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RESEARCH

Development of Polyelectrolyte-Coated Liposomes as Nanostructured Systems for Nisin Delivery: Antimicrobial Activity and Long-Term Stability

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Abstract
The use of natural antimicrobial peptides is a viable preservation alternative in the production of safe and good-quality products for consumption. Nanoliposomes containing nisin were prepared by film hydration with phosphatidylcholine (PC) and cholesterol, and coated with the polyelectrolytes (PEs) chitosan, cationic maltodextrin or poly-L-lysine (PLL), and characterized in their physical, thermal, functional and storage stability properties. As results, nisin encapsulation efficiency was around 90% for all formulations. The average diameter varied between 93.2 and 115.8 nm, with an increase in size after incorporation of PE, and the zeta potential ranged from + 11.3 to + 23.9 mV. These physical parameters showed good stability during 60 days of refrigeration (4 °C). The thermal characteristics of the liposomes were studied by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). An improvement in the thermal stability of liposomes coated with PEs was observed. Infrared spectroscopy (FTIR) revealed predominantly PC peaks as the bulk component of the nanostructures, but representative peaks of PEs and nisin suggested their presence on the surface of liposomes. Finally, antimicrobial activity was observed against Gram-positive bacteria (*L. monocytogenes*, *S. aureus*, and *B. cereus*) and Gram-negative bacteria (*E. coli* and *S. enterica*), in brain heart infusion (BHI), whole, and skimmed milk agar. The formulations containing PEs and nisin maintained the physical properties and antimicrobial activity after 60 days of storage. Therefore, liposomes coated with cationic PEs have the potential to deliver antimicrobial peptides to reduce undesirable bacteria in foods.

Keywords Nanotechnology · Liposomes · Nisin · Polyelectrolytes · Antimicrobial activity

Introduction

The use of natural compounds for improving food quality and safety has gained interest due to the increased demand for healthy and clean-label foods. This change in consumption patterns has increased the challenge for researchers and food industries. Due to the microbiological susceptibility of various food matrices, innovative processing technologies to extend shelf life and improve food safety have been proposed [1]. In this regard, natural antimicrobial agents are a promising alternative to control foodborne microorganisms [2, 3].

Among preservation technologies, bioconservation consists of the application of protective microbiota and/or its antimicrobial compounds, including antimicrobial peptides known as bacteriocins, which can be used to reduce and/or prevent pathogenic and spoilage microorganisms in foods [4, 5]. Different bacteriocins, such as nisin, pediocin, lacticin, and enterocin, among others, have been studied in food preservation. They are cationic peptides showing amphipathic properties and their target is often the bacterial membrane [6]. Nisin is the most used bacteriocin in the food

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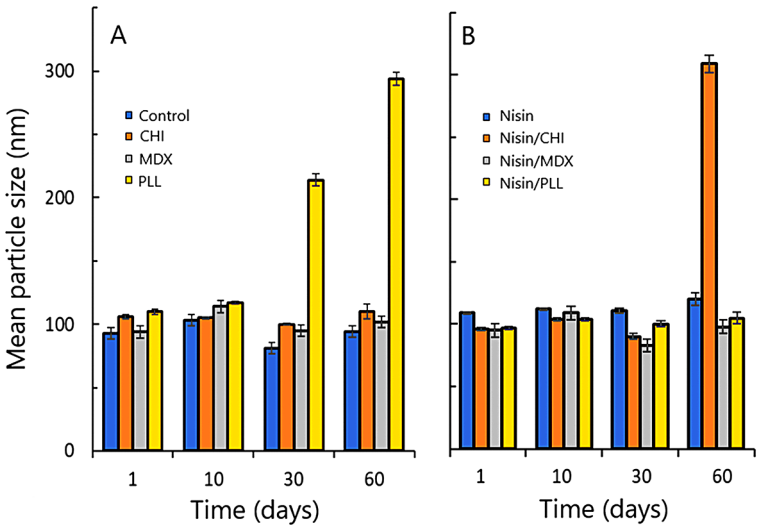

Fig. 1 Mean particle size of liposomes during long-term storage. Liposome formulations were prepared without (**A**) and with nisin encapsulation (**B**) and stored at 4ºC for up to 60 days. Control, liposomes prepared with PC/cholesterol; CHI, chitosan; MDX, maltodextrin; PLL, poly-L-lysine. Values are means ± standard deviations of three independent experiments

industry, recognized as safe by the Food and Drug Administration (FDA), European Food Safety Authority (EFSA), and other regulatory agencies, and potentially active against a large number of Gram-positive bacteria [7]. However, its effectiveness can be reduced when directly applied to food, due to undesirable interactions with food constituents during processing and storage, such as degradation by proteases or interaction with fat [8].

In this context, nanotechnology becomes an interesting alternative, often increasing the stability of encapsulated antimicrobials in foods when compared to their unencapsulated forms [9, 10]. The encapsulating material must be biodegradable, biocompatible, and stable in food during processing and storage, with phospholipids being one of the most studied materials for nanoencapsulation. Liposomes are colloidal structures formed by phospholipid membrane bilayers and an aqueous internal core, making them excellent carriers for the controlled release of active compounds [11]. In this regard, the encapsulation of nisin into liposomes can be effective in controlling pathogenic bacteria in food models and real foods [12, 13].

However, liposomes have limited colloidal stability, and their application can be compromised under some conditions of food processing and storage resulting in the burst release of incorporated ingredients [11]. In this regard, the use of coating materials such as biomacromolecules is an effective way to increase the stability of liposomes, reducing the damage to the lipid membrane and leakage of the encapsulated compound [4, 14]. Charged polysaccharides such as chitosan and pectin become an interesting alternative for covering the liposome surface to improve stability during food processing and storage [15–17]. However, the use of polysaccharides as a coating material for phospholipid membranes presents some limitations, such as high solubility in low pH, and the necessity of thickener compounds such as sodium alginate [16, 18]. Besides studies on chitosan, other cationic polyelectrolytes (PEs) with antimicrobial properties such as poly-L-lysine (PLL) or cationic maltodextrin have been poorly investigated as coating for liposome formulations.

Thus, the present work aims to develop phospholipid nanostructures with the incorporation of PEs (chitosan, cationic maltodextrin and poly-L-lysine) as coating material for the delivery of the bacteriocin nisin, carry out their characterization, storage stability, and evaluate their efficiency in controlling food-related pathogenic bacteria.

Materials and Methods

Chemicals

Nisin was obtained from the commercial product Nisaplin™ (Danisco do Brasil, Cotia, SP, Brazil). Chitosan (from crab shells, 85% deacetylated), poly-L-lysine (PLL, MW 1000–4000, 30000–70000 and > 300000 Da), maltodextrin, cholesterol, and glycidyl trimethylammonium chloride were from Sigma-Aldrich (St. Louis, MO, USA). Phospholipon 90G® was obtained from Lipoid (Ludwigshafen, Germany).

