International Journal of Peptide Research and Therapeutics (2021) 27:1751–1762
https://doi.org/10.1007/s10989-021-10207-x

Effects of Substituting Arginine by Lysine in Bovine Lactoferricin Derived Peptides: Pursuing Production Lower Costs, Lower Hemolysis, and Sustained Antimicrobial Activity

Karen Johanna Cárdenas-Martínez[1] · Derly Grueso-Mariaca[2] · Yerly Vargas-Casanova[3] · Laura Bonilla-Velásquez[4] · Sandra Mónica Estupiñán[2] · Claudia Marcela Parra-Giraldo[3] · Aura Lucía Leal[5] · Zuly Jenny Rivera-Monroy[6] · Javier Eduardo García-Castañeda[1]

Accepted: 24 March 2021 / Published online: 8 April 2021

Abstract

Monomeric, dimeric, palindromic, and tetrameric peptides derived from bovine lactoferricin (LfcinB) containing the RRWQWR or the KKWQWK sequence were synthesized via SPPS- Fmoc/tBu strategy, and Lys-peptides showed higher synthesis efficiency. The antibacterial activity of the peptides against *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* ATCC reference strains and a multidrug resistant clinical isolate was evaluated. Lys peptides exhibited similar antibacterial activity to the Arg peptides, suggesting that the Arg for Lys substitution does not significantly affect the antibacterial activity. The synergy assays showed that the covalent dimeric Lys peptide decreased the MIC value of the ciprofloxacin up to 32 times, and LfcinB (20-25), and one of the Lys-peptides [K]-LfcinB $(20\text{-}25)_{Pal}$ presented a synergistic effect. Additionally, Lys peptides lowered the hemolytic activity by 2 to 7 times. These results suggest that short peptides derived from Lys-LfcinB could be a cost-effective alternative for the development of drugs or combinatory treatments against bacterial infections.

Keywords Antimicrobial peptides · Arginine · Lysine · Hemolysis

Introduction

According to the World Health Organization (WHO), antimicrobial resistance (AR) is generated when microorganisms undergo changes or mutations that make the drugs commonly used to treat infections cease to be effective (World Health Organization (WHO) 2015). It is estimated that more than 50% of antibiotics are used incorrectly and improperly in many countries (World Health Organization (WHO) 2019). The WHO has reported an increase in pathogens that show resistance to conventional antibiotics and recommends investigating and developing new molecules that respond to current and future needs, focusing on activity, no resistance induction, and cost/benefit production, guaranteeing equitable access to treatment (World Health Organization (WHO) 2015; Wang et al. 2018). The WHO classified the bacteria into three priority groups: the critical group (*Acinetobacter*, *Pseudomonas* and several enterobacteria; *E. coli*, *Serratia* spp, and *Klebsiella* spp), high-priority bacteria (*S. aureus*, *E. faecium*, *H. pylori*, *Campylobacter*, *Salmonella* spp, and *N. gonorrhoeae*), and the medium priority tier (*S. pneumoniae*, *H. influenzae*, and *Shigella* spp) (Asokan et al. 2019). Specifically, *Escherichia coli* that causes urinary tract infections has exhibited resistance to ciprofloxacin in 8 to 65% of the cases (World Health Organization (WHO) 2018), Methicillin-resistant *Staphylococcus aureus*

✉ Javier Eduardo García-Castañeda
jaegarciaca@unal.edu.co

1 Pharmacy Department, Universidad Nacional de Colombia, Bogota, Colombia

2 Bacteriology Department, Universidad Colegio Mayor de Cundinamarca, Bogota, Colombia

3 Microbiology Department, Pontificia Universidad Javeriana, Bogota, Colombia

4 Biotechnology Institute, Universidad Nacional de Colombia, Bogota, Colombia

5 Medicine Faculty Institution, Universidad Nacional de Colombia, Bogota, Colombia

6 Chemistry Department, Universidad Nacional de Colombia, Bogota, Colombia

(MRSA) could cause longer hospitalization and an increase in hospital mortality (Inagaki et al. 2019), and *Pseudomonas aeruginosa* has now been related to high resistance to carbapenems, one of the few available treatment alternatives (Xu et al. 2020).

Antimicrobial peptides (AMPs) are naturally found as part of the first line of defense of living organisms, exhibiting activity against diverse microorganisms, such as bacteria, yeast, and fungi (Patel and Akhtar 2017; Tang et al. 2018; Ciumac et al. 2019). They have a low possibility of developing resistance and in general a good toxicity profile (Ageitos et al. 2017; Kumar et al. 2018; Ciumac et al. 2019; Lei et al. 2019). Nevertheless, AMPs are susceptible to proteolytic degradation and rapid clearance, and some of them exhibit severe hemolytic activity and have a high cost of production. This last aspect could increase specifically for longer sequences or peptides that contain high quantities of some amino acids such as arginine (Arg), thus making the production of pharmaceutical forms based on AMPs challenging (Hancock and Sahl 2006; Vrettos et al. 2018; Gao et al. 2018; Lei et al. 2019).

Bovine lactoferrin (LFB) is an iron-binding glycoprotein that possesses a highly positively charged N-terminal region (Gifford et al. 2005) and has exhibited antibacterial activity against Gram-positive and Gram-negative bacteria (Bellamy et al. 1992). The pepsin hydrolysis of LFB releases a 25 residues antimicrobial peptide: Bovine Lactoferricin (LfcinB: ^{17}FKCRRWQWRMKKLGAPSITCVRRAF^{41}), that is located at the N-terminal side of lactoferrin and is more active than its parent protein (LFB). LfcinB forms an intramolecular disulfide bond (^{19}Cys- ^{36}Cys) thus generating a looped structure (Gifford et al. 2005). Its minimal antimicrobial motif is RRWQWR (Quintieri et al. 2013; Huertas et al. 2017). This protein and its derivative synthetic peptides have been widely studied and exhibit well-known antibacterial activity (Huertas et al. 2017; Hao et al. 2018; Zarzosa-Moreno et al. 2020). The suggested mechanism of action of lactoferricin-derived peptides starts with their cationic character (due to cationic amino acids), which leads to recognition of anionic molecules located in the pathogen. This electrostatic interaction is mainly developed by the Arg residues of the LfcinB, followed by a hydrophobic interaction of Trp residues that leads to membrane destabilization and cell lysis (Farnaud et al. 2004b; Huertas et al. 2017).

Solid-Phase Peptide Synthesis (SPPS) is used for obtaining these peptides. SPPS allows peptide chain elongation by a covalent attachment of the first amino acid to a resin, followed by multiple coupling and deprotection steps, processes based on orthogonal protecting groups that allow the specific formation of the peptide bond (Fields 2001). Our group has synthesized AMPs by the SPPS-Fmoc/tBu strategy and we have studied the multivalent approach (Huertas et al. 2017), which is based on the repetition of short active motives within a peptide, thus increasing binding sites interactions and increasing the antimicrobial activity or stability to proteolysis of peptides, through a rise of cationic and hydrophobic clusters, (Liu et al. 2010; Scorciapino et al. 2017; Huertas et al. 2017). Polyvalent or multivalent peptides can present multiple structures, such as palindromic or linear multiple sequences, covalent dimeric or two-branched peptides, and covalent tetrameric or tetra-branched peptides. Within the article, covalent dimers and tetramers will be referred as dimeric and tetrameric peptides. Dimeric peptides can be synthesized using the methodology developed by (Tam and Zavala 1989); with a lysine core molecule, that has unprotected α and ε amino groups and allows the double elongation of the peptide chain. According to (Rivera et al. 2002), the establishment of intermolecular disulfide bonds by oxidation of dimeric peptides containing Cys amino acids, forms tetra-branched structures.

Several authors have evaluated the biological activity of peptides derived from LfcinB, showing that modifications in the sequences can enhance the antibacterial activity (Hao et al. 2018). In previous studies, we reported that monomeric, dimeric, and tetrameric peptides derived from the minimal motif of LfcinB exhibited high activity against ATCC strains of Gram-positive bacteria such as *S. aureus* and *E. faecalis* and Gram-negative bacteria such as *E.coli* and *P. aeruginosa*, and were considered as promising therapeutic agents (Vargas Casanova et al. 2017, 2019). But it has also been seen that dimeric and tetrameric peptides exhibit higher hemolytic activity (Vargas-Casanova et al. 2019), which could cause a problem when they are administered. Generally, AMPs present electrostatic interactions due to the cationic charge given by the high content of arginine; it has been seen in other studies that a substitution of arginine for lysine can help decrease the percentage of hemolysis, preserving the antibacterial activity while also reducing manufacturing costs (Sitaram et al. 1992; Chen et al. 2000; Tokunaga et al. 2001). In addition, short peptides derived from LfcinB that contain Lys residues have been synthesized. For example, the peptide LTX-315 (KKWWKK-Dip-K), which was designed based on a structure-activity relationship and has exhibited a cytotoxic effect against cancerous cells in vitro and induced an immunological response upon being injected locally into tumors of immunocompetent mice (Sveinbjørnsson et al. 2017). Previous results have shown that Arg/Lys substitutions in several Trp-rich AMPs retained or slightly decreased the antimicrobial activity of the peptides against *E. coli* ATCC 25922, and that shorter Lys analogs such as ornithine or 2,3-diaminopropionic acid improved the stability to trypsin digestion (Arias et al. 2018), in fact, some of these peptides have cytotoxic effect against Jurkat cells. Furthermore, Lys modifications have shown an increase in selectivity (peripheral blood mononuclear cells)

and ornithine derivatives have exhibited increased anticancer potency (Arias et al. 2020).

