Nisin and lacticin 481 coproduction by *Lactococcus lactis* strains isolated from raw ewes' milk

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ABSTRACT

Bacteriocin-producing lactococci were isolated from raw ewes' milk samples obtained from 5 different Protected Designation of Origin Zamorano cheese manufacturers. Thirteen isolates with antimicrobial activity against *Lactococcus lactis* HP were selected. Eleven were identified by a PCR technique as *L. lactis* ssp. *lactis* and 2 were identified as *L. lactis* ssp. *cremoris*. They were grouped under 4 different pulsed-field gel electrophoresis patterns. The presence of structural genes of both nisin and lacticin 481 was detected in 10 *L. lactis* ssp. *lactis* isolates belonging to 2 different pulsed-field gel electrophoresis patterns. Coproduction of nisin and lacticin 481 was confirmed after semipurification by using selective indicators. The production of 2 bacteriocins by the same strain is an uncommon property, with relevance in food safety. Nisin and lacticin 481 *L. lactis*-producing strains might be used as adjunct cultures to the commercial starter in the manufacture of dairy products.

Key words: lactococcus, nisin, lacticin 481, raw ewes' milk

INTRODUCTION

Lactococcus lactis strains are used as starter cultures in the manufacture of cheese. The isolation and characterization of lactococci from dairy products manufactured by using traditional procedures, without added commercial starters, to extend the availability of cultures has been considered in the search for industrially important properties (Cogan et al., 1997). On the other hand, the demand for safe foods with a prolonged shelf life and minimal processing technologies has considerably enhanced the interest in biopreservation. Raw milk products represent a source of new strains of lactic acid bacteria (LAB) with the potential to inhibit

undesirable microbiota (Cogan et al., 1997; Rodríguez et al., 2000).

Many LAB produce bacteriocins, ribosomally synthesized antibacterial peptides with activity against related species (Cleveland et al., 2001). Although many bacteriocins from LAB have been characterized and exhibit a large spectrum of food applications, the lantibiotic nisin is the only bacteriocin industrially exploited as a food preservative. The potential applications for other lactococcal lantibiotics have been reviewed by Guinane et al. (2005), mainly focusing on the 2-component lacticin 3147 and its activity to control nonstarter LAB and accelerate cheese ripening and as food biopreservative. The properties of lacticin 481 have recently been reviewed by Dufour et al. (2007). Lacticin 481 has been used successfully in flavor enhancement, in the acceleration of cheese ripening (Oumer et al., 2001; Garde et al., 2006), and, combined with high pressure, in the reduction of pathogen levels in cheese (Rodríguez et al., 2005).

The application of bacteriocins in foods would allow a significant reduction in the concentration of additives, the intensity of physical treatments currently applied in food manufacture, or both. However, their use is limited by the proteolytic activity of food enzymes, by their adsorption onto fat in foods, and by the appearance of resistant variants in sensitive strains. The potential emergence of resistant populations could be counteracted by the use of LAB that produce multiple bacteriocins (Deegan et al., 2006) because bacteriocins in combination may overcome these shortcomings (Hanlin et al., 1993).

The development of bacterial strains exhibiting overexpression of bacteriocins by genetic manipulation and the introduction of this characteristic into different organisms have received particular attention (Settanni and Corsetti, 2008), although the application of these technologies would be limited by restrictive legal regulations and the lack of acceptance by consumers. Transconjugant lactococcal starters coproducing 2 lantibiotics, lacticin 481 and the 2-component lacticin 3147, were constructed by O'Sullivan et al. (2003). On the other hand, natural multiple-bacteriocin produc-

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tion has been reported for some LAB. Nonantibiotic multiple-bacteriocin production has been described for *Carnobacterium piscicola* LV17, which produces 3 carnobacteriocins (Quadri et al., 1994), or *Enterococcus faecium* L50, which produces 3 different enterocins (Cintas et al., 2000). Garde et al. (2001) detected the genetic determinants of nisin Z and lacticin 481 in a *L. lactis* ssp. *lactis* isolate from Manchego cheese.

In the present work, based on previous results concerning multiple-bacteriocin production, our main objective was to study the production of nisin and lacticin 481 by wild lactococci isolated from raw ewes' milk used in the manufacture of Protected Designation of Origin (PDO) Zamorano cheese, made in central Spain without added starter cultures.

