

# Antibacterial Activity of cLFchimera and its Synergistic Potential with Antibiotics against Some Foodborne Pathogens Bacteria

**Sahar Roshanak**

Ferdowsi university of mashhad

**Zana Pirkhezranian**

Ferdowsi University of Mashhad

**Fakhri Shahidi**

Ferdowsi University of Mashhad

**Mohammad hadi Sekhavati** (✉ [sekhavati@ferdowsi.um.ac.ir](mailto:sekhavati@ferdowsi.um.ac.ir))

Ferdowsi University of Mashhad

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## Research

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# Abstract

**Background:** Frequent and unlimited use of antibiotics caused the development of antibiotic resistance by microorganisms. Therefore, there is an urgent need to discover novel antibacterial agents or a combination of agents as a safe treatment strategy for various infections. The aim of the present study was to evaluate the synergistic effects of cLFchimera, an antimicrobial peptides (AMPs), and antibiotics on several foodborne bacterial strains.

**Methods:** A checkerboard method was used to determine the synergistic effects of cLFchimera and several antibiotics (Gentamicin, Cefazolin and Ceftazidime) on bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*).

**Results:** The combination of cLFchimera and antibiotics generated a total and partial synergistic interaction for all foodborne bacterial strain used in the present study (FIC= 0.25 to 0.77). In most cases, the effect of peptide and antibiotic synergist on release of cellular content was not different compared to antibiotics when they used alone, but the count of viable cells significantly decreased in combination peptide and antibiotics treatments. Generally, antibacterial dynamics of the combination of peptide and antibiotics showed an increase and stable trend after reaching the peak point for *E. coli*, *P. aeruginosa* and *S. typhi*, respectively. Scanning electron microscopy analysis showed that bacterial cells treated with the combination Gentamycin and cLFchimera were markedly damaged, and most of the outermost layer of the bacterial cells disappeared.

**Conclusion:** Overall, our results may suggest that cLFchimera mediated its synergistic activity independent to antibiotics mode of action by disrupting the cell membrane and intramolecular mechanisms.

## Introduction

Antibiotic resistance has emerged as serious public health problem. The wide use of antibiotics has resulted in the emergence of resistant bacteria[1]. Therefore, an imperative need to propose new and safe antimicrobial agents, including antimicrobial peptides (AMPs), as alternatives to existing antibiotics [2]. AMPs play an important role in natural defense mechanism for destroying microbial infections [2]. AMPs have a net positive charge and an amphipathic structure and usually contain 12–50 amino acid residues[3, 4]. More recently, a chimeric form of peptide named cLFchimera has been expressed and purified in *E. coli* [5] in our lab. The results of *in vitro* studies showed that this peptide has antibacterial [5–7], antiviral [8], and anticancer [9] properties. Furthermore, the results of an *in vivo* experiment showed that supplementing *E. coli* challenged broilers with cLFchimera improved villi morphology in the jejunum, restored microbial balance in the ileum, and improved gene expression of cytokines and tight junctions in the jejunum of challenged birds [10]. These results revealed that this peptide could be nominated as an alternative for growth promoter antibiotics.

For enhancing the efficacy, restoring the sensitivity and reducing the minimum effective dose of antibiotics, their combined with other antimicrobial agents is one of the promising strategies [11, 12]. It has also been demonstrated that the combined use of APMs and antibiotics increase the bacterial killing of antibiotics regardless of the antibiotics' mode of action [13–15]. AMPs permeabilize and interact with the membrane of microorganisms in a very different manner than antibiotics [16], this perturbation in or pore formation on the bacterial cell wall may enhance the uptake of antibiotics and increase their antibacterial effect [17].

The aim of the present study was to evaluate the combined effects of cLFchimera and some current antibiotics compared with peptide alone, against some Gram-negative foodborne bacteria. The final goal of the present study was to determine the synergistic effects of these compounds to decrease the effective dose of antibiotics, thereby minimizing the potential toxic side effects of these drugs and reducing the chance of antibiotic resistance.

## Material And Methods

### Preparation of cLFchimera and antibiotics concentrations

The cLFchimera recombinant peptide was prepared from our previously study. A concentration of 1000 mg/ml of cLFchimera peptide was prepared in a sterile culture medium, filtered with 0.22  $\mu\text{m}$  and used as a stock to prepare other dilutions [18]. The antibiotics including, gentamicin, ceftazidime and cefazolin, which were prepared from Jaber Ebne Hayyan Pharmaceutical Company, Tehran, Iran used according to CISI [19].