In the present investigation, we evaluated the antibacterial activity of LfcinB-derived peptides in which Arg residues were substituted for Lys residues to establish if these changes affect the antibacterial and hemolytic activity and the synthetic process. Additionally, we wanted to know if the antibacterial activity exhibited in reference strains was similar in a multi-drug resistant clinical isolate of *E. coli*.

Materials and Methods

Reagents and Materials

Mueller-Hinton Agar, Mueller-Hinton Broth (MHB), Brain Heart Infusion Broth (BHI), Tryptic Soy Agar (TSA), and peptonate water were obtained from OXOID, bacterial strains *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 11775 were obtained from ATCC (Manassas, VA, USA), The clinical isolate *E. coli* 301755 was obtained from the Instituto Nacional de Cancerología E.S.E, located in Bogotá, Colombia. Sterile distilled water, Ciprofloxacin (Bayer), Vancomycin (Hospira. Inc- Pfizer), *N,N*-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), 1,2-ethanedithiol (EDT), 4-methylpiperidine, and ninhydrin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rink amide resin, Fmoc-amino acids, 6-chloro-1-hydroxy-benzotriazole (6-Cl-HOBt), and *N,N*-dicyclohexylcarbodiimide (DCC) were purchased from AAPPTec (Louisville, KY, USA). Methanol, diethyl ether, *N,N*-dimethylformamide (DMF), absolute ethanol, dichloromethane (DCM), acetonitrile (ACN), isopropyl alcohol (IPA), and trifluoroacetic acid (TFA) were obtained from Honeywell-Burdick & Jackson (Muskegon, MI, USA). All reagents were used without further purification.

LfcinB-Derived Peptide Synthesis

The designed peptides were synthesized using manual solid-phase peptide synthesis (SPPS-Fmoc/tBu) (Behrendt et al. 2016; Rodríguez et al. 2019). Briefly, Rink amide resin (0.46 meq/g) was used as a solid support. Resin swelling was carried out with *N,N*-Dimethylformamide (DMF) for 2 hours. As shown in Fig. 1S (Step 1) Fmoc group removal was carried out twice through treatment with 2,5% 4-methylpiperidine in DMF and then, washed with DMF (6×) and DCM (3×); (Step 2) For the coupling reaction, Fmoc-amino acids were pre-activated with DCC/6-Cl-HOBt in DMF at room temperature (RT) for 10 minutes; Fmoc group elimination and the incorporation of each amino acid was confirmed by the Kaiser test (Kaiser et al. 1970) (Step 3) Side-chain deprotection reactions and peptide separation from the resin were carried out with a cleavage cocktail containing TFA/water/TIPS/EDT (93/2/2.5/2.5 v/v/v). The reaction was stirred for 4 or 8 h at RT, and then the resin-peptide was filtered and the solution was collected. Crude peptides were precipitated by treatment with cold ethyl ether, dried at RT, and finally washed with ethyl ether five times. After purification of the dimeric precursor (Step 4), the tetrameric peptides were obtained by the oxidation of precursor dimeric peptides (Step 5) $(RRWQWR)_2$K-Ahx-C and $(KKWQWK)_2$K-Ahx-C in accordance with (León-Calvijo et al. 2015; Vargas Casanova et al. 2017); The oxidation of the molecules is carried out in Dimethyl Sulfoxide (DMSO) 10% (pH:7,5) during 24hrs, then the tetramer molecules were desalted using an RP-SPE cartridge.

Reverse-Phase HPLC

The analysis was performed on a Merck Chromolith® C18 (50 × 4.6 mm) column using an Agilent 1200 liquid chromatograph (Omaha, NE, USA) with UV-Vis detector (210 nm). For peptide analysis, a linear gradient was applied from 5 to 50% solvent B (0.05% TFA in ACN) in solvent A (0.05% TFA in water) for 8 min at a flow rate of 2.0 mL/min at RT. 10 µL samples were injected with an approximate concentration of 1.0 mg/mL of crude or purified molecules (Vargas Casanova et al. 2017).

Peptide Purification

The molecules were purified in accordance with the method reported by (Insuasty Cepeda et al. 2019), using solid-phase extraction columns (SUPELCO LC-18 with 2.0 g resin). SPE columns were activated prior to use with 30 mL acetonitrile (containing 0.1% TFA) and equilibrated with 30 mL water (containing 0.1% TFA). The crude peptides were passed through the column, and a gradient was used for their elution. The collected fractions were analyzed using RP-HPLC (as described above). The fractions that contained pure products were lyophilized (Insuasty Cepeda et al. 2019).

Activation, Confirmation, and Maintenance of Bacterial ATCC Strains

Reference strains *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *E. coli* ATCC 11775, and *P. aeruginosa* ATCC 27853 were activated according to the protocol established by the provider. Three serial passes were made until the optimum growth rate was obtained; to confirm the purity of the microorganisms, Gram staining was performed. When isolation of the bacteria in the TSA was obtained, the strains were stored at 4 °C for a maximum of 60 days. After this time,

or upon observing contamination in the medium, the strains were discarded and activated again as described above. The strains were stored at − 70 °C in 20% glycerol.

Antibiogram of the *E.coli* Clinical Isolate

The determination of the resistance profile of the clinical isolate was performed by Idime (Instituto de Diagnóstico Médico) according to its internal protocols.

Antibacterial Activity Assay

The minimal inhibitory concentration (MIC) was determined using the Broth microdilution method (Vargas-Casanova et al. 2019), according to the standards of the Clinical & Laboratory Standards Institute (CLSI) (Weinstein 2018). 90 µL of Mueller-Hinton (MH) broth were added in a 96-well microtiter plate, followed by 90 µL of peptide (concentration of 200 µg/mL to 6.2 µg/mL). Then 10 µL of bacterial inoculum (5×10^6 CFU mL^{-1}) was added to all the wells. Finally, the plate was incubated at 37 °C for 24 h and the absorbance was measured (620 nm). The minimal bactericidal concentration (MBC) was determined according to the National Committee for Clinical Laboratory Standards (NCCLS) standard (Clinical and Laboratory Standards Institute 1999). After measuring the absorbance, a subculture of each of the wells where there was no growth was made in MH Agar. MBC was defined as the when bacterial growth was reduced by 99.9%. Each of these tests was performed twice (n = 2).

Hemolysis Assay

5 mL of peripheral blood in EDTA was centrifuged at 500 rpm for 15 min; the erythrocytes were separated and washed three times with 0.9% saline solution (SS). Then 100 µL of peptide (final concentrations of 200 to 6.2 µg/mL) was mixed with 100 µL of erythrocytes (2% hematocrit) and incubated at 37 °C for 2 h. It was then centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was measured at 450 nm. Two controls were used; positive control: distilled water and negative control: SS 0.9% (Solarte et al. 2015). All experiments were approved by the Universidad Nacional de Colombia ethical committee (code number 04-19 2019). Informed consent of all participating subjects was obtained.

Time-Kill Curve

The time-kill curves were performed as described in the CLSI protocol (Clinical and Laboratory Standards Institute 1999), with some modifications. 270 µL of peptide (0.5 MIC, MIC and 2 MIC) in Mueller Hinton Broth and 30 µL of inoculum (5×10^6 CFU mL^{-1}) were added to a 100-well microtiter plate; this was incubated at 37 °C for 48 h in the Bioscreen C equipment. The absorbance (600 nm) was measured every hour (n = 3).

