooley, R.T., 2022. Phage therapy for antibiotic-resistant bacterial infections. Annu. Rev. Med. 73, 197–211. https://doi.org/10.1146/annurev-med-080219-122,208.

Holger, D.J., Lev, K.L., Kebriaei, R., Morrisette, T., Shah, R., Alexander, J., Lehman, S.M., Rybak, M.J., 2022. Bacteriophage-antibiotic combination therapy for multidrug-resistant *Pseudomonas aeruginosa*: *in vitro* synergy testing. J. Appl. Microbiol. 133, 1636–1649. https://doi.org/10.1111/jam.15647.

Holloway, B., Egan, J., Monk, M., 1960. Lysogeny in *Pseudomonas aeruginosa*. Aust. J. Exp. Biol. Med. Sci. 38, 321–330. https://doi.org/10.1038/icb.1960.34.

Horcajada, J.P., Montero, M., Oliver, A., Sorlí, L., Luque, S., Gómez-Zorrilla, S., Benito, N., Grau, S., 2019. Epidemiology and treatment of multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa* infections. Clin. Microbiol. Rev. 32, e00031-19. https://doi.org/10.1128/CMR.00031-19.

Huff, W.E., Huff, G.R., Rath, N.C., Balog, J.M., Donoghue, A.M., 2004. Therapeutic efficacy of bacteriophage and Baytril (enrofloxacin) individually and in combination to treat colibacillosis in broilers. Poult. Sci. 83 (12), 1944–1947. https://doi.org/10.1093/ps/83.12.1944.

Juan, C., Peña, C., Oliver, A., 2017. Host and pathogen biomarkers for severe *Pseudomonas aeruginosa* infections. J. Infect. Dis. 215 (Suppl. 1), S44–S51. https://doi.org/10.1093/infdis/jiw299.

Karaynir, A., Salih, H., Bozdoğan, B., Güçlü, Ö., Keskin, D., 2022. Isolation and characterization of Brochothrix phage ADU4. Virus Res. 321, 198902. https://doi.org/10.1016/j.virusres.2022.198902.

Kaur, S., Harjai, K., Chhibber, S., 2012. Methicillin-resistant *Staphylococcus aureus* phage plaque size enhancement using sublethal concentrations of antibiotics. Appl. Environ. Microbiol. 78, 8227–8233. https://doi.org/10.1128/AEM.02371-12.

Khong, E., Oh, J.J., Jimenez, J.M., Liu, R., Dunham, S., Monsibais, A., et al., 2024. A simple solid media assay for detection of synergy between bacteriophages and antibiotics. Microbiol. Spectr. 12, e03221-23. https://doi.org/10.1128/spectrum.03221-23.

Kim, J., Kim, J.C., Ahn, J., 2022. Assessment of bacteriophage-encoded endolysin as a potent antimicrobial agent against antibiotic-resistant *Salmonella Typhimurium*. Microb. Pathog. 168, 105576. https://doi.org/10.1016/j.micpath.2022.105576.

Knezevic, P., Curcin, S., Aleksic, V., Petrusic, M., Vlaski, L., 2013. Phage-antibiotic synergism: A possible approach to combatting *Pseudomonas aeruginosa*. Res. Microbiol. 164, 55–60. https://doi.org/10.1016/j.resmic.2012.08.008.

Kutateladze, M., 2015. Experience of the Eliava Institute in bacteriophage therapy. Virol. Sin. 30, 80–81. https://doi.org/10.1007/s12250-014-3557-0.

Loganathan, A., Bozdogan, B., Manohar, P., Nachimuthu, R., 2024. Phage-antibiotic combinations in various treatment modalities to manage MRSA infections. Front. Pharmacol. 15, 1356179. https://doi.org/10.3389/fphar.2024.1356179.

Melo, L.D., Veiga, P., Cerca, N., Kropinski, A.M., Almeida, C., Azeredo, J., Sillankorva, S., 2016. Development of a phage cocktail to control *Proteus mirabilis* catheter-associated urinary tract infections. Front. Microbiol. 7, 1024. https://doi.org/10.3389/fmicb.2016.01024.

Morris, T.C., Reyneke, B., Khan, S., Khan, W., 2025. Phage-antibiotic synergy to combat multidrug resistant strains of Gram-negative ESKAPE pathogens. Sci. Rep. 15, 17235. https://doi.org/10.1038/s41598-025-01489-y.

Moryl, M., Szychowska, P., Dziąg, J., Różalski, A., Torzewska, A., 2024. The combination of phage therapy and β-lactam antibiotics for the effective treatment of *Enterococcus faecalis* infections. Int. J. Mol. Sci. 26, 11. https://doi.org/10.3390/ijms26010011.

Neter, E.R., Clark, P., 1944. Salmonella enterocolitis in infants and children. Am. J. Dig. Dis. 11, 323–325. https://doi.org/10.1007/BF02997963.

Nicholls, P., Clark, J.R., Gu Liu, C., Terwilliger, A., Maresso, A.W., 2023. Class-driven synergy and antagonism between a *Pseudomonas* phage and antibiotics. Infect. Immun. 91, e00065-23. https://doi.org/10.1128/iai.00065-23.

