

Comparative Combinatorial Effects of Endolysins LNT103 with Ten Conventional Antibiotics against Gram-Negative Bacterial Pathogens

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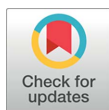
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Bacteriophage-derived endolysins have emerged as promising antibacterial agents; however, their combinatorial interactions with conventional antibiotics remain insufficiently characterized across Gram-negative bacterial species. Here, we systematically evaluated synergistic, additive, or indifferent interactions between the Gram-negative-targeting endolysin LNT103 and ten clinically relevant antibiotics across 15 *Escherichia coli*, 10 *Pseudomonas aeruginosa*, and 5 *Acinetobacter baumannii* strains. Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration indices (FICIs) were determined using checkerboard assays. Among 300 combinations tested, synergistic interactions were observed with chloramphenicol (three cases), sulfamethoxazole (three cases), colistin (two cases), and trimethoprim (one case), indicating pronounced strain dependence. When synergistic and additive interactions were considered together, positive effects were most frequent in *P. aeruginosa* (76.0%), followed by *E. coli* (54.6%) and *A. baumannii* (40.0%). Notably, these trends were inversely correlated with median endolysin MIC values (72, 16, and 4 µg/mL, respectively). In contrast, no correlation was observed between intrinsic antibiotic MICs and combination outcomes, and no antagonism was detected across all combinations. Time-kill assays performed on representative strains further demonstrated that endolysin concentration exerted a greater influence on bactericidal activity than antibiotic concentration. Collectively, these results indicate that intrinsic endolysin susceptibility is a primary determinant of combination efficacy and provide a rational framework for optimizing endolysin-antibiotic adjunctive therapies against multidrug-resistant Gram-negative pathogens.

Keywords: Antibiotic resistance, Bacteriophage endolysin, Combination treatment, Synergy



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Introduction

The global rise of multidrug-resistant (MDR) bacterial pathogens has intensified efforts to develop adjunctive strategies that restore or enhance the efficacy of existing antibiotics. Among emerging alternatives, bacteriophage-derived endolysins—peptidoglycan-degrading enzymes that induce rapid bacterial lysis—represent some of the most advanced protein-based antibacterial candidates currently under development [1-6]. Their rapid bactericidal activity, target specificity, and low propensity for conventional resistance have prompted growing interest in endolysin-antibiotic combination therapies, particularly for difficult-to-treat Gram-negative infections [3].

In Gram-negative bacteria, the outer membrane constitutes a major barrier to exogenously applied endolysins and plays a critical role in determining combination outcomes with antibiotics. Hong et al. engineered the *Escherichia coli* phage endolysin EC340 into LNT113 by incorporating outer-membrane-penetrating elements and systematically evaluated its interactions with standard-of-care antibiotics. Checkerboard analyses revealed clear synergy with colistin, whereas combinations with meropenem, tigecycline, chloramphenicol, azithromycin, and ciprofloxacin were predominantly additive [7]. Mechanistic studies further demonstrated that antimicrobial peptide fusion enhances outer-membrane disruption, facilitates access to peptidoglycan, and complements both membrane-active and intracellularly acting antibiotics.

Consistent findings have been reported for combinations of Gram-negative endolysins with polymyxins. Blasco *et al.* showed that combinations of colistin with the GH108 endolysin ElyA1 reduced colistin MICs, enhanced bactericidal activity in time–kill assays, and improved survival in *Galleria mellonella* infection models [8]. More recently, Soontarach *et al.* demonstrated that LysAB1245 restored colistin susceptibility in colistin-resistant isolates, enabling effective killing at reduced polymyxin doses [9]. Together, these studies suggest that synergy in Gram-negative systems most consistently arises when endolysins are paired with agents that permeabilize the outer membrane or otherwise facilitate antibiotic uptake.

LNT103 is an engineered endolysin with demonstrated activity against a broad range of Gram-negative pathogens, including *E. coli*, *P. aeruginosa*, *A. baumannii*, and *Klebsiella pneumoniae* [10]. Its antibacterial activity is partially attributable to a C-terminal amphipathic helix [11, 12], and further enhancement was achieved through site-directed mutagenesis and N-terminal fusion of an antimicrobial peptide. Although numerous endolysin–antibiotic combinations have been reported, most studies have focused on limited strain sets or individual drug partners, and generalizable principles governing combination efficacy remain poorly defined. Accordingly, recent reviews have emphasized the need for systematic, multi-species analyses to identify determinants of successful endolysin–antibiotic combination therapy [13].

In this study, we present a systematic evaluation of combinatorial effects between the engineered endolysin LNT103 and ten conventional antibiotics against three different major Gram-negative pathogens.