### Preparation of inoculum

The bacterial strains were procured from microbial collection, Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad. Strains included were *Pseudomonas aeruginosa* PTCC 1707, *Escherichia coli* ATCC 25922, and *Salmonella typhi* PTCC 1609. Before the antimicrobial tests, The microbial strains were cultured 24 hours To standardize the microbial strains from the 0.5 McFarland standard, it was used according to its administration instructions, which was equivalent to  $1.5 \times 10^8$  CFU/mL of microorganism [20].

### Determination of minimum inhibitory concentration

MIC was performed using the micro broth dilution method as suggested by the Clinical and Laboratory Standards Institution [21]. Dilutions of cLFchimera (1, 2, 4, 8, 16, 32, 64, 128, 256, 512  $\mu\text{g/ml}$  L) were prepared in sterile Muller Hinton Broth (MHB) (Sigma-Aldrich). 10  $\mu\text{L}$  of microbial suspensions with an optical density of 630 nm ( $\text{OD}_{630}$ ) equal to 0.08–0.13, was added to 190  $\mu\text{L}$  each dilution in the 96 microwell plates. The microwell plates were incubated at 37 °C for 24 h. To determine the MICs, the absorbance was considered at 630 nm by ELISA reader model BioTek ELx808. The above protocol was repeated for each microorganism at same concentrations using gentamicin, ceftazidime and cefazolin,

separately. Growth medium without inoculum was used for negative controls [22]. In order to confirm the results, the experiments were repeated in three times.

## Determination of minimum bactericidal concentration

10 µL of the culture from each well including cLFchimera and Antibiotics in which the microbial growth was not observed, was cultured on Mueller Hinton Agar (Sigma-Aldrich). Then, the plates were incubated at 37 °C for 24 h and MBC was defined as the lowest concentration at which no growth of microorganism was observed [23]. For confirm the results, the experiments were repeated in three times.

## Synergistic Interaction between cLFchimera and Antibiotic by Checkerboard Assay

The synergistic interaction between cLFchimera peptide and any of the antibiotics, were measured using checkerboard method [11, 12]. Then, seven number of two-fold serial dilutions (from 2MIC to MIC/32) of the cLFchimera and the antibiotics were prepared; according to obtained MIC in the previous section for each microorganism. To obtain a fixed amount of both antimicrobial peptides, equal amount (25 µl) of each dilution were poured into 96-well microplates. Therefore, each row (and column) contained a fixed amount of the first agent and increasing amounts of the second one. A total of 50 µl of fresh bacteria suspension ( $10^8$  CFU/ml) were added to each wells and cultured at 37°C. The Fraction Inhibitory Concentration index ( $FIC_I$ ) was calculated using the following formula:

$$FIC_I = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B}$$

The above formula,  $MIC_A$  and  $MIC_B$  belong to compound A and B respectively.  $MIC_{A/B}$  belongs to the MIC of compound A in combination with B. Total synergism ( $FIC_I \leq 0.5$ ), partial synergism ( $0.5 < FIC_I \leq 0.75$ ), Indifference ( $0.75 < FIC_I \leq 2$ ) or antagonism ( $FIC_I > 2$ ) between the two compounds were obtained using  $FIC_I$  [24].

## Antimicrobial Dynamics of the Antimicrobial Agents

The antibacterial dynamics were determined using a liquid culture inhibition assay according Liu *at al.* with minor modifications [11]. A total of 50 µl of fresh bacteria ( $10^6$ - $10^8$  CFU/ml) were added to the wells of 96-well microplates, also 50 µl of the antimicrobial agent (at MICs or  $FIC_5$  concentrations), was added to each well. The bacterial strains were cultured at 37°C for 24 h and the absorbance was measured at 630 nm for 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 and 36 hours after incubation. 50 µl of the microbial suspensions without antimicrobial agents was used as a control group. The antibacterial activity was calculated using the following formula:

$$U = \sqrt{\frac{A_0 - A}{A_0}}$$

The above formula  $A_0$  is the absorbance of the control group and  $A$  is the absorbance of the tested group.

## Survival Curve

The effect of cLFchimera and any of the antibiotics were evaluated separately and in combination on the growth of microbial strains using draw a survival curve [25]. The final concentration of suspension of the strains (adjusted to  $10^6$ - $10^8$  CFU/ml) were added to the wells of 96-well microplates, and 50  $\mu$ l of the antimicrobial agent (at MICs or  $FIC_s$  concentrations), was added to each well. The bacterial strains were cultured at 37°C for 36 h. After incubating, a 50  $\mu$ l liquid from each dilution were spread on the surface of the agar plates and were incubated at 37 °C for 24 h, eventually, the number of CFU/ml was counted. It is worth noting 50  $\mu$ l of the microbial suspensions without antimicrobial agents was used as a control group. Finally, survival curves were constructed by plotting the log number of CFU/ml against time (h).

