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Antilisterial activity and stability of nanovesicle-encapsulated antimicrobial peptide P34 in milk

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ABSTRACT

Bacillus sp. P34, a strain isolated from aquatic environments of Brazilian Amazon basin, produces a bacteriocin-like substance (BLS) which was encapsulated in nanovesicles prepared from partially purified soy lecithin. The efficiency of free and encapsulated BLS P34 to control the development of *L. monocytogenes* and maintenance of antimicrobial activity was assessed over time in milk. The antimicrobial activity of free and encapsulated BLS P34 decreased approximately 50% after 4 days of storage (<4 °C) in skim and whole milk. After this period there was not significant loss of activity up to 21 days. The viable counts of *Listeria monocytogenes* in skim and whole milk containing 3200 AU/ml of free or encapsulated BLS P34 were always lower than those observed in controls without bacteriocin at both 30 °C and 7 °C. At 1600 AU/ml concentration, free and encapsulated BLS P34 were inhibitory to *L. monocytogenes* in skim milk, when compared with the control at 7 days. Nanovesicle-encapsulated and free BLS P34 shows potential use as biopreservative for application in milk-derived products.

1. Introduction

Bacteriocins are antimicrobial peptides widespread produced among bacteria that may show varied antimicrobial spectra (Cotter, Hill, & Ross, 2005). Those produced by Gram-positive bacteria, particularly by lactic acid bacteria, have been largely studied with the perspective of food protection against pathogenic and spoilage microorganisms (Arauz, Jozala, Mazzola, & Penna, 2009; Cleveland, Montville, Nes, & Chikindas, 2001). Their proteinaceous nature implies a putative degradation in the gastro-intestinal tract of human and animals, suggesting that some bacteriocin-producing lactic acid bacteria or purified bacteriocins could be used as natural preservatives in foods (Cleveland et al., 2001). Bacteriocins are also produced by several other classes of bacteria (Riley & Wertz, 2002).

Bacillus has been reported as a safe and interesting bacterial genus for utilization in food industry (Pedersen, Bjørnvad, Rasmussen, & Petersen, 2002). Production of bacteriocins or bacteriocin-like substances (BLS) has been described for many species of the genus *Bacillus* including *B. subtilis* (Zheng, Yan, Vederas, & Suber, 1999), *B. thuringiensis* (Kamoun et al., 2005), *B. amyloliquefaciens* (Lisboa, Bonatto, Bizani, Henriques, & Brandelli, 2006), *Bacillus licheniformis* (Cladera-Olivera, Caron, & Brandelli, 2004) and *B. cereus* (Bizani & Brandelli, 2002). *Bacillus* sp. strain P34 was isolated from aquatic environments of Brazilian Amazon basin, and its antimicrobial activity was described as a BLS active against important Gram-positive pathogenic bacteria like *Listeria monocytogenes* and *Bacillus cereus* (Motta, Cladera-Olivera, & Brandelli, 2004). This antimicrobial substance has a molecular mass of 1456 Da, was relatively heat stable and sensitive to proteolytic enzymes (Motta, Cannavan, Tsai, & Brandelli, 2007a). In cytotoxicity tests, BLS P34 shows similar behavior to nisin, a bacteriocin wide accepted for utilization in food industry (Vaucher, Motta, & Brandelli, 2010).According to its properties of size and protein stability data, BLS P34 could be associated with the group of Listeria-active class Ib bacteriocins (Motta, Lorenzini, & Brandelli, 2007b). This antimicrobial substance has the cell membrane as target of action, promoting loss of protoplasmic material and consequently the death of the cell (Motta, Flores, Souto, & Brandelli, 2008).

Bacteriocins or bacteriocin-like substances can lose their antimicrobial activity in many food products for a variety of reasons. Interference and cross-reactions of the antimicrobial with various food constituents, such as protein and fat, are difficult to overcome and often require large amounts of antimicrobial in order to gain significant reductions in the pathogen load in a product (Taylor, Bruce, Weiss, & Davidson, 2008). The efficiency of these peptides in food products may be increased by their incorporation into

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nanovesicles, enhancing the stability by a protective effect against endogenous food proteases or binding to food compounds (Laridi et al., 2003; Malheiros, Daroit, & Brandelli, 2010; Malheiros, Daroit, Silveira, & Brandelli, 2010; Teixeira, Santos, Silveira, & Brandelli, 2008; Were, Bruce, Davidson, & Weiss, 2003). Liposome, a closed phospholipid bilayer membrane, has a nano-order interface harboring hydration layer and non-polar (hydrophobic) layer on its surface. Due to the presence of both lipid and aqueous phases in the structure of lipid vesicles, they can be utilized in the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials (Khosravi-Darani, Pardakhty, Honarpisheh, Rao, & Mozafari, 2007; Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008).

