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Full Length Research Paper

Antimicrobial activities of the bacteriocin-like substances produced by lactic acid bacteria isolated from Moroccan dromedary milk

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A total of 450 different colonies, isolated from 25 samples of dromedary milk collected from Laâyoune region of Morocco, were tested for antimicrobial compounds production. Out of these, 30 were determined to be lactic acid bacteria (LAB) and able to inhibit the growth of the indicator strain *Listeria innocua* CECT 4030. Seven isolates were selected by the large and clear zones of inhibition when tested by the agar well diffusion assay. They were classified by phenotypic and biochemical analysis as two *Enterococcus durans* (E204 and E214), two *Lactococcus lactis* (R75 and R76), one *Enterococcus faecium* R111, one *Lactococcus cremoris* R112 and one *Enterococcus avium* R122. Their antimicrobial compounds were detected in cell-free culture supernatant fluids under conditions that eliminate acid and hydrogen peroxide inhibition. The antimicrobial activity was altered after treatment with trypsin, α-chymotrypsin, pepsin or papain which confirms the proteinaceous nature of the inhibition. It was heat stable even at autoclaving temperature (121°C for 15 min) and also active over a wide pH range (2 to 10). This fact suggests that bacteriocin-like produced by the seven LAB strains may find application as biopreservatives in food products.

Key words: Dromedary milk, lactic acid bacteria, bacteriocin-like substances, antimicrobial activity.

INTRODUCTION

Lactic acid bacteria (LAB) can produce antimicrobial substances with capacity to inhibit the growth of pathogenic and spoilage microorganisms in foods. The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (Daeschel, 1989). In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds and high-molecular-mass compounds like bacteriocins (Jay, 1982; Klaenhammer, 1988; Piard and Desmazeaud, 1991, 1992).

Bacteriocins have attracted a great interest in food industry due to their application potentiality in food preservation. They are antimicrobial proteinaceous substances secreted by some bacteria against microorganisms that are usually closely related to the producer organism (klaenhammer, 1988). During the last few years, a large number of new bacteriocins produced by LAB have been identified and characterized. LAB-bacteriocins comprise a heterogeneous group of physicochemically diverse ribosomally-synthesized peptides or proteins showing a narrow or broad antimicrobial activity spectrum against Gram-positive bacteria. Bacteriocins are classified into separate groups such as the lantibiotics (class I); the small (<10 kDa) heat stable postranslationally unmodified non-lantibiotics (class II), further subdivided in the pediocin-like and anti-*Listeria* bacteriocins (subclass IIa), the two peptide bacteriocins (subclass IIb), and the sec-dependent bacteriocins (subclass IIc); the large (>30 kDa) heat-labile non-lantibiotics (class III) and complex bacteriocins containing chemical moieties such as lipid and carbohydrate (class IV) (Klaenhammer, 1993).

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In all cases, the producer cells exhibit specific immunity to the action of their own bacteriocins. They mostly act at the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipids bilayer (Montville et al., 1995). Nisin is the best defined, and the only purified bacteriocin produced by LAB that has been approved for use in food products (Hansen, 1994).

Data from previous studies showed that some LAB were able to control the growth of some pathogens microorganisms such as *Listeria monocytogenes* in food products (Callewaert et al., 2000; Mataragas et al., 2003; Schöbitz et al., 1999; Tantillo et al., 2002). *Listeria innocua*, a non-pathogenic species, may be used as a biological indicator for *L. monocytogenes* because of their similar response to physico-chemical and thermal treatments (Kamat and Nair, 1996).

In this study, we reported on the study of antibacterial activities and the characterization of antibacterial compounds of seven strains isolated from Moroccan dromedary milk against *L. innocua* CECT 4030. The antibacterial activities were tested against indicator microorganisms.

