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HEALTH SCIENCES

Peptides DLL37-1 and LL37-1, an alternative to inhibit biofilm formation in clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Abstract: *Staphylococcus aureus* and *Staphylococcus epidermidis* have microbial surface components recognizing adhesive matrix molecules (MSCRAMM) adhesion proteins that enhance their biofilm formation ability, as well as virulence factors that influence morbidity and mortality in hospital settings. In this work, four peptides analogous of the peptide LL-37 that were evaluated to inhibit biofilm formation and its action potential on the expression of MSCRAMM proteins in clinical isolates through different tests, such as crystal violet, PCR and qPCR. In total, 96.8% of *S. aureus* were strong in biofilm formation in contrast to 48.4% of *S. epidermidis*. s*drG* and s*drF* genes were present in 100% of *S. epidermidis* strains and in all isolates. In *S. aureus,* specific genes that code for MSCRAMM proteins were detected: *clfA* (89%), *clFB, sdrC* and *fnBA* (94%). The peptides did not show hemolytic or cytotoxic activity. In this study, it was evidenced that of the peptides DLL37-1 at a 5 µM concentration was an efficacious antimicrobial agent and depicted greater biofilm inhibition in both bacterial species. Exhibiting a significant inhibition rate in *S. aureus,* this peptide caused a negative regulation in the expression of the genes *clfA* and *sdrC*, showed greater biological activity.

Key words: Biofilms, MSCRAMM, Peptides, *S. aureus, S. epidermidis*.

INTRODUCTION

Staphylococcus aureus is part of the human microbiota and a common agent in blood infections, specifically in the attachment and colonization of biotic and abiotic surfaces (Cervantes-Garcia et al. 2014, Wertheim et al. 2005). This microorganism has the capability to acquire resistance to multiple antibiotics of clinical use, and has led to the development of methicillin-resistant *S. aureus* (MRSA) (Vestergaard et al. 2019). Likewise, *S. epidermidis* can be an opportunistic pathogen that causes bacteremia in immunocompromised patients and its biofilm-forming capacity may contribute to bacterial resistance (Heilmann et al. 2019, Malachowa et al. 2016). *S. aureus* and *S. epidermidis* have a wide variety of virulence factors, mainly hemolysins, leukotoxins, enterotoxins and enzymes such as serine proteases, cysteine proteases and lipases (Betancourt et al. 2004, Osterson et al. 1999, Patti et al. 1994). These mechanisms are of importance in public health as they have a direct effect on the morbidity and mortality of hospital-acquired infections. *S aureus* and *S. epidermidis* have other virulence factors that are involved in biofilm

formation, including surface proteins that are covalently anchored to cell wall peptidoglycans, and can be classified based on structural and functional properties. The most prevalent are microbial surface components recognizing adhesive matrix molecules (MSCRAMM), and this family of proteins comprises fibrinogen-binding proteins, such as clumping factor A (*clfA*) and clumping factor B (*clfB*) (Patti et al. 1994); serine-aspartate repetitive proteins (*sdrC, sdrD, sdrE, sdrG and sdrF*); and fibrinogen/fibronectin binding proteins (FnBPA, FnBP*)* (Arora et al. 2016, Chagnot et al. 2012, Lianhua et al. 2014, Pei et al. 1999).

Additionally, the accumulation of a subpopulation of microorganisms that leads to the formation of biofilm may result in the development of an approximate 1000-fold antibiotic resistance (Del Pozo 2018), making antimicrobial therapy ineffective due to the capacity of the bacteria to remain viable in the host for a prolonged period of time. In addition, the ability of slow-growing bacteria to poorly or incompletely penetrate through polysaccharides can block directly antibiotics (Hall & Mah 2017, Lebeaux et al 2014, Sharma et al. 2019, Venkatesan et al. 2015).

Antimicrobial peptides (AMPs) are part of the host's defense system. In general, their immunomodulatory functions include reduction of the concentration of pro-inflammatory cytokines, modulation of chemokine expression, angiogenesis stimulation, leukocyte activation, and macrophages and leukocyte differentiation (Hilchie et al. 2013). AMPs may also act through direct interactions with the cell walls and membranes of bacteria without requiring specific receptors (Travkova et al. 2017). It has been also found that if the peptide sequence contains a hydrophobic fragment (i.e., nonpolar amino acids, such as A, V and other), and another hydrophilic fragment (i.e., charged or polar amino acids, such as R and K), this arrangement gives an amphipathic property and intensifies their activity and selectivity towards interaction of the phospholipids of the bacterial membrane, due to its anionic character (negative charge at a neutral pH) (Lebeaux et al. 2014).

AMPs with antibiofilm activity, such as human cathelicidin LL-37, have also been reported, as they have been shown to inhibit biofilm formation and/or dispersion processes in *Pseudomonas aeruginosa* (Overhage et al. 2008). Cathelicidin is a positively charged molecule (+6 at pH~7.4) with a high content of basic and hydrophobic amino acids (Ulaeto et al. 2016) and greater antibacterial activity when compared to defensins. Furthermore, it has a rapid bactericidal effect and antimicrobial action against clinical isolates (Lei et al. 2019); however, the optimization of molecular parameters, such as charge and/or structure, grants them a higher selectivity towards microbial cells and reduces cytotoxicity against host cells (Barrero et al. 2016, Hancock & Sahl 2006, Mishra & Wang 2012, Travkova et al. 2017).

L-amino acid-containing AMPs are susceptible to degradation by host enzymes (De la Fuente-Núñez et al. 2016); therefore, chemical modifications are necessary that allow them to overcome their limitations (Sim et al. 2019). D enantiomers depict great potential for the treatment of biofilms; specifically, modifications with D-amino acids such as D-tyrosine, D-methionine, D-tryptophan or D-leucine have been shown to result in derivatives that are able to inhibit biofilm formation in *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (De la Fuente-Núñez et al. 2016, Rabin et al. 2015). They also grant antimicrobial activity, proteolytic stability, decreased cytotoxicity to human cells, and increased immunomodulatory activity (Dean et al. 2011, Jiang et al. 2011).

This work considers four LL37 analogous peptides (LL37-1, ACLL37–1, ACLL37–2 and DLL37-1) as candidates to inhibit biofilm formation caused by *S. aureus* and *S. epidermidis* and their inhibition potential for the expression of MSCRAMM adhesion proteins was evaluated.

MATERIALS AND METHODS

Design of the peptides

The peptide LL37-1 is shorter than the peptide LL37 and was synthesized with the free amino group (NH_2) and the carboxyl group as the amide derivative (changes from having the last amino acid with COOH to the amide form [$CONH_2$]) (Table I). Seeking to enhance the biological activity, three modifications were generated from the sequence of this peptide. The first analog peptide, called ACLL37-1, was generated by the elimination of G (Glycine) and acetylation of the N-terminal group; and the second analog was named ACLL37-2 because the terminal amino group of the entire LL37-1 sequence was acetylated. Both peptides were synthesized with C-terminal amidation. Finally, for the third analog, the peptide called DLL37-1, the amino group was acetylated and the phenylalanine amino acid 21 (F) was changed from the L to D stereochemical configuration.

Synthesis and evaluation of the purity of the peptides

All peptides were synthesized on a solid matrix using the protocol previously described by Merrifield (1964), using one of the two peptide synthesis strategies, 9-fluoromethoxycarbonyl/tert-butyl (Fmoc/Tbu), in which protection of the amino group of the amino acid is used during synthesis with the Fmoc group and protection of the side chains of amino acids that can react with the Tbu group. The deprotection and coupling steps were monitored by the ninhydrin test. At the end of synthesis, the protecting groups of the side chains were removed and the peptide was released from the resin (Guzman et al. 2007). Using high performance liquid chromatography (HPLC) and mass spectrometry, the purity of the peptides and their molecular mass were determined. For the chromatography analysis, a Column Alltima™ C-18, 4.6 x 250 mm, was used, with the following conditions: pump A: 0.065% trifluoroacetic in 100% water (v/v), and pump B:

Table I. Sequences of peptides analogous to LL-37.

