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Effect of liposome-encapsulated nisin and bacteriocin-like substance P34 on *Listeria monocytogenes* growth in Minas frescal cheese

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ABSTRACT

The efficacy of liposome-encapsulated nisin and bacteriocin-like substance (BLS) P34 to control growth of *Listeria monocytogenes* in Minas frescal cheese was investigated. Nisin and BLS P34 were encapsulated in partially purified soybean phosphatidylcholine (PC-1) and PC-1-cholesterol (7:3) liposomes. PC-1 nanovesicles were previously characterized. PC-1-cholesterol encapsulated nisin and BLS P34 presented, respectively, 218 nm and 158 nm diameters, zeta potential of −64 mV and −53 mV, and entrapment efficiency of 88.9% and 100%. All treatments reduced the population of *L. monocytogenes* compared to the control during 21 days of storage of Minas frescal cheese at 7 °C. However, nisin and BLS P34 encapsulated in PC-1-cholesterol liposomes were less efficient in controlling *L. monocytogenes* growth in comparison with free and PC-1 liposome-encapsulated bacteriocins. The highest inhibitory effect was observed for nisin and BLS P34 encapsulated in PC-1 liposomes after 10 days of storage of the product. The encapsulation of bacteriocins in liposomes of partially purified soybean phosphatidylcholine may be a promising technology for the control of food-borne pathogens in cheeses.

1. Introduction

Encapsulation technology has been shown to protect antimicrobials from interfering food components, potentially enhancing their efficacy and stability. Among the materials used for the encapsulation of antimicrobials, liposomes appear as the most studied (Were et al., 2003; Laridi et al., 2003; Malheiros et al., 2010a; Sant'Anna et al., 2011). Liposomes are colloidal particles consisting of a membranous system formed by lipid bilayers encapsulating aqueous space(s). As liposomes contain both lipid and aqueous phases, they can be utilized for the entrapment, delivery, and release of water soluble, lipid-soluble, and amphiphilic materials (de Duve et al., 1978; Mozafari et al., 2008).

Bacteriocins are antimicrobial substances of polypeptidic nature that are not lethal to the producing cells (Cotter et al., 2005). These antimicrobial substances differ from traditional antibiotics by mechanisms of synthesis, mode of action, antimicrobial spectrum, toxicity and resistance mechanism (Montville and Chen, 1998; Sang and Blecha, 2008). The best studied bacteriocin is nisin, recognized as safe for food use by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives. Nisin is produced by strains of *Lactococus lactis* subsp. *lactis*, and its importance is due to its wide spectrum of activity against Gram-positive bacteria and also spores of bacilli and clostridia (Sobrino-López and Martín-Belloso, 2008; Arauz et al., 2009).

Besides nisin, other bacteriocins are being evaluated with respect to their potential for use in food. Production of bacteriocins or bacteriocin-like substances (BLS) has been described for many species of the genus *Bacillus* (Motta et al., 2004; Kamoun et al., 2005; Lisboa et al., 2006). *Bacillus* sp. P34, a strain isolated from the fish Piau-com-pinta (*Leporinus* sp.) of the Brazilian Amazon basin, produces a bacteriocin like substance (BLS P34) that inhibits a broad range of bacterial species, including *Listeria monocytogenes* (Motta et al., 2007a). Purified peptide P34 showed low in vitro toxicity to eukaryotic cells with a similar effect to that observed for nisin, showing great potential for safe application in foods (Vaucher et al., 2010).

Minas frescal cheese is a fresh cheese largely consumed in Brazil. It has high water activity, pH above 5.0, low salt content and contains no preservatives, resulting in good substrate for growth of microorganisms (Souza and Saad, 2009). Growth of molds and overgrowth of lactic acid bacteria causing sliminess, off-odors and acidity frequently occur after 10–12 days under refrigeration. In Brazil, Minas cheese is produced by large dairy industries and also by small manufacturing plants, where *L. monocytogenes* as an environmental contaminant is common (Barancelli et al., 2011). Several surveys have shown that *L. monocytogenes* is frequently detected in Minas cheese (Carvalho et al., 2007; Brito et al., 2008).

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Some studies have shown that application of bacteriocins to cheeses can be an additional hurdle to reduce the levels of *L. monocytogenes*. However, direct application of bacteriocins may result in decrease or loss of antimicrobial activity due to problems related to interaction with food components (Jung et al., 1992; Aasen et al., 2003; Chollet et al., 2008). To circumvent this problem, encapsulation of bacteriocins in liposomes may represent a promising alternative technology.

In this context, the aim of this study was to evaluate the inhibitory effect of commercial nisin and BLS P34 encapsulated in nanometric liposomes against *L. monocytogenes* in Minas frescal cheese.

2. Material and methods

2.1. Nisin preparation

Commercial nisin (Nisaplin) was purchased from Danisco Brasil Ltda (Pirapozinho, Brazil). Nisin was dissolved in 0.01 M HCl, and filter sterilized through 0.22 μm membranes. Before each experiment, nisin was diluted with 10 mM phosphate buffer (pH 6.4) to reach concentrations of 0.25 mg/ml of pure nisin.

2.2. Bacterial strains and growth conditions

Bacillus sp. P34, isolated from the intestine of the fish *Leporinus* sp., was used for production of BLS P34 (Motta et al., 2004). *L. monocytogenes* ATCC 7644 was used as the indicator organism for the bacteriocin activity assay. *L. monocytogenes* isolated from Minas frescal cheese (4 strains), Mozzarella cheese (1 strain), sausage (3 strains), salami (2 strains), chicken (3 strains), and the strain Scott A were used in inhibitory assays in milk agar plates. The strains were maintained on BHI agar plates at 4 °C, and sub-cultured periodically.

