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ORIGINAL ARTICLE

Characterization of bacteriocin from *Lactococcus* isolated from traditional Algerian dairy products

Amina Hellal · Linda Amrouche · Zoulikha Ferhat · Fatima Laraba

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Abstract Mesophilic strains of lactic acid bacteria belonging to the genus *Lactococcus* were isolated from traditional dairy products and selected for their ability to produce antimicrobial substances such as bacteriocins. Six strains: *Lactococcus lactis* ssp. *lactis* (strains Lc l1, Lc l3, Lc l5), *Lactococcus lactis* ssp. *cremoris* (strains Lc c4, Lc c6) and *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* (strain Lc d2) were selected for their antagonistic activities against different groups of microorganisms. The most active strain, *Lactococcus lactis* ssp. *lactis* (Lc l5), produced its inhibitory substance (Bac5) in M_{17} broth presenting an important inhibition mainly against *Listeria monocytogenes*. It had no effect on its producer strain. These properties suggested that the inhibitory substance could be considered as a bacteriocin-like substance. SDS-PAGE analyses indicated that it was a homogeneous protein of higher molecular weight of 67 kDa. The bacteriocin was stable over a wide range of pH and temperature; it was active after autoclaving and was not affected by storage at −20°C for 6 months. Moreover, it was sensitive to proteolytic enzymes confirming the proteinaceous nature of the inhibitory substance.

A. Hellal (✉) · L. Amrouche
Laboratory of Science and Environmental Techniques,
National Polytechnic School,
BP 182, Hassen Badi 16200 El Harrach,
Algiers, Algeria
e-mail: hellami25@gmail.com

Z. Ferhat
National Agronomic Institute,
Hassen Badi, 16200 El Harrach,
Algiers, Algeria

F. Laraba
University of Science and Technology Houari Boumediène,
BP 32 El Alia 16111 Bab-Ezzouar,
Algiers, Algeria

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Introduction

The lactic acid bacteria (LAB) are present in a large variety of foods and are responsible for the process of transformation that conditions the texture, flavour and quality of conservation of fermented products. They are routinely employed as starter cultures in the manufacture of fermented dairy, meat and vegetable products where they are responsible for acidification and production of aromas. This group of bacteria is mainly composed of the genera *Lactococcus, Lactobacillus, Pediococcus* and *Leuconostoc* (Ben Omar et al. 2000). In fermented foods, LAB display many antimicrobial activities mainly due to the production of organic acids, but also of other compounds, such as bacteriocins.

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: they are generally recognized as safe substances, they become inactivated by digestive proteases, having little influence on the gut microbiota, they are usually pH and heat-tolerant, and they have a relatively broad antimicrobial spectrum against many foodborne pathogenic and spoilage bacteria.

The bacteriocins have bactericidal activity towards homologous or more distant species. The accumulation of studies carried out in recent years clearly indicates that the application of bacteriocins in food preservation can offer several benefits. Thus, several bacteriocins with industrial potential have been purified and characterised (De Vuyst and Leroy 2007; Oelschlaeger 2010). All the studies underline the important role that functional bacteriocinogenic LAB strains may play in the food industry as starter cultures, co-cultures, or bioprotective cultures, to improve food quality and safety. In addition, antimicrobial produc-

tion by probiotic LAB might play a role during in vivo interactions occurring in the human gastrointestinal tract, hence contributing to gut health (Rbsslanda et al. 2005; De Vuyst and Leroy 2007).

Undoubtedly, the most characterised bacteriocin of the lactic acid bacteria is nisin, discovered by Rogers and Whittier in 1928. Currently, only this bacteriocin is industrially used as a food additive in order to prevent the development of harmful species responsible for sensory defects or presenting risks for the human health. This antagonistic compound is produced by *Lactococcus lactis* ssp. *lactis*; it presents a large activity because of its efficiency against pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Clostridium botulinum* and *Clostridium tyrobutyricum* (Lewus et al. 1991; Thuault et al. 1991).

In other ways, the perfect innocuousness of lactocoques and the development of knowledge on their capacity to secrete some varied inhibitory substances make these bacteria good candidates for the production of bacteriocins.

In this context, we studied the spectrum inhibitor activities of bacteriocins produced by *Lactococcus* strains isolated from traditional Algerian dairy products on different groups of microorganisms. The choice of this source is justified by the fact that the traditional dairy products are manufactured with raw milk presenting some considerable contamination risks.

In this work, we also report results on production, isolation, purification and antilisterial activity of a bacteriocin produced by the most active strain.

Materials and methods

Bacterial strains

The lactic acid bacteria of the *Lactococcus* species were isolated from traditional fermented milk called "leben" provided by a local dairy farm. Strains were isolated on M_{17} medium (Terzaghi and Sandine 1975) and were identified according to physiological and biochemical characteristics as described by Schillinger and Lücke (1987). The antagonistic microorganisms (Table 1) were obtained from the collection of the Pasteur Institute of Algiers. All strains were stored at −20°C in the appropriate medium containing 10% glycerol and regenerated twice before the use. Lactic acid bacteria were cultivated on MRS or M_{17} media, yeasts on Sabouraud, *Listeria* on Tryptone Soy Broth or Agar and the other bacteria on Muller Hinton medium.

Inhibitory activity

The assays were performed with supernatants produced by the six isolated *Lactococcus* strains:

- *Lactococcus lactis* ssp. *lactis* (strains Lc l1, Lc l3, Lc l5)
- *Lactococcus lactis* ssp. *cremoris* (strains Lc c4, Lc c6)
- *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* (strain Lc d2).