Name	Sequence
LL37 original	L G D F R K S K E K I G K E F K R I V Q R I K D F L R N L V P R T E S
LL37-1	**G** R K S **A K** I G K **R A** K R I V Q R I K D F L R Free Amino (C-terminal) Amidation
ACLL37-1	-R K S K E K I G K E F K R I V Q R I K D F L R **(N-terminal) Acetylation** (C-terminal) Amidation
ACLL37-2	**G** R K S **A K** I G K **R A** K R I V Q R I K D F L R **(N-terminal) Acetylation** (C-terminal) Amidation
DLL37-1	**(d-PHE) G** R K S **A K** I G K **R A** K R I V Q R I K D **(d-F)**L R **(N-terminal) Acetylation** (C-terminal) Amidation

Underlined: conserved native LL37 sequences. Modified amino acids.

0.05% trifluoroacetic in 100% acetonitrile (v/v); total flow: 1 ml/min; wavelength: 220 nm. The peptides were purified to a purity ≥ 95%. The molecular weight of the purified peptides was determined by mass spectrometry at the main peak fraction of the chromatogram using an Waters Alliance 2695 chromatography system equipped with Waters 995 photodiode array detector and ESI-MS Waters Micromass Detector ZQ (Waters, USA). The spectrum collection conditions were: nebulization gas flow, 1.5 L/min; temperature CDL, 250 °C; block temperature, 200 °C; interface bias (Bias interface), + 4.5 kV; drying gas flow, 5 L/min; T flow, 0.2 mL/min; and concentration of B, 50% water/50% MeOH.

The secondary structure assessment

The preferential secondary structure of the peptides was established by circular dichroism (CD) in a JASCO-J-810 (Jacso Incorporated, USA) spectropolarimeter at a final concentration of 200 µM in 30% trifluoroethanol (TFE) (Greenfield 2006).

Clinical isolates

Sixty-two clinical isolates of different samples and hospitals of the city of Bogota, Colombia were analysed. The purity of the isolates was verified in agar chromogenic BBL™ CHROMagar™ (Becton, Dickinson). They were genotypically characterized by Polymerase Chain Reaction (PCR), amplifying the *16s rADN* for *S. epidermidis* and the gene *nuc* for *S. aureus* (Brakstad et al. 1992, Zakrzewska-Czerwińska et al. 1992). Antimicrobial susceptibility was determined by the method of minimal inhibitory concentration (MIC), following the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2020). The antibiotics tested were oxacillin, gentamicin, vancomycin, chloramphenicol, ciprofloxacin, erythromycin, rifampicin, trimethoprim/sulfamethoxazole, tetracycline, clindamycin and linezolid.

Analysis of biofilm formation by Congo red and crystal violet methods

Phenotypic determination of biofilm production was performed using the Congo red agar (CRA) method described by Freeman et al. (1989). The strains of *S. epidermidis* ATCC 35984 and *S. aureus* USA300 were used as positive controls and *S. epidermidis* ATCC 12228, a non-biofilm former, as a negative control. Biofilm formation was assessed in polystyrene microtiter plates using the crystal violet technique described by Christensen et al. (1985). Briefly, 198 µL of brain heart infusion broth (BHI) with 1% glucose or BHI broth with 2% NaCl was inoculated with 2 µL of the inoculum at 0.5 McFarland scale, and subsequently incubated for 24 hours at 37 °C. The absorbance of the plates was read in a MicroElisa reader (Infinite 200 Pro, Tecan, Switzerland) at a wavelength of 492 nm and 570 nm. All tests were carried out in triplicate. To perform the classification, the cut-off point was calculated, and for this purpose, the standard deviation was multiplied by 3 and the value of the mean optical density of the BHI + glucose or BHI + NaCl was added at the same wavelength. The isolates were classified into three categories: non-adherent (NA), in which the optical density was equal to or less than the cut-off point; weakly adherent (WA), in which the optical density was greater than the cut-off point or equal to or less than twice that amount; and strongly adherent (SA), in which the optical density was greater than twice the cut-off point.

Determination of adhesion factors associated with the formation of biofilm by conventional PCR

Specific primers directed against the genes of interest were designed (Table II). The bacterial

Table II. Sequences of primers targeting the adhesion proteins ClfA, ClfB, FnbA, SdrG, SdrC, SdrF.

Name	Sequence	Length	Tm	%GC	Size pb	Gene
clfA-F	TGCTGCACCTAAAACAGACG	20	60.05	50.00	155	*clfA*
clfA-R	CAGTTACCGGCGTTTCTTCC	20	61.89	55.00		
clfB-F	AGCATTTACTACCGGTTCAGCA	22	60.03	45.45	96	*clfB*
clfB-R	CCACAAACGATTTCCAATGCGC	22	61.79	50.00		
sdrC-F	CCGTTTCTACTGGCAATGCT	20	60.27	50.00	148	*sdrC*
sdrC-R	GTTACATTGCCAACGCCTTT	20	60.00	45.00		
fnbA-F	TACCGCTATTTTGGCCACCT	20	61.71	50.00	180	*fnbA*
fnbA-R	AGCATGGTCAAGCACAAGGA	20	61.71	50.00		
sdrG-F	TCGCCATCAACTAACGCTCAA	21	60.34	47.62	188 pb	*sdrG*
sdrG-R	CCCTGTTCAGCAAGTTCGTC	20	59.13	55.00		
sdrF-F	GGACGATTACACGACGATACAGC	23	62.97	52.17	137 pb	*sdrF*
sdrF- R	CAAGTCACCTTGTCCTTGACCT	23	64.01	52.17		

DNA extraction was performed following the manufacturer's instructions (Wizard® Genomic DNA Purification Kit Technical Manual, Promega, USA). The genes *fnbA*, *clfA* and *clfB* of *S. aureus* were amplified by a single multiplex PCR reaction and *sdrG* and *sdrF* in another reaction. A conventional PCR was executed to detect the gene *sdrC* of *S. aureus*. DNA from ATCC bacteria strains was used as positive controls and negative controls in the reactions, USA300 was the positive control of amplification of *fnbA*, *clfA*, *sdrC* and *clfB* and negative control of *sdrG* and *sdrF*, the strains ATCC 35984 and ATCC 12228 were the positive control for genes *sdrG* and *sdrF* and were also used as a negative control for the specific genes of *S. aureus.*

Analysis of the hemolytic activity of the peptides

Human red blood cells (group O Rh +) at 3% were suspended in a solution containing 0.9% saline (Baxter Healthcare, USA) and five dilutions of peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1 were analyzed at a final concentration of 10, 7.5, 5, 2.5 and 1.25 μM in triplicate, and incubated in microtiter plates (Greiner Bio-one background U, USA) for 72 hours at 37 °C. Red blood cells without peptide were used as a negative control and erythrocytes were lysed with 20% Triton as a positive control. After incubation, microtiter plates were centrifuged at 3500 rpm for 10 minutes, and the absorbance of the microtiter plate reader was 540 nm in an ELISA plate reader (Multiskan EX, Thermo Scientific, USA). Subsequently, the percentage of hemolysis was calculated:

% hemolysis: ([absorbance exposed cells - absorbance negative control/absorbance positive control - absorbance negative control] x 100).

Cytotoxic effect of the peptides

L929 fibroblasts were cultured in DMEM medium (Life Technologies Corporation, USA) at 37 °C, after which the culture was exposed to an atmosphere of 5% CO_2 until reaching a confluence of 80–90%. In 96-well plates, 21,000 cells/per well were seeded in DMEM medium

and incubated for 24 hours at 37 °C. The culture medium was extracted and each of the peptides (LL37-1, ACLL37-1, ACLL37-2 and DLL37-1) were added at a concentration of 1.25, 2.5, 5 and 10 µM, in a final volume of 100 µL per well.

