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CHARACTERIZATION OF BACTERIOCINS PRODUCED BY *LACTOCOCCUS LACTIS* STRAINS

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ABSTRACT

Bacteriocins produced by fifteen strains of *Lactococcus lactis* (14 *L. lactis* subsp. *lactis* and one *L. lactis* subsp. *cremoris*) were heat resistant, sensitive to several proteolytic enzymes and active over a wide range of pH. Their resistance to the heating was greatly influenced by the pH. Only the strain *L. lactis* subsp. *lactis* ITAL 383 produced a bacteriocin with a wide activity spectrum, similar to nisin of *L. lactis* subsp. *lactis* ATCC 11454. This bacteriocin inhibited closely related species and other Gram-positive microorganisms including *Listeria monocytogenes* and *Staphylococcus aureus*, but it was not active against the Gram-negative bacteria tested. The identification of partially purified antimicrobial compounds by SDS-PAGE showed that bacteriocin produced by strain ITAL 383 had the same molecular weight of nisin produced by *L. lactis* subsp. *lactis* ATCC 11454.

Key words: bacteriocins, *L. lactis*, activity spectrum, physical-chemical characteristic

INTRODUCTION

Lactococcus lactis strains are widely used as starter cultures for several types of cheese, fermented milk products, and ripened cream butter. The composition of many mesophylic starters includes the acid-producing cultures (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) and the citric-acid-fermenting bacteria, *L. lactis* subsp. *lactis* var. *diacetylactis*. The fermentation of sugars, leading to a pH decrease is important for the clothing of the milk and reduction or prevention of adventitious microbial growth. Indeed, some cultures produce antimicrobial substances in smaller amounts, like hydrogen peroxide, diacetyl, organic acids, secondary reaction products, and bacteriocin (7). Competition for essential nutrients, accumulation of D-aminoacids, lowering of oxidation-reduction potential and coagregation may also be involved in antagonism (34).

By definition, bacteriocins are biologically active proteins or protein complexes displaying a bactericidal mode of action exclusively towards Gram-positive bacteria and particularly closely related species (36). They form a heterogeneous group with respect to the producing-bacterial species, molecular size, antibacterial spectrum, stability and physical and chemical properties, and mode of action (9, 10). Production of bacteriocin has been detected in all genera of lactic acid bacteria (9, 10). A large variety of bacteriocins occur within the species *L. lactis* and nisin is the most studied (16, 17). Two types are known, nisin A and Z (16, 27). Some bacteriocins characterized include diplococcin (8, 30), lactostrepcins (20), lactococcins (11, 13, 15, 29), dricin (35), lacticin (33) among others (4, 13, 19, 28).

In the last years, the bacteriocins of lactic acid bacteria have attracted much attention because of their potential to increase safety and to extend shelf life of food (12). Currently, only nisin has been granted Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA). The objective of this study was to determine the physical-chemical characteristics and antimicrobial spectrum of bacteriocins detected previously (26).

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MATERIALS AND METHODS

Microorganisms and culture media

Bacteriocin-producing *L. lactis* strains previously selected as well as other genus of lactic acid bacteria used in this study are listed in Table 1. Stock cultures were maintained in frozen storage at –20°C in 14% solids of sterile reconstituted skim milk powder. Before use, the cultures were transferred twice in M17 broth (Oxoid) supplemented with 0.5% (w/v) glucose (GM17) and incubated at 30°C for 16-18h.

The spoilage and food-borne microorganisms used in the experiments are listed in Table 2. These cultures were maintained at 4°C on agar slants. The culture media and growth conditions used were: brain heart infusion (BHI, Oxoid) for *Staphylococcus aureus (*37°C), triypticase soy broth (TSB, Difco) for *Salmonella typhimurium* (30°C), *Yersinia enterocolitica* (30°C) and *Bacillus subtilis* (37°C), triyptcase soy broth supplemented with 0,6% (w/v) yeast extract (TS-YE, Difco) for *Listeria* sp. (30°C) and reinforced clostridial medium (Oxoid) for *Bacillus* (37°C). A non-pathogenic *Listeria innocua* Lin 11 (Pasteur Institute, Paris, France) was used as the indicator microorganism for antimicrobial assays. The nisin-producing *L. lactis* subsp. *lactis* ATCC 11454 was used as positive control.

