

Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* DF04Mi isolated from goat milk: Application in the control of *Listeria monocytogenes* in fresh Minas-type goat cheese

Danielle N. Furtado, Svetoslav D. Todorov, Mariza Landgraf, Maria T. Destro, Bernadette D.G.M. Franco

Laboratório de Microbiologia de Alimentos, Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brazil.

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Abstract

Listeria monocytogenes is a pathogen frequently found in dairy products. Its control in fresh cheeses is difficult, due to the psychrotrophic properties and salt tolerance. Bacteriocinogenic lactic acid bacteria (LAB) with proven *in vitro* antilisterial activity can be an innovative technological approach but their application needs to be evaluated by means of *in situ* tests. In this study, a novel bacteriocinogenic *Lactococcus lactis* strain (*Lc. lactis* DF4Mi), isolated from raw goat milk, was tested for control of growth of *L. monocytogenes* in artificially contaminated fresh Minas type goat cheese during storage under refrigeration. A bacteriostatic effect was achieved, and counts after 10 days were 3 log lower than in control cheeses with no added LAB. However, this effect did not differ significantly from that obtained with a non-bacteriocinogenic *Lc. lactis* strain. Addition of nisin (12.5 mg/kg) caused a rapid decrease in the number of viable *L. monocytogenes* in the cheeses, suggesting that further studies with the purified bacteriocin DF4Mi may open new possibilities for this strain as biopreservative in dairy products.

Key words: bacteriocin, *Lc. lactis* subsp. *lactis*, biopreservation, fresh cheese, goat cheese.

Introduction

Listeriosis is a foodborne disease that affects pregnant women, the elderly, newborn and those who are immunocompromised. The causative agent is *Listeria monocytogenes*, a pathogen present in wide range of foods, including dairy products. Fresh cheeses pose a particularly high risk, as growth of *L. monocytogenes* is difficult to control due to the psychrotrophic characteristics and high salt tolerance (Kathariou, 2002; Gandhi and Chikindas, 2007; Swaminathan *et al.*, 2007). Several recent listeriosis outbreaks were linked to cheeses (Fretz *et al.*, 2010; Koch *et al.*, 2010).

A considerable body of experimental work on application of bacteriocins produced by lactic acid bacteria (LAB) for control of pathogens such as *L. monocytogenes* in food systems has accumulated in recent years (Riley and Wertz, 2002; Chen and Hoover, 2003; Cotter *et al.*, 2005; Deegan *et al.*, 2006; Galvez *et al.*, 2007, 2008, 2010; Garcia

et al., 2010;). Exploitation of bacteriocins as biopreservatives in dairy products is increasing, as they are an interesting technological alternative to conventional antimicrobial procedures. Biopreservation by bacteriocinogenic LAB fulfills the increased demand from consumers for foods that contain lower concentration of chemical preservatives, as bacteriocins are natural antimicrobials, produced by bacteria normally present in the milk. Additional claims of health-promoting benefits due to probiotic activity of bacteriocinogenic LAB bring extra value to these types of products. As probiotics, these bacteria can confer health benefits to the host such as reduction of gastrointestinal infections and inflammatory bowel disease, modulation of the immune system, and defense against colonization by pathogenic microorganisms (WHO, 2002; Oelschlaeger, 2010).

Several bacteriocin-producing LAB strains have been isolated from milk and dairy products, as recently reviewed by Franco *et al.* (2012). Nisin, produced by *Lactococcus*

lactis subsp. *lactis*, remains the best studied bacteriocin, and the use of commercial nisin in cheeses is permitted in many countries (Thomas *et al.*, 2000). Several other bacteriocins produced by *Lc. lactis* have been described, but are less well known (Piard, 1994; Ko and Ahn, 2000; Ferchichi *et al.*, 2001; Lee and Paik, 2001; Cheigh *et al.*, 2002; Mathara *et al.*, 2004; Todorov and Dicks, 2004; Aslom *et al.*, 2005; Ghrairi *et al.*, 2005; Alomar *et al.*, 2008; Nikolic *et al.*, 2008; Biscola *et al.*, 2013; Kruger *et al.*, 2013).