## Release of cytoplasmic material absorbing at 260 nm

The release of cytoplasmic material absorbing at 260 nm was measured according to the method described by Fadli et al. (2012). Viable cells in their exponential phase were collected using centrifugation (4000 rpm for 15 min.), washed three times, and resuspended in a saline buffer solution. Three milliliters of cell suspension of approximately  $10^8$  UFC/ml were incubated, under agitation, for 1 h at 37 °C in the presence of the antimicrobial agent (at MICs or  $FIC_s$  concentrations). After incubation, cells were centrifuged at 4000 rpm for 20 min, and the absorbance (260 nm) of the supernatant was determined using a WPA Lightwave S2000 UV/Vis Spectrophotometer (Richmond Scientific Ltd, England). The untreated cells (control) were corrected with buffer saline[12].

## Scanning Electron Microscopy

The bacterial strains cultured to the logarithmic phase in 100 ml of MHB at 37 C. The suspension was divided into four portions. Antimicrobials were added to three of the portions at at MICs or  $FIC_s$  concentrations. The remaining portion was left untreated as a control. The resuspension was incubated at 37 C for 3 h, and subsequently the cells from all four tubes were harvested through centrifugation and fixed with 2.5% glutaraldehyde overnight at 4 C. Subsequently, the cells were dehydrated using sequential ethanol concentrations ranging from 30 to 100%. The samples were gold covered through cathodic spraying. The morphology of the bacterial cells was observed through scanning electron microscopy (SEM, LAO-1450VP, Germany) [11].

## Results

### MIC determination

The MIC and MBC of each antibiotic and cLFchimera were determined using the broth microdilution method before the synergy test, and the highest concentration obtained was  $256 \mu\text{g ml}^{-1}$  (Table 1). cLFchimera showed weak activity against *P. aeruginosa* and *S. typhi* in the present study, while its activity was significantly better against *E. coli* ( $256$  vs.  $32 \mu\text{g ml}^{-1}$ , respectively).

Table 1  
The MIC/MBC values ( $\mu\text{g ml}^{-1}$ ) of peptide and antibiotics.

Microorganism	Sources	cLFchimera	Gentamicin	Cefazolin	Ceftazidime
<i>E. coli</i>		32/256	16/32	16/32	8/32
<i>P. aeruginosa</i>		256/512	32/64	32/64	32/64
<i>S. typhi</i>		256/512	16/32	32/64	32/64

MIC: Minimum inhibitory concentration

MBC: Minimum bactericidal concentration

### Synergy assay

We chose three commercially available antibiotics, Gentamicin, Cefazolin and Ceftazidime to investigate whether the combination of the cLFchimera with antibiotics provided a synergistic effect. In combination with a low peptide concentration equivalent to 4–32 of its MIC (Fig. 1, A), Gentamicin, Cefazolin, and Ceftazidime had improved antimicrobial activity, with 2–8-fold reduced MIC values for Gentamicin, 1–2-fold reduced MIC values for Cefazolin, and 2–16-fold reduced MIC values for Ceftazidime (Fig. 1, B, C and D). The microdilution checkboard method was carried out to evaluate the synergistic effects of the antibiotics combined with the cLFchimera, which were determined as FICIs (Table 2). The best antibacterial activity was obtained using a combination of cLFchimera + Cefazolin against *P. aeruginosa*, in which FIC index was 0.25. This combination generated a total synergistic effect on the tested microbes, except *E. coli* (FICI = 0.53), which showed a partial synergistic interaction. As the same way, the combination of cLFchimera + Gentamicin and cLFchimera + Ceftazidime generated a total synergistic effect on the tested microbes, except *S. typhi* and *E. coli* (FICI = 0.75 and 77, respectively), which showed a partial synergistic interaction.

Table 2  
The FIC Index of peptide and antibiotics against selected microorganism.

Microorganism	FIC index		
	cLFchimera + Gentamicin	cLFchimera + Cefazolin	cLFchimera + Ceftazidime
<i>E. coli</i>	0.5 <sup>a</sup>	0.53	0.77 <sup>b</sup>
<i>P. aeruginosa</i>	0.37 <sup>a</sup>	0.25 <sup>a</sup>	0.31 <sup>a</sup>
<i>S. typhi</i>	0.75 <sup>b</sup>	0.31 <sup>a</sup>	0.5 <sup>a</sup>
FIC: Fractional inhibitory concentration index			
<sup>a</sup> Total synergism.			
<sup>b</sup> Partial synergism.			

