BMC Microbiology

RESEARCH Open Access

Antimicrobial and anti-inflammatory effects of antimicrobial peptide Lf-KR against carbapenem-resistant *Escherichia coli*

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Abstract

Background Carbapenem-resistant *Escherichia coli* (CREC) is one of the most significant clinical pathogens, primarily emerging owing to the widespread use of broad-spectrum antibiotics. Antimicrobial resistance is a major global health challenge that prolongs treatment duration and increases healthcare costs. This study evaluated the antibacterial and anti-inflammatory effects of the antimicrobial peptide Lf-KR against CREC.

Methods Broth microdilution method, growth curve analysis, and time-kill assays were performed to evaluate the antibacterial activity of Lf-KR against CREC. The working mechanism of Lf-KR was elucidated using N-phenyl-1-naphthylamine, propidium iodide fluorochrome, and lipopolysaccharide-binding assays. qRT-PCR was used to assess the peptide's effects on the expression of pro-inflammatory cytokines expression during infection. Furthermore, the safety and stability of Lf-KR were assessed by testing its cytotoxicity, hemolytic activity, and antibacterial stability under various conditions. The *Galleria mellonella* infection model was applied to evaluate the in vivo activity of Lf-KR.

Results In vitro tests showed that Lf-KR exhibited potent antibacterial activity against CREC, with the minimum inhibitory concentrations of ranging from 4–8 μg/mL and minimum bactericidal concentrations 4–16 μg/mL. Mechanistically, Lf-KR induced bacterial cell death by disrupting the bacterial membrane. Furthermore, Lf-KR significantly reduced the expression of pro-inflammatory cytokine genes, including *IL-1β*, *IL-6*, and *TNF-α*, in RAW 264.7 macrophage cells infected with CREC. Lf-KR concentrations < 128 μg/mL showed no significant cytotoxicity or erythrocyte hemolytic activity. Lf-KR antibacterial activity was stable across a wide temperature range (− 80 °C to 65 °C), although it was more susceptible to inhibition by fetal bovine serum. The *G. mellonella* infection model further demonstrated the robust antimicrobial activity of Lf-KR.

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Conclusions This study demonstrated that the antimicrobial peptide Lf-KR is a highly promising antimicrobial and anti-inflammatory agent against CREC, with potential applications in combating multi drug-resistant bacterial infections.

Keywords Antimicrobial peptides, Lf-KR, Carbapenem-resistant *Escherichia coli*, Anti-inflammatory

Background

Escherichia coli is a highly significant pathogen worldwide, capable of causing various infections, including diarrhea, bloodstream infections, urinary tract infections, and sepsis [1]. Recently, carbapenem-resistant *Escherichia coli* (CREC) has emerged and continues to spread globally [2, 3]. Consequently, novel therapeutic strategies to combat CREC infections are urgently needed.

Antimicrobial peptides, also known as host defense peptides, are commonly produced by numerous organisms as part of the innate immune system, as the first line of defense against pathogens [4]. Typically, antimicrobial peptides have molecular weight of < 10 kDa and consist of relatively short amino acid sequences (5–50 amino acids) [5]. In addition to their antimicrobial properties, some antimicrobial peptides partly exhibit antibiofilm and immunomodulatory activities [6, 7]. The rational design of hybrid antimicrobial peptides is a promising area of research. This approach integrates distinct functional peptide domains to this approach significantly enhance antimicrobial efficacy, broaden the spectrum of activity, and reduce cytotoxic effects, thereby expanding their therapeutic potential [8, 9].

The hybrid peptide Lf-KR was designed through covalently linking two antimicrobial peptide sequences: LfcinB6 (RRWQWR-NH_2) and KR-12-a4 (KRIVKLIK-KWLR-NH_2), which are sequentially connected via a proline residue. This hybrid peptide exhibited enhanced physicochemical properties, including an increased net positive charge (+9), increased hydrophobicity (hydrophobic moment: 0.646 μH), and improved stability compared to its parent peptides. Structural analysis revealed a typical α-helical conformation, a molecular mass of 2647.28 Da, and a mass-to-charge ratio (m/z) of 662.8. Furthermore, Lf-KR exhibited broad-spectrum antimicrobial activity against both gram-negative and gram-positive bacterial strains, which is superior to those of the parent peptides [10].

In this study, the antimicrobial activity against CREC and underlying mechanisms of Lf-KR were systematically investigated through in vitro and in vivo experiments. Furthermore, the effects of Lf-KR on pro-inflammatory cytokines after bacterial stimulation were investigated. The therapeutic potential of Lf-KR was determined by evaluating the stability under various physiological conditions (including temperature and serum exposure), as well as its cytotoxicity and hemolytic activity. This study demonstrated an alternative approach to the clinical treatment of related infections and new insights into drug development.

Methods

Peptide and chemicals

Lf-KR was synthesized through solid-phase peptide synthesis by Nanjing Yuanpeptide Biotechnology Co., Ltd (Nanjing, China), yielding the amino acid sequence of RRWQWRPKRIVKLIKKWLR-NH_2 is the sequence of amino acid of Lf-KR [10]. Imipenem and meropenem were procured from Kangtai Biotechnology Co., Ltd (Wenzhou, China). Luria–Bertani (LB) and Mueller–Hinton (MH) broth were obtained from Oxoid.

Bacterial strains

Wenzhou Medical University First Affiliated Hospital provided 10 CREC strains for use in this study. The clinical information of CREC is detailed in Supplementary Table S1. Strains were stored in 30% glycerol and 70% LB broth at − 80 °C and incubated overnight at 37 °C on blood agar plates before use. *E. coli* ATCC 25922 was used as the quality control strain.

Minimum inhibitory concentration determination

The minimum inhibitory concentrations (MICs) of meropenem and imipenem against 10 CREC strains were examined using broth microdilution (BMD) method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [11]. To determine the MIC of Lf-KR, 100 μL MH broth was added to each well of a 96-well plate. Then, 100 μL of the prepared peptide was added to the last well, followed by serial dilutions across the remaining wells. Finally, 100 μL of the bacterial suspension (final concentration: 7.5×10^5 CFU/mL) was added to each well, and the plates were incubated for 16–18 h at 37 °C. The minimum concentration that entirely inhibited bacterial growth was designated as MIC, and the susceptibility or resistance of imipenem and meropenem was determined using the CLSI breakpoints [11].

Minimum bactericidal concentration determination

The minimum bactericidal concentrations (MBCs) of Lf-KR against 10 CREC strains were determined using CFU counts. Briefly, 10 μL suspensions from wells with varying drug concentrations in the BMD method were inoculated onto LB agar plates. After overnight incubation, the minimal concentration without bacterial growth on the plate was considered as MBC [12].

Growth curve analysis

To further understand the antimicrobial effect of Lf-KR, we performed 24 h growth curve monitoring assays, as described previously [13]. Using the same method for measuring MICs as described above, after adding the peptide preparation (1/4 – 2 × MIC) and bacterial suspension, the absorbance at 600 nm was examined at different time points (0, 2, 4, 6, 8, 10, 12, 20, and 24 h) with multifunctional microplate reader.