Activity spectrum

The well diffusion direct assay described by Tagg and McGiven (36) and modified by Benkerroum *et al*. (1) was utilized to detect the inhibition of the sensitive strain. The GM17 broth was supplemented with sodium β-glycerophosphate (2%) and catalase (Sigma Chemical Co., Dorset, England) at final concentration of 100U. Twenty milliliters of GM17 agar inoculated with 1% (10^5-10^6 cfu ml^{-1}) of stationary phase sensitive indicator cultures was poured in a sterile Petri dish and allowed to harden. Holes were punched out of the agar, by using a cork bore (4 mm of diameter). The base of each hole was sealed with 50 µl of GM17 soft agar (0.75% agar) and then filled with 50 µl overnight test strains. The inoculated plates were incubated at 30°C for 18-24h and checked for the presence of clear zones of inhibition as a result of antimicrobial activity.

Sensitivity to proteases

Sterile cell-free supernatants at pH 6.5 were treated with the following enzymes (0.2 mg ml^{-1}): proteinase K in 20 mM Tris-HCl, pH 7.0; ficin in 20 mM sodium phosphate, pH 7.0; trypsin in 40 mM Tris-HCl, pH 8.2; α-chymotrypsin in 20 mM Tris-HCl, pH 8.0; pronase E in 20 mM Tris-HCl, pH 7.8; pepsin in 0.002 N HCl; lipase in 0.1 M potassium phosphate, pH 6.0; and papain in 0.05 M sodium phosphate acetate (31). All these solutions were filter-sterilized through Millex (Millipore) GV 0.22 µm filters and then added to supernatants (v/v, 1/1). Controls consisted of enzyme solutions without bacteriocin and only bacteriocin in sodium 0.1 M phosphate buffer. The samples and controls were incubated at 37°C for 2h and heated at 100°C for 5 min to denature the enzymes. This treatment did not affect the antimicrobial activity of bacteriocins. The remaining bacteriocin activity was determined by serial twofold dilution assay described by Mayr-Harting *et al*. (23). The title was defined as the reciprocal of the highest dilution showing an inhibition of the indicator strain multiplied by 100 to express the results as activity units by milliliter ($AUml^{-1}$).

Cell-free supernatants from bacteriocin-producing strains were collected by centrifugation (7,500g/10 min, 4°C) of overnight GM17 broth cultures. The supernatants were neutralized to pH 6.5 with 10 N NaOH and sterilized through Millex-HV (0.22 µm, Millipore Corp.).

Activity of bacteriocins at different pH levels and effect of heat treatment

To determine the thermal stability at different pH levels, the sterile cell-free supernatants adjusted at different pH values (2.0, 4.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 12.0) were autoclaved at 121°C for 10 min. The treated and untreated control samples were assayed for activity by the critical dilution assay (23). Sterile cell-free supernatants of *L. lactis* subsp. *lactis* ITAL 383 and ATCC 11454 adjusted to pH 2.0 and 6.0 were also heated in a boiling bath. Residual activity was assayed at 10, 20, 30 and 60 min by the critical dilution assay (23) with appropriate controls.

To determine the activity of bacteriocins at different pH levels, sterile cell-free supernatants were adjusted with sterile NaOH or HCl to different pH values (2.0, 4.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 12.0). The activity of bacteriocins was assayed after 30 min. by the critical dilution assay of Mayer-Harting *et al*. (23).

Activity detection in SDS-PAGE

To estimate the molecular weight of partially purified bacteriocin of wide activity spectrum produced by ITAL 383 was analyzed by SDS-PAGE. A 200 ml aliquot of sterile cell-free supernatant was brought to 60% saturation by the addition of solid ammonium sulfate. The samples were stirred vigorously at 4°C over 4 h and then centrifuged at 20,000g at 4°C during 45 min. The pellets were suspended in 20 ml of 25 mM Sørensen buffer (pH 6.0) and treated with ammonium sulfate to 80% saturation. Following centrifugation (20,000g, 45 min), the pellet was ressuspended in 5 ml of Sørensen buffer (crude extracts) and assayed for both bacteriocin activity and protein content (32). To estimate the molecular weight, crude extracts of ITAL 383 were examined on 10-25% SDS-PAGE gradient gel. Samples (1.0 mg ml) were dissolved in buffer and loaded on to the gel. After electrophoresis at 40 mA for approximately 4 h, the gel was assayed for antimicrobial activity by the direct detection system previously described by Bhunia *et al.* (3). The nisin-producing *L. lactis* subsp. *lactis* ATCC 11454 was used as positive control.