Release of cytoplasmic material absorbing at 260 nm

## Antibacterial dynamics of the antimicrobials

The relationship between the antibacterial activity and time for cLFchimera and antibiotics used alone and in combination is presented in Fig. 3. The results showed that, for *E. coli* the antibacterial activity of Gentamicin gradually increased, peaking at 20 h and subsequently declined after 3 h, whereas the antibacterial activity of cLFchimera peaked at 26 h and subsequently declined after 4 h. The combination of Gentamicin and cLFchimera peaked at 33 h, and lasted unchanged after 3 h. However, the antibacterial activity of Cefazolin, Ceftazidime and cLFchimera peaking at 27 h and immediately declined, while for their combination with cLFchimera the antibacterial activity gradually increased till 36 h. For *P. aeruginosa*, the antibacterial activity of all antibiotics and cLFchimera sharply increased, peaking around 9 h and remained unchanged more than 20 h. And finally for *S. typhi*, the antibacterial activity of the combination of antibiotics with cLFchimera sharply increased, peaking around 11 h and remained unchanged more than 20 h. These results suggested that in the combination of antibiotics and cLFchimera, showed synergistic effect for *E. coli*, where under all circumstances, the trend antibacterial activity for their combination (red line, Fig. 3) was higher than the other lines (red line, Fig. 3). For other bacteria, this pattern was seen only in combination of Cefazolin and cLFchimera.

Morphological study of *P. aeruginosa* treated with peptide + Gentamycin

For better understanding the effect of cLFchimera, Gentamycin and their combination (cLFchimera + Gentamycin) on the bacterial morphology, the treated bacteria were observed using SEM (Fig. 4). *P. aeruginosa* was treated with cLFchimera (256  $\mu\text{g mL}^{-1}$ ), Gentamycin (32  $\mu\text{g mL}^{-1}$ ) (their MICs) and their combination (74 and 68  $\mu\text{g mL}^{-1}$  for antibiotic and peptide, respectively). The bacterial cells treated with cLFchimera at MIC values did not differ greatly compared with control group which had a smooth surface

(blank, Fig. 4, A and C) with the outer layer of the bacteria remaining relatively intact. In contrast, the damaging effect of Gentamycin and Gentamycin + cLFchimera to cell wall was stronger than that of cLFchimera (Figs. 4, B and C, respectively). Bacterial cells treated with the combination Gentamycin + cLFchimera were markedly damaged and exhibited viscosity, and most of the outermost layer of the bacterial cells disappeared which promoted aggregation.

## Discussion

Increase treatment difficulty and complexity regarding to MRSE strain due to incorrect antibiotics use is currently a global issue in human health. AMPs are one of the novel and promising class of potent antibacterial drugs that can be considered as an alternative to antibiotics because they have useful features such as broad-spectrum antimicrobial activity and distinct membrane action mechanisms and are less likely than current antibiotics to induce drug resistance [26–28]. Furthermore, the effects of AMPs combined with antibiotics as a combination therapy often exceed those of the individual drugs, reduce the dose of drugs to minimize adverse effects and thus be a way to overcome problems with toxicity and the development of resistance [29]. AMPs facilitate the passage of conventional small molecule antibiotics through the membrane by disrupting the cell membrane, increase access these components to the cell and exerts synergistic effects [30, 31]

The present study confirmed the synergistic effect between cLFchimera and the currently used antibiotics Gentamicin, Cefazolin, and Ceftazidime against *E. coli*, *P. aeruginosa* and *S. typhi* strain. When used as monotherapy, Gentamicin, Cefazolin, and Ceftazidime showed

weak antibacterial activity against the bacterial strain, with MIC values of 32–60  $\mu\text{g mL}^{-1}$ . However, their antibacterial activities were significantly improved in combination with the cLFchimera at a low concentration equivalent to 4–32 of the MICs, with 2–8-fold reduced MIC values for Gentamicin, 1–2-fold reduced MIC values for Cefazolin, and 2–16-fold reduced MIC values for Ceftazidime. In addition, a total and partial synergistic effect was also observed for the combination of the peptides and the all antibiotics against the bacterial strain in this study by using checkerboard assays. Gentamicin showed total synergistic activity with cLFchimera against *E. coli* and *P. aeruginosa* but a partial synergistic activity against *S. typhi*. Consists with our results, Wu et al., (2017) showed that Gentamicin had synergistic activity with combination of DP7 and CLS001 peptides against *A. baumannii*, *P. aeruginosa* and *E. coli*. Cefazolin and Ceftazidime exhibited total synergistic activity with cLFchimera against *P. aeruginosa* and *S. typhi*, but a partial synergistic activity against *E. coli* [32]. Previous study, demonstrated that Ceftazidime exhibited partial synergistic activity with the Trp-containing peptides against multidrug-resistant *Staphylococcus epidermidis* (MRSE) in vitro and in vivo [31].