1-h time-kill curve assay

To evaluate the bacteriostatic speed of Lf-KR against CREC, 1-h time-kill curve assays were performed, as described previously with minor modifications [14]. Briefly, a 3 mL MH broth containing bacterial suspension (1.5×10^6 CFU/mL) and peptide at varying concentrations (1/4 – 2 × MIC) was prepared in a 10 mL sterile tube and incubated at 37 °C with shaking at 180 rpm. The control group contained only bacteria and phosphate-buffered saline (PBS). Every 10 min, an aliquot was removed from the tube and serially diluted 10-fold, after which 10 µL of the diluted liquid was spotted onto LB plates. After incubation, colony counting was performed. The minimum detection limit was 2 $\log_{10}$ CFU/mL.

N-phenyl-1-naphthylamine and Propidium iodide uptake assay

N-phenyl-1-naphthylamine (NPN) and propidium iodide (PI) were used to detect the disruptive effects of Lf-KR on the outer and inner bacterial membranes, respectively [15]. DC 6729 and DC 11722 were used in these assays. A single colony was cultured in MH broth with shaking at 175 rpm at 37 °C until the logarithmic growth phase is attained. The bacterial suspension was then centrifuged at 5000 ×*g* for 3 min. The bacterial cells were resuspended in PBS, and the peptide preparation (final concentration: 1/4 – 2 × MIC) was added to each tube. The group without the peptide was designated as the control group. After 2 h incubation with shaking at 37 °C, 1 mL of each suspension was centrifuged at 5000 ×*g* for 3 min. NPN (30 µg/mL) or PI (50 µg/mL) staining solution was added, and the mixture was incubated for 30 min. To wash off the excess dye, the mixture was centrifuged at 5000 ×*g* for 3 min, and washed twice with PBS, and resuspended by adding PBS and mixing well. Finally, the fluorescence of NPN (350/420 nm) and PI (535/615 nm) was detected.

Lipopolysaccharide-binding test

The checkerboard experiment was used to perform the lipopolysaccharide (LPS)-binding assay, as described previously with modifications [16]. The DC 6729 and DC 11722 strains were used in this test. First, 50 µL of MH broth-solubilized LPS solution was added to each well of a 96-well plate, creating a twofold dilution gradient with a final concentration range of 0–160 µg/mL. Next, 50 µL of successive twofold dilutions of the Lf-KR solution was added to the wells, with a final concentration range of 1–64 µg/mL. This step would yield different combinations of LPS and Lf-KR concentrations. Finally, 100 µL MH broth-prepared bacterial suspension (final concentration: 7.5×10^5 CFU/mL) was added into each well, and the plates were incubated for 18 h, after which absorbance was measured at 600 nm.

qRT-PCR detection of pro-inflammatory cytokines

Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum was used to culture RAW 264.7 cells. To explore the effect of Lf-KR on inflammatory cytokines of RAW 264.7 macrophages, qRT-PCR was employed to detect the expression of pro-inflammatory cytokines [17]. Briefly, 1 mL of RAW 264.7 cells (2×10^6 cells/mL) was added to each well of the 6-well plates. Once the cells adhered, the medium was aspirated, and added with peptides (1/2 – 4 × MIC) dissolved in PBS. single colony of DC 11722 strain was selected from a blood agar plate and adjusted to a 0.5 McFarland's suspension with PBS. RAW 264.7 cells were infected with 2×10^7 CFU bacteria. The negative control was the group containing only cells and PBS without bacteria, while the positive control was the group containing cells and bacteria but not peptides. After 2 h of treatment, the medium was discarded, and washed three times with PBS. Total RNA of the cells was extracted using RNAiso Plus (TaKaRa, Dalian, China), and the quality of the RNA was determined by A260/280. Synthesizing cDNA using the PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China). The cDNA samples were then analyzed by quantitative real-time PCR using the ABI QuantStudio 5 (Thermo Fisher Scientific, USA) with TB Green Premix Ex TaqTM ‖ (Takara, Dalian, China). The Ct values for pro-inflammatory cytokine genes were quantified and analyzed using the $2^{-\Delta\Delta CT}$ method. The housekeeping gene used was *β-actin*. The primers used in this test are listed in Supplementary Table S2.

Cytotoxicity experiments and hemolysis tests

The cytotoxicity of Lf-KR was assayed using human kidney-2 (HK-2) cells to assess its safety [18], and HK-2 cells were cultured as described above. Subsequently, 100 µL of 1×10^5 HK-2 cells to the 96-well plate. Lf-KR of different concentrations dissolved in PBS was then added to the wells, with wells added with PBS alone serving as the control group. After 12 h of treatment, 10 µL of Cell Counting Kit 8 solution was added to each well, and the absorbance was measured at 450 nm.

The hemolysis test was performed as described previously with slight modifications [12]. Fresh sheep red blood cells were washed with PBS, and diluted to 6% concentration. Lf-KR solution at serial concentrations was

co-incubated with sheep red blood cells for 60 min at 37 °C. Hemolytic activity was determined by measuring the absorbance (540 nm) of supernatant after centrifugation. 0.1% Triton X-100 treatment was used as positive control, and PBS treatment was used as negative control.

Temperature and serum sensitivity tests

To evaluate the stability of Lf-KR under various conditions, the peptide was treated under various temperatures (– 80 °C, 0 °C, 4 °C, 37 °C, 50 °C, and 65 °C) for 3 h. Furthermore, Lf-KR was incubated with fetal bovine serum of different concentrations (0 °C, 5 °C, and 10%) for 24 h [19], after which the MICs of the peptide against CREC was determined with BMD method.

Galleria. Mellonella infection model

G. mellonella was employed to evaluate the in vivo antimicrobial efficacy of Lf-KR, as described previously with some modifications, as mentioned earlier [20]. The DC 6729 strain was used for this experiment. First, larvae weighing 250 – 350 mg was selected, single DC 6729 colony was selected and adjusted to a suspension of 1.5×10^7 CFU/mL. After that, 10 µL of the bacterial suspension was injected into the right leg of the larva using a microinjector. After 2 h of infection, 10 µL of peptides at different concentrations (MIC × 7 and 2 × MIC × 7, where × 7 represents the multiple of the drug diluted with larval hemolymph) were injected into the left leg of the larvae using a micro syringe. The control group received an equal volume of sterile normal saline. In total three groups were tested, with 10 larvae per treatment group.

Statistical analysis

GraphPad Prism 8.0 was used to perform data analysis. The data were expressed as mean ± standard deviation. Statistical significance was evaluated using one-way ANOVA Tukey's multiple comparisons tests and log-rank tests.

Results

Antimicrobial activity of Lf-KR and carbapenems against CREC

The MICs of Lf-KR, imipenem, and meropenem against 10 CREC strains, along with the MBCs of Lf-KR, are summarized in Table 1. All tested strains exhibited resistance to imipenem and meropenem, with MICs exceeding the clinical resistance breakpoint (≥ 4 µg/mL). Lf-KR showed good antimicrobial activity against CREC with, MICs 4–8 µg/mL and MBCs 4–16 µg/mL.