Materials and Methods

Bacterial Strains and Growth Conditions

The Gram-negative bacteria employed in this study included *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Strains originated from publicly curated repositories and collaborating laboratories. For *E. coli*, strains 8739 and 700926 were obtained from the American Type Culture Collection (ATCC, USA), whereas strains 939 and 1460 were obtained from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Republic of Korea). AIEC45 was a kind gift from Laurent Debarbieux (Institute Pasteur Paris). Clinical isolates F435, F459, F485, F541, F615, F690, F715, F722, F782 and F892 were kindly provided by Professor Kwan Soo Ko (Sungkyunkwan University). For *P. aeruginosa*, strains 2092, 2134, 2144, 2200, 2298, and 2326 were obtained from CCARM, strains 9027 and 15692 were obtained from ATCC, and clinical isolates 265, 315, and 341 were kindly provided by Professor Kwan Soo Ko (Sungkyunkwan University). PMM5 and PMM8 were kind a gift from Professor Youhee Cho (Cha University). For *A. baumannii*, strains ACB9, ACB11, ACB12, and ACB19 were clinical isolates and kindly provided by Professor Kwan Soo Ko, and strain 2508 was obtained from the Korean Collection for Type Cultures (KCTC, Republic of Korea). Bacteria were propagated in either Luria–Bertani (LB) broth or CAA medium (5 g/L casamino acids, 5.2 mM K_2HPO_4 , and 1 mM $MgSO_4$) at 37°C with agitation (200 rpm) and cultures were used once they reached an exponential phase suitable for antimicrobial testing.

Recombinant Protein Expression and Purification

A plasmid encoding the LNT103 endolysin was introduced into *E. coli* BL21 Star (DE3) (Invitrogen, USA), and the recombinant endolysin was expressed and purified as previously described [10]. Briefly, bacterial cultures were grown to early logarithmic phase ($OD^{600} = 0.3$) and induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; Duchefa, Netherlands), followed by incubation at 25°C for 5 h. Cells were harvested by centrifugation, and the pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 20 mM imidazole). Cell disruption was performed using a homogenizer (Ultrasonic processor, Sonics, USA). The lysate was centrifuged at $8,000 \times g$ for 20 min at 4°C, and the resulting supernatant was collected and filtered through a 0.2 μ m pore-size filter. Protein purification was carried out using fast protein liquid chromatography (FPLC; ÄKTA pure, Cytiva, UK). The purification workflow consisted of four sequential chromatographic steps: affinity chromatography (Capto Chelating, Cytiva), cation exchange chromatography (Capto S, Cytiva), anion exchange chromatography (Capto Q, Cytiva), and hydrophobic interaction chromatography (Butyl HP, Cytiva). The final eluate was buffer-exchanged into storage buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.0) by ultrafiltration and diafiltration.

Determination of Minimum Inhibitory Concentrations (MICs) of Endolysin and Antibiotics

Antibiotics ampicillin (DAEJUNG, Republic of Korea), ceftazidime (Sigma-Aldrich, Germany), kanamycin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), tetracycline (Duchefa, Netherlands), chloramphenicol (Sigma-Aldrich), sulfamethoxazole (Sigma-Aldrich), trimethoprim (Sigma-Aldrich), ciprofloxacin (MP Biomedicals, USA), and colistin (Research Products International, USA) were used. Minimum inhibitory concentrations (MICs) of the endolysin LNT103 and conventional antibiotics were determined using the broth microdilution method in accordance with CLSI guidelines [14]. LNT103 was prepared in CAA medium and serially twofold diluted in 96-well U-bottom microtiter plates to obtain final concentrations ranging from 1 to 512 μ g/mL. For antibiotic testing, the same broth microdilution procedure was applied. Tested agents included ampicillin (AMP), ceftazidime (CAZ), kanamycin (KAN), gentamicin (GEN), tetracycline (TET), chloramphenicol (CAP), sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CPFX), and colistin (CST), with final concentrations ranging from 0.0078 to 512 μ g/mL. Antibiotic stock solutions were prepared according to the manufacturers' instructions, and solvent concentrations were kept constant and below levels known to interfere with bacterial growth. Bacterial suspensions were adjusted to a 0.5 McFarland standard and diluted to yield approximately 1×10^5 CFU per well. Plates were incubated at 37°C for 18–20 h, and the MIC was defined as the lowest concentration showing no visible growth. All assays were performed in three independent biological replicates, and MIC values were interpreted according to CLSI criteria.