LAB from cow and goat milk and dairy products were widely investigated for antimicrobial activity of bacteriocins. A number of studies have been conducted for the isolation and characterization of lactic acid bacteria from camel milk (Benkerroum et al., 2003; Khedid et al., 2009) and on the antimicrobial activity of the camel milk proteins (El Agamy et al., 1992; Benkerroum et al., 2009). However, to our knowledge, the antimicrobial effects of bacteriocins produced by LAB isolated from the camel milk have never been investigated. Thus, the goal of this study was to characterize the bacteriocins-like compounds produced by LAB strains isolated from camel milk from Morocco.

MATERIALS AND METHODS

Sampling, isolation and preliminary characterization of isolates

25 samples of camel milk were analysed. The samples were stored at 4°C until analysis. Milk samples were vortexed and these were serially diluted in sterile saline solution (0.85% w/v NaCl) before being spread onto MRS (de Man, Rogosa and Sharpe, Biokar Diagnostic) agar plates. Plates were incubated under aerobic conditions at 30°C for 24 h to allow colonies development.

18 colonies were randomly transferred with the sterile toothpicks to two MRS agar plates and incubated at 30°C for 24 h. For the detection of antimicrobial activity, one plate was overlaid with 5 ml of molten BHI (Brain Heart Broth, Biokar Diagnostic) (0.75% agar) inoculated with 100 µl of an overnight culture of *L. innocua* CECT 4030 and was incubated at 37°C for 24 h. The antimicrobial activity was visually detected by observing clear zones around the producer strain. Then, the strains that showed antimicrobial activity were cultivated from the other plate and stored on MRS agar slants at 4°C for routine use.

Gram staining, morphology and catalase production with hydrogen peroxide as substrate were determined. Colonies of catalase negative and Gram-positive were presumed to be LAB. Such colonies were sub-cultured into fresh MRS agar and the isolates were stored at -20°C in 35% glycerol.

Screening for antagonistic activity and determination of antimicrobial spectra

For the detection of antimicrobial activity, agar spot test and agar well diffusion assay were used. 21 indicator strains was used to determine the spectrum of antimicrobial activity: 14 Gram-positive and 7 Gram-negative (Table 1).

Agar spot test

LAB strains were cultured in 5 ml MRS broth at 30°C for 18 h. Then, aliquots (3 µl) of the culture were spotted into agar plates containing 20 ml of MRS medium. After 24 h at 30°C, the plates were overlaid with 5 ml of BHI soft agar (0.75% agar) inoculated with 100 µl of an overnight culture of the indicator microorganism. The plates were incubated for 24 h and the appearance of inhibitory zones was observed.

Agar well diffusion assay

An agar well diffusion assay (AWDA) (Tagg and McGiven, 1971) was used for the detection of antagonistic activity. MHA (Mueller Hinton Agar, Biokar Diagnostic) plates were overlaid with 5 ml of molten BHI agar (0.75% agar) inoculated with 100 µl of an overnight culture of the indicator microorganism. Wells (6 mm in diameter) were cut in the plates. LAB strains were grown in MRS broth at 30°C for 18 h. Cultures were centrifuged at 4000 g for 20 min at 4°C, the cell-free supernatants (CFS) were collected, adjusted to pH 6.5 with 1 M NaOH, filtered through a 0.22 µm pore-size nitrocellulose membrane (Millipore, Ireland) and 80 µl of CFS of the potential producer strains was placed in each well. Plates were refrigerated (4°C) for 4 h to allow the radial diffusion of the compounds contained in the supernatants, and then incubated at 37°C for 24 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

In order to determine their antimicrobial spectra of activity, positive isolates from the preliminary screening were tested by the AWDA against different indicator strains.

