Permeabilizing action of an antimicrobial lactoferricin-derived peptide on bacterial and artificial membranes

O. Aguilera[a,b], H. Ostolaza[c], L.M. Quirós[b], J.F. Fierro[a,b,d,*]

[a] *Laboratory of Oral Microbiology, School of Stomatology, Oviedo, Spain*
[b] *Department of Functional Biology (Microbiology), Faculty of Medicine, University of Oviedo, C/Julián Clavería, s.n., 33006 Oviedo, Spain*
[c] *Unit of Biophysic (CSIC-UPV/EHU) and Department of Biochemistry, University of the Basque Country, 48080 Bilbao, Spain*
[d] *Service of Microbiology, Central Hospital of Asturias, Asturias, Spain*

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Abstract A synthetic peptide (23 residues) that includes the antibacterial and lipopolysaccharide-binding regions of human lactoferricin, an antimicrobial sequence of lactoferrin, was used to study its action on cytoplasmic membrane of *Escherichia coli* 0111 and *E. coli* phospholipid vesicles. The peptide caused a depolarization of the bacterial cytoplasmic membrane, loss of the pH gradient, and a bactericidal effect on *E. coli*. Similarly, the binding of the peptide to liposomes dissipated previously created transmembrane electrical and pH gradients. The dramatic consequences of the transmembrane ion flux during the peptide exposure indicate that the adverse effect on bacterial cells occurs at the bacterial inner membrane.

Key words: Lactoferricin; Lactoferrin; Liposome; Antimicrobial peptide; *Escherichia coli*

1. Introduction

The antimicrobial active N-terminal peptides derived from acid-pepsin hydrolysis of human lactoferrin (lactoferricin H) and bovine lactoferrin (lactoferricin B) and many synthetic peptides derived from them are promising candidates of therapeutic and industrial value due to their broad spectrum of antimicrobial activities. These peptides present an enhanced activity against a wider range of microorganisms than the native lactoferrin [1–4]. Lactoferricin H corresponds to amino acid residues 1–47 at the N-terminus of human lactoferrin, and includes the antimicrobial sequence (residues 20–37) and the amino acids (residues 28–34) involved in the high-affinity binding of the whole protein to lipopolysaccharides of *Escherichia coli* [1,5].

The possibility to use antimicrobial peptides as a therapeutical alternative due to the increase of the bacterial resistance to the classical antibiotics requires a detailed knowledge of the mechanism of action of each of these peptides. However, despite the fact that the mode of action of different classes of natural cationic antimicrobial peptides has been reported, studies including synthetic peptides are scarce [6].

Previous studies about the mechanism of action of lactoferricin and other derived peptides showed the ability of these peptides to bind to lipopolysaccharides of *E. coli*, followed by damage and permeability alterations of the bacterial outer membrane [3,7,8]. However, the complete mechanism of the antimicrobial action of the lactoferricin and derived peptides remains unclear.

In the present work we have investigated the ability of a lactoferrin H-derived synthetic peptide (Lfpep), which shows antimicrobial and antiprotozoal properties [9], to interact with bacterial inner membrane as well as phospholipid vesicles. The effects of Lfpep on the electrical and chemical proton gradients on *E. coli* cells were studied. In a similar way, peptide binding to liposomes, changes in transmembrane electrical potential, pH gradient, and permeability of *E. coli* phospholipid vesicles were also assayed.

2. Materials and methods

2.1. Materials

Materials were obtained from the following sources: The N-terminal antimicrobial peptide of human lactoferrin (residues 17–39) was synthesized by Chiron Mimotopes Pty. Ltd. (Clayton, Australia) with the following sequence: NH_2-TKCFQWQRNMRKVRGPPVSCIKR-COOH. Purity of the peptide was demonstrated by reverse-phase high-performance liquid chromatography (Chiron Mimotopes Pty.). [^{14}C]-acetic acid, [^{14}C]-methylinulin, tritiated water, and [^{3}H]TPP$^+$ were obtained from Amersham Corp. (Buckinghamshire, UK). Phospholipids of *E. coli* were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). ANTS, diS-C_3-(5), DPX, and pyranine were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Nigericin and valinomycin were supplied by Sigma Chemicals, Co., USA.

