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INHIBITION OF *LISTERIA MONOCYTOGENES* IN MINAS FRESCAL CHEESE BY FREE AND NANOVESICLE-ENCAPSULATED NISIN

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ABSTRACT

The effectiveness of free and nanovesicle-encapsulated nisin to control *Listeria monocytogenes* in Minas Frescal cheese was investigated. Commercial nisin was encapsulated into liposomes of partially purified soy lecithin. Free (0.1 mg/mL and 0.25 mg/mL) and nanovesicle-encapsulated nisin (0.25 mg/mL) were applied onto the surface of cheese samples, and *L. monocytogenes* was inoculated before incubation at 6-8°C for 28 days. A bactericidal effect was observed with 0.25 mg/mL free nisin; a bacteriostatic effect was observed for liposome-encapsulated nisin and 0.1 mg/mL free nisin. Free nisin was more efficient than nisin-loaded liposomes in controlling *L. monocytogenes*. Possible reasons for this behavior, and also the significance of nisin to soft cheeses are discussed. Nisin acted as a suitable barrier within hurdle technology, potentially extending the shelf-life and safety of fresh cheeses.

Key words: nisin, liposome, encapsulation, cheese, *Listeria monocytogenes*

INTRODUCTION

Food preservation and microbiological quality represent major concerns and challenges to the food industry. Much effort has been focused on the application of antimicrobial peptides as potential biopreservatives in hurdle technology. The most extensively studied bacteriocin is nisin, which is approved for food applications and has gained a widespread industrial significance. This bacteriocin, produced by strains of *Lactococus lactis* subsp. *lactis*, shows a broad inhibitory spectrum against Gram-positive bacteria like *Listeria* spp. and *Staphylococcus* spp., also inhibiting the outgrowth of spores of Bacilli and Clostridia (1). Nisin is frequently added directly to food systems as commercial products, an application in which loss of antimicrobial activity usually occurs over time because of enzymatic degradation and interactions with food components, such as proteins and lipids (19).

Nanotechnology is recognized as a potential source of novel products and processes for the food industry (17). However, only limited investigation in nanotechnology has been performed on foods and food-related products, and the global development of nanofoods seems to be on its initial stage (6). Thus, encapsulation technology is exploited as an alternative to protect antimicrobials, potentially enhancing their

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efficacy and stability in foods (11). Encapsulation into liposome, composed by one or more phospholipid bilayers encapsulating a volume of aqueous media, appears as promising alternative (13).

Fresh cheeses generally have a short shelf-life, even under refrigeration. Minas Frescal is an example of such cheese, presenting high pH (4.9-6.7), high moisture content (>55%), and low percentage of salt (1.4-1.6%) (4,5,18). These characteristics facilitate the development of bacteria that, in addition to limiting the product shelf-life, may pose a health hazard to consumers, since pathogenic bacteria could be found in such cheeses (15). Particularly, the ability to grow under refrigeration makes *Listeria monocytogens* a foodborne pathogen of special concern to the dairy industry, and this bacterium was previously found in fresh and soft cheeses (4,5,8). The aim of this study was to evaluate the effectiveness of free (unencapsulated) and nanovesicle-encapsulated nisin to control *L. monocytogenes* in Minas Frescal fresh cheese surfaces at 6-8°C.

MATERIALS AND METHODS

Nisin encapsulation

Commercial nisin (Nisaplin), containing 2.5% pure nisin A, was purchased from Danisco Brasil Ltda. A nisin solution was prepared with 0.01 M HCl and was filter-sterilized through 0.22 µm membranes (Millipore). Before each experiment, nisin was diluted with 10 mM phosphate buffer (pH 6.4) to reach concentrations of 0.1 and 0.25 mg/mL pure nisin.

Nisin encapsulation into partially purified soybean phosphatidylcholine liposomes was carried out by the thin-film hydration method (9). The size of the unilamellar nanovesicles was determined by light scattering (22). Encapsulated and unencapsulated nisin were separated by ultrafiltration through 10-kDa cut-off membranes (Ultracel YM-10 Membrane, Millipore). Entrapment efficiency (EE) was determined using the agar diffusion method, and calculated as previously reported (9).