Phenotypic and physiologic identification of isolates

Unidentified Gram-positive and catalase-negative bacteria that showed positive results from the AWDA were sub-cultured twice in MRS broth overnight at 30°C. Phenotypic and physiological tests were Gram reaction, cell morphology and catalase reaction, growth at 10, 40 and 45°C, in 4 and 6.5% NaCl, at pH 9.6 and CO_2 production from glucose in MRS broth. Growth in Kenner Fecal Agar (KF) (Scharlau Chemie SA, Barcelona, Spain) for streptococcus for 48 h at 37°C was examined. Bile Esculin Agar (Oxoid, Hapshire, UK) was used to determine hydrolysis of esculin and growth in the presence of 40% bile (Oxoid). Arginine hydrolysis was determined after 3 days incubation at 30°C in MRS broth supplemented with L-arginine monochlorohydrate at 0.3% and using Nessler reagent (Panreac Quimica S.A.U, Barcelona, Spain). Haemolytic activity was tested in fresh cultures of the isolates, streaked on Colombia Blood Agar (Oxoid, Hapshire, UK) plates containing 5% (v/v) horse blood, and incubated for 48 h at 37°C. Subsequent clearing around the colonies indicated the production of β-haemolysin. Carbohydrate fermentation patterns were determined by the API 20 Strep (API, BioMérieux, France) as described by the manufacturer and the identification was done by a

Table 1. Identification of the seven selected LAB isolates producing antimicrobial compounds.

Characteristic	Isolate						
	E. durans		*L. lactis* subsp. *lactis*		*L. lactis* subsp. *cremoris*	*E. faecium*	*E. avium*
	E204	E214	R75	R76	R112	R111	R122
Cell morphology	c	c	c	c	c	c	c
Gram reaction	+	+	+	+	+	+	+
Catalase reaction	-	-	-	-	-	-	-
Haemolysin production	-	-	-	-	-	-	-
CO_2 production from glucose	-	-	-	-	-	-	-
Hydrolysis of:							
Arginine	+	+	+	+	-	+	-
Esculin	+	+	+	+	-	+	+
Growth at:							
10°C	+	+	+	+	+	+	+
40°C	+	+	+	+	-	+	+
45°C	+	+	-	-	-	+	+
pH 9.6	+	+	+	+	-	+	+
Growth in the presence of NaCl							
4%	+	+	+	+	-	+	+
6.5%	+	+	-	-	-	+	+
Growth on:							
KF agar	+	+	-	-	-	+	+
Bile Esculin Agar (40% bile)	+	+	+	+	+	+	+
Production of acetoin	+	+	-	-	-	+	+
Utilization of carbohydrate							
L-arabinose	-	-	-	-	-	+	+
Ribose	+	+	+	+	-	+	+
Sucrose	-	-	+	-	-	+	+
Mannitol	-	-	-	+	-	+	+
D-raffinose	-	-	-	-	-	-	-
Trehalose	+	+	+	+	-	+	+
Lactose	+	+	+	+	+	+	+

computerized database programme provided by the same manufacturer.

Characterization of antimicrobial compounds

The isolates that exhibited antagonistic activity against the pathogenic organisms were investigated for their antimicrobial compounds following the procedure described by Ammor et al. (2006). These isolates were grown overnight at 30°C in 20 ml MRS broth. A sample of 1 ml from each culture broth was taken (sample 1). A cell-free supernatant was obtained by centrifuging the remaining 19 ml of culture broth at 4000 g for 20 min at 4°C (Beckman, J2-HS Centrifuge), followed by filtration of the supernatant through a 0.22 µm pore-size nitrocellulose membrane filter (Millipore, Ireland). Four samples (1 ml) from each filtered supernatant were taken (samples 2, 3, 4 and 5); samples 3 and 5 were adjusted to pH 6.5 with sterilized 2.5 M NaOH solution to avoid inhibition through the production of organic acids. Inhibitory activity from hydrogen peroxide was blocked by the addition of a sterile solution of catalase (2619 U/mg. sigma), dissolved in phosphate buffer at pH 7 and 1 mg/ml final concentration, to samples 4 and 5 at 25°C for 30 min. All samples were taken in duplicate. The antagonistic activities of the five samples were determined by the AWDA as described earlier.

