Annals of Microbiology, **58 (1)** 83-88 (2008)

Comparative analysis of *Lactococcus lactis* bacteriocins and preliminary characterisation of a new proteinase K resistant lactococcin member

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Received 17 October 2007 / Accepted 17 December 2007

Abstract - Detection of lactic acid bacteria (LAB) bacteriocins producers is of great significance for food industry to establish starter bacterial association and to improve food safety. Eighty one *Lactococcus lactis* strains, isolated from traditional Tunisian dairy products, were screened for their antibacterial activity. Bacteriocin production in the supernatant was demonstrated for twelve strains by the well diffusion assay, protease susceptibility and by direct detection of the activity on SDS-PAGE. By using PCR with primers targeting structural genes of nisin and lactoccocin 481, we were able to predict their presence in 10 and one strain respectively. No amplifications were recorded with primers targeting lactococcin A, lactococcin 972 and bacteriocin J46 of *L. lactis*. The remaining unidentified bacteriocin produced by strain BMG 6.25 and designed as lactococcin IAF 25, was further characterised. IAF 25 was shown to be a heat-stable proteinaceous inhibitory factor sensitive to papain and trypsin but resistant to proteinase K treatment. IAF 25 has an apparent molecular weight of 6 kDa and showed a narrow antimicrobial activity spectrum against closely related bacteria and genera. These original characteristics among *L. lactis* bacteriocins coupled with cross inhibition tests with bacteriocin producers reference strains, led to the assumption of the novelty of lactococcin IAF 25.

Key words: bacteriocins, *Lactococcus*, proteinase, activity spectrum.

INTRODUCTION

In addition to their implication in the fermentative process, production of aroma compounds and organoleptic characteristics, lactic acid bacteria (LAB) plays an important role in the conservation of foods by organic acid production, pH decrease and production of several compounds including hydrogen peroxide and bacteriocins (Juillard *et al.*, 1987). Bacteriocins are peptides or proteins, encoded by structural genes and ribosomally synthesised, with antagonistic activity, usually toward closely related bacteria, that can be extended to other genera. They have attracted increasing interest because of their potential use as food additives and their efficiency for the biological control of spoilage and pathogenic organisms (Klaenhammer *et al.*, 1978 and Klijn *et al.*, 1995; Corroler *et al.*, 1998).

On the basis of their amino acid composition, primary structure and mechanism of action, bacteriocins are divided into four classes I – IV, corresponding to lantibiotics (small posttranslationally modified peptides that contain unusual amino acid such as lanthionines), heat-stable non-lantibiotic peptides, heat-labile proteins and complex bacteriocins (with non proteinaceous moiety) (Klaenhammer, 1993; Nes *et al.* 1996; Drider *et al.* 2006). Most of the LAB bacteriocins belong to classes I and II. Among the numerous reported lactococcal bacteriocins, three are well characterised in term of genetics and chemical composition: Lactococcin A (Holo *et al.*, 1991) and two lantibiotics, nisin (Kaletta and Entian, 1989) and lacticin 481 (Piard *et al.*, 1993). While lacticin 481 related bacteriocins are currently attracting a growing interest (Dufour *et al.*, 2007), nisin remains the most studied bacteriocin and has been granted with the "*generally recognized as safe*" (GRAS) status by the US Food and Drug Administration and licensed for use as food preservative in many countries (De Vuyst and Vandamme, 1994; Drider *et al.*, 2006).

Recently, several other lactococcal bacteriocins were described including whose activity depends on the complementary action of two peptides such as lactococcin G (Nissen-Meyer *et al.*, 1992) and lactococcin Q (Zendo *et al.*, 2006); lantibiotics such as bacteriocin J46 (Huot *et al.*, 1996) and the two peptide lacticin 3147 (Ryan *et al.*, 1996 and Ryan *et al.*, 1999); class IIa bacteriocin MMFII (Ferchichi *et al.*, 2001) and other single active peptides, lactococcin 972 (Martinez *et al.*, 1999) and Lactococcin M and B (van Belkum *et al.*, 1991).

Identification of unknown bacteriocin requires the study of its characteristics, its purification, and the sequencing of

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its genetic determinants. These procedures are costly and not available for a relatively high number of isolates initially screened for their production of bacteriocins. Development of efficient and reliable methods would be useful for the detection of isolates that produce known or new bacteriocins.