Synergy Test

The synergistic effect of the antibacterial activity was determined by the chessboard test using Mueller-Hinton broth (Vargas-Casanova et al. 2019). Briefly, the following combinations were evaluated: Ciprofloxacin/[K]-LfcinB $(20\text{-}25)_{Pal}$, Ciprofloxacin/[K]-LfcinB $(20\text{-}25)_2$, LfcinB $(20\text{-}25)$/[K]-LfcinB $(20\text{-}25)_{Pal}$, LfcinB $(20\text{-}25)$/[K]-LfcinB $(20\text{-}25)_2$, [K]-LfcinB $(20\text{-}25)$/[K]-LfcinB $(20\text{-}25)_{Pal}$, [K]-LfcinB $(20\text{-}25)$/[K]-LfcinB $(20\text{-}25)_2$, Ciprofloxacin/[K]-LfcinB $(20\text{-}25)_{Pal}$, and Ciprofloxacin/[K]-LfcinB $(20\text{-}25)_2$. The peptides were evaluated at concentrations of 0, 0.06, 0.12, 0.25, 0.50, 1, and 2 times the MIC. The FIC (fractional inhibitory concentration) was determined according to the following equation: $[(A)/MIC_A] + [(P)/MIC_P] = FIC_A + FIC_P = FIC$ index, where the MIC_A and MIC_P are the MIC values of the individual antibiotic and the peptide, respectively, and (A) and (B) are the MIC evaluated in combination. Specifically, the values of FIC ≤ 0.5 indicate synergy, FIC between 0.5 and 1 additivity, between 1 and 4 indifference, and greater than 4 antagonism (Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Diseases 2000). Each of these tests was performed twice (n = 2).

Results and Discussion

AMPs derived from LfcinB have exhibited antimicrobial properties in both Gram-positive and Gram-negative ATCC strains (Huertas et al. 2017; Vargas-Casanova et al. 2019). In the present investigation, the activity of LfcinB-derived peptides with Arg/Lys substitution was evaluated with the aim of studying the synthetic viability, hemolytic activity, and antimicrobial effect against microorganisms that are part of the WHO priority group (Asokan et al. 2019): Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) reference strains, and an *E. coli* multidrug-resistant clinical isolate. Four Arg- peptides previously described (Vargas-Casanova et al. 2019) and four Lys-peptides newly designed, derived from LfcinB were synthesized, purified, and characterized via RP-HPLC and MALDI-TOF MS (Table 1 and Fig. 2S). In all cases chromatographic purity was above 91% (Fig. 3S) and the main MS signal corresponded to the *m/z* ratio of the [M+H]+ ion and was in accordance with the expected monoisotopic mass (Table 1 and Fig. 3, 4S).There were no significant changes in the hydrophobicity of the molecules, reflected in the retention times (t_R), determined in a chromatographic profile using RP-HPLC; the longer the

Table 1 Analytical characterization summary of LfcinB- derived peptides

Code	Sequence	Characterization	
		RP-HPLC t_R (Purity) min (%)	MS $[M+H]^+$ *m/z*
LfcinB (20-25)	RRWQWR	4.2 (96)	987.65
LfcinB $(21-25)_{Pal}$	RWQWRWQWR	5.9 (94)	1485.85
LfcinB $(20-25)_2$	$(RRWQWR)_2$K-Ahx	4.9 (99)	2194.00
LfcinB $(20-25)_4$	$((RRWQWR)_2$K-Ahx-C$)_2$	5.2* (99)	2295.10*
[K]-LfcinB (20-25)	KKWQWK	3.7 (99)	903.90
[K]-LfcinB $(21-25)_{Pal}$	KWQWKWQWK	5.7 (96)	1400.33
[K]-LfcinB $(20-25)_2$	$(KKWQWK)_2$K-Ahx	4.8 (99)	2025.34
[K]-LfcinB $(20-25)_4$	$((KKWQWK)_2$K-Ahx-C$)_2$	4.8* (91)	2127.82*

Molecules were synthesized via SPPS- Fmoc/tBu strategy, purified using RP-SPE, characterized by means of RP-HPLC, and analyzed via MALDI-TOF mass spectrometry

*Data of the precursor dimer that was oxidized to the tetramer (Supplementary material)

Ahx (6-aminohexanoic acid), *MS* mass spectrometry

retention time, the greater hydrophobicity is reflected. The palindromic peptides exhibited the highest hydrophobicity and the monomeric peptides the lowest. Both groups of molecules presented high purity and yields.

Lys peptides exhibited better synthetic efficiency represented by lower reagent consumption, shorter synthesis duration and lower costs, while maintaining high chromatographic purity. Our results are in agreement with previous reports that showed the difficulty of Arg coupling, requiring long reaction times and a high quantity of reagents (Huertas et al. 2017). The highly basic and nucleophilic guanidine group requires an electron withdrawal and a bulky protecting group, such as the Pbf (pentamethyl-2,3-dihydrobenzofuran-5 sulfonyl), which is voluminous and highly acid-stable, which makes it difficult to complete the coupling reaction of the Fmoc-Arg(Pbf)-OH and their removal in the cleavage process during SPPS (Seo and Silverman 2006). Lys is an amino acid that could introduce the positively charged character to the peptide chain, together with easier incorporation into the growing chain in SPPS-Fmoc/tBu synthesis. This is partly due to the fact that the Lys side chain is protected by the Boc group, which is less voluminous than Pbf, thus causing less steric hindrance and requiring less time and fewer reagents to complete the coupling reaction (Albericio et al. 2009; de la Fuente-Núñez et al. 2017).

Moreover, for Arg containing peptides, the number of coupling cycles was greater than for Lys-containing ones. For instance, for peptide [K]-LfcinB $(20-25)_2$ synthesis, twelve coupling reaction cycles were required, in contrast to twenty-three coupling reaction cycles required for peptide LfcinB $(20-25)_2$ synthesis. Besides, a 4-hour increase in the cleavage process was also required, which reaffirms that the synthesis of Arg peptides is more labor-intensive and represents higher costs (Fig 5S). Interestingly, the increase in coupling steps applies for the whole sequence and not just Arg cycles, as mentioned before, this behavior demonstrates the steric hindrance that Arg creates into the peptide chain. Each additional coupling reaction cycle represents higher expend on reagents, and currently, Arg (Fmoc-Arg(Pbf)-OH) costs at least twice as much as Lys (Fmoc-Lys(Boc)-OH). In addition, Lys-synthesis of the dimeric entity required approximately 48% less moles of amino acid, DCC and 6-Cl-HOBt and approximately 90% less volume of solvents (DMF and DCM), making this a green synthesis approach. The synthesis of the dimeric and tetrameric peptides was carried out using the methodology developed by Tam et al., The incorporation of Fmoc-Lys(Fmoc)-OH, allows the formation of two-branched peptide (dimer precursor), the oxidation of Cys residue located in the C-terminus leads to tetra-branched molecule (León-Calvijo et al. 2015) (Fig. 1S).

As shown in Table 2, monomeric peptides LfcinB (20-25) and [K]-LfcinB (20-25) exhibited the lowest antibacterial activity in the evaluated strains, suggesting that the Arg/Lys substitutions in the LfcinB (20-25): RRWQWR sequence did not notably increase the antibacterial activity of these linear peptides. On the contrary, the palindromic, dimeric, and tetrameric peptides exhibited higher antibacterial activity. This result is in agreement with previous reports by our research group, showing that palindromic and polyvalent sequences of the minimal motif of LfcinB enhance the activity compared to their monomeric counterparts (León-Calvijo et al. 2015; Huertas et al. 2017; Guerra et al. 2019; Vargas-Casanova et al. 2019). Furthermore, the tetrameric peptide LfcinB $(20-25)_4$ exhibited the highest antibacterial activity against all the reference strains, and we found that all the peptides exhibited better activity against the *E. coli* strains (ATCC 11775 and 25922) compared to the other bacterial strains. The palindromic peptide LfcinB $(21-25)_{Pal}$ exhibited higher antibacterial activity compared to the peptide

Table 2 Antibacterial activity of LfcinB-derived peptides against ATCC reference strains

Code	Antibacterial activity			
	E. coli		*P. aeruginosa*	*S. aureus*
	ATCC 11775 MIC/MBC	ATCC 25922 MIC/MBC	ATCC 27853 MIC/MBC	ATCC 25923 MIC/MBC
LfcinB (20-25)	203/> 203[a]	203/> 203[a]	203/> 203[b]	203/> 203[b]
LfcinB (21-25)$_{Pal}$	17/17[b]	17/34[b]	67/135[b]	135/> 135[b]
LfcinB (20-25)$_2$	23/23	6/11[a]	23/91[b]	91/91[b]
LfcinB (20-25)$_4$	11/22	11/11	11/22[b]	22/44[b]
[K]-LfcinB (20-25)	> 222/> 222	222/222	> 222/> 222	> 222/> 222
[K]-LfcinB (21-25)$_{Pal}$	36/72	36/36	143/143	143/> 143
[K]-LfcinB (20-25)$_2$	25/50	24/24	48/95	> 95/> 95
[K]-LfcinB (20-25)$_4$	23/47	47/47	47/47	47/47

