Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk

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Ángel Alegría[1], Susana Delgado[1], Clara Roces[2], Belén López[3], and Baltasar Mayo[1]*

[1]Departamento de Microbiología y Bioquímica and [2]Departamento de Tecnología y Biotecnología, Instituto de Productos Lácteos de Asturias (IPLA), Consejo Superior de Investigaciones Científicas (CSIC), Carretera de Infiesto, s/n, 33300-Villaviciosa, Asturias, Spain, and [3]Proquiga S.A., Polígono industrial de Bergondo, C/ Parroquia de Rois, S/N Parcela D-2, 15165-Bergondo, A Coruña, Spain

[*]Corresponding author:

Baltasar Mayo, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300-Villaviciosa, Spain

Tel.: 34+985 89 21 31

Fax: 34+985 89 22 33

E-mail address: baltasar.mayo@ipla.csic.es

ABSTRACT

Sixty bacterial strains were encountered by random amplification of polymorphic DNA (RAPD) and repetitive extragenic palindromic (REP) typing in a series of 306 *Lactococcus lactis* isolates collected during the manufacturing and ripening stages of five traditional, starter-free cheeses made from raw milk. Among the 60 strains, 17 were shown to produce bacteriocin-like compounds in both solid and liquid media. At a genotypic level, 16 of the strains were identified by molecular methods as belonging to *L. lactis* subsp. *lactis* and one to *L. lactis* subsp. *cremoris*. Among the *L. lactis* subsp. *lactis* strains, phenotypic and genetic data determined that eleven produced either nisin A (nine strains) or nisin Z (two strains), and that five produced lactococcin 972. Variable levels of the two bacteriocins were produced by the different strains. In addition, nisin was shown to be produced in inexpensive, dairy- and meat-based media, which will allow the practical application of its producing strains in industrial processes. Specific PCR and nucleotide and deduced amino acid sequence analysis identified as a lactococcin G-like bacteriocin the inhibitor produced by the single *L. lactis* subsp. *cremoris* isolate. Beyond the use of bacteriocins as functional ingredients for the biopreservation of foods, the newly identified bacteriocin-producing *L. lactis* strains from traditional cheeses may also be useful for designing starter cultures with protective properties and/or adjunct cultures for accelerating cheese ripening.

Keywords: Lactococcus lactis, bacteriocins, nisin, lactococcin 972, lactococcin G, starters, adjunct cultures, protective cultures, traditional dairy products

1. Introduction

Many microbial groups produce bacteriocins -peptides and proteins with bactericidal activity. The bacteriocins of some bacteria inhibit growth of closely related microbes, while others inhibit a much wider range of microorganisms, including food-borne pathogens and spoilage microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium tyrobutyricum* (Gálvez et al. 2008).

From a biochemical point of view, two types of bacteriocins have been identified in lactic acid bacteria (LAB), those characterized by the presence of dehydrated (dehydroalanine and dehydrobutyrine) and/or thioether amino acids (lanthionine and β-methyllanthionine), usually referred to as lanthibiotics (or class I), and those containing unmodified amino acids (non-lanthibiotics) (Jack et al. 1995). Non-lanthibiotics are divided into classes II through IV depending on their size and the presence of non-protein moieties. Both lanthibiotics and non-lanthibiotics are synthesized via a ribosomal pathway, but the former are later modified enzymatically. In the last 25 years, intensive research into the bacteriocins produced by LAB has been undertaken with the aim of improving the microbial quality and safety of fermented products (de Vuyst and Leroy 2007).

Lactococcus lactis strains are the majority LAB components of commercial starter cultures used by the dairy industry for the manufacture and ripening of cheese and fermented milks (Limsowtin et al. 1995). Lanthibiotic and non-lanthibiotic bacteriocins produced by *L. lactis* from different sources have been identified and characterized (Venema et al. 1995). The first bacteriocin isolated from *L. lactis* was nisin (Mattick and Hirsch 1947), a 34-amino acid lanthibiotic. This is currently approved and exploited in over 50 countries as a food additive (code E234) (Delves-Broughton et al. 1996). To date, five natural nisin variants (A, Z, Q, U, and F) have been identified (de Kwaadsteniet et al. 2008). Other lanthibiotics produced by *L. lactis* include the single peptide lacticin 481 and the two-component system lacticin 3147 (de Vuyst and Leroy 2007). Non-lanthibiotic bacteriocins from *L. lactis* include pediocin-like bacteriocins (class IIa) such as lactococcin MMFII, two-peptide component bacteriocins (class IIb) such as lactococcin G and M, thiol-activated bacteriocins (class IIc) such as lactococcin B, and heat-labile, lactococcus-specific bacteriocins (class IId) such as lactococcin A (diplococcin) and lactococcin 972 (Venema et al. 1995; Oppegård et al. 2007).

The incorporation of bacteriocin-producing lactococci as starter or adjunct cultures in the manufacture of fermented foods provides an attractive and economic alternative to the addition of purified bacteriocins (indeed, metabolic compounds produced during fermentation are no longer considered additives). Bacteriocin-producing *L. lactis* has therefore been experimentally tested in the manufacture of several cheese varieties (Ryan et al. 1996; Martínez-Cuesta et al. 2001; O'Sullivan et al. 2003; Rilla et al. 2003; Garde et al. 2006) and other fermented products (Diop et al. 2009). Following its addition, starter lysis is increased (O'Sullivan et al. 2003) and peptidolytic and transamination activities, key factors in the formation of aroma and taste compounds, may also be enhanced (Martínez-Cuesta et al. 2003; Fernández de Palencia et al. 2004). In addition to its technological applications, bacteriocin-producing *L. lactis* has been assayed for the treatment of mastitis in cows (Ryan et al. 1999; Twomey et al. 2000; Klostermann et al. 2009), and is being evaluated as an antipathogenic agent in human gastrointestinal infections (O'Connor et al. 2006; Millette et al. 2008).

The aim of the present work was to screen for bacteriocin production in a large number of *L. lactis* strains isolated during the manufacturing and ripening stages of different batches of five traditional, Spanish, starter-free cheeses made from raw milk. Efforts were also made to identify these antimicrobial compounds by searching for bacteriocin-encoding genes. Of the 17 bacteriocin producers detected, phenotypic and genetic analyses identified eleven as nisin producers, five as lactococcin 972 producers, and a single producer of lactococcin G.