Nanovesicles to delivery of bioactive components with substantiated health benefits of the foods will be required to meet the challenges in developing healthy foods, which are aimed at reducing the risks of target diseases in a population (Sanguansri & Augustin, 2006). A microorganism that causes disease is *L. monocytogenes,* which is capable of surviving environmental conditions that are normally fatal to many other bacteria, enabling this pathogen to remain active in foods and eventually establish infection after consumption (Cunningham, O'Byrne, & Oliver, 2009). The aim of the present study was to assess the effect of free and nanovesicles-encapsulated BLS P34 against *L. monocytogenes* in milk. Thereafter, the maintenance of antimicrobial activity was assessed over time in milk.

2. Materials and methods

2.1. Bacterial strains and media

Bacillus sp. P34, an isolate from intestine of the teleost Piau-com-pinta (*Leporinus* sp.) from Amazon basin, was the producer strain and was previously characterized (Motta et al., 2004). *L. monocytogenes* ATCC 7644 was used as the indicator organism for the bacteriocin activity assay. The strains were maintained on BHI agar plates at 4 °C, and subcultured periodically. Before each experiment, this strain was grown in BHI medium at 37 °C for 18–24 h in a rotary shaker (180 rpm).

2.2. Production of BLS P34

For the production of BLS, the producer strain was aerobically cultivated in 150 ml BHI medium in orbital shaker at 180 rpm for 24 h. The culture was centrifuged at 10,000 *g* for 15 min at 4 °C, and the supernatant was sterilized with a 0.22 μm membranes. The filtrate was precipitated with ammonium sulfate at 20% saturation. The precipitate was dissolved in 10 mmol/l sodium phosphate buffer pH 7.0. This solution was further purified by gel filtration chromatography on a Sephadex G-100 column, and active fractions were pooled. The partially purified BLS P34 was stored in sterile flasks at 4 °C until used (Motta et al., 2007b).

2.3. BLS P34 encapsulation

Encapsulation of BLS P34 in nanovesicles of partially purified soybean phosphatidylcholine (PC-1; Mertins, Sebben, Schneider, Pohlmann, & Silveira, 2008) was carried out by the thin-film hydration method according to Malheiros, Micheletto, Silveira & Brandelli, 2010. Summarizing, PC-1 was dissolved in chloroform and the solvent was removed by a rotary evaporator until a thin film was formed on the flask walls. The dried lipid film was dispersed by the addition of sodium phosphate buffer containing BLS P34. These mixtures were then agitated (60 °C) and sonicated in bath-type ultrasound (40 kHz, Unique USC 700). The size of nanovesicles was determined by light scattering (Teixeira et al., 2008). The entrapment efficiency (EE) was determined using antimicrobial activity assay. Discrimination of encapsulated from non-encapsulated BLS P34 was performed by ultrafiltration (Ultracel YM-50 Membrane, Millipore) and the EE was calculated (Malheiros, Micheletto, Silveira, et al., 2010).

2.4. Antimicrobial activity assay

Antimicrobial activity was determined essentially as described elsewhere (Motta & Brandelli, 2002). BLS P34 was diluted by serial two-fold dilution method (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128), and an aliquot of 10 μl each dilution was applied onto BHI agar plates inoculated with a swab submerged in *L. monocytogenes* ATCC 7644 (approximately 7 log CFU/ml). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and was expressed as activity unit (AU) per milliliter.

2.5. Determination of BLS P34 activity in milk

UHT skim milk (0% fat) and UHT whole milk (3.25% fat) were purchased from a local market. Free and nanovesicles-encapsulated BLS P34 was independently applied to skim and whole milk to produce an initial activity of 800 AU/ml. The samples were kept under refrigeration (4 °C) and the antimicrobial activity was evaluated by 21 days as described above.