The strains were individually cultivated on M_{17} broth, inoculated with a fresh appropriated subculture (1%), for 24 h at 30°C. After centrifugation of the cultures at 4,000 *g* for 20 min at 4°C, the two phases were recuperated and tested separately for the research of the inhibitory substance. The supernatants were first adjusted to pH 6.2 and filtered through a millipore membrane of 0.45 μm. The residues were washed twice with the distilled water and resuspended in methanol solution (2/3, v/v), then centrifuged at 4,000 *g* foring 20 min, the new supernatants being termed cellular extract.

The activity was performed by the agar well diffusion method (Tagg et al. 1976) based on the diffusion of the antibacterial agent in an appropriate agar medium inoculated on surface with a target microorganism (approximately 10^7 CFU/ml). Wells of 6 mm in diameter were dug sterilely in the agar medium and filled with 100 μl of the supernatant of culture or cellular extract. The Petri dishes so prepared were maintained at 4°C for 24 h to allow the diffusion of the antibacterial compounds before incubation at the appropriate growth temperature.

In order to eliminate the action of the lactic acid on the test organisms, the tested supernatants were adjusted to pH 6.0 with NaOH and treated with catalase (5 mg/ml) to exclude the inhibition due to hydrogen peroxide production (Gies et al. 1983). The diameter of inhibition zone formed around the wells was calculated as the difference between the diameter of the total inhibition zone and the diameter of the well. The inhibition is noted positive if the diameter is superior to 2 mm (Thompson et al. 1996).

Purification of bacteriocin Bac5

An 18-h-old culture of *Lactococcus lactis* ssp. *lactis* (Lc l5) strain was inoculated (1%, v/v) into M_{17} broth. Incubation was at 30°C, without agitation, for 24 h. The cells were harvested (4,000 *g*, 20 min, 4°C) and the peptide precipitated from the cell-free supernatant with 80% saturated ammonium sulphate (De Kwaadsteniet et al. 2005). The precipitate was resuspended in 0.1 M ammonium acetate (pH 5.85), desalted against distilled water by using a 1,000-Da cut-off dialysis membrane at 4°C for 72 h, and then separated by gel filtration chromatography on Sephadex G-100. The bacteriocin was eluted with 0.1 M ammonium acetate (pH 5.85). The active fractions were further separated by cation-exchange chromatography on carboxymethyl-Sephadex C-50 with a 0.1–2 M

Table 1 Activity spectrum of the inhibitory agents of the isolated *Lactococcus* species; diameter (mm) of inhibition zones

Target microorgamisms		*Bac1*	*Bac2*	*Bac3*	*Bac4*	*Bac5*	*Bac6*
Lactic acid bacteria	*Lactococcus lactis* ssp. *lactis* IPA32	6	-	-	17	24	13
	Lactococcus lactis ssp. *cremoris* IPA34	-	15	11	13	12	9
	Pediococcus acidilactici IPA11	-	8	-	6	-	11
	Pediococcus ssp. IPA10	4	9	20	2	23	6
	Leuconostoc citrovorum IPA74	18	-	10	9	15	8
Cocci Gram +	*Streptococcus α hemolytic* IPA61	11	6	13	18	20	9
	Streptococcus β hemolytic IPA63	12	10	6	22	23	11
	Staphylococcus aureus IPA100	-	11	-	-	-	10
	Staphylococcus aureus IPA119	4	-	-	13	19	-
	Staphylococcus aureus IPA103	3	23	18	12	14	18
	Staphylococcus aureus IPA209P	-	-	-	-	-	-
	Staphylococcus aureus IPA36	19	15	24	19	24	14
Yeasts	*Candida albicans* IPA1	-	-	-	-	-	-
	Candida albicans IPA2	-	-	-	-	-	-
	Candida tropicalis IPA80	-	-	-	-	-	-
	Candida ssp. IPA9	-	-	-	-	-	-
Bacilli Gram +	*Listeria monocytogenes* IPA1	17	23	16	-	25	14
	Listeria ivanovii IPA1b	2	-	18	-	14	17
	Bacillus polymyxa IPA20p	-	-	-	-	-	-
	Bacillus cereus IPA21c	4	-	-	-	11	-
	Bacillus lentimorbis IPA21l	-	-	-	-	-	10
	Bacillus thuringiensis IPA21t	-	-	14	-	-	-
	Bacillus brevis IPA21b	-	-	3	-	-	-
	Bacillus megaterium IPA21mt	-	6	-	-	-	
	Bacillus macerans IPA21mc	-	-	-	-	12	-
Enterobacteria and Bacilli Gram -	*Pseudomonas aeruginosa* IPA	-	-	-	-	10	-
	Pseudomonas fluorescens IPA64L(−)	15	-	13	-	17	-
	Pseudomonas ssp. IPA80s	-	-	-	-	17	-
	Acinetobacter IPA76	-	-	-	-	-	-
	Escherichia coli IPA27	18	12	-	-	16	-
	Escherichia coli IPA83	-	-	-	-	17	-
	Escherichia coli IPA124	-	-	-	-	-	-
	Escherichia coli IPA128	-	-	-	-	-	-
	Escherichia coli IPA136	3	-	12	-	20	7
	Klebsiella oxytoca IPA881k	-	15	-	-	-	-
	Klebsiella pneumoniae IPA455	-	-	-	-	19	-
	Klebsiella pneumoniae IPA885k	-	-	-	-	-	-
	Citrobacter sp. IPA91c	-	-	-	-	-	-
	Salmonella IPA90s	-	-	-	-	-	-
	Vibrio IPA002	-	-	-	-	-	-
	Proteus vulgaris IPA902v	-	-	12	-	16	-
	Proteus mirabilis IPA902m	-	-	-	-	-	-
	Enterobacter IPA6	-	4	16	-	10	-