Checkerboard Assay for Endolysin–Antibiotic Combination Testing

The interaction between endolysin and antibiotics was evaluated using the checkerboard microdilution assay [15–18]. Endolysin was serially diluted along the horizontal axis, while antibiotics were serially diluted along the vertical axis of 96-well plates. Each well was inoculated with bacterial suspension to a final concentration of 1×10^5 CFU per well in a 100 μ L volume. Plates were incubated at 37°C for 18–20 h, and growth inhibition was assessed visually. The MIC in combination was defined as the lowest concentration of each agent that prevented visible growth when used together. Experiments were performed in triplicates.

Calculation and Interpretation of Fractional Inhibitory Concentration Index (FICI)

Synergistic interactions were quantified using the fractional inhibitory concentration index (FICI), calculated as: (MIC endolysin in combination/MIC endolysin alone) + (MIC antibiotics in combination/MIC antibiotic alone). Interpretation of FICI values: $FICI \leq 0.5$: synergy, $0.5 < FICI \leq 1.0$:

additive, $1.0 < \text{FICI} \leq 4.0$: indifferent, or $\text{FICI} > 4.0$ antagonistic interaction [17, 19]. FICI values were visualized as heatmaps using a three-level green color scale, where strong synergy ($\text{FICI} \leq 0.5$) appears as dark green, additive interactions ($0.5 < \text{FICI} \leq 1.0$) as medium green, and indifferent interactions ($1.0 < \text{FICI} \leq 4.0$) as light green.

Time-Kill Assay

Time-kill assay was conducted to examine the combined antimicrobial activity of the LNT103 with sulfamethoxazole (*E. coli* strains ATCC8739 and clinical F435), with chloramphenicol (*P. aeruginosa* strains ATCC 15692 and CCARM 2326), or with trimethoprim (*A. baumannii* clinical strains 11 and 19). Bacteria were streaked on LB agar and incubated at 37°C for 18–24 h. Three to five colonies were suspended in CAA media and the turbidity was adjusted to a 0.5 McFarland standard. The suspension was subsequently diluted 1:1200 in fresh CAA media to obtain the working inoculum. LNT103 and antibiotics were freshly prepared in CAA media on the day of the experiment. Solutions were prepared at the desired final concentrations immediately before mixing with the bacterial suspension. Equal volumes of the antibiotic solution and the standardized bacterial suspension were mixed, and 100 µl of the mixture was dispensed into each well of a sterile 96-well microtiter plate. A growth control (bacteria without antibiotic) was included in each assay. Plates were incubated at 37°C, and samples were collected at 0, 1, 2, 4, 6, and 24 h. Aliquots were transferred into sterile microtubes for enumeration. Serial 10-fold dilutions (10^{-1} – 10^{-12}) were prepared in sterile phosphate-buffered saline (PBS). A 10-µl aliquot from each dilution was spotted onto LB agar plates and incubated at 37°C for 18–24 h. Plates with 30–300 colonies were used for enumeration, and counts were expressed as colony-forming units per milliliter (CFU/mL). When no growth was detected, the detection limit of the assay was recorded. Bacterial survival was expressed as $\log_{10}$ (CFU/mL) as a function of time. All experiments were performed in triplicate.

Statistical Analysis

All experiments were performed independently in triplicate. Data are presented as the mean $\pm$ standard deviation (SD). Statistical comparisons between groups were performed using Student's *t*-test.

Results

Antibiotic Susceptibility Profiles Vary Widely across Gram-Negative Species and Strains

We first determined the susceptibility of all test strains to ten conventional antibiotics, together with the engineered endolysin LNT103. A total of 15 *E. coli*, 13 *P. aeruginosa*, and 5 *A. baumannii* strains were analyzed (Tables 1–3). As expected, substantial inter-strain variability in antibiotic

Table 1. Minimum inhibitory concentrations (MICs) of endolysin LNT103 or 10 different antibiotics against 15 different strains of *E. coli*.