Table 1. Origin of lactic acid bacteria strains.

Number of strains	Species	Origin
1	*L. lactis subsp. lactis* 11454	ATCC
1	*L. lactis subsp. lactis* 150	CNRZ
38	*L. lactis subsp. lactis*	Raw buffalo milk
10	*L. lactis subsp. lactis*	Cheese whey
10	*L. lactis subsp. lactis*	Commercial cultures
12	*L. lactis subsp. lactis*	Cheese starters cultures
30	*L. lactis subsp. lactis*	Regional cheese
8	*L. lactis subsp. lactis*	INRA
2	*L. lactis subsp. cremoris*	Raw milk
8	*L. lactis subsp. cremoris*	Commercial cultures
1	*L. lactis subsp. cremoris*	INRA
38	*L. lactis subsp. cremoris*	Cheese starters cultures
10	*L. lactis subsp. lactis* biovar. *diacetylactis*	Commercial cultures
4	*Lactobacillus acidophilus*	Commercial cultures
4	*Lab. casei subsp. casei*	Commercial cultures
	Lab. bulgaricus	Commercial cultures
20	*Lab. plantarum*	Forrage
1	*Leuconostoc cremoris*	Commercial cultures
2	*Pediococcus pentosaceus*	Commercial cultures

ATCC: American Type Culture Collection; Rockville, MD, USA; INRA: Institut National de Recherches Agronomiques, Jouy-en-Josas, France; ITAL: Instituto de Tecnologia de Alimentos, Campinas, SP, Brazil

RESULTS AND DISCUSSION

Inhibitory spectrum

The inhibitory spectrum of bacteriocins against several Gram-positive and Gram-negative microorganisms is shown in Table 3. This is an important characteristic in order to evaluate the possibility of using the bacteriocin-producing strains as an additional barrier against spoilage and/or food-borne microorganisms in foods. Only 1 (6.7%) out of 15 bacteriocin-producing strains exhibited wide activity spectrum against pathogenic and spoilage microorganisms tested. This result is similar to that of Gupta and Batish (14) who found 1.5% of bacteriocin-producing lactococci among 600 strains isolated of buffalo milk. The antimicrobial spectrum of the bacteriocin of ITAL 383 was similar to nisin of *L. lactis* subsp. *lactis* ATCC 11454. It showed a wide spectrum of inhibitory activity affecting closely related species and all other Gram-positive microorganisms examined. The only difference between the antimicrobial spectra of ITAL 383 and ATCC 11454 was the inhibition of *E. faecalis* and *B. subtilis* by the last one. It is interesting to observe the resistance of *Lb. plantarum* strains to bacteriocin of ITAL 383 and nisin of ATCC 11454. Among the 20 strains tested, 40% were inhibited by ITAL 383 and 30% by ATCC 11454. Specific nisin-inactivating enzymes or nisinases are produced by nisin-resistant strains of *Lb. plantarum* (10). Otherwise, bacteriocin of ITAL 383 and nisin of ATCC 11454 inhibited all *Listeria* strains examined. The sensitivity difference of each strain was previously demonstrated (25). Bacteriocin produced by 12 strains (11 *L. lactis* subsp. *lactis* and 1 of *L. lactis* subsp. *cremoris*) exhibited a narrow spectrum of activity related only to bacteria belonging to the same species. One culture of *L. lactis* subsp. *lactis* ITAL 403 showed activity towards related bacteria belonging to the same genera and a limited numbers of *Lactobacillus* strains (3 *Lb. acidophilus*, 4 *Lb. casei* subsp. *casei* and 1 *Lb. delbrueckii* subsp. *bulgaricus*). The bacteriocins did not inhibit any of the Gram-negative tested. This inability of the bacteriocins over some microorganisms was previously reported (2, 5, 6, 14, 19, 24, 31, 37).