2. Material and Methods

2.1. Strains, media and culture conditions

A series of 306 lactococcus-like isolates collected during the manufacture and ripening of five Spanish traditional, starter-free cheeses made from raw milk were grouped by typing and identified by partial ARDRA, sequencing and sequence comparison. These isolates came from *Casín* (80), *Cabrales* (106), *Genestoso* (63), *Peñamellera* (44), and *Valle del Narcea* (13) cheeses. Representative isolates of the 60 different strains found were tested for the production of antimicrobial compounds against a series of Gram-positive indicator bacteria. The indicator strains included *L. lactis* subsp. *cremoris* MG 1363, *L. lactis* subsp. *lactis* NCDO 497 (nisin producer), *L. lactis* subsp. *lactis* IPLA 972 (lactococcin 972 producer), *Lactobacillus sakei* CECT 906^T, *Lactobacillus plantarum* LL 441 (plantaricin C producer), *Listeria innocua* 86/26 and *Staphylococcus aureus* CECT 86^T. Cryopreserved cultures of cheese isolates and control strains in glycerol were recovered on M17 agar plates (lactococci), de Man, Rogosa and Sharpe (MRS) agar plates (lactobacilli), or in tryptone soy broth (TSB) (*L. innocua* and *S. aureus*), and incubated at the corresponding optimum temperature for 24 h. *Micrococcus luteus* CECT 245 (=ATCC 10240) was used as the indicator strain for measuring nisin activity. This strain was grown in nutrient broth (NB) with shaking at 37ºC for 24 h.

2.2. Identification and typing of isolates

Total genomic DNA from isolates was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. Electrophoresis was performed in 1% agarose gels, and the bands stained with ethidium bromide (0.5 μg/mL) and photographed under UV light. Isolates were grouped by repetitive extragenic palindromic (REP) fingerprinting employing the polymerase chain reaction (PCR) and the primer BoxA2-R (Table 1), as reported by Koeuth et al. (1995), followed by random amplification of polymorphic DNA (RAPD) typing with the primer M13 (Table 1), as reported by Rossetti and Giraffa (2005). Reproducibility studies of the combined REP and RAPD techniques showed a percentage similarity of over 95%.

Representative isolates of the REP and RAPD groups were identified by partial ARDRA, followed by sequencing of representative amplicons and comparison of the sequences obtained against those in databases. For ARDRA, the 16S rRNA genes were almost completely amplified using the universal primers 27-F and 1492-R (Table 1). Amplicons were purified using GenElute™ PCR Clean-Up columns (Sigma-Aldrich), digested with the restriction enzymes *Hae*III and *Hin*fI (Invitrogen Ltd., Paisley, UK), and electrophoresed as above. When required, amplicons were sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared to those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), and to those held by the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

2.3. Antimicrobial activity

Antimicrobial activity was successively examined by an agar spot test and a well-diffusion assay. For the former, overnight cultures of isolates were spotted (5 µl) on the surface of M17, MRS and TSB agar plates and incubated at 30ºC for 24 h. Spots were then covered with 10 ml of soft agar (0.75%) inoculated at 0.25% with indicator bacteria. These plates were then incubated under the conditions required by the indicator species. Positive cultures were subjected to a well-diffusion assay with neutralized, filter-sterilized supernatants, essentially as reported by Schillinger and Lücke (1989). Briefly, 20 ml of agar medium at 45°C were vigorously mixed with 200 µl of an overnight culture of the indicator strain and poured into Petri dishes. Supernatants from overnight cultures of the producing strains were neutralized to pH 6.5-7.0 with NaOH 0.1 M, centrifuged at 14,000 rpm for 5 min, and filter-sterilized through a 0.20 µm pore membrane (Millipore, Bedford, MA, USA). Aliquots of 50 µl of each supernatant were placed in wells excavated into the agar. The inhibition of indicator growth was examined after incubation for 24 h under appropriate culture conditions.

2.4. Search for bacteriocin-encoding genes by PCR

Genes coding for the most common bacteriocins produced by *L. lactis* strains were sought by specific PCR. Based on published sequences and sequences on the databases, primers were designed for genes encoding nisin, lacticin 3147, lacticin 481, lactococcin 972, lactococcin A, lactococcin B, lactococcin G, lactococcin M, and lactococcin Q (Table 1).

Amplifications were all conducted under standard conditions at an annealing temperature of 50ºC. Then, amplicons were purified and sequenced, and their sequences compared as above.

2.5. Quantification of bacteriocin production

Nisin released in MRS broth was quantified and its activity expressed in international standard units per mL (IU/mL) by comparing the activity of the supernatants with that of commercial nisin (Nisaplin®, Danisco, UK) dilutions. Cultures were centrifuged at 12,000 x g for 10 min and the supernatants adjusted to pH 2.0 with 0.02 N HCl, heated at 80ºC for 5 min, and centrifuged once again under the same conditions. Dilutions of these supernatants were made in 0.02 N HCl and 50 μl deposited in wells made in NB agar plates previously inoculated with approximately 1.0×10^{8} colony forming units (cfu)/mL of *M. luteus* CECT 245. The diameter of the inhibition halos was measured and concentrations determined against a standard curve for commercial nisin dilutions prepared in the same way.

Lactococcin 972 was quantified by a non-competitive enzyme-linked immunoassay (NCI-ELISA) with rabbit polyclonal antibodies raised against the purified bacteriocin, which were supplied by the Immunotechnology External Service of the University of Oviedo (Spain). NCI-ELISA was essentially performed as described by Sánchez et al. (2008). Briefly, flat-bottom polystyrene microtiter wells (Maxisorp; Rochester, NY, USA) were coated with culture supernatants or different concentrations of pure lactococcin 972, washed and incubated with the primary (1:1,000) and the secondary (1:40,000) antibody goat anti-rabbit IgG peroxidase conjugate (Sigma). Plates were revealed with 2,2-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS; Sigma-Aldrich) as the substrate and the absorbance at 405 nm recorded in a Benchmark Plus microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Production of nisin in dairy- and meat-based media

The production of nisin in industrial media mimicking dairy- and meat-derived products was analyzed in reconstituted skim milk (10% w/v) supplemented with 0.5% whey protein concentrate (RSM-WPC) and in meat-extract medium (8% w/v) supplemented with soy-extract 2.25% (ME-SY), respectively. In both cases, the basal medium was supplemented with NaCl (2%), potassium sorbate (0.05%), and yeast extract (0.025%), and the pH adjusted to 6.4. The release of nisin in RSM-WPC and ME-SY media was quantified as above, using as a control commercial nisin dilutions and the bacteriocin produced in MRS.