Growth curves

We measured the 24 h growth curves based on the MICs for each strain. Compared with the control group, the curve trend indicated that Lf-KR slightly inhibited bacterial growth in the early stage at 1/4 × MIC (Fig. 1). At 1/2 × MIC, bacterial growth was inhibited for 12 h, while above the MIC, Lf-KR significantly inhibited bacterial growth for 24 h.

1-h time-kill curve

To further explore the antibacterial kinetic of Lf-KR, 1-h time-kill curve assays were plotted. Bacterial growth was hardly inhibited at 1/4 × MIC, displaying a trend similar trend to that of the control group (Fig. 2). As the concentration increased, the bactericidal effect became more pronounced and rapid. Therefore, the antibacterial and bactericidal effects of Lf-KR was dose-dependent.

Effect of Lf-KR on bacterial membrane permeability

NPN and PI staining experiments were performed to determine the disruptive effect of Lf-KR on bacterial membrane integrity. As shown in Fig. 3, Lf-KR significantly increased the permeability of the outer bacterial membrane at 1/2 × MIC (Fig. 3a) and significantly increased inner membrane permeability at above 1 × MIC (Fig. 3b). Thus, the antibacterial activity of Lf-KR is

Table 1 Antimicrobial susceptible test and β-lactamase of carbapenem resistant *E. coli*

strains	MIC (µg/mL)			MBC (µg/mL)	β-lactamase
	Lf-KR	IPM	MEM	Lf-KR	
DC 5147	8	8^R	8^R	8	KPC and CTX-M
DC 6729	4	4^R	4^R	4	NDM, TEM, and CTX-M
DC 7956	8	64^R	128^R	8	NDM, TEM, and CTX-M
DC 8439	4	8^R	32^R	8	NDM
DC 10495	4	8^R	32^R	4	NDM
DC 11712	4	16^R	8^R	4	CMY-42, TEM, CTX-M, and OXA
DC 11722	4	32^R	16^R	4	CMY-42, TEM, CTX-M, and OXA
DC 11723	8	16^R	32^R	16	NDM
DC 12843	8	4^R	16^R	16	NDM
DC 13281	8	8^R	32^R	8	NDM
ATCC 25922	4	0.06^S	0.03^S	8	/

IPM, imipenem; MEM, meropenem; MIC, the minimum inhibitory concentration; MBC, the minimum bactericidal concentration; R, resistant (≥ 4 µg/mL); S, susceptible (≤ 1 µg/mL)

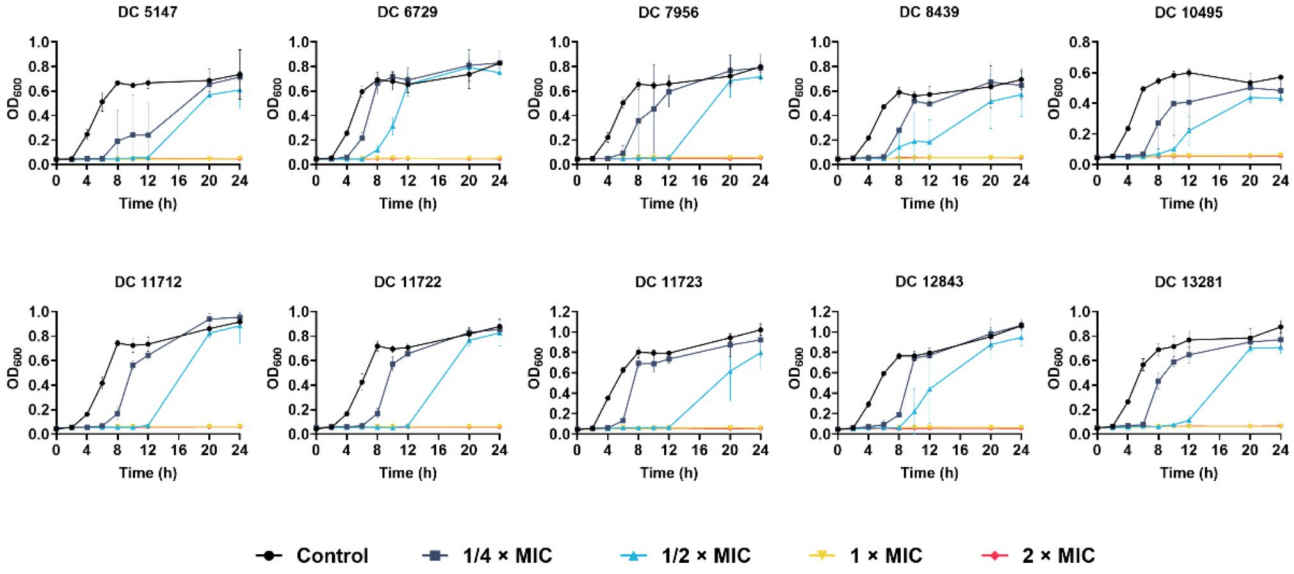

Fig. 1 Growth curves of 10 CREC strains treated with Lf-KR at different concentrations. The absorbance at optical density 600 nm (OD_{600}) of strains was measured over a 24 h period to monitor bacterial growth. The control groups represented the condition without Lf-KR. The Lf-KR concentrations ranged from 1/4 × MIC to 2 × MIC