Nisin and Polyelectrolytes Preparation

The nisin stock solution was prepared as described previously [13], where 1 g of Nisaplin was dissolved in 10 mL of 0.1 M hydrochloric acid (HCl), subsequently filtered through 0.22 µm membranes, transferred to a sterilized tube and stored under refrigeration (4ºC). In the preparation of nanoencapsulated nisin, an initial concentration of 2.5 mg/mL of nisin was used, resulting in a final concentration of 0.8 mg/mL of nisin in the liposome formulations.

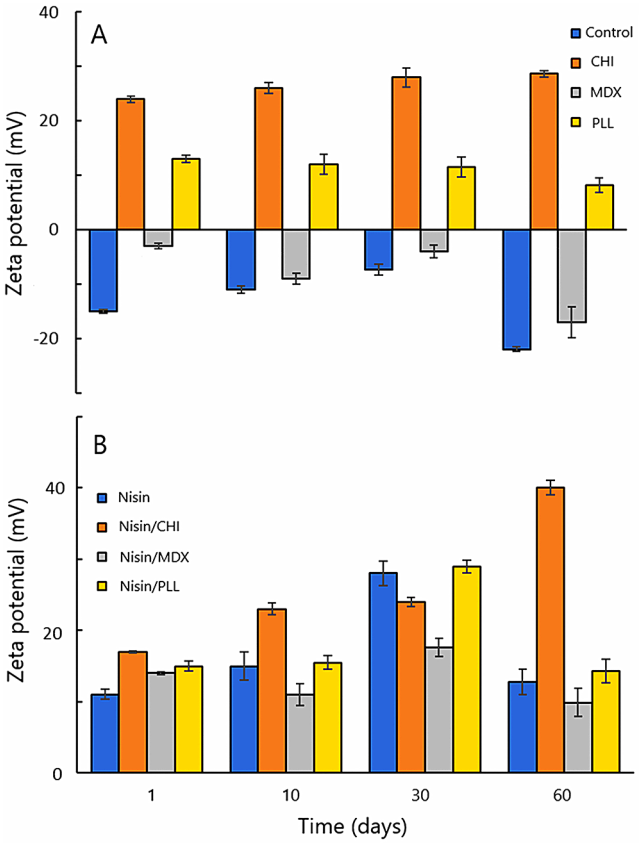

Fig. 2 Zeta potential (ζ) of liposomes during long-term storage. Liposome formulations were prepared without (**A**) and with nisin encapsulation (**B**) and stored at 7ºC for up to 60 days. Control, liposomes prepared with PC/cholesterol; CHI, chitosan; MDX, maltodextrin; PLL, poly-L-lysine. Values are means ± standard deviations of three independent experiments

The preparation of chitosan was adapted from a previous study [16]. Chitosan (0.3 g) was dissolved in 100 mL of 1.0% (v/v) acetic acid, under magnetic stirring at room temperature, for 24 h, generating a concentration of 3 mg/mL. The final concentration of chitosan in the liposome was 1.1 mg/mL.

Cationic maltodextrin was prepared from neutral maltodextrin by reaction with glycidyl trimethylammonium chloride, as described elsewhere [19] with some modifications, where 0.3 g of neutral maltodextrin was dissolved in 100 mL of 0.1% (v/v) acetic acid under magnetic stirring at room temperature for 2 h. An amount of 0.230 mL of glycidyl trimethylammonium chloride (hydroxycholine, cationic ligand) was added and mixed for 1 h to form the cationic polysaccharide. The initial concentration of cationic maltodextrin was 3 mg/mL, resulting in a final concentration of 1.1 mg/mL of maltodextrin in the liposome.

The preparation of PLL was adapted from a previous study [20], by dissolving 0.006 g of PLL in 6 mL of 10 mM sodium phosphate buffer (pH 7.0), under magnetic stirring at room temperature, for 3 h. For initial tests, three PLLs of different molecular weights (1–4 kDa, 30-70 kDa and > 300 kDa) were used. The initial concentration of PLL was 1 mg/mL, resulting in a final concentration of 0.4 mg/mL of PLL in the liposome.

Development of Liposomes

The encapsulation of nisin into liposomes was carried out using the thin film hydration method, adapted from a previous report [21]. Initially, a mixture of 0.076 g of purified phosphatidylcholine (PC, Phospholipon 90G®) and 0.0076 g of cholesterol were added to 15 mL chloroform and placed in a round-bottom flask. The solvent was evaporated by a rotary evaporator at 40ºC until obtaining a lipid film. The flask containing the lipid film was stored overnight in a desiccator to remove possible traces of solvent. The next step was the hydration of the film, with the addition of 2.5 mL of the solution containing nisin (2.5 mg/mL) and 2.5 mL of 10 mM sodium phosphate buffer (pH 7.0). Control liposomes were prepared using 5 mL of phosphate buffer only. Then, the flask with the mixture was subjected to brief heating (60 °C) followed by vigorous shaking in order to completely dilute the film. At the end of this process, the mixture was transferred to a test tube.

The test tube went through a sequence of heating in a water bath under stirring, at 60ºC, followed by vortexing and resting, respectively, totaling 3 cycles of 1 min each. After that, the PEs were incorporated into the liposomal dispersion by adding 3 mL of chitosan or cationic maltodextrin at a concentration of 3 mg/mL or PLL (30-70 kDa) at 1 mg/mL. Then, the size of the liposome particles was reduced and standardized, by using an ultrasound device (Unique OF S500, São Paulo, Brazil), operating at 50 kHz frequency, power 250 W, with 5 cycles performed 1 min at 50% power, under ice bath. Finally, the nanoliposomes were filter-sterilized with 0.22 µm membranes and stored under refrigeration (4ºC). Samples of liposomes covered with PEs but without nisin were produced in order to verify the antimicrobial properties of the nanostructured PE.

Particle Size, PDI and Zeta Potential (ζ)

The size, polydispersity index (PDI) and zeta potential (ζ) analyses of nanoliposomes were determined by dynamic light scattering and electrophoretic mobility techniques, using a Zetasizer® Nanoseries (Malvern Instruments, United Kingdom). To verify the stability of the liposomes over time, an aliquot was analyzed immediately after preparation, and after 10, 30, and 60 days under refrigeration. For the analysis, the samples were diluted in purified water (filtered through 0.45 µm membranes) in a ratio of 1:100 (v/v).

Encapsulation Efficiency (EE)

The encapsulation efficiency (EE) of nisin in the PE coating liposomes was determined by high-performance liquid chromatography (HPLC) with UV detection. The encapsulated nisin was separated from the non-encapsulated nisin by ultrafiltration (Ultracel YM-10 Membrane, Millipore, Burlington, MA, USA), at 4ºC and 10,000 rpm for 30 min. The amount of uncapsulated bacteriocin was established in the filtrate by HPLC based on a previously performed calibration curve of pure nisin, and using Eq. 1 to calculate the EE.

$$\%EE = \frac{\text{Nisin (used in preparation} - \text{in filtrate)}}{\text{Nisin used in preparation}} \times 100 \quad (1)$$

The HPLC analysis was carried out using an XBridge® C18 column (150×4.6 mm, 5 µm) (Waters, Milford, MA, USA) and the setup of the equipment was the same as reported previously [22].

