Brazilian Journal of Microbiology 45, 4, 1541-1550 (2014)
ISSN 1678-4405

Research Paper

Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* DF04Mi isolated from goat milk: Characterization of the bacteriocin

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Submitted: July 12, 2013; Approved: April 17, 2014.

Abstract

Lactic acid bacteria capable of producing bacteriocins and presenting probiotic potential open innovative technological applications in the dairy industry. In this study, a bacteriocinogenic strain (*Lactococcus lactis* subsp. *lactis* DF4Mi) was isolated from goat milk, and studied for its antimicrobial activity. The bacteriocin presented a broad spectrum of activity, was sensitive to proteolytic enzymes, resistant to heat and pH extremes, and not affected by the presence of SDS, Tween 20, Tween 80, EDTA or NaCl. Bacteriocin production was dependent on the components of the culture media, especially nitrogen source and salts. When tested by PCR, the bacteriocin gene presented 100% homology to nisin Z gene. These properties indicate that this *L. lactis* subsp. *lactis* DF4Mi can be used for enhancement of dairy foods safety and quality.

Key words: bacteriocin, probiotic, *Lc. lactis* subsp. *lactis,* biopreservation, goat milk.

Introduction

It is well known that many lactic acid bacteria (LAB) are capable of producing a variety of antimicrobial compounds, which may contribute to their colonization of habitats and their competitive advantage over other bacteria (Garriga *et al.*, 1993; Todorov, 2009). Besides production of lactic acid, which causes a drop in pH enough to inhibit certain strains, as its non-dissociated form triggers a lowering of the internal pH in sensitive bacteria that causes a collapse in the electrochemical proton gradient resulting in a bacteriostatic or bactericidal effect (O'Keeffe *et al.*, 1999; Todorov, 2009), LAB can produce other organic acids, diacetyl, hydrogen peroxide, and bacteriocins (Nes and Johnsborg, 2004; Parente and Ricciardi, 1999).

Several bacteriocin-producing LAB strains have been isolated from milk and dairy products (Franco *et al.*, 2011), and particularly from goat milk (Cocolin *et al.*, 2007; Casla *et al.*, 2008; Schirru *et al.*, 2012). However nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis,* remains the best studied bacteriocin (Sobrino-Lopez and Martin-Belloso, 2008). 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; Aslim *et al.*, 2005; Ghrairi *et al.*, 2005; Alomar *et al.*, 2008; Nikolic *et al.*, 2008; Kruger *et al.*, 2013). It is generally accepted that bacteriocins produced by LAB are more active against Gram positive bacteria, however KSA2386, ST34BR and lacticin NK24 are bacteriocins produced by *Lc. lactis* that present a larger spectrum of activity, being active against Gram-negative bacteria as well (Piard, 1994; Ko and Ahn, 2000; Todorov and Dicks, 2004).

Optimal bacteriocin production for technological applications in dairy products remains a challenge, as production does not always correlate with the increase in cell mass or growth rate of the producer strain. Higher bacteriocin levels are produced in the absence of growth stimulating nutrients, or at temperatures and pH conditions lower than required for optimal growth (Matsusaki *et al.*, 1996; Kim *et al.*, 1997; Bogovic-Matijasic and Rogelj, 1998; Krier *et al.*, 1998; Aasen *et al.*, 2000; Todorov *et al.*, 2000; Schirru *et al.*, 2012). Optimal bacteriocin production is often recorded

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in media with limiting concentrations of sugars, nitrogen sources, vitamins and potassium phosphate, or when the medium pH is regulated (Vignolo *et al.*, 1995; Schirru *et al.*, 2012).

Application of bacteriocinogenic strains for dairy product preservation is in agreement with consumers' demands for foods that are naturally preserved. In this study we report results on the isolation of a bacteriocinogenic *Lactococcus lactis* subsp. *lactis* strain (*Lc. lactis* DF04Mi) from raw goat milk and the characterization of the bacteriocin.