2.7. Analysis of plasmid content

Plasmid DNA from *L. lactis* was extracted and purified following the procedure of O'Sullivan and Klaenhammer (1993). Plasmid preparations were electrophoresed in 0.75% agarose gels, stained with ethidium bromide (0.5 μg/mL) and photographed.

3. Results and Discussion

3.1. Identification and typing of L. lactis isolates

Typing analysis of the 306 isolates by the combined REP and RAPD techniques gave 60 different fingerprinting patterns with lower percentage similarities than those recorded in a reproducibility study (Supplemented Material 1). Consequently, these 60 profiles were considered different strains and thus subjected to identification by partial ARDRA, sequencing and comparison of the sequences. A single ARDRA profile was obtained with either *Hae*III and *Hin*fI, indicating they all belonged to a single species. Sequencing of 21 16S rRNA amplicons representative of all strains showing a Spearman's coefficient of similarity in their REP/RAPD profiles of over 0.52% (Supplementary material 1) indicated that they all could be assigned to the *L. lactis* species. The sequences of six amplicons, corresponding to ten strains (Supplementary material 1, codes 14, 15, 16, 44, 46, 47, 49, 50, 54 and 58), were shown to match the 16S rRNA sequence of *L. lactis* subsp. *cremoris*; all others were shown to be identical to those of *L. lactis* subsp. *lactis*. Sequencing of all 10 isolates of the supposed *cremoris* subspecies and 20 more amplicons at random from the *lactis* subspecies further confirmed the identity and number of strains at the subspecies level. As reported for many other traditional cheeses (Callon et al. 2004; Delgado and Mayo 2004; Psoni et al. 2007; Nieto-Arribas et al. 2009), the genetic diversity found among the *L. lactis* isolates from the five raw-milk cheeses was rather high. However, the presence of (genetic) *L. lactis* subsp. *cremoris* strains in such cheeses has only rarely been reported (Gaya et al. 1999; Delgado and Mayo 2004; Nieto-Arribas et al. 2009).

3.2. Antimicrobial activity of L. lactis strains

The production of inhibitory compounds by representative isolates of the different strains against a group of indicator bacteria including well recognized food-borne pathogens was first analyzed by an agar spot test. A variable number of the 60 strains inhibited the different indicator organisms. *L. sakei* CECT 906^T, a strain reported to be very susceptible to bacteriocins and other antimicrobials (González et al. 1994), was inhibited by 37 strains (61.66%). In contrast, *S. aureus* CECT 86^T was inhibited by only 11 (18.33%); additionally, in most cases only faint halos were seen. *L. lactis* subsp. *cremoris* MG 1363, *L. innocua* 86/26, *L. plantarum* LL 441 and *L. lactis* subsp. *lactis* NCDO 497 were inhibited by 22, 18, 14 and 13 strains, respectively. Strains with antibacterial activity against any of the indicators were subsequently subjected to the well-diffusion assay. Under the conditions of this test (which requires neutralized, filter-sterilized supernatants), the number of positive strains was severely reduced, as only 17 strains showed clear inhibitory effects (Table 2). These results were not surprising; many authors have reported that confirmation in liquid media of the inhibition detected by the agar spot test is not always obtained (Schillinger and Lücke 1989; Larsen et al. 1993; Martínez et al. 1995; Hernández et al. 2005). Several colony-associated antimicrobial compounds, including fatty acids and H_20_2, have been considered responsible for the inhibitory effects observed in solid media (de Vuyst and Leroy 2007). Strains inhibiting the indicators used in this study were as follows: *L. sakei* CECT 906^T - 17 strains, *L. lactis* subsp. *cremoris* MG 1363 - 17 strains, *L. innocua* 86/26 - 10 strains, *L. plantarum* LL 441 - 9 strains, *S. aureus* CECT 86^T (weak inhibition) - 9 strains, and *L. lactis* subsp. *lactis* NCDO 497 - 7 strains. In the present work, the inhibitory strains were all shown to belong to *L. lactis* subsp. *lactis,* except for 2A27 which proved to be a *L. lactis* subsp. *cremoris* strain. All these 17 strains showed distinct typing profiles, as depicted in Figure 1 in which the REP patterns obtained with primer BoxA2-R are summarized.

Careful inspection of Table 2 shows that 11 strains did not inhibit the nisin producer indicator NCDO 497 (except for a small inhibition by strain 1AA17), suggesting that some strains might be nisin producers. In fact, the nisin production phenotype has been widely found among *L. lactis* strains from many ecosystems (Martínez et al. 1995; Rodríguez et al. 1995; Ayad et al. 2002; Park et al. 2003; Beasley and Saris 2004; Millette et al. 2007; Dal Bello et al., 2010). At the same time, the five strains on the right of the table produced bacteriocin-like substances that inhibited only the *L. sakei* strain and two *L. lactis* indicators (strains MG 1363 and NCDO 497). The availability of *L. lactis* subsp. *lactis* IPLA 972, the lactococcin 972 producer (Martínez et al. 1995; Martínez et al. 1999), allowed all antimicrobial producers to be assayed using this strain as an indicator. Table 2 shows that IPLA 972 was inhibited by most strains, including *L. lactis* subsp. *cremoris* 2A27, but not by these five *L. lactis* subsp. *lactis* strains. Therefore, these strains might produce lactococcin 972, a phenotype that has only been reported for strain IPLA 972 (Martínez et al. 1995).