Test developed in accordance with the Broth microdilution method (Vargas-Casanova et al. 2019). MIC: Minimal inhibitory concentration (μM); MBC: Minimal bactericidal concentration (μM)

In accordance with the results of [a](Vargas Casanova et al. 2017); [b](Vargas-Casanova et al. 2019)

[K]-LfcinB (21-25)$_{Pal}$; however, the difference between MIC values for these palindromic peptides corresponded to one peptide dilution in all cases, suggesting that the Arg/Lys substitutions do not significantly affect the antibacterial activity. It can be deduced that the antibacterial activity of palindromic, dimeric, and tetrameric Lys peptides for the *E. coli* reference strains was similar (MICs between 23 and 47 μM). For dimeric and tetrameric peptides containing Lys, the MIC values were higher (corresponding to one or two peptide concentration dilutions) than those of the dimeric and tetrameric peptides containing Arg residues. The tetrameric peptides LfcinB (20-25)$_4$ and [K]-LfcinB (20-25)$_4$ exhibited higher antibacterial activity against the *S. aureus* strain, confirming that the polyvalence enhances the antibacterial activity of sequences with moderate antibacterial activity and that the Arg/Lys substitutions do not significantly affect the antibacterial activity.

Peptides [K]-LfcinB (20-25)$_2$ and [K]-LfcinB (20-25)$_4$ exhibited significant antibacterial activity against *P. aeruginosa* and *S. aureus* strains. As mentioned above, in a manner similar to the RRWQWR motif, the polyvalence of the KKWQWK motif also improved the antibacterial activity, because the RRWQWR and KKWQWK peptides alone do not exhibit significant antibacterial activity. Our results are in agreement with reports that showed that the polyvalence of sequences derived from LfcinB significantly increases the antibacterial activity (Azuma et al. 2008; León-Calvijo et al. 2015; Vargas Casanova et al. 2017; Vargas-Casanova et al. 2019).The improvement of the antibacterial activity by the polyvalence of these short sequences possibly occurred because of the increase in the number of amino acids with positively-charged side chains (Arg or Lys); both Arg and Lys are basic residues that have a net charge of +1 under physiological conditions. This fact is in accordance with previous studies, which suggest that the antibacterial activity of AMPs is associated with their self-assembly, forming polymeric structures that interact with bacterial membranes (Farnaud and Evans 2003; Farnaud et al. 2004a; Azuma et al. 2008; Tian et al. 2015). In addition, positive charges are essential in order for the peptide to approach the cell membrane through electrostatic interactions with the negative charges located on cell surface, so that the hydrophobic side chains of the peptide can then interact with the lipid bilayer, causing the membrane to rupture (Schibli et al. 1999; Farnaud and Evans 2003; Nguyen et al. 2005; Chan et al. 2006). Dimeric and tetrameric peptides have multiple chains that are attached to an arm containing the unnatural amino acid; 6-aminohexanoic acid (Ahx), which may confer greater stability against proteolytic degradation.

The differences between the antibacterial activity of the Arg peptides and the Lys peptides could be due to the nature of the positive charge in the side chain. The Lys side chain has a primary amine, while Arg contains a guanidine group, causing it to interact differently with negatively charged molecules on the plasmic membrane. In the guanidine group, the positive charge could be dispersed, due the resonance effect and that it can form contact like-charge ion pairs in water, which avoids the repulsion of positive charges and favors the penetration across cellular membranes (Yang et al. 2003; Vazdar et al. 2018). Furthermore, other studies with Arg/Lys substitution observed that Arg peptides tend to exhibit better activity, which can be explained by the hydrogen bridge stabilization of the Arg-phosphate clusters. There is no clear correlation with the antimicrobial activity, due to the complexity of the peptide interactions (charge distribution, hydrophobicity, and secondary structure) (Kang et al. 1996; Li et al. 2013; Arias et al. 2018; Luong et al. 2018; Mann et al. 2018).

There are previous reports on Arg/Lys substitution in 11-residue peptides derived from LfcinB (Kang et al. 1996), where a decrease in the activity of Lys peptides was also observed. Other authors report reduction in the antimicrobial activity of Lys peptides, presumably due to the lower ability to induce membrane permeabilization in *E. coli* strains. On the other hand, Arias et al. evaluated the antimicrobial activity of some AMPs against *E. coli* ATCC 25922, and the shortest sequence that was presented and retained the activity of the Arg counterpart was VKKFAWWWAFLKK, with a MIC of 2 µM. Most of the Arg- and Lys-derivative peptides exhibited a strong correlation between the severity of the cytoplasmic membrane disruption in *E. coli* and the antibacterial activity, suggesting that the antibacterial activity of Arg and Lys peptides is related to the same action mechanism (Falciani et al. 2007; Arias et al. 2018).

To establish the bacteriostatic and/or bactericidal effect of Lys peptides, we developed time-kill curve assays (Fig 1). Previously, the time-kill curves of peptides LfcinB $(20\text{-}25)_2$ and LfcinB $(20\text{-}25)_4$ were reported (Vargas-Casanova et al. 2019). Peptides [K]-LfcinB $(20\text{-}25)_2$ and [K]-LfcinB $(20\text{-}25)_4$ were chosen for this assay because they exhibited the best results against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853 strains. In the *E. coli* ATCC 25922 strain, the dimeric peptide exhibited a bacteriostatic effect at all the evaluated concentrations (12, 24, and 48 µM that correspond to 0.5 MIC, MIC and 2 times the MIC) at 48 h, decreasing the bacterial growth to 20%, 50%, and 80%, respectively. In addition, this peptide prolonged the adaptation stage to between 7 and 11 h, compared with the control growth, which was 3 h (Fig. 1a). The dimeric peptide against *P. aeruginosa* ATCC 27853 (Fig. 1b) also prolonged the adaptation stage up to 6 h at 24 µM, but in this strain, a bactericide effect was achieved with peptide concentrations at 48 and 95 µM at 48 h. In *S. aureus* ATCC 25923, the tetrameric peptide exhibited a bacteriostatic effect at 24 µM, and the adaptation stage of the microorganism was prolonged up to 11 h, compared with the adaptation stage of the control (bacteria without the peptide), which was 5 h. Also, this peptide exhibited a bactericidal effect at 47 and 94 µM for 48 h of treatment (Fig. 1c). These results are in agreement with (Vargas-Casanova et al. 2019). The peptides LfcinB $(20\text{-}25)_4$ and [K]-LfcinB $(20\text{-}25)_4$ exhibited the same bactericidal effect against *S. aureus* ATCC 25923 (44 µM), while the dimers LfcinB $(20\text{-}25)_2$ (6-24 µM) and [K]-LfcinB $(20\text{-}25)_2$ (24-94 µM) exhibited a bacteriostatic effect against *E. coli* ATCC 25922. In addition, it was possible to observe the bactericidal and bacteriostatic (24/48 µM) effect of the peptide [K]-LfcinB $(20\text{-}25)_2$ against *P. aeruginosa* ATCC 27853. This means that the Lys and Arg peptides exhibited similar behavior with respect to their antibacterial activity up to 48 h of treatment.

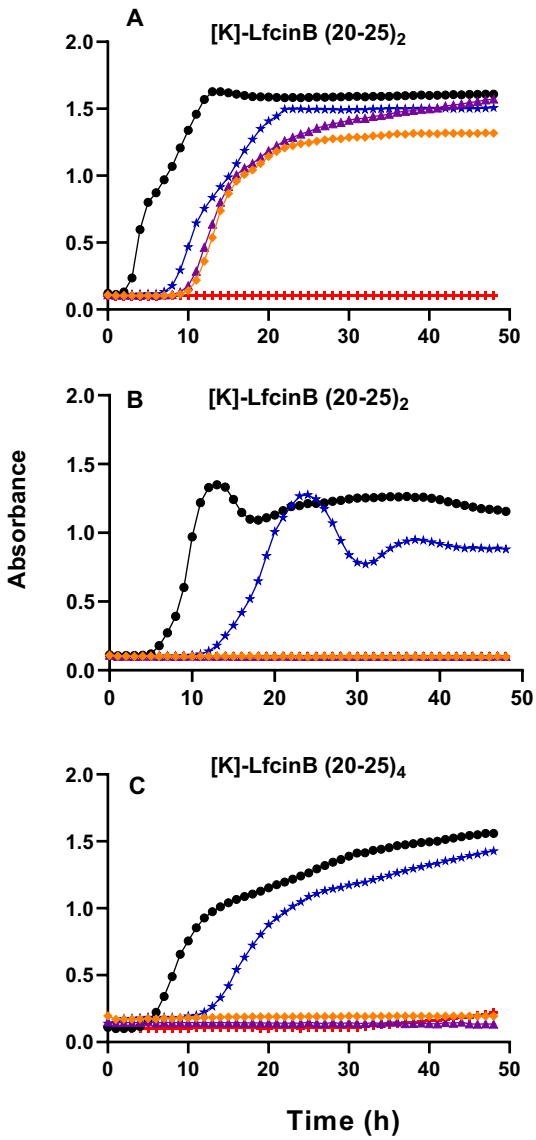

Fig. 1 Time-kill curve of peptides derived from LfcinB rich in Lys. A. [K]-LfcinB $(20\text{-}25)_2$ against *E. coli* 25922. B. [K]-LfcinB $(20\text{-}25)_2$ against *P. aeruginosa* 27853. C. [K]-LfcinB $(20\text{-}25)_4$, and against *S. aureus* 25923. ● Growth control, + Antibiotic, ★0.5MIC ◆MIC ▲2MIC