Materials and Methods

Isolation and identification of bacteriocin producing strains

Screening for bacteriocin-producing isolates from goat milk was carried out according to the triple-agar-layer method used by Todorov *et al.* (2010). Samples of goat milk obtained from a producer in Ibiúna, SP, Brazil, were submitted to serial decimal dilution with sterile saline (0.85%, w/v NaCl) and plated onto MRS agar (Difco, USA). A second layer of MRS agar (1.7%, w/v) was applied to the plates to provide anaerobic condition for growth of LAB. The plates were incubated at 30 °C for 24 h under anaerobiosis (Anaerocult, Merck, Darmstadt, Germany). Plates containing single colonies were overlaid with a third layer of Brain Heart Infusion (BHI) suplemented by 1% (w/v) agar (Difco) seeded with *Enterococcus faecalis* ATCC 19443, *Lactobacillus sakei* ATCC 15521 or *Listeria monocytogenes* 711 (10^6 cfu/mL), and incubated at 37 °C for 24 h. Colonies surrounded by a growth inhibition zone of at least 2 mm in diameter were transferred to MRS broth (Difco) and incubated for 24 h at 30 °C. Pure cultures, obtained by streaking onto MRS agar (Difco), were submitted to identification by carbohydrate fermentation reactions using API50CHL and API20Strep test strips (Biomérieux, Marcy-I'Etoile, France) and identification confirmed by amplification of genomic DNA with primers F8 and R1512, as described by Felske *et al.* (1997). The amplified fragments were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), sequenced, and compared to sequences in GenBank using BLAST, Basic Local Alignment Search Tool. Six strains were selected and submitted to bacteriocin bioassay. The strains were inoculated (2%, v/v) into 100 mL MRS broth and incubated at 30 °C for 24 h. Cells were harvested (1000 x g, 15 min, 4 °C), the pH of the cell-free supernatants adjusted to 6.0 with sterile 1 M NaOH, heated for 10 min at 80 °C, and then filter-sterilized (0.20 μm, Millipore). Bacteriocin activity was tested against target organisms listed in Table 1, using the agar-spot test method. Target microorganisms included a variety of Gram positive strains isolated from various foods, belonging to the collection of the Food Microbiology Laboratory of University of São Paulo and eight ATCC reference strains. Antimicrobial activity was expressed as arbitrary units (AU)/mL, calculated as follows: a^b x 100, where "a" represents the dilution factor and "b" the last dilution that produced an inhibition zone of at least 2 mm in diameter. Strain DF04Mi, identified as *Lc. lactis* subsp. *lactis*, presented the largest spectrum of activity, and therefore was selected for further bacteriocin characterization.

Characterization of the bacteriocin DF04Mi

Sensitivity of bacteriocin to proteolytic enzymes

One ml of a cell-free supernatant of strain DF04Mi, prepared as described before, was tested for sensitivity to the following enzymes (0.1 and 1.0 mg/mL): α-amylase (Sigma, St. Louis, MO, USA), lipase (Sigma), catalase (Sigma), proteinase K (Roche, Indianapolis, IN, USA), type II bovine α-chymotrypsin (Sigma), type XIV protease from *Streptomyces griseus* (Sigma) and pepsin (Sigma). The mixtures were incubated at 30 °C for 30 min, heated at 95-97 °C for 5 min, cooled and then tested for activity using *E. faecalis* ATCC 19443 as sensitive strain.

Effect of pH, temperature and chemicals on stability of bacteriocin DF04Mi

The effect of pH was measured adjusting the pH of 10 mL of cell-free supernatant of the culture of *Lc. lactis* DF04Mi to 2.0, 4.0, 6.0, 8.0 or 10.0 with 1 M HCl or 1 M NaOH and incubating at 30 °C for 1 h. The effect of temperature on activity was determined incubating supernatants at 30, 37, 45, 60, 80 and 100 °C for 30 min and 2 h, and autoclaving at 121 °C for 20 min. The effect of chemicals was tested adding 10 mg/mL Tween 20 (Synth, Sao Paulo, Brazil), Tween 80 (Synth), NaCl (Synth), SDS (Sigma) or EDTA (Sigma) to supernatants and incubating for 30 min at 30 °C. Before testing for activity, the pH of all samples was adjusted to 6.0. Activity was tested using *E. faecalis* ATCC 19443 as sensitive strain.

Effect of pH and components in MRS broth on bacteriocin production

Cells of *Lc. lactis* DF04Mi were washed two times with sterile saline and suspended in sterile saline to the original volume. The resulting cell suspension (100 μL) was used to inoculate 10 mL of MRS broth with pH adjusted to 4.5, 5.0, 5.5, 6.0 and 6.5, using 1 M NaOH or 1 M HCl. The cell suspension (100 μL) was also used to inoculate 10 mL of formulated MRS broth (Difco), in which original components were replaced by other chemicals or were added of new ingredients. In the first set of experiments, the concentration of glucose and lactose of formulated MRS broth was adjusted to 5.0, 10.0, 20.0, 30.0 and 50.0 g/L. In a second set of experiments, glucose of MRS broth was replaced by L-rhamnose, D-manitol, galactose or mannose (20.0 g/L). In a third set of experiments, organic nitrogen sources in MRS broth were replaced by 20.0 g/L tryptone (Difco) or meat extract (Difco) or yeast extract (Difco), or

Table 1 - Spectrum of activity of the bacteriocin produced by *Lc. lactis* subsp. *lactis* DF04Mi isolated from raw goat milk.