Polymerase Chain Reaction (PCR) is the most used molecular technique for rapid identification of the genetic determinants of proteins. PCR-based screening was previously used to detect and predict LAB bacteriocin content, such as nisin (De Vos *et al.*, 1993; Rodriguez *et al.*, 1995a), lacticin S (Rodriguez *et al.*, 1995b) and pediocin PA-1 (Rodriguez *et al.*, 1997). However, the risk of false positive amplification that could originate from analog or non functional gene makes necessary additional biochemical tests for bacteriocin production screening.

In this paper, we outline the selection and characterisation of bacteriocin producing strains isolated from Tunisian traditional fermented milk and we describe a comparative useful scheme, associating PCR and biochemical characterisation, to predict potentially new bacteriocin member.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Lactococcus lactis* strains used in this study were isolated from various Tunisian dairy products (cheese, Ricotta) and traditional fermented milk (Leben and Raieb) and identified by biochemical and molecular methods in previous studies (Ouzari *et al.*, 2002, 2006). Indicator strains used in this study and their growth conditions are listed in Table 1. Lactococcal strains were routinely maintained at 4 °C after growth at 28 °C for 12 or 24 h in M17 medium supplemented with 0.5% (w/v) glucose (GM17). For long term storage, stock cultures were kept at -70 °C in GM17 broth containing 30% glycerol.

Bacteriocin assay and antimicrobial spectrum. The well diffusion method (Jack *et al.*, 1995) was used to examine the antibacterial activity of lactococcal strains against *L. lactis* subsp. *cremoris* ATCC 11603, considered as the indicator strain because of its high sensitivity. A lawn of 5 ml of soft agar (0.7%) containing 0.1 ml of the indicator organism (10^6 CFU ml^{-1}) was poured on M17 agar plate. After cooling, wells with a 6 mm diameter were cut into the agar plates. Culture supernatants of producing strains were centrifuged at 8000 *x g* for 15 min, neutralised to pH 6.5 and filtered through a 0.22 µm filter. One hundred microlitres of the resulting cell-free supernatants (CFS) were placed in the prepared wells. The plates were incubated overnight and the inhibition zone diameters were measured.

The activity spectra of the bacteriocin producer's strains were determined by the same method against several Gram-positive and Gram-negative bacteria. All strains used as indicator organisms were previously subcultured in their growth medium before inoculation of the soft agar (Table 1).

TABLE 1 - Inhibitory spectrum of cell-free supernatants from bacteriocin producing strains

Indicator strains	Origin*	Medium - Incubation temperature (°C)**	Producer strains***			
			L. lactis ATCC11454 and nis-group strains (10)	*L. lactis* CNRZ481 and BMG 6.95	BMG 6.25	*L. lactis* MIMlac
Lactococcus lactis CNRZ 481 (*lct*+)	CNRZ	M17 - 28	+	–	+	+
Lactococcus lactis subsp. *lactis* (*nis*+)	ATCC	M17 - 28	–	+	–	+
Lactococcus lactis MIMlac (*lca*+)	DISTAM	M17 - 28	++	++	++	–
Lactococcus lactis BMG 6.25	LMBA	M17 - 28	–	+	–	+
Leuconostoc mesenteroides	LMBA	MRS - 30	ND	+	+	–
Enterococcus faecalis	LMBA	BHI - 37	++	–	+	–
Enterococcus sp.	LMBA	BHI - 37	–	–	–	–
Streptococcus pyogenes S1	LMBA	TSB - 37	+	–	–	–
Streptococcus pyogenes S2	LMBA	TSB - 37	–	ND	–	–
Staphylococcus aureus 799	DSMZ	TSB - 37	+	ND	–	–
Staphylococcus aureus	LMBA	TSB - 37	+	ND	–	–
Listeria monocytogenes MACa1	DISTAM	BHI - 37	+	–	–	–
Bacillus thuringiensis HD22	BGSC	TSB - 30	+	ND	–	–
Bacillus cereus 14579^T	BGSC	TSB - 30	+	+	+	–
Bacillus maroccanus	LMBA	TSB - 30	++	+	++	–
Pseudomonas aeruginosa S1	LMBA	TSB - 37	–	–	–	–
Pseudomonas aeruginosa S2	LMBA	TSB - 37	–	–	–	–
Escherichia coli DH5α	LMBA	LB - 37	–	–	–	–

* Origin of strains: CNRZ, Centre National de Recherches Zootechniques, INRA, France; ATCC, American Type Culture Collection; DISTAM, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Milano, Italy; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMBA, Laboratoire Microorganismes et Biomolécules Actives, Tunis, Tunisia; BGSC, *Bacillus* Genetic Stock Center. *B. thuringiensis* and *B. cereus* strains were kindly provided by Prof. D. R. Zeigler.
** M17: Lactococci medium; MRS: De Man Rogosa and Sharpe medium, De Man *et al.* (1984); TSB: Tryptic Soy Broth; NB: Nutrient Broth; LB: Luria Broth; BHI: Brain Heart Infusion.
*** –: absence of inhibition; +: diameter of the inhibition zone < 20 mm; ++: diameter of the inhibition zone > 20 mm.; ND: not determined.