Release of cellular content in treated samples with cLFchimera was significantly lower than the antibiotic-treated groups. These results led us to hypothesize that cLFchimera exerts its antibacterial activity from other pathways instead membrane disruption. Moreover, the results of SEM analysis showed that cLFchimera had no visible damaging effect on the outer layer of the bacteria, suggesting that maybe a

molecular-level mechanism plays an important role in the synergistic action of cLFchimera. In this regards, Reyes-Cortes et al., (2017) showed that this chimeric peptide mediated its antibacterial activity by entering the cytoplasm through translocation across the bacterial membrane and possibly interacting with internal organelles [33]. Consistent with these results, Pirkhezranian et al., (2020 a, b) using molecular simulation analysis showed that cLFchimera and its derivatives had a higher affinity for DNA interaction and hypothesized this chimeric peptide mediated its activity by intramolecular mechanisms which interference DNA related pathways such as DNA replication[34, 35]. However, treatment of all bacteria with antibiotics and their combinations (antibiotics + peptide) resulted significantly increased the release of cellular contents. Ceftazidime and Cefazolin inhibits cell wall biosynthesis by adhering to bacterial penicillin-binding proteins which are involved in peptidoglycan synthesis and thereby inhibiting cell wall synthesis [36]. Therefore, our results fit well with the mode of action of Ceftazidime and Cefazolin. In the other hand, Gentamicin inhibits the bacterial protein synthesis by binding to 30S ribosomes[36]. Negatively impacting protein synthesis by Gentamicin could be consider as the activation of the cell wall stress response.

## **Conclusion**

Our results suggest that the cLFchimera may be used as promising synergistic agents to improve the antibacterial effectiveness of the selected antibiotics against *E. coli*, *P. eruginosa* and *S. typhi* strain and to reduce the therapeutic dose of antibiotics, thus minimizing their toxic side effects. Overall, our results may suggest that cLFchimera mediated its synergistic activity independent to antibiotics mode of action by disrupting the cell membrane and intramolecular mechanisms which requires more investigation in future studies.

## **Declarations**

### **Acknowledgments**

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### **Authors' contributions**

SR carried out the project. ZP carried out the project, wrote the manuscript. FSH was the contributed to manuscript preparation. MHS was the corresponding author and contributed to manuscript preparation. All authors have read and approved the manuscript.