The plates were incubated for 72 hours at 37 °C, and subsequently, the reduction of tetrazolium (XTT) (EZcount™ XTT Cell Assay Kit, HiMedia Laboratories, India) reagent was added. The plates were incubated again for two to four hours, shaken for 15 seconds and read in an ELISA Reader using 450 nm as primary filter and 620 nm as differential filter. The experimental values were normalized with the results of the negative control red blood cells without peptide (0% hemolysis) and 20% Triton X-100 (100% hemolysis). The percentage of cytotoxicity was calculated:

% cytotoxicity = ([absorbance untreated cells - absorbance treated cells/absorbance treated cells] x 100).

Assessment of the effect of analogous peptides on bacterial growth and biofilm formation

The impact of the peptides on the bacterial growth curves was tested on 37 of the isolates. For this purpose, the strains were grown in triplicate in LB broth, at 0.5 McFarland scale, in the presence of the peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1 at a final concentration of 5 µM. Optical density readings were made at 620 nm every hour for 24 hours with continuous stirring at 37 °C in the Bioscreen C® equipment (Oy Growth Curves Ab Ltd, Finland).

Seventeen *S. epidermidis* isolates (14 biofilm formers, two non-biofilm formers, and one strain sensitive to all antibiotics) and 19 biofilm-forming *S. aureus* isolates with resistance to at least one antibiotic were analyzed. The strains were cultured in 96-well plates in BHI broth with 1% glucose, following the protocol described for biofilm evaluation. Incubation times of 6, 12 and 24 hours were evaluated at 37 °C in the presence of the peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1 at a final concentration of 5 µM. The absorbance of the plates was read at a wavelength of 492 nm and 570 nm in the MicroELISA reader and the percentage of biofilm inhibition exerted by the peptide was calculated (Costa et al. 2018):

Percentage inhibition = (100 - [Abs nm experimental well with the peptide/Abs nm control well without the peptide] x 100).

Assay of the effect of analogous peptides on the expression of MSCRAMM adhesion proteins

Two analogous peptides, LL37-1 and DLL37-1, were selected since they showed the best results in the hemolysis and cytotoxicity assays. These peptides were challenged at a concentration of 5 µM in a 96-well plate assay of the bacterial culture of two strains of *S. aureus* and *S. epidermidis* in LB broth after an incubation period of 8, 10 and 12 hours at 37 °C. For this task, 1.5 mL of the bacterial culture was centrifuged at 10000 rpm for five minutes at a temperature of 4 ºC, the supernatant was removed and the pellet was suspended with lysozyme (2.5 mg/mL), lysostaphin (0.2 mg/mL), buffer lysis and proteinase K (25 U/mL), followed by incubation at 37 ºC for four hours. Subsequently, the RNA extraction protocol was followed by the Trizol® Max™ (Ambion Life Technologies, USA) and Chomczynski method (Chomczynski & Sacchi 2006). The extracted RNA was quantified in a Nanodrop 2000 (Technoscientific, USA) and 10^2–10^{10} copies were taken as an RNA template, and the recommendations of the ImProm-II ™ Reverse Transcription System kit (Promega, USA) were followed for cDNA synthesis. Finally, cDNA was amplified using the GoTaq® qPCR Master Mix Kit (Promega, USA) and different specific primers (Table II) targeting the genes of the adhesion

proteins *clfA, fnbA, sdrC, sdrG* and *sdrF*. qPCR was performed using the BioRad CFX96 thermal cycler Touch™ Real Time PCR System (Bio-Rad Laboratories, USA) and the USA300, ATCC12228, and ATCC 35984 were used as controls. Ct (cycle threshold) data were normalized to the expression of the reference genes *rrn* and *gyrB* and analyzed by the method $2^{-\Delta\Delta}$Ct (Pfaffl 2001).

Statistical analysis

All statistical analyses were conducted using SPSS version 19.0 (Ibm, USA) and GraphPad Prism 8 (GraphPad Software Inc, USA). The p-values < 0.05 were considered statistically significant. The Kendall's Tau-b correlation coefficient, chi-square test, Fisher's exact test and comparison test (Bonferroni, one-way ANOVA, two-way ANOVA) were used.

RESULTS

Designed peptides are of high purity

The four peptides under study, LL37-1, ACLL37–1, ACLL37–2 and DLL37-1, each had a single chromatographic peak on HPLC, indicating they are high purity products. Electrospray spectrometry analysis confirmed that the molecular mass corresponded to the expected species and revalidated the purity of the product (data not shown).

Secondary structure of analogous peptides that may have antibiofilm activity

The CD applied to the peptides LL37-1, ACLL37–1, ACLL37–2 and DLL37-1 was used to evaluate the preferential secondary structure that each one of them adopts in solution. The CD spectra of the four peptides showed maximum absorption in the spectrum at 193 nm, minimum absorption at 208 nm and 222 nm, which are the characteristic peaks when the peptide presents as an alpha helix structure (Fig. 1) (Lau et al. 1984). To corroborate the structure found experimentally, secondary structure prediction was also made with the sequence of the peptides using bioinformatics programs, such as the PSSpred software, confirming that the sequences predicted had alpha helix structures. This secondary structure has been related to the antimicrobial and antibiofilm activity of peptides.

The peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1 did not demonstrate hemolytic or cytotoxic activity

The analogous peptides, in 1.5, 2.5 and 5 µM concentrations, presented a hemolysis of less than 5% at 72 hours of incubation, while at a 10 µM concentration, they could induce 15–17% hemolysis. All peptides at the 10 µM concentration showed a statistical difference with respect to their counterpart in different concentrations and the other peptides. No differences were observed in hemolysis between peptides at concentrations of 7.5, 5 and 1.5 µM (Fig. 2). Additionally, the LL37-1 and DLL37-1 peptides at 1.5 µM to 5 µM concentrations displayed a tendency to low cytotoxicity (less than 25%) even after 72 hours of incubation; peptides ACLL37-1 and ACLL37-2 exhibited a tendency to higher cytotoxicity, however no statistical differences were found in any of the comparisons (Fig. 2).

Clinical isolates belong to the genus *Staphylococcus* spp. and show antimicrobial resistance

In this study, 62 clinical isolates from blood cultures (45.1%), 27 from secretions (43.5%) and seven samples of unknown origin (11.4%) were evaluated. A total of 31 were characterized as *S. epidermidis,* 31 were *S. aureus,* and the purity of all the isolates was verified. Eleven antimicrobials were tested and this susceptibility analysis