Sensitivity to proteolytic enzymes and heat treatment. To detect the susceptibility of the different bacteriocins to proteolysis, CFS samples of producing strains were treated for 1h with various enzymes (trypsin, papain, lysozyme, catalase and proteinase K) at a final concentration of 1 mg ml^{-1}. All enzymes were dissolved in buffers as recommended by the supplier. Upon incubation, the enzymes were heat inactivated (3 min at 100 °C). For each test, untreated CFS plus buffer, CFS plus buffer treated for three min at 100 °C, buffer alone, and enzyme solutions served as controls. To determine the effect of heat on the inhibitory activities, aliquots of CFS of producing strains were exposed to heat treatment of 50, 60, 70, 80, 90, 95 and 100 °C for 30 min. Residual activities were determined using the described well-diffusion method against indicator organism *L. lactis* subsp. *cremoris* ATCC 11603. All the experiments were done in duplicate.

Direct detection of bacteriocin activity on SDS-PAGE. In order to estimate the molecular mass and confirm the proteinaceous nature of the antibacterial compounds, CFSs from producing strains and a molecular mass marker were subjected to SDS-PAGE according to the standard protocol of Laemmli (1970). Polyacrylamide concentration in the stacking and separating gel were 3.9 and 15%, respectively. Duplicate samples of CFSs (20 µl) were loaded simultaneously with the following standard proteins: myosine (205 kDa), b-galactosidase (116 kDa), phosphorylase B (97 kDa), BSA (66 kDa), albumine (45 kDa), pepsine (34.7 kDa), trypsinogène (24 kDa), lysozyme (14.3 kDa) and aprotinin (6.5 kDa). Electrophoresis was conducted at a constant voltage of 50 V for one hour and 100 V for five hours. After electrophoresis, the gel was cut vertically into slices corresponding to the different loaded CFSs. A first part of slices containing CFS samples and standard proteins were stained with Coomassie brilliant blue G-250 (Sigma-Aldrich, Steinheim, Germany) to determine bacteriocin molecular weight (Fig. 1A). The second part containing only CFS samples was assayed for direct detection of activity as described previously (Cherif *et al.*, 2003). Briefly, the gel slices were fixed for 1 h (25% isopropanol and 10% acetic acid), washed for 30 min with sterile distilled water and placed carefully onto M17-agar surface that was pre-overlaid with soft agar containing *L. lactis* ATCC 11603 as indicator strain. The Petri dish was incubated at 28 °C for 12 h and observed for the presence of inhibition zone (Fig. 1B).

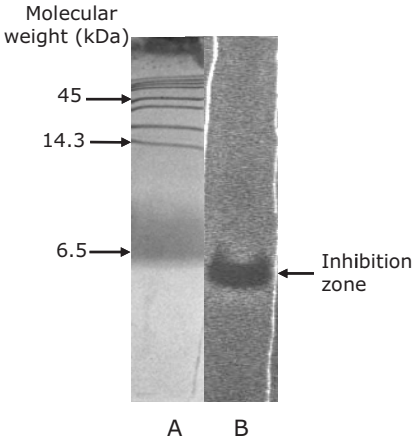

FIG. 1 - SDS-PAGE analysis and direct detection of lactococcin IAF 25 activity. (A) Coomassie blue-stained marker: myosine, 205 kDa; b-galactosidase, 116 kDa; phosphorylase B, 97 kDa; BSA, 66 kDa; albumine, 45 kDa; pepsine, 34.7 kDa; trypsinogène, 24 kDa; lysozyme, 14.3 kDa and aprotinin 6.5 kDa. (B) Part of the gel containing the BMG 6.25 cell-free supernatant and placed on M17-agar surface overlaid with *L. lactis* ATCC 11603 as indicator.