Target microorganism	Medium and growth temperature	Inhibition zone (mm)
Listeria monocytogenes ATCC 7644	BHI, 37 °C	-
L. monocytogenes Scott A	BHI, 37 °C	20
L. monocytogenes serological group 4b		
Strain 101	BHI, 37 °C	15
Strain 211	BHI, 37 °C	17
Strain 302	BHI, 37 °C	15
Strain 620	BHI, 37 °C	14
Strain 703	BHI, 37 °C	17
Strain 724	BHI, 37 °C	17
L. monocytogenes serological group 1/2a		
Strain 103	BHI, 37 °C	15
Strain 104	BHI, 37 °C	19
Strain 106	BHI, 37 °C	15
Strain 409	BHI, 37 °C	21
Strain 506	BHI, 37 °C	20
Strain 709	BHI, 37 °C	15
L. monocytogenes serological group 1/2b		
Strain 426	BHI, 37 °C	17
Strain 603	BHI, 37 °C	16
Strain 607	BHI, 37 °C	20
L. monocytogenes serological group 1/2c		
Strain 408	BHI, 37 °C	20
Strain 422	BHI, 37 °C	17
Strain 637	BHI, 37 °C	15
Strain 711	BHI, 37 °C	20
Strain 712	BHI, 37 °C	20
Listeria innocua ATCC 33090	BHI, 37 °C	-
Enterococcus faecalis ATCC 19443	MRS, 30 °C	9
E. faecium ST88Ch	MRS, 30 °C	-
E. hirae DF105	MRS, 30 °C	15
Lactobacillus acidophilus LA14	MRS, 30 °C	-
Lb. acidophilus La5	MRS, 30 °C	-
L. acidophilus LAC4	MRS, 30 °C	-
Lactobacillus delbrueckii subsp. *bulgaricus* B1	MRS, 30 °C	10
Lb. delbrueckii subsp. *bulgaricus* B15	MRS, 30 °C	12
Lb. delbrueckii subsp. *bulgaricus* B16	MRS, 30 °C	14
Lb. delbrueckii subsp. *bulgaricus* B2	MRS, 30 °C	12
Lb. delbrueckii subsp. *bulgaricus* B5	MRS, 30 °C	-
Lactobacillus paracasei LBC82	MRS, 30 °C	-
Lb. paracasei subsp. *paracasei* DF60Mi	MRS, 30 °C	-
Lactobacillus sakei 2a	MRS, 30 °C	-
Lb. sakei ATCC 15521	MRS, 30 °C	18
Lactococcus lactis subsp. *lactis* B17	MRS, 30 °C	14
Lc. lactis subsp. *lactis* DF02Mi	MRS, 30 °C	12
Lc. lactis subsp. *lactis* DF03Mi	MRS, 30 °C	12
Lc. lactis subsp. *lactis* MK02R	MRS, 30 °C	10
Lc. lactis subsp. *lactis* R704	MRS, 30 °C	15
Lc. lactis subsp. *lactis* V69	MRS, 30 °C	12
Leuconostoc lactis DF05Mi	MRS, 30 °C	12
Staphylococcus aureus ATCC 25923	BHI, 37 °C	-
S. aureus ATCC 29213	BHI, 37 °C	-
S. aureus ATCC 6538	BHI, 37 °C	-
Bacillus cereus ATCC 11778	BHI, 37 °C	-

- = no inhibition observed or inhibition zone < 2 mm diameter.

by these protein sources in the following combinations: 12.5 g/L tryptone plus 7.5 g/L meat extract or 12.5 g/L tryptone plus 7.5 g/L yeast extract, or 10 g/L meat extract plus 10 g/L yeast extract, or 10 g/L tryptone plus 5.0 g/L yeast extract plus 5.0 g/L meat extract. In a fourth set of experiments, MRS broth was added of increasing concentrations of glycerol (0, 2.0, 5.0, 8.0, 10.0 or 20.0 g/L). In the last set of experiments, formulated MRS was prepared adding KH_2PO_4 or K_2HPO_4 or tri-ammonium citrate or sodium acetate or Tween 80 (2.0, 5.0, and 10.0 g/L). All cultures were incubated at 30 °C for 24 h and bacteriocin activity was tested against *L. monocytogenes* 711.

Adsorption of bacteriocin to the producer cells

Adsorption of bacteriocin DF04Mi to producer cells was studied according to Yang *et al.* (1992). The pH of an 18 h-old culture of *Lc. lactis* DF04Mi in MRS broth was adjusted to 5.0 and 10.0 mL were centrifuged at 8000 x *g*, 15 min, 4 °C. The cells were harvested, washed with an equal volume of sterile 0.1 M phosphate buffer (pH 6.5) and the pellet was re-suspended in 10 mL 100 mM NaCl, pre-adjusted to pH 2.0 with 1 M HCl, and stirred for 1 h at 4 °C. Cells were harvested again (3000 x *g*, 30 min, 4 °C) and the cell-free supernatant adjusted to pH 7.0 with sterile 1 M NaOH. Antimicrobial activity in the treated supernatant was tested as previously described.

Effect of bacteriocin on growth of L. monocytogenes 711

The effect of bacteriocin on growth of *L. monocytogenes* 711 was measured adding a 20 mL aliquot of bacteriocin-containing filter-sterilized (0.22 μm, Millipore) supernatant (pH 6.0) to 100 mL culture of the pathogen in early exponential and middle exponential phases. The cultures were incubated for 12 h and optical density readings at 600 nm were recorded at 1 h intervals.

