

Inhibition of *Listeria* and *Staphylococcus aureus* by Bovicin HC5 and Nisin Combination in Milk

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Abstract

The aim of this work was to evaluate the effect of the bacteriocins bovicin HC5 and nisin against *Listeria* and *Staphylococcus aureus* in synthetic media and in milk. Growth of *Listeria monocytogenes*, *Listeria innocua* and *S. aureus* was carried out at 37°C in tryptic soy broth (TSB) and in ultra-high temperature whole milk containing bovicin HC5 and nisin added either individually or in combination. Concentrations above 100 AU ml⁻¹ of bovicin HC5 or 50 AU ml⁻¹ of nisin inhibited the growth of *Listeria* species in TSB. Bacteriocins at concentrations of at least 50 AU ml⁻¹ clearly increased the lag phase, but did not prevent the growth of *S. aureus*. The combination of both bovicin HC5 and nisin in TSB inhibited the growth of *Listeria* and *S. aureus* Embrapa 4018 at lower concentrations than the bacteriocins added separately. Bactericidal effect against *L. monocytogenes* and *S. aureus* cells was observed when both bacteriocins were added together in milk in concentrations larger than 400 AU ml⁻¹ of each one. The present results demonstrate that bovicin HC5 and nisin were effective against *Listeria* and *S. aureus* assessed in milk, especially when used in combination.

Keywords: food pathogen, bovicin HC5, nisin, milk

1. Introduction

Outbreaks of listeriosis resulting from consumption of dairy products contaminated with *L. monocytogenes* have prompted concern about the behavior of this microorganism during processing and subsequent storage of various dairy products (Silva, Almeida, Alves, & Almeida, 2003). *L. monocytogenes*, a ubiquitous foodborne pathogen, can be potentially introduced in raw milk in a dairy industry environment. Growth of *Listeria* in dairy products is often favored by its psychrotrophic nature and tolerance to high salt concentration and relatively low pH values (Farber & Peterkin, 1991). *L. monocytogenes* causes disease in high-risk groups, including pregnant women, neonates, and immunocompromised adults, and has a high mortality rate (Arques, Rodriguez, Nunez, & Medina, 2008).

Food from animal origins, such as milk, is naturally susceptible to contamination by *Staphylococcus aureus*, an important pathogen able to grow in a wide range of temperatures, pH and sodium chloride concentration up 15%, and then able to produce enterotoxins. These toxins are thermostable and maintain their stability even after thermal treatments (Dinges, Orwin, & Schlievert, 2000; Le Loir, Baron, & Gautier, 2003).

Although *Listeria* and *S. aureus* are inactivated under normal conditions of pasteurization, problems can arise from post-pasteurization contamination, representing a risk to consumers, making necessary an effective control during the steps of food production.

Bacteriocins from lactic acid bacteria are widely studied and have been suggested as a potential biological alternative to improve food safety (Cleveland, Montville, Nes, & Chikindas, 2001). Nisin is a well known broad spectrum bacteriocin that can inhibit gram-positive bacteria and prevent the outgrowth of spores of Bacilli and Clostridia associated with food (Arques et al., 2004; de Arauz, Jozala, Mazzola, & Penna, 2009). Although nisin has been widely used in food industries to increase the shelf life of food products, previous studies indicated that many sensitive gram-positive bacteria have developed resistance to nisin (Arques et al., 2008; Zapico, Medina, Gaya, & Nunez, 1998).

Bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5, has a broad spectrum of activity (Mantovani, Hu, Worobo, & Russell, 2002). Previous works demonstrated the ability of bovicin HC5 to inhibit *L. monocytogenes* (Mantovani & Russell, 2003), and prevent the growth and spore germination of strains of *Bacillus cereus* and *Bacillus thuringiensis* (de Carvalho, Costa, Mantovani, & Vanetti, 2007), *Clostridium tyrobutyricum* (de Carvalho, Mantovani, & Vanetti, 2007), and *Alicyclobacillus acidoterrestris* (de Carvalho, Vanetti, & Mantovani, 2008). Since bacteria that can readily become resistant to nisin did not become significantly more resistant to bovicin HC5 after they were repeatedly transferred with sublethal doses, it appeared that bovicin HC5 had important characteristics (Mantovani & Russell, 2003).