One of the most popular dairy products in Brazil is Minas cheese (*Queijo Minas*), a fresh cheese prepared with bovine milk. Due to the high water activity, pH above 5.0, low salt content and absence of preservatives, this product has a short shelf-life and is an excellent substrate for growth of microorganisms (Souza and Saad, 2009). Contamination with pathogens, such as *L. monocytogenes* and *Staphylococcus aureus*, is frequently reported (Silva *et al.*, 2001; Silva *et al.*, 2004; Brito *et al.*, 2008; Zocche *et al.*, 2010). Gálvez *et al.* (2008), reviewed the application of bacteriocins in several types of foods, including dairy products, indicating that they can be used successfully for improvement of their safety and quality. However, Nascimento *et al.* (2008), reported that the counts of *L. monocytogenes* and *S. aureus* in Minas cheese prepared with three bacteriocinogenic cultures did not differ significantly from those in cheeses not containing these strains. Thus, the effectiveness of bacteriocins on the control of pathogens in Minas cheese is controversial.

In recent years, cheeses prepared with goat milk have gained market in Brazil, as value-added and sophisticated dairy products, in consequence of their unique nutritional and health properties. However, little information is available on the microbiological aspects of these novel cheeses made in Brazil. In this study we report results on the control of *L. monocytogenes* in Minas-type fresh goat cheese during storage under refrigeration by a bacteriocinogenic *Lactococcus lactis* subsp. *lactis* strain (*Lc. lactis* DF04Mi) isolated from raw goat milk. Results were compared to those obtained in cheeses added of a non-bacteriocinogenic *Lc. lactis* strain, and cheeses added of commercial nisin.

Materials and Methods

Bacterial strains

The study was conducted with a bacteriocinogenic *Lactococcus lactis* subsp. *lactis* strain (*Lc. lactis* DF04Mi) isolated from raw goat milk (Furtado *et al.* 2014), and a non-bacteriocinogenic *Lc. lactis* (culture R704, from Ch. Hansen). *L. monocytogenes* Scott A was used for indication of antilisterial activity in the cheeses.

Preparation of inocula for experimental contamination of the cheeses

The cultures of *L. monocytogenes* Scott A, grown in TSB-YE broth for 24 h at 37 °C, and *Lc. lactis* DF04Mi and *Lc. lactis* R704, grown in MRS broth for 24 h at 30 °C, were centrifuged at 6000 x g for 10 min at 10 °C, and washed three times with 0.85% sterile saline. The final suspensions were submitted to decimal serial dilutions and plated on TSA-YE or MRS agar for determination of the number of viable cells.

For preparation of cheeses containing *Lc. lactis* DF04Mi or *Lc. lactis* R704, the two cultures were added to the goat milk after the pasteurization step (63 °C for 30 min), to reach a level of 10⁶ cfu/mL. Microbial examination of the pasteurized milk has been performed in similar approach as described in section “Microbial examination of the cheeses”. Pasteurized milk has been tested for presence of *Listeria* spp. and *Lactococcus* spp. by *Listeria* Selective Agar Base (Oxford Formulation, Oxoid) supplemented with *Listeria* Selective Supplement (Oxoid) and incubated at 37 °C for 24 h and MRS agar and incubated at 30 °C for 24 h, respectively. For contamination with *L. monocytogenes* Scott A, the culture was added to the salted curd at the agitation step, as described below, to reach a level of 10³ cfu/g.

Fresh Minas-type goat cheese manufacturing

Minas-type goat cheese was manufactured according to Scholz (1995), following the diagram presented in Figure 1. For each batch of cheese, ten liters of raw goat milk, provided by a producer in Ibiuna, SP, Brazil, were pasteur-