2.6. Inhibitory effect of free and encapsulated BLS P34 in milk

L.monocytogenes culture (9 log CFU/ml) was diluted in saline solution (8.5 g/l NaCl) to count approximately 6 log CFU/ml, and 500 μl of this suspension was added to 10 ml of whole and skim milk. After, 1 ml of free and nanovesicles-encapsulated BLS P34 (3200 AU/ml) was added to tube. Controls were inoculated with *L. monocytogenes* and sodium phosphate buffer (10 mmol/l pH 7.0). The tubes were incubated for 0, 2, 4, 6 and 24 h at 30 °C.

The effect of 1600 AU/ml and 3200 AU/ml of free and encapsulated BLS P34 was assessed in whole and skim milk at refrigeration temperature (7 ± 1 °C). Counts of *L. monocytogenes* were estimated at 0, 7 and 14 days for 1600 AU/ml and 0, 2, 5, 8, 10 and 14 days for 3200 AU/ml (Malheiros, Daroit, Silveira, et al., 2010). The quantification of *L. monocytogenes* was performed by the drop culture method (Malheiros, Brandelli, Noreña, & Tondo, 2009). The colonies were counted in BHI agar plates after 24 h of incubation at 37 °C.

2.7. Statistical analysis

All experimental treatments were tested in duplicate, and averages were calculated for treatments at each time point. Obtained counts were compared using test *t* of Tukey. Data analyses were performed with the software Statistica 7.1, and differences were considered significant at $P < 0.05$.

3. Results

3.1. Encapsulation of BLS P34

BLS P34 was encapsulated into nanovesicles of partially purified soybean phosphatidylcholine by hydration film method. Nanovesicles presented a mean diameter of 160 nm and the entrapment efficiency was 100%.

3.2. Antimicrobial activity of free and encapsulated BLS P34 in milk

The antimicrobial activities of free and encapsulated BLS P34 were evaluated for a period of 21 days in milk (Fig. 1). It was observed that there was no significant difference ($P > 0.05$) in antimicrobial activity between skim and whole milk. There was a loss of approximately 50% of antimicrobial activity after 4 days of storage at 4 °C in both samples containing free or encapsulated BLS P34. After this period there was no significant loss of activity for up to 21 days for all samples and treatments.

3.3. Inhibitory effect of free and encapsulated BLS P34 in milk

Free BLS P34 (3200 AU/ml) caused a decrease in *L. monocytogenes* counts below the detection limit of the method on skim milk at 30 °C during 6 h; however, after 24 h of incubation the microbial counts were of approximately 4 log CFU/ml. When encapsulated BLS P34 was tested, it was observed 2 log reduction in counts of *L. monocytogenes* at 6 h without significant increase until 24 h (Fig. 2A). A decrease of 4 log cycles in *L. monocytogenes* counts was observed for free and encapsulated BLS P34 versus control at 24 h in skim milk at 30 °C. In the case of whole milk at 30 °C, it was not observed significant growth of *L. monocytogenes* for free and encapsulated BLS P34 until 6 h (Fig. 2B). Only after 24 h there was significant ($P < 0.05$) growth of *L. monocytogenes* (for both treatments), but 1-2 log cycles lower than the control.

Two concentrations of BLS P34 were tested for refrigeration temperature (7 ± 1 °C). At lower concentration (1600 AU/ml) in skim milk, free and encapsulated BLS P34 were inhibitory to *L. monocytogenes* cells compared to the control (~2.5 log CFU/ml reduction) at 7 days (Fig. 3A). In whole milk both treatments tested showed no inhibitory effect on the pathogen for up to 14 days (Fig. 3B).

A concentration of 3200 AU/ml of free or encapsulated BLS P34 was very effective in reducing viable counts in skim milk (Fig. 4A). In the second day of incubation encapsulated BLS P34 showed a decrease of 2.5 log CFU/ml compared to the control, whereas free BLS P34 caused a decrease in *L. monocytogenes* counts below the