Inhibition of *L. monocytogenes* by bacteriocins such as bovicin HC5; nisin (Bozariar & Nychas, 2006); curvaticin 13 (Bouttefroy & Milliere, 2000); cerein 8A (Bizani, Morrissy, Dominguez, & Brandelli, 2008); reuterin (Arques et al., 2008; El-Ziney & Jakobsen, 2009); enterocin (Ghraiiri, Frere, Berjeaud, & Manai, 2008) and pediocin AcH (Loessner, Guenther, Steffan, & Scherer, 2003) demonstrates that this may be a useful strategy for food processing to ensure microbiological safety.

A combination of preservative methods may work synergistically or at least provide greater protection than a single method alone, thus improving the safety and quality of food (Deegan, Cotter, Hill, & Ross, 2006). Based on this assumption, a combination of bacteriocins has been tested in order to increase antimicrobial activities and improve food safety (Galvez, Abriouel, Lopez, & Ben Omar, 2007).

The objective of the present work was to study the combined effect of bovicin HC5 and nisin on *L. monocytogenes*, *L. innocua* and *S. aureus* in whole milk. Although the effect of nisin combined with other antimicrobial agents has already been extensively studied, this is the first study evaluating the effect of nisin combined with bovicin HC5 on foodborne pathogens.

2. Materials and Methods

2.1 Microorganisms and Growth

S. bovis HC5 was cultured anaerobically as previously described (Mantovani & Russell, 2003). *Lactococcus lactis* ATCC 19435 was cultivated aerobically in de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) at 37°C.

L. monocytogenes ATCC 7644, *L. monocytogenes* Scott A, *L. innocua* LMA83 (isolated from a dairy industry), *L. innocua* LMA84 (isolated from Minas cheese), *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 and *S. aureus* Embrapa 4018 (isolated from bovine mastitis) were cultivated in trypticase soy broth (TSB) (Merck, Germany) and incubated at 37°C. The identity of all bacterial strains was confirmed by biochemical tests.

2.2 Preparation and Activity of Bovicin HC5 and Nisin

Extracts of bovicin HC5 were prepared as described by Mantovani et al. (2002). Bovicin HC5 concentration was estimated by serial two-fold dilutions of extract followed by spotting 25 µl on MRS agar using *L. lactis* ATCC 19435 as the indicator organism. Plates were incubated at 37°C for 24 h. One arbitrary unity (AU) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5 mm diameter.

Nisin solution (Nisaplin[®], Danisco, Copenhagen, Denmark) was prepared in phosphate buffer (pH 2.0) and the bacteriocin activity was determined as described earlier.

2.3 Effect of Bovicin HC5 and, or Nisin on the Growth of *Listeria* and *S. aureus* in TSB

To determine the concentration of bovicin HC5 and nisin (individually or combined) that could inhibit *Listeria* and *S. aureus* we performed *in vitro* studies in 96-well microtiter plates. Strains of *L. monocytogenes*, *L. innocua* and *S. aureus* were activated in TSB and incubated at 37°C for 18 h. The cells were harvested by 3000 g centrifugation, washed with 0.1% salt peptone water, and then resuspended in TSB. *Listeria* and *S. aureus* cultures (10^6 CFU ml⁻¹) were treated with bovicin HC5 and nisin at concentration of 10, 50, 100 and 150 AU ml⁻¹ for the tests with isolated bacteriocins. In the assays using bovicin HC5 and nisin combined, the concentrations varied from 10 to 50 AU ml⁻¹.

The bacteria were incubated at 37°C and the growth was monitored via changes in optical density at 630 nm in an ELISA reader (Thermo Plate, model TP-Reader) for up to 10 h of incubation. Control treatments were performed in TSB inoculated with bacterial cultures without bacteriocins.

2.4 Effect of Bovicin HC5 and, or Nisin on the Growth of *Listeria* and *S. aureus* in Whole Milk

Cultures of *L. monocytogenes* Scott A, *L. innocua* LMA83, and *S. aureus* ATCC 6538 were selected because they presented more resistant after being treated with bacteriocins in TSB. Cells were activated in 5 ml TSB and incubated at 37°C for 18 h, harvested by centrifugation at 3000 g for 15 min, washed with 0.1% salt peptone

water and then, resuspended in 1 ml of ultra-high temperature (UHT) whole milk to reach 10^7 CFU ml⁻¹. Bovicin HC5 and nisin were added individually (400, 800, and 1200 AU ml⁻¹) or in combination (400, 600, and 800 AU ml⁻¹ of each bacteriocin).