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

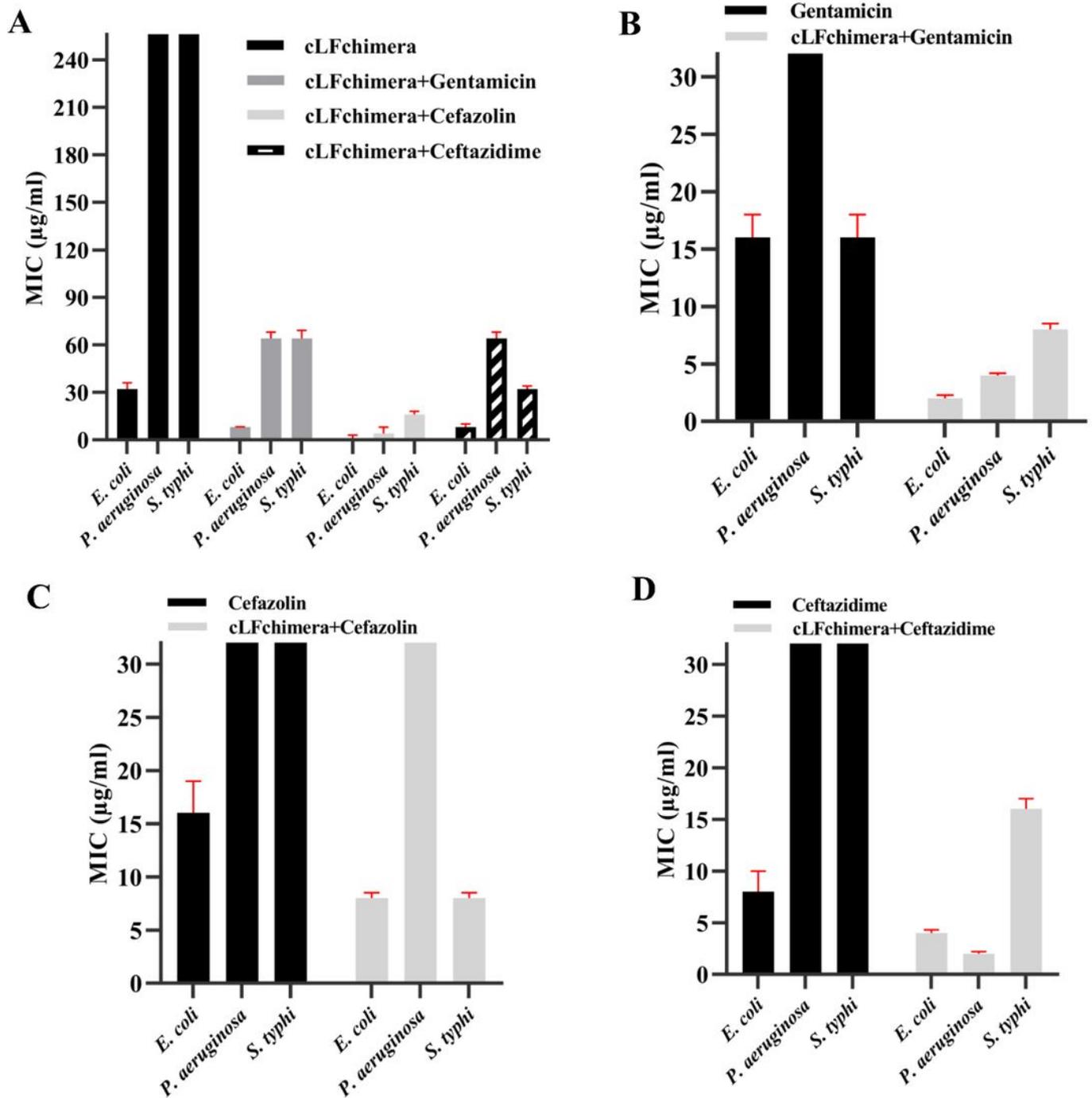
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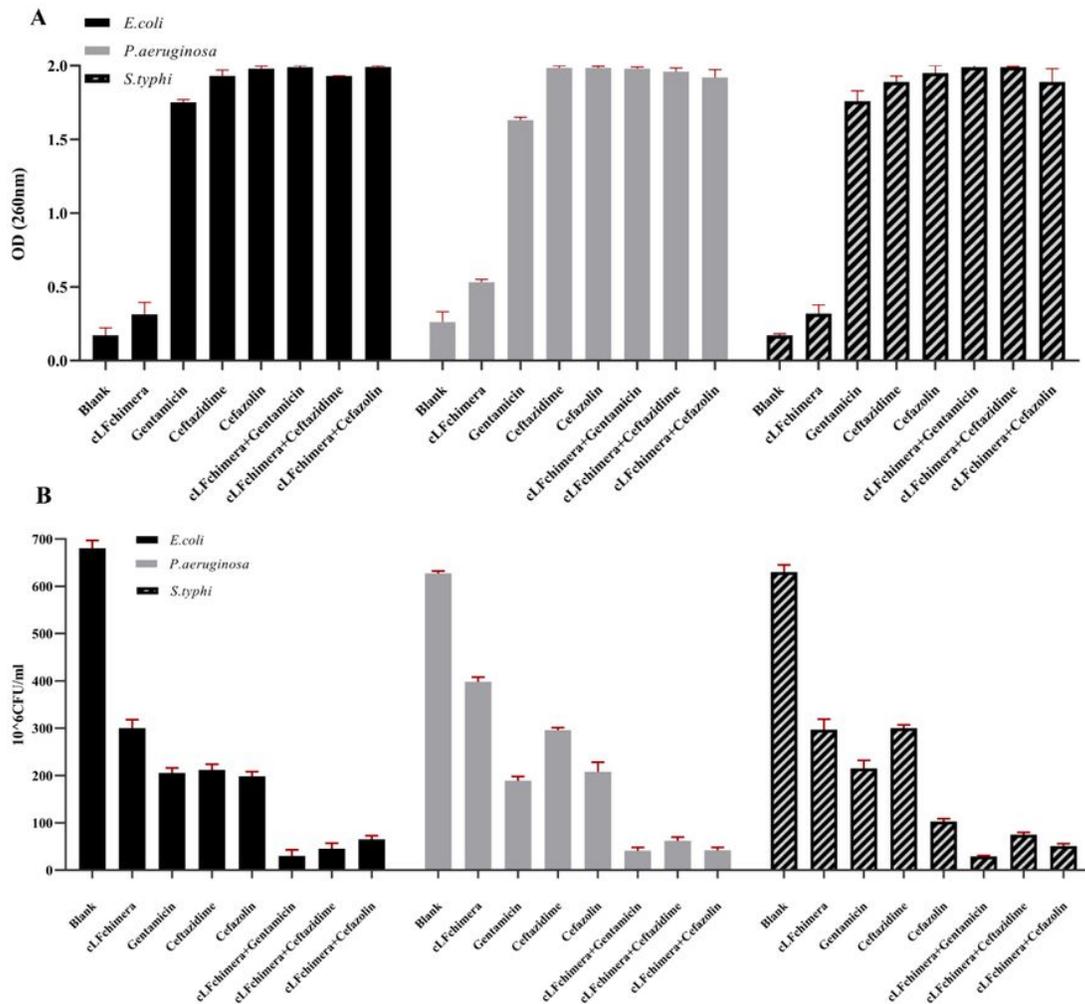
## Figures