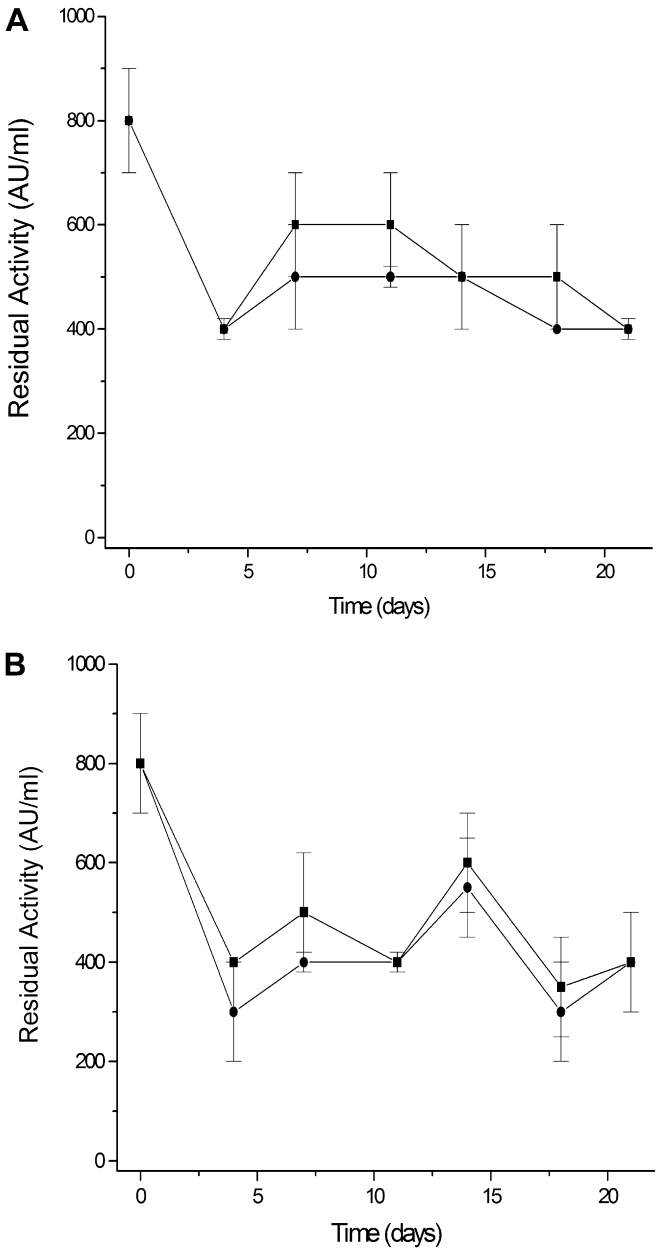

Fig. 1. Residual antimicrobial activity of free and encapsulated BLS P34 in skim milk (A) and whole milk (B) as a function of storage time at 4 °C. (■) Free BLS P34 and (●) encapsulated BLS P34. Values are means of two independent experiments.

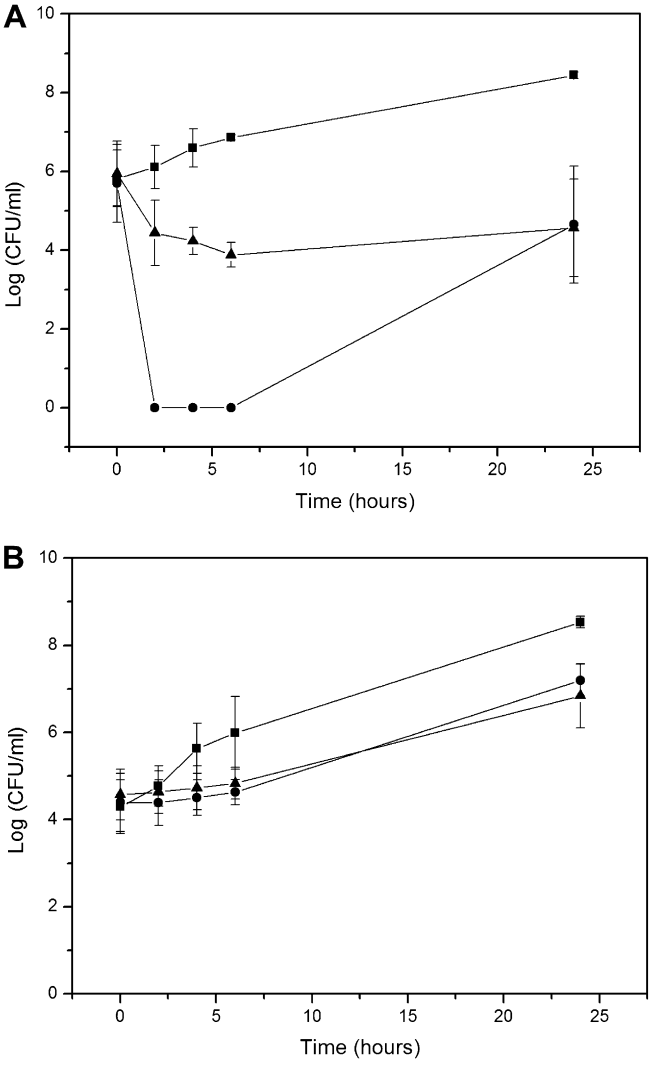

Fig. 2. Viable cell counts of *Listeria monocytogenes*, confronted to 3200 AU/ml of free BLS P34 (●), encapsulated BLS P34 (▲) and control (■) in skim milk (A) and whole milk (B) at 30 °C.