The tubes were incubated at 37°C and samples were taken at 0, 3, 6, 9, and 12 h for determination of viable cell number by microdrops (Herbert, 1990) plating aliquots of 25 µl in tryptic soy agar (Oxoid, England). Plates were incubated at 37°C for 12 or 24 h. Control treatments were performed in milk inoculated with bacterial cultures without bacteriocins.

2.5 Statistics

Each experiment was performed at least two times in duplicate. The log of the absorbance and colony forming units was plotted versus time (SigmaPlot version 11.0, USA) and the error bars presented indicate the standard deviation of the mean (shown only in the positive direction).

3. Results

3.1 Effect of Bovicin HC5 and/or Nisin on *Listeria* and *S. aureus* in TSB

Table 1. Effect of bovicin HC5 or nisin on the growth of *Listeria* and *S. aureus*

Microorganism	Bacteriocin Concentration (AU ml ⁻¹)	Specific growth rate (h ⁻¹)		Lag phase duration (h)	
		Bovicin HC5	Nisin	Bovicin HC5	Nisin
<i>L. monocytogenes</i> ATCC 7644	0	0.60	0.60	0	0
	10	0.26	-	0	>11
	50	0.21	-	0	>11
	100	-	-	>11	>11
	150	-	-	>11	>11
<i>L. monocytogenes</i> Scott A	0	0.55	0.55	0,5	0
	10	0.32	-	0	9
	50	0.21	-	0	10
	100	-	-	>11	>11
	150	-	-	>11	>11
<i>L. innocua</i> LMA84	0	0.61	0.61	0	0,5
	10	-	-	>11	>11
	50	-	-	>11	>11
	100	-	-	>11	>11
	150	-	-	>11	>11
<i>L. innocua</i> LMA83	0	0.59	0.59	0	0
	10	0.30	0.84	0	8
	50	0.28	-	0	>10
	100	-	-	>11	>11
	150	-	-	>11	>11
<i>S. aureus</i> Embrapa 4018	0	0.84	0.84	0	0
	10	0.82	1.13	8	8
	50	0.75	0.60	8	8
	100	-	-	>11	10
	150	-	-	>11	>11
<i>S. aureus</i> ATCC 25923	0	0.39	0.39	0	0
	10	0.30	0.26	0	2
	50	0.27	-	0	>11
	100	0.28	-	0	>11
	150	0.28	-	0	>11
<i>S. aureus</i> ATCC 6538	0	0.43	0.43	0	0
	10	0.21	0.54	1	6
	50	0.19	0.50	1	7
	100	0.40	0.61	8	8
	150	-	0.57	>11	8

-, no growth; AU, activity units.

The specific growth rate of *L. monocytogenes* ATCC 7644 and *L. monocytogenes* Scott A in TSB was reduced in the presence of bovicin HC5 at concentrations of 10 and 50 AU ml⁻¹, while growth was completely inhibited in the presence of 100 and 150 AU ml⁻¹ of this bacteriocin (Table 1). *L. monocytogenes* ATCC 7644 and Scott A were inhibited in TSB even at the smallest nisin concentration of 10 AU ml⁻¹ (Table 1). The effect of the bacteriocins bovicin HC5 and nisin was different on *L. innocua*: while *L. innocua* LMA84 was completely inhibited by bovicin HC5 or nisin at 10 AU ml⁻¹, concentrations up to 50 AU ml⁻¹ allowed the growth of *L. innocua* LMA83 after a lag phase period (Table 1).

Absorbance reduction of cultures of *L. monocytogenes* and *L. innocua* was observed when both bacteriocins were combined at the concentrations 10 (data not shown) and 50 AU ml⁻¹ (Figure 1), suggesting a bactericidal effect. As the behavior pattern was similar for both strains of *L. monocytogenes* and *L. innocua*, only data from *L. monocytogenes* Scott A and *L. innocua* LMA83 are shown in Figure 1.