MATERIALS AND METHODS

Isolation of Bacteriocin-Producing Bacteria

Bacterial strains were isolated from raw ewes' milk obtained from 5 different manufacturing plants producing PDO Zamorano cheese. Refrigerated (4 to 6°C) bulk ewes' milk samples (500 mL) from mixed evening and morning milkings were collected approximately 2 to 3 h after the morning milking and transported to the laboratory at 4°C. Curds from 2 milk samples per plant were prepared upon arrival under aseptic conditions to enrich them for LAB. Rennet (Maxiren 15L, Gist Brocades NV, Delft, the Netherlands) was added to 200 mL of milk at 30°C. Curds were cut 40 min after rennet addition, drained, and held at 25°C for 18 h. Curd samples (10 g) were homogenized in 90 mL of sterile 2% sodium citrate solutions, and decimal dilutions in peptone (0.1%) water were inoculated on the surface of de Man, Rogosa, Sharpe (MRS) agar plates (Difco Laboratories, Detroit, MI) with 2% (wt/vol) β -glycerophosphate (Merck, Darmstadt, Germany) by using an Spiral system (Interscience, Saint Nom La Brteche, France). Duplicated plates were incubated at 30°C for 48 h and then overlaid with 3 mL of APT broth (Difco) with 0.75% (wt/vol) agar inoculated with 0.1 mL of an 18-h culture in APT broth of *L. lactis* ssp. *cremoris* HP. After 24 h of incubation at 30°C, colonies surrounded by clear inhibition areas were isolated. They were maintained at -80°C as stock cultures in MRS broth supplemented with 30% (wt/vol) glycerol until further characterization and were activated by 2 successive transfers before use.

Antimicrobial activity of the isolates was confirmed by the agar well diffusion assay (Schillinger and Lcke, 1989). Neutralized supernatants (25 μ L) from cultures grown in MRS broth were filtered (Millex GV, Millipore, Bedford, MA) and placed in triplicate into wells

made in plates seeded with an overnight culture of *L. lactis* HP or *Lactobacillus buchneri* St2A. Plates were incubated at 30 or 37°C, respectively, and examined for the presence of zones of growth inhibition.

Genotypic Characterization of *Lactococcus* spp.

Isolates showing inhibition against *L. lactis* HP were purified on MRS agar plates by several transfers. Gram-positive and catalase-negative cocci were identified by the PCR-based technique of Beimfohr et al. (1997) by using 3 pairs of primers complementary to the histidine biosynthesis operon of *L. lactis*. Genomic DNA was prepared from 2 colonies of each isolate grown on MRS agar. Colonies were suspended in 20 μ L of sterile double-distilled water in microcentrifuge tubes, mixed gently, and heated at 85°C for 10 min. This preparation was used as a template. Amplification was carried out in a Gene ATAQ Controller thermal reactor (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as described by Beimfohr et al. (1997).

Pulsed-Field Gel Electrophoresis Typing

Strain characterization was carried out by means of pulsed-field gel electrophoresis (PFGE), according to a modification of the method of Le Bourgeois et al. (1989). Genomic DNA was prepared in agarose blocks and treated with *Sma*I (30 IU) restriction endonuclease (New England Biolabs, Ipswich, MA). Electrophoresis was carried out at 14°C in a Chef DRII apparatus (Bio-Rad Laboratories, Madrid, Spain) in a 1% agarose gel containing 0.5 $\times$ Tris, borate, EDTA buffer at 6 V/cm at 1 to 5 s, with a linear increase over 22 h. The gels were stained in ethidium bromide solution (0.5 μ g/mL) and analyzed by using the Diversity Database program (Bio-Rad Laboratories). The similarity of the band profiles was calculated by means of the Dice coefficient and clustered by the unweighted pair group method with arithmetic mean.

Characterization of Bacteriocins

Polymerase chain reaction amplification to detect the structural genes of nisin, lacticin 481, and lactococcin A was carried out with the primers and conditions indicated by Rodriguez et al. (2000). Reaction parameters were 92°C for 2 min, 41°C for 2 min, and 72°C for 2 min, for a total of 26 cycles for nisin primers; 92°C for 30 s, 51°C for 30 s, and 72°C for 1 min, for a total of 26 cycles for lacticin 481 primers; and 92°C for 2 min, 38°C for 2 min, and 72°C for 2 min, for a total of 30 cycles for lactococcin A primers. Amplified bands were visualized on 1% agarose gels by ethidium bromide

staining by using a 100-bp ladder (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) as the molecular weight standard.