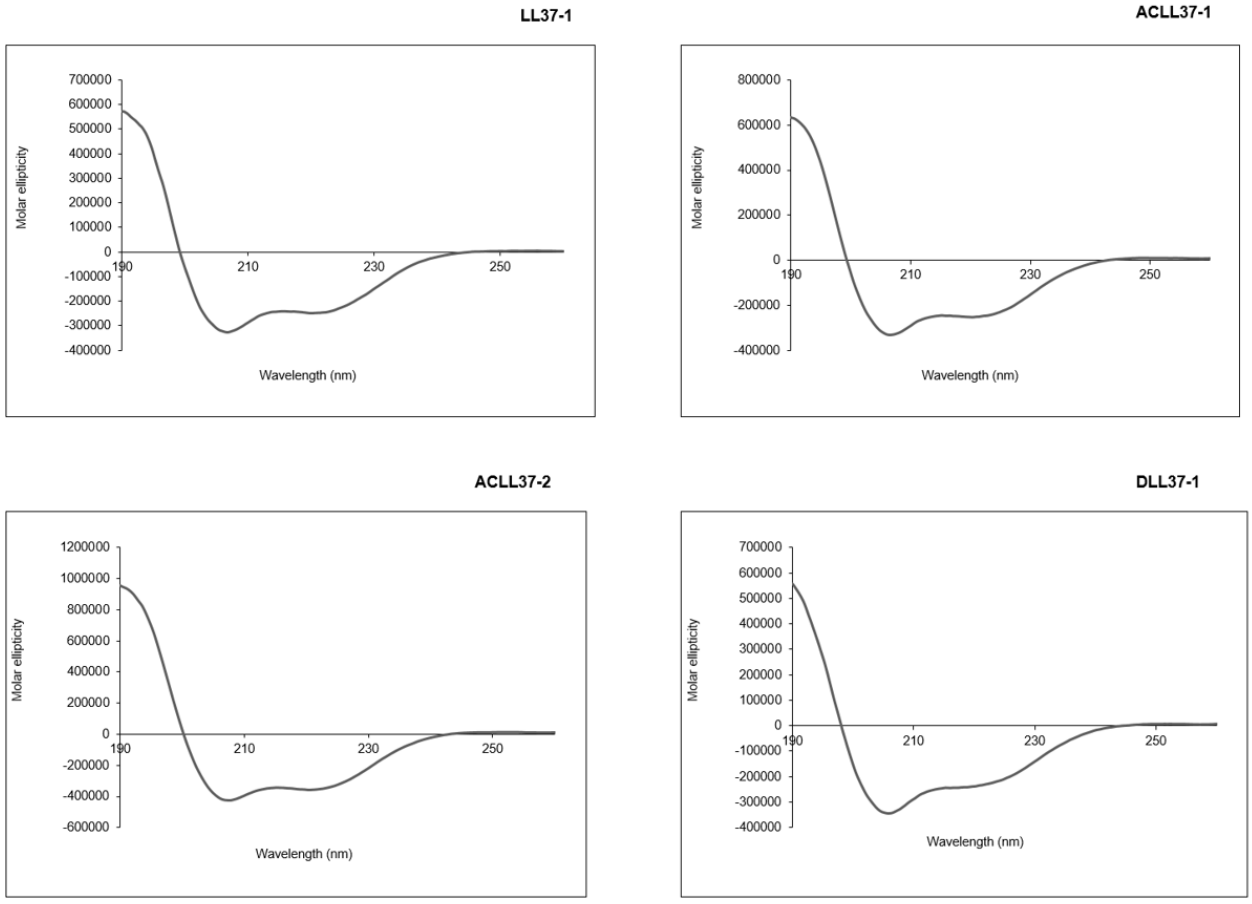

Figure 1. Preferential structure of the analogous peptides. Circular dichroism in 30% TFE at room temperature of the peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1.

evidenced that four (6.5%) isolates were not resistant to any antibiotic and 58 (93.5%) were resistant to at least one antibiotic. For *S. aureus*, the resistance to oxacillin was 100%, and for *S. epidermidis*, resistance was 64.5%.

S. aureus and *S. epidermidis* strains from clinical isolates have the capacity to form biofilm

The CRA test showed that 67.8% of *S. aureus* isolates and 35.5% of *S. epidermidis* are biofilm formers, with a significant difference observed in these proportions (p = 0.011) (Fig. 3a). Therefore, *S. aureus* strains have a greater capacity to produce biofilm compared to *S. epidermidis*. The isolates were classified according to the Christensen method (Christensen et al. 1985), evidencing that 1% glucose stimulates biofilm formation (Fig. 3b), according to the correlation coefficient of Kendall's Tau-b of 0.52 ($p \leq 0.001$). In this study, 96.8% of *S. aureus* isolates were strong biofilm formers and 48.4% of *S. epidermidis* would form film as well, if BHI + 1% glucose were found (Fig. 3c–d).

Genes coding for MSCRAMM adhesion proteins are present in microbiological isolates

A total of 100% of the *S. epidermidis* isolates had *sdrG* and *sdrF* genes and all the *S. aureus* isolates displayed specific MSCRAMM, such as: *clfA* (89%), *clFB*, *sdrC* and *fnBA* (94%) in several *S. epidermidis* isolates. These genes were also amplified (29–35%) (Fig. 4).

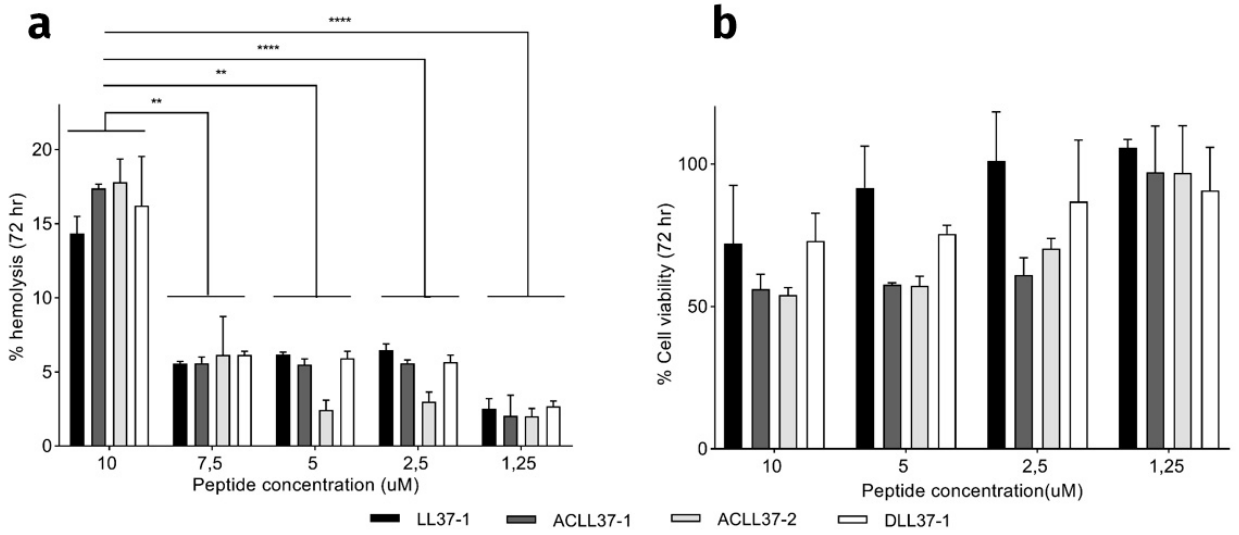

Figure 2. Hemolytic and cytotoxic effects of the analogous peptides. (a) Percentage of hemolysis at 72 hours of incubation of the peptides at concentrations of 1.5–10 µM. (b) Percentage of cell viability of L929 cells after 72 hours of incubation with the peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1, at four different concentrations. The data shown corresponds to the mean and standard error of the mean of tests carried out in triplicate. Data analyzed by Two-way ANOVA test, Tukey's post test, ** p < 0.01, **** p < 0.001.