Our results agree with the majority of reports of previous Arg/Lys substitution, but with emphasis on the fact that the synthesis of Lys peptides can provide a better equilibrium between costs and activity, so it was decided to evaluate the synergistic effects of peptides in combination with antibiotics or peptides at different concentrations. The most sensitive strain was selected for the trials, in this case *E. coli* ATCC 25922. In addition, a multidrug-resistant clinical isolate (301755) was chosen to evaluate the synergy between the peptide and ciprofloxacin. The resistance profile of clinically isolated *E. coli* 301755 was determined, exhibiting resistance to 7 (including ciprofloxacin) of the 10 evaluated

antibiotics, indicating that this clinically isolated bacterium was classified as multidrug resistant (Table 3). Peptides [K]-LfcinB $(20\text{-}25)_{Pal}$ and [K]-LfcinB $(20\text{-}25)_2$ exhibited significant antibacterial activity against *E. coli* multidrug-resistant clinical isolate 301755, with MIC values of 18 and 25 µM, respectively (Table 3). These results are in agreement with reports that showed that the peptides LfcinB $(21\text{-}25)_{Pal}$ and LfcinB $(20\text{-}25)_4$ exhibited antibacterial activity against clinical isolates of *E. faecium*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* (Vega et al. 2018).

Furthermore, we studied the synergy of LfcinB-derived peptides with ciprofloxacin. Indeed this fluoroquinolone, which is effective against Gram-negative bacteria, such as *E. coli*, and exhibits low adverse effects, has raised resistance over the years and it is important to overcome this issue (Goswami et al. 2006). Moreover, the availability in the group of an *E. coli* clinical isolate resistant to this antibiotic permitted to compare the results in the reference strains and in the clinical isolate. This resistance is understood microbiologically as the inhibition of the growth of the microorganism at higher doses than that observed in non-resistant strains (Pfaller 2012). The MIC of the antibiotic in *E. coli* ATCC 25922 was 0.6 µM, in contrast to the MIC value of 189 µM for the *E. coli* clinical isolate, which is in accordance with the resistant profile, indicating that the isolate is resistant to ciprofloxacin. Our results support the great potential of the peptides [K]-LfcinB $(20\text{-}25)_{Pal}$ and [K]-LfcinB $(20\text{-}25)_2$ for developing combined therapies using peptides mixture or peptide mixed with antibiotics to treat bacterial infections caused by resistant strains.

The synergy test was evaluated with peptide combinations of [K]-LfcinB $(20\text{-}25)_{Pal}$ and [K]-LfcinB $(20\text{-}25)_2$ because both exhibited higher activity against the reference strains. These peptides also showed additivity effect when were mixed with ciprofloxacin against *E. coli*, lowering the antibiotic MIC values by 2 and 8 times, respectively. Some of the mechanisms of resistance to ciprofloxacin are related to a decrease in drug concentration, which could be due to an increase in the mechanism of drug expulsion out of the microorganism or due to difficulties for the entrance of the drug in order to exert its function. Focusing on this last aspect, some antimicrobial peptides, especially those derived from lactoferricin, could have a permeabilizing action in the microorganism membrane (Aguilera et al. 1999; Midura-Nowaczek and Markowska 2014). For the clinical isolate, the combination of ciprofloxacin with [K]-LfcinB $(20\text{-}25)_{Pal}$ or [K]-LfcinB $(20\text{-}25)_2$ caused a decrease of 16 and 32 times the MIC of the antibiotic, respectively. Surprisingly, these results are even better than those found with the reference strains and bear out the great potential of these antimicrobial peptides. These results suggest that peptides [K]-LfcinB $(20\text{-}25)_{Pal}$ and [K]-LfcinB $(20\text{-}25)_2$ can be considered for developing a combined therapy with ciprofloxacin in bacterial infections caused by *E. coli* and strengthens the validity of the study of peptide-based approaches against multidrug-resistant pathogens.

We also assessed synergy effect using peptides mixture (*E. coli* ATCC 25922); when peptide [K]-LfcinB $(20\text{-}25)_{Pal}$ or [K]-LfcinB $(20\text{-}25)_2$ was combined with LfcinB (20-25) or [K]-LfcinB (20-25), additivity effect was observed in all

Table 3 Synergy test

Bacterial strain	A	B	MIC_A/ MIC_B	C_A/C_B	FIC	MIC_A/C_A	Activity
E. coli ATCC 25922	Ciprofloxacin	[K]-LfcinB $(20\text{-}25)_{Pal}$	0.6/36	0.30/75	1.0	2	A
	Ciprofloxacin	[K]-LfcinB $(20\text{-}25)_2$	0.6/25	0.08/75	0.6	8	A
	LfcinB (20-25)	[K]-LfcinB $(20\text{-}25)_{Pal}$	101/71	0.03/51	0.5	4000	S
	LfcinB (20-25)	[K]-LfcinB $(20\text{-}25)_2$	101/49	0.03/101	1.0	4000	A
	[K]-LfcinB (20-25)	[K]-LfcinB $(20\text{-}25)_{Pal}$	222/36	0.03/55	1.0	8000	A
	[K]-LfcinB (20-25)	[K]-LfcinB $(20\text{-}25)_2$	222/12	0.03/28	1.0	8000	A
E. coli 301755	Ciprofloxacin	[K]-LfcinB $(20\text{-}25)_{Pal}$	189/18	12/9	0.6	16	A
	Ciprofloxacin	[K]-LfcinB $(20\text{-}25)_2$	189/25	6/25	1.0	32	A

Antibiotic	ESBL	AM	SAM	CIP	NOR	AK	GEN	ETP	MER	NIT	STX
Resistance profile	S	R	R	R	R	S	R	S	S	R	R
MIC (µg/mL)	–	> 32	> 32	> 4	> 16	–	> 16	–	–	> 128	> 320

Antibacterial activity of peptide-peptide and peptide-ciprofloxacin combinations. A (Antibiotic or monomeric peptide). B (Lys- peptides). MIC_A/ MIC_B (MICs of molecule alone), C_A/C_B (concentrations at which the MIC was found in the synergy test), FIC (fractional inhibitory concentration). The peptides were evaluated at concentrations of 0, 0.06, 0.12, 0.25, 0.50, 1, and 2 times the MIC and the test was developed in duplicate

AM amplicillin, *SAM* ampicillin/sulbactam, *CIP* ciprofloxacin, *NOR* norfloxacin, *AK* amikacin, *GEN* gentamicin, *ETP* ertapenem, *MER* meropenem, *NIT* nitrofurantoin, *STX* trimethoprim/sulfamethoxazole, *ESBL* extended spectrum betalactamase. Resistant (R), Susceptible (S)

Activity: Synergy (S), Additivity (A)

the cases except for LfcinB (20-25) and [K]-LfcinB (20-25)$_{Pal}$ that presented synergistic effect. In this case, both MICs decreased, which could be considered as an interesting approach to enhance the antibacterial activity of monomeric peptides. The Lys-derivative peptides are costless compared with longer peptides or Arg- derivative peptides, therefore, it broadens the possibility of developing peptide-based therapies at lower costs.

