Peptide lipidation and shortening optimises antibacterial, antibiofilm and membranolytic actions of an amphiphilic polylysine-polyphenyalanine octapeptide

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Peptide lipidation and shortening optimises antibacterial, antibiofilm and membranolytic actions of an amphiphilic polylysine-polyphenylalanine octapeptide

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ABSTRACT

The demand for broad-spectrum antibacterial agents continues with increasing rates of resistance of microbial pathogens to traditional antibiotics. Peptides and lipopeptides are gaining traction as promising novel, class-reference antibiotics for tackling difficult-to-treat infections caused by multi-drug resistant bacteria. To identify novel candidates and expand treatment options in clinical settings, we explored the *in vitro* antibacterial potential and mode of action of a short octapeptide combining a cationic block of four lysines and a highly hydrophobic segment of four phenylalanines (K4F4), and two K4F4-inspired lipopeptides (Palmitoyl-K4F4 and K4-NH-Palmitoyl). Preliminary AI-based screening had revealed the antimicrobial potential of the K4F4 peptide coupled with limited haemolytic activity. Broth dilution and haemolytic assays have confirmed these *in silico* predictions. Overall, our lipidated peptides were more active at lower MIC values compared to non-lipidated species, indicating the beneficial impact of tailing lipidation on design of peptide-based antimicrobials. An integrated view of the membrane-active mechanism of these novel therapeutic templates was obtained using a combination of flow cytometry, fluorescence microscopy and dye-based permeabilization assays. K4F4 and its lipidated derivatives act via a fast-disrupting mechanism without inducing bacterial resistance mechanisms in a long-term exposure assay. A K4F4-inspired lipopeptide together with its shorter version (K4-NH-Palmitoyl), were more stable in environments closer emulating physiological conditions, showing a higher antibacterial response in physiological salts and serum than their parent peptide. Our findings reveal the antibacterial and antibiofilm potential of a novel polylysine-polyphenylalanine peptide and highlight the significant contribution of lipidation and shortening as molecular engineering strategies to improve and guide the future design of next-generation membrane-targeting antibiotics.

1. Introduction

For close to a century, antibiotics have successfully controlled bacterial infections (Ventola, 2015). However, due to the multiple resistance mechanisms evolved by both Gram-positive and Gram-negative bacteria and the ineffectiveness of some antimicrobial agents, previously treatable conditions can now lead to severe life-threatening complications and even death (Prestinaci et al., 2015; Pulingam et al., 2022). With the number of therapeutic options available now severely limited, there has been a significant push towards the isolation and study of novel nature-inspired agents, the re-engineering of existing approved medication and the use of computer-aided (AI) technologies for the development of future antibiotic drugs (Almeida et al., 2018; Dutescu and Hillier, 2021). From the discovery and design of penicillin derivatives inspired by a short, cyclic molecule consisting of two amino acids, peptides and their chemically modified versions, including lipopeptides, have been added to the FDA-approved database of drug candidates for advancement to clinical trials (Ribeiro da Cunha et al., 2019). Peptides and their derivatives are now considered one of the therapeutic pillars of the core package of promising strategies to tackle antimicrobial resistance (AMR) (Goyal and Ramakrishnan, 2023).

Peptides are short and versatile molecules that offer a wide and diverse chemical backbone that can both be rationalised and engineered through computer-aided technologies (Diller et al., 2015; Zhu et al.,

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2023). In terms of antimicrobial activity, cationic and hydrophobic amino acids, such as lysine and phenylalanine stand out as key, active elements in the primary sequence of peptides and approved antibacterial drugs (Robles-Loaiza et al., 2021; Tan et al., 2021). Despite their value in drug research and development, many of these molecules can be broken down by naturally occurring proteolytic enzymes or inactivated by salts, which leads to lowered activity in clinical and physiological scenarios (Al Musaimi et al., 2022). On the other hand, they constitute easy-to-synthesise templates with flexible manufacturing capabilities that can be chemically modified using an array of strategies (Al Musaimi et al., 2022; Gutman et al., 2022). Several research groups have successfully designed different types of peptide-mimetic antimicrobials capable of replicating the cationic amphiphilic nature of AMPs and their effective bactericidal mechanisms (Lai et al., 2008; Mowery et al., 2009; Scott et al., 2008; Takahashi et al., 2013). These elaborate structures which include peptide backbone mimetics, and functional and topographical mimetics can fine-tune the conformation, potency, selectivity and stability of AMPs (Vagner et al., 2008). These advancements overcome traditional challenges and mark a step forward in peptide application. In addition to the 20 naturally occurring amino acids available for chemical substitution and as part of the peptidomimetic toolbox, more potent peptide-based agents can be obtained by combining them with other biomolecules, such as lipids (Kowalczyk et al., 2017; Menacho-Melgar et al., 2019). Our study screened the antibacterial, antibiofilm and haemolytic effects of an 8-mer synthetic peptide composed of four cationic lysines and four hydrophobic phenylalanines (K4F4), and two lipopeptides engineered around the K4F4 peptide backbone (Palmitoyl-K4F4 and K4-NH-Palmitoyl) against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. We also evaluated their impact on bacterial membrane integrity, stability and their abilities to induce bacterial resistance after long-term exposure.

2. Material and Methods

2.1. Design of an antimicrobial peptide (AMP) template guided by computational approaches

Inspired by previous studies reporting strong antibacterial and antibiofilm activities of short peptide sequences integrating four cationic and four hydrophobic amino acids, particularly blocks comprising arginine and phenylalanine residues (Agüero-Chapin et al., 2023; Edwards-Gayle et al., 2020), and considering the high abundance of lysine in membranolytic peptide-based antibiotics (Cutrona et al., 2015; Decker et al., 2022), we used a computational-driven approach to confirm the potential of K4F4 as an antimicrobial template. As far we know, this amino acid sequence has not been evaluated for its antimicrobial and antibiofilm activity. Initially, we used two web-servers: AMPfun (Chung et al., 2019) and Antimicrobial Peptide Scanner vr.2 (Veltri et al., 2018) to gain insights into its possible antimicrobial properties. Posteriorly, to anticipate potential toxicity issues we employed HemoPI (Chaudhary et al., 2016), HemoPred (Win et al., 2017) and HAPPENN (Timmons and Hewage, 2020) to analyse the haemolytic tendency of our amphiphilic peptide template. Finally, we designed two lipidated peptides based on our backbone K4F4 peptide sequence to characterise their antibacterial, antibiofilm and haemolytic effects *in vitro*. To construct the first lipopeptide, named here as Palmitoyl-K4F4 (C16-K4F4), we incorporated a saturated hydrocarbon tail of 16 carbons to the primary structure of K4F4. In the second case, we explored whether a shorter amino acid sequence can still retain the therapeutic action, improve stability, and reduce toxicity. In this context, we synthesised the lipopeptide K4-NH-Palmitoyl (K4-NH-C16) retaining only the cationic block comprising lysine residues conjugated with a palmitoyl tail.

2.2. Obtention of K4F4 and K4F4-based lipopeptides

K4F4 and K4F4-based lipopeptides were purchased from Peptide Protein Research Ltd (Hampshire, UK). Identity and purity of each synthetic product were confirmed and assessed, respectively, using High Pressure Liquid Chromatography (HPLC) and mass spectrometry. The area under the curves in the chromatographic profiling of pure peptide confirmed the high purity levels of K4F4, C16-K4F4 and K4-NH-C16 (**Supplementary material Figure S1**). All peptides showed a minimum purity of 98.00 %. The molar mass of both the amphiphilic peptide and K4F4-based lipopeptides were confirmed by electrospray ionisation mass spectrometry (**Supplementary material Figure S2**), indicating the successful obtention of the synthetic peptide and lipidated derivatives.