The effect of bacteriocin on reduction on the number of viable cells of *L. monocytogenes* 711 was also measured. Early stationary phase (18 h-old) cultures of the pathogen were harvested (5000 x *g*, 5 min, 4 °C), washed twice with sterile saline and re-suspended in 10 mL saline. Equal volumes of L. monocytogenes 711 cell suspensions and bacteriocin-containing filter-sterilized supernatant (pH 6.0) were mixed, and counts of viable *L. monocytogenes* were performed before and after incubation for 1 h at 37 °C by plating onto MRS agar and BHI agar. Cell suspensions of *L. monocytogenes* 711 with no added bacteriocin served as controls.

Adsorption of the bacteriocin to L. monocytogenes 711, E. faecalis ATCC 19433 and Lb. sakei ATCC 15521

Adsorption of bacteriocin DF04Mi to the target pathogens was tested using the method described by Todorov (2008). The strains were grown overnight in BHI broth (*L. monocytogenes* 711) or MRS (*E. faecalis* ATCC 19433 and *Lb. sakei* ATCC 15521) at 37 °C and 30 °C, respectively, and centrifuged (8000 x *g*, 15 min, 4 °C). Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended to the original volume in the same buffer. The pH was adjusted to 6.5 with sterile 0.1 M NaOH. Each cell suspension was mixed with an equal volume of bacteriocin-containing filter-sterilized supernatant (pH 6.5) and incubated at 37 °C for 1 h. After removal of the cells (8000 *g*, 15 min, 25 °C), the activity of unbound bacteriocin in the supernatant was determined as previously described. All experiments were done in duplicate.

The percentage of bacteriocin adsorption to target cells was calculated according to the following formula:

$$\% \text{ adsorption} = 100 - \frac{\text{bactericon activity after treatment}}{\text{original bacteriocin activity}} \quad (1)$$

The effect of pH and temperature on the adsorption of bacteriocin to the target cells was tested by adding cell-free supernatant to cell suspensions of *L. monocytogenes* 711, *E. faecalis* ATCC 19433 and *Lb. sakei* ATCC 15521, as described before, with incubation for 1 h at 4, 25, 30 and 37 °C at pH 6.0, and at 37 °C at pH 4.0, 6.0, 8.0 and 10.0. Cells were harvested (8000 x *g*, 15 min, 25 °C), and the pH of the supernatant was adjusted to 6.0 with sterile 1 M NaOH. Bacteriocin activity was determined as described before. The experiments were done in duplicate.

The effect of inorganic salts and organic compounds on adsorption of bacteriocin to the target cells was also tested. Cell suspensions of *L. monocytogenes* 711, *E. faecalis* ATCC 19433 and *Lb. sakei* ATCC 15521 were prepared as described before and 1% (m/v) NaCl, Tween 80, glycerol or SDS was added to the cell suspensions. The pH of all samples was adjusted to 6.5 with 1 M NaOH or 1 M HCl. Bacteriocin was added to the treated cells, as described before, and incubated for 1 h at 37 °C. The cells were harvested (8000 *g*, 15 min, 25 °C) and the activity of bacteriocin in the cell-free supernatant determined as previously described. The experiments were done in duplicate.

Search for the nisin gene in Lc. lactis DF04Mi

Total DNA of *Lc. lactis* DF04Mi was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer protocol. Total isolated DNA was amplified with primers NisF, 5-ATG AGT ACA AAA GAT TTC AAC TT-3, and NisR, 5-TTA TTT GCT TAC GTG AAC GC-3, targeting the structural gene of nisin Q as previously reported by de Kruger *et al.* (2013). The amplification conditions were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 45 s, 48 °C for 30 s, and 72 °C for 45 s, and 72 °C for 7 min. Generated PCR product were cleaned with QIAquick PCR Purification Kit (Qiagen) and sequenced with an ABI Prism 377 DNA sequencer (PE Biosystems SA). The exact molecular size of mature bacteriocin DF04Mi (without the leader peptide) was determined from the amino acid sequence deduced from the DNA sequence. DNA homology to sequences listed in GenBank was determined using the BLAST program

(http://blast.ncbi.nlm.nih.gov/blast.cgi). Determination of the temperature responsible for activation/expresion of the nisin operon, including the immunity gene, was done testing growth of *Lc. lactis* DF04Mi in presence of 1% solution of nisin (Sigma) at 30 °C and 37 °C for 24 h, using the agar-spot test.

Results and Discussion

The average count of LAB in the raw goat milk samples used as source for isolation of bacteriocinogenic strains was 3.1 x 10^4 cfu/mL. Among the LAB screened for antimicrobial activity, six isolates were active against the three indicator strains used in the screening test. One of them, identified as *Lactococcus lactis* subsp. *lactis* (*Lc. lactis* DF04Mi), presented a broad spectrum of activity, and therefore was selected for the characterization of the bacteriocin.

The neutralized cell-free supernatant from 24 h-old cultures of Lc. *lactis* DF04Mi inhibited the growth of all tested *L. monocytogenes* strains, except the ATCC 7644 strain (Table 1). Most *Lc. lactis, Lb. delbruecki* and *Leuconostoc* spp. strains were inhibited, however all *L. acidophilus* strains were resistant. *Staphylococcus aureus* and *Bacillus cereus* strains were not affected by studied bacteriocin. Interestingly, most food isolates were sensitive to the cell-free supernatant of *Lc. lactis* DF04Mi while almost all ATCC strains were resistant, regardless the species.