The PCR-generated fragments with the primers for nisin and lactacin 481 were extracted from agarose gels and purified with a QIAquick PCR purification kit (Qiagen, Crawley, UK) according with the instructions of the supplier. Purified PCR fragments were sequenced by using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Waltham, MA). The sequences were compared with the Basic Local Alignment Search Tool programs of the National Center for Biotechnology Information.

Nisin A-producing *L. lactis* ssp. *lactis* ATCC 11454, lactacin 481 producing *L. lactis* ssp. *lactis* CNRZ 481, and lactococcin A producing *L. lactis* ssp. *lactis* WM4, as well as *L. lactis* ssp. *lactis* INIA 626 producing nisin Z, *L. lactis* ssp. *lactis* INIA 639 producing lactacin 481, and *L. lactis* ssp. *lactis* INIA 415 with the genetic determinants of both nisin Z and lactacin 481, from the INIA Culture Collection, were used as positive controls for bacteriocin characterization. *Lactococcus lactis* HP was used as a negative control for bacteriocin production. *Lactococcus lactis* HP sensitive to nisin and lactacin 481, and *Lb. buchneri* St2A sensitive to nisin and resistant to lactacin 481 were used as selective indicators.

SDS-PAGE Analysis

Tricine SDS-PAGE (Schägger and von Jagow, 1987) was performed by using a miniVE system (Hoefer Inc., San Francisco, CA). After electrophoresis, the gel was divided vertically into 2 parts. One part was stained with silver stain (Bio-Rad Laboratories). The second part of the gel was used for the detection of antimicrobial activity according to Bhunia et al. (1987) after 2 washing cycles with sterile distilled water for 1.5 h each. The gel was placed into a Petri dish and overlaid with 7 mL of MRS agar (0.7%) containing 10^6 cells/mL of the indicator strain *L. lactis* HP. The dish was then incubated at 30°C for 18 h and examined for inhibition zones.

Prepurification of Bacteriocins

Bacteriocin-producing strains were inoculated at 1% and cultured at 30°C overnight in 400 mL of M17 broth (Biolife Italiana S.r.l., Milano, Italy) or reconstituted (10%) skim milk. Cultures were centrifuged at $6,000 \times g$ for 15 min at 4°C. Supernatants were filtered through a Whatman No. 2 filter (Whatman Ltd., Maidstone, UK) and sterilized by passage through a Millex 0.22- μ m HV filter (Millipore). The solutions were loaded onto Sep-Pak C₁₈ cartridges (Waters, Milford, MA). Cartridges

Table 1. Polymerase chain reaction identification and amplification with nisin or lactacin 481 structural gene primers of bacteriocin-producing lactococci isolated from raw ewes' milk

PCR identification of <i>Lactococcus lactis</i>	Nisin	Lactacin 481
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z11	—	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z101	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z102	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z103	+	+
<i>L. lactis</i> ssp. <i>cremoris</i> INIA Z105	—	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z106	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z108	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z109	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z110	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z111	+	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z112	+	+
<i>L. lactis</i> ssp. <i>cremoris</i> INIA Z113	—	+
<i>L. lactis</i> ssp. <i>lactis</i> INIA Z114	+	+
<i>L. lactis</i> ssp. <i>lactis</i> ATCC 11454	+	—
<i>L. lactis</i> ssp. <i>lactis</i> CNRZ 481	—	+

were equilibrated with 2 mL of methanol, followed by 2 mL of distilled water. The antibacterial compounds were eluted with successive steps of methanol:water mixtures (from 10 to 100% MeOH). Antimicrobial activity in fractions was assayed by the agar well diffusion method, with *L. lactis* HP and *Lb. buchneri* St2A as indicator strains.

RESULTS AND DISCUSSION

Identification of Bacteriocin-Producing Isolates

Mean counts of LAB in curds made from raw milk samples from 5 different PDO Zamorano cheese-manufacturing plants were approximately log 9 cfu/g. Thirteen isolates, which exhibited antimicrobial activity against *L. lactis* HP, were selected. The production of bacteriocin-like antimicrobial compounds was confirmed by the agar well diffusion assay against *L. lactis* HP.

All bacteriocin-producing gram-positive catalase-negative cocci were identified by PCR as *L. lactis* (Table 1). Eleven isolates exhibited the pattern characteristic of *L. lactis* ssp. *lactis* and 2 isolates were identified as *L. lactis* ssp. *cremoris*.

