Antimicrobial properties of lactic acid bacteria isolated from Uruguayan artisan cheese

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Abstract

Uruguayan artisan cheese is elaborated with raw milk and non-commercial starters. The associated native microbiota may include lactic acid bacteria and also potentially pathogenic bacteria. Lactic acid bacteria were isolated from artisan cheese, raw milk, and non-commercial starter cultures, and their potential bacteriocin production was assessed. A culture collection of 509 isolates was obtained, and five isolates were bacteriocin-producers and were identified as *Enterococcus durans, Lactobacillus casei*, and *Lactococcus lactis.* No evidence of potential virulence factors were found in *E. durans* strains. These are promising results in terms of using these native strains for cheese manufacture and to obtain safe products.

Keywords: lactic acid bacteria; bacteriocins; artisan cheese.

1 Introduction

The production of artisan cheese is traditional in Uruguay and represents an important source of income for farmers. This kind of cheese is produced using raw, pasteurized, or thermised milk exclusively produced on farms (BAGNATO, 2004). In this type of production, the so called "whey ferment" (serum of the batch form previous cheese vat) is used instead of commercial starter cultures. Therefore, lactic acid bacteria (LAB) that mediate these processes derive from this serum and are complex and variable in composition (COGAN et al., 1997; VELJOVIC et al., 2007).

Artisan cheese production sometimes lacks strict food-safety control and regulations, which are very important for their improvement and to reach acceptable quality (O'BRIEN et al., 2009). To improve these standards, native LAB could be used to inhibit pathogenic or spoilage microorganisms. LAB have been extensively studied as preservation agents since they can exert antimicrobial activity against pathogens and survive for long periods without altering the quality or organoleptic properties of the product (ALI, 2010; DEVLIEGHERE; VERMEIREN; DEBEVERE, 2004; GARCÍA et al., 2010; LEROY; DE VUYST, 2004; ZACHAROF; LOVITT, 2012). Antibacterial compounds produced by these bacteria, such as bacteriocins (ZACHAROF; LOVITT, 2012), can inhibit pathogens like *Listeria monocytogenes*, *Staphylococcus aureus,* or *Escherichia coli*, and they also play an important role in food preservation and are considered an extremely effective tool for food safety applications (DEEGAN et al., 2006; GALVEZ et al., 2007).

The aim of this study was to perform a collection of LAB isolated from artisan cheese, raw milk, and non-commercial starter cultures from a group of farms located in Nueva Helvecia, Colonia, Uruguay, to evaluate their potential bacteriocin production and to select those with promissory potential for cheese elaboration.

2 Materials and methods

2.1 Samples

Raw milk (M), cheese (C), and non-commercial starter cultures (S) samples were obtained from dairy farms located in Nueva Helvecia, Colonia, Uruguay (Lat 34° 17' 60 S; Long 57° 13' 60 W, Altitude 41 m). Farmers produce artisan cheese with their own milk without any heat treatment using non-commercial starters. Starter cultures are kept and maintained by daily addition of cheese whey.

Raw milk and starter culture samples (n = 87 and n = 89 respectively) were collected between October 2007 and July 2008, while cheese samples (n = 83) were obtained between November 2007 and August 2008. All samples were kept at 4 °C until analysis.

2.2 Sample processing and LAB isolation

One milliliter of samples S or M was diluted in 9 ml of sterile phosphate buffered saline (PBS) and serially diluted in PBS (10^{-1}-10^{-8}), while 1 g of C samples was processed with 10 ml of sterile PBS in a Stomacher 80 Lab Blender (Seward, UK). Subsequently, serial dilutions were performed in PBS (10^{-1}-10^{-8}). Plate counts were performed on de Man Rogosa Sharpe agar (MRS, Oxoid) in order to obtain isolated LAB colonies. Incubations were performed in a candle jar at 28-30 °C for 24 to 48 hours until growth was evident. Three to five colonies were randomly selected and grown onto MRS plates for isolation and characterization. LAB isolates were kept in MRS-glycerol (15%, w/v) at –80 °C.

2.3 Well diffusion agar assay

Production of bacterial-growth inhibitory substances was assessed by agar well diffusion assay (FRAGA et al., 2008).

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Fifteen ml of culture medium, melted and tempered at 45 °C, were inoculated with a strain of *Escherichia coli* or *Streptococcus bovis,* which were used as Gram negative and Gram positive indicators, respectively, at 10^6 CFU (Colony forming units)/ml and poured into Petri dishes that had 10 mm-diameter metalic cilinders to form wells (FRAGA et al., 2008). The wells were filled with 100 µl of 48 h-culture-supernatants of the tested isolates obtained after centrifugation (10,000 × g × 20 minutes, Beckman Microfuge™ 11, USA), The Plates were incubated aerobically at 37 °C for 24 hours. Nutrient agar was used for *E. coli* and MRS for *S. bovis* cultures.