Endolysin LNT103 or antibiotic	MIC against <i>E. coli</i> strains (µg/mL)															Median MIC
	ATCC 8739	ATCC 700926	CCARM 939	CCARM 1460	AIEC 45	F435	F459	F485	F541	F615	F690	F715	F722	F782	F892	
LNT103	8	16	128	8	8	64	16	16	16	16	32	128	16	16	64	16
Ampicillin	2	1	>512	512	2	>512	512	2	>512	>512	>512	512	4	>512	2	512
Ceftazidime	0.015	0.015	32	0.062	0.031	32	0.031	0.015	512	512	>512	0.06	0.015	32	0.06	0.062
Kanamycin	0.25	0.5	1	>512	0.25	0.5	2	0.25	32	256	0.25	512	0.25	16	1	1
Gentamicin	0.015	0.06	8	64	0.015	0.015	8	0.015	0.031	0.25	0.0078	0.015	0.015	32	0.125	0.023
Tetracycline	0.5	1	1	1	0.5	0.5	128	1	2	128	64	128	1	64	64	1
Chloramphenicol	2	1	2	64	2	4	2	2	4	2	4	2	2	2	2	2
Sulfamethoxazole	1	1	2	>512	128	128	>512	128	64	64	128	32	2	256	1	64
Trimethoprim	0.5	0.5	1	>512	1	4	>512	1	0.25	1	128	0.25	0.5	1	0.25	1
Ciprofloxacin	0.0078	0.015	4	64	0.0078	32	16	0.015	128	32	64	0.125	0.0078	64	0.015	10
Colistin	1	1	1	0.5	1	1	1	1	1	1	1	0.25	1	1	1	1

Table 2. Minimum inhibitory concentrations (MICs) of endolysin LNT103 or 10 different antibiotics against 13 different strains of *P. aeruginosa*.

Endolysin LNT103 or antibiotic	MIC against <i>P. aeruginosa</i> strains (µg/mL)													Median MIC
	ATCC 15692	ATCC 9027	CCARM 2134	CCARM 2144	CCARM 2200	CCARM 2298	CCARM 2326	265	315	341	CCARM 2092	PMM5	PMM8	
LNT103	4	16	8	8	128	32	128	16	128	8	>128	>128	>128	72
Ampicillin	128	64	>512	128	>512	>512	>512	>512	>512	256	N/D	N/D	N/D	>512
Ceftazidime	4	1	8	1	512	256	256	128	>512	1	N/D	N/D	N/D	64
Kanamycin	64	32	>512	16	>512	64	256	64	128	16	N/D	N/D	N/D	64
Gentamicin	0.5	0.06	64	32	>512	2	64	32	256	0.5	N/D	N/D	N/D	32
Tetracycline	32	32	128	4	32	16	8	4	16	8	N/D	N/D	N/D	16
Chloramphenicol	16	64	128	128	>512	16	8	128	512	16	N/D	N/D	N/D	96
Sulfamethoxazole	64	64	128	256	512	64	32	128	>512	32	N/D	N/D	N/D	96
Trimethoprim	32	32	512	32	256	64	16	64	256	32	N/D	N/D	N/D	48
Ciprofloxacin	0.5	0.125	32	0.06	64	8	16	1	1	0.125	N/D	N/D	N/D	4.5
Colistin	1	1	1	1	0.5	1	0.5	0.5	0.5	1	N/D	N/D	N/D	1

(N/D; Not determined)

Table 3. Minimum inhibitory concentrations (MICs) of endolysin LNT103 or 10 different antibiotics against 5 different strains of *A. baumannii*.

Endolysin LNT103 or antibiotic	MIC against <i>A. baumannii</i> strains (µg/mL)					Median MIC
	ACB 9	ACB11	ACB12	ACB19	KCTC2508	
LNT103	4	4	8	4	16	4
Ampicillin	16	>512	>512	>512	128	>512
Ceftazidime	8	>512	256	>512	32	256
Kanamycin	32	256	256	512	2	256
Gentamicin	64	64	16	256	1	64
Tetracycline	128	8	32	4	2	8
Chloramphenicol	64	128	64	128	64	64
Sulfamethoxazole	512	512	512	512	512	512
Trimethoprim	32	128	>512	128	64	128
Ciprofloxacin	16	128	8	64	1	16
Colistin	1	2	1	2	4	2

susceptibility was observed across all species. Among *E. coli* strains, resistance exceeding 512 µg/mL was frequently detected for ampicillin and ceftazidime, and several strains also exhibited high-level resistance to kanamycin, sulfamethoxazole, and trimethoprim (Table 1). Notably, certain strains displayed multidrug resistance profiles, with elevated MICs across multiple antibiotic classes (e.g., CCARM1460). Similarly, *P. aeruginosa* strains exhibited pronounced resistance to β-lactams, with multiple isolates showing MICs >512 µg/mL for ampicillin and ceftazidime (Table 2). High-level resistance was also observed for kanamycin, gentamicin, chloramphenicol, and sulfamethoxazole in selected strains. In *A. baumannii*, resistance to sulfamethoxazole and trimethoprim was uniformly high, whereas susceptibility to other antibiotics varied among strains (Table 3). Overall, *A. baumannii* exhibited higher median antibiotic MICs than *E. coli* and *P. aeruginosa*, consistent with its well-established multidrug-resistant phenotype.