2.3. Antimicrobial activity screening

In vitro susceptibility testing of antimicrobial peptide-based candidates and a commercial broad-spectrum antibiotic (Ciprofloxacin) was performed employing the classical Broth-dilution method (Wiegand et al., 2008). In brief, a serial dilution of each tested compound (K4F4, C16-K4F4 and K4-NH-C16) was used to determine their Minimum Inhibitory Concentration (MIC) against enterohaemorrhagic *Escherichia coli* O157:H7 strain EDL933, *E. coli* (K12), a multi drug (carbapenems, β-lactam, aminoglycosides and quinolones) resistant strain of *Pseudomonas aeruginosa* NCTC13437, *P. aeruginosa* (PA01) and *Staphylococcus aureus* (ATCC 12600). Bacterial cultures, stored in 30 % glycerol stocks at −80 °C, were inoculated onto Lysogeny agar (LA) and incubated overnight at 37 °C. A single isolated colony was then transferred into 5 mL of Lysogeny broth (LB) and cultured overnight on an orbital shaker (250 rpm) at 37 °C. A suspension of 5×10^6 CFU mL^{-1} mid-log phase bacteria was placed onto a round-bottom 96-well microtiter plate containing 150 μL Mueller-Hinton broth (MHB) (Sigma) and incubated together with K4F4 and lipidated peptides in a series of concentrations ranging from 0 to 500 μM for 24 h at 37 °C. MIC values were determined as the lowest concentration of the peptides at which bacterial growth was completely inhibited, as compared to the untreated control (media with PBS alone) using a Tecan Spark® microplate reader at 600 nm. Bacterial CFU counts in agar plates were conducted to determine the Minimum Bactericidal Concentration (MBC). For this, 10 μL aliquots from the MIC incubation plates were transferred onto MH agar plate followed by incubation for 24 h at 37 °C. MBC values were determined as the lowest concentration where there was no visible growth. All experiments were employed in triplicate from three independent experiments.

2.4. Time-kill kinetic analysis of K4F4 and lipidated peptides

Time-kill kinetic curves were carried out to monitor the dynamic bactericidal effects of K4F4 and lipidated peptides over time against *E. coli*, *P. aeruginosa* and *S. aureus*. Briefly, 5×10^6 CFU mL^{-1} mid-log phase of bacterial cells were incubated with a series of 2-fold dilutions of MIC peptides. Then, 10 μL aliquots were collected and resuspended into 90 μL MHB at 0, 15, 30, 45, 60, 75, 90 and 120 min. Subsequently, cell suspensions were serially diluted and spread onto MH agar for colony enumeration after incubation (24 h at 37 °C). The data obtained were from three independent assays.

2.5. An integrated assessment of K4F4 and lipidated peptides induced bacterial membrane damage

2.5.1. Bacterial membrane depolarization

A fluorescence-based microplate assay was employed to study real-time changes in bacterial membrane depolarization following treatment with K4F4 and lipidated peptides. The voltage-sensitive and membrane-permeable fluorescent dye 3,3′-Dipropylthiadicarbocyanine

iodide $DiSC_3(5)$ was employed to assess whether K4F4 and lipidated peptides were able to depolarise the bacterial cytoplasmic membrane (Te Winkel et al., 2016). For this, early-log phase cells of *E. coli* and *S. aureus* were resuspended in 5 mM HEPES Buffer (supplemented with 5 mM glucose and 0.1 M KCl) at OD_{600} of 0.1 and incubated with 0.5 μM $DiSC_3(5)$ for 15 min. Then, cells were exposed to K4F4 and lipidated peptides (2-fold MIC), and a positive (1 % Triton-X) and negative control (Phosphate-buffered saline, PBS) for 12 min. Dye uptake was assessed using a SpectraMax M Series (Molecular Devices) multimode microplate reader (excitation λ = 622 nm, emission λ = 670 nm).

2.5.2. Outer membrane permeability

The fluorescent dye N-*Haemolyticactivity*$(\%) : \frac{(Ab_s - Ab_0)}{(Ab_{100} - Ab_0)} \times 100$ Phenyl-1-naphthylamine (NPN) was employed to determine the impact of K4F4 and lipidated peptides on the bacterial outer membrane (Eckert et al., 2006). Early-log phase cells of *E. coli* and *P. aeruginosa* were resuspended in 5 mM HEPES Buffer (supplemented with 5 mM glucose) and adjusted to an OD_{600} of 0.1. Then, cells were placed onto black round-bottom 96-well microtiter plate and incubated with 10 μM NPN for 15 min at 37 °C. After this, 1 and 2-fold peptides at established MIC levels were added and incubated for 30 min. Positive and negative controls consisted of 1 % Triton-X and PBS, respectively. The fluorescence was measured using a SpectraMax M Series (Molecular Devices) multimode microplate reader (excitation λ = 350 nm, emission λ = 420 nm) and expressed by the formula:

$$\textbf{NPN release}\,(\%) : \frac{(F_{pep} - F_0)}{(F_{100} - F_0)} \times 100$$

where, F_{pep} is peptide fluorescence intensity, F_0 is growth control fluorescence intensity, and F_{100} is 1 % Triton-X fluorescence intensity. All experiments were performed in triplicate and data derived from three independent experiments.

2.5.3. Validating peptide-target interactions using flow cytometry and microscopy

To validate the findings observed on the previous fluorescence-based assays, we applied specific dyes and assessed the membrane-disrupting activities of K4F4 and lipidated peptides integrating flow cytometry and microscopy. In the first assessment, a total of 5×10^6 CFU mL^{-1} mid-log phase cells of *E. coli* were incubated with 1 and 2-fold MIC peptides in MHB for 1 h at 37 °C. Subsequently, cell suspensions were washed three times and resuspended in 0.85 % NaCl Buffer for subsequent processing using a Bacteria Live/Dead Staining kit (Promokine) for 15 min at 22 °C. Positive and negative controls were assessed using 1 % Triton-X and PBS, respectively. Data were collected and analysed from 10 k events generated in an Accuri C6 Plus (BD) flow cytometry.

In the second approach, bacterial membrane disruption by peptides was assessed using fluorescence and scanning electron microscopes (SEM). Regarding both techniques, cultures of *E. coli* grown in the mid-log phase were carefully resuspended in MHB and diluted to the final concentration at 5×10^6 CFU mL^{-1}. Subsequently, 1-fold MIC peptides were incubated with bacterial suspension for 1 h at 37 °C. For fluorescence sample preparation, cells were harvested by centrifugation and resuspended in 0.85 % NaCl, followed by simultaneous staining with DMAO and EthD-III (Promokine). Coverslips were mounted with 5 μL of staining suspension and analysed under a Nikon Eclipse Ti inverted microscope. For SEM sample preparation, cells were resuspended in PBS and placed into poly-l-lysine-coated coverslips. Subsequently, cells were fixed with 2.5 % glutaraldehyde overnight at 4 °C, then serially dehydrated in ethanol (50, 70, 80, 90, 95, and 100 %) for 15 min. After being coated with gold, samples were observed using a Cambridge Instruments Stereoscan 360 microscope.

2.6. Biofilm inhibition and eradication by peptide-based antimicrobials

The potential of K4F4 and lipidated peptides to inhibit biofilm formation and/or eradicate mature biofilms was evaluated using a crystal violet (CV) assay (Au – O'Toole, 2011). To assess the biofilm inhibition, mid-log phase cells of *P. aeruginosa* and *S. aureus* were diluted to 5×10^6 CFU mL^{-1} in M63 medium minimal (supplemented with 1 mM $MgSO_4$ and 23 μM L-arginine). Cultures were seeded onto a flat-bottom 96-well microtiter plate and incubated with a series of concentrations of peptides ranging from 0 to 500 μM for 24 h at 37 °C. Additionally, PBS and MIC of ciprofloxacin were added as controls. Then, the wells were washed three times with PBS, followed by staining with 0.1 % CV (w/v) for 30 min. Dye excess was removed by washing, and samples were left to dry overnight at room temperature. Finally, wells were homogenized with 30 % acetic acid (w/v), transferred to a new round-bottom 96-well microtiter plate, and measured at 595 nm using a microplate reader (Tecan Spark). Absorbances were expressed as a percentage of biofilm inhibition as compared to control without treatment. To evaluate biofilm eradication, cells of *P. aeruginosa* and *S. aureus* were diluted to an OD_{600} of 0.01 in M63 medium (supplemented with 1 mM $MgSO_4$ and 23 μM L-arginine) and placed onto flat-bottom 96-well microtiter plate for 48 h at 37 °C. Then, planktonic cells were carefully removed by washing with PBS and the mature biofilm was incubated with peptides (0 to 500 μM) for 24 h at 37 °C. Subsequently, the suspension was removed, stained, and measured as described above.