The analysis of PFGE patterns of the chromosomal DNA restriction patterns generated by *Sma*I digestion showed low genetic diversity within this group of lactococci (Figure 1). Pulsed-field gel electrophoresis divided the 13 bacteriocin-producing isolates into 4 pulsotypes (P1, P2, P3, and P4). *Lactococcus lactis* ssp. *lactis* INIA Z11 was classified into pulsotype P1, *L. lactis* ssp. *cremoris* INIA Z105 and INIA Z113 into P2, *L. lactis* ssp. *lactis* INIA Z103 and INIA Z106 into P3, and the rest of the *L. lactis* ssp. *lactis* isolates (INIA Z101, INIA Z102, INIA Z108, INIA Z109, INIA Z110, INIA Z111, INIA Z112, and INIA Z114) into P4. All isolates except

Figure 1. *Sma*I pulsotypes and dendrogram obtained with 13 isolates of bacteriocin-producing lactococci from raw ewes' milk.

L. lactis ssp. *lactis* INIA Z11 were obtained from milk from the same cheese-manufacturing plant. The plant makes cheese from milk produced by its own flock of Castellana breed sheep. Good hygiene practices were observed in milk and cheese production.

Lactococci commonly isolated from artisanal cheese varieties mostly belong to the species *L. lactis* (Cogan et al., 1997), with *L. lactis* ssp. *lactis* as the predominant subspecies in many cheese varieties (Nuñez et al., 1989). A high genetic diversity within *L. lactis* strains isolated from raw ewes' milk cheese was revealed by random amplification of polymorphic DNA analysis (Gaya et al., 1999). Approximately 70% of lactococci were identified by these authors as *L. lactis* ssp. *lactis*, with *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* biovar diacetylactis in a lower proportion. Bacteriocin-producing LAB from raw milk have largely been isolated and characterized. Bacteriocin-producing lactococci were the majority among the bacteriocinogenic LAB isolated from raw milk (Rodríguez et al., 2000) and were also common among raw milk cheese isolates (Martínez et al., 1995; Cardinal et al., 1997). The production of bacteriocins provides an ecological advantage that enables producers to withstand competition against other bacteria in milk.

Characterization of Bacteriocins

Bacteriocins produced by the 13 isolates were characterized by using nisin, lactacin 481, and lactococcin A structural gene primers. Deoxyribonucleic acid from 10 of the 13 isolates amplified the expected 898-bp fragment containing the structural gene of nisin (Table 1). Lactacin 481 genetic determinants were detected in the 13 bacteriocin-producing isolates studied. The expected 366-bp fragment containing the structural gene *lct* of lactacin 481 (Piard et al., 1992) was observed in all the isolates and in the reference strain *L. lactis* CNRZ 481. Consequently, the structural genes of nisin and lactacin 481 were present in 10 isolates. On the other hand, the structural gene of lactococcin A was not detected by PCR.

The 3 isolates carrying the structural gene of lactacin 481, but not the structural gene of nisin, were *L. lactis* ssp. *lactis* INIA Z11, *L. lactis* ssp. *cremoris* INIA Z105, and *L. lactis* ssp. *cremoris* INIA Z113. *Lactococcus lactis* INIA Z11 amplified a band of 980 bp with the nisin primers instead of the expected 898-bp band. The sequence of this amplified fragment did not show homology with sequences of nisin or any other known bacteriocin. The other 2 isolates were those identified


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Nisin A      MSTKDFNLDLVSUSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK 57
Nisin Z      MSTKDFNLDLVSUSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK 57
Nisin Q      MSTKDFNLDLVSUSKTDSSGASTRITSISLCTPGCKTGVLMMGCNLKTATCNCSVHVSK 57
Nisin F      MSTKDFNLDLVSUSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK 57
Nisin INIA-Z108 MSTKDFNLDLVSUSKKNSGASPRITSISLCTPGCKTGAVMMGCNMKTATCNCSSIHVSK 57
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Figure 2. Amino acid sequence of nisin from *Lactococcus lactis* ssp. *lactis* INIA Z108, deduced from the DNA sequence and compared with the sequences of nisin A, nisin Z, nisin Q, and nisin F. Differences in AA are indicated by underlined letters.

as *L. lactis* ssp. *cremoris*, and were classified into the same PFGE group.