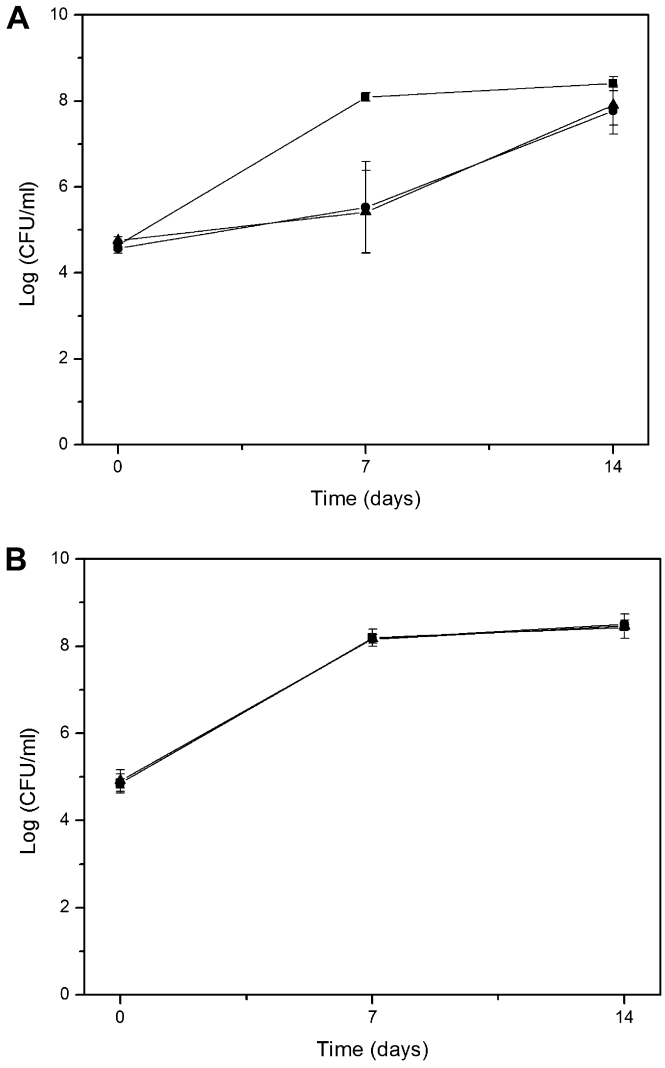

Fig. 3. Viable cell counts of *Listeria monocytogenes* in skim milk (A) and whole milk (B) at 7 ± 1 °C, confronted to 1600 AU/ml of free BLS P34 (●), encapsulated BLS P34 (▲) and control (■).