3.3.- Targeting the bacteriocin-encoding genes by PCR

PCR analyses were undertaken using specific primers for genes of the most common lactococcal bacteriocins, i.e., nisin, lacticin 3147, lacticin 481, lactococcins A, B, G, and M, as well as specific primers for lactococcin 972. Amplicons of the expected size for lacticin 3147, lacticin 481, and lactococcins A, B, and M, were never obtained. Sequencing of eventually-produced amplicons showed non-specific amplification of *L. lactis* genes. In contrast, 11 of the 17 strains produced an amplicon of the expected size for nisin (lines 1 through 11 in Fig. 2A) as did five for lactococcin 972 (lines 13 to 17 in Fig. 2B). Amplicons were all sequenced to prove unequivocally they corresponded to their respective bacteriocin-encoding gene. A nucleotide difference was observed in the sequences of the nisin structural gene in two strains (1AA17 and 2BB9) with respect to the nisin A structural gene of the other nine strains. This nucleotide change corresponded to the sequence of the structural gene of nisin Z (Table 2) (Mulders et al., 1991).

The sequences obtained for the lactococcin 972 gene were shown to be identical to one another as well as to the sequence from *L. lactis* subsp. *lactis* IPLA 972 (Martínez et al.

1999). Positive amplification with the *L. lactis* subsp. *cremoris* 2A27 strain was only obtained when using specific primers for the genes encoding the two-peptide, related bacteriocins lactococcin G and lactococcin Q. Analysis of nucleotide and amino acid deduced sequences indicated that this strain produced a bacteriocin almost identical to lactococcin G, although small changes at the nucleotide level leading to a few amino acid changes in both α and β peptides were noted (Supplementary material 4).

The slight inhibition of *L. lactis* subsp. *lactis* NCDO 497 by 1AA17 strain is intriguing, since they both are nisin producers. The latter strain might co-produce a second, undetected bacteriocin, as has been reported recently for other *L. lactis* strains (Topisirovic et al. 2006; Bravo et al. 2009; Dal Bello et al., 2010). All five lactococcin 972 producers have recently been isolated during the microbial characterization of *Casín* cheese (Alegría et al. 2009). Since the lactococcin 972 structural gene has been found in plasmid pBL1 (11 kbp) (Martínez et al. 1999), the plasmid content of the lactococcin-producing strains was analyzed. The plasmid profiles of the different lactococcin producers varied (Supplemented material 3), and none of the bands was shared by all strains. This further strengthens the view of the typing results, and suggests these isolates are indeed different strains and that the lactococcin operon may be located in plasmids of variable size.

3.4. Bacteriocin production in laboratory and industrial media

The activity of nisin released into the culture medium by the different producers was measured by comparing the inhibition halos against a standard curve for commercial nisin (Supplementary Material 2), using *M. luteus* CECT 245 as the indicator. Nisin activity ranged from <20 to about 125 IU/mL (Table 2). Activity of the major producers was comparable to or higher than that of *L. lactis* subsp. *lactis* NCDO 497 (85 IU/mL), and those reported on the literature for wild *L. lactis* isolates (Ayad et al., 2002). Nisin activity was further assayed and quantified in industrial media simulating dairy (RSM-WPC) and meat products (MS-YS). The quantification of nisin in these two media showed a general decrease of around 10% in bacteriocin production in RSM-WPC (average 67.3 IU/mL; range 16.7-118 IU/mL). On the contrary, production of nisin in MS-YS was shown to be greatly enhanced in all strains. As compared to that in MRS, nisin activity in this latter medium showed, depending on the strain, a 2-4 fold increase (average 196 IU/mL; range 97-346 IU/mL). Nisin production shows primary metabolite kinetics and is only produced during the exponential growth phase (de Vuyst and Vandamme, 1992). Accordingly, strains 2BB9 and 3AA28 were shown to reach the highest cell density and were the best nisin producers in all media and under all conditions assayed. The production of nisin in low-cost media would facilitate the practical application of the producers for the industrial manufacture of nisin as a food preservative, but also their inclusion as starters or adjunct cultures for the preservation of dairy and meat fermented products.

Variable amounts of lactococcin 972 were also measured in the supernatant of the producing strains by an immunoassay (Table 2). Two strains, Q1-6 and T2-43, were shown to produce two-fold bacteriocin as compared to the original producer. *L. lactis* resistant strains to lactococcin 972 have never been reported, except for the immunity of producers (Martínez et al., 1995; 1999). This fact would allow the use of producing-strains as the components of adjunct cultures, which may contribute to accelerate cheese ripening by increasing lysis of starter cells, as it has been proposed for producers of other bacteriocins (Martínez-Cuesta et al., 2001; Fernández de Palencia et al., 2004). In addition to their technological value, these strains could also serve as a suitable source of lactococcin 972 for molecular studies aimed to unravel its atypical mode of action (Martínez et al., 2008).

4. Conclusions

In conclusion, 17 bacteriocin producers were identified in a collection of 60 lactococcal strains from traditional cheeses made from starter-free raw milk, indicating that this phenotype is well spread among wild dairy *L. lactis* strains. Besides the discovering of new bacteriocins, it is also important to identify strains producing higher amounts of the antimicrobials (particularly those with broad inhibitory spectrum such as nisin), which would lead to their commercial application. As the bacteriocin production trait is widely spread among *L. lactis* from artisanal, traditional cheeses made of raw milk, these products could be a good source of strains displaying enhanced outputs. The structural gene of nisin was identified by PCR in 11 strains, which produced nisin at variable concentrations. A remaining set of five strains harboured the lactococcin 972 structural gene and variable amounts of this inhibitory peptide were measured in the culture medium. Finally, specific PCR and analysis of the amplicons strongly suggested that the *L. lactis* subsp. *cremoris* 2A27 produces a two peptide, lactococcin G-like bacteriocin. Because of their broad inhibitory activity, nisin-producing strains might be of interest in the development of protective starter cultures for cheese and other fermented products. The inhibitory activity of lactococcin 972 and lactococcin G against lactococci alone renders them of interest in the design of adjunct cultures aimed at improving and accelerating cheese ripening. Autochthonous starters and adjunct cultures composed by bacteriocin-producing strains may further help to reinforce tipycity and originality of traditional cheeses.