2.2. Determination of membrane potential ($\Delta\Psi$) and ΔpH in *E. coli*

The transmembrane electrical potential ($\Delta\Psi$) and ΔpH of glucose-energized cells were estimated from the transmembrane equilibrium distribution of the lipophilic cation [^{3}H]TPP$^+$ and [^{14}C]-acetic acid, respectively [10]. Bacterial suspensions of *E. coli* 0111 ATCC 33780 (0.5 to 1 mg dry weight per ml) were centrifuged, washed twice, and suspended in 10 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM glucose. To determine the transmembrane electrical potential, 0.5 μCi/ml of [^{3}H]TPP$^+$ were added to 1 ml aliquots and incubated for 5 min. Samples were taken before the peptide addition as well as 5 and 15 min later. These samples were centrifugated ($8000 \times g$, 1 min) and aliquots from the supernatant as well as the pellet were removed and the radioactivity was determined using a liquid scintillation counter [11]. [^{3}H]TPP$^+$ uptake was corrected for non-specific binding as described [12]. Nernst equation was used to calculate the membrane electrical potential [10]. Internal volume of *E. coli* cells was measured using the non-permeant hydroxy [^{14}C]-methylinulin (1 μCi/ml) and tritiated water (2 μCi/ml) as described [12].

*Corresponding author. Fax: (34)-8-5103534.
E-mail: ffr@sauron.quimica.uniovi.es

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylene-bis-pyridiniumbromide; diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine iodide; K-PIPES, potassium piperazine-*N*,*N*′-bis-(ethanosulfonate); Pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; [^{3}H]TPP$^+$, [^{3}H]-tetraphenylphosphonium bromide

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The ΔpH changes across the cytoplasmic membrane were determined in a similar way from the distribution of [^{14}C]-acetic acid (0.1 μCi/ml), which was added to cell suspensions and the mixture incubated for 5 min at 25°C. The *E. coli* internal pH was calculated as described [10] using the equation:

$$pH_{in} = \log\left[\frac{A_{in}}{A_{out}}(10^{pK_a} + 10^{pH_{out}}) - 10^{pK_a}\right]$$

where A_{in} and A_{out} are the internal and external acetic acid concentration, respectively.

2.3. Antibacterial assays

A standard microdilution technique [13] was used to determine the minimal inhibitory concentration (MIC) of the Lfpep for *E. coli* 0111. Assays were performed in sterile 96-well microtiter plates by using 1% Bacto peptone (Difco) as growth medium. The peptide was serially diluted to give concentrations between 0.8 μM and 180 μM in a final volume of 100 μl. The plates were incubated at 37°C for 24 h. The MIC was taken as the lowest peptide concentration at which observable growth was inhibited. Cellular viability at visible growth inhibitory concentrations was assessed by serial dilution and drop counting on Nutrient broth (Difco).

2.4. Preparation of liposomes

Total lipids from *E. coli* were mixed in chloroform:methanol (9:1, vol/vol) and extensively evaporated. For binding and electrical potential assays, the lipid film was suspended by vortex mixing in a 50 mM potassium phosphate buffer (pH 6.0) to obtain a final lipid concentration of 20 mg/ml. The suspension was sonicated in an ice bath, using a titanium tip ultrasonicator to obtain small unilamellar vesicles (SUVs) as previously described [14]. Titanium debris were removed by centrifugation. For ΔpH assays pyranine-containing SUVs were prepared using a pyranine buffer solution (20 mM potassium phosphate, pH 6.0; 100 mM potassium acetate) including 100 μM pyranine, which was added to the dried lipid film before sonication. Non-encapsulated fluorescent probe was separated from the vesicle suspension through a Sephadex G-50 gel filtration column (Pharmacia, Uppsala, Sweden) of 25×0.5 cm and eluted with buffer 20 mM potassium phosphate (pH 6.0) containing 100 mM K-PIPES. For assays of vesicle leakage, large unilamellar vesicles (LUVs) were prepared by the extrusion method [15] using polycarbonate filters with a pore size of 100 nm (Nucleopore, Pleasanton, CA, USA). These vesicles contained 12.5 mM ANTS, 45 mM DPX, and 20 mM Tris-HCl (pH 7.4). When required, strictly isotonic conditions were ensured by checking the osmotic pressure of intra- and extravesicular solutions in an Osmomat 30 osmometer (Gonotec, Berlin, Germany). Phospholipid concentrations were determined by inorganic phosphorus analysis [16].