Intrinsic Susceptibility to Endolysin LNT103 is Species Dependent

In contrast to antibiotic susceptibility patterns, the MIC distribution of LNT103 revealed a distinct species-dependent trend. The median MIC of LNT103 was 16 µg/mL for *E. coli*, 72 µg/mL for *P. aeruginosa*, and 4 µg/mL for *A. baumannii* (Tables 1–3). Substantial intra-species variability was also observed. Notably, three *P. aeruginosa* strains exhibited endolysin MICs exceeding 128 µg/mL and were therefore excluded from subsequent combination analyses, as fractional inhibitory concentration indices could not be reliably calculated under these conditions. These results indicate that intrinsic susceptibility to LNT103 varies markedly among Gram-negative species and strains and is independent of conventional antibiotic resistance profiles.

Endolysin–Antibiotic Combinations Exhibit Species- and Strain-Specific Interaction Patterns

Combination testing of LNT103 with ten antibiotics across 15 *E. coli* strains yielded a total of 150 strain–drug pairs (Fig. 1A). Of these, five combinations (3.3%) met the criteria for synergy (FICI ≤ 0.5), 77 combinations (51.3%) were classified as additive, and 55 combinations (36.7%) were indifferent. No antagonistic interactions were detected. Synergistic effects were observed exclusively with chloramphenicol (two cases), sulfamethoxazole (two cases), and colistin (one case). When synergistic and additive interactions were considered together, 82 of 150 combinations (54.6%) exhibited positive effects. Strains CCARM939 and F715 showed the fewest positive interactions and also exhibited the highest MICs for LNT103, suggesting that reduced endolysin susceptibility limited the efficacy of combination treatments. In contrast, no consistent relationship was observed between antibiotic MIC values and the likelihood of synergy or additivity.

In *P. aeruginosa*, 100 combinations were evaluated across 10 strains (Fig. 1B). Two combinations (2.0%) demonstrated synergy, 74 combinations (74.0%) were additive, and 10 combinations (10.0%) were indifferent. As observed for *E. coli*, antagonism was absent across all combinations. Both synergistic interactions occurred in strain ATCC15692, which displayed the lowest MIC for LNT103 among tested *P. aeruginosa* strains. Overall, 76 of 100 combinations (76.0%) exhibited positive effects. These findings further support a dominant role for intrinsic endolysin susceptibility in determining combination outcomes, whereas antibiotic susceptibility alone did not predict interaction profiles.

Combination testing in *A. baumannii* included 50 strain–drug pairs across five strains (Fig. 1C). Two combinations (4.0%) exhibited synergy, 18 combinations (36.0%) were additive, and 24 combinations (48.0%) were indifferent. No antagonistic interactions were observed. When synergistic and additive interactions were combined, 20 of 50 combinations (40.0%) showed positive effects. Consistent with observations in *E. coli* and *P. aeruginosa*, the proportion of positive interactions inversely correlated with median LNT103 MIC values across species. In contrast, antibiotic MICs showed no direct association with combination outcomes. Among the 300 total strain–drug pairs tested, 30 combinations were unevaluable (N/A) because FICI values could not be determined under the assay conditions. Detailed FICI values are provided in Tables S1–S3.

Time–Kill Assays Reveal Endolysin Concentration as the Primary Determinant of Bactericidal Efficacy

To validate checkerboard-derived interaction patterns, time–kill assays were performed using two representative strains from each species that exhibited synergistic or strongly additive interactions. Combinations were tested at fractional MICs of both LNT103 and the corresponding antibiotic (Fig. 2). For *E. coli* strains ATCC8739 and F435, treatment with 0.5× MIC of LNT103 combined with 0.5× MIC of sulfamethoxazole resulted in complete bacterial killing (Fig. 2A). Notably, reducing the antibiotic concentration to 0.25× MIC while maintaining LNT103 at 0.5× MIC preserved bactericidal activity. In contrast, reducing the LNT103 concentration to 0.25× MIC while maintaining the antibiotic at 0.5× MIC led to diminished killing, indicating that endolysin concentration exerted a greater influence on antibacterial outcome. A similar trend was observed for *P. aeruginosa* strains ATCC15692 and CCARM2326. Treatment with 0.5× MIC of LNT103 and 0.5× MIC of chloramphenicol initially reduced viable counts, followed by limited regrowth (Fig. 2B), consistent with the relatively lower intrinsic susceptibility of *P. aeruginosa* to LNT103. Importantly, combinations containing higher endolysin concentrations consistently outperformed those with higher antibiotic concentrations but reduced endolysin exposure. For *A. baumannii* strains ACB11 and ACB19, combinations of 0.5× MIC of LNT103 and 0.5×