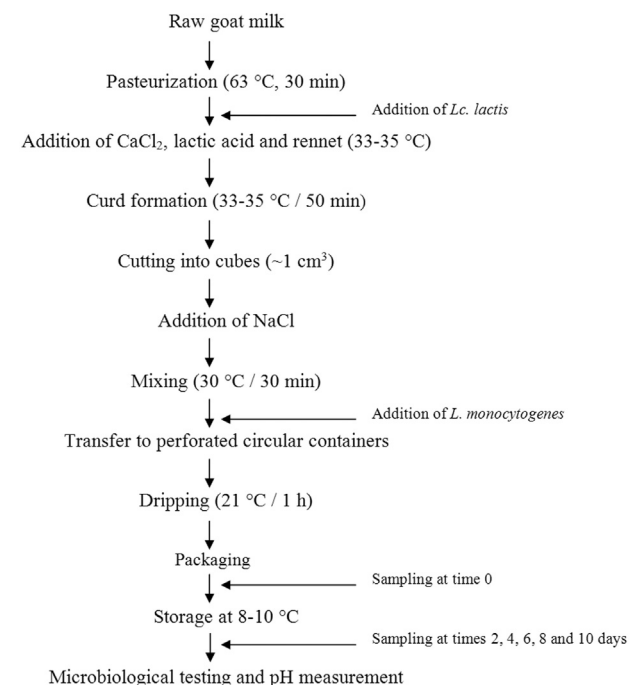


Figure 1 - Protocol for fresh Minas-type goat cheese manufacturing.

ized by heating at 63 °C for 30 min, cooled to 35 °C, and added of 2.5 mL of saturated CaCl₂ solution, 2.5 mL of 85% lactic acid (Chemco Indústria e Comércio de Produtos Químicos Ltda, Brazil) and 9.0 mL of commercial rennet (Fábrica de Coalhos e Coagulantes Bela Vista Produtos Enzimáticos Indústria e Comércio Ltda., Brazil). After 50 min, the curd was cut both vertically and horizontally into cubes of approximately 1 cm³ using a plastic spatula. Cooking grade NaCl (2%) was added and the salted curd was agitated slowly for 30 min at 21 °C. The curd was transferred to perforated sterile plastic circular cheese containers (appr. 15 cm diameter) and maintained at 21 °C for 1 h for dripping. The cheeses were unmolded, packed in plastic bags, and stored under refrigeration (8-10 °C). Each package contained approximately 170 g of cheese.

Six different batches of fresh Minas-type goat cheeses were prepared:

(A) Cheeses prepared with pasteurized goat milk containing no added *Lc. lactis*, experimentally contaminated with *L. monocytogenes* Scott A, added to the salted curd;

(B) Cheeses prepared with pasteurized milk containing *Lc. lactis* DF4Mi, and experimentally contaminated with *L. monocytogenes* Scott A, added to the salted curd;

(C) Cheeses prepared with pasteurized milk containing *Lc. lactis* R704, and experimentally contaminated with *L. monocytogenes* Scott A, added to the salted curd;

(D) Cheeses prepared with pasteurized milk containing 12.5 mg/kg pure nisin (Sigma-Aldrich), experimentally contaminated with *L. monocytogenes* Scott A, added to the salted curd;

(E) Cheeses prepared with pasteurized milk containing *Lc. lactis* DF4Mi, non-contaminated with *L. monocytogenes* Scott A;

(F) Control cheeses, containing no added cultures or nisin.

Microbial examination of the cheeses

Experimentally contaminated cheeses were submitted to counts of *L. monocytogenes* and *Lc. lactis*, as appropriate, on time zero and every two days, up to ten days of storage. Twenty five grams of each sample were stomached with 225 mL of 0.1% peptone water, submitted to decimal serial dilutions in the same diluent and pour-plated (1 mL) with TSA-YE. After solidification, plates were overlaid with 10 mL *Listeria* Selective Agar Base (Oxford Formulation, Oxoid) supplemented with *Listeria* Selective Supplement (Oxoid) and incubated at 37 °C for 24 h. For enumeration of *Lc. lactis*, the decimal serial dilutions were plated on MRS agar, and incubated at 30 °C for 24 h. Non-contaminated control cheeses were also tested for *L. monocytogenes* and *Lc. lactis*, using the described procedures. Growing colonies were counted and results expressed as CFU/g. The experiments were repeated three times in separated occasions.

pH monitoring

At each sampling for bacterial counts, the cheese homogenates in 0.1% peptone water were submitted to pH measurement, using a DMPH2 potentiometer (Digimed, Brazil).