Thermal Analyses

The thermal stability of the nanostructures was evaluated on a TGA Pyris 1 thermogravimetric analyzer (Perkin Elmer, Shelton, CT, USA). The analysis temperature was set at 20 °C to 600 °C, at a rate of 20 °C/min under a nitrogen atmosphere (flow rate of 40 mL/min).

The DSC analysis was carried out on the DSC Q8500 equipment (Perkin Elmer, Shelton, CT, USA). Approximately 10 mg of each sample were heated steadily from 0 °C to 200 °C in a hermetically sealed aluminum pan at a heating rate 10 °C/min under a nitrogen atmosphere with flow rate of 40 mL/min. As a reference, a clean and empty pan sealed with its lid was used. For both analyses (TGA and DSC), the liposome samples were previously lyophilized.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed to evaluate the intermolecular interactions between the nisin, lipid membrane and PE used in the formulations. FTIR spectra of all samples were obtained using a Shimadzu 8300 FTIR spectrophotometer (Shimadzu, Kyoto, Japan). The analyses were carried out using the KBr disk method and lyophilized samples, in the scan range of 4000 to 400 cm^{-1}, with a resolution set at 4 cm^{-1}, totaling 64 scans.

Antimicrobial Activity Evaluation

Antimicrobial activity was determined by diffusion test on BHI agar as described elsewhere [23]. In addition, the antimicrobial activity was investigated on milk agar to simulate a food system. This analysis was carried out using agar plates containing 10% (v/v) whole or skimmed UHT milk [12, 15]. The indicator microorganisms used for this analysis were *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 9634, *Staphylococcus aureus* ATCC 19095, *Escherichia coli* ATCC 25922 and *Salmonella enterica* sv. Enteritidis ATCC 13076. Serial dilutions of each liposome formulation were performed and 10 µL aliquots were applied to BHI, whole milk or skim milk plates previously inoculated in the surface, with the indicator microorganism (10^7 CFU/mL). The plates were incubated at 37 °C for 24 h. The activity unit (AU) per mL was given by the value resulting from the highest dilution that produced an inhibition zone.

To verify the possible decrease in the antimicrobial activity over the storage time, samples were analyzed immediately after preparation and during refrigeration storage at 4ºC for 10, 30 and 60 days.

Statistical Analysis

The results were analyzed by one-way ANOVA and Tukey means comparison test at 5% significance level, using the Statistica® software (StatSoft. Inc., Tulsa, USA) version 10.

Table 1 Physical characterization and encapsulation efficiency (EE) of liposomes

Liposome formulation	Size (nm)	Polydispersity (PDI)	Zeta potential (mV)	EE (%)
Control	93.2 ± 1.1^{c}	0.23 ± 0.01^{c}	-15.2 ± 0.4^{f}	-
Nisin	109.5 ± 0.7^{a}	0.32 ± 0.02^{a}	$+11.3 \pm 0.7^{d}$	88.9 ± 7.0
Chitosan	106.4 ± 1.4^{a}	0.27 ± 0.01^{b}	$+23.9 \pm 0.6^{a}$	-
Nisin/Chitosan	95.9 ± 0.5^{b}	0.20 ± 0.03^{b}	$+16.6 \pm 0.1^{c}$	94.2 ± 0.3
Maltodextrin	93.9 ± 0.7^{c}	0.27 ± 0.03^{b}	-3.0 ± 0.5^{e}	-
Nisin/Maltodextrin	$94.7 \pm 0.4^{b,c}$	0.20 ± 0.03^{c}	$+17.4 \pm 0.2^{b}$	93.8 ± 0.8
PLL *	110.1 ± 2.1^{a}	0.35 ± 0.02^{a}	$+12.6 \pm 0.7^{d}$	-
Nisin/PLL	96.6 ± 0.7^{b}	0.34 ± 0.01^{a}	$+15.2 \pm 0.7^{c}$	93.4 ± 0.1

Values are means ± standard deviations of three independent experiments. Different superscript letters indicate significant differences at $P < 0.05$

* PLL, poly-L-lysine; results of analysis (size and PDI) with 1/10 dilution. A bimodal distribution was observed, including particles on micrometric scale