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Figure

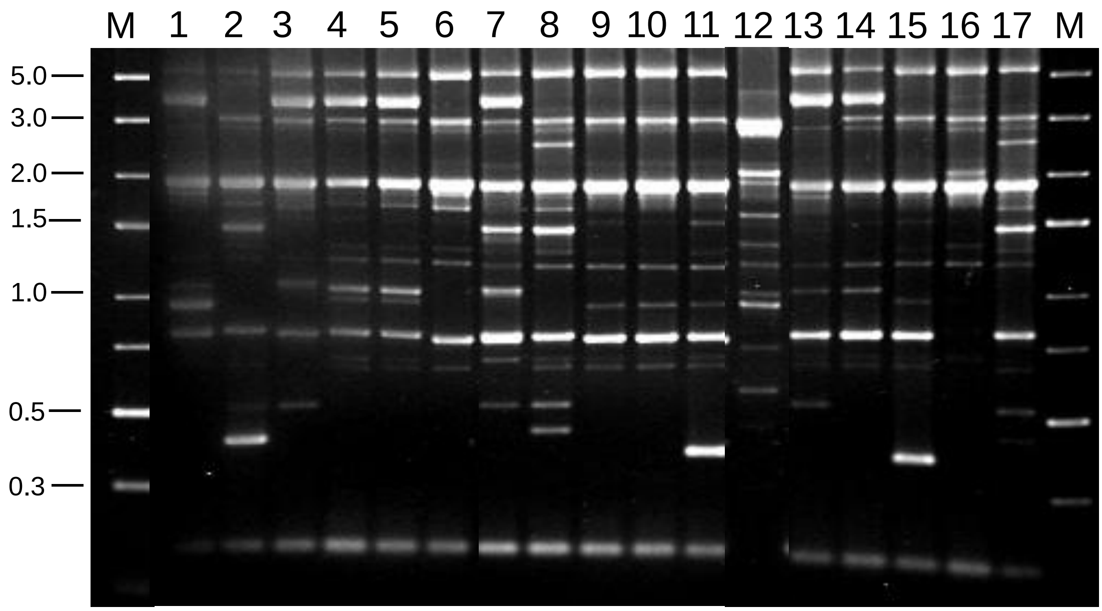


