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# Molecular View by Fourier Transform Infrared Spectroscopy of the Relationship between Lactocin 705 and Membranes: Speculations on Antimicrobial Mechanism[▽]

Patricia Castellano,[1] Graciela Vignolo,[1] Ricardo Norberto Farías,[2]
José Luis Arrondo,[3] and Rosana Chehín[2]*

*Centro de Referencia para Lactobacilos (CERELA/CONICET), Chacabuco 145 Tucumán, Argentina[1]; Departamento de
Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas (INSIBIO) and Instituto de
Química Biológica Dr. Bernabé Bloj (CONICET-UNT), Chacabuco 461 (4000) Tucumán,
Argentina[2]; and Unidad de Biofisica (CSIC-UPV) and Departamento de
Bioquímica y Biología Molecular, Bilbao, España[3]*

Lactocin 705 is a bacteriocin whose activity depends upon the complementation of two peptides, termed Lac705α and Lac705β. Neither Lac705α nor Lac705β displayed bacteriocin activity by itself when the growth of sensitive cells was monitored. To obtain molecular insights into the lactocin 705 mechanism of action, Fourier transform infrared spectroscopy was used to investigate the interactions of each peptide (Lac705α and Lac705β) with dipalmitoylphosphatidylcholine liposomal membranes. Both peptides show the ability to interact with the zwitterionic membrane but at different bilayer levels. While Lac705α interacts with the interfacial region inducing dehydration, Lac705β peptide interacts with only the hydrophobic core. This paper presents the first experimental evidence that supports the hypothesis that Lac705α and Lac705β peptides could form a transmembrane oligomer. From the obtained results, a mechanism of action of lactocin 705 on membrane systems is proposed. The component Lac705α could induce the dehydration of the bilayer interfacial region, and the Lac705β peptide could insert in the hydrophobic region of the membrane where the peptide has adequate conditions to achieve the oligomerization.

In the last decade, there has been a growing interest in biopreservation through the use of microorganisms and/or their metabolites to prevent food spoilage and to extend the shelf life of foods (7, 34, 42). Lactic acid bacteria (LAB) are of particular interest as biopreservative organisms. The preserving effects of these organisms are due to the production of antimicrobial substances, including hydrogen peroxide, organic acid, and bacteriocins (16, 36). Bacteriocins are ribosomally synthetized antimicrobial peptides active against closely related bacteria. The major classes of bacteriocins produced by LAB include: lantibiotics, small heat-stable peptides, large heat-labile proteins, and complex proteins. Most of the bacteriocins produced by LAB belong to class II, which can be subdivided into *Listeria*-active peptides (IIa), two-peptide bacteriocins (IIb), *sec*-dependent bacteriocins (IIc), and bacteriocins that do not belong to the other subgroups (IId) (27). The potential application of LAB bacteriocins as food preservatives requires an in-depth knowledge of how they exert their bactericidal effects. Many bacteriocins appear to elicit their lethal effects by permeabilizing the cell membrane of target organisms, in certain cases by targeting intermediates of cell wall biosynthesis (10, 44) or possibly proteins of the sugar phosphotransferase systems (24, 39). The meat isolate *Lactobacillus curvatus* CRL705 (formerly identified as *Lactobacillus casei* CRL705) produces lactocin 705, a small antimicrobial sub-

stance that belongs to the class IIb bacteriocins, whose activities depend upon the complementation of two peptides, termed Lac705α (GMSGYIQGIPDFLKGYLHGISAANKHK KGRLGY; pI = 9.87) and Lac705β (GFWGGLGYIAGRVG AAYGHAQASANNHHSPING; pI = 8.61) (17, 18). Lactocin 705 exerted an inhibitory effect on the indicator strain *Lactobacillus plantarum* CRL691 with an optimal Lac705α/Lac705β peptide ratio of 1 to 4. Neither Lac705α nor Lac705β displayed bacteriocin activity by itself when the growth of sensitive cells was monitored (18). Both peptides were required to dissipate the proton motive force of energized cells of *Lactobacillus plantarum* CRL691 (15). However, the mechanism by which lactocin 705 interacts with bacterial membrane is not clearly established. A way to provide insight into this research area is to study the interactions of each peptide (Lac705α and Lac705β) with membrane model systems using Fourier transform infrared (FTIR) spectroscopy. This technique is known to be a versatile and powerful tool to investigate protein and lipid structures and their interactions (5). In this work, some evidence that could help with understanding the mechanism of action of the two-component peptides of lactocin 705 is provided.