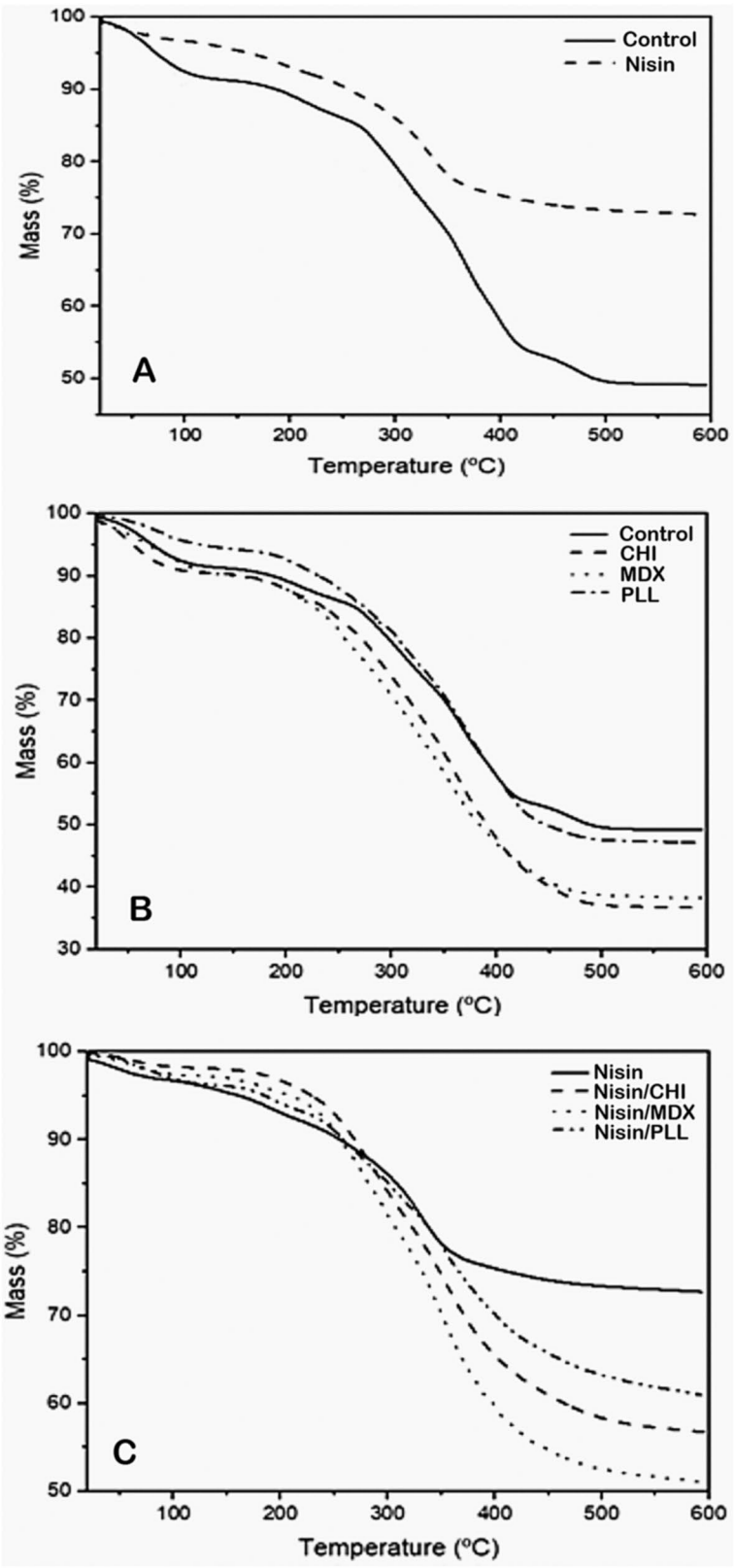

Fig. 3 TGA analysis of (**A**) liposomes control and with nisin encapsulation, (**B**) liposomes without nisin encapsulation, with polyelectrolyte coating, and (**C**) liposomes with nisin encapsulation and polyelectrolyte coating. Control, liposomes prepared with PC/cholesterol; CHI, chitosan; MDX, maltodextrin; PLL, poly-L-lysine

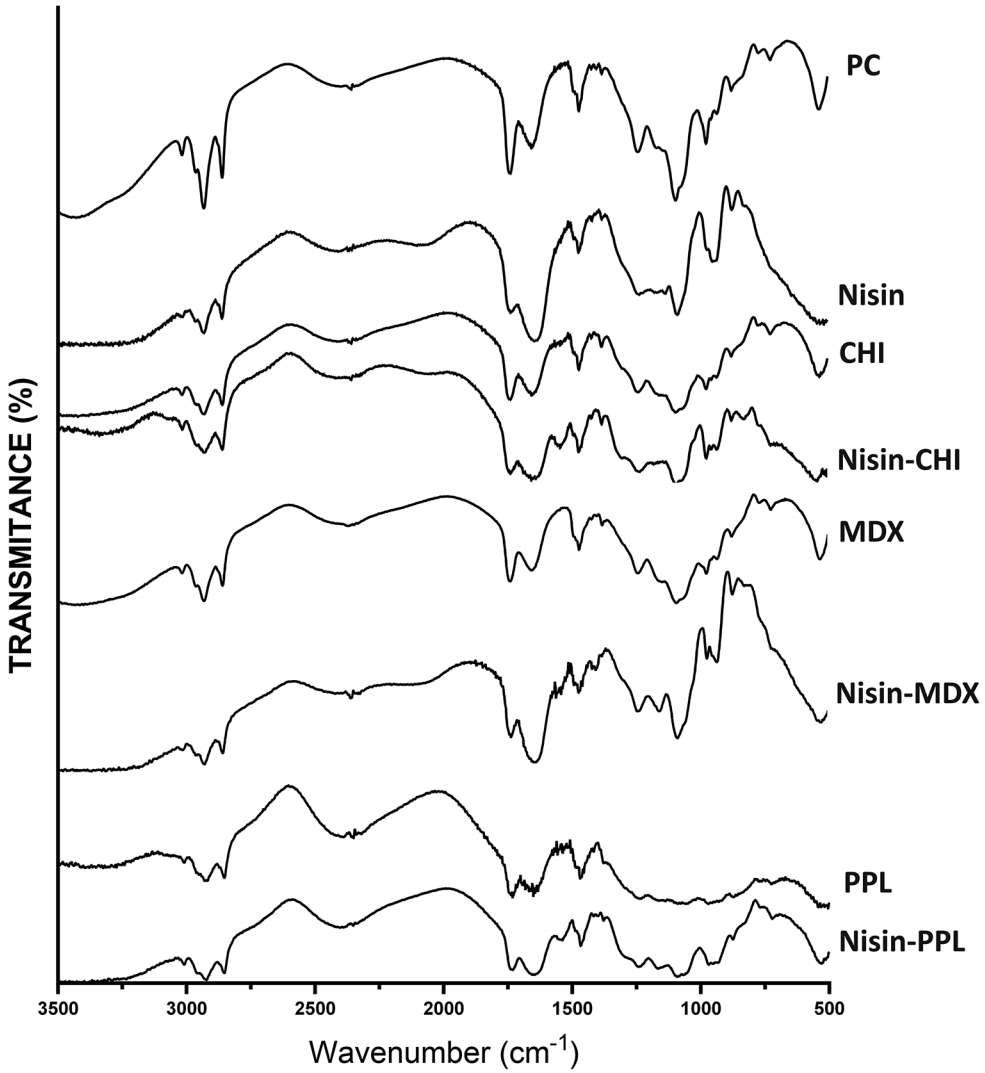

Fig. 4 FTIR spectrum analysis of unloaded liposomes (PC/cholesterol), liposomes containing nisin, liposomes with nisin and covered with chitosan (Nisin-CHI), cationic maltodextrin (Nisin-MDX) or poly-L-lysine (Nisin-PLL), and liposomes covered with the polyelectrolytes without nisin (CHI, MDX or PLL)

Results and Discussion

Production and Characterization of Liposomes

PLL is recognized as an antimicrobial compound, and commercially available in different molecular weight ranges: 1–4 kDa, 30–70 kDa, and ~300 kDa. To determine which PLL is more suitable for nisin-liposome formulations, a preliminary test was carried out, producing liposomes loaded with nisin and covering with the different PLLs.

This initial test resulted in liposomes with higher antimicrobial activity against Gram-positive bacteria observed for PLL 30–70 kDa (Figure S1). The antimicrobial activity of PLL vary according to its molecular weight (MW), and reason for the changes of activity related to the MW ranges remains unclear, however, the PLL activity can be improved with the presence of biocompatible polymers like chitosan, or food additives, such as nisin, glycine and organic acids [24–26]. Based on the results, formulations with PLL 30–70 kDa were selected for further experiments.

After the initial test for PLL selection, eight liposome formulations consisting of PC and cholesterol were produced, with or without nisin encapsulation, and they were coated with the PEs chitosan, cationic maltodextrin or poly-L-lysine. These liposomes were subjected to physical characterization, and the results of analyses performed at time 0 (shortly after the obtainment of liposomes) are summarized in Table 1.