- no inhibition

Bac1, Bac3, Bac5 are related to *Lactococcus lactis* ssp. *lactis* Lc l1, Lc l3, Lc l5, respectively

Bac4, Bac6 are related to *Lactococcus lactis* ssp. *cremoris* Lc c4, Lc c6, respectively

Bac2 is related to *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* Lc d2

IPA Institut Pasteur d'Alger

NaCl gradient elution. Peptides were detected spectrophotometrically at 280 nm.

Estimation of the molecular weight

The molecular weight was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bhunia et al. 1987). Protein solutions were previously submitted to heating at 100°C for 5 min in presence of SDS (10%), and then 1 µl of bromophenol 0.01% was added to every sample. A volume of 5 µl of each sample was put down on the gel introduced in a separation room where the migration was carried out. After migration, polypeptides were fixed with a glutaraldehyde solution 5%, followed by colouring with silver nitrate solution 0.4%. The colouring development was obtained with formaldehyde solution 2% in Na_2CO_3 2.5% followed by the discolouration in an acetic acid solution 10%. SDS–PAGE Molecular Weight Standards were used simultaneously.

Bactericidal action

To investigate the bactericidal effect of the inhibitory substance on the target bacterium, *L. monocytogenes* IPA1 was incubated overnight at 30°C in Trypton Soy Broth (TSB) (Difco). Cells were then harvested by centrifugation (3,000 *g*, 20 min, 4°C), washed twice aseptically with sterile ammonium-acetate buffer (50 mM, pH 6.5) and resuspended in the same buffer (approximately 10^6 CFU/ml). Assays were performed by adding 10 ml of purified bacteriocin (500 AU/ml) to 50 ml of cell suspension. As control, a sample without inhibitor was performed. At 0, 6, 12, 18 and 24 h of incubation at 30°C, samples were withdrawn and the enumeration of viable cells was determined by plating samples on Trypton Soy Agar (TSA). The colonies were counted after 48 h at 30°C (Mao et al. 2001).

Study of the stability of the antagonistic compound

The purified bacteriocin Bac5 was tested for sensitivity to pH, heat and proteases.

The sensitivity of the active substance to different pH values was estimated by adjusting the pH of aliquots of the purified substance between 2 and 11. After 1 h 30 min of incubation at room temperature, the various samples were adjusted to pH 6.2 and tested for their residual activity assayed against *L. monocytogenes* IPA1 (approximately 10^6 CFU/ml) by the agar well diffusion method (Gomez et al. 1997).

To test the heat sensitivity of the purified inhibitory substance, samples were incubated at different temperatures 60, 90 and 100°C for 1 h and residual activity was determined as described above. Another sample, autoclaved at 121°C for 20 min, was tested in the same conditions. The purified compound was also preserved at −20°C for 6 months then tested for its activity. All the tests were carried out after adjustment of the pH to 6.2.

Aliquots of the purified compound were submitted to the action of the proteolytic enzymes (pepsin: 3,200–4,500 U/mg; trypsin: 13,000–20,000 U/mg; α-chymotrypsin: 40–60 U/mg; and pronase E: 4 U/mg). The enzymes were prepared in sodium-phosphate buffer (100 mM, pH 7.5) except for the pepsin which was dissolved in sodium-acetate buffer (50 mM, pH 3.5) at a concentration of 2 mg/ml. Each enzyme is added at equal volume of 250 µl to the purified bacteriocin (500 AU). After 1 h of incubation at 37°C, enzyme activity was stopped by heating at 100°C for 5 min. Untreated samples were used as control. The residual bacteriocin activity was assayed against the target strain as described above (Gomez et al. 1997; Delgado et al. 2001).

Results and discussion

Antimicrobial spectrum

Among the isolated and identified lactic acid bacteria, six strains belonging to the *Lactococcus* genus were retained in the present study for their antimicrobial activities:

- *Lactococcus lactis* ssp. *lactis* (strains Lc l1, Lc l3, Lc l5)
- *Lactococcus lactis* ssp. *cremoris* (strains Lc c4, Lc c6)
- *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* (strain Lc d2).

None of these six strains has sensitivity to its own antibacterial agent confirming the hypothesis of an immunity system that protects the microorganisms against their own product (Jack et al. 1995).

Table 1 indicates that all bacteriocins are active on bacterial strains, but none has an inhibiting action towards yeasts *(Candida*). These results approach those obtained by Henning et al. (1986) on the nisin which possesses an inhibitory action on several microorganisms with the exception of *Candida.*

According to Gies et al. (1983), in the present study bacteriocins of the *Lactococcus* species form a very heterogeneous group and possess a spectrum of activity which varies between the strains of the same species. However, in accordance with the classification of Klaenhammer (1988), we can classify the tested bacteriocins according to their spectrum of activity into three groups:

- **Group I**: contains bacteriocins with narrow spectrum of activity (action limited to the taxonomically related microorganisms). Such is the case of the bacteriocin

Bac4 of *Lactococcus lactis* ssp. *cremoris* (Lc c4) whose action is limited to the cocci Gram-positive. Similar results were obtained for the diplococcin resulting from *Streptococcus cremoris* 346 (Davey and Richardson 1981), plantaricin W from *Lactobacillus plantarum* (Holo et al. 2001), lactococcin G of *Lactococcus lactis* (Moll et al. 1996) and lacticin 3147 of *Lactococcus lactis* (Ryan et al. 1996).