## MATERIALS AND METHODS

**Bacteriocin synthesis.** The synthesis of the 33-amino-acid Lac705α peptide and of the 33-amino-acid Lac705β peptide was performed according to the methods of Palacios et al. (37) and Cuozzo et al. (17) by Gemini Biotech (Alachua, FL) and Bio-Synthesis (Lewisville, TX), respectively. Peptide stock solutions were prepared in 20 mM HEPES-D₂O, pD 7.4 (pD = pH + 0.4 pH unit).

**Vesicles preparation.** The zwitterionic phospholipid dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Birmingham, AL). An

* Corresponding author. Mailing address: INSIBIO-Chacabuco 461 (4000) Tucumán, Argentina. Phone and fax: 54-381-4248921. E-mail: rosana@fbqf.unt.edu.ar.

appropriate amount of DPPC was dissolved in chloroform-methanol (2:1, vol/vol) and dried under nitrogen onto the wall of a Corex glass tube and then placed in a vacuum oven to completely remove any remaining solvent. The lipid was then rehydrated in 20 mM HEPES-D$_2$O, pD 7.4, and the large multilamellar vesicles formed were sonicated on ice under nitrogen with a probe-type sonifier. Cycles of sonication (1-min pulse) and cooling (1 min) were repeated up to 15 times until the initially cloudy lipid dispersion became translucent. To remove titanium debris, the suspension was centrifuged for 15 min at 1,100 × $g$ to obtain a pure DPPC small unilamellar vesicle suspension (21).

**Samples for FTIR spectra preparation.** For solution spectra, peptides were dissolved to a final concentration of 10 mg ml$^{-1}$ in 20 mM HEPES-D$_2$O, pD 7.4. For samples in the presence of lipids, each peptide was added to liposomes to give a molar ratio of 20:1 (lipid to protein). The peptide-vesicle mixtures were incubated for 10 min at room temperature (20 to 22°C) before data acquisition. Phospholipid and peptide concentrations were measured by standard methods (1, 30).

**FTIR spectroscopy.** The samples were recorded in a Nicolet Magna II spectrometer equipped with an MCT detector (Thermo Nicolet, Madison, WI) using a demountable liquid cell (Harrick Scientific, Ossining, NY) with calcium fluoride windows and 50-µm spacers. A tungsten-copper thermocouple was placed directly onto the window, and the cell was placed in a thermostatized cell mount. Thermal analysis was performed by heating continuously in the range of 20 to 80°C, with a heating rate of 1°C/min. Spectra were collected by using rapid-scan software running under OMNIC (Nicolet). Usually, 310 scans/sample were taken, averaged, apodized with a Happ-Genzel function, and Fourier transformed to give a final resolution of 2 cm$^{-1}$. The contribution of D$_2$O in the amide I′ region was eliminated by subtracting the buffer spectra from that of the solution at the same temperature to obtain a flat baseline of between 2,000 and 1,700 cm$^{-1}$. Liposome analyses either in the absence or in the presence of the peptides were repeated three times with fresh new samples to test the reproducibility of the measurements. In all cases, the differences among the three experiments were lower than 5%. The error in estimation of the percentage of secondary structure depends mainly on the removal of spectral noise, and it was estimated to be 2% (20). For the measurement of the tyrosine ring vibration wave number, an exponential baseline was subtracted. The wave number of the symmetric C—H stretching mode ($v_s$ [CH$_2$]) of the acyl chain methylene groups of the lipids was determined after a linear baseline was subtracted in the 3,050- to 2,750-cm$^{-1}$ region. The deconvolution, band position determination, and bandwidth measurement, together with the curve fitting of the original amide I′ band, were performed as reported previously (2, 3). Briefly, band component positions were obtained from deconvolution and derivation. Since the results obtained after iterations may not be unique, the following restrictions were applied: (i) from initial guesses, the band position could not diverge more than the distance between data points, and (ii) the width of the bands should be less than one-half of the amide I′ bandwidth. The use of several spectra recorded at different temperatures below the thermal denaturation reduces the error of the quantification procedure to around 3% (8).