Sensitivity to proteases

Since bacteriocins are by definition proteinaceous substances they must be sensitive to at least one proteolytic enzyme. Consequently, protease sensitivity is a key criterion

in their characterization. The effect of various proteolytic and lipolytic enzymes over the bacteriocins is shown in Table 4. The loss of the antimicrobial activity after treatment with enzymes indicated the sensitivity of the active compounds secreted by *L. lactis* strains. All bacteriocins, including nisin of ATCC 11454, were fully or partially inactivated by proteinase K, pronase E, and α-chymotrypsin. Exception was noted in ITAL 104, which was fully resistant to pronase E. Some authors distinguish the nisin from other bacteriocins based on the strength of their sensitivity to α-chymotrypsin. However, the

Table 2. Origin of spoilage and/or food-borne microorganisms.

Species	Origin	Species	Origin
B. subtilis	Commercial cultures	*L. welshimeri* LW1	Dairy industries INRA
Citrobacter sp.	Commercial cultures	*L. welshimeri* LW2	Cheese INRA
E. aerogenes	Commercial cultures	*L. welshimeri* LW3	Processed meat FEA
E. coli	Commercial cultures	*L. welshimeri* LW4	Cheese FEA
L. monocytogenes V7	Raw milk	*Listeria* sp. L1	INRA
L. monocytogenes Scott A SA	Clinical material	*Listeria* sp. L2	INRA
L. monocytogenes Ohio OH	Cheese	*Listeria* sp. L3	INRA
L. monocytogenes Califórnia CA	Cheese	*Listeria* sp. L4	INRA
L. monocytogenes type 1 LM1	Milk, INRA	*Listeria* sp. L5	INRA
L. monocytogenes type 1 LM2	Milk, INRA	*Listeria* sp. L6	INRA
L. monocytogenes type 1 LM3	Milk, INRA	*Listeria* sp. L7	INRA
L. monocytogenes type 1 LM4	Milk, INRA	*S. aureus* S8	INRA
L. monocytogenes type 1 LM5	Cheese, INRA	*S. aureus* S9	INRA
L. monocytogenes type 1 LM6	Cheese, INRA	*S. aureus* S10	INRA
L. monocytogenes type 4 LM7	Milk, INRA	*S. aureus* S11	INRA
L. monocytogenes type 4 LM8	Cheese, INRA	*S. aureus* S12	INRA
L. monocytogenes type 4 LM9	Cheese, INRA	*S. aureus* S15	INRA
L. monocytogenes type 4 LM10	Milk, INRA	*S. aureus* S18	INRA
L. innocua LIN1 type 6A	Material meat, INRA	*S. aureus* S22	INRA
L. innocua LIN2 type 6B	Cheese, INRA	*S. aureus* S23	INRA
L. innocua LIN3	Meat, INRA	*S. aureus* S26	INRA
L. innocua LIN4	Milk, FEA	*S. aureus* Sa1	ITAL
L. innocua LIN5	Meat, FEA	*S. aureus* Sa2	ITAL
L. innocua LIN6	Cheese, FEA	*S. aureus* Sa3	ITAL
L. innocua LIN7	Processed meat, FEA	*S. aureus* Sa4	ITAL
L. innocua LIN11	Milk, INRA	*S. aureus* Sa5	ITAL
L. ivanovii LIV	Dairy industries, INRA	*S. aureus* Sa6	ITAL
L. murrayi LM	Meat, FEA	*S. aureus* Sa8	ITAL
L. seeligeri tipo 6B LS1	Meat, INRA	*S. typhimurium*	ITAL
L. seeligeri tipo 1-2 LS2	Meat, INRA	*V. parahemoliticus*	ITAL
L. seeligeri LS3	Processed meat, FEA	*Y. enterocolítica*	ITAL
L. seeligeri LS4	Cheese, FEA		

ITAL: Instituto de Tecnologia de Alimentos, Campinas, S.P., Brasil; INRA: Institute National de Recherches Agronomiques, Jouy-en-Josas, France; FEA: Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, S.P. Brasil; Strains V7, SA e CA: Dr. E. RYSER, University of Wisconsin, E.U.A.; Strains *L. monocytogenes* (LM1 a LM10): Dr. AUDURIER, Faculté de Médicine de Tours, France.