Figure 1

Figure

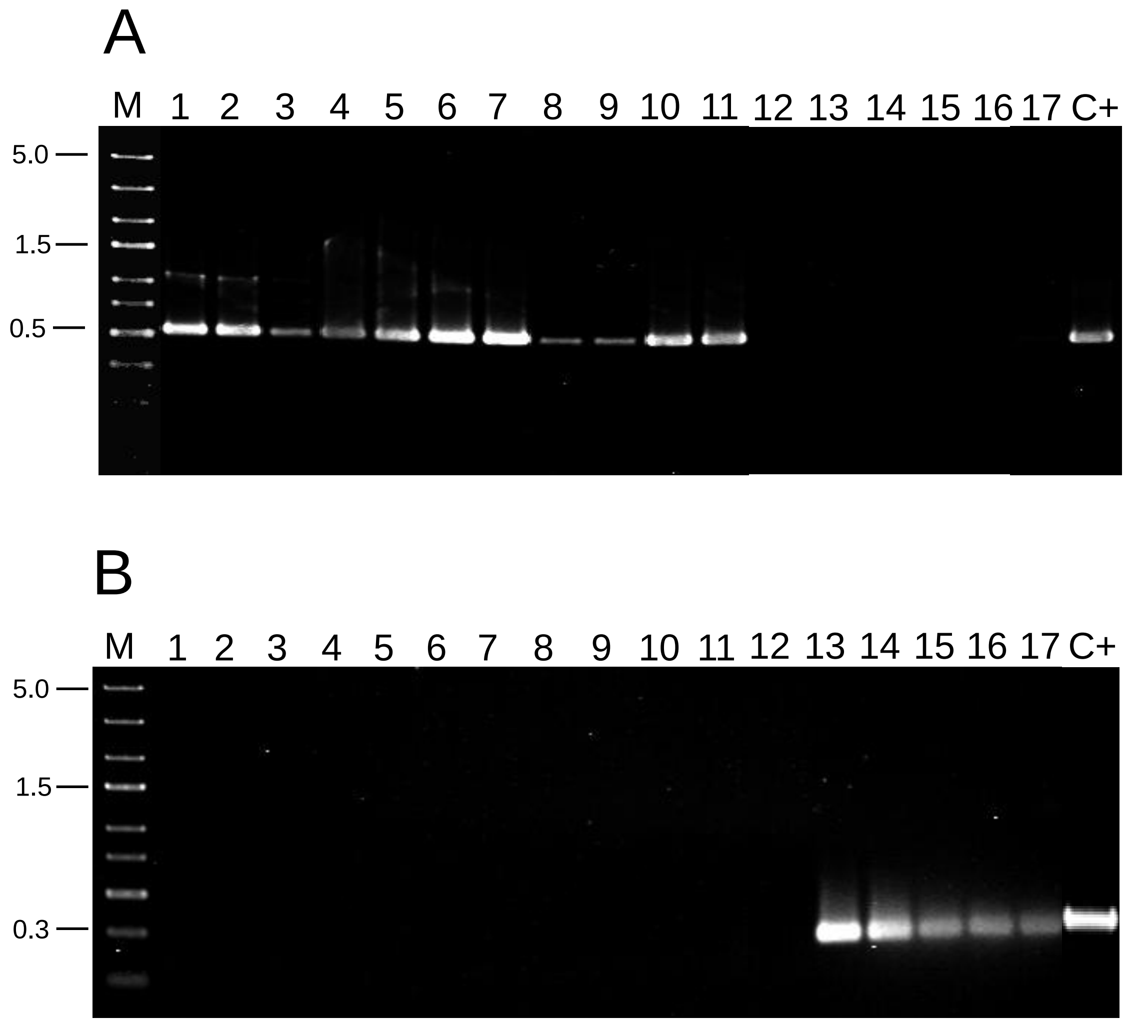
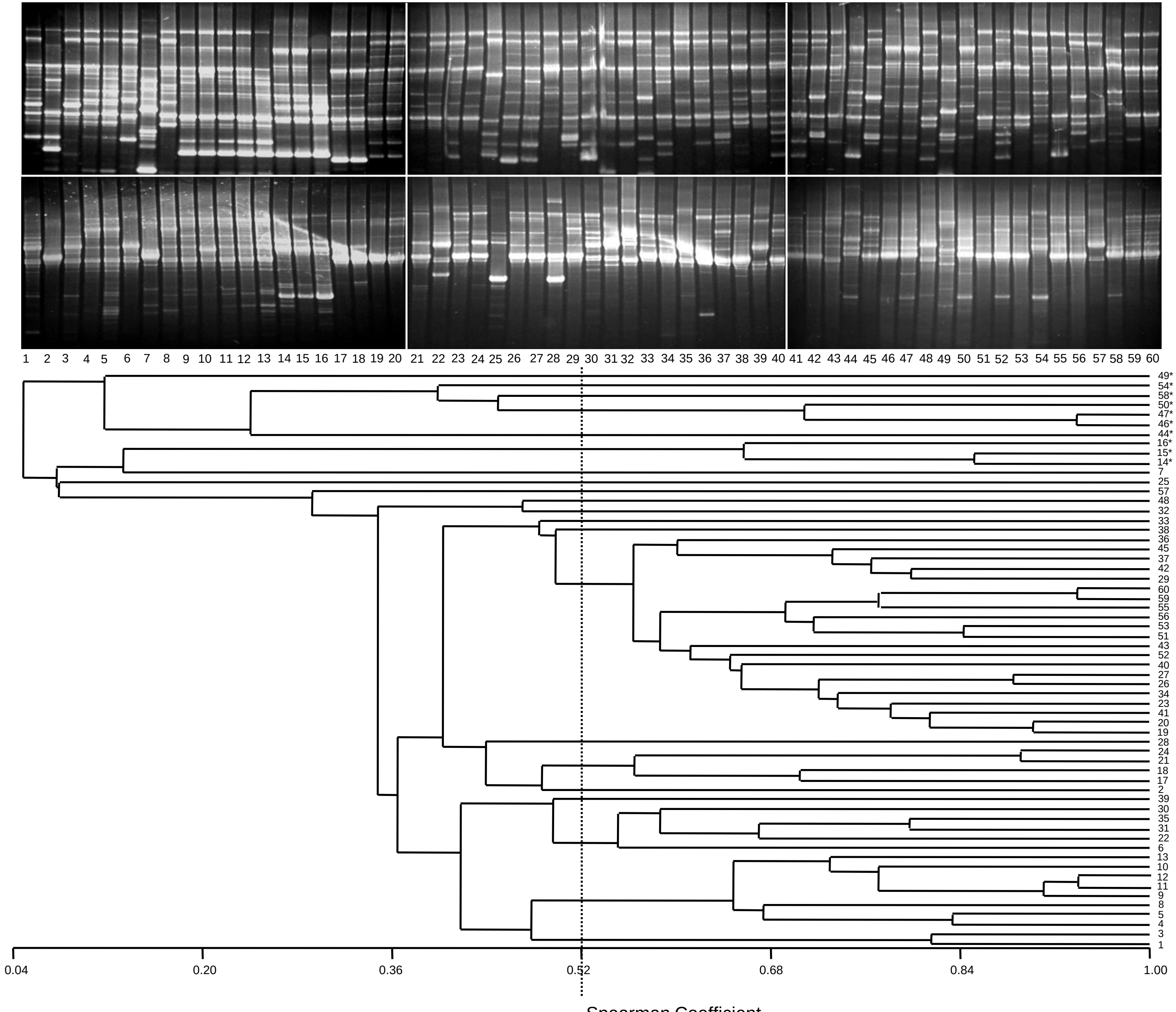