Antimicrobial activity assay

Antimicrobial activity of free and encapsulated nisin was detected by the agar diffusion assay performed in BHI agar plates inoculated with *L. monocytogenes* ATCC 7644, and expressed as activity units per mL (AU/mL) (9).

L. monocytogenes ATCC 7644 was maintained on Brain-Heart Infusion (BHI) agar plates at 4ºC, and subcultured periodically. Before each experiment, this strain was grown in BHI broth at 37ºC for 18-24 h in an orbital shaker (125 rpm).

Inhibition of *L. monocytogenes* in Minas cheese

Free (0.1 mg/mL and 0.25 mg/mL) or liposome-encapsulated nisin (0.25 mg/mL) were applied onto the surface of Minas Frescal cheese cubes of approximately 10 g, and maintained for 30 min. An overnight culture of *L. monocytogenes* was diluted in saline solution (8.5 g/L NaCl). Then, 500 µL of this suspension (~4.5 log CFU/mL) was inoculated into the cheese surface before incubation at 6-8°C for 28 days. At defined intervals, cheese samples (10 g) were homogenized with 90 ml of Listeria Enrichment Broth (Acumedia, Baltimore, MA, USA) in a blender for 60 s, followed by decimal serial dilutions. Counts were carried out in duplicate on Oxford Listeria selective agar (Acumedia) plates.

Recovery of nisin activity

For recovery of nisin activity, free and nanovesicle-encapsulated nisin (0.25 mg/mL) were applied onto the surface of 5 g cheese cubes and two methods were tested: (a) the cubes were homogenized with 10 mM phosphate buffer (pH 6.4); and (b) the cubes were homogenized with 10 mM phosphate buffer (pH 6.4) containing 0.2% (w/v) Tween 80 and the mixture was heated for 15 min at 60 °C in a water bath (2). Then, for both methods, the homogenates were centrifuged at 10,000 × g for 30 min, and the antimicrobial activity was assessed by the agar diffusion assay.

RESULTS

The antimicrobial activity of 0.25 and 0.1 mg/mL free

nisin was 400 and 200 AU/mL, respectively, and liposome-encapsulated nisin showed an antimicrobial activity of 400 AU/mL. Nisin-loaded liposomes presented a mean diameter of 140 nm, with an EE of 100% (data not shown). Initial counts of *L. monocytogenes* inoculated into Minas Frescal cheese were around 2.7 log CFU/mL. In control treatments (without nisin), counts increased to approximately 5 log CFU/mL after 28 days of incubation under refrigeration (Table 1).

Table 1. Counts of inoculated *Listeria monocytogenes* in Minas Frescal cheese under refrigeration (6-8°C), treated with free or liposome-encapsulated nisin[a]

Time (days)	Control	Free nisin (0.10 mg/ml)	Free nisin (0.25 mg/ml)	Encapsulated nisin (0.25 mg/ml)
0	2.87 ± 0.10	2.79 ± 0.00	2.87 ± 0.10	2.87 ± 0.10
7	3.03 ± 0.40	1.98 ± 0.14	n.d.	1.97 ± 0.04
14	3.75 ± 0.76	2.60 ± 0.22	n.d.	2.19 ± 0.28
21	4.15 ± 0.30	2.48 ± 0.57	n.d.	3.02 ± 0.12
28	5.07 ± 0.41	3.21 ± 0.98	n.d.	3.42 ± 0.60

[a] The values represent mean log CFU/mL ± SD of two replicates; n.d., not detected.

Treatment of cheese with nisin (free or encapsulated forms) prior to inoculation with *L. monocytogenes* resulted in lower counts when compared to the controls. Regarding free nisin, the higher concentration (0.25 mg/mL) exhibited a bactericidal effect against *L. monocytogenes*. The inferior detection limit of the method employed in microbial counts (~1.69 log CFU/mL) (12) was reached after 7 days, and such effect was maintained until day 28 (Table 1). At lower concentration (0.1 mg/mL), free nisin presented a bacteriostatic effect throughout the experiment, although *L. monocytogenes* counts tended to increase as time progressed. Similarly to 0.1 mg/mL free nisin, liposome-encapsulated nisin also presented a bacteriostatic effect (Table 1).

Although attempts to recover nisin from the cheese samples were not successful (data not shown), it appeared that the rate of nisin release from liposomes was sufficient to inhibit *L. monocytogenes* growth in comparison to the control (Table 1).