Table 3.Evaluation of inhibition of Gram-positive and Gram-negative microorganisms by the well direct diffusion assay.

Microorganisms	*L. lactis* strains															
	N*	104	175	185	187	383	387	402	403	404	408	435	436	437	438	PC***
Gram-positive																
Bacillus cereus	1	0**	0	0	0	1	0	0	0	0	0	0	0	0	0	1
B. subtilis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Enterococcus faecalis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
E. faecium	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Lactobacillus acidophilus	3	0	0	0	0	3	0	0	3	0	0	0	0	0	0	3
Lb. casei	4	0	0	0	0	4	0	0	4	0	0	0	0	0	0	4
Lb. bulgaricus	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1
Lb. plantarum	20	0	0	0	0	8	0	0	0	0	0	0	0	0	0	6
Lactococcus lactis subsp cremoris	20	13	15	13	17	20	15	18	10	17	11	10	13	15	17	13
L. lactis subsp. lactis	20	6	8	5	7	6	5	6	5	7	8	7	5	4	9	10
L. lactis subsp. lactis var. *diacetylactis*	5	2	3	1	2	2	1	0	1	1	2	3	2	3	1	3
Leuconostoc mesenteroides	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Listeria innocua	6	0	0	0	0	6	0	0	0	0	0	0	0	0	0	6
L. ivanovii	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
L. murayi	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
L. seeligeri	3	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3
L. weshimeri	3	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3
L. monocytogenes	14	0	0	0	0	14	0	0	0	0	0	0	0	0	0	14
Listeria spp.	7	0	0	0	0	7	0	0	0	0	0	0	0	0	0	7
Staphylococcus aureus	17	0	0	0	0	14	0	0	0	0	0	0	0	0	0	8
Pediococcus pentosaceus	2	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
Gram-negative																
Citrobacter sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Escherichia coli	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter aerogenes	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella dublin	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. enteridis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. havana	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. rhrizonae	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. infantis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio paraahemoliticus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V. cholerae	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yersinia enterocolitica	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*N: Number of strains examined
** Number of strains sensitive.
***PC: *L. lactis* subsp. *lactis* ATCC 11454 used as positive control

resistance of nisin to α-chymotrypsin (4, 21) and their sensitivity to trypsin (34, 36), pronase (4, 19, 21, 35, 38, 39) and ficin (4) was demonstrated. Bacteriocins produced by four strains of *L. lactis* (ITAL 187, ITAL 404, ITAL 437 and ITAL 438) and nisin of ATCC 11454 were inactivated by ficin. Both sensitivity (4) and resistance (31) of nisin to ficin were reported. Seven bacteriocins showed sensitivity to trypsin (ITAL 104, ITAL 179, ITAL 185, ITAL 403, ITAL 435, ITAL 437 and ITAL 438). Likewise, bacteriocins produced by *L. lactis* subsp. *lactis* S50 (19) and bacteriocins of groups I, II, III, IV, V and VII (13) were sensitive to this enzyme. The resistance to trypsin was detected in four bacteriocins (ITAL 179, ITAL 185, ITAL 387 and ITAL 408) and nisin of ATCC 11454. This characteristic was related to nisin produced by *L. lactis* subsp. *lactis* NP45 (19) and ATCC 11454 (21, 35, 39) and commercial nisin (4, 39) and others bacteriocins (4, 13, 14, 37). Treatment with pepsin promoted inactivation of the bacteriocins produced by seven strains (ITAL 104, ITAL 179, ITAL 185, ITAL 187, ITAL 404, ITAL 437 and ITAL 438) and nisin of ATCC 11454. Likewise, nisin produced by *L. lactis* subsp. *lactis* S50 was sensitive (19). The resistance to this enzyme was detected for bacteriocins produced by ITAL 383, ITAL 387, ITAL 403, ITAL 408, ITAL 435 and ITAL 436 strains. Bacteriocins resistant to trypsin reported in the literature are the ones produced by *L. lactis* subsp. *lactis* NP45 (19) and ATCC 11454 (4), commercial nisin (4) and the bacteriocins of seven *L. lactis* strains. Bacteriocins of nine strains (ITAL 104, ITAL 179, ITAL 187, ITAL 383, ITAL 387, ITAL 403, ITAL 404, ITAL 437 and ITAL 438) and nisin of ATCC 11454 were fully or partially inactivated after treatment with lipase. They can have a lipid moiety in their chemical composition (10). Bacteriocin activity is frequently associated with large aggregates in cell free extracts, which include not only proteinaceous component, but also lipids and other macromolecule (28).