2.7. MIC determination after long-term exposure to K4F4 and lipidated peptides

An *in vitro* resistance development assay was employed to determine whether the peptides were able to induce resistance in bacterial cells after consecutive passages (Hong et al., 2016). In this context, sub-MIC (0.5-fold MIC) concentrations of peptides were incubated with mid-log phase (5×10^6 CFU mL^{-1}) cells of *E. coli*, *P. aeruginosa* and *S. aureus* for 24 h at 37 °C. Subsequently, the MIC was measured at 600 nm using a Tecan Spark® microplate reader. Following that, 15 μL of suspension was collected and diluted to a final concentration of 5×10^6 CFU mL^{-1} for further incubation with sub-MIC concentrations in the same conditions. This procedure was repeated and recorded over 16 days.

2.8. Assessing red blood cell lysis induced by K4F4 and lipidated peptides

The haemolytic activity of peptides was assessed using a previously established method (Peña-Carrillo et al., 2021). Briefly, fresh human red blood cells (hRBCs) were washed three times with PBS and resuspended in the same buffer at a final concentration of 0.5 % (v/v). Concentrations of peptides ranging from 0 to 2 mM were serially diluted in PBS and incubated with 0.5 % hRBC suspensions on flat-bottom 96 well-microtiter for 1 h at 37 °C. Triton X-100 (1 %) and PBS were employed as positive and negative controls, respectively. After incubation, samples were centrifuged at 1000g for 10 min at 4 °C, the supernatant was then transferred to a new round-bottomed 96-well microtiter plate and OD_{414} was measured (Tecan Spark®). Haemolytic activity (%) was expressed using the following equation:

$$\textbf{Haemolytic activity}\,(\%) : \frac{(Ab_s - Ab_0)}{(Ab_{100} - Ab_0)} \times 100$$

where, A_s is the absorbance value of peptide group, Ab_0 is the absorbance value of the untreated control (PBS), and Ab_{100} is the absorbance value of the positive control (1 % Triton X-100). The data were obtained from three independent assays.

2.9. Impact of saline solutions and serum on K4F4 and lipidated peptides-mediated antimicrobial effect

Peptide sensitivity was evaluated using different concentrations of physiological salts and serum. For this, K4F4, C16-K4F4 and K4-NH-C16 were incubated with mid-log phase cells of *E. coli*, and *S. aureus* in the presence of 145 mM NaCl, 4.2 mM KCl, 0.8 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1 mM $FeCl_3$, 25 % or 40 % bovine heat-inactivated serum for 24 h at 37 °C. Then, MICs were recorded and compared with PBS controls. The assays were conducted independently in triplicate.

2.10. Statistical analysis

Experimental results are shown as mean ± standard deviation (SD), and all statistical analyses were conducted using GraphPad Prism 8.0. The significance test of control (bacteria only) and peptide-treated bacteria groups was estimated using a one-way analysis of variance (ANOVA) with Tukey's posthoc tests, P-values < 0.05 were considered statistically significant (** $p < 0.0001$, * $p < 0.001$, * $p < 0.05$).

3. Results

3.1. In silico tools suggest the antimicrobial potential of a K4F4 peptide

A combined use of computational tools for antimicrobial activity, including Antimicrobial Peptide Scanner vr.2 and AMPfun, have suggested that the short polylysine-polyphenyalanine octapeptide template is a potential AMP encouraging further *in vitro* assessment (**Supplementary Table S1**). Another key initial evaluation relies on peptide-induced toxicity to build a more realistic view of the full potential. For this purpose, we employed different computational-based approaches to predict the haemolytic properties of our peptide template, such as HemoPI, HemoPred and HAPPENN. In general, the *in silico* screening for the ability of peptides to cause red blood lysis was not consistent (**Supplementary Table S2**). While K4F4 is presumed to be toxic to erythrocytes by HemoPred, other webservers, including HAPPENN and HemoPI classifies this short cationic and hydrophobic sequence as non-haemolytic. Balancing effective antimicrobial activity and low toxicity is a challenge in peptide drug discovery initiatives that can be addressed by promising approaches such as attaching fatty acid chains (Kang et al., 2022). This AMP engineering is also a strategy to counteract stability issues. Motivated by this, we explored the *in vitro* antibacterial, anti-biofilm and haemolytic profiles of K4F4, and two engineered peptides: Palmitoyl-K4F4 and a shorter version maintaining only the positively charge block of the amino acid sequence, K4-NH-Palmitoyl.

3.2. K4F4 is a broad-spectrum AMP peptide and its lipidation enhances the antibacterial activity

In agreement with the bioinformatic tools, K4F4 showed promising *in vitro* antibacterial effects against Gram-positive and Gram-negative bacteria with activity in the micromolar range (125–250 μM). Interestingly, C16-K4F4 and K4-NH-C16 exhibited lower MIC and MBC values (31.25–250 μM) for most of the strains (Table 1). The shorter lipidated peptide stands out as the most active antibacterial agent, showing the lowest MIC and MBC values (31.25–62.5 μM). In general lines, in most of the cases, Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were more susceptible than *S. aureus* to the trialled compounds.

3.3. Activity rate of K4F4 and lipidated peptides is rapid

The kinetics of the time-kill change of peptide-based antimicrobials against three different bacteria is shown in Fig. 1. The findings underscore their rapid killing actions, particularly C16-K4F4 and K4-NH-C16, which lead to the eradication of bacterial strains tested in shorter periods (less than 60 min following *in vitro* incubations). At 2-fold MIC they exhibited fast-acting bactericidal effects, except for K4F4 against *S. aureus*. The parent peptide showed weaker activity and limited capacity to completely kill Gram-positive bacteria within the 90 min of the assay at 2-fold MIC. The bactericidal properties were significantly enhanced by peptide lipidation and shortening.

3.4. K4F4 and lipidated peptides compromise the bacteria membrane integrity

The monitoring of the targets and mechanistic details of peptide-based antimicrobials is key to understanding the action, selectivity, and further structural and functional optimizations. To decipher the molecular mechanisms underlying the antibacterial effects of K4F4 and lipidated peptides, we initially combined fluorescent-based assays to analyse the membrane depolarization and outer membrane permeability. Fluorometric assessment evidenced that lipidated peptides cause significant membrane depolarization in both Gram- negative and Gram-positive bacteria in a rapid manner (Fig. 2**A and B**). K4F4 induced a lower membrane depolarization at this concentration. Fluorescent measurements of *E. coli* and *P. aeruginosa* bacterial permeabilization demonstrated a high uptake of the hydrophobic dye (NPN) after treatment with amphiphilic K4F4 peptide and lipidated peptides (Fig. 2**C and D**). In summary, this NPN-based assay indicated a strong and rapid structural weakening of the outer membrane of *E. coli* and *P. aeruginosa*. Bacteria incubated with K4-NH-C16 were more affected and showed high levels of uptake of the dye, suggesting the positive contribution of peptide shortening and lipidation to membrane-damaging properties, similarly to the higher effects observed in the antimicrobial screening.