The hemolytic effect of the peptides was evaluated at concentrations between 0 and 200 µg/mL. Monomeric peptides LfcinB (20–25) and [K]-LfcinB (20–25) exhibited a similar hemolytic effect at the evaluated concentrations, corresponding to 0.5% at the maximum concentration (Fig. 2a). In the same way, dimeric peptides LfcinB (20-25)$_2$ and [K]-LfcinB (20-25)$_2$ exhibited a hemolytic effect of 2% and 1%, respectively, at 200 µg/mL (Fig. 2b). These results suggest that Arg/Lys substitution does not significantly affect the hemolysis of these sequences. Nevertheless, for the palindromic peptides at 200 µg/mL, the hemolytic activity of [K]-LfcinB (21-25)$_{Pal}$ was approximately four times lower than for LfcinB (21-25)$_{Pal}$ (Fig. 2d). This enables the use of the palindromic peptide [K]-LfcinB (21-25)$_{Pal}$ at high concentrations (200 µg/mL).

The hemolytic effect of tetrameric peptide LfcinB (20-25)$_4$ was significantly higher than for the tetrameric peptide [K]-LfcinB (20- 25)$_4$ (Fig. 2c). The peptide LfcinB (20-25)$_4$ exhibited a hemolytic effect between 2% and 11%, while peptide [K]-LfcinB (20-25)$_4$ exhibited a hemolytic activity between 0.5% and 1.5%. At a concentration of 200 µg/mL (LfcinB (20-25)$_4$: 44 µM and [K]-LfcinB (20-25)$_4$: 47 µM), where the tetrameric peptides exhibited the highest antibacterial activity against the three ATCC strains, it was observed that [K]-LfcinB (20-25)$_4$ decreased the hemolysis percentage by approximately 7 times in comparison with the Arg analog. These results indicate that the therapeutic index (TI = peptide concentration that produces 10% hemolysis/MIC) (Vishnepolsky et al. 2019) for peptide [K]-LfcinB (20-25)$_4$ was presumably higher than for LfcinB (20-25)$_4$ in all cases, where higher therapeutic indexes are desired in antibiotics research (Maturana et al. 2017). For peptide LfcinB (20-25)$_4$, the TI for the strains *E. coli* ATCC 25922, *E. coli* ATCC 11775, and *P. aeruginosa* ATCC 27853 was 3.6, while for *S. aureus* ATCC 25923 it was 1, 8 and for the peptide [K]-LfcinB (20-25)$_4$ the TI was > 1 for all the strains except *E. coli* ATCC 11775, which had a value of > 2.

In previous studies (Vargas Casanova et al. 2017, 2019), the antibacterial activity of the minimum motif of LfcinB was improved with the synthesis of polyvalent peptides, nevertheless it was also evident that for the tetra-branched sequence the hemolytic activity also increased. This disadvantage was solved within the present study through Arg/Lys substitutions, decreasing the hemolytic activity, specifically for the peptides LfcinB (20-25)$_{Pal}$ and LfcinB (20-25)$_4$.

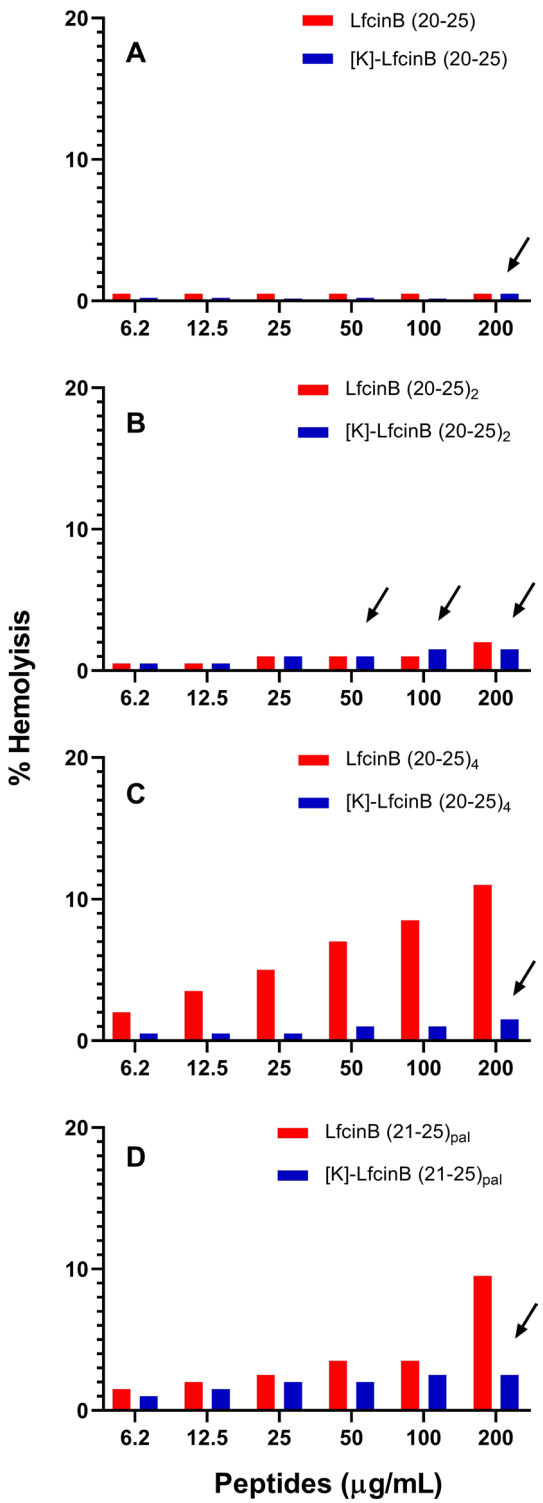

Fig. 2 Comparison of the hemolytic activity between peptides derived from LfcinB rich in Arg (red bar) and the analogs rich in Lys (blue bar). **a** Monomeric peptides, **b** dimeric peptides, **c** tetrameric peptides, **d** palindromic peptides. The arrows indicate the concentration where the Lys peptide exhibited the highest antibacterial activity

In general, it has been described that Arg, and Lys, have a strong effect on the hemolytic activity (Oddo and Hansen 2017), but is also worthwhile to note that Lys exhibits a lower hemolytic effect than Arg. (Sitaram et al. 1992) found that peptide SPFK at 9 μM with Glu/Lys substitution exhibited less hemolysis (10%) than the original peptide (15%) at the same concentration. In addition, (Yang et al. 2003) reported a significant decline in the hemolytic activity of peptides Tritrpticin and SYM1 (37% and 24%, respectively) with Arg/Lys substitution at 100 μg/mL. The Lys peptides TRK and SYM11KK did not exhibit hemolysis (0%) at the same concentration, which agrees with our results.

The decrease in the affinity of Lys for mammalian cells over bacterial cells is because the membrane of mammals, like erythrocytes, contains a higher number of zwitterionic lipids (phosphatidylcholine, sphingomyelin, and others such as cholesterol) than anionic lipids, and furthermore, the negatively charged lipids are located in the inner face of the membrane and are less exposed to interaction with the AMPs. On the contrary, bacteria membranes are mainly composed of anionic lipids such as PG and cardiolipin(Matsuzaki 1999; Glukhov et al. 2005). Thus it has been reported that Arg interacts strongly with anionic and zwitterionic phospholipids and Lys interacts weakly with zwitterionic phospholipids (Yang et al. 2003); therefore, Lys residues exhibit less attraction for erythrocytic membranes than do Arg residues. It is important to highlight that the hemolysis assessment is a widely used initial toxicity test, previous reports indicate a strong correlation between membrane affinity and hemolytic activity, being the main reason of peptide hemolytic activity which is related to the ability to bind the erythrocyte membrane (Maturana et al. 2017). Nevertheless, these results are considered as the starting point to select the peptides to determine the correlation of the hemolytic results with toxicity *in vivo* activity (Greco et al. 2020).

These promissory results need to be further studied in more bacterial species. And since LfcinB derived peptides have shown anticancer activity, Lys-peptides should be tested against cancer cell lines, such as MDA-MB-468 or MDA- MB- 231 (breast cancer cell lines) to evaluate the effect of Arg to Lys substitution, as it was reported for Arg-peptides by (Vargas Casanova et al. 2017), and it is needed to further study toxicity and stability. These approaches will be useful to enlarge Lys-LfcinB peptides potential and settle the impact of Arg/Lys changes.

Conclusion

We studied Arg/Lys substitution in peptides derived from LfcinB. Our preliminary results indicate that the polyvalence of Lys peptides derived from KKWQWK increases the antibacterial activity against Gram-positive and Gram-negative bacteria, as it was confirmed in ATCC reference strains and an *E. coli* multidrug-resistant clinical isolate. In addition, Lys peptides exhibited similar antibacterial activity to the Arg peptides in reference strains, suggesting that the Arg for Lys substitution does not significantly affect the antimicrobial activity. The synergic assays and the hemolytic profile of Lys peptides suggest that they can be candidates for developing combined therapies with a lower cost of synthesis, which opens new doors for the use of these peptides at higher scales or for the development of cheaper drug delivery systems. We consider that the Lys polyvalent peptides [K]-LfcinB $(20\text{-}25)_{Pal}$ and [K]-LfcinB $(20\text{-}25)_2$ are antimicrobial molecules that could be taken into consideration in further studies of the optimization of lead molecules and/ or in combined therapeutic approaches.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10989-021-10207-x.