- **Group II**: concerns bacteriocins with a large spectrum of activity (action exerted on Gram-positive as well as on Gram-negative, on cocci and bacilli). Among the bacteriocins tested, Bac2, Bac3 and Bac5 correspond to this condition. Nevertheless, Bac5 of *Lactococcus lactis* ssp. *lactis* (Lc l5) is the most active and shows an inhibiting action towards 56% of the target bacteria with variable inhibiter diameters between 10 and 25 mm which justifies the choice of this strain in the further study. In the literature, *Lactococcus lactis* ssp. *lactis* produces several bacteriocins from which the action extends to the phylogenetically different bacteria (bacilli Gram-positive: *Bacillus* and *Listeria*; bacilli Gram-negative: *Pseudomonas, Enterobacteria*; cocci Gram-positive: *Staphylococcus* and *Streptococcus* (Maldonado-Barragán et al. 2009; Dalié et al. 2010).
- **Group III**: contains bacteriocins with a fairly wide spectrum of activity. Bac6 and Bac1 can be included in this group; their action is much more marked on the cocci Gram-positive compared to the bacilli Gram-positive and to least effect on the bacilli Gram-negative. Indeed, Bac6 inhibits only one strain among the 18 tested Enterobacteria and bacilli Gram-negative. These results agree with those obtained by Lee and Païk (2001) whm described lacticin NK24, a bacteriocin resulting from *Lactococcus lactis* NK24 showing an inhibiting action against the Gram-positive bacteria (bacilli and cocci) and some Gram-negative strains (*Pseudomonas, E. coli*).

Figure 1 shows some examples of antimicrobial activities of the isolated *Lactococcus* strains, by the agar well diffusion method.

Determination of the molecular weight of the bacteriocin Bac5

The bacteriocin Bac5 obtained from the selected strain *Lactococcus lactis* ssp. *lactis* (Lc l5) was purified. An electrophoresis on polyacrylamide gel in the presence of SDS was used in order to determine the homogeneity of the purified protein and to estimate its molecular weight. Several samples during the purification were used (the supernatant of culture of *Lactococcus lactis* ssp. *lactis* (Lc l5), the ammonium sulphate precipitate, the chromatographic fraction resulting from the molecular filtration on Sephadex G-100 presenting an inhibitory activity, as well as the molecular weight markers) (Fig. 2). The electrophoretic profile reveals 7 bands in the crude culture supernatant (lane 3), 5 in the precipitate with the sulphate of ammonia (lane 4), 3 in the chromatography of filtration on gel (lane 2) and only one in the exchanging chromatography of ions (lane 5). The various stages of purification thus permitted to eliminate contaminant proteins contained in the crude supernatant and to recover the fraction containing the antagonistic activity.

The evaluation of its molecular mass was determined in comparison with proteins of known molecular weights. It appears that this substance agrees with the Bovine Serum Albumin (BSA) protein whose molecular weight is 67 kDa (Fig. 2). In the literature, bacteriocins present variable molecular weights according to producing strains. The isolated

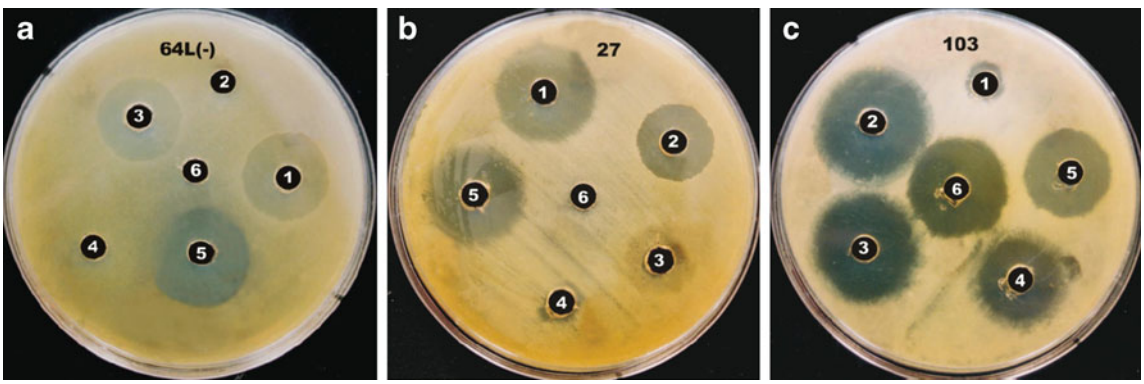

Fig. 1 Examples of antimicrobial activities of six strains of isolated *Lactococcus* by the agar well diffusion method. Target microorganisms: left *Pseudomonas fluorescens* IPA64L(−), centre *Escherichia coli* IPA27, right *Staphylococcus aureus* IPA103. Wells contain the crude culture supernatants of lactococci. Wells *1, 3, 5* correspond to the crude culture supernatants of *Lactococcus lactis* subsp. *lactis* Lc l1, Lc l3, Lc l5), respectively. Wells *4, 6* correspond to the crude culture supernatants of *Lactococcus lactis* ssp. *cremoris* Lc c4, Lc c6), respectively. Well *2* corresponds to the crude culture supernatant of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* Lc d2).