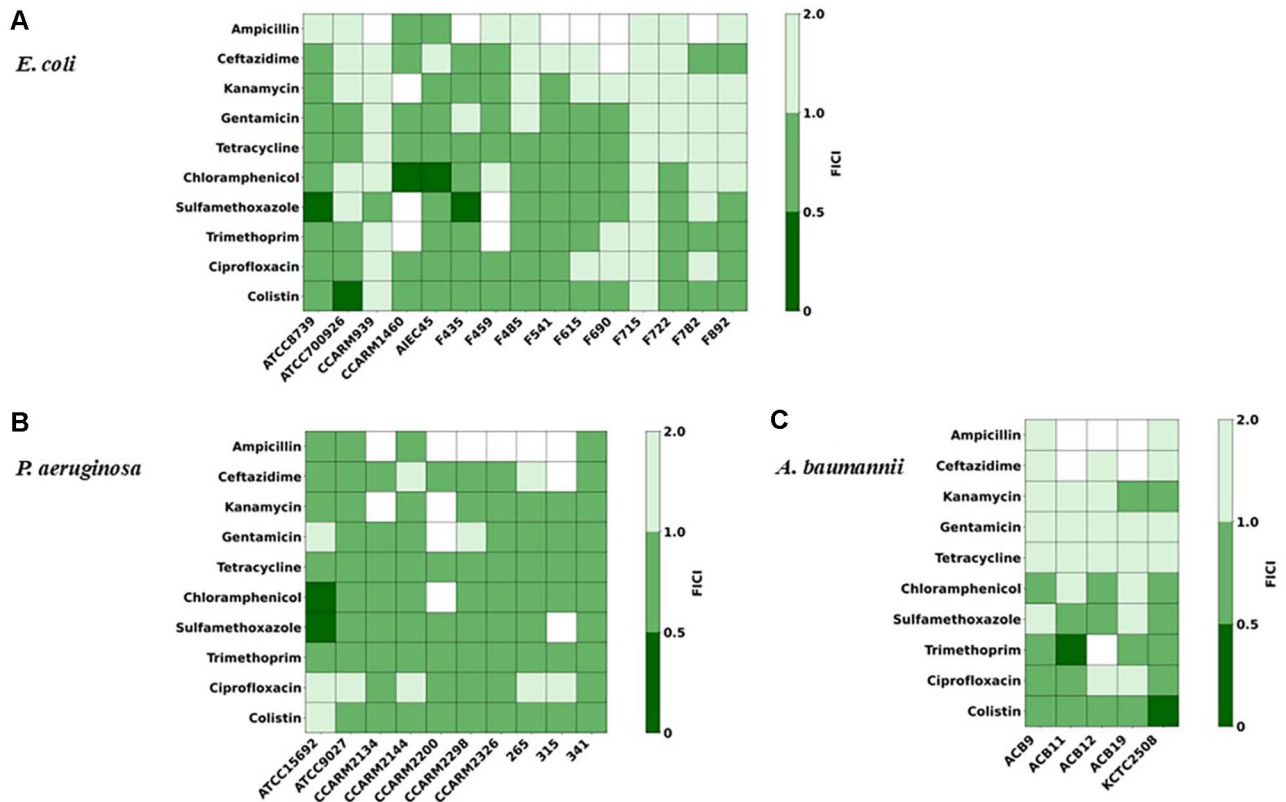


Fig. 1. Outcomes of endolysin–antibiotic combination treatments against Gram-negative pathogens. (A) *Escherichia coli* (15 strains), (B) *Pseudomonas aeruginosa* (10 strains), and (C) *Acinetobacter baumannii* (5 strains). Heatmaps depict fractional inhibitory concentration index (FICI) values: $FICI \leq 0.5$: synergy, $0.5 < FICI \leq 1.0$: additive, $1.0 < FICI \leq 4.0$: indifferent, or $FICI > 4.0$ antagonistic interaction. Unevaluable cases are shown as white squares.