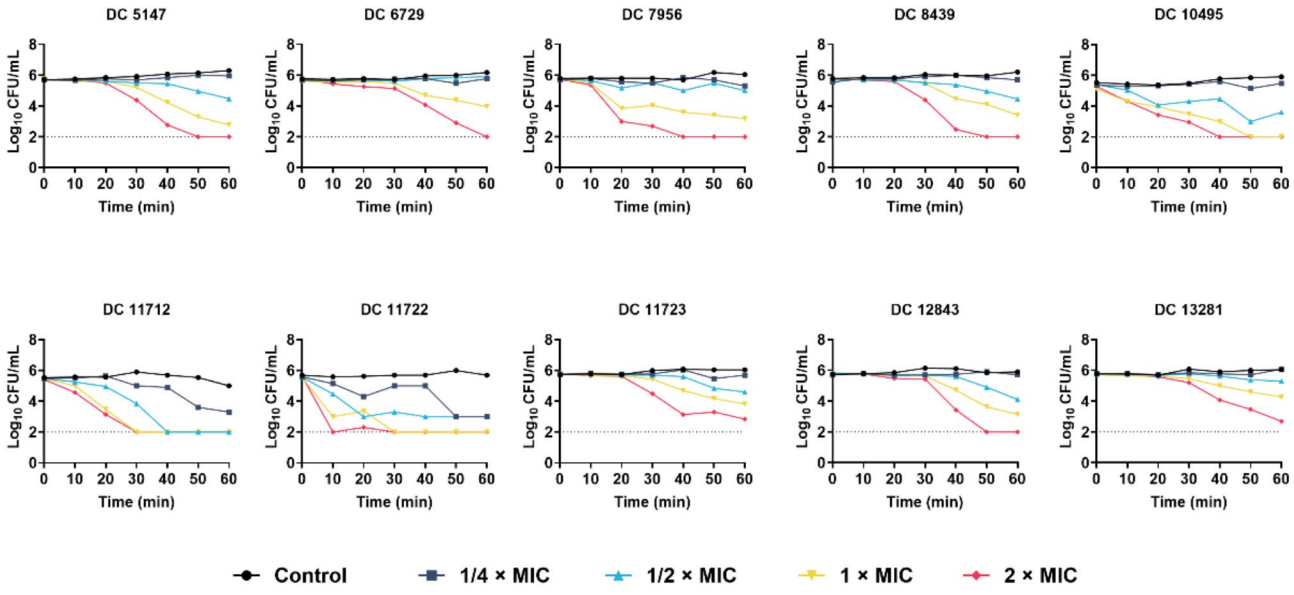

Fig. 2 The 1-h time-kill curves of 10 CREC strains treated with Lf-KR at different concentrations. Colony-forming unit (CFU) counts were performed every 10 min. The dashed line represented the lower limit of detection, which is 2 $\log_{10}$ CFU/mL. The control groups represented the condition without Lf-KR. The Lf-KR concentrations ranged from 1/4 × MIC to 2 × MIC

mediated through the disruption of bacterial membrane integrity.

Effect of Lf-KR on bacterial LPS

The interplay among LPS and Lf-KR was further explored to reveal the underlying mechanism of action. The MIC of Lf-KR increased gradually with the LPS concentration (Fig. 4), indicating that Lf-KR can react with LPS to alter bacterial membrane permeability.

Effect of Lf-KR on the inflammatory response

As bacterial infections tend to trigger a series of inflammatory responses, we performed qRT-PCR to detect the effect of Lf-KR on the inflammatory response in the macrophages during CREC infection (Fig. 5). The mRNA expression of pro-inflammatory cytokines *IL-6*, *IL-1β* and *TNF-α* in macrophages were significantly inhibited after treatment with Lf-KR, compared with the positive control.

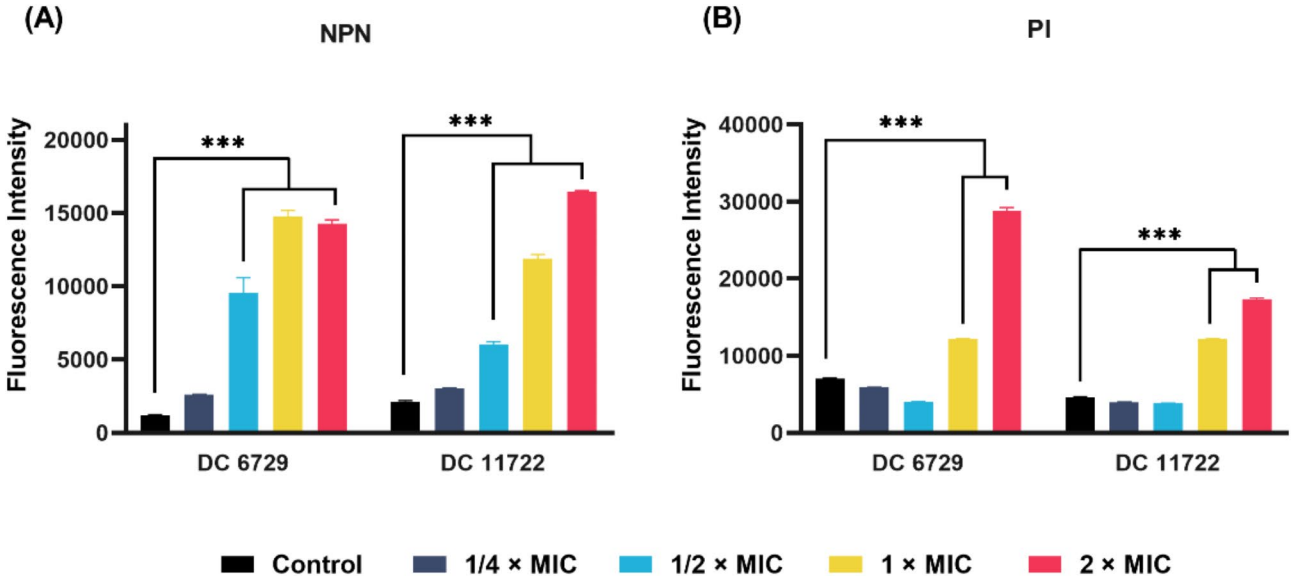

Fig. 3 Fluorescence intensity of NPN (**A**) and PI (**B**) staining for DC 6729 and DC 11722 strains treated with varying Lf-KR concentrations. ***$P < 0.001$

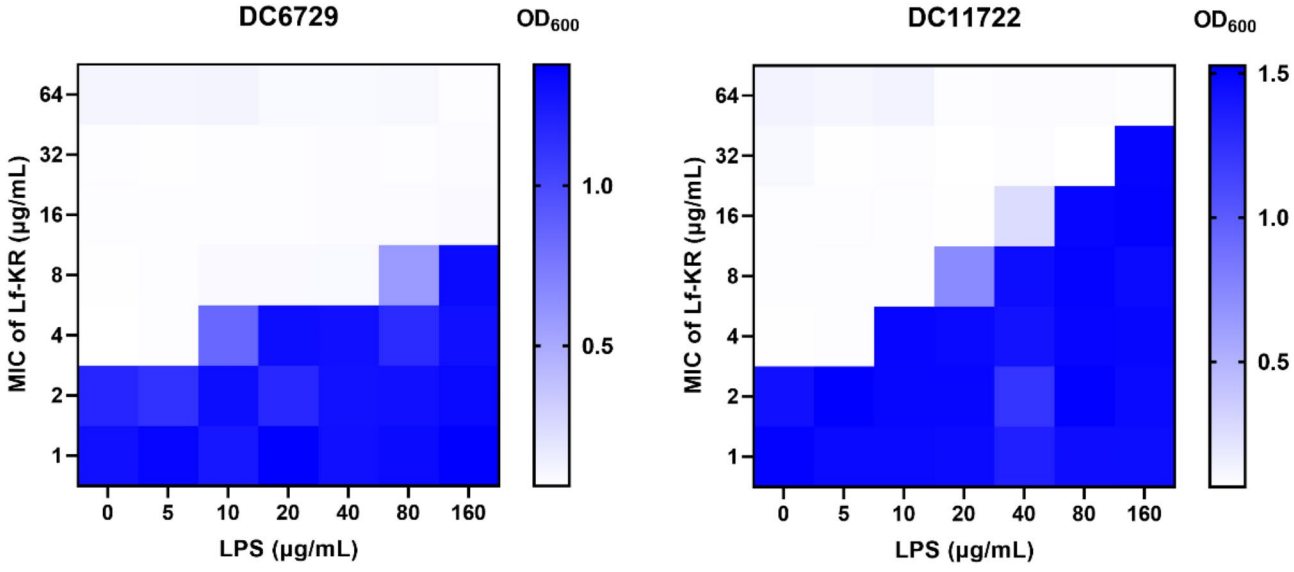

Fig. 4 Heat map of the LPS-binding assay. MICs of Lf-KR for DC 6729 and DC 11722 strains at different LPS concentrations were determined by measuring the absorbance at an optical density of 600 nm (OD_{600})

Safety of Lf-KR

Lf-KR exhibited no significant cytotoxicity at concentrations of ≤ 64 μg/mL (8 or 16 × MIC) compared to the control group (Fig. 6A). However, cell viability significantly decreased at 128 μg/mL (16 or 32 × MIC).