In the next step, we gained more insight into the membranolytic action of these antimicrobial candidates by examining their influence on plasma-membrane permeability using flow-cytometry and microscopy. In both approaches, we used a fluorescence staining that explored the differential membrane permeability and colour of two different dyes DMAO and EtD-III. DMAO is a cell-permeable fluorogenic DNA dye that stains both live and death cells green. EtD-III is a red, cell impermeable dye that selectively binds to DNA in solution (i.e. following cellular membrane disruption and DNA release). Control cells which were not exposed to membranolytic agents did not show any uptake of impermeant dye or stain red. In turn, treatment with Triton X (positive control) resulted in a significant change evidenced by a drastic disruption of

Table 1
Broad-spectrum antibacterial effect of K4F4 template and engineered lipopeptides. MICs/MBCs of peptides and commercially available antibiotic (positive control) against different human pathogenic bacteria were expressed in μM.

	MIC (μM)			MBC (μM)		
	E. coli (EDL933 – K12)	*P. aeruginosa* (NCTC13437 – PA01)	*S. aureus* (ATCC 12600)	*E. coli* (EDL933 – K12)	*P. aeruginosa* (NCTC13437 – PA01)	*S. aureus* (ATCC 12600)
K4F4	250	125	250	500	250	250
C16-K4F4	62.5	62.5	125	125	62.5	250
K4-NH-C16	31.25	31.25	62.5	62.5	31.25	62.5
Ciprofloxacin	2.35	MDR – 2.35	1.17	2.35	MDR – 4.7	2.35

MDR: Multi Drug Resistence.

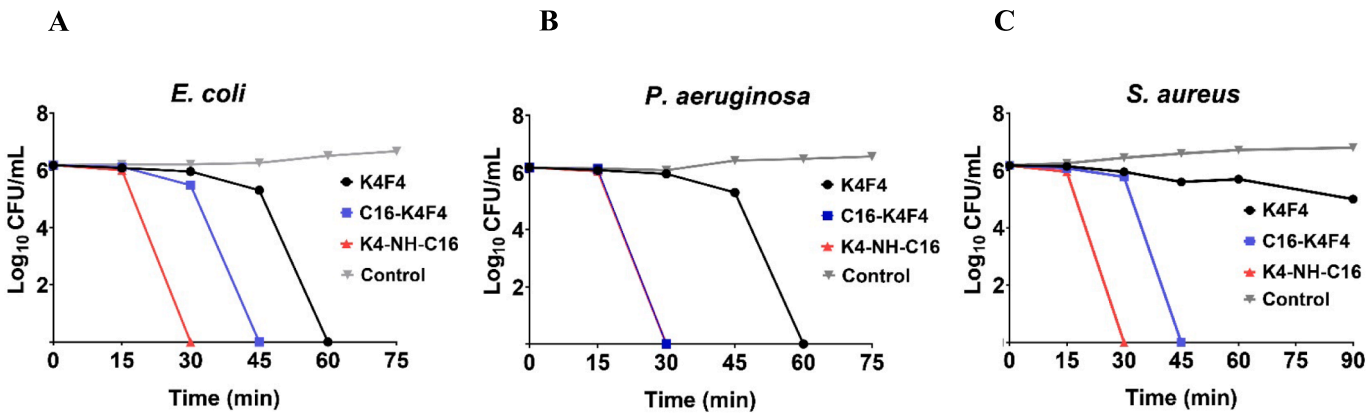

Fig. 1. Time-killing kinetic profiles of K4F4 and lipidated peptides against pathogenic bacteria. Representative curves of bactericidal kinetics of 2-fold MIC peptides on **(A)** *E. coli* (K12), **(B)** *P. aeruginosa* (PA01) and **(C)** *S. aureus* (ATCC 12600) over 90 min.

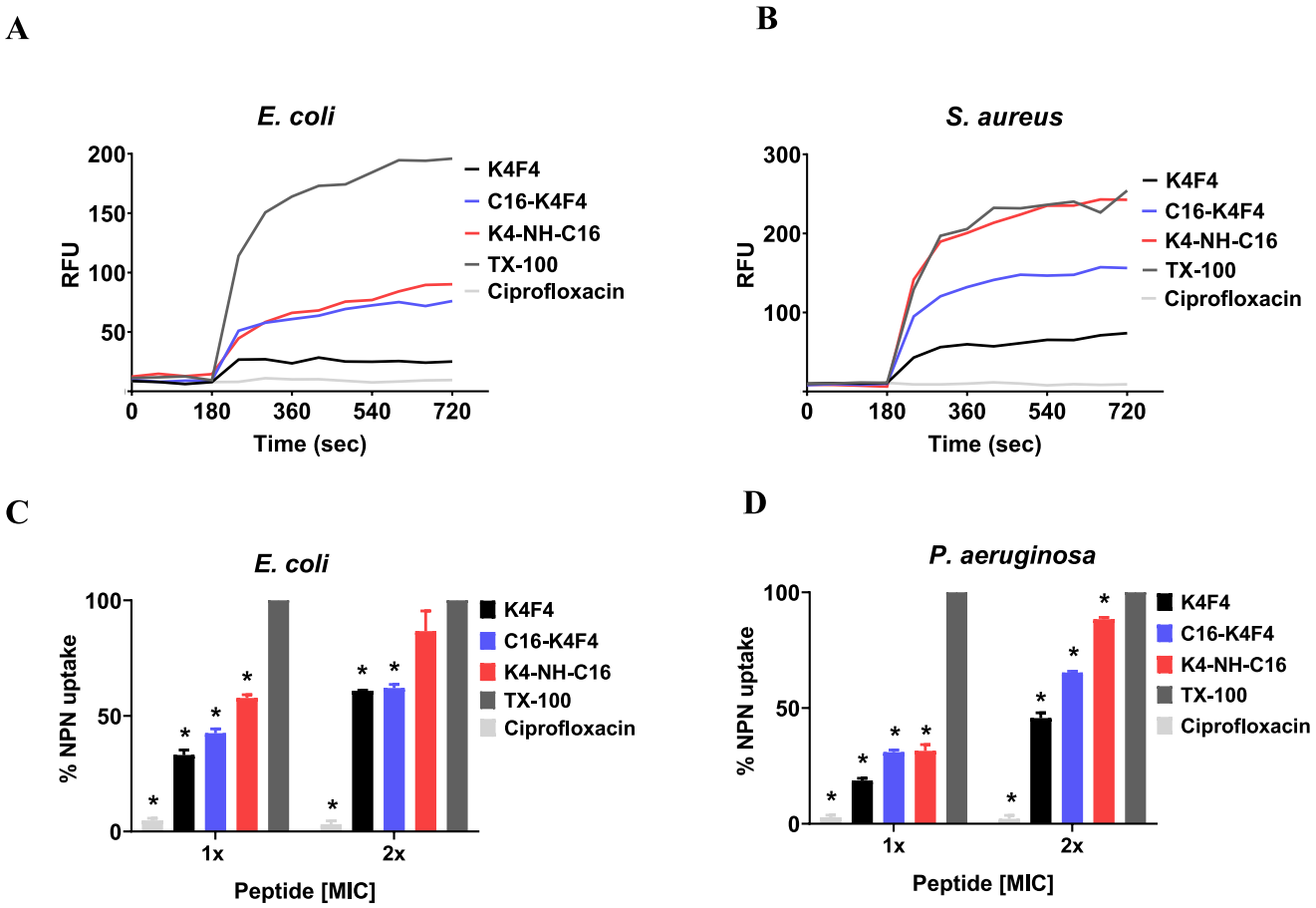

Fig. 2. Fluorometric assessment of bacterial membrane depolarization and permeabilization caused by K4F4 and lipidated peptides. The antimicrobial peptide-based candidates induced bacterial membrane alterations leading depolarization of **(A)** Gram-negative and **(B)** Gram-positive reference strains. NPN uptake-based assay evidenced the impact of peptide treatments on outer membrane of **(C)** *E. coli* and **(D)** *P. aeruginosa*. Statistical significance was established by one-way analysis of variance (ANOVA) using Tukey test comparison test and asterisks are indicated as $^{*}P < 0.0001$.

the bacterial plasma membrane and cell viability. Like the detergent, K4F4 and lipidated peptides induced a considerable damage of bacterial membranes in a concentration-based manner. Shorter cationic lipopeptide exhibited highest membrane-disrupting activities (Fig. 3**A**). Microscopic imaging analysis coincide with the previous fluorescence-based findings, confirming the membrane-active properties of K4F4 and lipidated peptides (Fig. 3**B**).