Effect of different treatments on the antimicrobial compounds produced by the seven strains

To prepare cell-free culture supernatants of 7 isolates, the strains were grown in MRS broth at 30°C for 16 h. The cultures were centrifuged at 4000 g for 20 min at 4°C, and the supernatants were neutralized to pH 6.5 with 1 M NaOH. The *L. innocua* CECT 4030 was used as indicator strain in these experiments. After each treatment, the residual antimicrobial activities were determined by the AWDA. All evaluations were carried out in duplicate.

Enzyme sensitivity

The sensitivity of the active substances to proteolysis and other enzymes was assayed by incubating the neutralized CFS of each strain (1 h at 37°C) with: pepsin A (3100 U/mg), trypsin (13600 U/mg), α-chymotrypsin (60 U/mg), papain (18 U/mg), lipase Type VII (901 U/mg) and α-amylase Type VII-A. The enzymes (all from Sigma, St Louis, MO, USA) were used at a final concentration of 1 mg/l in 10 mmol sodium phosphate buffer at pH 7.0, except for the pepsin (pH 2). The CFS in buffer without enzymes was exposed to the same conditions. The residual antimicrobial activities were determined using the AWDA.

Stability of the antimicrobial compounds at different temperatures and pH values

Sensitivity of the antimicrobial compounds to heat was investigated by treating the neutralized CFS of each strain in water bath for 30 min at 30, 60, 80 and 100°C, and by autoclaving the supernatant for 15 min at 121°C followed by immediate cooling at 4°C. Then, the residual antimicrobial activities were determined by the AWDA as described earlier. Untreated samples were included as controls.

In order to determine the sensitivity of the antimicrobial compounds to pH, the supernatant of each strain culture was adjusted to the pH levels ranging from 2 to 10 (intervals of 1.0) with 1 N HCl and 1 M NaOH. After incubation at 37°C for 5 h, the pH was readjusted to 6.5 before the evaluation of the residual antimicrobial activity. The supernatants of unadjusted pH were used as controls.

RESULTS

Screening for antimicrobial activity

A total of 450 strains, isolated from 25 samples of camel milk, were initially screened for antagonistic activity against *L. innocua* CECT 4030 by the agar-spot deferred method. Of the 450 strains tested, 30 produced an inhibition zone. They were characterized as LAB. In this step, the possible inhibitory effect of the organic acids and hydrogen peroxide was not excluded. Subsequently, the cell free supernatants of the 30 strains were treated with catalase, neutralised, sterilised by filtration and tested by the agar well diffusion assay against *L. innocua* CECT 4030. They showed a measurable clear zone around the well. Only seven isolates (R204, E214, R75, R76, R111, R112 and R122) were selected by the large zone of inhibition for their identification at species level.

Identification of the seven LAB isolates

Phenotypic and biochemical identification of the seven selected isolates was carried out according to the characteristics shown in Table 1. All isolates were cocci Gram positive, catalase negative and were found to be negative for haemolysin.

Enterococcus isolates were identified on the basis of their growth on KF agar showing typical colonies (Reuter, 1995) and by forming black colonies on Bile Esculin Agar containing 40% bile.

Lactococcus was identified on the basis of their growth at 10°C but not at 45°C and their incapacity to grow in the presence of 6.5% NaCl. Lactococcal isolate was arginine and esculin positive and grew very well in the presence of 4% NaCl (Jokovic et al., 2008).

Sugar fermentation patterns are considered to be reliable methods of distinguishing *Enterococcus* ssp. (Mundt, 1986; Klein, 2003). From the sugar fermentation profiles and arginine catabolism (Table 1), the isolates were identified; E204 and E214 as *Enterococcus durans* (raffinose (-), arabinose (-), mannitol (-) and arginine (+)), R111 as *Enterococcus faecium* (raffinose (-), arabinose (+), mannitol (+) and arginine (+)) and R122 as *Enterococcus avium* (arabinose (+), mannitol (+) and arginine (-)).