Acknowledgements Yerly Vargas Casanova thanks COLCIENCIAS for financing her Ph.D. studies (Programa de Becas de Excelencia Doctoral del Bicentenario).

Authors Contribution Conceptualization: KJCM, YVC, JEGC, ZJRM; Methodology: YVC, SME, CMPG, ALL, JEGC; Formal analysis and investigation: KJCM, DGM, LDBV, YVC; Writing—original draft preparation: KJCM, DGM, YVC full name, LDBV, JEGC; Writing—review and editing: JEGC, ZJRM; Funding acquisition: CMPG, ALL, ZJRM; Supervision: JEGC, ZJRM.

Funding This work was supported by COLCIENCIAS, Code 110184466986, contract RC-N° 845-2019

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval All experiments were approved by the Universidad Nacional de Colombia ethical committee (code number 04-17-007). Informed consent of all participating subjects was obtained.

References

Ageitos JM, Sánchez-Pérez A, Calo-Mata P, Villa TG (2017) Antimicrobial peptides (AMPs): ancient compounds that represent novel weapons in the fight against bacteria. Biochem Pharmacol 133:117–138. https://doi.org/10.1016/j.bcp.2016.09.018

Aguilera O, Ostolaza H, Quirós LM, Fierro JF (1999) Permeabilizing action of an antimicrobial lactoferricin-derived peptide on bacterial and artificial membranes. FEBS Lett 462:273–277. https://doi.org/10.1016/S0014-5793(99)01545-8

Albericio F, Isidro-llobet A, Mercedes A (2009) Amino Acid-Protecting Groups. 2455–2504

Arias M, Piga KB, Hyndman ME, Vogel HJ (2018) Improving the activity of trp-rich antimicrobial peptides by Arg/Lys substitutions and changing the length of cationic residues. Biomolecules. https://doi.org/10.3390/biom8020019

Arias M, Haney EF, Hilchie AL et al (2020) Selective anticancer activity of synthetic peptides derived from the host defence peptide tritrpticin. Biochim Biophys Acta - Biomembr 1862:183228. https://doi.org/10.1016/j.bbamem.2020.183228

Asokan GV, Ramadhan T, Ahmed E, Sanad H (2019) WHO global priority pathogens list: A bibliometric analysis of medline-pubmed for knowledge mobilization to infection prevention and control practices in Bahrain. Oman Med. J. 34:184–193

Azuma M, Del Carpio CA, Kojima T et al (2008) Antibacterial activity of multiple antigen peptides homologous to a loop region in human lactoferrin. J Pept Res 54:237–241. https://doi.org/10.1034/j.1399-3011.1999.00090.x

Behrendt R, White P, Offer J (2016) Advances in Fmoc solid-phase peptide synthesis. J Pept Sci 22:4–27. https://doi.org/10.1002/psc.2836

Bellamy W, Takase M, Wakabayashi H et al (1992) Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J Appl Bacteriol 73:472–479. https://doi.org/10.1111/j.1365-2672.1992.tb05007.x

Chan DI, Prenner EJ, Vogel HJ (2006) Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. Biochim. Biophys. Acta - Biomembr. 1758:1184–1202

Chen J, Falla TJ, Liu H et al (2000) Development of protegrins for the treatment and prevention of oral mucositis: Structure-activity relationships of synthetic protegrin analogues. Biopolym - Pept Sci Sect 55:88–98. https://doi.org/10.1002/1097-0282(2000)55:1%3c88::AID-BIP80%3e3.0.CO;2-K

Ciumac D, Gong H, Hu X, Lu JR (2019) Membrane targeting cationic antimicrobial peptides. J Colloid Interface Sci 537:163–185. https://doi.org/10.1016/j.jcis.2018.10.103

Clinical and Laboratory Standards Institute (1999) M26-A methods for determining bactericidal activity of antimicrobial agents; approved guideline.

Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology E, Diseases I (2000) Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clin Microbiol Infect 6:503–508. https://doi.org/10.1046/j.1469-0691.2000.00149.x

de la Fuente-Núñez C, Silva ON, Lu TK, Franco OL (2017) Antimicrobial peptides: Role in human disease and potential as immunotherapies. Pharmacol Ther 178:132–140. https://doi.org/10.1016/j.pharmthera.2017.04.002

Falciani C, Lozzi L, Pini A et al (2007) Molecular basis of branched peptides resistance to enzyme proteolysis. Chem Biol Drug Des 69:216–221. https://doi.org/10.1111/j.1747-0285.2007.00487.x

Farnaud S, Evans RW (2003) Lactoferrin - A multifunctional protein with antimicrobial properties. Mol Immunol 40:395–405. https://doi.org/10.1016/S0161-5890(03)00152-4

Farnaud S, Spiller C, Moriarty LC et al (2004) Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity. FEMS Microbiol Lett 233:193–199. https://doi.org/10.1016/j.femsle.2004.01.039

Farnaud S, Patel A, Odell EW, Evans RW (2004) Variation in antimicrobial activity of lactoferricin-derived peptides explained by structure modelling. FEMS Microbiol Lett 238:221–226. https://doi.org/10.1111/j.1574-6968.2004.tb09759.x

Fields GB (2001) Introduction to peptide synthesis. Curr Protoc Protein Sci. https://doi.org/10.1002/0471140864.ps1801s26

Gao Y, Fang H, Fang L et al (2018) The Modification and design of antimicrobial peptide. Curr Pharm Des. https://doi.org/10.2174/1381612824666180213130318

Gifford JL, Hunter HN, Vogel HJ (2005) Lactoferricin: A lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cell. Mol. Life Sci. 62:2588–2598

Glukhov E, Stark M, Burrows LL, Deber CM (2005) Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes. J Biol Chem 280:33960–33967. https://doi.org/10.1074/jbc.M507042200

Goswami M, Mangoli SH, Jawali N (2006) Involvement of reactive oxygen species in the action of ciprofloxacin against Escherichia coli. Antimicrob Agents Chemother 50:949–954. https://doi.org/10.1128/AAC.50.3.949-954.2006

Greco I, Molchanova N, Holmedal E et al (2020) Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. Sci Rep. https://doi.org/10.1038/s41598-020-69995-9

Guerra JR, Cárdenas AB, Ochoa-Zarzosa A et al (2019) The tetrameric peptide LfcinB (20–25)4 derived from bovine lactoferricin induces apoptosis in the MCF-7 breast cancer cell line. RSC Adv 9:20497–20504. https://doi.org/10.1039/c9ra04145a

Hancock REW, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24:1551–1557. https://doi.org/10.1038/nbt1267

Hao Y, Yang N, Teng D et al (2018) A review of the design and modification of lactoferricins and their derivatives. BioMetals 31:331–341. https://doi.org/10.1007/s10534-018-0086-6

Huertas N, Monroy Z, Medina R, Castañeda J (2017) Antimicrobial activity of truncated and polyvalent peptides derived from the FKCRRQWQWRMKKGLA sequence against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Molecules 22:987. https://doi.org/10.3390/molecules22060987

Inagaki K, Lucar J, Blackshear C, Hobbs C (2019) Methicillin-susceptible and methicillin-resistant staphylococcus aureus bacteremia: nationwide estimates of 30-day readmission, in-hospital mortality, length of stay, and cost in the united states | clinical infectious diseases | oxford academic. Clin Infect Dis 69:2112–2118

Insuasty Cepeda DS, Pineda Castañeda HM, Rodríguez Mayor AV et al (2019) Synthetic peptide purification via solid-phase extraction with gradient elution: a simple, economical, fast, and efficient methodology. Molecules. https://doi.org/10.3390/molecules24071215

Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal Biochem 34:595–598. https://doi.org/10.1016/0003-2697(70)90146-6

Kang JH, Lee MK, Kim KL, Hahm KS (1996) Structure-biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin. Int J Pept Protein Res 48:357–363. https://doi.org/10.1111/j.1399-3011.1996.tb00852.x

Kumar P, Kizhakkedathu JN, Straus SK (2018) Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. Biomolecules. https://doi.org/10.3390/biom8010004

Lei J, Sun LC, Huang S et al (2019) The antimicrobial peptides and their potential clinical applications. Am J Transl Res 11:3919–3931

León-Calvijo MA, Leal-Castro AL, Almanzar-Reina GA et al (2015) Antibacterial activity of synthetic peptides derived from lactoferricin against *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212. Biomed Res Int 2015:1–8. https://doi.org/10.1155/2015/453826