Lactococcus lactis ssp. *lactis* INIA Z108, amplifying for the nisin and lactacin 481 structural genes, was selected as representative of the main pulsotype for bacteriocin confirmation. The nucleotide sequence of the 898-bp DNA fragment confirmed that the bacteriocin produced was nisin. The sequence of the 898-bp band (Figure 2) showed a 96% identity with the sequence of the nisin Z precursor (Mulders et al., 1991), 94% identity with the sequence of nisin A and nisin F precursors (de Kwaadsteniet et al., 2008), and 87% identity with the nisin Q precursor (Zendo et al., 2003). Nisins A, Z, Q, and F are variants produced by *L. lactis* isolates from different origins. Nisin Q was detected in an *L. lactis* strain isolated from river water in Japan, and nisin F was detected in the intestinal tract of a freshwater fish in South Africa. In addition, nisin U produced by *Streptococcus uberis*, which has 78% sequence identity to nisin A, has been reported (Wirawan et al., 2006). At the AA level, 2 polymorphisms were detected when nisin from *L. lactis* INIA Z108 was compared with the

AA consensus nisin sequence (Figure 2), asparagine instead of aspartic acid at position 17 (N₁₇ D₁₇), and valine instead of leucine at position 39 (V₃₉ L₃₉). Our results suggest the possibility of a new nisin variant, although both equivalent substitutions are considered unlikely to bring about substantial changes in gene expression. More research is needed for a detailed characterization and analysis of nisin from *L. lactis* INIA Z108. On the other hand, the sequence of the 366-bp amplified fragment from the lactacin 481 structural gene *lct A* revealed 100% identity with the corresponding sequence of lactacin 481.

The predominance of nisin-producing lactococci among wild lactococci isolated from raw milk was reported by Rodriguez et al. (2000). These authors also detected the presence of lactacin 481 producers. Recently, nisin genes were detected in DNA from 6 out of 9 Slovenian traditional cheeses, whereas the incidence of lactacin 481 determinants was lower (Trmcic et al., 2008). The presence of genes encoding nisin Z and lactacin 481 in the same strain has been proven so far only by Garde et al. (2001), in *L. lactis* ssp. *lactis* INIA 415

Table 2. Inhibitory activity of methanol fractions¹ obtained from supernatants of bacteriocin-producing lactococci isolated from raw ewes' milk, cultured in M17 broth or in milk, against 2 selective indicators²

Item	Nisin (10% methanol fraction; <i>Lactococcus lactis</i> HP/ <i>Lactobacillus buchneri</i> St2A)	Lactacin 481 (70% methanol fraction; <i>Lactococcus lactis</i> HP/ <i>Lactobacillus buchneri</i> St2A)
M17 broth		
<i>L. lactis</i> INIA Z101	+/+	-/-
<i>L. lactis</i> INIA Z103	+/+	+/-
<i>L. lactis</i> INIA Z108	-/-	+/-
<i>L. lactis</i> INIA 415	-/-	+/-
<i>L. lactis</i> INIA 626	+/+	-/-
<i>L. lactis</i> INIA 639	-/-	+/-
Milk		
<i>L. lactis</i> INIA Z101	+/+	+/-
<i>L. lactis</i> INIA Z103	-/-	+/-
<i>L. lactis</i> INIA Z108	+/+	+/-
<i>L. lactis</i> INIA 415 ³	+/+	-/-
<i>L. lactis</i> INIA 626 ³	+/+	-/-
<i>L. lactis</i> INIA 639 ³	-/-	+/-

¹Nisin eluted with methanol at 10% and lactacin 481 at 70%.

²*Lactococcus lactis* HP is sensitive to nisin and lactacin 481. *Lactobacillus buchneri* St2A is sensitive to nisin and resistant to lactacin 481.

³*Lactococcus lactis* INIA 415 has the structural genes of nisin and lactacin 481. *Lactococcus lactis* INIA 626 and *L. lactis* INIA 639 are nisin Z and lactacin 481-producing strains, respectively.

Figure 3. Separation of bacteriocins from culture supernatants by SDS-PAGE and overlaid with de Man, Rogosa, Sharpe agar (Difco Laboratories, Detroit, MI) with *Lactococcus lactis* HP as an indicator microorganism: 1) negative control, 2) *L. lactis* INIA Z103, 3) *L. lactis* INIA Z101, 4) *L. lactis* INIA Z108, 5) *L. lactis* INIA 626 + *L. lactis* INIA 639, 6) *L. lactis* INIA 626, 7) *L. lactis* INIA 639, 8) supernatant from *L. lactis* INIA Z103 after silver staining.

isolated from raw milk Manchego cheese. Interestingly, the simultaneous presence of both genetic determinants had not been observed previously among isolates from raw milk (Rodriguez et al., 2000) or different dairy products, including cheeses (Cogan et al., 1997). In the present work, bacteriocin-producing strains were obtained from curds in which a natural enrichment for bacteria took place. Such isolates predominating in curds might be technologically competitive in cheese making. The 10 isolates with the genetic determinants of both bacteriocins were ascribed to only 2 pulsotypes, a fact that points to their competitiveness.