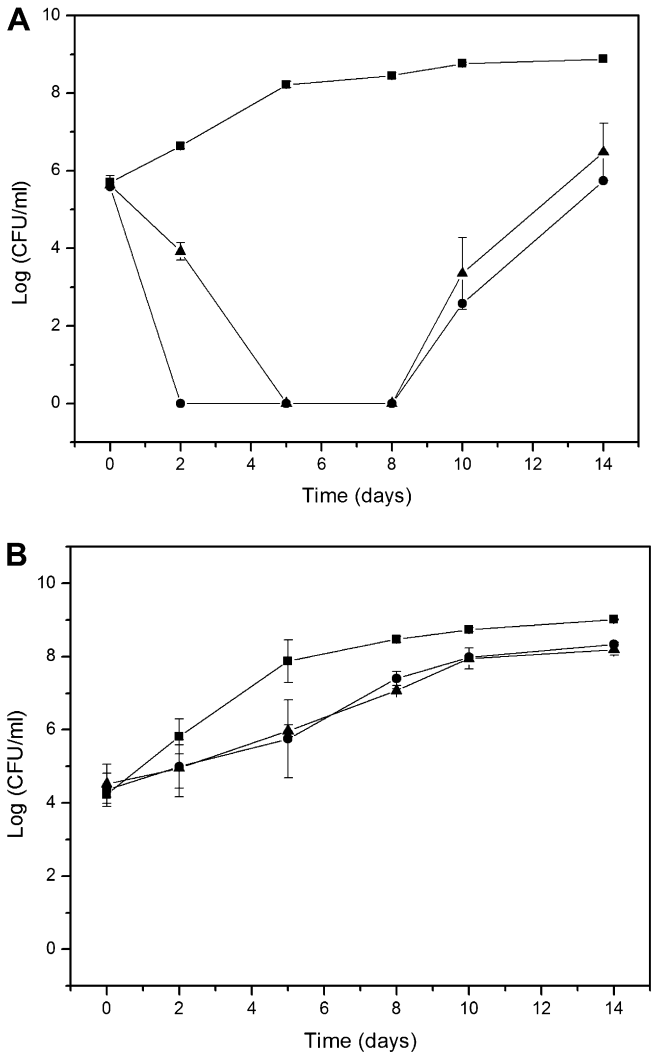

Fig. 4. Viable cell counts of *Listeria monocytogenes* in skim milk (A) and whole milk (B) at 7 ± 1 °C, confronted to 3200 AU/ml of free BLS P34 (●), encapsulated BLS P34 (▲) and control (■).

detection limit of the method. After, there were no significant differences ($P > 0.05$) between treatments. Free and encapsulated BLS P34 treatments consistently lowered *L. monocytogenes* counts below the detection limit of the method at days 5 and 8, increasing thereafter. In whole milk, free and encapsulated BLS P34 presented no significant reduction ($P < 0.05$) in bacterial population during the first 2 days. However, after 5 days both treatments decreased the population of *L. monocytogenes* by 1–2 log cycles compared to the control (Fig. 4B).

4. Discussion

We have earlier reported that BLS P34 was inhibitory to a broad spectrum of indicator strains, including several spoilage and pathogenic microorganisms (Motta, Cannavan, Tsai, & Brandelli, 2007a). Moreover, this antimicrobial substance presented low *in vitro* toxicity to eukaryotic cells with similar effect to that observed for nisin, suggesting to be safe for food use (Vaucher et al., 2010).

In this study, the antimicrobial activity of free and nanovesicles-encapsulated BLS P34 was assessed in skim and whole milk as a function of storage time at 4 °C. The initial decrease (after 4 days) of antimicrobial activity of BLS P34 in skim and whole milk may be associated to interaction with the milk proteins, which can bind somehow with BLS P34 as suggested for nisin (Jung, Bodyfelt, & Daeschel, 1992). It was observed that the milk fat content and the encapsulation in nanovesicles did not influence the antimicrobial activity of BLS P34. In contrast, the negative effect of fat on antimicrobial activity of nisin is extensively reported, caused by the peptide adsorption onto fat globules (Bhatti, Veeramachaneni, & Shelef, 2004; Chollet, Sebti, Martial-Gros, & Degraeve, 2008; Jung et al., 1992). Jung et al. (1992) reported that nisin activity against *L. monocytogenes* dropped by 33% in skim milk, by 50% in milk with 1.2% fat, and by 88% in milk with 12.9% fat. More recently, Chollet et al. (2008) observed that a 30% fat (w/w) level in gel gave rise to a significant decrease ($P < 0.01$) in nisin antimicrobial activity against *K. rhizophila*. Encapsulation of BLS P34 in liposomes maintains the same inhibitory effect against *L. monocytogenes* than free BLS P34. Teixeira et al. (2008) suggest controlled release of the antimicrobial peptide from the vesicle and/or slower diffusion on semisolid agar medium, since to these authors a higher dosage of liposome-encapsulated bacteriocin was necessary to obtain the same inhibitory effect observed for the free bacteriocin.

Although the residual antimicrobial activity of free and encapsulated BLS P34 was similar in skim and whole milk, the inhibition of *L. monocytogenes* by free and encapsulated BLS P34 was especially observed in skim milk. In accordance, the antimicrobial peptide nisin was more effective in reducing the initial population of *L. monocytogenes* in brain-heart infusion broth and in skim milk than fat milk (Jung et al., 1992). Therefore, higher concentration of nisin it is necessary to control the growth this bacteria in whole milk (Malheiros, Daroit, Silveira, et al., 2010).