The assessment of the inhibitory spectrum is an important characteristic when evaluating possible applications of bacteriocin-producing strains as potential probiotics or starter cultures, as their inhibitory activity plays a relevant role in the competition with other microorganisms in the gastrointestinal tract or food matrix, protecting them from the colonization by foodborne pathogens. The activity of cell-free supernatants of *Lc. lactis* DF04Mi against most tested *L. monocytogenes* strains is quite interesting, particularly because *L. monocytogenes* continues to be a relevant pathogen in dairy products, as several recent listeriosis outbreaks were linked to cheeses (Fretz *et al.*, 2010; Koch *et al.*, 2010).

Contrary to what was expected, bacteriocin produced by *Lc. lactis* DF04Mi did not inhibit the growth of species of bacteria that are known to belong to the same ecological niche, such as *Lactobacillus* spp. and *Enterococcus* spp. Similar findings were reported for the bacteriocins produced by other *Lc. lactis* subsp. *lactis* isolates from milk products (Nikolic *et al.*, 2008; Kruger *et al.*, 2013).

Treatment of the cell-free supernatant with proteinase K, protease, pepsin or α-chymotrypsin resulted in complete inactivation of the antimicrobial activity (Table 2). Treatment with catalase did not affect the activity against the target strains, discarding clearly the involvement of H_2O_2 in the antagonism process. Moreover, treatment with α-amylase and lipase did not affect the antimicrobial activity, suggesting that bacteriocin DF04Mi does not belong to the controversial group IV of the bacteriocins, which contain carbohydrates or lipids in the active molecule. According to De Vuyst & Vandamme (1994), most bacteriocins are polypeptides but others, like leuconocin S produced by *Leuconostoc paramesenteroides* (Lewus *et al.*, 1992), carnocin 54 produced by *Leuconostoc carnosum* (Keppler *et al.*, 1994) and bacteriocin ST63BZ produced by *Leuconostoc lactis* ST63BZ (Todorov, 2010) are typical examples of amylase-sensitive bacteriocins.

Bacteriocin DF04Mi activity was not affected by SDS, Tween 20, Tween 80, EDTA or NaCl (Table 2). The sensitivity of bacteriocins to detergents and NaCl seems to be bacteriocin-dependent and affected by the experimental conditions. Bioactivity of plantaricin C19 was not affected by the presence of SDS or Triton X-100 (Atrih *et al.*, 2001) contrarily to bacteriocins ST311LD (Todorov and Dicks, 2005), plantaricin 423 (Verellen *et al.*, 1998), pediocin PA-1/AcH (Biswas *et al.*, 1991), lactacin B (Barefoot and Klaenhammer, 1984) and lactocin 705 (Vignolo *et al.*, 1995). On the other side, bacteriocin HV219 was sensitive to SDS, Triton X-114 and Triton X-100, but resistant to Tween 20, Tween 80, urea or EDTA (Todorov *et al.*, 2006). Similar results were recorded for bozacin B14 produced by Lc. lactis subsp. *lactis* B14 (Ivanova *et al.*, 2000), and bacteriocin J46 produced by *Lc. lactis* subsp. *cremoris* (Huot *et al.*, 1996).

As shown in Table 2, bacteriocin DF04Mi is heat resistant (100 °C for 2 h, 121 °C for 20 min) and remains stable at pH ranging from 2.0 to 10.0 for 2 h. A number of studies have shown that heat and pH resistance of bacteriocins are also bacteriocin-dependent. Bacteriocins produced by other *Lactobacillus* and *Lactococcus* strains presented results similar to bacteriocin DF04Mi (Klaenhammer, 1988; Verellen *et al.*, 1998; Todorov *et al.*, 1999; Todorov and Dicks, 2004; Kruger *et al.*, 2013). However, lactocin NK24, produced by *Lc. lactis*, lost 87.5% of its activity after 30 min at 100 °C and was completely inactivated after 15 min at 121 °C (Lee and Paik, 2001). In the case of lactocin MMFII produced by *Lc. lactis*, only 8.3% of activity was recorded after 30 min at 110 °C or 25% after 30 min at 80 °C and 100 °C (Ferchichi *et al.*, 2001). Nisin, produced by *Lc. lactis* WNC20, was inactivated after 15 min at 121 °C when incubated at pH 7.0, but not when incubated at pH 3.0 at this temperature (Noonpakdee *et al.*, 2003). Bozacin B14, produced by *Lc. lactis*, was inactivated after 10 min at 90 °C (Ivanova *et al.*, 2000).