Statistical analyses

Results of microbial counts in the cheeses were submitted to variance analyses (ANOVA). The growth of *L. monocytogenes* during storage was evaluated using regression analyses. Statistical differences were detected by analyses of contrast ($p < 0.05$). The statistical analyses were performed using the Assistat (Assistat - Statistical Assistance, Version 7.5 beta, 2008) software.

Results and Discussion

Considering that psychrotrophic bacteria can survive in cheeses during manufacture, ripening and storage under refrigeration (Morgan *et al.*, 2001), the control of growth of *L. monocytogenes* is of great relevance and a big challenge for producers and consumers. One technological alternative to chemical additives is the use of bacteriocins produced by indigenous LAB present in cheese. Primary microbiological analysis of pasteurized milk showed not detectable levels of *Listeria* spp. and LAB. As shown in Table 1, *L. monocytogenes* can grow fast in fresh Minas-type goat cheese during storage under refrigeration. In the cheeses experimentally contaminated with 10³ cfu/g (experimental set "A"), the log counts of *L. monocytogenes* Scott A were 6.32 ± 0.08 cfu/g after 10 days under refrigeration. When the cheeses were prepared with added bacteriocinogenic *Lc. lactis* DF4Mi strain (experimental set "B"), the growth of *L. monocytogenes* Scott A was inhibited and the average counts after 10 days under refrigeration were almost 3log lower than in cheeses where the bacteriocinogenic strain was absent (3.76 ± 0.03 cfu/g). However, the same result was observed in the cheeses containing the non-bacteriocinogenic *Lc. lactis* strain (experimental set "C"). The differences in counts in both types of cheeses (experimental set "B" and "C") along storage were not significant ($p < 0.05$), showing that the inhibition of *L. monocytogenes* Scott A in cheese might have occurred due to another factor than the production of bacteriocin. In counterpart, addition of pure nisin at a level of 12.5 mg/kg caused a decrease in the number of viable *L. monocytogenes* Scott A cells (experimental set "D"), and in the second day under refrigeration the counts were below the detection level ($< 10^{-1}$ cfu/g). The pH of the cheeses containing the bacteriocinogenic and the non-bacteriocinogenic *Lc. lactis* strains dropped from initial 5.8 to 5.2 after 10 days. The inhibition of *L. monocytogenes* Scott A cannot be attributed to this decrease of pH, as this pathogen can grow well at pH 5.0, even under refrigeration (Gandhi and Chikinda, 2007).

Table 1 - Counts of *L. monocytogenes* ScottA in fresh Minas-type goat cheeses, during storage under refrigeration up to 10 days.

Cheeses containing (cfu/mL)	Storage time (days)					
	0	2	4	6	8	10
A. <i>L. monocytogenes</i> only	3.91 ± 0.05 ^c	3.70 ± 0.06 ^c	3.70 ± 0.02 ^{dc}	3.78 ± 0.02 ^{cd}	5.94 ± 0.02 ^b	6.32 ± 0.08 ^a
B. <i>L. monocytogenes</i> + bacteriocinogenic <i>L. lactis</i> DF4Mi	3.52 ± 0.03 ^b	3.27 ± 0.05 ^c	3.25 ± 0.04 ^c	3.31 ± 0.05 ^c	3.75 ± 0.03 ^a	3.76 ± 0.03 ^a
C. <i>L. monocytogenes</i> + non bacteriocinogenic <i>L. lactis</i> R704	3.78 ± 0.02 ^b	3.91 ± 0.02 ^a	3.90 ± 0.7 ^a	3.94 ± 0.01 ^a	3.97 ± 0.07 ^{ab}	3.98 ± 0.01 ^a
D. <i>L. monocytogenes</i> + nisin*	3.00 ± 0.03	< 1	< 1	< 1	< 1	< 1
E. <i>L. lactis</i> DF4Mi only	< 1	< 1	< 1	< 1	< 1	< 1
Without inocula	< 1	< 1	< 1	< 1	< 1	< 1

*12.5 mg/kg cheese; Preparation of the experimental cheeses is specified in section Material and methods (Fresh Minas-type goat cheese manufacturing).