## RESULTS

**Effect of Lac705α and Lac705β peptides on the CH$_2$ stretching vibration of DPPC.** The impact of each peptide component of lactocin 705 (Lac705α and Lac705β) on DPPC bilayers was investigated. The methylene stretching vibration of the lipid chains is shown in Fig. 1. Two main bands, around 2,800 and 3,100 cm$^{-1}$, dominate the spectra in this region and are assigned to the antisymmetric ($v_{as}$ [CH$_2$]) and symmetric ($v_s$ [CH$_2$]) methylene stretching modes, respectively. The thermotropism of lipids is characterized by the shift of the wave number of these modes that have been shown to be sensitive to the presence of gauche conformers (13, 14). Thus, they are useful probes for monitoring the lipid-phase transitions (31). Figure 1 presents the evolution of the $v_s$ [CH$_2$] wave number of DPPC acyl chains as a function of temperature in the absence and presence of Lac705α and Lac705β peptides. The $v_{as}$ [CH$_2$] wave number showed just the same behavior with the peptide addition (data not shown). For pure DPPC, the melting temperature (*Tm*) was 42°C, as deduced from the curve, which is
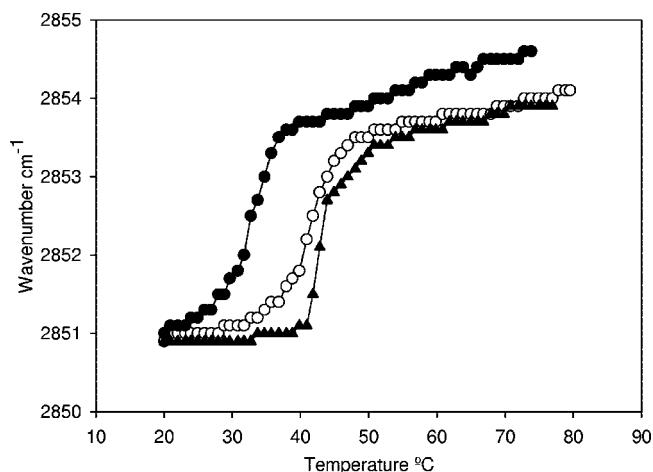


FIG. 1. Temperature dependence of the wave number of symmetric methylene stretching $v_s$ [CH$_2$] vibration bands of DPPC at pD 7.0 for a pure DPPC SUV liposome (○) and in the presence of Lac705β (●) or Lac705α (▲).

in agreement with the literature (19). In the presence of Lac705α, the *Tm* is slightly increased to about 2°C. However, there are no significant changes in the onset of the $v_{as}$ [CH$_2$] and $v_s$ [CH$_2$] above or below the phase transition, indicating that this peptide does not alter the conformation of the acyl chains. On the contrary, the Lac705β peptide modified the *Tm* from 42 to 33°C. This shift to lower temperatures may be taken as evidence of an interaction of the peptide with the hydrocarbon lipid chains (41). The more rapid increase (compared to the curve of pure DPPC) of the wave number with the change in temperature in the range preceding the abrupt increase due to the phase transition may also indicate a disturbance of the gel phase organization. Furthermore, the wave numbers of the methylene stretching band are higher in the presence of the Lac705β peptide than those observed for pure DPPC bilayers. These observations show that the presence of this peptide results in a decrease of the conformational order of the lipid acyl chains (41).

**Effect of Lac705α and Lac705β peptides on the hydration of the polar groups of DPPC.** The stretching vibration of the lipid carbonyl groups ($v$ [C=O]) can provide details concerning the hydration, the environment, and the conformation of the membrane interfacial region. The ester band of DPPC is known to be composed of two overlapped components located near 1,745 and 1,730 cm$^{-1}$, which can be evidenced by Fourier self-deconvolution. These are attributed to free (anhydrous) and hydrogen-bonded (hydrated) carbonyl groups, respectively (29). Thus, the study of this band as a function of temperature provides insights into the hydration level of the polar-apolar interface of DPPC bilayers. Table 1 presents an analysis for DPPC bilayers at different phase states in the absence and presence of Lac705α and Lac705β peptides. In the absence of peptides, there is a well-known intensity increase in the lower wave number component upon increasing temperature, interpreted as a rise in the amount of water molecules bound to the ester groups due to a higher penetration of water into the interfacial region above the phase transition. In the presence of the Lac705β peptide, the thermal behavior of the $v$ (C=O)

TABLE 1. DPPC carbonyl stretching vibration in the presence and in the absence of lactocin 705[a]

| Temp | DPPC | | DPPC + Lac705β | | DPPC + Lac705α | |
|---|---|---|---|---|---|---|
| | Band position | Area (%) | Band position | Area (%) | Band position | Area (%) |
| 25°C (gel phase) | 1743 | 34 | 1744 | 29 | 1741 | 62 |
| | 1730 | 66 | 1730 | 71 | 1727 | 38 |
| 50°C (liquid crystalline phase) | 1744 | 27 | 1745 | 23 | 1741 | 55 |
| | 1730 | 73 | 1730 | 77 | 1726 | 45 |

[a] Results show carbonyl $\nu$ (C=O) stretching vibration bands of DPPC at pD 7.0 for pure DPPC small-unilamellar-vesicle liposomes in the presence and in the absence of lactocin 705 in gel phase (25°C) and in liquid crystalline phase (50°C).