Low levels of activity (200 AU/mL) for bacteriocin DF04Mi was recorded after treatment of the producer cells with 100 mM NaCl at pH 2.0 suggesting adhesion of the bacteriocin to the surface of the producer cells. Similar result was reported for bacteriocin HV219 (Todorov *et al.*, 2006), while the opposite was reported for plantaricin ST31 (Todorov *et al.*, 2000) and bozacin B14 (Ivanova *et al.*,

Table 2 - Activity of bacteriocin produced by *Lactococcus lactis* subsp *lactis* DF04Mi after treatment with enzymes, pH, heat and detergents.

	Concentration	Activity of bacteriocin DF04Mi*
Enzymes	0.1 and 1.0 g/L	
α-amylase, lipase, catalase		+
Proteinase K, α-chymotrypsin, protease, pepsin		-
pH		
2-6		+
8-10		+
Temperature		
100, 80, 60, 45, 37, 30, 20, 4 °C for 1 h		+
100, 80, 60, 45, 37, 30, 20, 4 °C for 2 h		+
121 °C for 20 min		+
Detergents / chemicals	1% (w/v)	
Tween 20, Tween 80, SDS, NaCl, EDTA		+

* + = presence of inhibition zone (> 2 mm).
- = no inhibition.

2000). In the case of plantaricin C19, maximal adsorption to the producer cells was recorded between pH 5.0 and 7.0, with a complete loss of adsorption at pH 1.5 and 2.0 (Atrih *et al.*, 2001).

L. lactis DF04Mi grew well both at 30 °C and 37 °C, but bacteriocin production occurred only at 30 °C. The maximum bacteriocin production at this temperature was 3200 AU/mL (Table 3). *Lc. lactis* DF04Mi did not grow in MRS with an initial pH of 4.5, and consequently, no bacteriocin could be detected. Low levels of bacteriocin DF04Mi (400 AU/mL) were recorded in MRS broth adjusted to pH 5.0. In the same medium adjusted to pH 5.5, only 800 AU/mL of bacteriocin was recorded. The highest level (3200 AU/mL) was recorded after 18 h in MRS adjusted to an initial pH of 6.0 or 6.5. The final pH values of the cultures were approximately the same (pH 4.3), irrespective of the initial pH.

Growth of *Lc. lactis* DF04Mi in MRS containing rhamnose, manitol, mannose or galactose instead of glucose yielded reduced or absence of bacteriocin production (Table 3). When the concentration of glucose was increased from 5.0 g/L to 20.0 g/L, the production of bacteriocin increased from 400 AU/mL to 3200 AU/mL, but higher concentrations reduced the amount of bacteriocin in the medium. Similar results were reported for bacteriocin HV219 (Todorov *et al.*, 2006). When glucose was replaced by lactose (at concentrations from 0.5 to 50.0 g/L) production of bacteriocin increased from 800 AU/mL to 1600 AU/mL. Similar results were recorded for sakacin P (Aasen *et al.*, 2000) and nisin (De Vuyst and Vandamme, 1994).

As shown in Table 3, the maximum production of bacteriocin DF04Mi in MRS broth supplemented with tryptone, meat extract or yeast extract, or combinations of two of these ingredients, was 800 AU/mL. However, when the three organic nitrogen sources were present simultaneously, the amount of bacteriocin increased four-fold (3200 AU/mL). The influence of the nitrogen source in the culture medium on the production of bacteriocins was also observed in other studies. For bacteriocin HV219, yeast extract was the best nitrogen compound (Todorov *et al.*, 2006), while for plantaricin 423, highest production was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Verellen *et al.*, 1998).

Glycerol in MRS medium presented a marked influence in bacteriocin production. The highest production (3200 AU/mL) was obtained when glycerol was absent or when the concentration was below 5.0 g/L (Table 3). Glycerol above 5.0 g/L resulted in reduced bacteriocin production.

Presence of $MgSO_4$ and $MnSO_4$ and appropriate amounts of tri-ammonium citrate or sodium acetate were essential for production of bacteriocin DF04Mi. K_2HPO_4 is also an important ingredient for bacteriocin production in MRS. Lower levels of bacteriocin DF04Mi were recorded when KH_2PO_4 was used as replacement for K_2HPO_4 (Table 3). However, 5.0 and 10.0 g/L K_2HPO_4 yielded reduced levels of bacteriocin compared with 2.0 g/L K_2HPO_4, which corresponds to the concentration of K_2HPO_4 in commercial MRS medium. Previous studies conducted with bacteriocin HV219 have shown that increasing the concentration of K_2HPO_4 from 2.0 g/L to 10.0 g/L an increase of 400% in bacteriocin activity was achieved (Todorov *et al.*, 2006). In the case of the bacteriocin produced by *Lb. plantarum* UG1, a minimal level of 7.0 g/L K_2HPO_4 was required for bacteriocin production (Enan *et al.*, 1996). Similar results were reported for plantaricin ST31 (Todorov *et al.*, 2000).