DNA manipulation. Total DNA extraction was performed as previously described by Kalman *et al.*, (1993). Specific primers (Table 2) corresponding to lacticin 481 (Piard *et al.*, 1990), lactococcin A (Holo *et al.*, 1991), lactococcin 972 (Martinez *et al.*, 1999) and bacteriocin J46 (Huot *et al.*, 1996), were designed from the published sequences in Genbank using PC/PLAN included in PC/GENE software while primers for nisin gene were those used by Klijn *et al.* (1995). The PCR reactions were performed in 25 µl using up to 100 ng of genomic DNA (1 µl), 1x Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1 µM for each primer and 1 U Taq DNA polymerase. The reaction mixtures were incubated in a thermocycler (Perkin Elmer) at 94 °C for 5 min, subjected to 30 cycles consisting of 94 °C for 45 s, annealing temperature for 45 s and 72 °C for 90 s. Finally, mixtures were incubated at 72 °C for 10 min. Five microlitres of each amplification mixture were analysed by agarose (1.5% w/v) gel electrophoresis according to standard procedures (Sambrook *et al.*, 1989). For sequencing, PCR products were purified with a QIAquik PCR Purification Kit (QIAGEN™ GmbH, Hilden, Germany) and nucleotide sequences were determined using a Dye Terminator Sequencing kit and ABI PRISM 310 DNA sequencer (Perkin Elmer).

TABLE 2 - Specific primer pairs directed to the detection of bacteriocin known genes

Primer	Specificity	Sequence (5′ à 3′)	Tm (°C)	Expected size (bp)	Reference
P8 (A)	Nisin	CGCGAGCATAATAAACGGCT	55	319	Klijn *et al.* (1995)
P9 (S)		GGATAGTATCCATGTCTGAAC			
LCNP 8	Lacticin 481	TGACAGAAAGTGTATTGCCC	58	445	This study
LCNM 9		CCTCTGGTGTATTGCCC			
LCAF	LactococcinA	AAGAACTTTCAGAAGCTAACGGAGG	58	606	This study
LCAR		TGCTTAATCAATGGCACG			
972 F	Lactococcin 972	CCAAGTCTCTCGTATTGGCA	58	231	This study
972 R		AGTTACGTCCAACAGTAGCT			
J46 F	Bacteriocin J46	TGGACCTTATTTTAGGTGCA	52	95	This study
		GCAAGTAAATACAAAGTTCCAGCT			

RESULTS AND DISCUSSION

A total of 81 *Lactococcus lactis* isolates from traditional Tunisian dairy products (Ouzari *et al.*, 2002, 2006) were screened for their capacity to produce inhibitory substances in the culture supernatant, using the well diffusion method. Twelve *L. lactis* strains (15% of the collection) were found to produce bacteriocin-like substances with large inhibition zone, a lower percentage respect to those reported by Piard *et al.* (1990) in *Lactococcus* strains (20%) and Rodriguez *et al.* (2000) in different lactic acid bacteria (24%). Inhibitory spectra were determined against different Gram-positive and Gram-negative bacteria and towards reference bacteriocin producer strains, *L. lactis* ATCC 11454 producing nisin, *L. lactis* CNRZ 481 producing lacticin 481 and *L. lactis* MIMlac expressing lactococcin A (Table 1). Ten of the selected strains, called here nis-group strains, (BMG 6.14, BMG 6.121, BMG 6.89, BMG 6.26B, BMG 6.D1, BMG 6.26A, BMG 6.7B, BMG 6.B1, BMG 6.27 and BMG 28A) exhibited inhibitory spectra similar to the nisin producer strain. They were active against most of the tested bacteria from different genera including *Bacillus*, *Staphylococcus* and *Listeria* but not towards Gram-negative bacteria and nisin producer strain *L. lactis* subsp. *lactis* ATCC 11454. The two remaining strains BMG 6.95 and BMG 6.25 showed narrower inhibitory spectra comparable to that of lacticin 481 producer strain. They inhibited only closely-related bacteria of the genera *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Bacillus*. Beside, strain BMG 6.25 showed antagonistic activity against *L. lactis* CNRZ 481 and *Enterococcus faecalis* strain but was not active against nisin producer strains (Table 1). Basing on the activity spectra and cross inhibition test, the bacteriocin produced by *L. lactis* BMG 6.25, appears to be diverse from nisin, lactococcin 481 and lactococcin A.