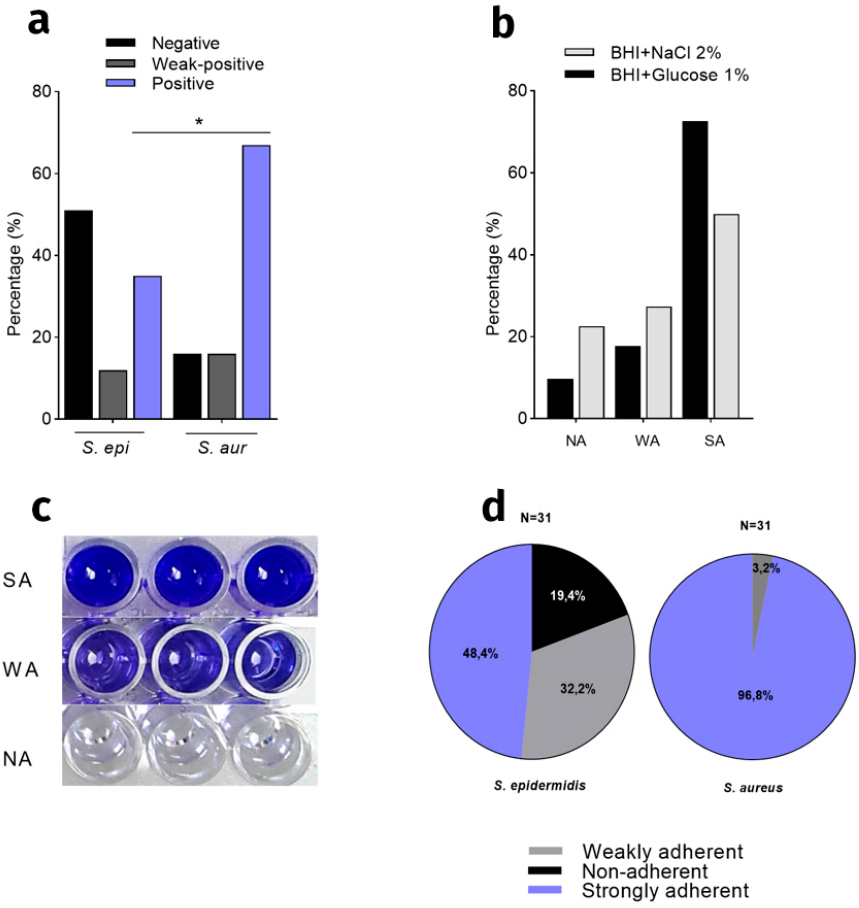

Figure 3. Biofilm formation ability assessment of the clinical isolates. (a) Distribution of the results of the Congo red agar (CRA) test, data analyzed by the Chi-square test (X2), *p = 0.011 (b) Determination of biofilm formation per microplate in the presence of brain heart infusion (BHI) + 1% glucose. NA: non-adherent. WA: weakly adherent. SA: strongly adherent. (c) Representative figure formation of biofilm crystal violet method. (d) Classification of biofilm production according to the Christensen method for isolated *Staphylococcus* spp.

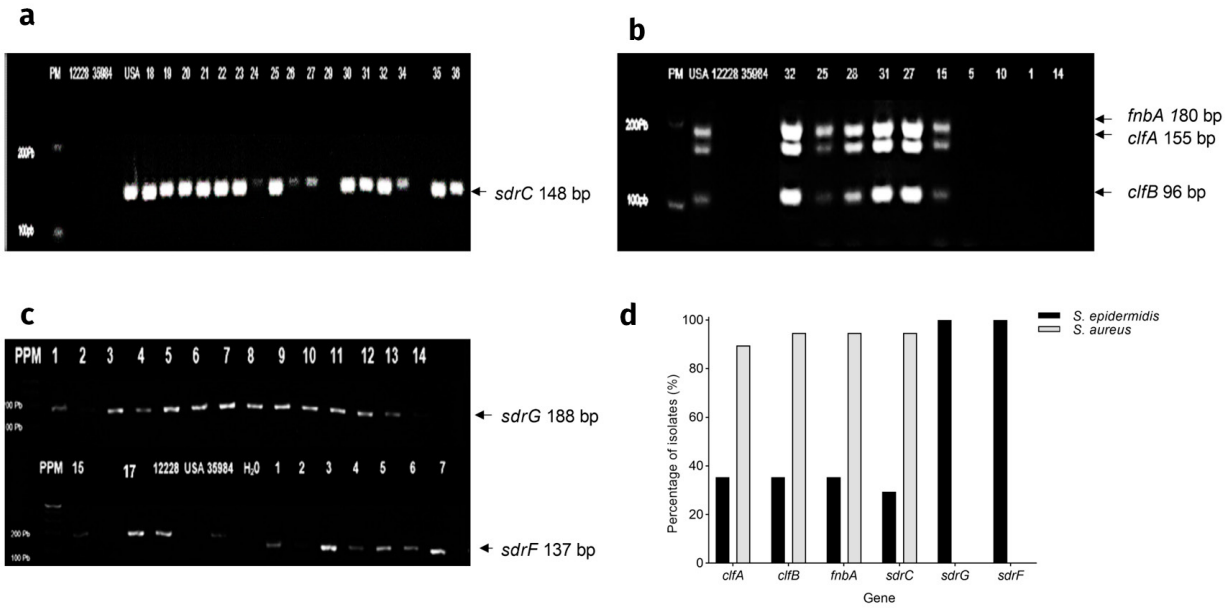

Figure 4. Detection of the genes encoding the MSCRAMM adhesion proteins. (a) *sdrC* gene amplification from *S. aureus* (b) Multiplex PCR, *fnbA* 180 bp, c*lfA* 155 bp and c*lfB* 96 bp amplified in *S. aureus* strains (c) Amplicons of the 188 bp *sdrG* and 137 bp *sdrF* genes in clinical isolates of *S. epidermidis,* USA300 positive control of *S. aureus*, ATCC 35984 positive control of *S. epidermidis*. ATCC 12228 non-producing negative control biofilm. Molecular Weight (MW): 100bp. (d) Percentage of isolates positive for the s*drG, sdrF, sdrC*, *fnbA, slfA* and c*lfB* genes by species.

The peptides LL37-1, ACLL37-1, ACLL37-2 and DLL37-1 affect bacterial growth

In a previous analysis through the cell continuous flow cabinet methodology, it was observed that the peptide LL37-1 has the ability to eradicate 100% of the aggregation of live bacterial cells from preformed biofilm in the ATCC 35984 strain at 24 hours of treatment in a concentration of 5 µM. The peptide at a lower concentration did not trigger the dispersal of biofilm cells (data in process of publication). Considering this result, a decision was made to carry out the next experiments with this concentration of the peptides.

The growth curves displayed statistical differences ($p < 0.0001$) when compared with their equivalent subjected to treatment with peptide at a 5 µM concentration (two-way ANOVA with Sidak posthoc test). It was proven these analog peptides inhibited bacterial growth at 24 hours of incubation, with the exception of a clinical isolate corresponding to *S. epidermidis,* with the ACLL37-1 peptide at 5 µM (Fig. 5). The results present a prolongation of the latency phase (*lac*) for the peptide LL37-1, with *S. aureus* that lasts approximately 10 hours. With ACLL37-1 and ACLL37-2, there were no differences observed with respect to the control. DLL37-1 lengthened the adaptation phase to almost 10 hours in the clinical isolation of *S. epidermidis* (Fig. 5).

Analogous peptides showed antibiofilm activity

The percentage differences in biofilm inhibition with significant differences ($p < 0.001$; Bonferroni test) in the optical density (OD) of strains with and without treatment with a 5 µM peptide concentration were analyzed. The bacterial species evaluation depicted a high percentage of biofilm inhibition, especially at 24 hours (Fig. 6), indicating this activity could be incubation time-dependent. DLL37-1 displayed a consistent effect in both bacterial species (inhibition