Addition of 3200 AU/mL bacteriocin DF04Mi to a 3 h-old culture of *L. monocytogenes* 711 resulted in growth

Table 3 - Effect of pH and components added to formulated MRS broth on the production of bacteriocin DF04Mi at 30 °C for 24 h.

pH or medium component	Concentration (g/L)	bacteriocin activity* (AU/mL)
pH 4.5		0
pH 5.0		400
pH 5.5		800
pH 6.0		3200
pH 6.5		3200
Glucose	50.0	200
Lactose	5.0	800
Lactose	10.0	800
Lactose	20.0	1600
Lactose	30.0	1600
Lactose	50.0	800
L-Rhamnose	20	0
D-Manitol	20	400
Galactose	20	0
Mannose	20	400
Tryptone	20	800
Meat extract	20	400
Yeast extract	20	800
Tryptone and meat extract	12.5 + 7.5	400
Tryptone + yeast extract	12.5 + 7.5	800
Meat extract + yeast extract	10.0 + 10.0	800
Tryptone + yeast extract + meat extract	10.0 + 5.0 + 5.0	3200
Glycerol	0	3200
Glycerol	2.0	3200
Glycerol	5.0	3200
Glycerol	8.0	1600
Glycerol	10.0	1600
Glycerol	20.0	800
KH_2PO_4	2.0	400
KH_2PO_4	5.0	400
KH_2PO_4	10.0	400
K_2HPO_4	0	0
K_2HPO_4	2.0	3200
K_2HPO_4	5.0	800
K_2HPO_4	10.0	800
$MgSO_4$	0	400
$MgSO_4$	0.1	3200
$MnSO_4$	0	800
$MnSO_4$	0.05	3200
Tri-ammonium citrate	0	0
Tri-ammonium citrate	2.0	3200
Tri-ammonium citrate	5.0	800
Sodium acetate	0	800
Sodium acetate	2.0	400
Sodium acetate	5.0	3200
Tween 80	0	3200
Tween 80	2.0	3200
Tween 80	5.0	3200
Tween 80	10.0	3200

*test microorganism *Listeria monocytogenes* 711.

inhibition for at least 12 h (data not shown). When cells at stationary phase were treated with bacteriocin DF04Mi, no viable cells of *L. monocytogenes* 711 were detected after 1 h contact time, regardless the amount of cells submitted to treatment (data not shown). Other bacteriocins, such as those produced by *Pediococcus acidilactici* HA-6111-2 and *E. faecium* ST5Ha, presented a similar behaviour (Albano *et al.*, 2007; Todorov *et al.*, 2010).

Adsorption of bacteriocin DF04Mi to *L. monocytogenes* 711, *Lb. sakei* ATCC 15521 and *E. faecalis* ATCC 19433 varied according to the pH, temperature and chemicals in the culture medium (Table 4). Optimal adsorption to *L. monocytogenes* 711 and *Lb. sakei* ATCC 15521 was recorded at pH 4.0 and 6.0. Adsorption to *E. faecalis* ATCC 19443 was lower when compared to the two other microorganisms, regardless the pH. Various studies, conducted with other bacteriocins, have reported different levels of adsorption to different target cells. In the case of buchnericin LB, optimal adsorption to *Lb. plantarum* was recorded at pH 5.0 - 8.0 (Yildirim *et al.*, 2002). Optimal adsorption of plantaricin 423 to *E. faecium* HKLHS was recorded between pH 8.0 and 10.0, and to *Lb. sakei* DSM 20017 between pH 2.0 and 6.0 (Todorov and Dicks, 2006). These results suggest that application of bacteriocin DF04Mi at neutral or moderately acid pH is more effective than at other pH. Differences in adsorption affected by pH rates may be due to specific interaction between bacteriocin DF04Mi and the target strain or structural pH-dependent modification of the bacteriocin receptors on the target cell surface.

As observed for bacteriocin DF04Mi, bacteriocin HV219, produced by *Lc. lactis* subsp. *lactis* HV219, presented the highest levels of adsorption to sensitive strains compared to strains resistant to bacteriocin (Todorov *et al.*, 2007). In the case of plantaricin 423, adsorption ranged from 17% for *Streptococcus caprinus* ATCC 700066 to 67% for *Lb. plantarum* LMG 13556, *Lb. curvatus* DF38, *L. innocua* LMG 13568 and *Lb. sakei* DSM 20017. Strains sensitive to plantaricin 423 presented a stronger adsorption,

Table 4 - Effect of pH, temperature, and chemicals on adsorption of bacteriocin DF04Mi to *Listeria monocytogenes* 711, *Lactobacillus sakei* ATCC 15521 and *Enterococcus faecalis* ATCC 19433.

	L. monocytogenes 711	*L. sakei* ATCC 15521	*E. faecalis* ATCC 19433
Control	50*	50	25
pH			
4.0	50	75	50
6.0	50	50	25
8.0	25	25	25
10.0	0	25	0
Temperature (°C):			
4	75	75	50
25	75	75	50
30	50	50	25
37	50	50	25
Chemicals (1%)			
glycerol	25	50	25
NaCl	75	75	50
SDS	50	50	25
Tween 20	25	25	25
Tween 80	25	25	25

* results presenting the % of adsorption of bacteriocin produced by *L. lactis* DF04Mi to *Listeria monocytogenes* 711, *Lactobacillus sakei* ATCC 15521 and *Enterococcus faecalis* ATCC 19433, respectively.

probably due to the presence of the recognition site on the cell surface on sensitive strains (Todorov and Dicks, 2006).