**Figure 1**

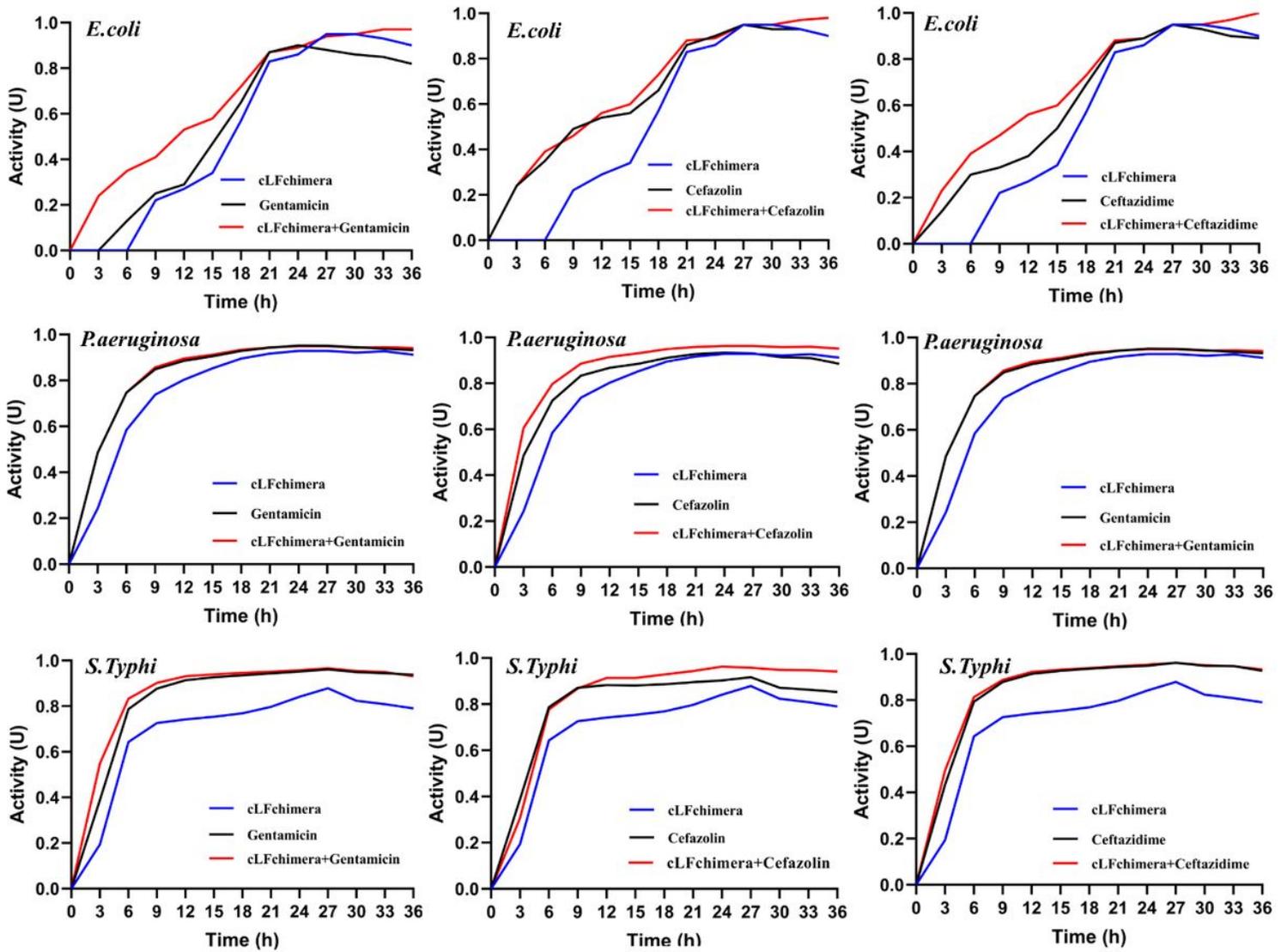
. Antibacterial activity of antibiotics in combination with cLFchimera. Bacterial cultures were treated with a series of concentration of antibiotics in the presence of a low peptide concentration equivalent to 4-32 of its MIC at 37° C overnight (A). Gentamicin, Cefazolin, and Ceftazidime had improved antimicrobial activity, with 2–8-fold reduced MIC values for Gentamicin (B), 1–2-fold reduced MIC values for Cefazolin (C), and 2–16-fold reduced MIC values for Ceftazidime (D). The OD600 was recorded using a microtiter

plate reader. The MIC was defined as the lowest antibiotics concentration that inhibited 95% of the bacterial growth. Each data point represents an average of six independent experiments.