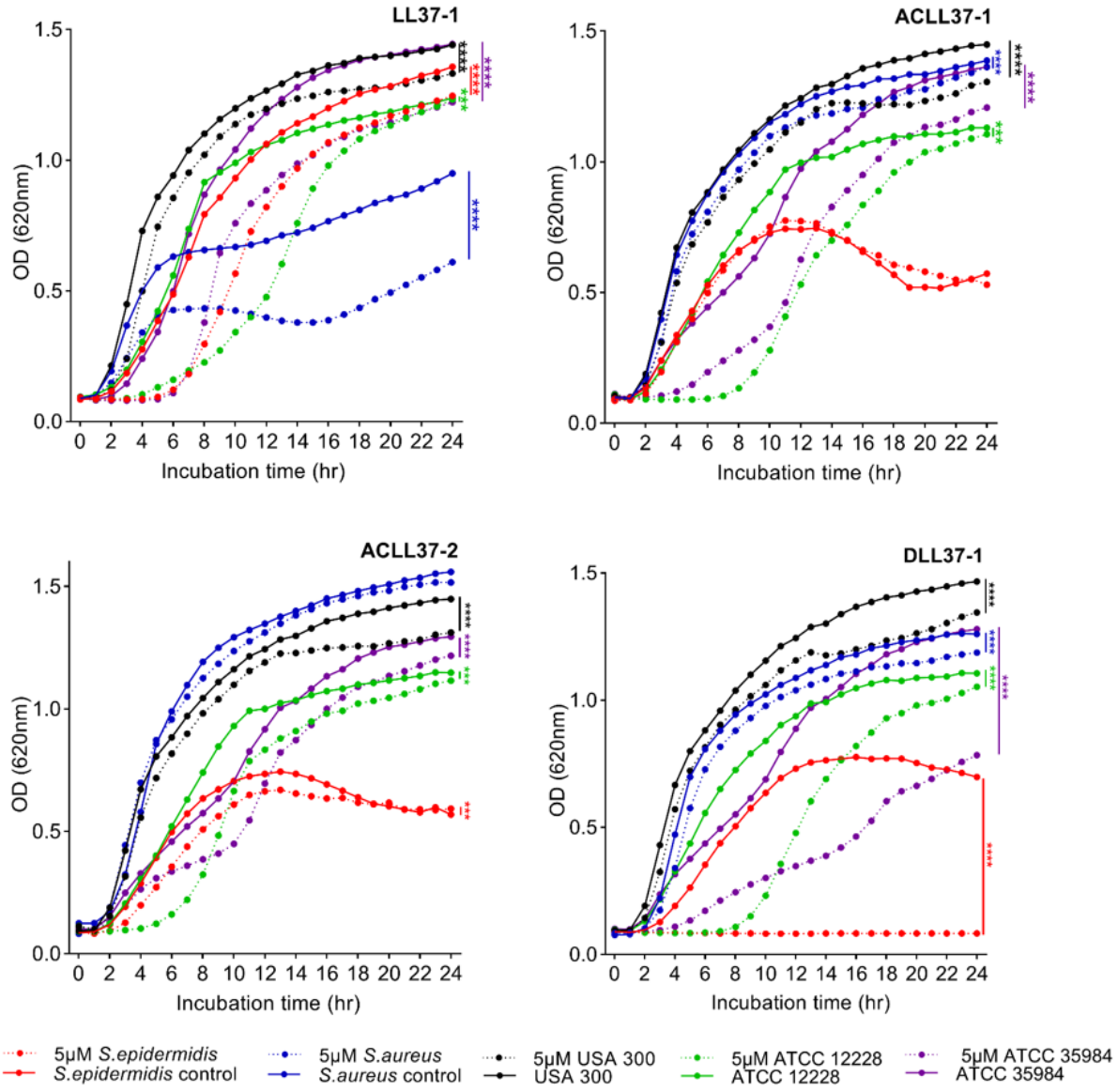

Figure 5. Effect of synthetic peptides at a concentration of 5 μM into growth kinetics of clinical isolates of *S. aureus* and *S. epidermidis* and of the controls, without treatment. The data shown correspond to the mean OD of tests performed in triplicate of measurements performed every hour for 24 hours. Data analyzed by Two-way ANOVA test, Tukey's post test, ** p < 0.01, ** p = 0.002, **** p < 0.001.

percentage of 52–58%) and LL37-1 presented a significant inhibition rate in *S. aureus* (52.9%).

Peptides affect the expression of the MSCRAMM adhesion proteins in *S. aureus* and *S. epidermidis*

Fig. 7 shows the mRNA expression level coding genes for the adhesion proteins *clfA*, *fnbA*, and *sdrC* of *S. aureus* in a clinical isolate and in the control strain USA300, which was previously subjected to treatment with the peptides LL37-1 and DLL37-1 (at 5 μM). LL37-1 decreases the expression of the *clfA* gene at 10 and 12 hours of incubation in the USA300 strain. In clinical isolation, it is notorious that after 12 hours of treatment, gene expression is stimulated. DLL37-1 did not cause alterations in the protein expression of the strain from the hospital

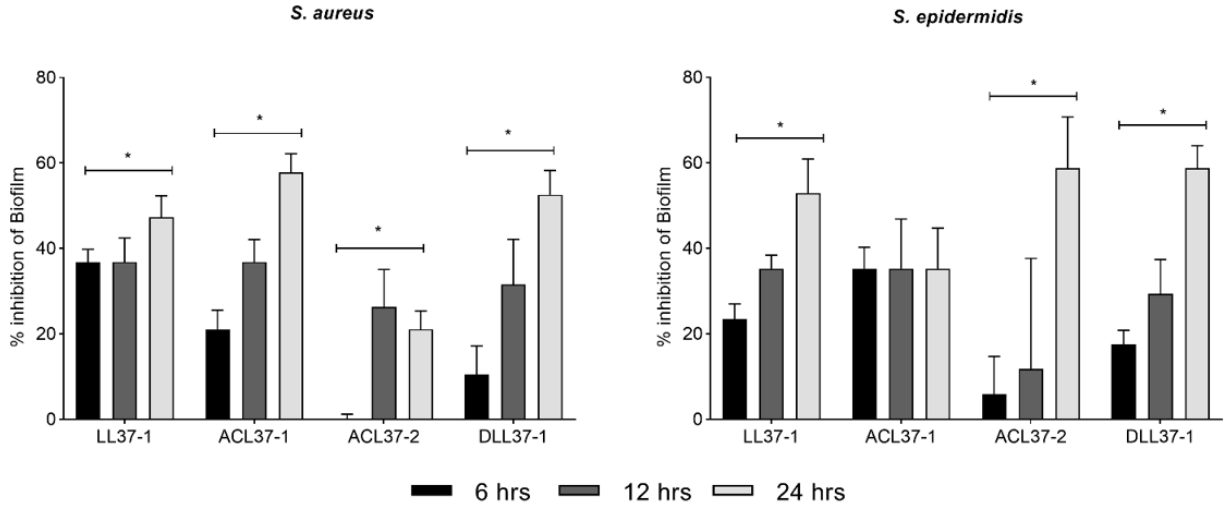

Figure 6. Biofilm inhibition of analogous peptides at concentration of 5 µM in clinical isolates of *S. aureus* and *S. epidermidis* The data shown correspond to the mean of the % inhibition of the clinical isolates by species carried out in triplicate. The inhibition percentage was calculated with respect to the absorbance obtained from the isolation without peptide. Data analyzed by Two-way ANOVA test, Tukey's post test, * p < 0.05.

environment, but it negatively regulated its expression at 12 hours in the USA300 strain. In relation to the *fnbA* gene, the peptides stimulated expression at 10 and 12 hours of incubation in clinical isolation. For the s*drC* gene, its expression is increased after treatment with LL37-1 at eight hours for the control strain, and at 10 and 12 hours for the *S. aureus* isolate; however, with DLL37-1, the opposite effect was observed at eight hours for the USA300 strain.

The expression levels of the *sdrG* and *sdrF* genes of *S. epidermidis* (Fig. 8) indicated that LL37-1 positively regulates the expression of *sdrG* in the two ATCC strains at eight hours of exposure, while in clinical isolation, there is negative regulation. The DLL37-1 peptide was found to increase the expression of the *sdrG* gene at 12 hours of treatment only in the non-biofilm-forming strain ATCC 12228, but the clinical isolation of *S. epidermidis* had gene expression inhibition at 8, 10 and 12 hours of treatment. The LL37-1 peptide was shown to affect the expression of the *sdrF* gene at 10 hours (ATCC 12228) and 12 hours of incubation (in all strains). With DLL37-1, there was a decrease in the expression of this gene in clinical isolation, mainly at eight and 10 hours.

DISCUSSION

The increasing number of nosocomial infections associated with the appearance of conventional antibiotic-resistant microorganisms is one of the main concerns of clinical institutions (Galdiero et al. 2019). In this study, it was found that 93.5% of clinical isolates presented resistance to at least one antibiotic. A total of 96.8% of *S. aureus* and 48.4% of *S. epidermidis* were biofilm formers. This finding is highly relevant, as these microorganisms are among the most associated with medical device-related infections (Arciola et al. 2006, Evans & Bold 2019, Haddad et al. 2018).