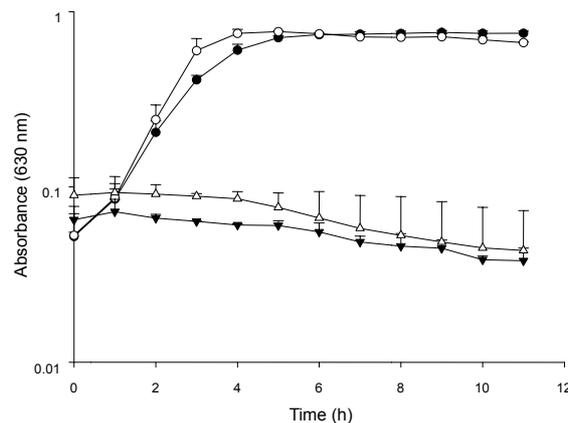


Figure 1. Effect of bovicin HC5 and nisin in combination at 50 AU ml⁻¹ of each one on the growth of *L. monocytogenes* Scott A (▼) or *L. innocua* LMA83 (△) in TSB. Control without bacteriocins with *L. monocytogenes* Scott A (●) or *L. innocua* LMA83 (○) is also shown

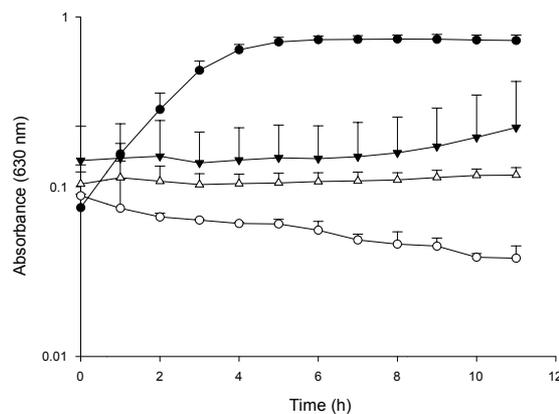


Figure 2. Effect of bovicin HC5 and nisin in combination at 50 AU ml⁻¹ of each one on the growth of *S. aureus* Embrapa 4018 (○) or *S. aureus* ATCC 25923 (▼) or *S. aureus* ATCC 6538 (△). Control without bacteriocins with *S. aureus* (●) is also shown

Although the presence of bovicin HC5 or nisin has clearly different impact on the specific growth rate of *S. aureus* Embrapa 4018, ATCC 25923 and ATCC 6538 (Table 1), all three strains were more resistant against the bacteriocins than *Listeria*. Bovicin HC5 at 150 AU ml⁻¹ was sufficient to inhibit the growth of two of the three *S. aureus* strains tested.

Bovicin HC5 and nisin combined was more effective than the separate bacteriocins to inhibit the growth of *S. aureus* in TSB. Although the addition of bacteriocins in the combined concentration of 10 AU ml⁻¹ (data not shown) has reduced the specific growth rate, inhibition was greater when 50 AU ml⁻¹ of each bacteriocin was

added (Figure 2). The addition of bovicin HC5 and nisin combined at the concentration of 50 AU ml⁻¹, exerted a greater inhibitory effect on *S. aureus* Embrapa 4018 and ATCC 6538 than the isolated addition of each bacteriocin in the same concentration (Table 1).