2.5. Fluorescence measurements

Fluorescence measurements were made using a Perkin-Elmer LS-50 spectrofluorometer. Binding of the peptide to vesicles was monitored through changes in the intrinsic tryptophan fluorescence of the peptide, which includes a single tryptophan (Trp-22). A fixed amount of peptide (10 μM) was incubated overnight with LUVs composed of phospholipids from *E. coli* at different phospholipid/peptide (L:P) molar ratios at room temperature. Fluorescence spectra were recorded using 5 mm path quartz cuvettes with excitation at 280 nm and emission from 300 to 400 nm, and slit widths of 2.5 nm. The spectra were corrected for light scattering by subtracting the corresponding blank spectra without the peptide [17].

In the quenching experiments, the peptide was added to a SUVs suspension (0.2 mg/ml, final concentration) in 50 mM potassium phosphate buffer (pH 6.0). No corrections were made for changes in osmolarity, as little change in 90° light scattering indicated little change in vesicle structure. Small aliquots of the stock solutions of quenchers (8 M acrylamide; 5 M potassium iodide) were added to vesicle suspensions. Fluorescence intensity was measured for potassium iodide (KI) or acrylamide after the signal had stabilized during 30 s. The samples containing KI or acrylamide were excited at 280 nm and 295 nm, respectively. The emission wavelength was recorded in a range from 310 to 380 nm. Results were fitted by linear regression using the program Statistica (Statsoft, Inc., USA) to the Stern-Volmer equation ($F_o/F = 1 + K_{sv}\cdot Q$), where F and F_o are the fluorescence intensities with and without quencher, K_{sv} is the Stern-Volmer constant, and Q is the quencher concentration. Contributions of static quenching were generally unimportant below approximately 80–100 mM acrylamide as determined by plotting $(F_o/F-1)/Q$ versus Q. Lack of upward curvature of plots below 80–100 mM acrylamide supported this assumption [18,19].

2.6. Monitoring of transmembrane potential (ΔΨ) and ΔpH in liposomes

The reaction mixture (1 ml) contained 100-fold diluted liposomes in a 50 mM sodium phosphate buffer (pH 6.0) and 1 μM of the fluorescent potential-sensitive dye diS-C_3-(5). A transmembrane K^+-diffusion potential (negative inside) was generated in the phospholipid vesicles by addition of valinomycin (1 μM), a potassium ionophore [20]. Changes of transmembrane potential in the liposomes were continuously monitored by the fluorescence quenching of diS-C_3-(5) using a spectrofluorometer. Experiments were performed at 25°C at a wavelength of excitation and emission of 616 nm and 676 nm, respectively.

Changes in the internal pH of liposomes was monitored from the fluorescence of pyranine entrapped within the liposomes [20]. Liposomes were diluted 100-fold in 20 mM potassium phosphate (pH 6.0) containing 100 mM K-PIPES for the generation of a pH gradient. The assays were carried out at 25°C at a wavelength of excitation and emission of 450 nm and 508 nm, respectively.

3. Results

3.1. Effect of Lfpep on membrane potential (ΔΨ) and ΔpH in E. coli

Glucose-energized cells of *E. coli* 0111 were used to investigate the Lfpep effects on ΔΨ and ΔpH of *E. coli* 0111. Determination of ΔΨ was performed using the lipophilic cation [^{3}H]TPP$^+$ as described in Section 2. The ΔΨ value in the control assays was estimated at −110 mV. The addition of different concentrations of the peptide (0.2, 2, 10, 20, 45, 90, 180 μM) resulted in a immediate releasing of the previously accumulated [^{3}H]TPP$^+$ at concentrations ≥20 μM, indicating the dissipation of ΔΨ. In control experiments, the addition of *n*-butanol (7%) to the cellular suspension also induced a complete dissipation of the ΔΨ of *E. coli*.

In a similar way, the ΔpH was determined by the distribution of [^{14}C]-acetic acid as described in Section 2. The internal pH of *E. coli* was estimated in 8.3 units at a external pH of 7.0. The Lfpep addition to the cells caused a total loss of ΔpH, after 15 min, when the concentrations assayed were ≥90 μM.

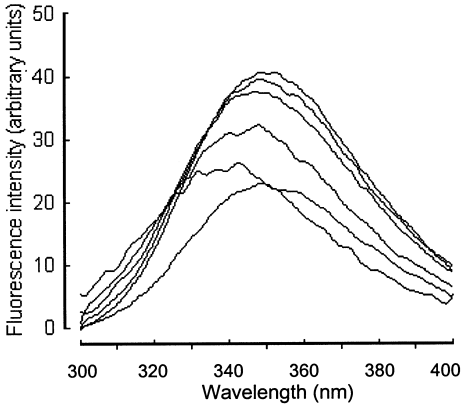

Fig. 1. Changes in tryptophan fluorescence of Lfpep bound to *E. coli* phospholipid liposomes. A fixed concentration of Lfpep was mixed with various amounts of liposomes to obtain a phospholipid:peptide ratio (mol:mol) of 2.5, 5, 10, 25, 50, 0 (from top to bottom).