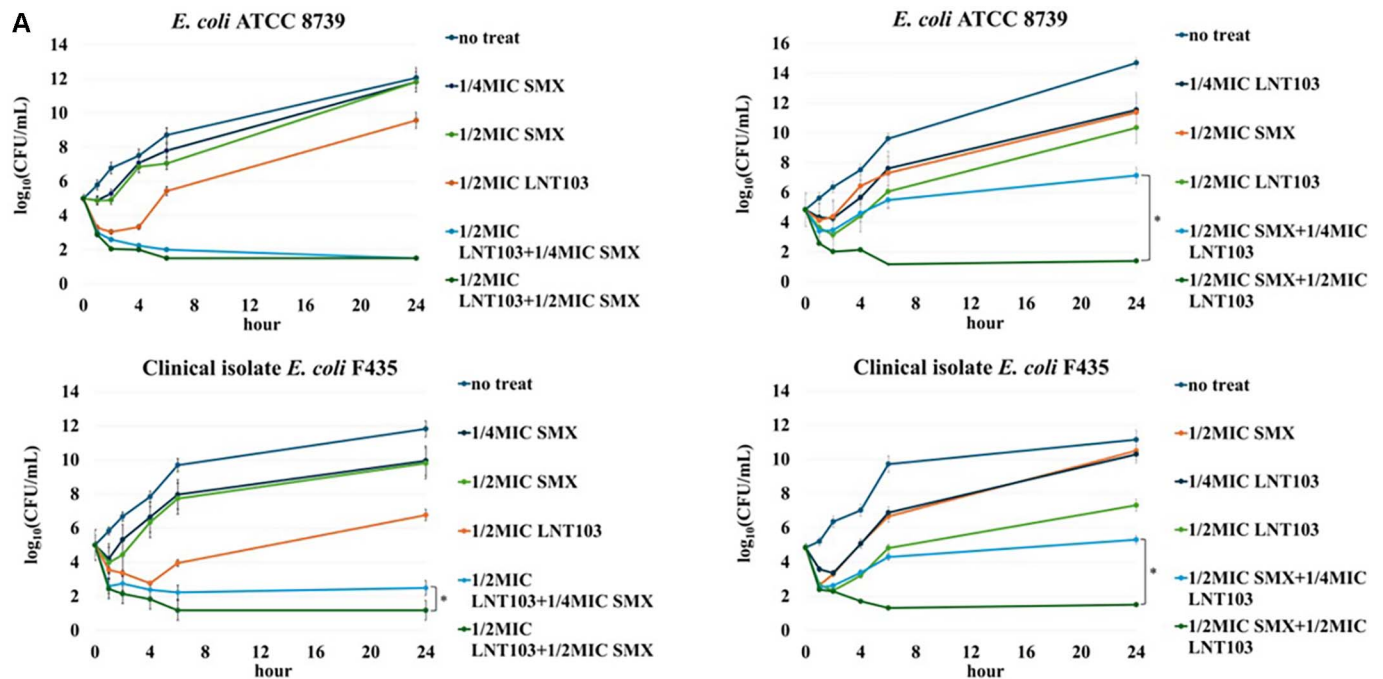


Fig. 2. Time-kill assays of representative endolysin–antibiotic combinations. (A) LNT103 with sulfamethoxazole against *E. coli* ATCC8739 and clinical isolate F435. (B) LNT103 with chloramphenicol against *P. aeruginosa* ATCC15692 and CCARM2326. (C) LNT103 with trimethoprim against *A. baumannii* ACB11 and ACB19. Experiments were performed in triplicate. Statistical significance was defined as follows: ns, $p \geq 0.05$; *, $p < 0.05$; and **, $p < 0.01$.