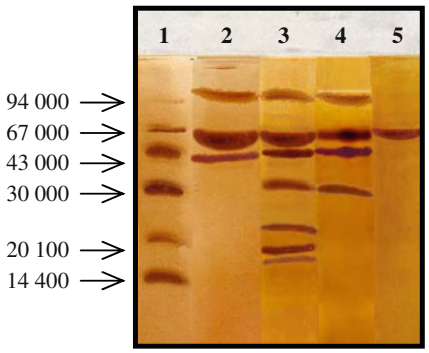

Fig. 2 Gel SDS-PAGE analysis of purified bacteriocin Bac5. *Lane 1* molecular weight markers Phosphorylase b : 94,000, Bovin serum albumin : 67,000, Ovalbumine : 43,000, Carbonic anhydrase : 30,000, soybean trypsin inhibitor : 20,100 and α–Lactalbumin : 14,400). *Lane 2* fraction resulting from the molecular filtration on Sephadex G-100. *Lane 3* culture supernatant of *Lactococcus lactis* ssp *lactis* Lc l 5). *Lane 4* the precipitate with sulphate of ammonia. *Lane 5* active fraction derived from the exchanging chromatography of ions.

bacteriocin Bac5 is a larger molecule compared to the 2.7-kDa molecule of pediocin AcH/PA-1 and the 4-kDa of pediocin SJ-1, both produced by *P. acidilactici* strains (Bhunia et al. 1987; Schved et al. 1994). Nevertheless, Piva and Headon (1994) identified a pediocin of 80 kDa from *Pediococcus pentosaceus* whereas Gobetti et al. (1997) isolated a bacteriocin from *Pseudomonas fluorescens* of 67 kDa.

Action of bacteriocin Bac5 on the growth of *Listeria monocytogenes*

Listeria monocytogenes is a psychrotrophic foodborne pathogen causing listeriosis. It has been recognized as major foodborne pathogen with the ability to survive in various environmental conditions, such as refrigeration, at low pH in foods, in high salt concentrations and at high temperatures. This microorganism has also been encountered in fermented products (cheese, yoghurt, fermented milk, sausage) made from raw materials contaminated with the organism. Thus, traditional methods of preservation are not sufficient to prevent growth of *L. monocytogenes* in foods. Therefore, bacteriocins elaborated by several bacteria are generally regarded as safe, and investigations of bacteriocins which inhibit pathogens, such as *L. monocytogenes*, are thus becoming attractive for the food industry as food additives. On the other hand, several approaches were developed to evaluate the antagonistic effect of various bacteriocins against *L. monocytogenes*. These studies provide interesting information to develop strategies to suppress or control *L. monocytogenes* (Guerrieri et al. 2009; Molinos et al. 2009; Ananou et al. 2010). Therefore, bioactive bacteriocins are currently being tested against foodborne bacterial pathogens in different types of foods (Molinos et al. 2009; Rivera-Espinoza and Gallardo-Navarro 2010).

In the present study, the efficiency of the antimicrobial peptide Bac5 to control the development of *L. monocytogenes* in Trypton Soy Broth was investigated. Figure 3 indicates the effect of bacteriocin Bac5 on the survival of the target bacterium *L. monocytogenes* IPA1. The number of viable cells decreases over time and is cancelled at the end of 24 h, which suggests a bactericidal effect on this microorgamism. The choice of *L. monocytogenes* as antagonistic microorganism is justified by the fact that this bacterium is expected to contaminate dairy products manufactured with raw milk and thus to be at the origin of serious consequences on human health. Otherwise, the strain *L. monocytogenes* IPA1 has been retained as target bacterium because of its high sensitivity (presents the biggest diameter of inhibition, 25 mm) and because of the serious problems that are generated by the presence of this species in the agro-food industries.

Most lactic acid bacteria bacteriocins have a bactericidal action on pathogenic microorganisms from where the interest carried to this type of bacteriocin and their use in the preservation of foods (Schöbitz et al. 1999; Lee and Paik 2001). Recently, Ghalfi et al. (2010) described the antilisterial peptides properties produced by *Lactobacillus curvatus* in MRS broth. Previously, Martinez and De Martinis (2005) have studied antilisterial activity of a crude preparation of *Lactobacillus sakei* bacteriocin and its influence on *L. monocytogenes* haemolytic activity.

Taking into account its antagonism against *L. monocytogenes, Lactococcus lactis* ssp. *lactis* (Lc l5) might be a good candidate for future use to prevent growth of these pathogens in dairy food products. Thus, the antimicrobial peptide Bac5 obtained in this study presents a potential use as a biopreservative for application in dairy products mainly manufactured with raw milk. Moreover, the bacteriocinogenic strain can be used as a protective adjunct

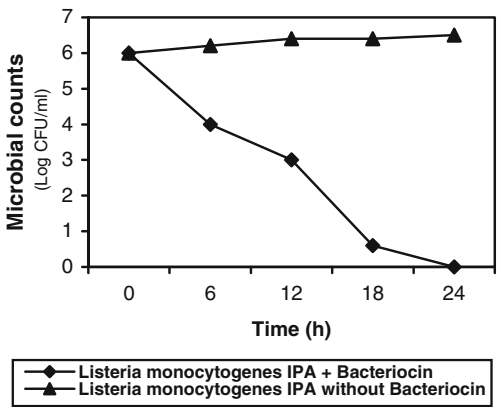

Fig. 3 Effect of bacteriocin Bac5 addition 500 AU/ml) on the viability of *Listeria monocytogenes* IPA1. Data points represent the average of at least three experiments

culture. As an example, inoculation of milk with an enterocin AS-48 producer enterococcal strain as adjunct culture in combination with a commercial starter culture for cheese manufacture had no effect on growth of the starter or the physicochemical properties of the produced cheese. At the same time, enough bacteriocin was produced in the cheese to ensure inhibition of *Bacillus cereus* (Muñoz et al. 2004).