Identification of the detected bacteriocins was further performed by PCR using whole DNA preparation as matrix and specific primers targeting known lactococcal bacteriocins; nisin, lacticin 481, lactococcin A, lacticin 972 and lacticin J46. The expected fragment of nisin gene (319 bp) was amplified from DNA of the nis-group strains (Fig. 2A) who showed the same inhibitory spectrum as the nisin producer strain. Similarly, strain BMG 6.95 that showed an inhibitory spectrum analogous of that of *L. lactis* CNRZ 481, gave an amplicon of the expected size (445 bp) with lacticin 481 primers (Fig. 2B). Sequencing of the amplified fragments yielded 100% of similarity with the corresponding regions in *nis Z* and lacticin 481 genes, respectively (Mulders *et al.*, 1991; Piard *et al.*, 1993). No DNA amplifications were obtained, using primers specific to lactococcin A, bacteriocin 972 and J46, indicating their limited distribution in lactococcal strains as reported previously (Rodriguez *et al.*, 2000).

The high occurrence of nisin production is in agreement with that reported by De Vos *et al.* (1993) and Cai *et al.* (1997), indicating that nisin is widespread among lactococcal strains. This feature could be related to the genetic determinant nature of this bacteriocin, which was characterised as a conjugative transposon (Rauch and de Vos, 1992; Horn *et al.*, 1991) and could be responsible for horizontal transfer of *nis Z* gene within *L. lactis* strains in the same environment. Except for lacticin 481 operon, associated in same cases to a composite transposon Tn*5721*

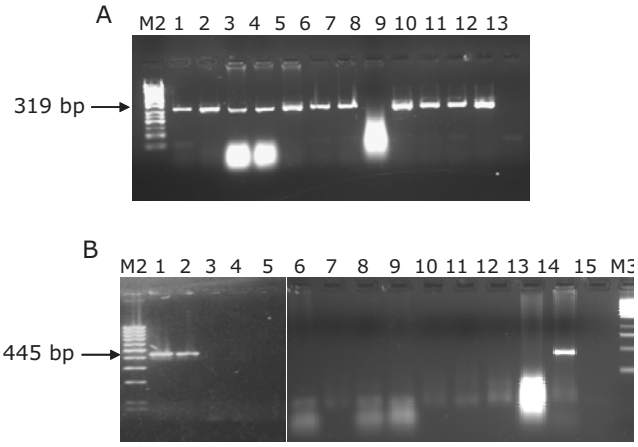

FIG. 2 - Agarose gel electrophoresis of PCR products from various bacteriocin producing strains. (A) Amplification of nisin DNA fragments using p8 and p9 primers. Lanes: 1, nisin A producing strain of *L. lactis* subsp. *lactis* ATCC 11454; lanes 2-13, BMG 6.14, BMG 121, BMG 89, BMG 26B, BMG D1, BMG 26A, BMG 25, BMG 7B, BMG B1, BMG 27, BMG 28A and BMG6.95 strains; lane 14, negative control. (B) Amplification of lacticin 481 DNA fragments using LCNP8 and LCNM9 primers. Lanes: 1 and 14, Lacticin 481 producing strain *L. lactis* CNRZ 481; lanes 2-13: BMG 6.95, BMG 6.14, BMG 121, BMG 89, BMG 26B, BMG D1, BMG 26A, BMG 25, BMG 7B, BMG B1, BMG 27 and BMG 28A strains; lane 15, negative control. M1, M2 and M3: 50 bp, 100 bp and 1 kb DNA ladder marker respectively.

within a 70 kb plasmid (Dufour *et al.*, 2000), most of the other identified *L. lactis* bacteriocins operons were linked to non-conjugative plasmids (van Belkum *et al.*, 1992; Huot *et al.*, 1996; Martinez *et al.*, 1999) and related to a specific ecological niche.

Biochemical characterisation of the detected bacteriocins was performed using cross-inhibition tests, protease and heat treatments. As bacteriocin production is greatly affected by medium composition (Coetzee *et al.*, 2007; Todorov and Dick, 2007), all producer strains were grown in the same medium and under the same conditions. The nis-group and BMG6.95 strains were considered as nisin and lacticin 481 producers, respectively, while BMG6. 25 presented a diverse profile related to the production of other antimicrobial substance called lactococcin IAF 25. IAF 25 activity was not affected by catalase, lysozyme and interestingly resistant to proteinase K, whereas it was inactivated by trypsin and partially by papain. No inactivation was observed after heating at 60, 70 and 80 °C. Residual activity was about 60% after treatment at 100 °C for 15 min. Proteinaceous nature of lactococcin IAF 25 was further confirmed by direct detection of the inhibitory activity after SDS-PAGE analysis and assay toward *L. lactis* subsp. *cremoris* ATCC 11603 (Fig. 1B). Its apparent molecular weight was estimated to 6 kDa (Fig. 1A).