Highest absorption of bacteriocin DF04Mi to target cells was observed at 4 °C or 25 °C than at 30 °C or 37 °C (Table 4). Previous studies have also detected the influence of temperature on adsorption of bacteriocins to the surface of bacteria, in a bacteriocin dependent manner. Higher adsorption to *E. faecium* HKLHS at lower temperatures was also observed for bacteriocin HV219 but not for plantaricin 423 (Todorov and Dicks, 2006), but adsorption of bacteriocin AMA-K to *L. innocua* LMG 13568, *L. monocytogenes* Scott A and *L. ivanovii* subsp. *ivanovii* ATCC 19119 was higher at 45 °C than at 30 °C and 37 °C, while low temperatures (4 °C and 15 °C) resulted in reduction of adsorption (Todorov, 2008).

Tween 20 and Tween 80 caused a decrease in adsorption of bacteriocin DF04Mi to *L. monocytogenes* 711 and *Lb. sakei* ATCC 15521, but not to *E. faecium* ATCC 19433 (Table 4). SDS did not affect the adsorption to any of the tested organisms, while glycerol caused a reduced adsorption to *L. monocytogenes* 711 but not to the other strains. NaCl caused an increase in the absorption of bacteriocin DF04Mi to all tested strains. This may have a positive effect if the bacteriocin will be applied in cheese production, because of the presence of NaCl in the food matrix. Other studies with bacteriocins AMA-K and HV219 indicated that surfactants and solvents such as Tween 20, Tween 80, Triton X-100, β-mercapto-ethanol, ethanol, methanol, and chloroform, and salts such as NaCl, sodium nitrate, KH_2PO_4, $MgCl_2$, sodium acetate, Na_2CO_3, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl and others affect the adsorption in different ways, depending on the bacteriocin and the target bacteria (Todorov *et al.*, 2007; Todorov, 2009).

In the search for the nisin gene in *Lc. lactis* DF04Mi, the sequenced PCR product presented 100% homology to nisin Z (Figure 1). Variants of nisin producing *Lc. lactis* have been previously reported (Aso *et al.*, 2008). However, when we tested the effect of 1% commercial nisin on growth of *Lc. lactis* DF04Mi (by spot-on-lawn method) at 30 °C and 37 °C we have observed that strain DF04Mi was sensitive only if the test was performed at 37 °C. As been previously pointed in this study, *Lc. lactis* DF04Mi produces bacteriocin only if it was cultivated at 30 °C. Taking in consideration that bacteriocin structural gene and the immunity gene are belong to the same operon, we can conclude that most probably bacteriocin immunity gene is not expressed neither at 37 °C. This observation, combined with the results for the effect of commercial nisin on *Lc. lactis* DF04Mi at 30 °C and 37 °C pointing that most probably *Lc. lactis* DF04Mi expressing a nisin. Future studies on purification of the bacteriocin produced under different growth conditions, followed by identification by mass spectrometry and partial or complete amino acid sequencing, are necessary to provide evidence that the gene responsible for synthesis of the bacteriocin produced by Lc. *lactis* DF04Mi is expressed. Several studies have shown that ex-

	-20	-10	1	10	20	30
DF04Mi:	MSTKDFNLDLV	SVSKKDS	GASPRITSIS	LCTPGCKTGA	LMGCNMKTAT	CNCS_IHVSK
Nisin Z:	MSTKDFNLDLV	SVSKKDS	GASPRITSIS	LCTPGCKTGA	LMGCNMKTAT	CNCS_IHVSK
Nisin F:	MSTKDFNLDLV	SVSKKDS	GASPRITSIS	LCTPGCKTGA	LMGCNMKTAT	CNCSVHVSK
Nisin A:	MSTKDFNLDLV	SVSKKDS	GASPRITSIS	LCTPGCKTGA	LMGCNMKTAT	CHCS_IHVSK
Nisin Q:	MSTKDFNLDLV	SVSKTOS	GASPRITSIS	LCTPGCKTGV_	LMGCN__LKTAT	CNCSVHVSK

Figure 1 - Amino acid sequence of the bacteriocin produced by *Lactococcus lactis* subsp *lactis* DF04Mi, compared to nisin Z, nisin F, nisin A and nisin Q. The leader peptide of each nisin variant consists of 13 amino acids (from position -13 to -1), followed by amino acids encoding the mature protein (amino acid positions 1 to 34) Differences in amino acids are indicated in bold.

pression of structural bacteriocin genes is affected by the conditions in which the bacteriocin producing strain is cultivated (Vignolo *et al.*, 1995; Matsusaki *et al.*, 1996; Krier *et al.*, 1998; Aasen *et al.*, 2000; Poeta *et al.*, 2007).

Acknowledgments

This research was supported by grants from FAPESP (Sao Paulo, SP), CAPES (Brasilia, DF) and CNPq (Brasilia, DF), Brazil.

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