Particle size and Polydispersity (PDI)

The particle size is a key property of any delivery system since it is related to the surface-to-volume ratio and may influence the release kinetics of the active compound. Nanostructures with low diameters tend to provide good solubility and bioavailability of the compounds due to the highest surface area [27].

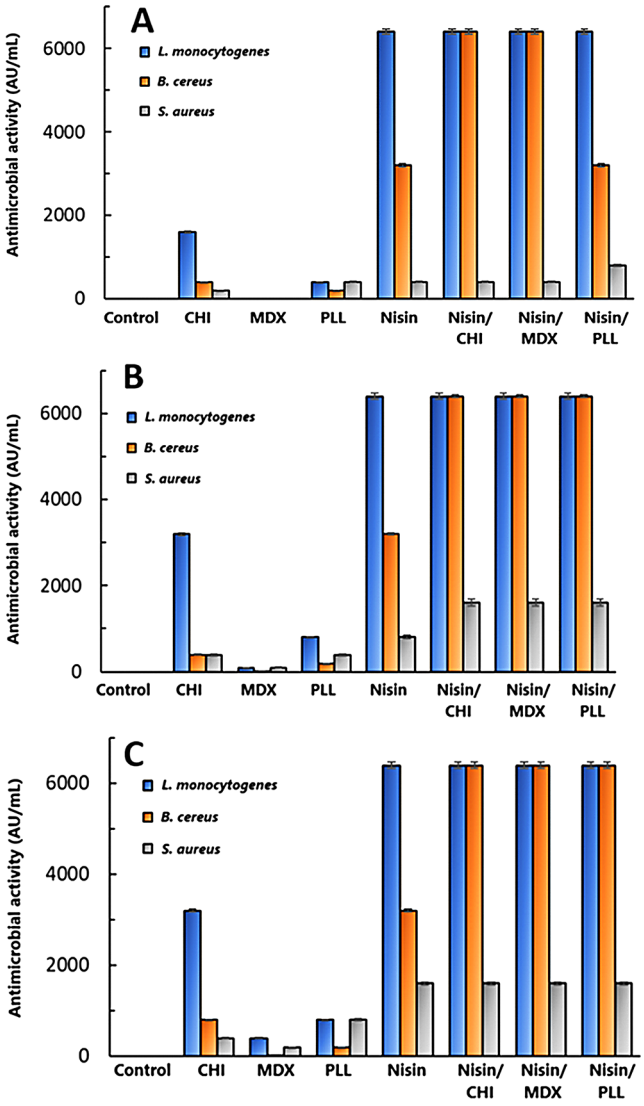

Fig. 5 Antimicrobial activity of polyelectrolyte-coated liposomes against Gram-positive bacteria, just after formulation. (**A**) BHI agar, (**B**) Whole milk agar, (**C**) skimmed milk agar. Control liposomes prepared with PC/cholesterol; CHI, chitosan; MDX, maltodextrin; PLL, poly-L-lysine. Values are means ± standard deviations of three independent experiments

In general, liposomes had an average diameter in the range between 93 and 110 nm, as well as PDI between 0.2 and 0.3, indicating a narrow size distribution for the samples. These values are similar to those described in previous studies, which developed polysaccharide-covered liposomes with an average diameter between 88 and 135 nm and PDI between 0.2 and 0.3 [13, 28]. Different results were reported in the chitosan coating of dipalmitoyl phosphatidylcholine (DPPC) liposomes, which resulted in a peripheral increase in the diameter of the structures, adding a layer of 92 ± 27 nm [29]. PDI values are related to the size distribution of nanostructures, and values between 0.2 and 0.3 are considered adequate for peptide encapsulation, indicating a good homogeneity of the nanostructures regarding size [30].

Liposomes containing nisin showed larger size than control liposomes (PC). This can be attributed to antimicrobial peptide retention at the nucleus and phospholipid bilayers. The same can be observed in liposomes with PEs, where an increase in nanostructure size due to coating has been described [15, 16, 31].

The liposome with PLL coating showed a higher mean diameter and PDI, regardless the MW of PLL used. From this initial result, the analysis of this formulation was carried out in a less diluted form, where a multimodal distribution was observed in two peaks, suggesting a mixture of micrometer-sized structures (2 μm) and nanometric structures of 110 nm for PLL (Table 1). This is likely due to the irregular coverage of the liposome surface, enhancing electrostatic interactions that drove the system to aggregation. Similar patchy adsorption of the cationic PLL was reported previously on the surface of bovine serum albumin (BSA) nanoparticles [32]. In addition, the increase in particle size of negatively charged liposomes by PLL has been previously demonstrated, showing aggregation of molecules and size distribution in the micrometric scale [33]. The interaction between liposomes and PLL could result in the formation of either single PLL-coated vesicles or PLL-vesicle aggregates, which is strongly affected by the molecular mass of PLL and the PLL/lipid molar ratio [34]. However, it is important to mention that nisin was essential in this type of structure, as reduced the formation of aggregates in the liposomes containing PLL.

Zeta Potential (ζ)

The zeta potential, an important parameter for the stability of nanostructures, is related to the surface charges of the particles. The values obtained for the different liposome formulations are presented in Table 1. Most nanostructures showed positive zeta potential (between + 11 mV and + 23 mV), which was expected due to the use of cationic PEs and/or incorporation of nisin, a cationic peptide [35, 36]. Negative values were found for the control liposome (PC) (-15.2 mV), similar to those found in other studies for PC liposomes without cholesterol [13] or containing cholesterol [21]. PC nanoliposomes may present this result due to the negative charge of the phosphate groups.

The maltodextrin-coated liposomes showed negative values of zeta potential (-3.0 mV), which was initially not expected, due to the use of cationic PE. However, this may be associated with the predominance of negative charges present in the control liposome (PC), indicating that the degree of ionic interactions between maltodextrin and the liposome were not sufficient to counterbalance the charge