Low levels of bacteriocin production by *Lc. lactis* DF4Mi have been detected when strain have been cultured in sterile 10% reconstructed milk (Difco). However, by applying similar approach, no detection of bacteriocin produced by *Lc. lactis* DF4Mi have been recorded, when strain have been grown in cheese, prepared as described before. Most probably bacteriocin is expressed in low levels, related to viability of the essential nutrient factors important for growth and production of this antimicrobial protein. In addition interaction between bacteriocin and milk protein/s and lipids is possible scenario as well.

These results confirm previous findings suggesting that the efficacy of bacteriocins in culture media is not always reproducible in food systems (*in situ*) (Schillinger *et al.*, 1996). Several factors present in the food can influence the inhibitory effect, such as interaction with additives/ingredients, adsorption to food components, and inactivation by food enzymes and pH changes in the food. Low solubility and uneven distribution in the food matrix and limited stability of bacteriocin during food shelf life are additional factors that influence the activity of bacteriocins in foods. The food microbiota has an important role, especially the microbial load and diversity, as sensitivity to the bacteriocins is variable among bacteria and even among strains belonging to the same species. Microbial interactions in the food system may be responsible for changes in the sensitivity to the bacteriocins. The target microorganisms play also an important role, depending on the physiological stage (growing, resting, starving or viable but non-culturable cells, stressed or sub-lethally injured cells, endospores), the protection by physico-chemical barriers (microcolonies, biofilms, slime) and the development of resistance/adaptation (Galvez *et al.*, 2008).

Despite the wide knowledge on nisin produced by *Lc. lactis* and on bacteriocins produced by other LAB as preservatives in cheeses, little information is available on activity of bacteriocins produced by other *Lactococcus* spp. strains in dairy products (Galvez *et al.*, 2008). Detection of bacteriocin activity in complex food matrixes based on antagonistic approach may be influenced by presence of

lipids and other proteins. In addition, previously have been shown that even if bacteriocin/s are produced in sterile milk medium, antagonistic effect against *L. monocytogenes* frequently is influenced by pH, presence of NaCl, temperature and other ingredients of the fresh or matured cheeses. These factors have effect on interaction on adsorption of bacteriocin/s to *L. monocytogenes* (Pingitore *et al.*, 2012).

Nisin has been used for many years in cheeses to prevent gas blowing caused by *Clostridium tyrobutiricum* (De Vuyst and Vandamme, 1994), proliferation of surviving endospore formers (*Clostridium botulinum* and other clostridia), and control of post-processing contaminant pathogens, mainly *L. monocytogenes* and *S. aureus* (Galvez *et al.*, 2008). Nisin producing strains have been reported to inhibit *Listeria* in several types of cheeses (cottage, camembert, manchego) (Galvez *et al.*, 2008). Nevertheless, nisin producing strains may not offer the technological properties required for cheese making, such as fast acidification and proteolytic activity (O'Sullivan *et al.*, 2002).

Studies on the application of bacteriocins produced by *Lc. lactis* strains in cheeses indicate that results may vary according to the bacteriocinogenic strain and the type of cheese. Lacticin 3147, a two-peptide bacteriocin produced by *Lc. lactis*, inactivated *L. monocytogenes* in cottage cheese (Morgan *et al.*, 2001). Liu *et al.* (2008), also obtained a decrease in *L. monocytogenes* levels in cottage cheese using a strain of *Lc. lactis* with heterologous production of enterocin A. Bacteriocin producing lactococci inhibited or suppressed *L. monocytogenes* in Jben, a Moroccan fresh cheese (Benkerroum *et al.*, 2000). In counterpart, not so good results were observed for Brazilian Minas cheese, as counts of *L. monocytogenes* in samples containing bacteriocinogenic strains did not differ from those in samples containing non bacteriocinogenic LAB (Nascimento *et al.*, 2008).

In the case of fresh Minas-type goat cheese and control of *L. monocytogenes* by bacteriocinogenic *Lc. lactis* DF04Mi, results reported here suggest that the bacteriocinogenic strain is less effective than the bacteriocin on the antilisterial activity. Further studies with semi-purified or

pure bacteriocin produced by this strain, associated to other antimicrobial hurdles, are necessary to evaluate the application of this strain/ bacteriocin for the improvement of safety and quality of this type of cheese.

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