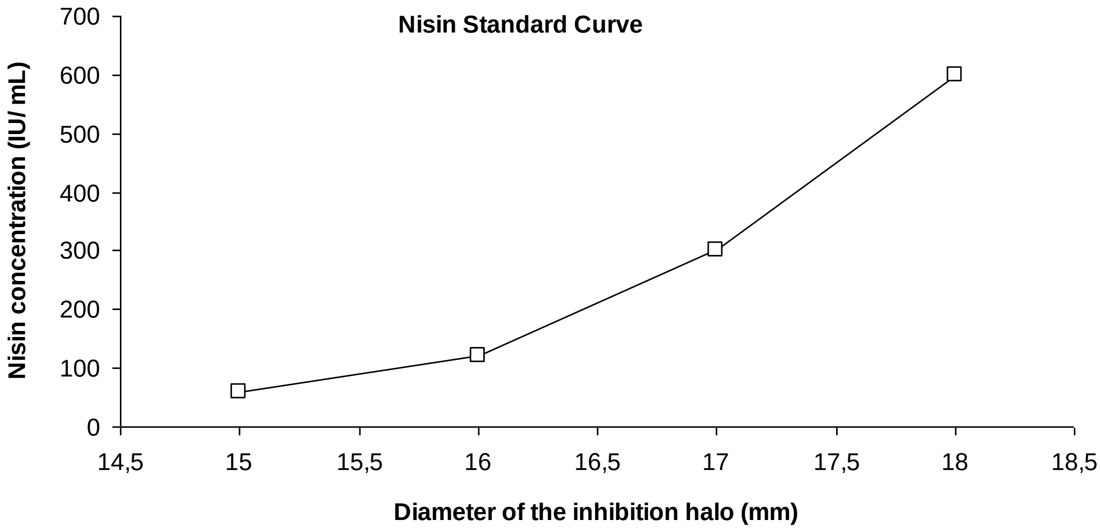
Figure 2

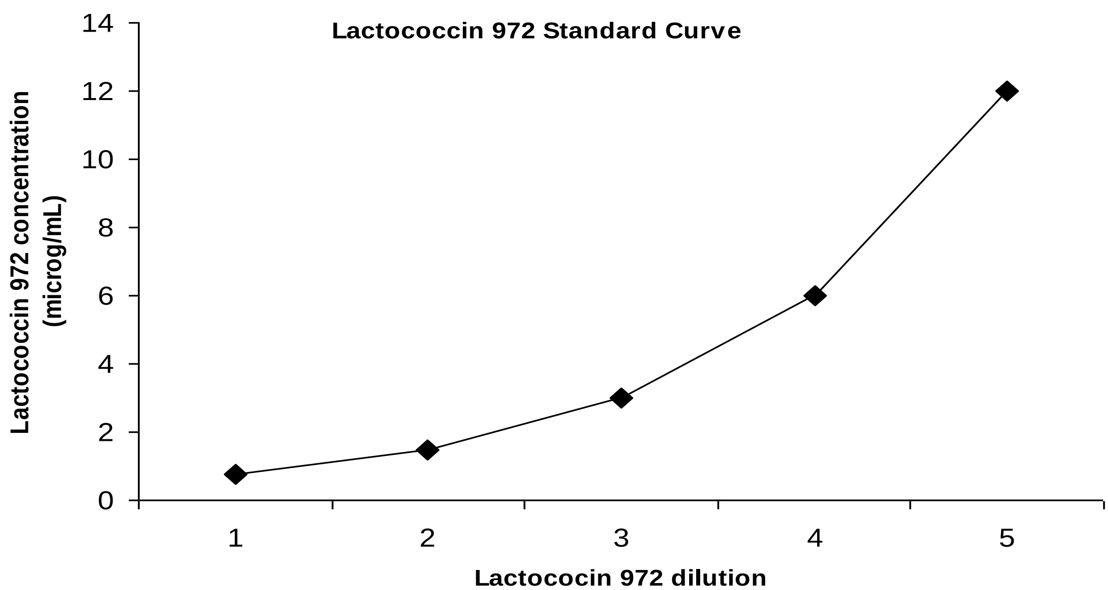
Figure



Supplementary material 1

Figure





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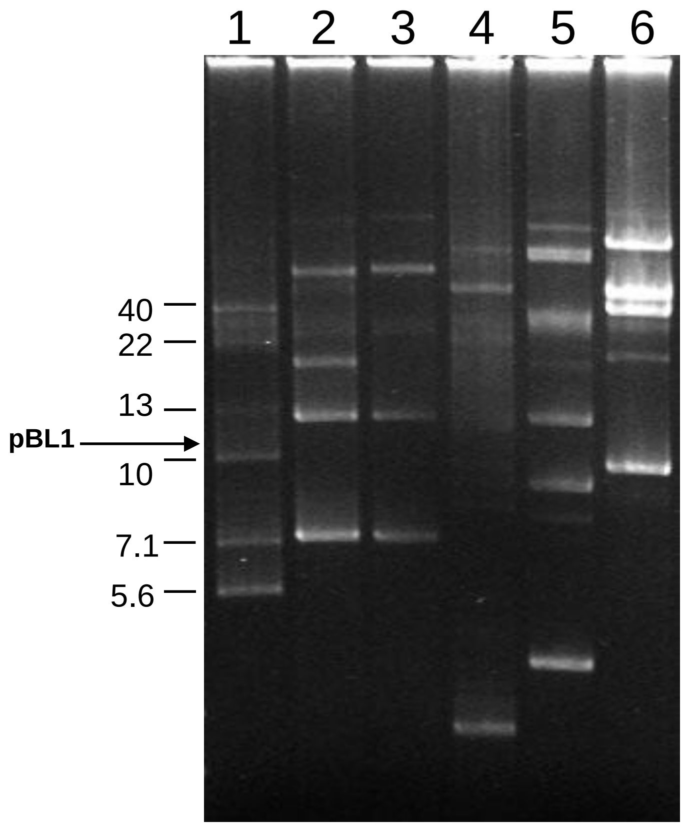

Supplementary material 3

Figure

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Lactococcin Gα:     MKELSEKELRECVGG SIWGDIGQGVGKAAYWVGKAMGNMSDVNQASRINRKKKH
Lactococcin 2A27α:  ??????KELRECVGG GAWGDIGQGVGKAAYWVGKAMGNMSDVNQASRINRKKKH
Lactococcin Qα:     MKELSEKELRECVGG GTWDDIGQGIGRVAYWVGKAMGNMSDVNQASRINRKKKH

Lactococcin Gβ:     MKNNNNNNFFKDMEIIEDQELVSITGG KHKKWGWLAWVEPAGEFLKGFGKGAIKEGNKDKWKNI
Lactococcin 2A27β:  MKNNNNNNFFKDMEIIEDQELVSITGG --KKWGWLAWVEPAAAFLKGFGKGAIKEGNKDKW???
Lactococcin Qβ:     MK-NNNNNFFKGMEIIEDQELVSITGG KHKKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI
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Supplementary material 4

FIGURE LEGENDS

Figure 1.- REP-PCR Typing of the seventeen *L. lactis* subsp. *lactis* strains bacteriocin producers with the primer BoxA2R. Order, lines 1 through 11 nisin producer strains 1A6, 1A8, A16, 1A38, 1AA16, 1AA17, 1AA48, 2BB9, 3AA28, L30, and P83A; line 12, *L. lactis* subsp. *cremoris* 2A27; lines 13 through 17 lactococcin 972 producers Q1-2, Q1-6, Q1-8, T2-26, and T2-43. M, Molecular weight marker (Gene Ruler Express^{TM} DNA ladder, Fermentas Gmbh., Germany); molecular weight (kbp) of key bands is indicated.

Figure 2.- Specific PCR amplification of the nisin structural gene (**Panel A**) and that of lactococcin 972 (**Panel B**) using total DNA of the wild *L. lactis* subsp. *lactis* strains producing inhibitory substances as a template. Order, lines 1 through 11 nisin producer strains 1A6, 1A8, A16, 1A38, 1AA16, 1AA17, 1AA48, 2BB9, 3AA28, L30, and P83A; line 12, *L. lactis* subsp. *cremoris* 2A27; lines 13 through 17 lactococcin 972 producers Q1-2, Q1-6, Q1-8, T2-26, and T2-43; line C+, positive reaction using as a template total DNA from *L. lactis* subsp. *lactis* NCDO 497 and *L. lactis* subsp. *lactis* IPLA 972, respectively; line M, Molecular weight marker, indicating molecular weight of key bands in kbp.