DISCUSSION

The antimicrobial effect of nisin and other bacteriocins in cheeses is mainly investigated through the direct addition of the bacteriocin during cheese manufacture or post-production, or by inoculation of cheese milk with bacteriocinogenic starters (3,16,19). However, the successful utilization of encapsulated nisin in reducing *L. monocytogenes* counts in fluid model systems (10,20,22) also suggests its potential usefulness in (semi-)solid matrices, such as cheeses. Liposomes were selected as nisin carriers due to the presence of both lipid and aqueous phases in its structure, allowing the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials (13). As nisin is a cationic amphiphilic peptide, this bacteriocin could be both encapsulated in the inner aqueous phase of liposomes and immobilized into liposome membranes (11).

Differences in the effect of free (bactericidal) and encapsulated nisin (bacteriostatic) at 0.25 mg/mL might indicate that nisin have been strongly associated to the phospholipidic vesicles, as previously suggested for fluid systems (10), being gradually released from liposomes. Insertion of nisin into liposomes of PC, a lipid not commonly found in prokaryotic membranes, might cause the stabilization of the nanovesicles, possibly through a lowering of curvature stresses (21). Accordingly, nisin-loaded PC liposomes showed the slowest apparent release of antimicrobial when compared to liposomes manufactured with PC:phosphatidylglycerol (60:40) (20). Additionally, lecithin was showed to inhibit nisin activity, possibly through the formation of stable nisin-phospholipid complexes (7).

Presence of *L. monocytogenes* in soft, fresh cheeses is usually related to the utilization of unpasteurized contaminated milk or, when pasteurization is employed, postprocessing contamination (8,18). As counts of *L. monocytogenes* found in such cheeses are probably lower than that employed in this experiment, nisin in its free or encapsulated forms might efficiently inhibit the growth of this pathogen during refrigerated storage. In this context, control of *L. monocytogenes* growth for 28 days is well correlated with the shelf-life of Minas Frescal cheeses (25-33 days) (5).

Nascimento *et al* (15) reported that nisin application in Minas Frescal cheese gains further importance since counts of *L. monocytogenes* inoculated into this cheese, manufactured using a commercial starter with adjunct bacteriocinogenic cultures, showed no significant differences when compared to cheeses produced without bacteriocinogenic cultures. On the other hand, according to Naldini *et al* (14), *L. monocytogenes* counts remained almost unchanged during storage (25 days at 5 or 10°C) of Minas Frescal cheeses manufactured with the addition of starter culture (traditional process), whereas an increase in counts of 2-3 log cycles was observed in cheeses manufactured by direct acidification (14). These results were attributed to pH lowering through lactic acid production, and competition of the lactic culture with *L. monocytogenes*. *Listeria* spp. was found in 22.6% and 12.9% of Minas Frescal cheeses manufactured by direct acidification and by the traditional process, respectively (5). Therefore, both free and encapsulated nisin forms might be employed in Minas Frescal cheeses manufactured either by the traditional process or direct acidification, aiming to restrict *L. monocytogenes* growth if contamination occurs, and adding to the effective safety of these products. Also, the storage of Minas Frescal cheese at 10°C increased *L. monocytogenes* growth when compared to storage at 5°C (14), reinforcing the significance of nisin's antimicrobial activity within the hurdle technology concept.

Little information is available on the effect of antimicrobial loaded liposomes in cheese. Previously, liposome-entrapped nisin was reported to improve nisin stability and its inhibitory action against *L. monocytogenes* in Cheddar cheese matrix during ripening (2). These results, divergent from those obtained in the current study, might be related to differences in the materials and protocols employed: for instance, purified phospholipids and purified nisin Z were utilized by Benech *et al.* (2) to produce nisin-loaded liposomes, and the nisin-loaded liposomes were added to milk during cheese manufacture.

Due to the scarce information available on this topic, the current investigation might contribute to assess the feasibility and suitability of applying nisin-loaded nanovesicles in semi-solid food matrices, such as cheeses. As encapsulation was observed to protect the starter cultures from the detrimental action of nisin, not affecting the fermentation process during cheese production (2), the possibility to apply nisin-loaded liposomes to milk during the manufacture of Minas Frescal cheese through the traditional process (which employs starter cultures) warrants further investigation.

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