3.2 Effect of Bovicin HC5 and/or Nisin on *Listeria* and *S. aureus* in Milk

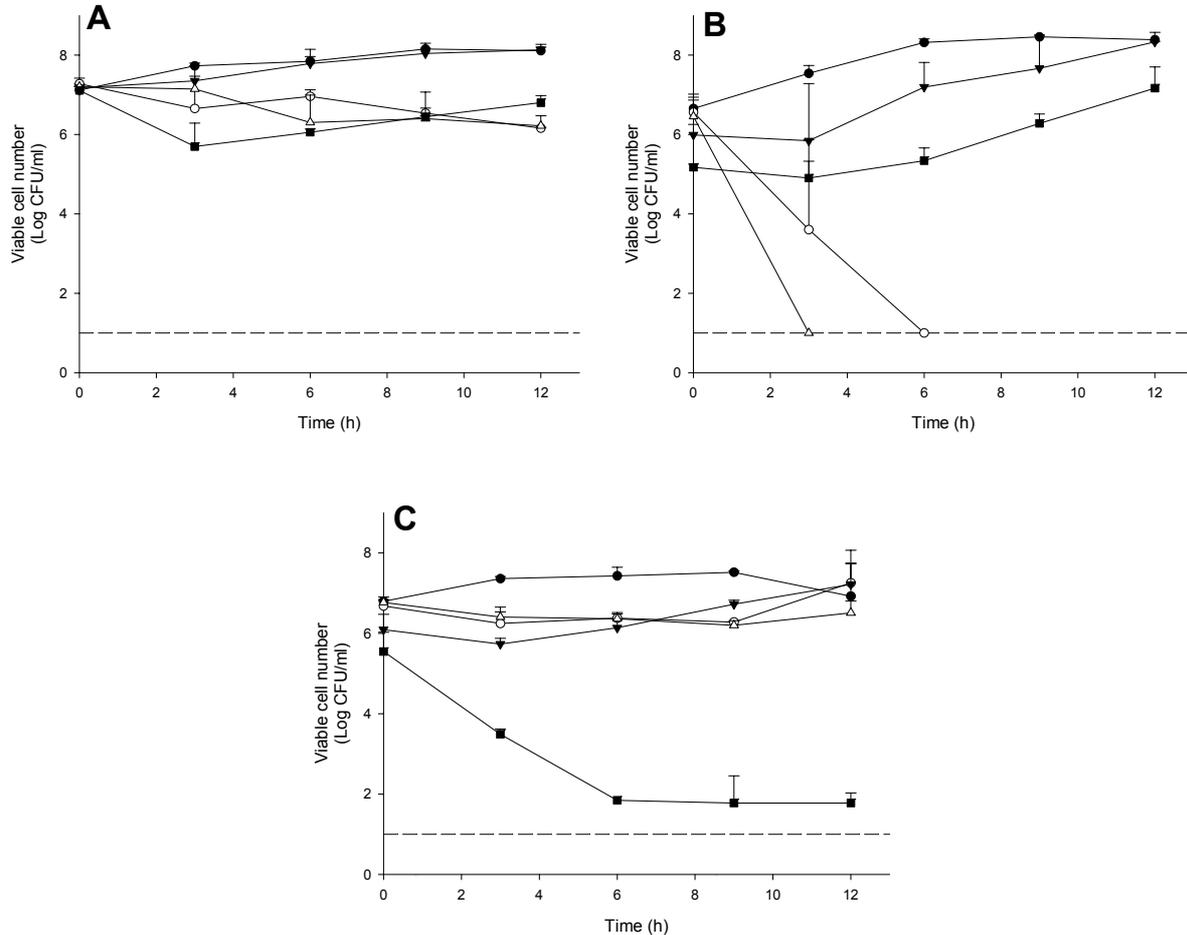


Figure 3. Effect of bovicin HC5 at concentration of 800 AU ml⁻¹ (○) and 1200 AU ml⁻¹ (△) or nisin at concentration of 800 AU ml⁻¹ (▼) and 1200 AU ml⁻¹ (■) on the growth of *L. monocytogenes* Scott A (A), *L. innocua* LMA83 (B) and *S. aureus* ATCC 6538 (C) in whole milk. Control without bacteriocins is shown (●). The dotted line shows the detection limit of the enumeration technique used

L. monocytogenes Scott A, *L. innocua* LMA83 and *S. aureus* ATCC 6538 strains were selected to evaluate the effect of bacteriocins in milk. The addition of bovicin HC5 at 400 (data not shown), 800 and 1200 AU ml⁻¹ to milk reduced the viable cell number of *L. monocytogenes* (Figure 3A). Although a pronounced effect was observed when 1200 AU ml⁻¹ of nisin was used, the growth was resumed after 3 h of incubation (Figure 3A). The lethal effect of nisin and bovicin HC5 combined on *L. monocytogenes* in whole milk was observed at concentrations of 600 and 800 AU ml⁻¹ of each, with a sharp reduction in the cell number to below the detection limit of the technique, which is 10¹ CFU ml⁻¹ (Figure 4A).