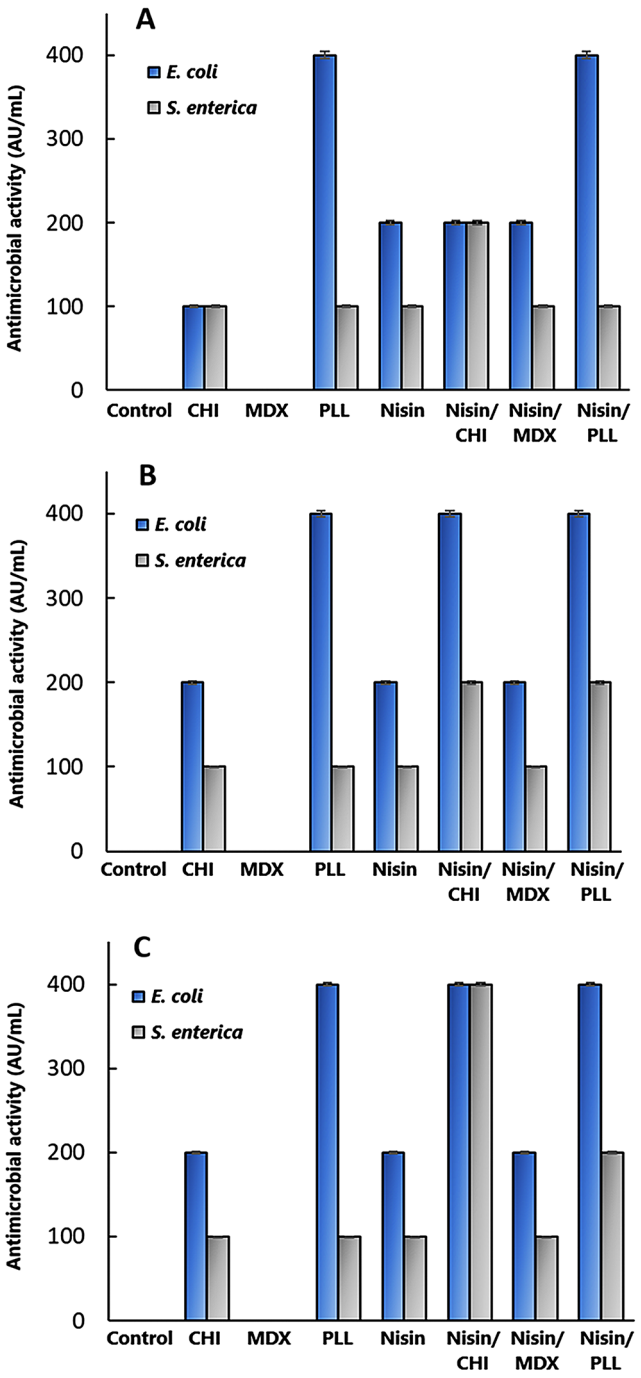

Fig. 6 Antimicrobial activity of polyelectrolyte coated liposomes against Gram-negative bacteria, just after formulation. (**A**) BHI agar, (**B**) whole milk agar, (**C**) skimmed milk agar. Control, liposomes prepared with PC/cholesterol; CHI, chitosan; MDX, maltodextrin; PLL, poly-L-lysine. Values are means ± standard deviations of three independent experiments

density on the liposome surface. This result could be explained by the hypothesis of the presence of a gradient of counterions, which finds support in the theory of "soft" particles [37]. During the synthesis of cationic maltodextrin, cationic groups provided by the quaternary ammonium graft were associated with anionic counterions [19], and as result of the liposome preparation, cationic groups could be oriented to interact with anionic phosphate groups of the PC, generating a gradient of anionic counterions towards the particle surface.

Zeta potential values above ±30 mV suggest electrostatic repulsion between particles and increased stability of the nanostructure, while values close to 0 mV indicate lower particle repulsion, therefore, the possibility of aggregation, which tends to decrease its stability [38]. However, good stability of nanoparticles, even with a zeta potential value closer to neutrality, has already been reported for other liposome formulations [39, 40].

Encapsulation Efficiency (EE)

The analysis of EE, an important parameter providing an indication of the amount of bioactive compound that is successfully entrapped/adsorbed in the nanoparticle, was performed on liposomes with nisin encapsulation. The EE was 88.9% for nisin encapsulated in PC liposomes, and similar values were observed for those with PE cover, ranging from 93.4 to 94.2% (Table 1). The liposome formulations included cholesterol, which has demonstrated a stabilizing effect on liposomes through its interaction with phospholipid molecules, making the nanostructure more rigid, thus reducing the possibility of particle aggregation [41]. In this regard, the EE of nisin in PC liposomes was increased from 80.7 to 90.7% when cholesterol was included in the formulation [21]. In general, the EE values observed in this study were superior as compared to other reports of nisin encapsulation in pectin-coated and chitosan-coated liposomes, which reported values around 75% and 77%, respectively [13, 16]. However, nisin encapsulation into liposomes covered with PLL and cationic maltodextrin has not been reported.

Evaluation of Physical Parameters during long-term Storage

The size of the liposomes was evaluated after 10, 30 and 60 days, under refrigeration at 4ºC, and the results are summarized in Fig. 1.

Over two months, the average diameter of the liposomes remained unchanged, except for the sample with PLL coating, which increased in mean particle size during the period (Fig. 1A). Among the formulations with nisin encapsulation, only liposomes with chitosan coating increased in size after 60 days under refrigeration (Fig. 1B). The PDI values of most liposomes remained in the range of 0.2 to 0.35 during storage (Figure S2). However, the liposomes that showed an increase in size also had an increase in PDI, particularly

those with PLL coating without nisin encapsulation (Figure S2).

Low temperature tends to delay the oxidative degradation of fatty acids present in the lipid bilayer of liposomes, but particle size modification can be caused by structural changes due to the hydrolysis of phospholipids [21]. The encapsulation of nisin in polysaccharide-coated liposomes was evaluated during storage at 7ºC, and the results demonstrated degradation and instability, with an increase in PDI, after 28 days [15]. The encapsulation of nisin and extract of garlic in PC liposomes underwent size change after 3 months under refrigeration [21]. It is important to highlight that changes in physical properties do not always result in the collapse of the colloidal suspension, therefore, only the change in the size of the nanostructure is not sufficient to demonstrate loss of activity.

During the period evaluated, the nanostructures demonstrated some changes in zeta potential (Fig. 2). As result, the absence of nisin in the formulation resulted in a high variation of surface charge in the liposomes during the storage, varying from positive to negative values (Fig. 2A). On the other hand, the presence of nisin resulted in liposomes that, besides changes in the values, maintained the initial positive surface charge (Fig. 2B). A previous study reported that incorporation of nisin can significantly alter the surface charges of liposomes due to structural rearrangements during storage leading to surface exposure of nisin [42], leading to modification of the zeta potential, as already observed for PC liposomes with encapsulated nisin after 14 days at 7 °C or after 7 days at 25 °C [15]. Thus, the phenomenon of charge rearrangement in the liposomes without nisin may be driven by weak interactions between the cationic coatings and the cholesterol. These interactions were likely improved by the nisin due to H-bonding between N-H group of nisin to the O-H groups of the cationic coatings, resulting in stronger peptide-polymer intermolecular interactions [43].

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was performed to evaluate the thermal stability of the nanostructures, and the results are presented in Fig. 3. The data from Fig. 3A indicate that in the first stage at a temperature of up to 100ºC, the loss of residual moisture corresponded to 3 to 13% of mass. The second stage, where degradation of the phospholipids occurs, took place at a temperature above 130ºC, with a mass loss of 27% for the nisin liposome and up to 50% for the control liposome. From this, it can be observed that the presence of nisin considerably improved the thermal stability of the nanostructure, with a lower mass loss when compared to the control liposome (Fig. 3A).