In this study, the bacteriocin produced by *L. lactis* subsp. *lactis* ITAL 383 showed sensitivity to proteases similar to that found in nisin of *L. lactis* subsp. *lactis* ATCC 11454 except for lipase and pepsin. However, several factors can have an effect on antimicrobial activity including the interaction between bacteriocin and constituents from the cells or the growth medium (28), purity and concentration of enzyme (31) and the technique used to test for enzymatic sensitivity (31). The inactivation of antimicrobial activity by proteases suggested that the substances evaluated in this study could be antimicrobial peptides or bacteriocins. The proteases themselves did not produce any visible alteration of indicator strains.

Activity of bacteriocins at different pH levels and effect of heating treatment

Bacteriocins differ greatly with regard to their sensitivity to inactivation by changes in pH and temperature. Many are stable only in acid and neutral conditions, and are even inactivated at pH 8.0, for example, nisin and lactostrepcins.

The stability of bacteriocins in different pH level is shown in Table 5. The strains ITAL 104 and ITAL 185 were active in a wide range of pH from 2.0 up to 12.0. This result was similar to bacteriocin S50 produced by *L. lactis* subsp. *lactis* S50 (19). All the others bacteriocins were fully or partly active at range of pH 2.0-10.0 and completely inactivated at pH 12.0. Similar to nisin of ATCC 11454, bacteriocin of ITAL 383 was stable at neutral and acid pH (2.0-6.0), partly active at pH 6.0-10.0 and completely inactive at pH 12.0. Nisin is the most stable at pH 2.0 and its activity decreases drastically or is lost at pH > 7.0 at room temperature (16).

Table 4. Sensitivity of bacteriocins produced by *L. lactis* strains and nisin of *L. lactis* subsp. *lactis* ATCC 11454 to treatment with proteolytic and lipolytic enzymes

Enzymes	L. lactis													
	104	179	185	187	383	387	403	404	408	435	436	437	438	PC
Control*	6.4**	0.8	1.6	6.4	0.8	6.4	0.8	6.4	3.2	1.6	1.6	6.4	1.6	1.6
Pronase E	6.4	0.0	0.0	0.0	0.0	3.2	0.0	0.0	1.6	0.0	0.4	0.0	0.0	0.0
Pepsin	0.8	0.4	0.4	3.2	0.8	6.4	0.8	1.6	3.2	1.6	1.6	1.6	0.8	0.8
Ficin	6.4	0.8	1.6	0.8	0.8	6.4	0.8	3.2	3.2	1.6	1.6	1.6	0.8	0.8
Lipase	0.4	0.0	1.6	0.8	0.4	3.2	0.0	1.6	3.2	1.6	1.6	1.6	0.0	1.6
Trypsin	1.6	0.0	0.4	3.2	0.8	6.4	0.0	3.2	3.2	0.0	0.8	0.2	0.0	1.6
Proteinase K	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
α-chymotrypsin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Phosphate buffer + sterile cell-free supernatants;
**Results expressed in activity (arbitrary unity x $10^3 ml^{-1}$);
***PC: *L. lactis* subsp. *lactis* ATCC 11454 used as positive control

Table 5. Activity of bacteriocins of *L. lactis* subsp. *lactis* strains at different pH levels.