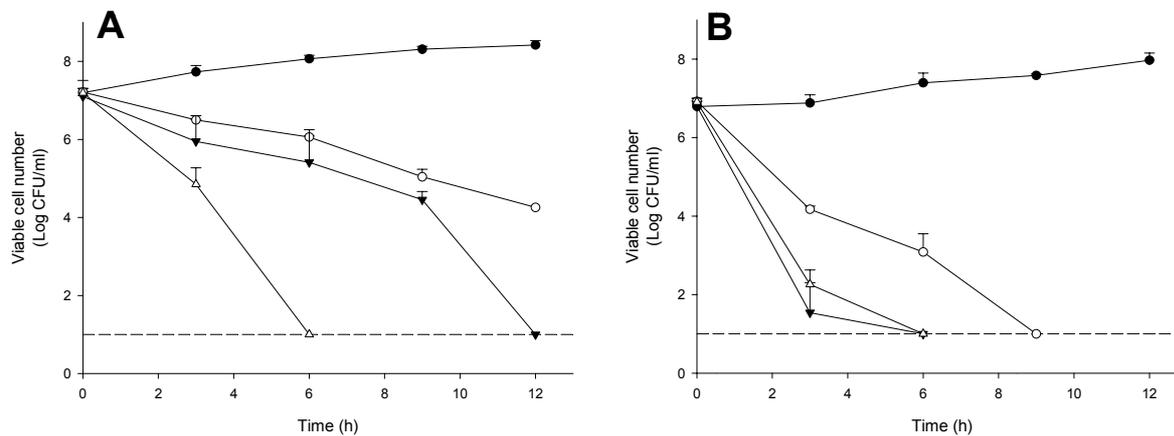


Figure 4. Effect of the combination of bovicin HC5 and nisin at 400 AU ml⁻¹ (○), 600 AU ml⁻¹ (▲) or 800 AU ml⁻¹ (△) of each one on the growth of *L. monocytogenes* Scott A (A) and *S. aureus* ATCC 6538 (B) in whole milk. Control without bacteriocins is shown (●). The dotted line shows the detection limit of the enumeration technique used

Bovicin HC5 concentrations from 400 to 1200 AU ml⁻¹ in milk were also bactericidal to *L. innocua* and the viable cell number was reduced by more than 5 log cycles. Although nisin has exerted an inhibitory effect on *L. innocua* by increasing 3 h in the lag phase, the growth was resumed after this period (Figure 3B).

The major resistance of *S. aureus* to the bacteriocins effect observed in TSB was confirmed in milk and the concentration of 800 AU ml⁻¹ did not prevent the growth of *S. aureus* ATCC 6538 (Figure 3C). At the highest concentration (1200 AU ml⁻¹), bovicin HC5 exerted a bacteriostatic effect while nisin initially exerted a bactericidal effect during 6 h after exposition followed by a bacteriostatic effect (Figure 3C). However, the combination of bovicin HC5 and nisin reduced the viable cell number of *S. aureus* ATCC 6538 in UHT milk to below the detection limit of the technique, within 9 h of incubation (Figure 4B).

4. Discussion

Bacteriocins have been used as a food preservative, mainly against foodborne pathogens. However, our finding that *Listeria* and *S. aureus* growth can be resumed after the lag phase indicates selection of resistant bacteria to bacteriocins and this is a problem to be addressed before the adoption of a food preservation process. Another study also found a transient antimicrobial effect of 100 AU ml⁻¹ of nisin or 320 AU ml⁻¹ of curvaticin 13, a bacteriocin produced by *Lactobacillus curvatus* SB13, against *L. monocytogenes* ATCC 7644 being the growth resumed after approximately 9 h of incubation at 37°C (Bouttefroy & Milliere, 2000). *L. monocytogenes* resistant to antimicrobial agents showed an increase in the proportion of saturated fatty acids that can increase the rigidity of the cell membrane, making it less fluid and thus preventing the penetration of molecules of bacteriocins (Naghmouchi, Belguesmia, Baah, Teather, & Drider, 2011; Naghmouchi, Kheadr, Lacroix, & Fliss, 2007). The resistance of some species of *Staphylococcus* to nisin is associated with increased amounts of D-alanine in the structure of teichoic acids present in the peptidoglycan layer of these bacteria becoming the cells more positively charged (Peschel et al., 1999). These changes may hinder the interaction of nisin, positively charged, with the surface of cells.