Figure 3B shows TGA data from liposomes with a PE coating, without encapsulated nisin. It can be observed that the loss of residual moisture, which occurs up to 100ºC, was 4 to 9% by mass. The degradation of phospholipids occurred at temperatures above 170ºC, with a mass loss of 50 to 63%, similarly to that observed for liposomes covered with PEs. Comparing the three PEs evaluated, PLL showed better thermal stability.

Figure 3C illustrates the thermal stability of liposomes containing nisin and coated with PEs. The loss of residual moisture, which occurs up to 100ºC, was 2 to 4% by mass. The degradation of phospholipids occurred at temperatures above 200ºC, with a mass loss of 27 to 49%. It can be observed that the degradation of coated liposomes started at a higher temperature (> 200ºC), related to those without PE coating, indicating an improvement in thermal stability when using the PEs. Comparing the three PEs evaluated, it can be seen that nisin-loaded liposomes covered with chitosan showed better thermal stability up to 300ºC when compared to other formulations.

In all experiments, the presence of PEs improved the stability of the nanostructures at temperatures below 300ºC. A similar increase in thermal stability was observed in the TGA analysis of PC liposomes containing oleic acid compared to pure PC liposomes, suggesting that intercalation of oleic acid into the bilayer promotes a reduction in lipid mobility in the bilayer [44]. Similar degradation curves were previously observed for chitosan-coated liposomes encapsulating nisin Z [16]. In this sense, the insertion of PE molecules intercalated in the liposome bilayers could cause a certain reduction in the molecular mobility of the lipid membrane, causing an increase in its rigidity.

Differential Scanning Calorimetry (DSC)

DSC analysis is a versatile analytical technique that allows the evaluation of the thermal stability of nanostructures. It is an important analysis for nanostructures containing PEs, as it can be used in a wide heating kinetic range and isothermal conditions [45]. The results of the DSC analysis are illustrated in Figure S3.

DSC is a technique that allows study the effects of coatings or stabilizers on liposome preparation and also helps to determine their phase transition temperature (T_m), which reflects the transition of molecules in the lipid bilayer from a covered all-trans hydrocarbon conformation to a phase in which there is an increase in the rotational freedom of the lipid [21]. Anhydrous PC liposomes have T_m values around 100ºC but shifts to higher temperatures in the multilamellar vesicle arrangement can be observed [46, 47]. The DSC thermograms of liposomes with PEs did not show defined peaks, similar to

that described for amorphous systems containing polysaccharides and/or proteins [48, 49]. In general, this behavior can be associated with cryogenic cooling after the first scan, the microcrystalline interactions of macromolecules do not have enough time to be formed resulting in amorphous materials [50]. The presence of multiple components such as lipids, peptides, and carbohydrates in the formulation can result in complex phase structures and different interactions that influence the main phase transition temperature of the resulting nanostructures. Similar DSC scans were also reported in DPPC liposomes coated with 5 mg/mL chitosan [29], and for PC/phosphatidylglycerol liposomes covered by PLL [33].

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is considered an important tool for analyzing structural changes produced by the entrapment of different compounds present in the liposome, in different parts of the lipid bilayer, by detecting different functional groups of the compounds present in the sample [44]. The nanostructures were analyzed by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra obtained for the liposomes are shown in Fig. 4.

The FTIR spectrum of the control liposome (PC, Fig. 4) presented peaks at 2924 and 2852 cm^{-1} that correspond to the stretching of the C-H bond of fatty acids. At 1732 cm^{-1}, peaks related to the ester bond between glycerol and fatty acids were observed, while the peak observed at 1465 cm^{-1} is typical of the amide group. The peak at 1232 cm^{-1} corresponds to the stretching of the C-N bond of the choline functional group, while the peak at 1089 cm^{-1} is characteristic of the phosphate group [51]. The FTIR spectra obtained for liposomes containing nisin with PE coating (Nis-CHI, Nis-MDX and Nis-PLL) showed the typical peaks of PC, that represent the bulk component of the nanostructures. The predominance of typical PC peaks could be expected, as its quantity is higher than the concentration of nisin in the formulations [52].

The spectra of liposomes containing nisin, with or without PE coating, showed similar intensity in the peak corresponding to the amide group at 1465 cm^{-1} and the high intensity of the peak at 1640 cm^{-1}, which is attributed to bending of primary amines, this fact indicate the presence of nisin on the liposome surface. In general, the FTIR spectra of nanoliposomes showed most of the peaks at close wavenumbers. However, some differences in the regions of 1000–1250 cm^{-1} and 1600–1750 cm^{-1} suggest that the presence of PEs on the surface of the liposomes has caused intermolecular interactions between these substances, the phospholipids, and the peptide nisin.

Antimicrobial Activity

The antimicrobial capacity of the liposomes was investigated using BHI agar and agar plates containing two types of milk, in order to simulate a complex food matrix and evaluate changes in antimicrobial activity depending on the fat content in the media.

The antimicrobial activity of the liposomes was monitored at time 0 (immediately after formulation) and after 10, 30 and 60 days of storage under refrigeration (4 °C). The inhibition of Gram-positive bacteria *L. monocytogenes*, *S. aureus* and *B. cereus*, as well as the Gram-negative bacteria *E. coli* and *S. enterica* sv. Enteritidis, was evaluated.

Activity against Gram-positive bacteria

Liposomes encapsulating nisin showed antimicrobial activity against *L. monocytogenes*, *S. aureus* and *B. cereus*, regardless of the medium used for evaluation (Fig. 5). The activity against *L. monocytogenes* for all liposomes containing nisin was 6400 AU/mL, the maximum value for antimicrobial activity observed in this study. Furthermore, antimicrobial activities of 1600 AU/mL and 400 AU/mL were recorded for unloaded liposomes coated with chitosan or poly-L-lysine, respectively, while maltodextrin did not show any activity against *L. monocytogenes* in BHI medium (Fig. 5A). The liposomes containing nisin showed an activity of 3200 AU/mL against *B. cereus* but increased to 6400 AU/mL when coated with chitosan or maltodextrin (Fig. 5A). Moreover, the highest antimicrobial activity against *S. aureus* was observed for the nanostructure containing nisin with poly-L-lysine coating, showing 800 AU/mL. Thus, some antimicrobial activity could be associated with the PE coating, as some liposomes without nisin showed activity, and improved effects were observed against *B. cereus* and *S. aureus* for liposomes containing nisin.

The antimicrobial activity of unencapsulated nisin is usually similar to encapsulated nisin, however unencapsulated nisin tends to have poor performance when directly added to food, due to interactions with components such as fat and proteins, resulting in reduced antimicrobial activity [53, 54]. In agreement with our results, nisin Z encapsulated in liposomes covered with chitosan demonstrated good antimicrobial activity against *L. monocytogenes* and increased activity against *S. aureus*, as compared with the unencapsulated nisin [16].