3.5. K4F4 and lipidated peptides prevent biofilm formation and partially affect existing biofilms

Gram-positive and Gram-negative bacteria can form extensive multicellular biofilm structures that play key roles in their pathogenicity, immune evasion strategies and ability to resist antibiotic treatments (Mirghani et al., 2022; Uruén et al., 2020; Vestby et al., 2020). For this reason, we assessed whether the amphiphilic octapeptide and

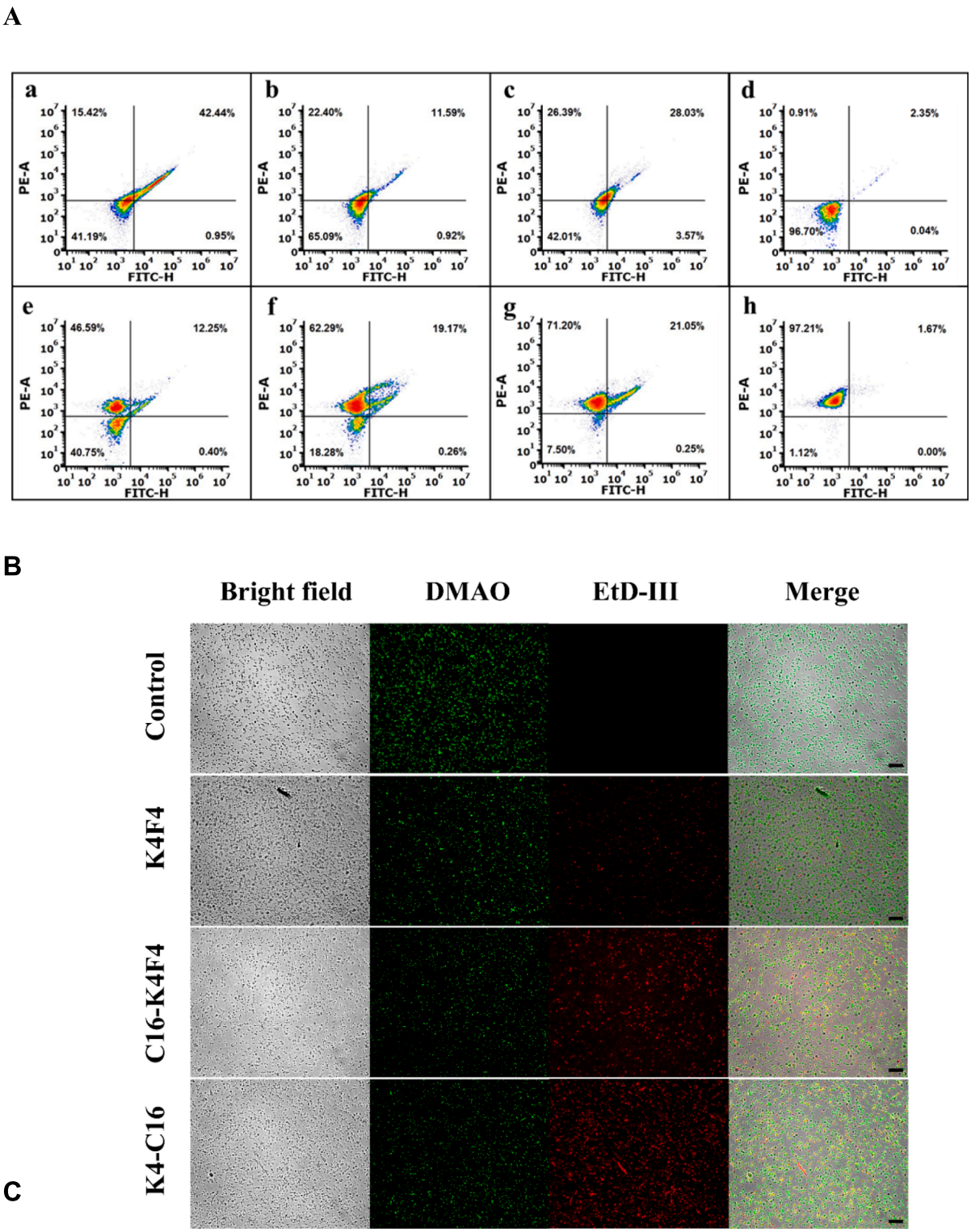

Fig. 3. Membrane-disrupting activities of K4F4 and lipidated peptides. **(A)** Flow cytometry plots confirmed the damage to the *E. coli* membrane following incubation with peptide for 1 h. **(a-c)** 1-fold MIC and **(e-g)** 2-fold MIC of K4F4, C16-K4F4 and K4-NH-C16 show cell permeabilization in a dose-dependent manner, where the shift to the first left quadrant represents dead cells. **(d)** PBS (−ve control) and **(e)** 1 % Triton X (+ve control). **(B)** The significant increase of red-fluorescent dye binding nucleic acid in microscopic images of peptide-incubated bacteria is a biomarker of membrane disruption. **(C)** SEM images support the membranolytic action of K4F4 and lipidated peptides. **(a)** Membrane of *E. coli* is intact in the presence of PBS, while significant changes are observed in treatment groups. **(b)** K4F4 induces a clear pore formation. **(c)** Arrows indicate changes in membrane surface caused by C16-K4F4 evidencing the lytic action. **(d)** Leakage of intracellular content caused by K4-NH-C16. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

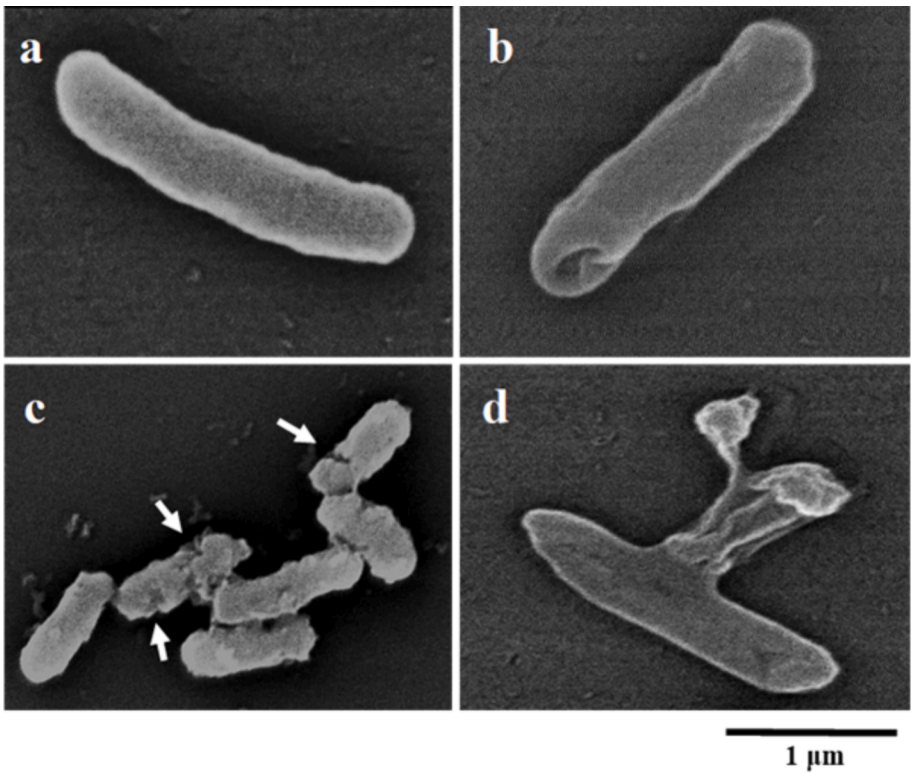

Fig. 3. (*continued*).

lipopeptides can inhibit biofilm formation and eradicate pre-established biofilms created by complex bacterial communities of the important human pathogens *P. aeruginosa* and *S. aureus*. In the first assessment, the template peptide and lipopeptides exhibited a broad-spectrum biofilm-formation prevention activity (Fig. 4**A and B**). In agreement with the antibacterial and membrane-active properties, the shorter cationic lipopeptide presented the strongest inhibition of biofilm growth for both bacteria at lower concentrations. In the second assessment, pre-formed biofilms were exposed to antimicrobial candidates. These findings evidenced that K4-NH-C16 also showed highest biofilm disruption (Fig. 4**C and D**). Despite the higher levels of destruction of biofilm architecture, we observed only a partial elimination of biofilms following treatment with K4F4 and lipidated peptides. Interestingly, the potential antimicrobial candidates showed higher biofilm inhibition than biofilm eradication activities at lower molar concentrations.