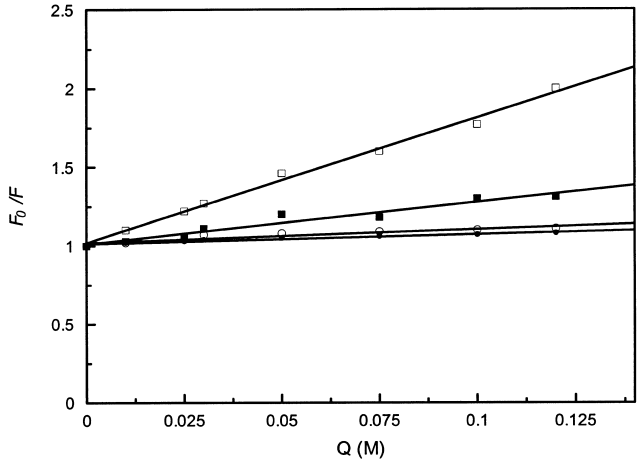

Fig. 2. Acrylamide and iodine quenching of Lfpep tryptophan. Acrylamide quenching in absence (■) and presence (□) of liposomes. Iodine quenching in absence (●) and presence (○) of liposomes.

3.2. Antibacterial assays

The ability of the Lfpep to inhibit the growth of *E. coli* was determined using a broth microdilution assay. The number of cells present in each well was adjusted to obtain a similar number of CFU/ml as in transmembrane electrochemical gradient determination assays. Results showed that the minimum concentration of the Lfpep inhibiting growth (MIC) was 90 μM. This concentration was bactericidal and no growth was observed on counting plates.

3.3. Intrinsic tryptophan fluorescence studies

Intrinsic tryptophan fluorescence changes derived from binding of Lfpep to liposomes were estimated. A fixed concentration (10 μM) of the peptide was mixed with various amounts of LUVs and the fluorescence spectra in the equilibrium state were measured. The addition of increasing amounts of vesicles caused a blue shift in the maximum wavelength (Fig. 1).

Changes in the fluorescence quenching of the Trp residue microenvironment were investigated using iodine, as ionic quencher, and acrylamide as an uncharged quencher. Fig. 2 shows Stern-Volmer plots for intrinsic tryptophan fluorescence quenching by both quenchers in absence and presence of liposomes. In absence of liposomes, iodine quenching was minimal with a Stern-Volmer quenching constant (K_{sv}) of 0.57 M^{-1} and no significant changes were observed when liposomes were added ($K_{sv} = 0.6$ M^{-1}). Intrinsic tryptophan fluorescence was quenched more efficiently by acrylamide than iodine in absence of liposomes ($K_{sv} = 3.6$ M^{-1}), but the Trp-22 was more accessible to acrylamide, with a quenching constant of 11.3 M^{-1}, in presence of liposomes.

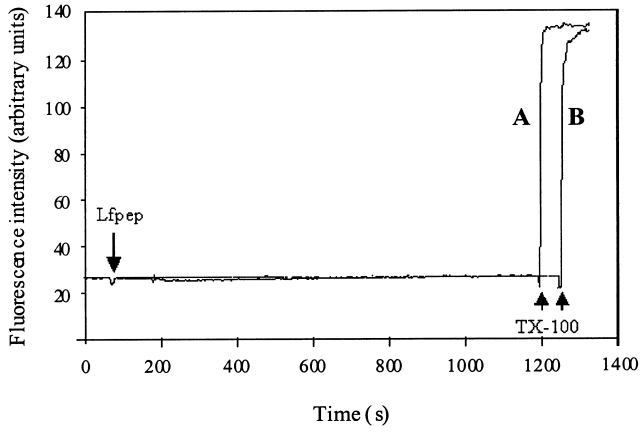

Fig. 3. Effect of Lfpep on the ANTS/DPX leakage from *E. coli* liposomes. Lfpep at different concentrations (A) 1 μM, or (B) 20 μM, and Triton X-100 (TX-100) were added at indicated times. Leakage is marked by an increase in fluorescence intensity.