Stability of the antagonistic compound Bac5

Effect of the pH

The stability of the antagonistic compound Bac5 was studied at variable pH from 2 to 11 at ambient temperature for 1 h 30 min. The calculated residual activities were compared with the activity of the witness. Figure 4 shows that the inhibiting activity of the substance Bac5 on *Listeria monocytogenes* IPA1 is not affected by the variations of pH from 2 to 8 whereas the alkaline pH deteriorates it by 45%. This reduction is probably due to a modification of ionisation and/or a partial denaturation of the molecule. In general, the bacteriocins of lactococci are very sensitive to the variations of the medium pH. Indeed, they are active at acid pH and become unstable with neutral and alkaline pH. Such is the case of nisin (Rogers and Whittier 1928; Liu and Hansen 1990) and diplococcin (Davey and Richardson 1981). However, Piard and Desmazeaud (1992) identified the lacticin 481 (from *Lactococcus lactis*) whose activity remained stable with ranges of pH from 2 to 8. Similar observations were reported by Yildrim and Johnson (1998) for the lactococcin produced by *Lactococcus lactis* ssp. *cremoris*. In the same way, Rodriguez et al. (2002) characterised a bacteriocin from *Streptococcus sobrinus* which was stable at varying pH from 2 to 7. Bacteriocins generated by lactic acid strains are generally stable at acid or neutral pH, indicating that these substances are well adapted to the environment produced by these bacteria. From a technological point of view, the high pH stability observed at low pH range is an important factor, allowing their use in fermented foods or in preserved foods.

Effect of the temperature

Figure 5 represents the residual antagonistic activities of Bac5 after thermal treatments at 90, 60 or 100°C. This activity remains stable for 30 min at 60°C; beyond that, it decreases. Indeed, it decreases quickly to reach 22 and 7% of the maximum activity, respectively, after 100 and 90°C treatment. In addition, no inhibiting activity is detected after autoclaving at 121°C for 20 min. On the other hand, this activity was perfectly preserved at −20°C for 6 months.

Bacteriocins have a variable sensitivity to heat treatments according to their molecular weight and structure (Kalaenhammer 1988). The bacteriocins of weak molecular weights are generally heat resistant; such is the case of the nisin, lactostrepsin and lacticin (Aslim et al. 2005) except for the diplococcin which is inactivated at 100° C (Piard and Desmazeaud 1992). However, those which have high molecular weights are heat-sensitive, such as the bacteriocin-like compound resulting from mesophilic lactic streptococci which is sensitive to heating to 100 and 121°C (Gomez et al. 1997). Purified pediocin produced by *Pediococcus acidilactici,* is heat stable for up to 60 min at 121°C (Anastasiadou et al. 2008). The ecological adaptation and growth characteristics of cultures in food products will determine their effectiveness as biocontrol cultures. Thus, differences in resistance to heat treatments observed in our case compared to bacteriocins of lactic acid bacteria reported by other authors could be attributed to differences in ecological adaptation and environmental conditions.

The bacteriocin produced by *Lactococcus lactis* ssp. *lactis* (Lc l5) is heat stable, and therefore would be a very useful characteristic if it was to be used as a food preservative, because many food-processing procedures involve a heating step.

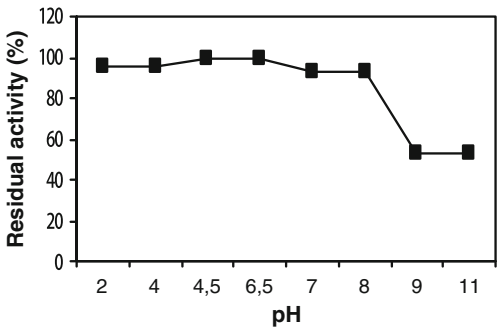

Fig. 4 Antibacterial residual activity of Bac5 on *Listeria monocytogenes* IPA1 after exposure at different pH for 1 h 30 min. Data points represent the average of at least three experiments

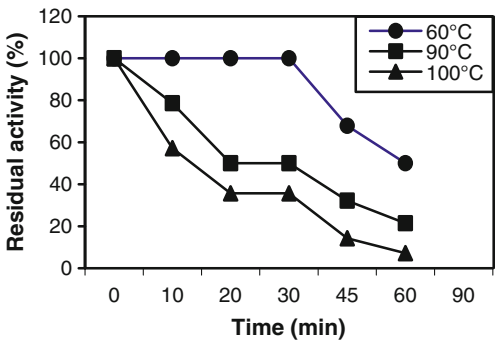

Fig. 5 Residual antibacterial activity of Bac5 on *Listeria monocytogenes* IPA1 after different thermal treatments. Data points represent the average of at least three experiments

Effect of the proteolytic enzymes

The active compound Bac5 was exposed to the proteolytic enzymes action (pepsin, trypsin, α-chymotrypsin and pronase E) which completely deteriorate the bactericidal activity of this substance confirming the proteinaceous nature of the inhibitory substance. It is well established now that all the bacteriocins are of proteinaceous nature, or at least contain a peptide which is responsible for their bactericidal function (Lima et al. 2002). However, their sensitivity to the proteolytic enzymes is variable. The diplococcin is completely inactivated by trypsin, pronase and α-chymotrypsin (Davey and Richardson 1981) whereas lacticin NK24 is sensitive only to the action of the two enzymes proteases IX and XIV. No modification of its activity was observed after treatment with trypsin, α-chymotrypsin, papain and pepsin (Lee and Paik 2001).