R75 and R76 were able to grow in pH 9.6 and in 4% but not in 6.5% NaCl broth. Both were able to hydrolyse esculin and produce NH_3 from arginine. The strains formed acid from lactose and ribose but acid production from mannitol and sucrose was strain dependant. The phenotypic characteristic of the strains (Table 1) showed close resemblance to *Lactococcus lactis* subsp. *lactis* (Sharpe, 1979; Schleifer et al., 1985; Teuber et al., 1991).

Table 2. Inhibitory spectrum of the seven LAB isolates tested by the agar well diffusion assay.

Indicator strain	Isolate						
	E. durans		*L. lactis* subsp. *lactis*		*L. lactis* subsp. *cremoris*	*E. faecium*	*E. avium*
	E204	E214	R75	R76	R112	R111	R122
L. innocua CECT 4030	15[a]	13	14	16	13	13	15
L. monocytogenes CECT 4032	14	13	13	14	13	14	14
L. ivanovii BUG 496	18	10	13	16	10	11	16
S. aureus CECT 794	-	-	10	-	9	10	8
S. aureus CECT 976	-	9	10	8	9	9	-
S. aureus MBLA	-	-	-	-	-	-	-
E. faecium CECT 410	13	12	11	12	-	10	12
E. faecium CECT 419	15	14	11	15	9	2	15
E. faecium CECT 420	15	11	12	14	11	11	15
E. faecium CECT 510	16	-	9	14	-	-	15
E. faecalis	13	12	11	12	11	10	12
Lactococcus IH	8	8	8	8	10	8	10
B. subtilis DCM 6633	12	12	12	10	12	10	10
B. lactoporus	14	12	-	12	-	11	15
P. fluorosens CECT 378	-	-		-	-	-	-
P. aerugenosa IH	-	-	-	-	-	-	-
P. aerugenosa CECT 110	-	-	-	-	-	-	-
P. aerugenosa CECT 118	-	-	-	-	-	-	
P. vulgaris CECT 484	-	-	-	-	-	-	-
Escherichia coli MBLA K12	-	-	-	-	-	-	-
Escherichia coli CECT 4076	-	-	-	-	-	-	-

[a]: Diameter in millimetres ± 1 mm; -: no activity; CECT, Spanish Type Culture Collection, Spain. DCM, German collection of microorganisms; MBLA, Laboratory of Food Microbiology, Belgium ; IH, obtained from Hygiene Institute, Rabat, Morocco; BUG, Institute Pasteur, Paris, France.

Strain R112 had negative reactions for hydrolysis of arginine and esculin, the production of acetoin and CO_2 and for growth at 40°C in the presence of 4% NaCl. The characteristics of strain R112 are consistent with those of *L. lactis* subsp*. cremoris*.

Inhibitory spectra

A total of 7 strains produced inhibition zones against some indicator microorganisms. Inhibitory spectra of these isolates are presented in Table 2. These strains isolated from dromedary milk revealed a strong inhibitory activity towards pathogenic microorganisms and other closely-related species. The antimicrobial activities of these isolates have been evaluated by measuring the diameter of the inhibition zones around the wells.

Characterization of the antimicrobial compounds

The 7 selected LAB isolates, exhibiting antagonistic activities against *L. innocua* CECT 4030, were characterized for their antimicrobial compounds. NaOH (2.5 M) and/or catalase were added to avoid acid and hydrogen peroxide inhibitions. All isolates exhibited inhibition zones for sample 1 (culture broth). They also displayed similar inhibition zones for sample 2 (filtered supernatant) and for sample 4 (filtered supernatant with the addition of catalase). The inhibition zones for sample 3 (filtered supernatant adjusted to pH 6.5) and sample 5 (filtered supernatant adjusted to pH 6.5 and with the addition of catalase) were more large than that displayed by sample 1.