3.6. No resistance was developed against long-term exposure to K4F4 and lipidated peptides

Bacteria have evolved remarkable strategies to counteract the action of drugs designed to target them (Pulingam et al., 2022; Uruén et al., 2020). Overuse of antibiotics is one of the leading causes associated with the development of multiple drug resistance (Ventola, 2015). Therefore, discovering antimicrobials with a decreased likelihood for emergence of resistance is a priority. From this perspective, we monitored the MIC values for three synthetic peptide-based antimicrobials after 16 consecutive passages. MIC values for all studied bacteria were unchanged over time (Fig. 5). This consistency indicated that K4F4, C16-K4F4 and K4-NH-C16 were not able to induce the selection of resistant mutants against Gram-negative or Gram-positive bacterial strains after prolonged exposure (16 consecutive passages).

3.7. Lipidated peptides exhibit greater toxicity towards bacteria compared to human red blood cells

Of particular clinical significance, *in vitro* assessment of peptide-induced erythrocyte lysis anticipates low selectivity issues associated with side effects. K4F4 is moderately haemolytic towards hRBCs (at the highest concentration evaluated) (Fig. 6), confirming one of the initial *in silico* haemotoxicity predictions. C16-K4F4 and K4-NH-C16 showed higher haemolytic properties than their parent peptide (K4F4), showing that lipidation and shortening can also impact on the effects induced in other cell types. However, the analysis of cell toxicity only describes one dimension. Overall, the peptide-based antimicrobials evaluated here (both parent peptide and engineered peptides) are not haemolytic in the antimicrobial range. Hence, we expand this view by calculating the selectivity index, considering the toxicity to hRBCs (HC50; concentration of the peptide inducing 50 % haemolysis) and MIC values (**Supplementary material Table S3**). Under this scenario, K4F4 and lipopeptides exhibited promising toxicity-to-MIC ratio (3.69–21.38). Despite increased rates of haemotoxicity, lipopeptides still displayed appropriately selective and higher preference for bacteria than hRBCs.

3.8. Lipidated peptides remain active under physiological conditions

The presence of salts and proteolytic enzymes in real-world scenarios present additional barriers to the effectiveness and mode of action based on electrostatic interactions of antimicrobial peptide-based agents. Changes in MIC values for K4F4 illustrate the impact of NaCl and enzymes essentially found in FBS on its antibacterial activity. Interestingly, C16-K4F4 remains active in most conditions, exhibiting the same MIC values, except at the highest concentration of FBS (40 %). Finally, the MICs for K4-NH-C16 were unchanged, suggesting its higher stability in the presence of anions, cations, and serum components (Table 2).

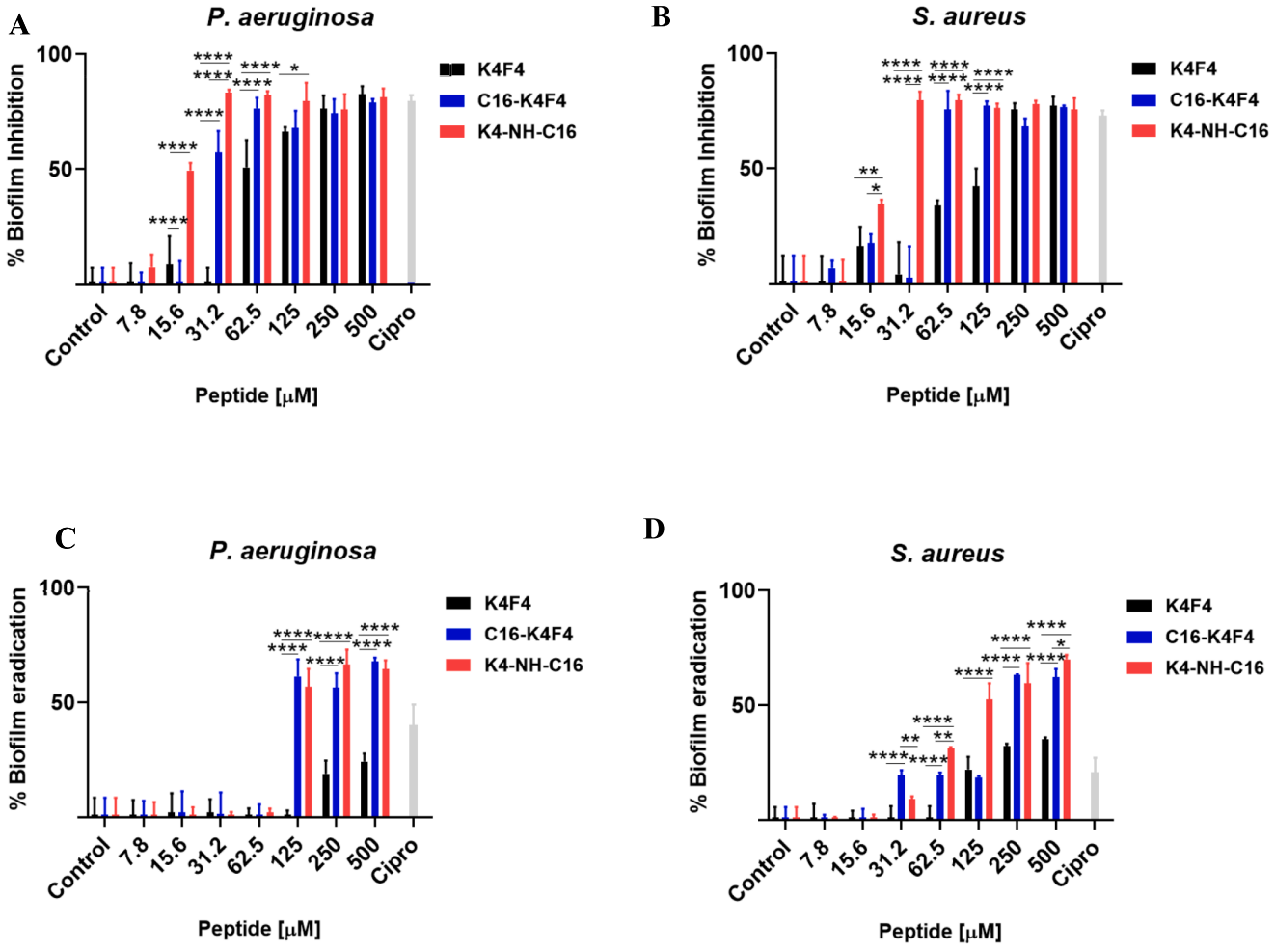

Fig. 4. Antibiofilm action of K4F4 and engineered peptides. Peptides exhibited an *in vitro* biofilm inhibition activity, affecting the development of biofilms from **(A)** Gram-negative and **(B)** Gram-positive bacteria. They also presented ability of disintegrating preformed biofilms of both **(C)** *P. aeurginosa* and **(D)** *S. aureus*. Statistical significance was established by one-way analysis of variance (ANOVA) using Tukey test comparison test and asterisks are indicated as $^{*}P < 0.01$, $^{**}P < 0.001$, $^{****}P < 0.0001$.