TABLE 2. Lac705β amide I′ band components[a]

| Parameter | Position (cm$^{-1}$) | % Area |
|---|---|---|
| Lac705β at 25°C | 1687 | 4 |
| | 1678 | 1 |
| | 1670 | 13 |
| | 1655 | 29 |
| | 1639 | 35 |
| | 1620 | 18 |
| Lac705β at 50°C | 1687 | 5 |
| | 1677 | 4 |
| | 1670 | 13 |
| | 1655 | 28 |
| | 1639 | 33 |
| | 1621 | 17 |
| Lac705β + DPPC (25°C) | 1687 | 12 |
| | 1677 | 4 |
| | 1666 | 13 |
| | 1654 | 29 |
| | 1642 | 7 |
| | 1626 | 35 |
| Lac705β + DPPC (50°C) | 1688 | 13 |
| | 1677 | 2 |
| | 1666 | 10 |
| | 1654 | 32 |
| | 1642 | 5 |
| | 1628 | 38 |

[a] Results show amide I′ band decomposition parameters corresponding to Lac705β peptide in D$_2$O buffer in the presence and in the absence of DPPC liposomes.

band did not reflect changes in the water accessibility compared to that observed in the absence of the peptide. However, after the addition of the Lac705α peptide, an increase in the higher wave number component in either the gel or in the liquid crystalline lipid phase was observed.

**Effect of DPPC bilayer on Lac705β amide I′ vibration.** Fig. 2 presents a representative spectrum of the Lac705β amide I′ band in the absence and in the presence of DPPC liposomes. The peptide spectrum in aqueous solution at 25°C as well as 50°C is centered at about 1,650 cm$^{-1}$, which is typical of an α-helix structure. Furthermore, the peptide could be heated up to 50°C without aggregation in the amide I′ band shape being observed. In the presence of DPPC liposomes, the maximum of the spectrum shifts to 1,628 cm$^{-1}$ at both temperatures, indicating that structural modification occurs. It is important to note that the bilayer acyl chain conformational order influences the magnitude of the peptide structural changes, because the shift from 1,650 to 1,628 is increased in the liquid crystalline phase.

To observe the underlying components of the broad amide I′ band, Fourier self-deconvolution and derivative methods have been applied to the original envelope (6, 26). We have identified six different component bands at frequencies of about 1,687, 1,678, 1,670, 1,655, 1,639, and 1,620 cm$^{-1}$ for Lact705β in solution at pD 7.0, for both 25 and 50°C (Table 2). To assign the component bands to specific structural features and estimate the percentage of each component, the guides given in Materials and Methods were followed and compared with val-
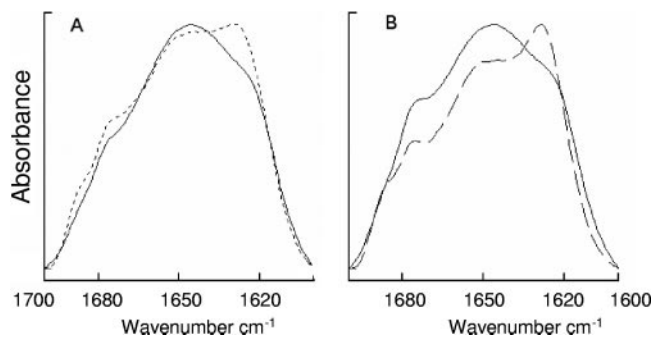


FIG. 2. FTIR absorption spectra in the amide I′ region of the Lac705β peptide (solid line) and the Lac705β peptide with DPPC liposomes (dashed lines) at 25°C (A) and 50°C (B).