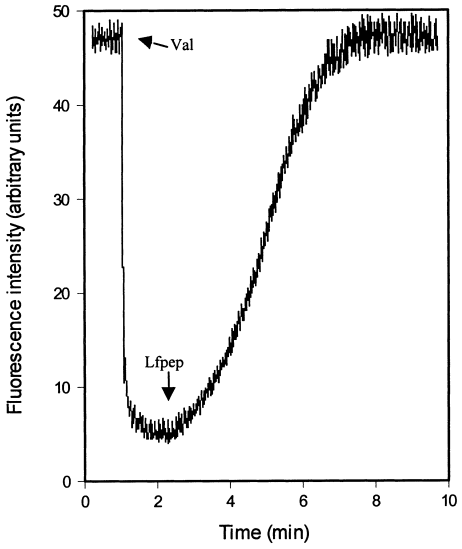

Fig. 4. Effect of Lfpep on the diS-C_3-(5) fluorescence quenching induced by valinomycin in *E. coli* phospholipid liposomes. Valinomycin (Val; 1 μM) and Lfpep (0.2 μM) were added at indicated times.

3.4. Effect of Lfpep on ΔΨ and ΔpH in liposomes

The Lfpep effect on the valinomycin-induced transmembrane potential in phospholipid vesicles of *E. coli* was determined using diS-C_3-(5), a membrane potential-sensitive probe. The addition of the different concentrations (0.1, 0.2, 0.4, 0.8, 4, 8 μM) of the peptide to the valinomycin-hyperpolarized liposomes completely reversed the fluorescent quenching, and the minimal concentration of Lfpep necessary to achieve the potential loss was 0.2 μM (Fig. 3). However, no changes in the fluorescence quenching were detected when the peptide was added to the liposomal suspension before the addition of the ionophore (data not shown).

The effect of Lfpep on internal pH of *E. coli* phospholipid liposomes containing pyranine was investigated. An artificial pH gradient was generated as described in Section 2, and the changes experimented by the addition of peptide were monitored measuring the fluorescence of the entrapped fluorescent probe. The initial ΔpH was estimated in 1.2 units using nigericin (1 μM), in previous assays. Different peptide concentrations from 0.1 to 8 μM were assayed, resulting in 0.2 μM minimal concentration that induced a total dissipation of the previously generated pH gradient (Fig. 4).

3.5. Vesicle leakage studies

The ANTS/DPX complex leakage induced by Lfpep (1 and 20 μM) from liposomes composed of total lipids from *E. coli* was monitored. The peptide was unable to induce leakage of the fluorescent probes at the concentrations tested (Fig. 5).

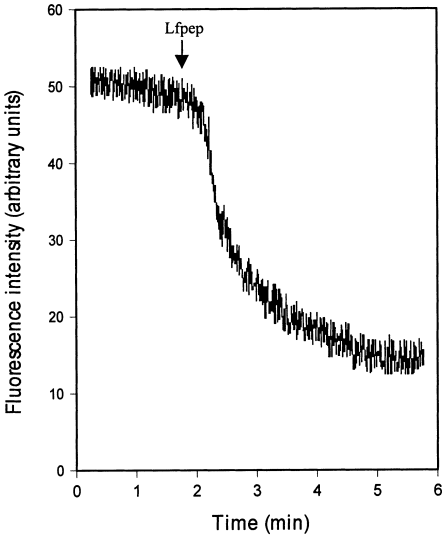

Fig. 5. Effect of Lfpep on ΔpH in *E. coli* phospholipid liposomes. Lfpep (0.2 μM) was added at the indicated time. Changes of the ΔpH were monitored by the fluorescence changes of the liposome entrapped pyranine, used as pH indicator.

Nevertheless, 100% release was achieved when 5 μl of 10% (w/v) Triton X-100 was added at the end of the assay as a probe-releasing control.

4. Discussion

Lactoferricin is a single antimicrobial peptide generated by the lactoferrin acid-pepsin hydrolysis, an iron-binding protein found in several body fluids of mammals and mainly secreted by polymorphonuclear neutrophils. Disruption of the bacterial outer membrane mediated by a derived peptide has been reported [8], however the complete antimicrobial mechanism of action of the lactoferricin and derived peptides is unknown.