Li L, Vorobyov I, Allen TW (2013) The different interactions of lysine and arginine side chains with lipid membranes. J Phys Chem B 117:11906–11920. https://doi.org/10.1021/jp405418y

Liu SP, Zhou L, Lakshminarayanan R, Beuerman RW (2010) Multivalent antimicrobial peptides as therapeutics: design principles and structural diversities. Int J Peptide Res Ther 16(3):199–213

Luong HX, Kim DH, Lee BJ, Kim YW (2018) Effects of lysine-to-arginine substitution on antimicrobial activity of cationic stapled heptapeptides. Arch Pharm Res 41:1092–1097. https://doi.org/10.1007/s12272-018-1084-5

Mann B, Athira S, Sharma R, et al (2018) Bioactive peptides from whey proteins. Elsevier Inc.
Matsuzaki K (1999) Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochim. Biophys. Acta - Biomembr. 1462:1–10
Maturana P, Martinez M, Noguera ME et al (2017) Lipid selectivity in novel antimicrobial peptides: Implication on antimicrobial and hemolytic activity. Colloids Surf B Biointerfaces 153:152–159. https://doi.org/10.1016/j.colsurfb.2017.02.003
Midura-Nowaczek K, Markowska A (2014) Antimicrobial peptides and their analogs: searching for new potential therapeutics. Perspect Medicin Chem 6:73–80. https://doi.org/10.4137/PMC.S13215
Nguyen LT, Schibli DJ, Vogel HJ (2005) Structural studies and model membrane interactions of two peptides derived from bovine lactoferricin. J Pept Sci 11:379–389. https://doi.org/10.1002/psc.629
Oddo A, Hansen PR (2017) Hemolytic activity of antimicrobial peptides. Methods Mol Biol 1548:427–435
Patel S, Akhtar N (2017) Antimicrobial peptides (AMPs): the quintessential 'offense and defense' molecules are more than antimicrobials. Biomed Pharmacother 95:1276–1283. https://doi.org/10.1016/j.biopha.2017.09.042
Pfaller MA (2012) Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med 125:S3–S13. https://doi.org/10.1016/j.amjmed.2011.11.001
Quintieri L, Pistillo BR, Caputo L et al (2013) Bovine lactoferrin and lactoferricin on plasma-deposited coating against spoilage Pseudomonas spp. Innov Food Sci Emerg Technol 20:215–222. https://doi.org/10.1016/j.ifset.2013.04.013
Rivera Z, Granados G, Pinto M et al (2002) Double dimer peptide constructs are immunogenic and protective against Plasmodium falciparum in the experimental Aotus monkey model. J Pept Res 59:62–70. https://doi.org/10.1046/j.1397-002x.2001.00001_957.x
Rodríguez V, Román JT, Fierro R et al (2019) Hydrazine hydrate: a new reagent for Fmoc group removal in solid phase peptide synthesis. Tetrahedron Lett 60:48–51. https://doi.org/10.1016/j.tetlet.2018.11.054
Schibli DJ, Hwang PM, Vogel HJ (1999) The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. FEBS Lett 446:213–217. https://doi.org/10.1016/S0014-5793(99)00214-8
Scorciapino MA, Serra I, Manzo G, Rinaldi AC (2017) Antimicrobial dendrimeric peptides: structure, activity and new therapeutic applications. Int J Mol Sci. https://doi.org/10.3390/ijms18030542
Seo J, Silverman RB (2006) Synthesis of arginine-containing hydroxamate dipeptidomimetics. Tetrahedron Lett 47:4069–4073. https://doi.org/10.1016/j.tetlet.2006.03.190
Sitaram N, Chandy M, Pillai V, Nagaraj R (1992) Change of glutamic acid to lysine in a 13-residue antibacterial and hemolytic peptide results in enhanced antibacterial activity without increase in hemolytic activity. Antimicrob Agents Chemother 36(11):2468–2472
Solarte VA, Rosas JE, Rivera ZJ et al (2015) A tetrameric peptide derived from bovine lactoferricin exhibits specific cytotoxic effects against oral squamous-cell carcinoma cell lines. Biomed Res Int 2015:1–13. https://doi.org/10.1155/2015/630179
Sveinbjørnsson B, Camilio KA, Haug BE, Rekdal Ø (2017) LTX-315: a first-in-class oncolytic peptide that reprograms the tumor microenvironment. Future Med Chem 9:1339–1344. https://doi.org/10.4155/fmc-2017-0088
Tam JP, Zavala F (1989) Multiple antigen peptide. A novel approach to increase detection sensitivity of synthetic peptides in solid-phase immunoassays. J Immunol Methods 124:53–61. https://doi.org/10.1016/0022-1759(89)90185-3
Tang SS, Prodhan ZH, Biswas SK et al (2018) Antimicrobial peptides from different plant sources: isolation, characterisation, and purification. Phytochemistry 154:94–105. https://doi.org/10.1016/j.phytochem.2018.07.002
Tian X, Sun F, Zhou XR et al (2015) Role of peptide self-assembly in antimicrobial peptides. J Pept Sci 21:530–539
Tokunaga Y, Niidome T, Hatakeyama T, Aoyagi H (2001) Antibacterial activity of bactenecin 5 fragments and their interaction with phospholipid membranes. J Pept Sci 7:297–304. https://doi.org/10.1002/psc.317
Vargas Casanova Y, Rodríguez Guerra JA, Umaña Pérez YA et al (2017) Antibacterial synthetic peptides derived from bovine lactoferricin exhibit cytotoxic effect against MDA-MB-468 and MDA-MB-231 breast cancer cell lines. Molecules 22:1–11. https://doi.org/10.3390/molecules22101641
Vargas-Casanova Y, Rodríguez-Mayor AV, Cardenas KJ et al (2019) Synergistic bactericide and antibiotic effects of dimeric, tetrameric, or palindromic peptides containing the RWQWR motif against Gram-positive and Gram-negative strains. RSC Adv 9:7239–7245. https://doi.org/10.1039/C9RA00708C
Vazdar M, Heyda J, Mason PE et al (2018) Arginine "magic": guanidinium like-charge ion pairing from aqueous salts to cell penetrating peptides. Acc Chem Res 51:1455–1464. https://doi.org/10.1021/acs.accounts.8b00098
Vega SC, Martínez DA, del Chalá M, S, et al (2018) Design, synthesis and evaluation of branched RRWQWR-based peptides as antibacterial agents against clinically relevant gram-positive and gram-negative pathogens. Front Microbiol 9:329. https://doi.org/10.3389/fmicb.2018.00329
Vishnepolsky B, Zaalishvili G, Karapetian M et al (2019) De novo design and in vitro testing of antimicrobial peptides against gram-negative bacteria. Pharmaceuticals. https://doi.org/10.3390/ph12020082
Vrettos EI, Mező G, Tzakos AG (2018) On the design principles of peptide-drug conjugates for targeted drug delivery to the malignant tumor site. Beilstein J Org Chem 14:930–954. https://doi.org/10.3762/bjoc.14.80
Wang W, Arshad MI, Khurshid M et al (2018) Antibiotic resistance : a rundown of a global crisis. Infect Drug Resist 11:1645–1658
Weinstein MP (2018) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Clin Lab
World Health Organization (WHO) (2015) WHO library cataloguing-in-publication data global action plan on antimicrobial resistance
World Health Organization (WHO) (2018) WHO | High levels of antibiotic resistance found worldwide, new data shows. WHO
World Health Organization (WHO) (2019) In the face of slow progress, WHO offers a new tool and sets a target to accelerate action against antimicrobial resistance. https://www.who.int/news-room/detail/18-06-2019-in-the-face-of-slow-progress-who-offers-a-new-tool-and-sets-a-target-to-accelerate-action-against-antimicrobial-resistance. Accessed 1 Aug 2020
Xu C, Wang D, Zhang X et al (2020) Mechanisms for rapid evolution of carbapenem resistance in a clinical isolate of pseudomonas aeruginosa. Front Microbiol 11:1390. https://doi.org/10.3389/fmicb.2020.01390
Yang ST, Shin SY, Lee CW et al (2003) Selective cytotoxicity following Arg-to-Lys substitution in tritrpticin adopting a unique amphipathic turn structure. FEBS Lett 540:229–233. https://doi.org/10.1016/S0014-5793(03)00266-7
Zarzosa-Moreno D, Avalos-Gómez C, Ramírez-Texcalco LS et al (2020) Lactoferrin and its derived peptides: an alternative for combating virulence mechanisms developed by pathogens. Molecules 25:5763. https://doi.org/10.3390/molecules25245763