L. lactis	pH values						
	2.0	4.0	6.0	7.0	8.0	10.0	12.0
ITAL 104	6.4*	6.4	6.4	6.4	6.4	6.4	6.4
ITAL 179	6.4	6.4	3.2	0.8	0.0	0.0	0.0
ITAL 185	6.4	6.4	6.4	6.4	6.4	6.4	6.4
ITAL 187	6.4	6.4	6.4	6.4	6.4	0.0	0.0
ITAL 383	3.2	3.2	3.2	1.6	0.8	0.4	0.0
ITAL 387	6.4	6.4	6.4	6.4	6.4	6.4	0.0
ITAL 403	6.4	6.4	6.4	6.4	6.4	3.2	0.0
ITAL 404	6.4	6.4	6.4	6.4	6.4	3.2	0.0
ITAL 408	6.4	6.4	6.4	6.4	6.4	6.4	0.0
ITAL 435	6.4	6.4	6.4	3.2	3.2	1.6	0.0
ITAL 436	6.4	6.4	3.2	3.2	1.6	0.4	0.0
TAL 437	6,4	6.4	6.4	6.4	3.2	0.4	0.0
ITAL 438	3.2	3.2	3.2	3.2	3.2	0.4	0.0
ATCC 11454**	6.4	6.4	6.4	3.2	3.2	1.6	0.0

*Results expressed in activity (arbitrary unity x $10^3 ml^{-1}$);
***L. lactis* subsp. *lactis* ATCC 11454 used as positive control

Table 6. Effect of heat treatment (121°C for 10 minutes) on activity of bacteriocins of *L. lactis* subsp. *lactis* strains at different pH levels.

L. lactis	pH values													
	2.0		4.0		6.0		7.0		8.0		10.0		12.0	
	C*	RA**	C	RA	C	RA	C	RA	C	RA	C	RA	C	RA
ITAL 104	6.4***	6.4	6.4	6.4	6.4	6.4	6.4	1.6	6.4	0.0	6.4	0.0	6.4	0.0
ITAL 179	6.4	6.4	6.4	6.4	3.2	3.2	0.8	0.8	0.0	0.0	0.0	0.0	0.0	0.0
ITAL 185	6.4	1.6	6.4	1.6	6.4	1.6	6.4	0.2	6.4	0.0	6.4	0.0	6.4	0.0
ITAL 187	6.4	6.4	6.4	6.4	6.4	0.8	6.4	0.8	6.4	0.0	0.0	0.0	0.0	0.0
ITAL 383	3.2	3.2	3.2	0.8	3.2	0.4	1.6	0.0	0.8	0.0	0.4	0.0	0.0	0.0
ITAL 387	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	0.8	6.4	0.0	0.0	0.0
ITAL 403	6.4	3.2	6.4	3.2	6.4	0.8	6.4	0.2	6.4	0.0	3.2	0.0	0.0	0.0
ITAL 404	6.4	6.4	6.4	6.4	6.4	1.6	6.4	0.8	6.4	0.2	3.2	0.0	0.0	0.0
ITAL 408	6.4	6.4	6.4	6.4	6.4	3.2	6.4	1.6	6.4	1.6	6.4	0.0	0.0	0.0
ITAL 435	6.4	6.4	6.4	6.4	6.4	0.8	3.2	0.0	3.2	0.0	1.6	0.0	0.0	0.0
ITAL 436	6.4	3.2	6.4	3.2	3.2	0.4	3.2	0.0	1.6	0.0	0.4	0.0	0.0	0.0
ITAL 437	6.4	6.4	6.4	3.2	6.4	0.2	6.4	0.0	3.2	0.0	0.4	0.0	0.0	0.0
ITAL 438	3.2	3.2	3.2	3.2	3.2	0.2	3.2	0.0	3.2	0.0	0.4	0.0	0.0	0.0
ATCC 11454	6.4	6.4	6.4	3.2	6.4	1.6	3.2	0.0	3.2	0.0	1.6	0.0	0.0	0.0

*Control: bacteriocin activity before heat treatment;
**Residual activity after heat treatment;
***Results expressed in activity (arbitrary unity x $10^3 ml^{-1}$); Measured by critical dilution method

The results of resistance of bacteriocins to heat treatment at 121°C for 10 minutes at different pH levels are shown in Table 6. All bacteriocins, except ITAL 104, ITAL 403 and ITAL 436, were stable to heating at pH 2.0 with-reduced activity at range of pH 4.0 to 7.0, with complete loss of its activity in alkaline conditions (pH ≥ 8.0). In this last situation the only exception was ITAL 408 that was active at pH 8.0. Nisin produced by ATCC 11454 lost 50% activity at pH 4.0 and 75-100% at pH 6.0 and 7.0, respectively. ITAL 383 showed loss of 75% at pH 6.0 and 100% at pH 7.0. These results indicated that the active substances were thermal stable. The activity was lost irreversibly, and it could not be regained upon lowering the pH to 7.0. Irreversible inactivation can be the result of a combination of denaturation and chemical modifications of the molecule (22). According to Hurst (16) nisin solutions can be boiled in diluted hydrochloride acid at pH 2.5 or less without any loss of activity. Moreover, nisin remains stable after autoclaving at 115.6°C at pH 2.0, but loses 40% of its activity at pH 5.0 and more than 90% at pH 6.8 (16).