Conclusion

Lactococcus strains isolated from traditional Algerian fermented milk, principally *Lactococcus lactis* ssp. *lactis* (Lc l5), may be further used as biocontrol cultures directly added to milk products, ensuring that these lactic acid bacteria strains are well ecologically adapted. This fact is an important factor for their effectiveness as natural antimicrobial agents. The native *Lactococcus* isolated presented antagonistic activities against frequently foodborne pathogens with a potential role as food biopreservatives.

In our study, the antimicrobial activities exhibited by the selected strain *Lactococcus lactis* ssp. *lactis* (Lc l5) were found to be sensitive to proteinase treatments, thereby providing evidence of the proteinaceous nature of the substance responsible for the antimicrobial activity.

Moreover, the data reported in this study also support the idea that the antimicrobial activities were not caused by the bacterial acidification of the medium and also they were not related to the production of hydrogen peroxide. Such observations strongly suggest that the antimicrobial activities against indicator microorganisms used in this study would be based on the biosynthesis and secretion of bacteriocins.

The bacteriocin-like compound (Bac5) produced by *Lactococcus lactis* ssp. *lactis* (Lc l5) resisted freezing, and also retained high antimicrobial activity after storage at low temperature. The present results suggest that this bacteriocin may be interesting to apply in processed milk products which will be stored at refrigerated or freezing temperatures. Its use would be therefore be of interest especially in raw dairy products.

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References

Ananou S, Baños A, Maqueda M, Martínez-Bueno M, Gálvez A, Valdivia E (2010) Effect of combined physico-chemical treatments based on enterocin AS-48 on the control of *Listeria monocytogenes* and *Staphylococcus aureus* in a model cooked ham. Food Control 21:478–486

Anastasiadou S, Papagianni M, Ambrosiadis I, Koidis P (2008) Rapid quantifiable assessment of nutritional parameters influencing pediocin production by *Pediococcus acidilactici* NRRL B5627. Bioresource Technol 99:6646–6650

Aslim B, Yuksekdag ZN, Sarikaya E, Beyatli Y (2005) Determination of the bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish dairy products. LWT 38:691–694

Ben Omar N, Ampe F, Raimbault M, Guyot JP, Tailliez P (2000) Molecular diversity of lactic acid bacteria from cassava sour starch Colombia. Syst Appl Microbiol 23:285–291

Bhunia AK, Johnson MC, Ray B (1987) Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J Ind Microbiol 2:319–322

Dalié DKD, Deschamps AM, Richard-Forget F (2010) Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. Food Control 21:370–380

Davey GP, Richardson BC (1981) Purification and some properties of diplococcin from *Streptococcus cremoris* 346. Appl Environ Microbiol 41:84–89

De Kwaadsteniet M, Todorov SD, Knoetze H, Dicks LMT (2005) Characterization of a 3944 Da bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria. Int J Food Microbiol 105:433–444

De Vuyst L, Leroy F (2007) Bacteriocins from Lactic Acid Bacteria: Production purification and Food Applications. J Mol Microb Biotech 13:194–199

Delgado A, Brito D, Fevererio P, Peres C, Marques JF (2001) Antimicrobial activity of *Lactobacillus plantarum* isolated from a traditional lactic acid fermentation of table olives. Lait 8:203–215

Ghalfi H, Benkerroum N, Ongena M, Bensaid M, Thonart P (2010) Production of three anti-listerial peptides by *Lactobacillus curvatus* in MRS broth. Food Res Inter 43:33–39

Gies A, Singh J, Teuber M (1983) Potential of lactic streptococci to produce bacteriocin. Appl Environ Microbiol 45:205–211

Gobetti M, Corsetti A, Smacchi E, Rossi J (1997) Purification and characterization of proteinaceous compound from *Pseudomonas fluorescens* ATCC 948 with inhibitory activity against some gram-positive and gram negative bacteria of interest. Lait 77:267–278

Gomez S, Cosson C, Deschamps AM (1997) Evidence for a bacteriocin – like substance produced by a new strain of *Streptococcus* sp, inhibitory to Gram positive food – borne pathogens. Res Microbiol 148:757–766

Guerrieri E, Niederhäusern S, Messi P, Sabia C, Iseppi R, Anacarso I, Bondi M (2009) Use of lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes* in a small-scale model. Food Control 20:861–865

Henning S, Metz R, Hammes WP (1986) Studies on the mode of action of nisin. Int J Food Microbiol 3:121–134

Holo H, Jeknic Z, Daeschel M, Stevanovic S, Nes IF (2001) Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology 147:643–651

Jack RW, Tagg RJ, Ray B (1995) Bacteriocins of Gram positive bacteria. Microbiol Rev 59:171–200