In an attempt to understand the antimicrobial mechanism of a lactoferricin-derived peptide (Lfpep) we investigated its ability to disrupt the *E. coli* inner membrane permeability. Energy transduction is one of the principal functions of the cytoplasmic membrane, which involves the generation of a proton-motive force (Δp). The Δp is composed of an electrical gradient ($\Delta\Psi$) and a proton gradient (ΔpH). In general, changes in the inner membrane integrity induce a loss of the $\Delta\Psi$ and/or ΔpH affecting to vital processes of bacteria, such as the Na^+ or K^+ gradients across the cytoplasmic membrane, ATP synthesis, or solute transport [21–24]. We performed assays to determine the effects of Lfpep on the transmembrane potential and pH gradient of *E. coli* 0111. Determination of the $\Delta\Psi$ indicated that the peptide was able to induce a drastic and quick loss of the bacterial transmembrane electrical potential. In addition, Lfpep was also able to induce the loss of the ΔpH as was determined by the cytoplasmic accumulation of the [^{14}C]-acetic acid.

The observed effects on *E. coli* cells were further investigated using *E. coli* phospholipid liposomes. Binding of Lfpep to liposomes was monitored measuring the intrinsic fluorescence of the single Trp residue of the peptide. Study of intrinsic fluorescence provides insights into the environment, structure and dynamics of the fluorophores [25]. In presence of liposomes, the Trp-emission wavelength was shortened (blue shift) suggesting a decrease of the environment polarity associated with the binding of the peptide to the membranes. Peptide binding was also analyzed performing quenching experiments using the iodine and acrylamide quenchers in presence or absence of liposomes. Changes in the exposure of tryptophan can be determined using low molecular weight quenching agents that are able to decrease the fluorescence intensity due to a direct interaction with the excited indole group. These variations can be produced by binding to other molecules or by peptide conformational changes [18]. In our assays, iodine was not an effective quencher, and no significant modifications were observed after the liposome addition. This could be due to the fact that iodine is an ionic quencher and therefore charged and very hydrated, what makes it able to quench only surface tryptophan residues, and sensitive to electrostatic effects that may influence its quenching action. In contrast, acrylamide is an uncharged molecule and consequently its quenching effect is very sensitive to the tryptophan exposure [19]. Acrylamide was a more efficient quencher than iodine, and its effect was greatly enhanced after the peptide binding to liposomes, indicating an increased exposure of the tryptophan to the quencher. Quenching assays suggest that Lfpep is able to bind to liposomes. Interestingly, this peptide is very basic ($pI = 12$) with a high positive charge (+7) and forms an amphipathic alpha-helix in the native protein surface. That features are very common in peptides showing antibacterial activity independently of the sequence homology [6,26]. The increase in the exposure of the Trp in presence of liposomes could be reflecting changes in the bound peptide structure.

The consequences of this interaction with respect to the previous generated $\Delta\Psi$ and ΔpH in liposomes were monitored using the fluorescent probes diS-C_3-(5) and pyranine, respectively. The Lfpep was able to induce a total dissipation of the generated valinomycin-dependent electrical potential. However, when the Lfpep was added before the ionophore the generation of the electrical potential was not observed. On the other hand, the peptide was able to dissipate a previously generated pH gradient in liposomes. All the above results show the ability of the peptide to permeate different ions. To determine if other high molecular weight agents could be permeated, the leakage of entrapped ANTS/DPX from the vesicles was determined. At high concentrations (i.e. inside the vesicles) ANTS and DPX form a low-fluorescence complex, the breakdown of the vesicle membrane releases both molecules resulting in the complex decomposition and a high ANTS fluorescence [27]. In our case, Lfpep binding did not cause enough membrane disruption to induce the leakage of encapsulated ANTS/DPX, even though the peptide concentrations used in this assay (1 and 20 μM) were 5 and 100 times greater than the dose needed to induce membrane depolarization (0.2 μM). These results support the ability of Lfpep to permeate ions, excluding molecules of high molecular weight, without a generalized membrane disintegration.

The overall result supports the ability of Lfpep to interact with the cytoplasmic membrane of *E. coli* and liposomes producing a non-specific ion permeation, and inducing a $\Delta\Psi$ dissipation and loss of the ΔpH in both models. The dissipation of both $\Delta\Psi$ and ΔpH could impair some vital cell membrane functions, such as the production of ATP, causing the cellular death. This suggestion could explain the bactericidal effect of Lfpep on *E. coli*, and is supported by the similar peptide

concentration necessary to induce the loss of the ΔpH and the inhibition of the cellular growth (MIC). Despite the fact that Lfpep includes the antimicrobial homologous sequence present in lactoferrin, the results obtained here can not be extrapolated to explain the antimicrobial action of the whole molecule of lactoferrin, which exerts only a bacteriostatic effect on *E. coli* [7].

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