Compared with the negative control, Lf-KR exhibited no significant hemolytic activity at concentrations ≤ 64 μg/mL (8 or 16 × MIC; Fig. 6B) compared to the negative control. At a concentration of 128 μg/mL (16 or 32 × MIC), despite a significant change ($P < 0.05$), the hemolysis rate remained below 5%. These results indicate that Lf-KR exhibited good selectivity toward bacteria and showed potential utility for in vivo applications.

Stability of Lf-KR

We also investigated the stability of Lf-KR under varying temperatures and serum conditions. Lf-KR exhibited consistent MIC values against the DC 6729 and DC 11722 strains across a wide temperature range (– 80 °C to 65 °C), demonstrating excellent thermal stability (Tables 2 and 3). However, when exposed to increasing fetal bovine serum concentrations (0%, 5%, and 10%), the MIC for DC 11722 gradually increased, signifying that the serum stability of Lf-KR required further optimization.

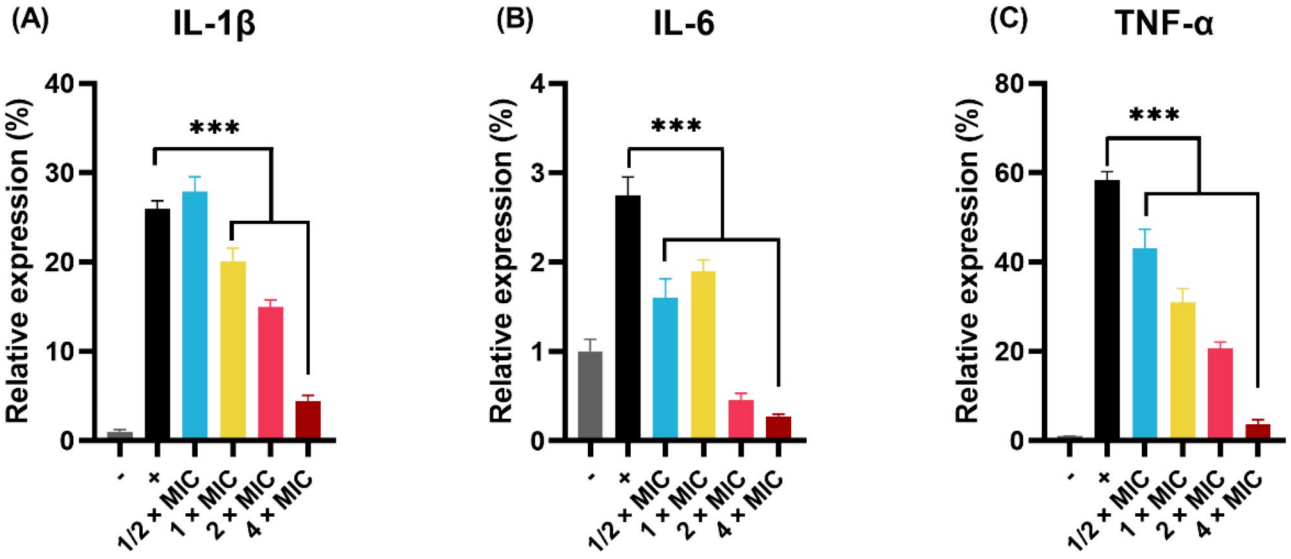

Fig. 5 Lf-KR inhibited the relative expression of pro-inflammatory factors, including *IL1β* (-**A**), *IL-6* (**B**), and *TNF-α* (**C**), in RAW 264.7 cells following DC 11,722 infection. ***$P < 0.001$; (-), only cells and PBS; (+), cells and bacteria

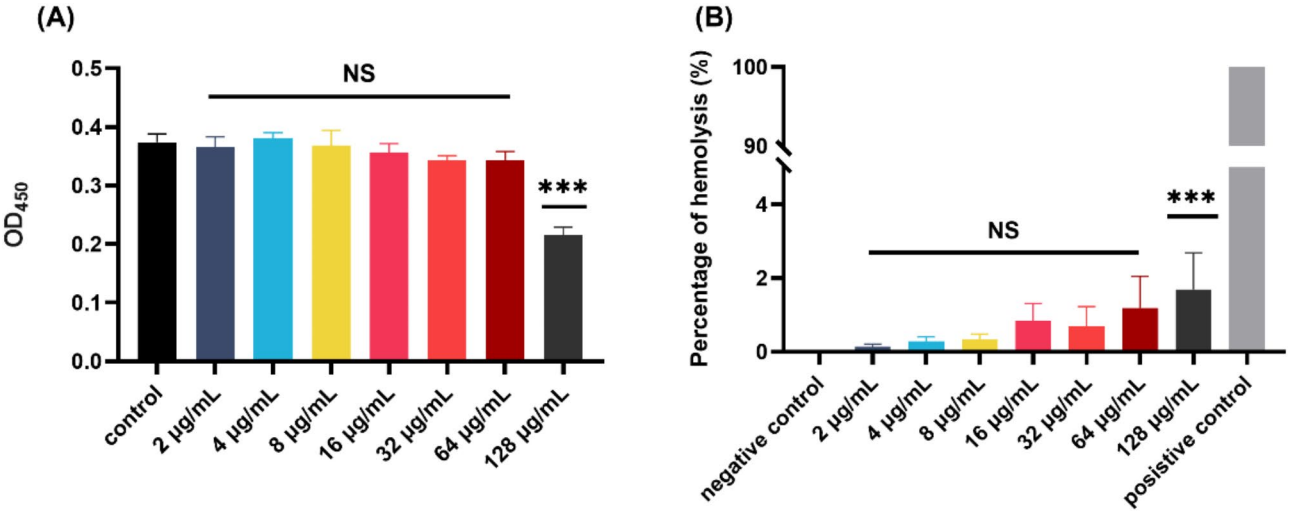

Fig. 6 Toxicity of Lf-KR. (**A**) Cytotoxicity of Lf-KR with different concentrations in the HK-2 cell line. (**B**) Hemolytic rate of Lf-KR against sheep red blood cells. NS, not significant, **$P < 0.01$, ***$P < 0.001$

Table 2 Antibacterial activity of Lf-KR at various temperatures [MIC (μg/mL)]

Strains	-80 °C	-20 °C	4 °C	37 °C	50 °C	65 °C
DC 6729	4	4	4	4	4	4
DC 11722	4	4	4	4	4	4

Table 3 Antibacterial activity of Lf-KR at varying prenatal serum concentrations [MIC (μg/mL)]

Strains	Control	5% Fetal bovine serum	10% Fetal bovine serum
DC 6729	4	4	4
DC 11722	4	8	16

Therapeutic effect in vivo

The *G. mellonella* infection model was established to assess the therapeutic effect of Lf-KR *in vivo*. The survival rate of the *G. mellonella* injected with Lf-KR was significantly improved (70–80%; $P < 0.05$) compared with the control group, for which survival rate was 10% (Fig. 7). The results suggested that Lf-KR holds promise for in vivo applications.