ues found in the literature (6). Still, some of the bands can be unambiguously assigned, while for others, reasonable approximations can be made by comparisons with data from other techniques (2). The band at around 1,655 cm$^{-1}$ in D$_2$O corresponds to a canonical α-helix (2). The bands located around 1,666 to 1,688 cm$^{-1}$ arise from β turns (12, 28). The band at 1,677 cm$^{-1}$ may also arise from a small contribution of the high-frequency vibration of the antiparallel β-strand (12). Bands appearing at about 1,639 cm$^{-1}$ are assigned in D$_2$O to unordered conformation (2, 12). The band located at 1,620 cm$^{-1}$ could be attributed to an intermolecular hydrogen bond resulting from the aggregation of proteins and peptides (40). These results, taken together, indicate that, in deuterated solution and at 25°C, Lac705β consists of 29% α-helix, 35% unordered, and 14% β structure (Table 2). Even though the amide II region between 1,600 and 1,500 cm$^{-1}$ is not as well documented as is the amide I region, it also provides valuable information on the secondary structure of proteins. In our D$_2$O condition, and in the range of 20 to 80°C, the amide II band completely disappears, indicating a full deuteration of the amide nitrogen (data not shown). This H/D exchange between Lact705β and the aqueous medium is rapid, occurring during the sample preparation, and suggests that the solvent can easily access the NH groups of the protein. These results, together with the broad contour of the amide I spectrum, are characteristic of proteins or peptides with loose secondary structures (40). In the presence of DPPC liposomes in liquid crystalline phase, the band located at 1,621 cm$^{-1}$ shifts to 1,628 cm$^{-1}$, increasing its area significantly. This shift has already been attributed to an extended antiparallel β sheet (4). However,

TABLE 3. Lac705α amide I′ band decomposition

| Parameter | Position (cm$^{-1}$) | % Area |
|---|---|---|
| Lac705α (50°C) | 1688 | 7 |
| | 1677 | 12 |
| | 1656 | 44 |
| | 1640 | 26 |
| | 1625 | 11 |
| Lac705α + DPPC (50°C) | 1689 | 6 |
| | 1676 | 20 |
| | 1656 | 39 |
| | 1640 | 26 |
| | 1625 | 9 |

[a] Results show amide I′ band decomposition parameters corresponding to Lac705α peptide in $D_2O$ buffer in the presence and in the absence of DPPC liposomes.

this band increases together with the aggregation band located at 1,688 cm$^{-1}$, suggesting the formation of weaker intermolecular hydrogen bonds (25). The formation of such aggregations has already been observed for magainin-2 and related peptides, and it is expected to occur for polypeptide chains that retain some secondary structure, preventing a close alignment of chains (25). Another noticeable change observed in the presence of DPPC liposomes is the shifting of the band located at 1,639 cm$^{-1}$ to 1,642 cm$^{-1}$, with a significant decrease in its intensity. The conformational changes of Lac705β described above depend on the lipid phase of the phospholipids because in liquid crystalline they are greater than in the gel phase, suggesting the influence of the hydrophobic region packing in the membrane-peptide interaction. Moreover, the full width at 0.75 height diminishes from 41 to 32 cm$^{-1}$ when the lipids go from gel to liquid crystalline phase.

**Effect of DPPC bilayer on Lac705α amide I′ vibration.** The amide I′ band decomposition of Lac705α shows six different component bands at frequencies of about 1,688, 1,677, 1,656, 1,640, and 1,625 cm$^{-1}$. For the assignment and quantification of each band, the same criteria described above were used. Table 3 shows that the amide I′ of Lac705α in $D_2O$ solution at 50°C is composed of 44% α-helix, 26% unordered, and 19% β structure. It is important to point out that the Lac705α amide I′ band did not show significant differences in the presence or in the absence of the DPPC liposome in the temperature range from 20 to 80°C (data not shown).

**Effect of DPPC on the peptides' tyrosine vibration.** Lac705α and Lac705β contain three and two tyrosine residues, respectively. Tyr is a relatively strong infrared absorber due to its polar character, and its most intense band is due to the aromatic ring ($v$ C—C) located around 1,515 cm$^{-1}$ (9). However, this wave number is affected by the tyrosine's local environment being different according to whether it is exposed to water or to a hydrophobic environment. For example, it increases upon peptide unfolding (22, 38). Therefore, this vibration mode may represent a marker providing information relative to the peptide surroundings (9). The tyrosine wave number of pure Lac705β peptide in $D_2O$ solution shows its maximum at 1,515.14 cm$^{-1}$, without any change when the temperature increases from 20°C to 80°C. When the Lac705β peptide was incubated with DPPC vesicles, a decrease in the wave number to 1,511.5 cm$^{-1}$ was observed. This decrease

could be related to a more hydrophobic environment (9) induced by peptide folding or by the peptide insertion to the hydrophobic region of the membrane. The Lac705α peptide shows its maximum at 1,514 cm$^{-1}$ without any change in the presence of DPPC liposomes in the range of 20 to 80°C (data not shown).