Antimicrobial Activity

As indicated previously, supernatants from 10 isolates inhibited both *L. lactis* HP, which is sensitive to nisin and lactacin 481, and *Lb. buchneri* St2A, which is sensitive to nisin and resistant to lactacin 481 indicators. Nisin production was thus confirmed in all 10 isolates with the genetic determinants for nisin and lactacin 481. However, lactacin 481 production had to be demonstrated.

Supernatants of bacteriocin-producing *L. lactis* ssp. *lactis* INIA Z103, *L. lactis* ssp. *lactis* INIA Z108, and *L. lactis* ssp. *lactis* INIA Z114 with the genetic determinants of nisin and lactacin 481 and from *L. lactis* ATCC 11454 (nisin) and *L. lactis* CNRZ 481 (lactacin 481) were fractionated by SDS-PAGE. Antimicrobial activities were detected on the gel with the soft agar layer inoculated with the sensitive strains of *L. lactis* HP (Figure 3). Higher inhibition halos were observed in the presence of supernatants from isolates with both genetic determinants, although individual activities were not separated into the expected 2 bands. When the supernatants of *L. lactis* ATCC 11454 (nisin) and *L. lactis* CNRZ 481 (lactacin 481) were mixed at 50%, 2 halos corresponding to nisin and lactacin 481 were clearly differentiated. Protein bands corresponding to

bacteriocins were not easily distinguished on the SDS-PAGE gels after staining with silver stain, probably because of the high number of metabolites in the supernatant without further treatment.

After prepurification by Sep-Pak cartridges, the bacteriocins in supernatants were recovered in different fractions. Nisin eluted at 10 to 20% methanol and lactacin 481 eluted at 70 to 90%, in agreement with the high proportion of hydrophobic residues in the latter. Larger halos were observed for the 10 and 70% methanol fractions for nisin and lactacin 481 activities, respectively. Activities of these fractions from cultures of 3 isolates in M17 broth and milk, against the 2 selective indicator strains *L. lactis* HP and *Lb. buchneri* St2A, are shown in Table 2. Coproduction was confirmed in the semipurified preparations of *L. lactis* INIA Z101, INIA Z103, and INIA Z108, although it was dependent on the growth medium. The simultaneous presence of nisin and lactacin 481 was observed in the supernatants of *L. lactis* INIA Z103 grown in M17 broth and in the supernatants of *L. lactis* INIA Z101 and INIA Z108 grown in milk. As expected, nisin and lactacin 481 were detected in supernatants from *L. lactis* INIA 626 and *L. lactis* INIA 639, respectively. With respect to the previously studied *L. lactis* ssp. *lactis* INIA 415, lactacin 481 was the only bacteriocin produced in M17 broth and nisin was the only one produced in milk. Bacteriocin production can be influenced by environmental conditions, such as the composition of the culture medium, pH, and the temperature of incubation (Gänzle et al., 1999). Differences in bacteriocin production in MRS and milk medium supplemented with yeast extract were reported by Avonts et al. (2004) in probiotic *Lactobacillus* strains. According to those authors, factors that may contribute to the low bacteriocin titers in milk medium were associated with the adsorption of bacteriocins to CN, the scarce induction of production by medium components, or lower biomass formation. In our work, the undetectable nisin or lactacin 481 activities found in M17 broth or in milk might be attributed to reduced bacteriocin production.

The use of lactococcal bacteriocins as tools to control microbial populations in food systems was reviewed by Guinane et al. (2005). In the present work, *L. lactis* strains coproducing nisin and lactacin 481 in broth and in milk were obtained. To extend the potential applications for lactococcal bacteriocins, optimization of bacteriocin production by our isolates under industry conditions should be investigated.

CONCLUSIONS

In the present work, bacteriocin coproduction was detected in new *L. lactis* isolates from raw ewes' milk.

The coproduction of nisin and lactacin 481 would constitute a useful system for the biological control of spoilage or pathogenic microorganisms. These new *Lactococcus* isolates can have a potential impact on the development of new industrially important cultures for their application in the manufacture of dairy products.

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