Comparative analysis of lactococcin IAF 25 with the known bacteriocins produced by *Lactococcus lactis* is summarized in Table 3. Lactococcin IAF 25 differs from nisin and other lactococcal bacteriocins in several properties. While nisin and lacticin 3147 present a wide antibacterial spectrum (Drider *et al.*, 2006), other reported lactococcins, mainly lactococcin A, B, M G Q and 972 had inhibitory activ-

TABLE 3 - Biochemical characteristics of the known lactococcal bacteriocins

Bacteriocin	Proteases effect*			Heat stability* (100 °C/ 60 min)	MW (kDa)	Activity spectrum	Reference
	Proteinase K	Trypsin	Papain				
Nisin	–	++	++	++	3.4	Broad	Olasupo *et al.* (1999); Noonpakdee *et al.* (2003); This study
Lacticin 481	–	++	+	++	2.9	Narrow (Genera closely related to *Lactococcus*)	Piard *et al.* (1990); Piard *et al.* (1993); This study
Lactococcin A	–	–	–	ND	5.778	Restricted to *L. lactis*	Holo *et al.* (1991); This study
Lacticin 972	ND	ND	ND	–	7.5	Restricted to *L. lactis*	Martinez *et al.* (1999)
Lacticin J46	ND	+	++	++	3	Narrow	Huot *et al.* (1996)
MMFII	–	–	–	+	4.142	broad	Ferchichi *et al.* (2001)
Lactococcin M and B	ND	ND	ND	ND	3.4	Restricted to *L. lactis*	van Belkum *et al.* (1991, 1992)
Lacticin 3147	–	–	ND	++	3.322-2.847	Broad	Ryan *et al.* (1996, 1999)
Lactococcin Q	ND	ND	ND	ND	4.260-4.018	Restricted to *L. lactis*	Zendo *et al.* (2006)
Lactococcin G	ND	ND	ND	ND	4.376-4.109	Restricted to *L. lactis*	Zendo *et al.* (2006); Nissen-Meyer *et al.* (1992)
Lactococcin IAF25	++	–	+	+	6	Narrow (Genera closely related to *Lactococcus*)	This study

*Residual activity. ++: 100%; +: 25 to 50%; –: loss of activity; ND: not determined.

ity only against *L. lactis* strains (Holo *et al.,* 1991; Morgan *et al.,* 1995; Moll *et al.,* 1996 and Zendo *et al.*, 2006) and could be used for species identification and typing. Lactococcin IAF 25 is rather similar to lacticin 481 and J46, characterised by an inhibitory spectrum including various species of *Enterococcus* and *Bacillus*. These narrow spectrum-bacteriocins, could play a promising role in food industry since they inhibit pathogenic and spore forming bacteria; i.e. *Bacillus* for IAF 25 and *Clostridium* for lacticin 481 and J46 (Piard *et al.*, 1990; Huot *et al.*, 1996; Dufour *et al.*, 2007). Conversely, lactococcin IAF 25 diverge from lacticin 481 and J46 in molecular weight, heat and proteases sensitivity with the particularity to be resistant to proteinase K treatment.

Indeed protease sensitivity is a key criterion for the characterisation of an inhibitory substance such as bacteriocin since it reflects amino acid composition and in some cases a specific conformational state. Proteinase K is a highly active endopeptidase with a broad spectrum of action towards peptide bonds, preferentially next to the carboxyl group of N-substituted hydrophobic aliphatic and aromatic amino acids such as leucine, phenylalanine, tryptophane and tyrosine. *In silico* analysis targeting cleavage sites of proteinase K within the known lactococcal bacteriocin, revealed the presence of at least two potential sites of proteinase K in each bacteriocin. Likewise, sensitivity to proteinase K was reported for most of the identified *L. lactis* bacteriocins (Olasupo *et al.*, 1999; Piard *et al.*, 1990; Ryan *et al.*, 1996; Ferchichi *et al.*, 2001; Dufour *et al.*, 2007).

The low molecular weight, resistance to proteinase K, activity spectrum and heat stability corroborate the novelty of bacteriocin lactococcin IAF 25 produced by the local isolate of *L. lactis* BMG 6.25. To our knowledge, this is the first description of a proteinase K-resistant L*. lactis* bacteriocin.

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