## DISCUSSION

The present work provides evidences of the mechanism of action of the two-component bacteriocin lactocin 705, whose activity relies upon the complementation of Lac705α and Lac705β peptides. The FTIR studies presented in this paper show that Lac705α and Lac705β peptides can interact with zwitterionic DPPC bilayers and that each peptide exerts its effect on different regions of the membrane. The Lac705β peptide interacts with only the hydrophobic core. This fact is proved by the decrease of the conformational order of the DPPC acyl chains without significant change in the membrane interfacial region upon Lac705β peptide addition (41). The peptide's tyrosine wave number shifting from 1,515.14 cm$^{-1}$ to 1,511.5 cm$^{-1}$ is also evidence of the peptide's change to a more hydrophobic environment (9, 38). These results support the hypothesis that this peptide could insert into the DPPC bilayer (22). As a consequence, the FTIR spectrum of Lac705β in the amide I′ region reveals an important conformational reorganization upon the interaction with DPPC vesicles (Fig. 2). The shifting of the band located at 1,620 cm$^{-1}$ to 1,628 cm$^{-1}$ is notable (Table 2). Therefore, it is possible that two types of Lac705β aggregate exist, one in aqueous medium with a band at 1,620 cm$^{-1}$ and the other one at 1,628 cm$^{-1}$ in a hydrophobic environment due to different hydrogen bond patterns (25). Bands at around 1,625 cm$^{-1}$ have been associated with intramolecular interactions, such as monomer-monomer contacts (32). It has also been described for membrane proteins that helix-helix interaction can occur through a surface similar to an intramolecular β sheet (43), which would agree with the observed increase in the 1,628 cm$^{-1}$ band. Even though the Lac705β peptide is relatively short and may not be able to completely span a bilayer, its aggregation into the membrane can form oligomers involved in membrane permeabilization (25).

On the other hand, in an aqueous medium, the main component of the amide I′ suggests that Lac705β adopts a mainly unordered structure. However, bands corresponding to the α-helix have been observed. These results are in good agreement with previous circular dicroism studies on plantaricin E/F and J/K and lactococcin G, bacteriocins which share the same subclass as lactocin 705. The above peptides showed an unstructured conformation under aqueous conditions but, in the presence of trifluoroethanol and micelles of dodecylphosphocholine, they adopted some α-helical structure (23, 35). Regarding Lac705α, the peptide was not able to induce any modification in the hydrophobic region of the membrane or tyrosine wave number shift. However, it induces strong dehydration of the carbonyl region, suggesting that Lac705α can play a part in interfacial interactions. Moreover, the spectrum of Lac705α in the amide I′ region did not show any conformational reorganization upon interaction with DPPC vesicles (Fig. 2). Thus, we suggest that this peptide could interact with

the surface without penetration into the zwitterionic bilayer. Lac705α, as well as Lac705β, remains essentially in random conformation upon heating up to 80°C. This property has direct implications for the potential use of these antimicrobial peptides as food preservatives. It is interesting to note that purified lactocin 705 and the synthetic peptides, Lac705α and Lac705β, have the same MIC (40 nM), suggesting that the peptides are correctly ensembled in vitro.

There are three models to explain the antimicrobial activity of peptides: the toroidal pores, the barrel stave, and the carpet-like structures. Bacteriocins in class II may function by creating barrel stave-like pores or a carpet mechanism (33). In the barrel stave model, the attached peptides aggregate and insert into the hydrophobic core of the bilayer. On the contrary, in the carpet mechanism, the peptides do not aggregate but bind to the surface of the lipid bilayer to form a closely packed layer or "carpet" of peptide, which renders the membranes permeable (11). Regarding the Lac705α and Lac705β peptides, interfacial and hydrophobic interaction occurs; thus, the carpet-like mechanism does not explain our results. Taking together the results presented in this work, it is possible to hypothesize the differential roles of the two-component peptides of lactocin 705 in their interaction with the membrane model. Lac705α would dehydrate the interfacial region of the bilayer, while Lac705β would insert in the hydrophobic core.

This paper shows the first experimental evidence that supports the hypothesis that Lac705α and Lac705β peptides could form a transmembrane oligomer (pore?) partially responsible for the previously reported bactericidal effects induced by this bacteriocin in sensitive cells, such as increases in permeability, efflux of ions, and changes in the membrane potential and pH gradient (15).

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