Sensitivity to enzymes, heat and pH

The effects of various enzymes on the inhibitory agent were investigated (Table 3). The antimicrobial compounds from all strains were completely inactivated by treatment with α-chymotrypsin. Pepsin and trypsin affected the activity of cell-free supernatants derived from strains R76 and R122 but not strains E204, E214, R75, R111 and R112. Papain inhibited the activity of the supernatant derived only from R122. However, catalase had no effect, indicating that hydrogen peroxide did not account for the observed inhibition. The antimicrobial

Table 3. Sensitivity of the antimicrobial compounds of LAB isolates to enzymes.

Enzyme	Isolate						
	E. durans		*L. lactis subsp. lactis*		*L. lactis subsp. cremoris*	*E. faecium*	*E. avium*
	E204	E214	R75	R76	R112	R111	R122
Control	-	-	-	-	-	-	-
Pepsin	-	-	-	+	-	-	+
Trypsin	-	-	-	+	-	-	+
α-Chymotrypsin	+	+	+	+	+	+	+
Papain	-	-	-	-	-	-	+
Lipase a	-	-	-	-	-	-	-
α-Amylase	-	-	-	-	-	-	-

+: Antimicrobial compound inactivation after treatment; -: antimicrobial compound resistance after treatment; Control: untreated CFS.

activities expressed by these strains were sensitive to proteolytic enzymes, indicating that the active compounds are of proteinaceous nature, a general characteristic of bacteriocin. Such proteinaceous compounds that inhibit closely related bacteria can be included in the category of bacteriocins (Tagg et al., 1976; Jack et al., 1995). Because molecular characterization of the compounds has not yet been done, they will be referred to as bacteriocin-like substances. The insensitivity to lipase A and α-amylase suggests the absence of a quaternary structure in which a lipidic or glucosidic moiety could be present (Klaenhammer, 1993).

Heating for 30 min at 30, 60, 80 and 100°C or 15 min at 121°C (autoclaved) did not affect the antimicrobial activity since the residual activity was detected by the AWDA (Table 4). The results suggest that all strains produced thermostable compounds.

Stability of antimicrobial activity, at different pH values, was evaluated after incubation of the CFS at 37°C for 5 h and its readjustment at pH 6.5. All samples retained full antimicrobial activity against *L. innocua* CECT 4030 in the pH range (2 to 10) (data not shown). The antimicrobial compounds can be used in foods because they are stable at pH and temperatures chosen on the basis of their usual levels in foods and processing operations.

DISCUSSION

By considering their phenotypic and physiological characteristics, the LAB strains isolated from Moroccan camel milk belonged to *E. durans*, *E. faecium*, *E. avium*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris.*

The presence of *E. faecium* and *E. durans* in raw milk and raw milk cheese is common (Freitas et al., 1999; Rodríguez et al., 1995). *E. avium* has also been identified in León cheese (Rodríguez et al., 1995) and in artisanal Mexican Fresco cheese (Torres-Llanez et al., 2006).

Enterococcal strains, mainly those of *E. faecium* which frequently occur in various food systems and whose technological and probiotic benefits are widely recognised (Giraffa, 1995), are good candidates for potential applications against *L. monocytogenes* in foods. Enterocin 81 produced by *E. faecium* and isolated from cheese exhibited a narrow spectrum against *Listeria* spp. including *L. monocytogenes* (Ennahar et al., 1998).

L. lactis subsp. lactis are characterised by their ability to hydrolyse arginine, to metabolise a number of sugars and to grow at 40°C in the presence of 4% NaCl. These performances are relevant for certain cheeses, which are cooked to high temperatures or contain relatively high salt concentrations. Members of the subspecies *cremoris*, however, are not able to grow under these conditions (Salama et al., 1991; Cogan, 1996).