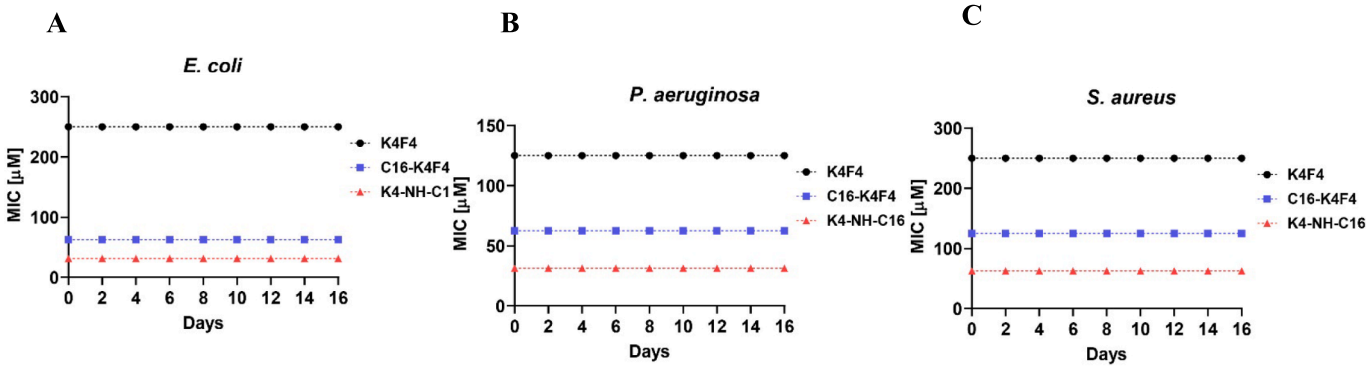

Fig. 5. Monitoring of bacterial peptide susceptibility over 16 consecutive passages. Sub-MIC exposure of **(A)** *E. coli*, **(B)** *P. aeruginosa* and **(C)** S. *aureus* to K4F4 and engineered lipopeptides did not alter bacterial peptide susceptibility as evidenced by no alterations in MIC values.

4. Discussion

The rise in antimicrobial-resistant infection-related fatalities, disabilities, and disease transmission is increasingly evident, attributable to the overuse and diminished efficacy of antibiotics (Mancuso et al., 2021). For this reason, a range of new antibacterial agents and therapeutic approaches have and are being explored to mitigate the impacts of this global burden on both human and animal health and the global economy (Bhandari and Suresh, 2022; Miethke et al., 2021; Mulukutla et al., 2024). Promising therapeutic alternatives which are proving highly successful in addressing this complex and critical problem include the development and application of cationic peptides and

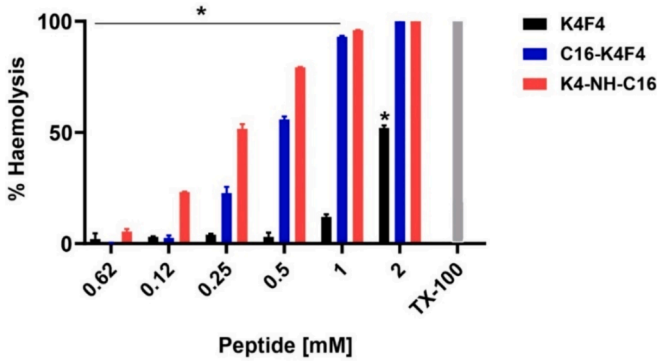

Fig. 6. ***In vitro*** **assessment of peptide-induced toxicity using red blood cells.** The release of haemoglobin after peptide treatments was spectrophotometrically quantified at 414 nm and compared to haemolytic Triton X-100. Low haemolytic rates were observed in the antimicrobial range of K4F4 and lipidated peptides. Statistical significance was established by one-way analysis of variance (ANOVA) using Tukey test comparison test and asterisks are indicated as $*P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Salt and serum sensitivity of K4F4, C16-K4F4 and K4-NH-C16 against *E. coli* (K12) and *S. aureus* (ATCC 12600).

	MIC (μM)					
	E. coli **(K12)**			***S. aureus*** **(ATCC 12600)**		
	K4F4	**C16-K4F4**	**K4-NH-C16**	**K4F4**	**C16-K4F4**	**K4-NH-C16**
Control	**250**	**62.5**	**31.25**	**250**	**125**	**31.25**
NaCl	>250	62.5	31.25	>250	125	31.25
KCl	250	62.5	31.25	>250	250	31.25
$MgCl_2$	250	62.5	31.25	250	125	31.25
$CaCl_2$	250	62.5	31.25	250	125	31.25
$FeCl_3$	250	62.5	31.25	250	125	31.25
FBS (25 %)	>250	62.5	31.25	>250	125	31.25
FBS (40 %)	>250	125	31.25	>250	>250	31.25

lipopeptides. Following years of preliminary research these agents are now entering into the final stages of antibiotic drug development to arrive, in the near future, as marketable products in the pharmaceutical industry (Greber et al., 2019; Ledger et al., 2022).

In this study, we first designed a short cationic octapeptide (K4F4) composed of a combination of two naturally occurring amino acids commonly found as residues in effective antimicrobial peptides and with strongly associated membranolytic and antibacterial properties (Cutrona et al., 2015; Edwards-Gayle et al., 2020). According to different *in silico* tools, the K4F4 peptide was predicted as an antimicrobial peptide with the ability to inhibit the growth of both Gram-positive and Gram-negative bacteria. Our *in vitro* screening has confirmed its bacterial killing activity, evidencing its potential as a core structure for the design of antibacterial peptide-based approaches. Conventionally, the discovery of new therapeutic peptides relies on time-consuming, laborious, wet-lab and high-cost processes with many steps, from DNA sequencing, peptide purification or chemical synthesis to *in vivo* assays (Craik and Kan, 2021; Wang et al., 2022). More recently, initial screening using AI-based technologies broadens this pipeline offering valuable insights that can help guide peptide design and increase success rate (Chang et al., 2024; Robles-Loaiza et al., 2022). However, ground-truthing using *in vitro* analysis remains critical before preclinical evaluation using animal models due to the possible biases or failures of current algorithms or software, which are all highly dependent on the scientific value included within the published data sets (Feijoo-Coronel et al., 2024; Vora et al., 2023). The successful use of the computer-aided approach was exemplified in our study, which led to the promising selection of an appropriate AMP scaffold. Despite this, most of the programs generate qualitative outputs, classifying the peptide sequences without a clear idea of the potency of the molecules. To illustrate this point, we observed that, despite its antimicrobial potential, K4F4 is less active than other previously reported potent peptides, such as microcin (Kaur et al., 2016) and melittin (Lima et al., 2022) which exert their effects at lower concentrations.

Combining peptides rich in hydrophobic and cationic amino acids with lipid chains has shown a beneficial impact on the antimicrobial potency of peptide-inspired agents (Lin et al., 2023; Myšková et al., 2023). Consequently, we combined K4F4 and palmitoyl to investigate the modulation of the bactericidal activity of the K4F4 peptide. *In vitro* assays have shown that this biomolecular engineering strategy significantly increases the antibacterial effect altering its ability to inhibit bacterial growth. The lipidated peptide showed 2 to 4 times lower MIC and MBC values for *P. aeruginosa* and *E. coli*. Our findings illustrate how the lipidation of cationic short peptides may be a useful route for obtaining higher active peptide-based antibiotics in agreement with accumulating evidence presented in other studies (Walker and Marty, 2022; Wenzel et al., 2016). For instance, some authors have reported lipopeptides with antimicrobial properties that were more effective at lower concentrations than the MIC described in our study (Mohanram and Bhattacharjya, 2014; Woodworth et al., 1992).