L. monocytogenes ATCC 7644 was chosen for experiments in Minas frescal cheese since this strain has been previously used as indicator strain for antimicrobial activity of both BLS P34 and nisin (Motta et al., 2007b; Malheiros et al., 2010b). For application in the Minas cheeses, cultures of *L. monocytogenes* ATCC 7644 obtained in Tryptic Soy Broth supplemented with 0.6% yeast extract (TSB-YE) (37 °C/18 h) were centrifuged at 5,000 ×*g* for 10 min to 4 °C. The supernatant fluid was discarded and the pellet was suspended in 0.1% peptone water, followed by centrifugation. After the supernatant removal, the pellet was suspended in 0.1% peptone water and the suspension was diluted in 0.1% peptone water to reach 10^4 CFU/ml. This suspension was added to 200 g of cheese mass in order to obtain the concentration of approximately 10^2 CFU/g.

2.3. Production of BLS P34

Partial purification of BLS P34 was described by Motta et al. (2007b). *Bacillus* sp. P34 was cultivated aerobically in BHI broth at 30 °C in an orbital shaker at 180 rpm for 24 h. Cells were harvested by centrifugation, and the resulting supernatant was filtered through 0.22 μm membranes. The culture filtrate was submitted to precipitation with ammonium sulfate at 20% (w/v) saturation. The resulting pellet was resuspended in 10 mM sodium phosphate buffer at pH 7.0 and applied to a gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala, Sweden) and eluted with 10 mM sodium phosphate buffer at pH 7.0. Fractions with antimicrobial activity were stored at 4 °C until needed.

2.4. Nisin and BLS P34 encapsulation

Encapsulation of nisin and BLS P34 in liposomes of partially purified soybean phosphatidylcholine (PC-1) and PC-1-cholesterol (7:3) was carried out by the thin-film hydration method, according to Mertins et al. (2008) and Malheiros et al. (2010b). PC-1 or PC-1-cholesterol was dissolved with chloroform in a round-bottom flask and the solvent was removed by a rotary evaporator until a thin film was formed on the flask walls. The resulting dried lipid film was dispersed by the addition of phosphate buffer containing nisin or BLS P34 under vigorous stirring at 60 °C. The preparation was sonicated in a bath-type ultrasound (40 kHz, Unique USC 700) for 30 min and filtered through 0.22 μm membranes. Size and polydispersity (PDI), encapsulation efficiency (EE), and zeta potential of bacteriocins encapsulated in PC-1-cholesterol liposomes were determined according to Malheiros et al., 2010b.

2.5. Antimicrobial activity assay

The antimicrobial activity was detected by agar diffusion assay (Motta and Brandelli, 2002). Aliquots of 10 μl of the testing materials were applied on BHI agar plates previously inoculated with a suspension of *L. monocytogenes* ATCC 7644 (10^7 CFU/ml). Plates were incubated at 37 °C for 24 h. The reciprocal value of the highest dilution where an inhibition zone was observed was taken as activity units per ml (AU/ml).

2.6. Inhibition of different L. monocytogenes strains in milk agar

Aliquots (15 μl, 1600 AU/ml) of free and encapsulated nisin or BLS P34 were applied onto milk agar plates (Riffel and Brandelli, 2006) previously inoculated with different *L. monocytogenes* strains (0.5 McFarland turbidity standard solution). The strains evaluated were isolated from Minas frescal cheese (4), Mozzarella (1), sausage (3), salami (2), and chicken (3). The strains Scott A and ATCC 7644 were included in this panel. A cocktail prepared with the strains isolated from cheese products was also tested. Plates were incubated at 37 °C for 24 h and the inhibition zones indicating the presence of antimicrobial activity were measured.

2.7. Minas frescal cheese preparation

Minas cheese was prepared according to Scholz (1995). Six liters of pasteurized cow's milk, purchased at a local market, was heated to 34 ± 1 °C, and 2.5 ml of lactic acid (2.5%), 3 ml $CaCl_2$ (saturated solution), and 5.4 ml of commercial rennet Estrella (85% bovine pepsin + 15% bovine chymosin; Chr. Hansen, Valinhos, Brazil) were added. After coagulation (about 40 min), curd cutting was performed for 30 min under low agitation. Sodium chloride (2.0 g/l) and free or encapsulated bacteriocins were added to the curd to reach 6.25 mg/g of cheese. After 30 min, a population of 4 log CFU/ml of *L. monocytogenes* was inoculated in the curd. The curd was transferred to perforated sterile plastic circular cheese containers (15 cm in diameter) and maintained at 21 °C for 1 h for dripping. The cheeses were unmolded, packed in plastic bags, and stored under refrigeration (6–8 °C). Each package contained approximately 200 g of cheese.

The experimental cheese groups were: (a) cheese containing only *L. monocytogenes*; (b) cheese containing free nisin and *L. monocytogenes*; (c) cheese containing nisin encapsulated in PC-1-cholesterol liposomes and *L. monocytogenes*; (d) cheese containing nisin encapsulated in PC-1 liposomes and *L. monocytogenes*; (e) cheese containing free BLS P34 and *L. monocytogenes*; (f) cheese containing BLS P34 encapsulated in PC-1-cholesterol liposomes and *L. monocytogenes*; (g) cheese containing BLS P34 encapsulated in PC-1 liposomes and *L. monocytogenes*; and (h) cheese without any addition.

2.8. Determination of pH, water activity and L. monocytogenes counts in the cheeses during storage

Physico-chemical parameters of the cheeses stored under refrigeration (7 °C) were monitored for 21 days, which corresponds to the claimed shelf-life of this product (Buriti et al., 2005a). The pH values of control cheeses (treatment H) were determined on triplicate samples (three different cheeses of the same trial) with a pH meter Analyser