Similar to nisin, bacteriocin of ITAL 383 was stable after treatment for 60 min at pH 2.0 and lost 50% of its activity after 30 min at pH 6.0 (Table 7). Klaenhaemmer (18) observed resistance to treatment at 100°C for 10 min, at acid conditions. Likewise, Vandenbergh *et al*. (38) showed that nisin produced by eight *L. lactis* strains isolated from vegetables did not loose their activities after treatment at 100°C for 2h.

Detection of activity in SDS-PAGE

To determine if the antimicrobial activity of partially purified bacteriocin produced by *L. lactis* subsp. *lactis* ITAL 383 was due to nisin, the detection and identification of antimicrobial peptides in SDS-PAGE were done. When the preparation of bacteriocin ITAL 383 was submitted to electrophoresis on 20% polyacrylamide gel in the presence of 0.1% SDS, one band revealed by silver staining had the same antimicrobial activity and molecular weight (MW) when compared to similar preparation from *L. lactis* ATCC 11454.

Table 7. Effect of heat treatment (100ºC for 10, 20, 30 e 60 minutes) on the activity of bacteriocins of *L. lactis* subsp. *lactis* ITAL 383 and nisin of *L. lactis* subsp. *lactis* ATCC 11454 at pH 2.0 and 6.0.

L. lactis	pH	Control	Time (minutes)			
			10	20	30	60
ITAL 383	2.0*	1.6	1.6	1.6	1.6	1.6
	6.0	1.6	1.6	1.6	0.8	0.8
ATCC 11454	2.0	1.6	1.6	1.6	1.6	1.6
	6.0	1.6	1.6	1.6	0.8	0.8

*Results expressed in activity (arbitrary unity x $10^3 ml^{-1}$)

This antimicrobial activity that corresponded to the band of nisin of preparations of *L. lactis* ATCC 11454 was demonstrated by clear zone of inhibition when the other half of the SDS-PAGE gel was overlaid with a layer of the sensitive indicator (*L. innocua* LIN 11) (results not shown).

Our results confirmed that all inhibitory substances produced by the tested *L. lactis* strains were bacteriocins. The sensitivity of these bacteriocins to proteolytic enzymes indicated their proteinaceous nature. Similar to nisin in molecular weight, the bacteriocin of strain ITAL 383 showed a wide spectrum of activity and heat resistance.

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RESUMO

Caracterização de bacteriocinas produzidas por linhagens de *Lactococcus lactis*

Bacteriocinas resistentes ao aquecimento produzidas por quinze linhagens de *Lactococcus lactis* (14 *L. lactis* subsp. *lactis* e 1 *L. lactis* subsp. *cremoris*) foram sensíveis à enzimas proteolíticas e ativas em uma ampla faixa de pH. A resistência dessas bacteriocinas ao aquecimento foi fortemente influenciada pelo pH do meio. Somente a linhagem *L. lactis* subsp. *lactis* ITAL 383 produziu uma bacteriocina com um amplo espectro de atividade, semelhante ao da nisina de *L. lactis* subsp. *lactis* ATCC 11454. Esta bacteriocina inibiu as espécies relacionadas e outros microorganismos gram-positivos, inclusive *Listeria monocytogenes* e *Staphylococcus aureus*, mas não as bactérias Gram-negativas examinadas. A identificação do composto antimicrobiano parcialmente purificado por SDS-PAGE revelou um peso molecular similar entre a bacteriocina ITAL 383 e a nisina de *L. lactis* subsp *lactis* ATCC 11454.

Palavras-chave: bacteriocinas, *L. lactis*, espectro de atividade, características físico-químicas

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