Additionally, *S. aureus* and *S. epidermidis* have adhesins associated to the building and maturation of biofilm, known as MSCRAMM (Speziale et al. 2014); thus, *clfA* was found in 89.47% of isolates and *clfB*, *fnbA* and *sdrC* were found in 94.73% of isolates in *S. aureus* samples. These results match those reported by Wang et

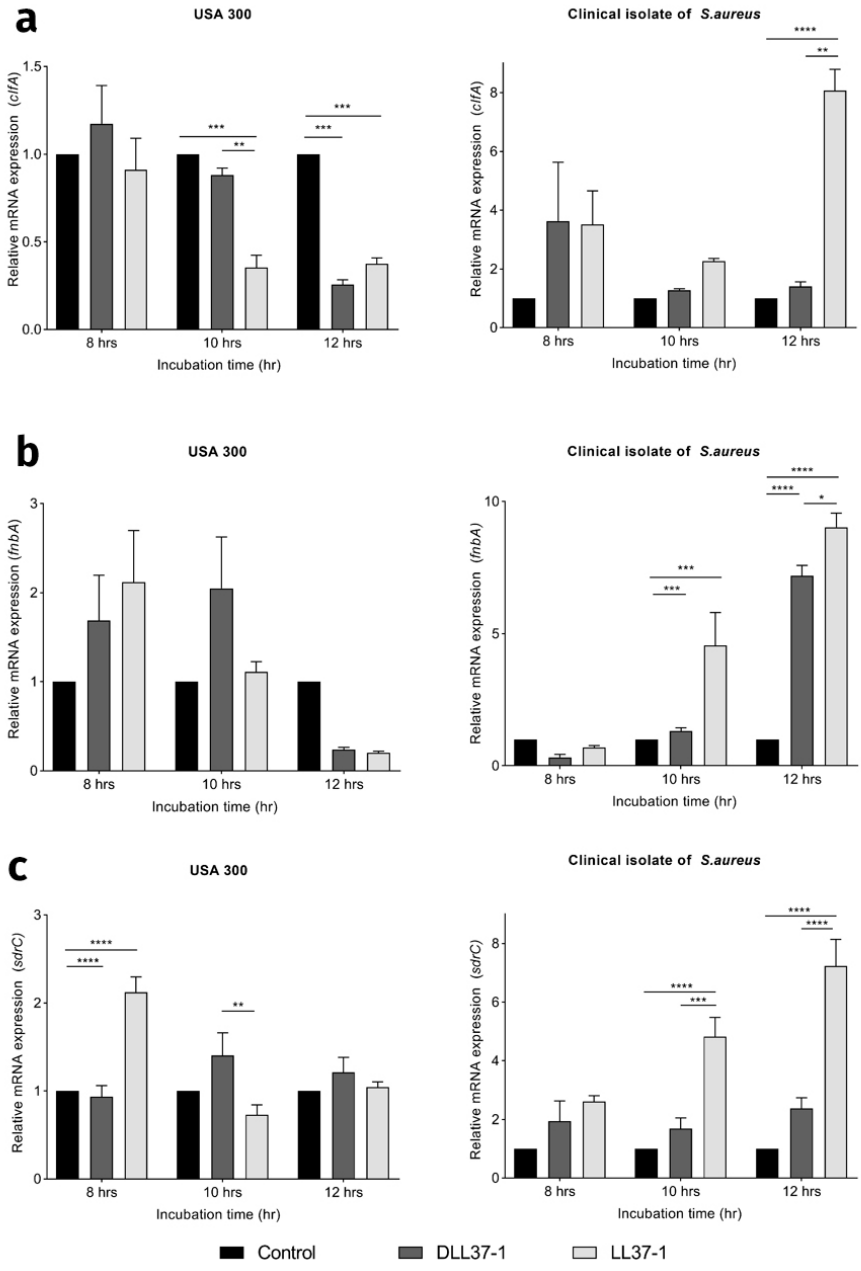

Figure 7. Relative expression of mRNA from genes (a) *clfA*, (b) *fnbA* and (c) *sdrC* of a clinical isolate of *S. aureus* and of the control strain USA300. The results are presented as mRNA expression of the bacterial strains treated with the LL37-1 and DLL37-1 peptides at concentration of 5 µM, in relation to the untreated bacterial strains. Relative levels of gene expression were calculated in relation to the bacterial reference gene *gyrB* of *S. aureus*, using the threshold cycle method (Ct). Two-way ANOVA test, with Tukey's multiple comparison post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are presented as the mean of each group ± SEM and are representative of at least three independent experiments.

al. (2019). In the case of *S. epidermidis*, it was determined that the genes *clfA, clfB, and fnbA* were in 35.29% and *sdrC* were found in 29.41% of isolates; this finding could indicate horizontal transfer of said genes between the species studied, and *S. epidermidis*, being an important genetic reservoir (Büttner et al. 2015, Méric et al. 2015), or due to the high homology amongst the identified genes. The *sdrG* and *sdrF* genes were detected in 100% of the isolates studied.

Cardile et al. (2014) found that exposing *S. aureus* to human plasma *in vitro* improves MSCRAMM expression and biofilm formation, confirming the relevant role of these proteins *in vivo* biofilm infections, as they favor anchorage to the cell wall and are involved in host colonization and invasion (De la Fuente-Núñez et al. 2016).

On the other hand, antimicrobial peptides have emerged as an alternative therapy in