Discussion

CREC is a significant global concern, with its rapid global spread highlighting the urgent need for new treatment strategies [21, 22]. Carbapenem resistance in *Enterobacteriaceae* is primarily mediated by carbapenemase genes

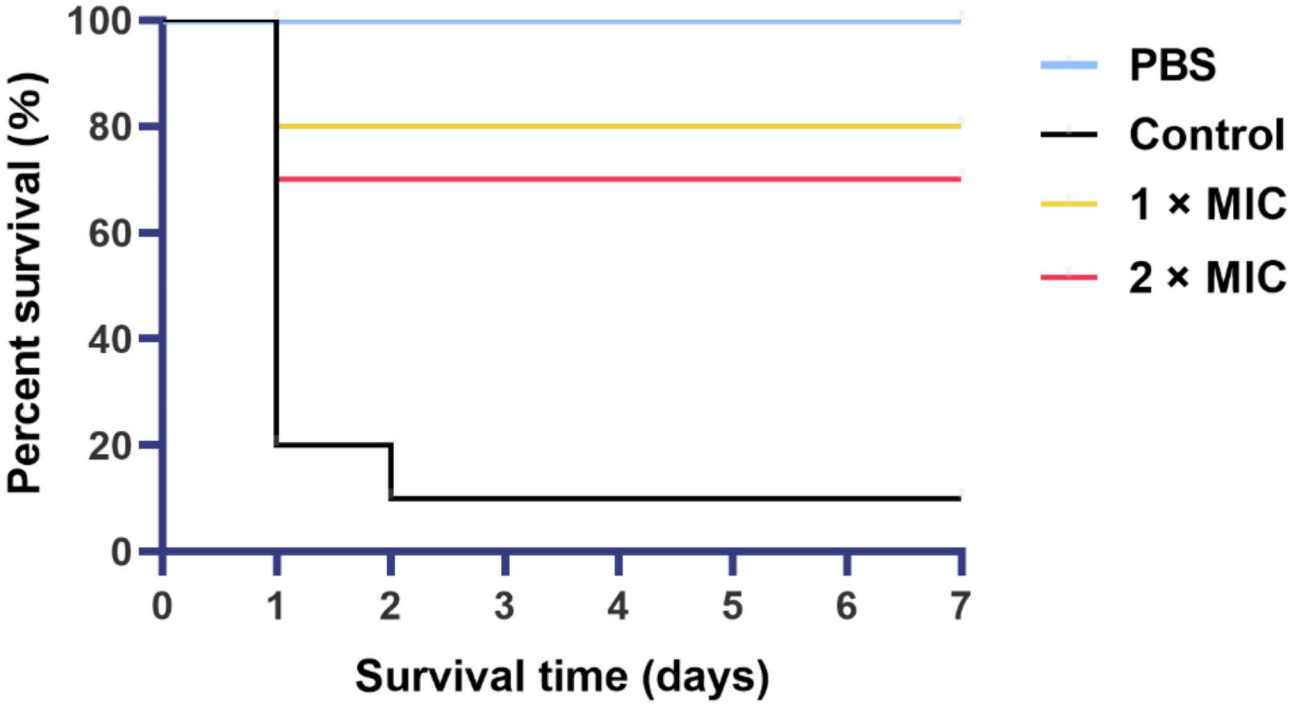

Fig. 7 Survival rates of *Galleria mellonella* after various treatments. Survival rate of larvae infected with DC 6729 were recorded for 7 days

located on plasmids. These include by class A carbapenemases (KPC) [23, 24], class B metallo-β-lactamases (NDM-1 and VIM-1) [25, 26], and class D oxacillinases (OXA-48 like carbapnemase) [27].

Antimicrobial peptides are a promising alternative to traditional antibiotics because of their broad-spectrum antimicrobial activity, ability to target bacterial membranes, and low probability of inducing drug resistance [28]. Lf-KR is a hybrid peptide derived from LfcinB6 and KR-12-a4. LfcinB6 (RRWQWR-NH_2) is the shortest peptide sequence exhibiting antimicrobial activity derived from the antimicrobial peptide LfcinB, which is a pepsin-digested product of bovine lactoferrin [29]. KR-12-a4 (KRIVKLIKKWLR-NH_2) is an analog of the antimicrobial peptide KR-12, which is the shortest sequence derived from LL-37 [30]. Previous studies have shown that the antibacterial activity of Lf-KR is comparable to melittin and is more potent than its parent peptides. It has exhibited significant antimicrobial activity against multidrug-resistant bacteria, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and multidrug-resistant *Pseudomonas aeruginosa* [10]. These studies highlighted the antimicrobial potential of Lf-KR. In the present study, we focused on CREC, which is a highly antimicrobial-resistant pathogen prevalent in hospital settings, highlighting the urgent need for novel antimicrobial agents to address this pose.

In this study, Lf-KR exhibited significant antibacterial activity against CREC in vitro and in vivo. The BMD method, growth curve analysis, and time-kill curve analysis revealed that the 10 CREC strains had MICs ranging from 4 to 8 μg/mL and MBCs ranging from 4 to 16 μg/mL, demonstrating antibacterial activity within 24 h. Generally, antimicrobial peptides rapidly act on bacterial membranes, exerting antibacterial effects within 15–90 min [31]. Our findings indicated that Lf-KR significantly reduced the bacterial loads within 40 min (> 3 $\log_{10}$ CFU/mL reduction). The *G. mellonella* model is commonly employed to explore the efficacy of newly developed drugs and drug combinations in vivo, such as the effectiveness of gentamicin and daptomycin or unconventional antibacterial agents like LL37 and quorum sensing inhibitors [32–34]. We established a *G. mellonella* infection model to explore the antimicrobial activity of Lf-KR in vivo. Lf-KR significantly reduced the mortality rate of larvae infected with CREC. Overall, the study findings demonstrate the antibacterial potential of Lf-KR against CREC and its potential for further development and applications in combating drug-resistant bacterial infections.

Antimicrobial peptides usually have positively charged properties that can interact with bacterial membranes [35]. NPN and PI staining experiments revealed that Lf-KR significantly disrupted the integrity of the bacterial membrane. Furthermore, LPS is a key component of the outer bacterial membrane and serves as a target for

certain cationic peptide antibiotics, such as polymyxin B [36]. The MIC of Lf-KR against CREC increased with the addition of exogenous LPS, demonstrating that LPS interacted with Lf-KR. Based on this result, we hypothesized that the mechanism of action of Lf-KR is similar to that of polymyxin. Presumably, Lf-KR bound to LPS attached on the outer membrane, subsequently disrupting both the inner and outer membranes, thereby inducing membrane impairment and bacterial cell mortality.