The antimicrobial activity of nisin is related to its abilities to bind specifically to lipid II, a precursor in the biosynthesis of cell wall, and to form pores leading to cell death due to loss of intracellular compounds (Breukink & de Kruijff, 2006; Breukink et al., 2003). As a lantibiotic, bovicin HC5 has a primary mode of action similar to nisin, which involves specific interaction with lipid II. However, some differences regarding the pore-forming capacity of both bacteriocins were observed in model membranes: the pore-formation by bovicin HC5 was clearly dependent on membrane thickness, being observed only in thinner membranes (Paiva, Breukink, & Mantovani, 2011). Independent on the pore formation, bovicin HC5 maintains its antibacterial activity by recruiting lipid II molecules in a prepore-like structure, and consequently preventing the use of such molecules in the cell wall synthesis (Paiva et al., 2011).

Another issue to consider is the variation of resistance to bacteriocins found among strains of the same species. For example, we noted that bovicin HC5 and nisin have exerted an inhibitory effect on *L. monocytogenes* but strain ATCC 7644 was more sensitive to nisin while *L. monocytogenes* Scott A was more sensitive to bovicin HC5. The sensitivity variation to nisin among different strains of *S. aureus* isolated from dairy products was also observed (Sudagidan & Yemenicioglu, 2012). Mantovani and Russell (2003) found that some bacteria resistant to nisin showed no significant resistance to bovicin HC5, even after repeated treatments with sublethal doses of that bacteriocin. Higher concentrations of bacteriocins could increase the bactericidal effect and reduce the chances of selecting resistant bacteria and to have an inhibitory effect on several strains that could contaminate food.

Another alternative to reduce the selection of resistant cells would be the combined use of bacteriocins. In this study we showed that the impact of bovicin HC5 and nisin can be greatly enhanced if both bacteriocins are applied in combination. In fact, concentrations as low as 10 AU ml⁻¹ of each bacteriocin in TSB resulted in reduction of the absorbance of cultures of *L. monocytogenes* Scott A and *L. innocua* LMA83 indicating cell lysis. The results showed that the combined effect of bovicin HC5 and nisin allow the use of lower dosages compared to the individual application.

According to Gálvez et al. (2007) when cells are exposed to a combination of antimicrobial factors, the intensity of the damage can be increased since these factors may act on different sites of the same target. The repair of multiple damages may require a high energy expenditure, resulting in energy depletion and cell death. The addition of nisin (0.5% w/v) in ready to eat salad did not achieved the complete inactivation of *L. monocytogenes* but the combination of this bacteriocin with enterocin AS-48 reduced the concentration of viable cells below the detection limit of the technique after 24 h of incubation (Molinos et al., 2009).

Although the addition of bacteriocins in milk used for consumption is not permitted, this product serves as an important system to evaluate the influence of chemical composition of milk on the activity of bacteriocins. Milk is a complex mixture of substances such as water, protein, lactose and fat, which can affect the effectiveness of bacteriocins. The addition of nisin at concentrations of 62.5, 125, 250 and 500 IU ml⁻¹ in skim milk had a significant effect on *L. monocytogenes*, while in raw milk, the inhibitory activity was only moderate, with a rapid reduction of the *L. monocytogenes* population during two days of incubation before growth was resumed (Kim, Choi, Bajpai, & Kang, 2008). These results demonstrate that the activity of bacteriocin is dependent on the fat content in milk, and the interaction between the lipids of milk and nisin may limit the application of this bacteriocin in dairy fat products (Sobrino-Lopez & Martin-Belloso, 2008).

No research to assess the synergistic effect of bovicin HC5 with another antimicrobial agent in milk was carried out so far. However, results from the synergistic effect of nisin and other antimicrobial substances can be exemplified. Nisin combined with garlic extract showed potential antilisterial activity with a synergistic effect in reducing the viable cell number of *L. monocytogenes* in milk and skim milk in 14 days (Kim et al., 2008). Synergistic effect was also found by Arqués et al. (2008) evaluating the combination of nisin and reuterin, antimicrobial produced by *Lactobacillus reuteri*, in milk. These authors reported a reduction of approximately two log cycles of *S. aureus* treated with these antimicrobials in combination after 24 h of incubation.

5. Conclusions

The present study demonstrates that the combination of bovicin HC5 and nisin against *Listeria* and *S. aureus* in TSB and milk was more effective than the addition of these bacteriocins individually. These results indicate their potential use as biopreservatives in food. However, the molecular basis of synergistic effect between bovicin HC5 and nisin are now under investigation. Furthermore, the combination of bovicin HC5 with others antimicrobial agents against foodborne pathogens is focus of further research.

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