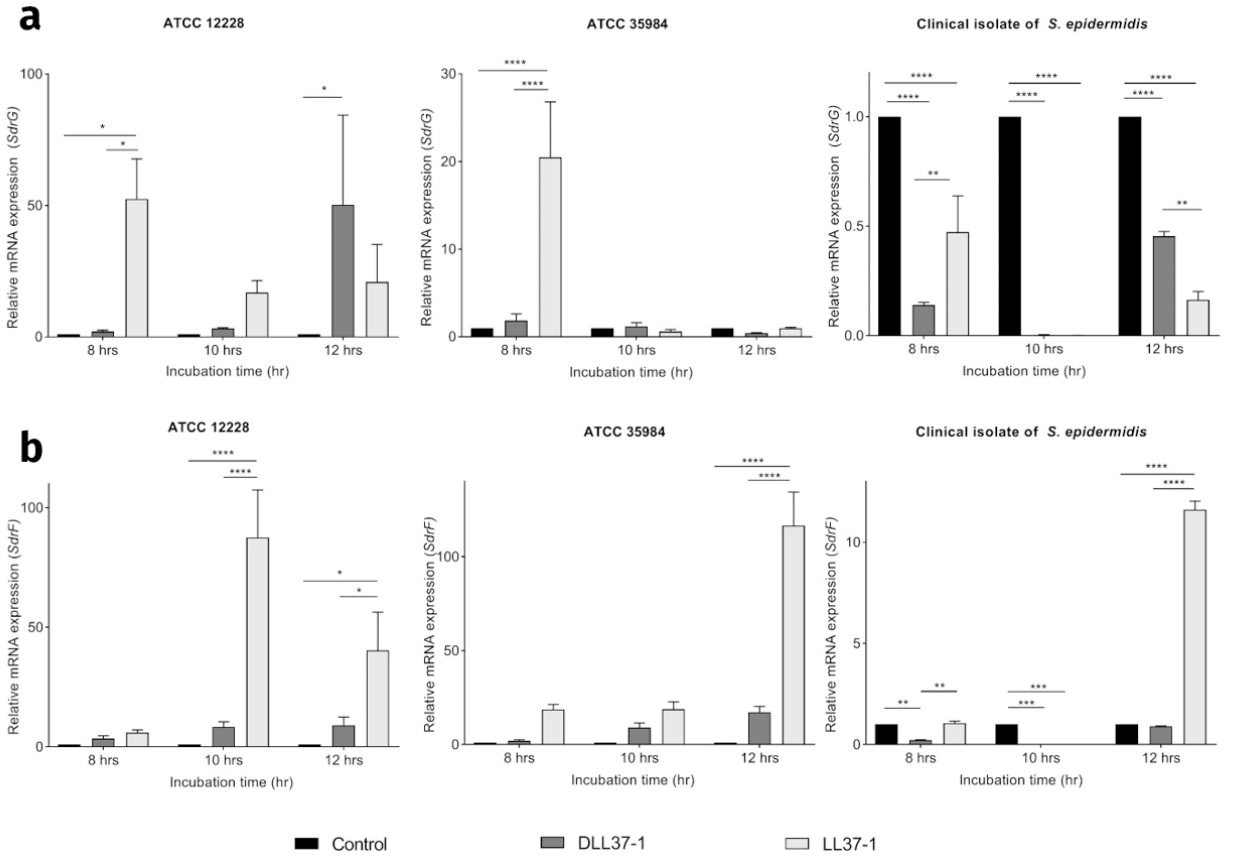

Figure 8. Level of expression of mRNA of the genes target (a) *sdrG* and (b) *sdrF* of a clinical isolate of *S. epidermidis* and the controls ATCC 12228 and ATCC 35984. The results are presented as mRNA expression of the bacterial strains treated with the LL37-1 and DLL37-1 peptides at concentration of 5 μM, in relation to the untreated bacterial strains. Relative levels of gene expression were calculated by reference to the bacterial *rrn* gene of S. epidermidis by the threshold cycle method (Ct). Two-way ANOVA test, Tukey's multiple comparison post test. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Data are presented as the mean of each group ± SEM and are representative of at least three independent experiments.

response to bacterial resistance, given their ability to inhibit growth and biofilm formation, even in drug-resistant microorganisms (Paharik & Horswill 2016). In this study, four peptides with helical structures were designed, which has been reported to favor biofilm inhibition (Klubthawee et al. 2020). Regarding the potential cytotoxic effect, peptides LL37-1 and DLL37-1 displayed the lowest toxicity against red blood cells and L929 cells at concentrations up to 5 μM.

It has been suggested that differences in the sequence and position of amino acids are important for the antimicrobial activity of the peptides (Klubthawee et al. 2020). In fact, in this research, it was found that peptides LL37-1, ACLL37-2 y DLL37-1 induced changes in the kinetic growth of strains by extending the latency or adaptation phase, which is where the microorganism adapts to a new environment (Yates & Smotzer 2007), and then initiates an increase in gene expression of relevant genes. A change in adaptation may be directly related to a decrease in the adherence ability of the bacterial population, reducing its virulence capacity (Lee et al. 2014). In some of the analyzed strains (mainly ATCC 12228), a recovery in growth rate was observed after 24 hours of incubation. This phenomenon can be explained as a collective

tolerance mechanism previously described in bacteria subjected to treatment with LL37, a mechanism by which the peptide eliminates a subpopulation of bacteria and absorbs it, decreasing its availability in the environment. As a result, they protect the remaining viable strains from death by peptide (Wu & Tan 2019).

The peptides with the highest biofilm inhibitory activity were LL37-1 and DLL37-1, with inhibition percentages of 58% and 57% in *S. epidermidis* and *S. aureus,* respectively. These results are consistent with those found by Hell et al. (2010), in which they described that a low concentration of the LL37 peptide significantly reduced the binding of bacteria on the surface. The mechanism by which the LL37 peptide acts is not yet well described, but it could penetrate biofilms and exhibit antimicrobial activity. Other mechanisms may include decreased adhesion of planktonic bacteria and regulation of quorum sensing, related to the development and maintenance of biofilm (Overhage et al. 2008, Segev-Zarko & Shai 2017). The D enantiomer cationic peptide, DJK-5, exerted antibiofilm activity against *E. faecalis* and oral biofilms of multiple species. It has great advantages, such as resistance to protease degradation, effective activity and that it is more powerful than other antimicrobial peptides (Wang et al. 2017), as well what was observed in this study.

The expression of the MSCRAMM proteins was evaluated in the clinical isolates after treatment with the analog peptides DLL37-1 and LL37-1. In relation to the *clfA* gene, the two peptides depicted a negative regulation in the strongly biofilm-building strain USA300; and with LL37-1, positive regulation was observed in clinical isolation. This last result could be related to the inhibition of biofilm formation and the proportion of sessile and planktonic cells available. It has been described that the transcription levels of this protein can vary when it binds to a solid substrate; therefore, clinical isolation may present a greater quantity of bacteria in the planktonic state (greater inhibition), and therefore, greater expression of the gene (Wolz et al. 2002). Additionally, the bacteria can change their expression profile as the infection progresses, or according to their survival needs (Jan-Roblero et al. 2017).

In the *fnbA* expression, a compensation mechanism was found; that is, in response to treatment with analogous peptides in *S. aureus,* the expression of this protein, which promotes the formation of biofilm on polystyrene surfaces, was stimulated (Beenken et al. 2004, Yeswanth et al. 2017). This change in expression is significant for the perpetuation and survival of bacteria in biofilm, which contributes to its permanence over time (Atshan et al. 2013). Regarding adhesion protein *sdrC*, Sitkiewicz et al. (2011) found that treatment with calcium and magnesium causes significant changes in gene expression, which is associated to the differential reactions of the strains to the environment (Sitkiewicz et al. 2011, Trivedi et al. 2017). Similarly, in a previous study, it was found that the role of *sdrC* in biofilm formation in clinical isolates is dependent on genetic and molecular factors of the strain and this could justify the variation in gene expression in the two strains evaluated (Barbu et al. 2014).

In the *S. epidermidis* strain ATCC (non-biofilm forming), an increase in the expression of *sdrG* and its inhibition was found during clinical isolation and in the positive control; this may be caused by negative counter-regulation and virulence factors in the presence of other biofilm inhibitor treatments (Swetha et al. 2019). In this sense, the DLL37-1 peptide could be postulated as a counter-regulator of this protein that plays a key role in the colonization of implanted biomaterials (Bowden et al. 2008).

Regarding the expression of *sdrF*, there was a correlation in the three bacterial strains, again showing an increase in the expression of these strains treated with the LL37-1 peptide. Treatment with the LL37-1 peptide showed a general tendency to positively regulate the expression of the genes of the MSCRAMM proteins in *S. aureus* and *S. epidermidis*, which could be correlated with bacterial survival mechanisms. In the DLL37-1 peptide, a more counter-regulatory mechanism of these proteins was observed, mainly in *clfA* and *sdrC*; however, research is required to understand the genetic and metabolic factors involved in the regulation of these genes *in vivo* and *in vitro*.

CONCLUSIONS

In this study, it was found that 93.5% of clinical isolates presented resistance to at least one antibiotic. A total of 96.8% of *S. aureus* and 48.4% of *S. epidermidis* were biofilm formers. LL37-1 and DLL37-1 peptides exhibited biofilm inhibitory activity with inhibition percentages of 58% and 57% in *S. epidermidis* and *S. aureus*, respectively are promising results. The D enantiomer cationic peptide DLL37-1 has great advantages, such as the lowest toxicity against red blood cells and L929 cells at concentrations up to 5 μM and they have no hemolytic effect. Therefore, the DLL-37 enantiomer peptide possibly has higher biological activity, making it a better candidate for the inhibition of biofilm formation and the containment of bacterial resistance.

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M.L.S carried out the experiments. A.T.D.R contributed to the interpretation of the results and took the lead in writing the manuscript. S.A.G assisted in the experiments. L.C.M.M, G.P.B and J.N.O conceived the presented idea and planned the experiments. L.M.S.P designed and synthesized the peptides. J.E.P provided the strains for this study. All authors provided critical feedback and helped shape the research, analysis and manuscript.