Shorter lipopeptides are usually more appropriate and valuable templates for peptide research and development due to their potential to lower production costs, facilitate synthesis and decrease susceptibility to proteolysis including the activity of serum enzymes (Azmi et al., 2016; Makovitzki et al., 2006). Moving forward, we investigated the antibacterial activity of K4-NH-Palmitoyl. This shorter lipidated peptide exhibited higher levels of bacterial inhibition. MIC and MBC values were 6 or 8 times lower than the AMP template. Similarly, Azmi et al (Azmi et al., 2016) and Armas et al (Armas et al., 2019) demonstrated the membrane-disrupting and antibacterial effects of short lipopeptides designed on cationic peptides. Shortening the sequence length can be a useful tool in molecular engineering for the obtention of more economically viable alternatives, however, this fine tuning is challenging and may lead to decreases in therapeutic potential as observed for example for some shortened peptides derived from PMAP-36 (Biondi et al., 2023). Additionally, our findings indicate that the position of C16 acyl N- or C-termini may also affect the properties of designed lipopeptides. In our study, we integrated shortening in peptide length and shifting in the lipid chain as synthetic tools to test for greater antimicrobial efficacy. However, analyzing analogs with the same proteogenic scaffold but differing only in the addition of a palmitoyl C_{16} tail is crucial for a more comprehensive understanding of the impact of the differential attachment of lipids.

Based on the multifunctionality of peptide-based agents, we evaluated the ability of K4F4 and lipidated derivatives to inhibit the growth of biofilms or eliminate existing ones. Both activities are fundamental pillars for the development of therapies focusing on biofilm-associated infections (Damyanova et al., 2024) which is often overlooked at the preferential lifestyle of most bacterial pathogens in clinical settings. Our assessment has demonstrated that K4F4 and lipidated peptides efficiently interrupt biofilm formation processes. In agreement with the initial *in vitro* antibacterial screening, lipidated peptides inspired by the cationicity of our octapeptide template exhibited higher impact on biofilm formation for both Gram-positive and Gram-negative bacteria than the octapeptide parent. Additionally, they also partially destroyed mature bacterial biofilms, although higher peptide concentrations were required for this. This is line with other studies reporting that the structure of established biofilms created barriers that made their removal more difficult than the inhibition of initial steps of their growth (Casciaro et al., 2017; Raheem and Straus, 2019). Mature biofilms are known to protect the encapsulated microbial community from external

factors through a complex network of extracellular polymeric substances (EPS) including polysaccharides, proteins, nucleic acids, and lipids (Flemming et al., 2007). The bifunctional activity of these peptide-based candidates on biofilms illustrates the potential of applying these peptides in the control or prevention of growth of bacterial biofilms, and especially those derived from important bacterial pathogens.

Bacterial membranes are multifunctional and act as dynamic boundaries that play critical roles in the survival of Gram-positive and Gram-negative bacteria (Silhavy et al., 2010; Strahl and Errington, 2017). For this reason, they pose as attractive targets in the design of novel antibiotic agents (Almeida et al., 2022). Peptides and lipopeptides are clear examples of membranolytic antibiotics (Upert et al., 2021) as many target and interact with the membrane as their primary site of activity (Meena and Kanwar, 2015). With this in mind, we carried out a series of assays to specifically investigate the membrane-disrupting activities of our designed peptides. Interestingly, data gathered from the combined applications of flow cytometry, fluorescence and electron microscopies and fluorescence-based assays all strongly suggest that K4F4 and its derivatives killed Gram-positive and Gram-negative bacteria through a membrane disrupting mechanism involving the formation of pores, leakage of intracellular content and alterations in membrane surface roughness together with creasing. Our lipidated peptides displayed even greater impact on membrane integrity and depolarization as visualised through increased degrees of pore formation and surface roughness; findings which coincide with their rapid killing patterns. This perturbing of vital functions by membrane-active peptides, ultimately leading to rapid cell death, is supported by numerous studies in the literature (Li et al., 2017; Sengkhui et al., 2023). Collectively, our results strongly suggest that the K4F4 peptide and its derived lipidated species are membrane-targeting molecules with antibiotic properties.

The development of antibiotics requires the overcoming of considerable barriers, notably the catalytic activity of proteases, effective stability in physiological conditions, induction of target resistance and non-target activity (i.e. toxicity to human cells) (He et al., 2023; Valdivieso-Rivera et al., 2022). The degradation by serum enzymes is one of the main factors that prevents AMPs, rich in positively charged amino acids, from entering clinical use (Mahlapuu et al., 2016; Wang et al., 2022). In our study we saw a significant reduction in the antibacterial properties of the K4F4 peptide in the presence of foetal bovine serum and NaCl. The discovery and optimization of salt-resistant antimicrobial peptide templates remain of great interest. For this reason, several researchers have conducted structural activity studies to gain insights to improve the action of peptide-based agents in more realistic environments (Mohanram and Bhattacharjya, 2016; To et al., 2023). These studies have highlighted some general guidelines for preparing stable and active molecules, but more approaches must be integrated to increase success. Salts can affect the initial interaction of peptides with bacterial membranes (Kandasamy and Larson, 2006), while serum is a rich source of protein-degrading enzymes that can impact the structure and function of positively charged peptides (Jenssen et al., 2006). Conversely, K4-NH-Palmitoyl exhibited greater resistance to proteolysis evidenced through the broth dilutions whereby MIC values remained constant. C16-K4F4 displayed greater resistance to proteolysis than K4F4, but its MIC values were altered in increased concentrations of FBS (40 %). This suggests a positive role of lipid tails on peptide stability, increasing the therapeutic value of these peptide-based agents. On the other hand, the MIC values for *E. coli* and *S. aureus* after long-term exposure to K4F4 and lipidated peptides were unchanged, corroborating the rapid membranolytic and antibacterial effects previously discussed which likely do not allow the bacteria to evolve resistance. Finally, we expanded the assessment by evaluating the toxicity to hRBCs. Haemolytic ability of K4F4 was closer to the designated antibacterial range, confirming its haemolytic tendency predicted by the *in silico* tools initially applied in our experimental design. On the other hand, our engineered lipopeptides were more toxic to hRBCs than the parent molecule, although, notably, at much higher concentrations then those recorded against bacteria; and thus rendering the lipopeptides suitable for clinical applications.

Self-assembly is a commonly observed biochemical phenomenon with potential implications for the action, activity and toxicity of lipidated peptides (Zapadka et al., 2017). Recent studies have shown that amphiphilic lipopeptides with similar structures to those reported here (combining C16 hydrophobic tail and positively-charged amino acids) can self-assemble through inter- and intra-molecular interactions to form a variety of structures, such as micelles, vesicles, or fibrillar structures (nanotubes, fibers) (Adak et al., 2024; Lee et al., 2019). Although relevant, this task is still pending and should be considered in future stages to better understand how to improve the structure–activity relationship of these bioactive lipopeptides.

5. Conclusions

In summary, this study evidenced the antimicrobial nature, antibiofilm activity and membrane-disrupting effects of two synthetic lipidated peptides inspired by a short cationic polylysine-polyphenyalanine octapeptide with broad-spectrum effects against a selection of Gram-positive and Gram-negative bacteria. Mechanistically, they affect bacterial survival by compromising their membranes at a highly effective rate with low probability of inducing pathogen resistance. In the complex world of peptide chemistry, our findings present a set of novel positively charged and lipidated peptide-based molecules with rapid action and significant antimicrobial activity in physiological conditions (presence of salts or serum). They extend the list of useful starting points to pilot viable therapies targeting bacterial infections whilst working towards better treatment options. Collectively, our results have important implications for the future design of bacterial infection-fighting strategies using peptides as scaffolds. Further *in vivo* and synergism studies (focusing on lipidated peptides in combination with antibiotics) will be instrumental in leading beneficial structural optimisations and modifications of potent and selective antibacterial compounds through a closer pre-clinical view.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2024.100240.

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