Nikolic, I., Vukovic, D., Gavric, D., Cvetanovic, J., Aleksic Sabo, V., Gostimirovic, S., Narancic, J., Knezevic, P., 2022. An optimized checkerboard method for phage-antibiotic synergy detection. Viruses 14, 1542. https://doi.org/10.3390/v14071542.

Nir-Paz, R., Gelman, D., Khouri, A., Sisson, B.M., Fackler, J., Alkalay-Oren, S., et al., 2019. Successful treatment of antibiotic-resistant, poly-microbial bone infection with bacteriophages and antibiotics combination. Clin. Infect. Dis. 69, 2015–2018. https://doi.org/10.1093/cid/ciz222.

Odds, F.C., 2003. Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. 52, 1. https://doi.org/10.1093/jac/dkg301.

Qin, S., Xiao, W., Zhou, C., Pu, Q., Deng, X., Lan, L., Liang, H., Song, X., Wu, M., 2022. *Pseudomonas aeruginosa*: Pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduct. Target. Ther. 7, 199. https://doi.org/10.1038/s41392-022-01056-1.

Salih, H., Karaynir, A., Yalcin, M., Oryasin, E., Holyavkin, C., Basbulbul, G., Bozdogan, B., 2022. Metagenomic analysis of wastewater phageome from a University Hospital in Turkey. Arch. Microbiol. 204, 353. https://doi.org/10.1007/s00203-022-02962-2.

Santos, S.B., Carvalho, C.M., Sillankorva, S., Nicolau, A., Ferreira, E.C., Azeredo, J., 2009. The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. BMC Microbiol. 9, 148. https://doi.org/10.1186/1471-2180-9-148.

Stachurska, X., Roszak, M., Jabłońska, J., Mizielińska, M., Nawrotek, P., 2021. Double-layer agar (DLA) modifications for the first step of the phage-antibiotic synergy (PAS) identification. Antibiotics 10, 1306. https://doi.org/10.3390/antibiotics10111306.

Suh, G.A., Lodise, T.P., Tamma, P.D., Knisely, J.M., Alexander, J., Aslam, S., et al., 2022. Considerations for the use of phage therapy in clinical practice. Antimicrob. Agents Chemother. 66, e02071-21. https://doi.org/10.1128/AAC.02071-21.

Summers, W.C., 2001. Bacteriophage therapy. Ann. Rev. Microbiol. 55, 437–451. https://doi.org/10.1146/annurev.micro.55.1.437.

Uyttebroek, S., Chen, B., Onsea, J., Ruythooren, F., Debaveye, Y., Devolder, D., Spriet, I., Depypere, M., Wagemans, J., Lavigne, R., Pirnay, J.P., Merabishvili, M., De Munter, P., Peetermans, W.E., Dupont, L., Van Gerven, L., Metsemakers, W.J., 2022. Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. Lancet Infect. Dis. 22, e208–e220. https://doi.org/10.1016/S1473-3099(21)00612-5.

Van Twest, R., Kropinski, A.M., 2009. Bacteriophage enrichment from water and soil. Methods Mol. Biol. 501, 15–21. https://doi.org/10.1007/978-1-60,327-164-6_2.

Wilkinson, R.C., Thomas, N.E., Bhatti, A., Burton, M.R., Joyce, N., Jenkins, R.E., 2025. Phage-encoded antimicrobial peptide gp28 demonstrates LL-37-like antimicrobial activity against multidrug-resistant *Pseudomonas aeruginosa*. PHAGE 6, 12–19. https://doi.org/10.1089/phage.2024.0009.

Yang, H., Liang, L., Lin, S., Jia, S., 2010. Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. BMC Microbiol. 10, 131. https://doi.org/10.1186/1471-2180-10-131.

Yazdi, M., Bouzari, M., Ghaemi, E.A., 2018. Isolation and characterization of a lytic bacteriophage (vB_PmiS-TH) and its application in combination with ampicillin against planktonic and biofilm forms of *Proteus mirabilis* isolated from urinary tract infection. J. Mol. Microbiol. Biotechnol. 28, 37–46. https://doi.org/10.1159/000487137.

Yerushalmy, O., Khalifa, L., Gold, N., Rakov, C., Alkalay-Oren, S., Adler, K., Ben-Porat, S., Kraitman, R., Gronovich, N., Ginat, K.S., Abdalrhman, M., Coppenhagen-Glazer, S., Nir-Paz, R., Hazan, R., 2020. The Israeli Phage Bank (IPB). Antibiotics 9, 269. https://doi.org/10.3390/antibiotics9050269.

Yilmaz, U., Coşkun, A.G., Özel, Y., Ünlü, M., Vardar-Ünlü, G., 2024. Synergistic interactions of essential oil components with antibiotics against multidrug-resistant *Corynebacterium striatum*. J. Appl. Microbiol. 135, lxae090. https://doi.org/10.1093/jambio/lxae090.