Bacterial infections usually triggers inflammatory responses, as LPS of stimulates macrophages and monocytes to produce high levels of pro-inflammatory cytokines [37]. Partial IL-1β activation is the key link to mediating the pro-inflammatory response, which invokes subprime inflammatory mediators, including IL-6 [38]. TNF-α is a pleiotropic pro-inflammatory cytokine involved in several diseases [39]. Our findings indicate that Lf-KR is capable of suppresses the expression of pro-inflammatory cytokine genes in macrophages infected with CREC, likely owing to its ability to interact with LPS.

We preliminarily assessed the safety of Lf-KR through hemolysis and cytotoxicity assays. The hemolysis test showed that the hemolytic activity of Lf-KR at different concentrations was negligible. Even at the highest concentration of 128 μg/mL, the hemolysis percentage was approximately 2%, which is minimal. Furthermore, our investigation of the cytotoxicity of Lf-KR against human HK-2 cells revealed that at the highest concentration of 128 μg/mL, Lf-KR significantly inhibited cellular activity. However, the Lf-KR is safe at the antimicrobial concentrations used in our experiments were safe for therapeutic use. Notably, previous studies reported that Lf-KR exhibited greater cytotoxicity toward mouse macrophage RAW264.7 cells than its parent peptides LfcinB6 and KR-12-a4. It decreased cell viability at 8 μM (approximately 21 μg/mL) [10]. This discrepancy may be due to the varying tolerance levels of different cell lines to the peptide. Further safety evaluations must be conducted across a broader range of cell types to confirm the overall safety profile of Lf-KR.

Subsequent stability tests revealed that Lf-KR's antimicrobial activity remained largely unaffected by temperature variations, indicating good thermal stability. However, its activity was slightly reduced in the presence of fetal bovine serum, signifying that serum components can affect peptide stability. Furthermore, the antimicrobial activity of Lf-KR remained stable in high salinity conditions. Several factors influencing antimicrobial peptides in serum are primarily the following: proteolytic enzymes, which hydrolyze antimicrobial peptides, directly compromising their structure; negatively charged serum proteins, such as albumin, which bind to positively charged antimicrobial peptides, reducing their effective concentration; and negatively charged host cell membranes, which compete with bacteria for binding antimicrobial peptides, thereby diminishing the antibacterial efficacy [40–42].

The limitations of this study mainly include the following: On one hand, we primarily focused on the antibacterial activity of Lf-KR against CREC, which is not sufficient to represent other multidrug-resistant pathogens. On the other hand, mammalian infection models were not used to evaluate the antibacterial effects of Lf-KR. Although we observed a significant antibacterial activity of Lf-KR against CREC, the complexity of the in vivo environment may affect its pharmacokinetics and distribution, thereby impacting its efficacy. Future studies should consider establishing mouse models of in vivo infection to obtain more accurate assessments of the clinical application potential of Lf-KR.

Conclusions

Our study demonstrates that Lf-KR exhibits significant antibacterial and bactericidal activity against CREC, and possesses anti-inflammatory properties. Lf-KR is a promising alternative treatment for CREC infections.

Abbreviations

CREC	Carbapenem-resistant Escherichia coli
Da	The average mass
m/z	The mass-to-charge ratio
LB	Luria-Bertani
MH	Mueller Hinton
LPS	Lipopolysaccharide
BMD	Broth microdilution
PI	Propidium iodide
NPN	N-phenyl-1-naphthylamine
PBS	Phosphate-buffered saline
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentrations
RT-qPCR	Real-Time quantitative polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03906-8.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

JCK and YW contributed equally to this work. JCK and YW participated in the experiments and paper writing. YL, WJC, YJH, HJZ, XDZ, and BBZ analysed the data and reviewed the manuscript. TLZ and JYZ designed the study and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

Funding

This work was funded by Key Laboratory of Clinical Laboratory Diagnosis and Translational Research of Zhejiang Province (2022E10022).

Data availability
The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 11 December 2024 / Accepted: 18 March 2025
Published online: 31 March 2025