L. lactis subsp. lactis and *L. lactis subsp. cremoris* are important in food technology (Salama et al., 1991; Garvie, 1984; Sandine, 1985). Bacteriocin producing lactococcal strains have been used successfully in starter cultures for cheese to improve the safety and quality of the cheese (Lipinska, 1973; Maisnier-Patin et al., 1992; Delves-Broughton et al., 1996; Ryan et al., 1996). Wouters et al. (2002) investigated the production of bacteriocins and bacteriocin-like compounds by the wild lactococci in raw milk. They found the well-known antimicrobial peptide nisin, diplococcin, lactococcin and some unidentified bacteriocin-like compounds.

Some papers reported that *L. monocytogenes* show a greater sensitivity towards some antimicrobial compounds as compared to *L. innocua* (Mataragas et al., 2003; Çon et al., 2001). The antimicrobial activities of the 7 LAB strains was assayed against *Listeria* spp., *Enterococcus* spp., *Staphylococcus* spp. and other Gram positive and negative bacteria. The seven strain were active against Gram-positive as *L. innocua* CECT 4030, *L. monocytogenes* CECT 4032, *L. ivanovii* BUG 496, *Staphylococcus aureus* CECT 794, *S. aureus* CECT 976, *E. faecium* CECT 410, *E. faecium* CECT 419, *E. faecium* CECT 420, *E. faecium* CECT 510, *Lactococcus* IH, *E. faecalis*, *Bacillus subtilis* DCM 6633 and *Bacillus lactoporus*, although, none was active against Gram-negative bacteria such as *Escherichia coli* species and

Table 4. Effect of temperature on the antimicrobial compounds of 7 LAB isolates.

Temperature	Isolate						
	E. durans		*L. lactis subsp. lactis*		*L. lactis subsp. cremoris*	*E. faecium*	*E. avium*
	E204	E214	R75	R76	R112	R111	R122
Control	15	13	14	16	13	13	15
30°C 30 min	15	13	13	15	12	12	15
60°C 30 min	15	13	13	15	12	12	15
80°C 30 min	15	13	13	15	13	13	15
100°C 30 min	14	13	13	14	12	13	15
121°C 15 min	14	13	13	14	12	13	15

Diameter of inhibition zone is measured in mm; Control: untreated CFS.

Pseudomonas aeruginosa. LAB strains are mostly inactive against Gram-negative bacteria due to the resistance conferred by the outer membrane. However, inhibitory effects of nisin (Cutter and Siragusa, 1995), bacteriocin produced by *Lactobacillus paracasei* subsp. *paracasei* (Caridi, 2002), bacteriocin ST151BR produced by *Lactobacillus pentosus* ST151BR (Todorov and Dicks, 2004), thermophylin produced by *Streptococcus thermophillus* (Ivanova et al., 1998) and some enterocins (Gálvez et al., 1989; Simonetta et al., 1997; Jennes et al., 1999) on Gram-negative bacteria through their synergetic effects with other antimicrobials has gained increased interest (Helander et al., 1997).

Treatment of the neutralized supernatants with catalase did not change their activities, indicating that the inhibition recorded was not acid and hydrogen peroxide. The samples 3 (filtered supernatant adjusted to pH 6.5) and 5 (filtered supernatant adjusted to pH 6.5 and with addition of catalase) showed greater activities because the adsorption of bacteriocin into indicator strain is maximal at pH 6.5 (Yang et al., 1992).

The activity of the supernatants was lost after treatment with proteolytic enzymes (pepsin, trypsin, α-chymotrypsin or papain) which indicates that the active compounds secreted were extracellulary and proteinaceous and provides evidence that growth inhibition of *L. innocua* CECT 4030 was caused by a bacteriocin. Furthermore, the inhibitory activity was not altered by neutralization of the supernatant. Treatment with lipase did not cause any loss of activity, probably because of the absence of lipid moiety in the compounds. Incubation with α-amylase had no effect on the antimicrobial activity recorded, suggesting that the carbohydrates are not bound on the molecules.