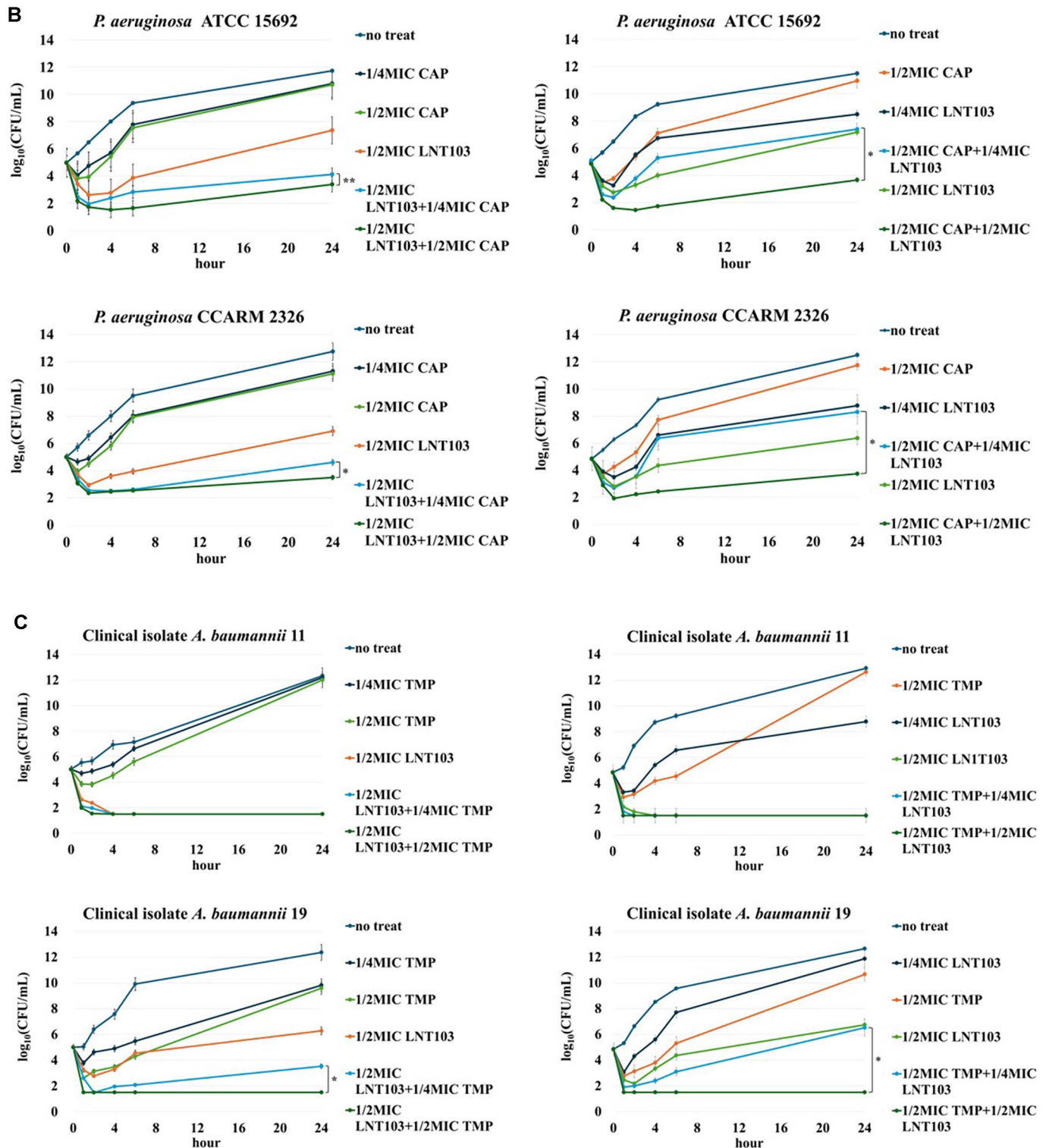


Fig. 2. Continued

MIC of trimethoprim resulted in complete killing (Fig. 2C). Reducing either agent to 0.25× MIC revealed strain-dependent differences; however, in both strains, reduced endolysin concentration was associated with a more pronounced loss of bactericidal activity than reduced antibiotic concentration.

Discussion

This study provides a comprehensive comparative analysis of endolysin–antibiotic interactions across three clinically important Gram-negative pathogens. A key finding is that positive combination effects were not associated with intrinsic antibiotic susceptibility but instead inversely correlated with endolysin MIC values. Thus, species and strains exhibiting lower intrinsic susceptibility to the endolysin tend to display a higher frequency of synergistic or additive interactions. However, no clear inverse relationship between endolysin MIC and FICI values was always observed, and endolysin MIC values did not define a distinct boundary between synergistic and additive effects in strains with lower intrinsic susceptibility to the endolysin, highlighting the importance of species- and strain-specific factors. Both synergistic and additive interactions are considered important, as their positive effects are not clearly distinguishable in *in vivo* animal models.

Importantly, the presence of synergy or additivity did not necessarily predict overall bactericidal efficacy. Time–kill assays revealed that endolysin concentration was the dominant determinant of antibacterial outcome, whereas increasing antibiotic concentration alone could not compensate for insufficient endolysin activity. These observations support a permeability-driven facilitation model, in which endolysin-mediated disruption of the bacterial envelope enhances antibiotic entry. Given that LNT103 contains an N-terminal cecropin A fusion domain, which is known to perturb bacterial membranes [10], adequate endolysin exposure appears essential to achieve sufficient envelope permeabilization. Below this threshold, antibiotics—even at higher concentrations—are unlikely to exert maximal activity.

Several endolysins have advanced into preclinical and clinical development [20–24], and co-administration with standard-of-care antibiotics is required in clinical trials. Our findings suggest that optimizing endolysin dosing, rather than escalating antibiotic exposure, may be critical for maximizing therapeutic benefit, particularly in the treatment of MDR Gram-negative infections.

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