Klaenhammer TR (1988) Bacteriocins of lactic acid bacteria. Biochimie 70:337–349

Lee NK, Paik HD (2001) Partial characterization of lactin NK 24 a newly identified bacteriocin of *Lactococcus lactis* NK 24 molted from Jeat gal. Food Microbiol 18:17–24

Lewus CB, Kaiser A, Montville TJ (1991) Inhibition of food- borne bacterial pathogens by bacteriocins from lactic acid bacteria solated from meat. Appl Environ Microb 57:1683–1688

Lima FL, Farias FF, Costa JE, Auxiadora MR, Alviano CS, Farias LM (2002) Bacteriocin production by *Actinobacillus actinomycetemcomitans* isolated from oral cavity of humans with periodontal diereses periodontal heathy subjects and marmosets. Res Microbiol 153:45–52

Liu W, Hansen N (1990) Some chemical and physiological properties of nisin a small-protein antibiotic produced by *Lactococcus lactis*. Appl Environ Microbiol 56:2551–2558

Maldonado-Barragán A, Caballero-Guerrero B, Jiménez E, Jiménez-Díaz R, Ruiz-Barba JL, Rodríguez JM (2009) Enterocin C a class IIb bacteriocin produced by *E faecalis* C901 a strain isolated from human colostrums. Int J Food Microbiol 133:105–112

Mao Y, Muriana PM, Cousin MA (2001) Purification and transpositional inactivation of Lacticin FS92 a broad-spectrum bacteriocin produced by *Lactococcus lactis* FS92. Food Microbiol 18:165–175

Martinez RCR, De Martinis ECP (2005) Antilisterial activity of a crude preparation of *Lactobacillus sakei* 1 bacteriocin and its lack of influence on *Listeria monocytogenes* haemolytic activity. Food Control 16:429–433

Molinos AC, Abriouel H, Lucas López R, Ben Omar N, Valdivia E, Gálvez A (2009) Enhanced bactericidal activity of enterocin AS-48 in combination with essential oils natural bioactive compounds and chemical preservatives against *Listeria monocytogenes* in ready-to-eat salad. Food Chem Toxicol 47:2216–2223

Moll G, Ubbink-Kok T, Hildeng-Hauge H, Nissen-Meyer J, Nes I, Konings WN, Drissen AJM (1996) Lactococcin G Is a Potassium ion-conduting two component bacteriocin. J Bacteriol 178:600–605

Muñoz A, Maqueda M, Gálvez A, Martínez-Bueno M, Rodríguez A, Valdivia E (2004) Biocontrol of Psychrotrophic Enterotoxigenic *Bacillus cereus* in a Nonfat Hard Cheese by an Enterococcal Strain–Producing Enterocin AS-48. J Food Protect 67:1517–1521

Oelschlaeger TA (2010) Mechanisms of probiotic actions – A review. Int J Med Microbiol 3001:57–62

Piard JC, Desmazeaud M (1992) Inhibiting factors produced by lactic acid bacteria 2- Bacteriocins and other antibacterial substances. Lait 72:113–142

Piva A, Headon RD (1994) Pediocin A a bacteriocin produced by *Pediococcus pentosaceus* FBB61. Microbiology 140:697–702

Rbsslanda E, Langsruda T, Granumb PE, Sbrhauga T (2005) Production of antimicrobial metabolites by strains of *Lactobacillus* or *Lactococcus* co-cultured with *Bacillus cereus* in milk. Int J Food Microbiol 98:193–200

Rivera-Espinoza Y, Gallardo-Navarro Y (2010) Non-dairy probiotic products. Food Microbiol 27:1–11

Rodriguez JM, Martinez MI, Kok J (2002) Pediocin PA-1 a wide spectrum bacteriocin from lactic acid bacteria. Crit Rev Food Sci Nut 42:91–121

Rogers LA, Whittier ED (1928) Limiting factors in lactic fermentation. J Bacteriol 16:211–229

Ryan MP, Rea MC, Hill C, Ross PR (1996) In application in cheddar cheese manufacture flora strain of *Lactococcus lactis* producing a novel broad spectrum bacteriocin Lacticin 3147. Appl Environ Microbiol 62:612–619

Schillinger U, Lücke FK (1987) Identification of lactobacilli from meat and meat products. Food Microbiol 4:199–208

Schöbitz R, Zaror R, Leon TO, Costa M (1999) A bacteriocin from *Carnobacterium pisicola* for the control of *Listeria monocytogenes* in vacuum packaged meat. Food Microbiol 16:249–255

Schved F, Henis Y, Juven BJ (1994) Response of spheroplasts and chelator-permeabilized cells of Gram-negative bacteria to the action of the bacteriocins pediocin SJ-1 and nisin. Int J Food Microbiol 21:305–314

Tagg JR, Dajani AS, Wannamaker LW (1976) Bacteriocins of Gram positive bacteria. Bacteriol Rev 40:722–756

Terzaghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. Appl Microbiol 29:807–813

Thompson JK, Collins MA, Merler WD (1996) Characterization of proteinaceous antimicrobial produced by *Lactobacillus helveticus* CNRZ 450. J Appl Bacteriol 80:338–348

Thuault D, Belliard E, Le Guern J, Bourgeois CM (1991) Inhibition of *Clostridium tyrobutyricum* by bacteriocin like substances produced by lactic acid bacteria. J Dairy Sci 74:1145–1150

Yildrim Z, Johnson MG (1998) Detection and characterization of a bacteriocin produced by *Lactococcus lactis* ssp. *cremoris* R isolated from radish. Lett Appl Microbiol 26:297–304