The antimicrobial compounds produced by the 7 LAB isolates were characterized based on their resistances to heat treatment, their susceptibility to proteolytic enzymes and their effects on indicator microorganisms. The antimicrobial compounds could be classified with the earlier mentioned characteristics into four groups: nisin, enterocin, lactococin and bacteriocin-like (unknown).

The proteolytic profile of the antimicrobial compounds of E204, E214 and R111 is similar to several *E. faecium* bacteriocins such as enterocin CRL 35 (Farias et al., 1994), and enterocin I (Floriano et al., 1998) which are resistant to at least one of the proteolytic enzymes tested in this study. However, the antimicrobial agent of the isolate R122, which displayed a broad spectrum of activity, had the common characteristic of enterococcal bacteriocins (Giraffa, 1995). Isolate R122 exhibited properties similar to other groups of bacteriocin such as enterocin 1146 (Parente and Hill, 1992), enterocin CRL504 (Farias et al., 1994), enterocin EL1 (Lyon et al., 1995), enterocin 81 (Ennahar et al., 1998) and enterocin P (Herranz et al., 1999) which are hydrolyzed by a wide range of proteolytic enzymes. The culture supernatants of *E. durans* isolates E204 and E214 lost completely their antimicrobial activity after treatment with α- chymotrypsin and maintained antimicrobial activity after autoclaving (121°C for 15 min). These antimicrobial metabolites had the same characteristics as durancin L28-1A produced by *E. durans* isolated from soil (Yanagida et al., 2005).

The antimicrobial compound produced by *Lactococcus* isolate was considered as nisin based on its resistance to heat treatment, inactivation by α-chemotrypsin treatment and based on its activity towards indicator strains, features which are typically attributed to nisin (Hurst, 1981; Gupta and Prasad, 1989).

The results of thermal treatment showed that inhibitory substances are resistant to heat. This resistance is also known for other bacteriocin produced by lactic acid bacteria: lactacin B (Barefoot and Klaenhammer, 1983), lactacin F (Muriana and Klaenhammer, 1987), nisin (Bailey and Hurst, 1971) and bacteriocin ST15 (De Kwaadsteniet et al., 2005). This resistance to heat is a common characteristic of bacteriocin (Tagg et al., 1976). The species of enterococci are found in dairy products but *E. faecalis* and *E. faecium* remain the prevailing ones. These strains are capable of producing a variety of enterocins with inhibitory activities against *L. monocytogenes*, *S. aureus* and *Clostridium* spp. (Floriano et al., 1998; Franz et al., 1999; Gelsomino et al., 2001). Several enterocins described to date belong to class II bacteriocins and most of them are identical either to

enterocin A or enterocin B that were initially described from *E. faecium* CTC492 and *E. faecium* T136 (Aymerich et al., 1996; Casaus et al., 1997) isolated from fermented sausages. These bacteriocins were considered to be like nisin and pediocin PA-1, among the most common LAB bacteriocins. Moreover, some enterococci strains produced two enterocins (Casaus et al., 1997; Ghrairi et al., 2007; Ibarguren et al., 2010) and three enterocins (Basanta et al., 2008).

The pH and temperatures used were chosen at their usual levels in foods and processing operations. The bacteriocins-like substances produced by the seven strains tested exhibited a broad pH range of activity against *L. innocua* CECT 4030. They inhibited *L. innocua* at 2 to 10 pH range. These data suggest that the bacteriocin-like compounds described in this study could be applied in both low and medium-acid fermented food products with final pH values in such range; this, includes a number of fermented and ripened dairy and meat products.

Conclusion

Non-identified bacteriocins-like substances produced by the seven LAB strains isolated from camel milk in this study have shown resistance to high temperatures, stability to a wide range of pH and a large inhibitory spectrum. These characteristics offer useful protection against eventual contamination of milk or curd with pathogenic or spoilage microorganisms. Thus, further investigations are needed to determine their molecular properties as novel bacteriocins.

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