Supplementary Material 1.- Different profiles found by combined typing by REP-PCR with primer BoxA2R and RAPD with primer M13 of the 306 wild *L. lactis* isolates. Below, dendogram of similarity of the 60 different typing patterns clustered by the UPGMA method using the Spearman coefficient. Representative strains showing a Spearman coefficient of similarity in their REP/RAPD profiles of over 0.52% (broken line) were identified by 16S rRNA amplification, sequencing and comparison of the sequences against those in GenBank and the Ribosomal Database Project (see the text). *Lactococcus lactis* subsp. *cremoris* strains are denoted by an asterisk.

Supplementary Material 2.- Standard curve of nisin concentration (in IU/mL) by a well diffusion assay using different dilutions of commercial nisin (Nisaplin®, Danisco, UK) and *M. luteus* CECT 245 as the susceptible indicator.

Supplementary material 3.- Agarose gel electrophoresis of plasmid DNA preparations from the *L. lactis* subsp. *lactis* strains producing lactococcin 972. Order: line 1, IPLA 972; line 2, Q1-2; line 3, Q1-6; line 4, Q1-8; line 5, T2-26, and line 6, T2-43. The arrow points out to the position of the bacteriocinogenic plasmid pBL1.

Supplementary material 4.- Alignment of deduced amino acid sequence from the lactococcin 2A27-encoding gene with the lactococcin G and lactococcin Q sequences. Amino acids differing in their respective sequences are colour coded. Arrows point out to the signal peptidase processing sites, whose cleavage gives rise to the mature, active bacteriocins. Dashes indicate not amino acid at a particular position, while question mark symbols denote non-determined amino acids.

Table 1.- Primers used throughout this study.

Name	Sequence (5'→ 3')	Technique/Amplification	Reference/GenBank Accession nº
BoxA2-R	ACGTGGTTTGAAGAGATTTTCG	REP-PCR typing	Koeuth et al. 1995
M13	GAGGGTGGCGGTTCT	RADP typing	Rossetti and Giraffa 2005
27-F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene	S-D-Bact-0008-a-S-20
1492-R	GGTTACCTTGTTACGACTT	16S rRNA gene	S-*-Univ-1492R-b-A-21
Nis-F	CGGCTCTGATTAAATTCTGAAG	Nisin genes	M65089
Nis-R	GGATTAGCTAGTAGTAACTGTTC	Nisin genes	M65089
Lact3147-F	GTCTTTGTGTTGTTTGGAGATG	Lacticin 3147 gene	AE001272
Lact3147-R	CAACTCCCGAAATAAATCATCG	Lacticin 3147 gene	AE001272
Lact481-F	CCAATGTCATTGCATCTGCAC	Lacticin 481 gene	X71410
Lact481-R	GTCCTTATGTTGCTATTCATC	Lacticin 481 gene	X71410
Lcn972-F	TTGTAGCTCCTGCAGAAGGAACATGG	Lactococcin 972 gene	Martínez et al. 1999
Lcn972-R	GCCTTAGCTTTGAATTCTTACCAAAAG	Lactococcin 972 gene	Martínez et al. 1999
LactABM-F	GAAGAGGCAATCAGTAGAG	Lactococcin A, B, and M genes	M90969, S38128, van Belkum et al. 1991
LactA-R	GTGTTCTATTTATAGCTAATG	Lactococcin A gene	M90969
LactB-R	CCAGGATTTTCTTTGATTTACTTC	Lactococcin B gene	S38128
LactM-R	GTGTACTGGTCTAGCATAAG	Lactococcin M gene	van Belkum et al. 1991
LactGQ-F	GAAAGAATTATCAGAAAAAG	Lactococcin G and Q genes	FJ938036, AB182406
LactGQ-R	CCACTTATCTTTATTTCCCTCT	Lactococcin G and Q genes	FJ938036, AB182406

Table 2.- Antimicrobial activity of *L. lactis* strains from traditional cheeses against of a series of indicator strains assayed with neutralized supernatants by a well-diffusion assay. Also included, representative genotype as determined by specific PCR and bacteriocin activity or bacteriocin production.

Indicator strain/ genes/bacteriocin production	***L. lactis*[a] strain**																
	1A6	1A8	1A16	1A38	1AA16	1AA17	1AA48	2BB9	3AA28	L30	P83A	2A27	Q1-2	Q1-6	Q1-8	T2-26	T2-43
L. lactis subsp. *cremoris* MG 1363	++	++	++	++	++[b]	++	+	++	++	+	++	+	++	++	++	++	++
L. lactis subsp. *lactis* NCDO 497	-	-	-	-	-	(+)	-	-	-	-	-	++	++	++	++	++	++
L. lactis subsp. *lactis* IPLA 972	++	++	++	++	++	++	-	++	++	+	++	++	-	-	-	-	-
Lactobacillus plantarum LL 441	++	++	++	++	++	++	-	++	++	-	+	-	-	-	-	-	-
Lactobacillus sakei CECT 906[T]	++	+++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	++	++	+	++	++
Listeria innocua 86/26	++	++	+	++	++	++	-	++	++	+	++	-	-	-	-	-	-
Staphylococcus aureus CECT 86[T]	+	(+)	+	(+)	(+)	+	-	+	+	-	+	-	-	-	-	-	-
Presence of *nisA*	+	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-
Presence of *nisZ*	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Presence of *lcn972*	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Presence of *lcnG*	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Bacteriocin production	45[c]	88[c]	75[c]	85[c]	50[c]	60[c]	<20[c]	125[c]	96[c]	70[c]	64[c]	Nd[d]	12.4[e]	5.6[e]	5.6[e]	8.1[e]	11.8[e]

[a]Genetically, all strains are *L. lactis* subsp. *lactis* except that of 2A27 which is a *L. lactis* subsp. *cremoris* strain.
[b]The number of crosses in the test is related to the diameter of the inhibition halo; in parenthesis, weak inhibition.
[c]Nisin activity is expressed as IU per mL of culture medium (MRS). Under the same experimental conditions, nisin production by *L. lactis* subsp. *lactis* NCDO 497 was shown to be 85 IU/mL.
[d]Nd, not determined.
[e]Production of lactococcin 972 was measured as µg of protein per ml of culture medium (M17). The original producer, *L. lactis* subsp. *lactis* IPLA 972, produces 4.9 µg/mL.