References

1. Riley LW. Distinguishing pathovars from nonpathovars: *Escherichia coli*. Microbiol Spectr. 2020;8(4).
2. Liang WJ, Liu HY, Duan GC, Zhao YX, Chen SY, Yang HY, Xi YL. Emergence and mechanism of carbapenem-resistant *Escherichia coli* in Henan, China, 2014. J Infect Public Health. 2018;11(3):347–51.
3. Mahmoud NE, Altayb HN, Gurashi RM. Detection of Carbapenem-Resistant Genes in *Escherichia coli* Isolated from Drinking Water in Khartoum, Sudan. J Environ Public Health. 2020;2020:l2571293.
4. Annunziato G, Costantino G. Antimicrobial peptides (AMPs): a patent review (2015–2020). Expert Opin Ther Pat. 2020;30(12):931–47.
5. Kardani K, Bolhassani A. Antimicrobial/anticancer peptides: bioactive molecules and therapeutic agents. Immunotherapy. 2021;13(8):669–84.
6. Luo Y, Song Y. Mechanism of antimicrobial peptides: antimicrobial, Anti-Inflammatory and antibiofilm activities. Int J Mol Sci. 2021;22(21).
7. Jiang SJ, Xiao X, Zheng J, Lai S, Yang L, Li J, Liu C, Yang Y, Mu Y. Antibacterial and antibiofilm activities of novel antimicrobial peptide DP7 against the periodontal pathogen *Porphyromonas gingivalis*. J Appl Microbiol. 2022;133(2):1052–62.
8. Wang C, Yang C, Chen YC, Ma L, Huang K. Rational design of hybrid peptides: A novel drug design approach. Curr Med Sci. 2019;39(3):349–55.
9. Juretić D. Designed multifunctional peptides for intracellular targets. Antibiot (Basel). 2022;11(9).
10. Ajish C, Yang S, Kumar SD, Kim EY, Min HJ, Lee CW, Shin SH, Shin SY. A novel hybrid peptide composed of LfcinB6 and KR-12-a4 with enhanced antimicrobial, anti-inflammatory and anti-biofilm activities. Sci Rep. 2022;12(1):4365.
11. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 34th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2024.
12. Zhang X, Shi S, Yao Z, Zheng X, Li W, Zhang Y, Wang L, Cao J, Zhou T. Antimicrobial peptide WAM-1: a promising antibacterial and anti-inflammatory drug against carbapenem-resistant *Klebsiella pneumoniae*. J Antimicrob Chemother. 2022;77(7):1903–11.
13. Ding R, Wang X, Fu J, Chang Y, Li Y, Liu Y, Liu Y, Ma J, Hu J. Design, synthesis and antibacterial activity of novel pleuromutilin derivatives with thieno[2,3-d] pyrimidine substitution. Eur J Med Chem. 2022;237:114398.
14. Zhang Y, Lin Y, Zhang X, Chen L, Xu C, Liu S, Cao J, Zheng X, Jia H, Chen L, et al. Combining colistin with furanone C-30 rescues colistin resistance of Gram-Negative bacteria in vitro and in vivo. Microbiol Spectr. 2021;9(3):e0123121.
15. Jiang M, Chen R, Zhang J, Chen F, Wang KJ. A novel antimicrobial peptide Spampcin(56–86) from scylla paramamosain exerting rapid bactericidal and Anti-Biofilm activity in vitro and Anti-Infection in vivo. Int J Mol Sci. 2022;23(21).
16. Wang M, Deng Z, Li Y, Xu K, Ma Y, Yang ST, Wang J. Antibiofilm property and multiple action of peptide PEW300 against *Pseudomonas aeruginosa*. Front Microbiol. 2022;13:963292.
17. de Campos GY, Oliveira RA, Oliveira-Brito PKM, Roque-Barreira MC, da Silva TA. Pro-inflammatory response ensured by LPS and Pam3CSK4 in RAW 264.7 cells did not improve a fungistatic effect on *Cryptococcus Gattii* infection. PeerJ. 2020;8:e10295.
18. Murphy RA, Stafford RM, Petrasovits BA, Boone MA, Valentovic MA. Establishment of HK-2 cells as a relevant model to study Tenofovir-Induced cytotoxicity. Int J Mol Sci. 2017;18(3).
19. Dong M, Kwok SH, Humble JL, Liang Y, Tang SW, Tang KH, Tse MK, Lei JH, Ramalingam R, Koohi-Moghadam M, et al. BING, a novel antimicrobial peptide isolated from Japanese Medaka plasma, targets bacterial envelope stress response by suppressing CpxR expression. Sci Rep. 2021;11(1):12219.
20. Thieme L, Hartung A, Makarewicz O, Pletz MW. In vivo synergism of ampicillin, gentamicin, Ceftaroline and ceftriaxone against *Enterococcus faecalis* assessed in the *galleria Mellonella* infection model. J Antimicrob Chemother. 2020;75(8):2173–81.
21. Luo Y, Luo R, Ding H, Ren X, Luo H, Zhang Y, Ye L, Cui S. Characterization of Carbapenem-Resistant *Escherichia coli* isolates through the Whole-Genome sequencing analysis. Microb Drug Resist. 2018;24(2):175–80.
22. Wang Q, Jin L, Sun S, Yin Y, Wang R, Chen F, Wang X, Zhang Y, Hou J, Zhang Y, et al. Occurrence of high levels of Cefiderocol resistance in Carbapenem-Resistant *Escherichia coli* before its approval in China: a report from China CRE-Network. Microbiol Spectr. 2022;10(3):e0267021.
23. Navon-Venezia S, Chmelnitsky I, Leavitt A, Schwaber MJ, Schwartz D, Carmeli Y. Plasmid-mediated imipenem-hydrolyzing enzyme KPC-2 among multiple carbapenem-resistant *Escherichia coli* clones in Israel. Antimicrob Agents Chemother. 2006;50(9):3098–101.
24. Hong T, Moland ES, Abdalhamid B, Hanson ND, Wang J, Sloan C, Fabian D, Farajallah A, Levine J, Thomson KS. Escherichia coli: development of carbapenem resistance during therapy. Clin Infect Dis. 2005;40(10):e84–86.
25. El-Herte RI, Araj GF, Matar GM, Baroud M, Kanafani ZA, Kanj SS. Detection of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* producing NDM-1 in Lebanon. J Infect Dev Ctries. 2012;6(5):457–61.
26. Baroud M, Dandache I, Araj GF, Wakim R, Kanj S, Kanafani Z, Khairallah M, Sabra A, Shehab M, Dbaibo G, et al. Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. Int J Antimicrob Agents. 2013;41(1):75–9.
27. Gülmez D, Woodford N, Palepou MF, Mushtaq S, Metan G, Yakupogullari Y, Kocagoz S, Uzun O, Hascelik G, Livermore DM. Carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from Turkey with OXA-48-like carbapenemases and outer membrane protein loss. Int J Antimicrob Agents. 2008;31(6):523–6.
28. Bechinger B, Gorr SU. Antimicrobial peptides: mechanisms of action and resistance. J Dent Res. 2017;96(3):254–60.
29. Mouritzen MV, Petkovic M, Qvist K, Poulsen SS, Alarico S, Leal EC, Dalgaard LT, Empadinhas N, Carvalho E, Jenssen H. Improved diabetic wound healing by LFcinB is associated with relevant changes in the skin immune response and microbiota. Mol Ther Methods Clin Dev. 2021;20:726–39.
30. Wang G. Structures of human host defense Cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. J Biol Chem. 2008;283(47):32637–43.
31. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 2005;3(3):238–50.
32. Dean SN, Bishop BM, van Hoek ML. Susceptibility of Pseudomonas aeruginosa biofilm to Alpha-Helical peptides: D-enantiomer of LL-37. Front Microbiol. 2011;2:128.
33. Brackman G, Cos P, Maes L, Nelis HJ, Coenye T. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and in vivo. Antimicrob Agents Chemother. 2011;55(6):2655–61.
34. Luther MK, Arvanitis M, Mylonakis E, LaPlante KL. Activity of daptomycin or linezolid in combination with Rifampin or gentamicin against biofilm-forming *Enterococcus faecalis* or E. faecium in an *invitro* pharmacodynamic model using simulated endocardial vegetations and an in vivo survival assay using *galleria Mellonella* larvae. Antimicrob Agents Chemother. 2014;58(8):4612–20.

35. Zeth K, Sancho-Vaello E. The human antimicrobial peptides Dermcidin and LL-37 show novel distinct pathways in membrane interactions. Front Chem. 2017;5:86.
36. Sabnis A, Hagart KL, Klöckner A, Becce M, Evans LE, Furniss RCD, Mavridou DA, Murphy R, Stevens MM, Davies JC et al. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. Elife 2021;10.
37. Singh AK, Jiang Y. Lipopolysaccharide (LPS) induced activation of the immune system in control rats and rats chronically exposed to a low level of the organothiophosphate insecticide, acephate. Toxicol Ind Health. 2003;19(2–6):93–108.
38. Chen S, Wang Y, Pan Y, Liu Y, Zheng S, Ding K, Mu K, Yuan Y, Li Z, Song H, et al. Novel role for Tranilast in regulating NLRP3 ubiquitination, vascular inflammation, and atherosclerosis. J Am Heart Assoc. 2020;9(12):e015513.
39. Zhao Y, Zhang K, Zou M, Sun Y, Peng X. gga-miR-451 negatively regulates *Mycoplasma gallisepticum* (HS Strain)-Induced inflammatory cytokine production via targeting YWHAZ. Int J Mol Sci. 2018;19(4).
40. Wang Y, Song M, Chang W. Antimicrobial peptides and proteins against drug-resistant pathogens. Cell Surf. 2024;12:100135.
41. Wimley WC. Application of synthetic molecular evolution to the discovery of antimicrobial peptides. Adv Exp Med Biol. 2019;1117:241–55.
42. Islam T, Tamanna NT, Sagor MS, Zaki RM, Rabbee MF, Lackner M. Antimicrobial peptides: A promising solution to the rising threat of antibiotic resistance. Pharm. 2024;16(12).

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