The antimicrobial activity was also evaluated in milk agar as a food-simulating model, using either whole UHT milk (Fig. 5B) or skimmed UHT milk (Fig. 5C). The antimicrobial activity of some liposomes was increased when tested on milk agar, in particular those without nisin encapsulation. Furthermore, the antimicrobial activity of

liposomes containing nisin reached 6400 AU/mL against *B. cereus* with poly-L-lysine coating and 1600 AU/mL against *S. aureus* with PE coating in either whole or skimmed milk agar. This increase in antimicrobial activity may be related to the variety of components present in milk, probably with synergism between the PE and some of the milk components, such as lysozyme and lactoferrin, which are natural antimicrobials present in this food [55]. In addition, the increased antimicrobial activity can be attributed to higher stability and the augmented EE of the PE-coated liposomes (Table 1) compared to the uncoated liposomes. Another possibility for this increased activity may be related to a greater release of the antimicrobial, which would be stimulated by the interaction of casein with liposomes. This interaction can be induced by the strong electrostatic attraction of negatively charged caseins with cationic PEs and between the hydrophobic domains of the casein and the lipid bilayer [56], causing structural instability and thus, modulating the release of nisin. However, due to most of the studies of nisin interaction with milk components being based in unencapsulated nisin [57], more in-depth studies on the nanostructured nisin interactions should be carried out in order to better understand this putative mechanism.

Activity against Gram-negative bacteria

Soon after formulation, liposomes encapsulated with nisin showed antimicrobial activity against the Gram-negative bacteria *E. coli* and *S. enterica* sv. Enteritidis, when applied to BHI agar, whole milk agar and skimmed milk agar, as shown in Fig. 6.

From the results presented in Fig. 6A, it can be seen that an antimicrobial activity of 200 UA/mL against *E. coli* was found for liposomes containing nisin, with or without the addition of PEs, but increasing to 400 AU/mL with PLL coating. Liposomes without nisin encapsulation showed an activity of 100 UA/mL and 400 UA/mL for chitosan and PLL coating, respectively, suggesting that the antimicrobial effect against Gram-negative bacteria was due to the PE in the case of PLL coating. When the same nanostructures were evaluated against *S. enterica*, an antimicrobial activity of 100 AU/mL was found for the liposomes containing nisin, and coating with chitosan PE improved the activity to 200 AU/mL, probably because chitosan itself already showed certain activity. Although nisin is recognized by the weak activity against Gram-negative bacteria due to nisin cannot penetrate the outer membrane barrier of Gram-negative bacteria. Some nanostructured nisin formulations have been described as inhibitory against *Escherichia coli* O157:H7, using nisin/γ-PGA/chitosan nanoparticles [58]; and *E. coli* O157:H7 and *S. Typhimurium* by nisin-loaded chitosan (N-CS) nanoparticles [59].

As seen in Fig. 6B and C, in milk agar as a food model the efficiency of the antimicrobial activity of some liposomes increase as compared with the BHI medium. When tested against *E. coli*, the improvement was observed for chitosan-coated liposomes, with or without nisin encapsulation. In the case of *S. enterica*, an improvement in activity was observed for nisin/chitosan liposomes (200 to 400 UA/mL) in skimmed milk agar, and for nisin/PLL (100 to 200 UA/mL) in whole milk agar.

Long-term Stability of Antimicrobial Activity

These same liposomes, stored under refrigeration at 4ºC, were evaluated after 10, 30 and 60 days against Gram-positive bacteria. The results are presented in Figure S4. Nisin-containing liposomes, with and without PE coating, maintained maximum activity (6400 UA/mL) against *L. monocytogenes* for up to 30 days, regardless of the medium in which it was analyzed. After 60 days under refrigeration, a reduction in antimicrobial activity was observed but at least 800 AU/mL was observed against *L. monocytogenes*.

The antimicrobial activity against *B. cereus* decreased during storage, although at least 800 AU/mL could be observed at 60 days in liposomes without PE coating. In general, a pronounced decrease occurred in the first 10 or 30 days depending on the formulation. However, higher values of antimicrobial activity were often observed for formulations containing PEs in all cultivation media tested. Furthermore, liposomes coated with PEs showed greater activity against *B. cereus* when tested in whole or skimmed milk agar as compared with BHI (Figure S4). The antimicrobial activity was lower against *S. aureus* and also decreased during storage in all liposome preparations. However, the liposomes with chitosan or PLL coatings showed activity even at 60 days, particularly in whole milk agar and skimmed milk agar.

Briefly, it can be highlighted that the best activities were observed for liposomes with PE coating, which often presented high activity even after 60 days under refrigeration. Thus, it was possible to observe that the use of PEs improved efficiency. Furthermore, the results from milk agar as food-simulating media indicate these liposomes could be successfully used in foods, demonstrating a possible improvement in antimicrobial activity. It is important to highlight that, even though there was a considerable loss of antimicrobial activity in some liposomes, they were maintained in liquid suspension for a long storage time, considering this type of nanostructure.

Similar to our results, nanoliposomes coated with pectin and encapsulating a mixture of nisin and lysozyme were used to control growth of *L. monocytogenes* in liquid whole and skimmed milk at refrigeration temperatures, resulted in

a better reduction of the pathogen in milk, as compare with the uncapsulated antimicrobials [13]. The antimicrobial stability of liposomes with nisin encapsulation and chitosan coating was observed against the gram-positive bacteria *L. monocytogenes* and *S. aureus*, evaluated for 7 days, suffering a reduction of up to 50% after this period [16]. This positive result is due to the synergistic antibacterial effect of nisin and chitosan, including the ability to increase stability and EE due to the improved bioavailability of the antimicrobial peptide.

The same evaluation was realized for gram-negative bacteria. In general, the antimicrobial activity was reduced by 50% for *E. coli* and *S. enterica* after 30 and 10 days, respectively. Complete activity loss was observed for *S. enterica* at day 60 for all liposomes tested, while activity against *E. coli* remained at 100–200 AU/mL in milk agar (data not shown).

Conclusion

Liposomes containing nisin, a natural antimicrobial peptide, and coated with chitosan, cationic maltodextrin and PLL, were obtained successfully, presenting around 100 nm of diameter, with high encapsulation efficiency of nisin. The incorporation of the PEs positively affected the thermal characteristics of the liposomes. Regarding the antimicrobial activity, nisin/chitosan liposome stood out, followed by the nisin/PLL liposome, both of which showed good activity against the bacteria *L. monocytogenes*, *S. aureus* and *B. cereus*. The activity against the bacteria *S. enterica* and *E. coli* was relatively lower. An improvement in activity was observed in the evaluation on milk agar, as a food system model. The antimicrobial activity of all liposomes demonstrated a continuous decrease in activity during the 60-day period under refrigeration, being observed a greater stability in the nanostructures containing PEs. The results found can be used as a starting point to obtain nanoliposomes with encapsulation of antimicrobial peptides covered with cationic PEs, with consequent release of bioactive compounds for a longer period of time.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Compliance with ethical standards This study does not include research involving human participants or animals.

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