Bacteriostatic effect was observed in skim milk at 30 °C using encapsulated BLS P34 (3200 AU/ml) treatment. However, when free BLS P34 was tested, *L. monocytogenes* were reduced to counts below the detection limit during the first 6 h. Therefore, the free BLS P34 inhibited the target pathogen in the first moment possibly to injure the bacteria, while encapsulated BLS P34 was possibly being released over time maintaining the same initial count by 24 h. Nisin, an antimicrobial peptide recognized as safe for food applications by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additivies, was encapsulated in liposomes and tested against *L. monocytogenes* in BHI medium and skim milk at 30 °C (Malheiros, Daroit, Silveira, et al., 2010). At such abuse temperature conditions, these authors found that free nisin showed better inhibitory than the liposomal counterparts. Taylor et al. (2008) evaluated the effect of nisin encapsulated in purified phosphatidylcholine liposomes to inhibition of *L. monocytogenes* growth. Those authors observed inhibition of about 60% of pathogens by free and encapsulated nisin after 48 h of incubation at 32 °C on double-strength BHI medium. Antimicrobial activity of bacteriocins in food matrix is affected by several factors, such as temperature to storage, fat and protein content and cation concentrations. This was demonstrated in the work from Branen and Davidson (2004), when nisin did not inhibited *L. monocytogenes* in 2% fat milk stored at 25 °C for 24.

Bacteriocins have been used to inhibit *L. monocytogenes* in fluid systems. The addition of the antimicrobial peptide cerein 8A (final concentration of 160 AU/ml) to UHT milk resulted in a decrease of 3 log cycles in viable cells of *L. monocytogenes* within the 14-day period at 4 °C (Bizani, Morrissy, Dominguez, & Brandelli, 2008). In this study free and encapsulated BLS P34 at final concentration of 160 AU/ml was bacteriostatic by 7 days to 7 °C in skim milk. However, according to Motta et al. (2007a) this same antibacterial substance was bactericidal and bacteriolytic (final concentration of 160 AU/ml) to *L. monocytogenes* after 6 h of growth in TSB medium at 37 °C. In whole milk the treatment tested was not effective to decrease of growth of *L. monocytogenes* to 7 °C.

Free and encapsulated BLS P34 at 3200 AU/ml consistently lowered *L. monocytogenes* counts below the detection limit of the method at days 5 and 8 in skim milk. In whole milk, after 5 days both treatments decreased the population of *L. monocytogenes* 1-2 log cycles compared to the control. Malheiros, Daroit, Silveira, et al., 2010a observed that in skim milk, encapsulated and free nisin (0.5 and 0.1 mg/ml) treatments consistently lowered *L. monocytogenes* counts below the detection limit (<1.69 log CFU/ml) during a period of 14 days, however, in whole milk, only the highest concentration (0.5 mg/ml) of nisin reduced bacterial counts. It is important to note that antilisterial activities of nisin are well documented; however chemical composition and treatment of a food may play an important role in its antilisterial effects (Bhatti et al., 2004).

Antimicrobials peptides could be present both in the aqueous and lipids phases of liposomes. Then, might be able to effectively provide both short-term (by release of encapsulated bacteriocin) and long-term (desorption of membrane-immobilized bacteriocin) antibacterial actions (Benech, Kheadr, Lacroix, & Fliss, 2002; Taylor et al., 2008).

5. Conclusions

The present study demonstrated that the milk fat had not a significant effect on the activity of free and encapsulated BLS P34, but influenced on the ability of bacteriocin to inhibit *L. monocytogenes* in fluid milk. The encapsulation of BLS P34 in nanovesicles of partially purified soy phosphatidylcholine was relatively inexpensive as required by the food industry. In general, free and encapsulated BLS P34 appeared to be effective to control *L. monocytogenes* in milk, especially skim milk, suggesting its potential use as biopreservative in food products. Therefore, the use of a bacteriocin in combination with nanotechnology was useful to control an important pathogen in the dairy industry. This research addresses the feasibility of the use of antimicrobial peptides and nanotechnology to improve the quality and safety of food. Further study, regarding the maintenance of antimicrobial activity and ability of free and encapsulated BLS P34 to inhibit *L. monocytogenes* over time, will be conducted for fluid milk and frescal cheese.

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