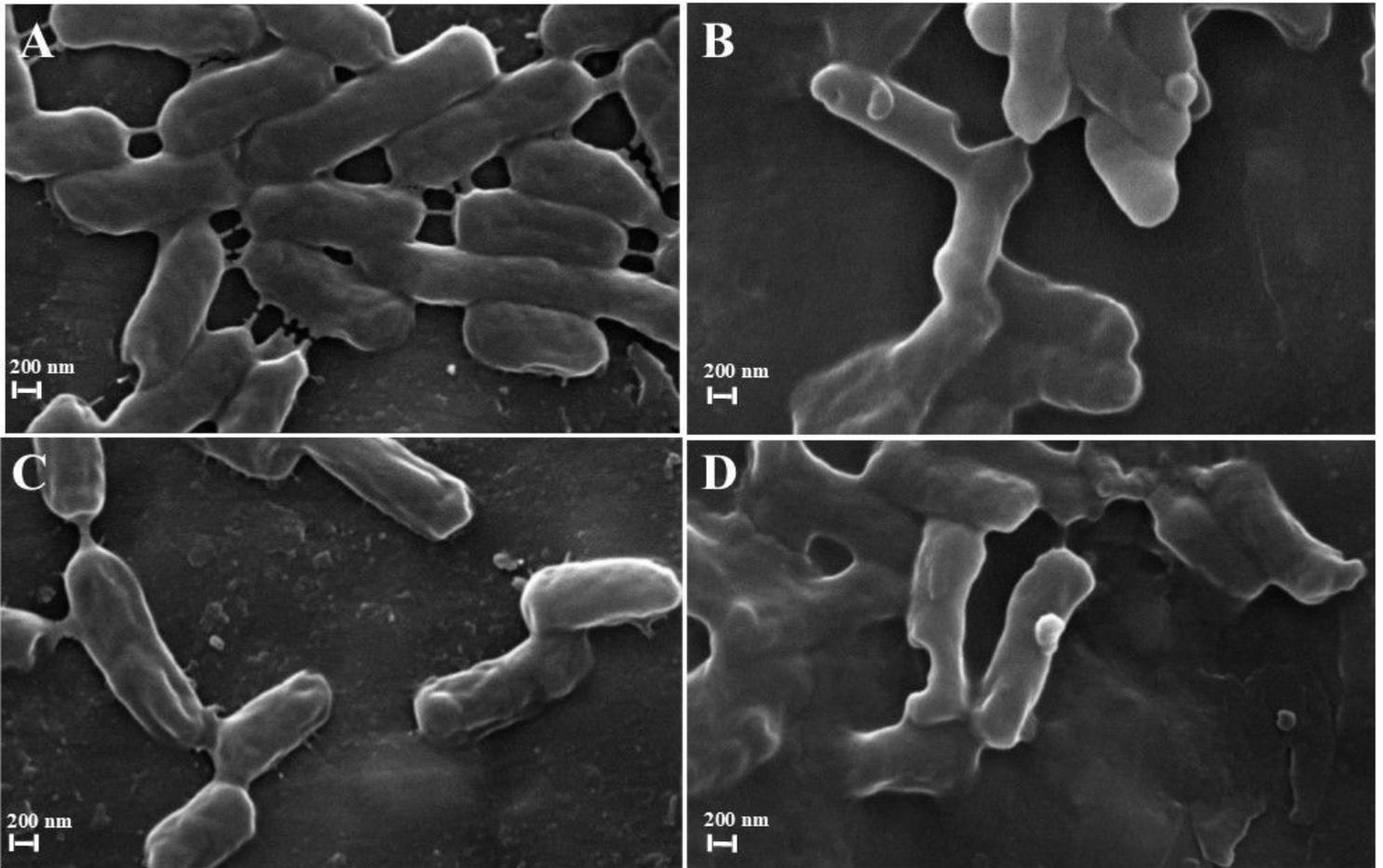
**Figure 2**

Leakage of cytoplasmic material (OD at 260 nm) (A) and viable cell concentration in CFU/ml after incubation (B). Blank: without any treatment.



**Figure 3**

The antibacterial dynamics of the antimicrobials against the bacterial strains in the present study.



**Figure 4**

SEM of *P. aeruginosa* without (A) and with 32 µg mL<sup>-1</sup> Gentamycin (B), 256 µg mL<sup>-1</sup> cLFchimera (C), and the combination of 74 cLFchimera µg mL<sup>-1</sup> and Gentamycin 68 µg mL<sup>-1</sup> (D).