Isolates that inhibited the growth of at least one indicator strain were subsequently tested in order to study the nature of the inhibitory substances. The methodology described above was used, but the supernatants were neutralized with NaOH 10 M. Isolates that inhibited the growth of the indicator strains after neutralization were subsequently tested in order to assess the proteinaceus nature of the inhibitory substances.

In this case, neutralized supernatants were treated with proteolytic enzymes (pepsin, proteinase K, α quimiotrypsin or pronase E; Sigma, St. Louis, Mo, USA) at 1 mg/ml, 37 °C for 1 hour or boiled for 15 minutes (PERELMUTER; FRAGA; ZUNINO, 2008) in all cases. All assays were performed in duplicate.

Bacterial identification

In order to identify the isolates that showed promising results as bacteriocin producers, a molecular approach was adopted performing PCR followed by sequencing of the almost complete 16S rRNA gene amplified with primers 27F 5’-AGAGTTTGATYMTGGCTCAG-3’ and 1492R 5’-TACCTTGTTACGACTT-3’. Genomic DNA was extracted (Gene elute bacterial genomic DNA extraction kit, Sigma, USA). PCR reactions were performed (T1 thermocycler, Biometra, Germany) as follows: an initial cycle of 94 °C for 3 minutes; 30 cycles of 94 °C for 1 minute, 50 °C for 1 minute, 72 °C for 1.5 minute; and final extension at 72 °C for 10 minutes. The sequences were obtained from the sequencing service of Macrogen Inc. (South Korea) and compared to the NCBI database (ALTSHUL et al., 1997).

2.4 Analysis of Enterococcus *spp. virulence factors*

Enterococcus spp. could have pathogenic attributes (FOULQUIÉ-MORENO et al., 2006); thus, isolates 11Q9A and 11Q9D identified as *Enterococcus durans* were further studied to evaluate the absence of a series of virulence factors.

Haemolytic activity. Fresh cultures were seeded onto brain heart infusion agar (Himedia, India) supplemented with 5% ovine blood, and the plates were incubated for 48 hours at 37 °C. *Staphylococcus aureus* ATCC 6538 and *Listeria innocua* ATCC 33091 were used as positive and negative controls, respectively (RIVAS et al., 2012).

Gelatinase activity. Isolates were cultured in tryptic soy agar supplemented (Himedia, India) with 0.6% (w/v) yeast extract and 1.6% (w/v) gelatine (Himedia, India). Incubation was performed for 24 hours at 37 °C, and the plates were developed with trichloroacetic acid at 35% (MEDINA; BARESI, 2007). *S. aureus* ATCC 6538 and *L. monocytogenes* ATCC 19111 were used as positive and negative controls, respectively.

Vancomycin resistance. To determine potential vancomycin resistance of the selected strains, a diffusion test onto Mueller Hinton agar was performed. The procedures were performed according to Bauer et al. (1966) and vancomycin disks were used (30µg, BBL® Sensi-disc®, Becton Dickinson and Co., Sparks, MD). *Enterococcus faecalis* ATCC 29212 was used as positive control.

Genes related to virulence of E. durans.

In order to evaluate the presence of genes potentially related to *Enterococcus* spp. virulence, a PCR approach was performed. Specific primers for the amplification of genes that code for the sex pheromone (*cpd*), cytolisin transporter (*cylB),* aggregation protein (*agg*) and gelatinase (*gelE*) were used, as proposed by Eaton and Gasson (2001). *E. faecalis* ATCC 29212 was used as a positive control.

3 Results and discussion

3.1 LAB strains characterization and identification

Raw milk counts ranged from 1×10^2 to 1×10^8 CFU/ml; counts of non-commercial starter samples ranged from 1×10^4 to 3×10^9 CFU/ml, while cheese LAB counts ranged from 1×10^4 to 2.4×10^8 CFU/g. LAB counts values were similar to those reported in Brazilian raw milk and soft cheeses (ORTOLANI et al., 2010) and lower than those reported in fresh soft cheese samples (3.3 log CFU/g to 10.5 log CFU/g) from three different regions in Croatia (GOLI’C et al., 2013).

A total of 509 LAB strains were isolated during the study. Three hundred and three of these strains showed antagonistic effect against *E. coli,* while 191 showed growth inhibition against *S. bovis;* none had significant activity against *E. coli* after neutralization, except for five of them when tested with *S. bovis* (Table 1). These five isolates were further identified by sequencing the almost complete 16S rRNA gene. When the sequences were compared with the *GeneBank* (NCBI) database, sequence similarity values of 99% or more were obtained. Based on this values, the isolates were classified as *Lactococcus lactis* (two isolates from milk), *Enterococcus durans* (two isolates from cheese samples), and *Lactobacillus casei* (isolated from cheese; Table 1) Sequences were deposited under accession numbers GU967435 to GU967439.

Some enterococci are associated with food borne illnesses, and among their virulence factors are adherence to host tissues, toxin secretion, modulation of immune response, synthesis of hydrolytic enzymes, and invasion and abscess formation (ALTSHUL et al., 1997; FOULQUIÉ-MORENO et al., 2006; GIRAFFA; CARMINATI; NEVIANI, 1997; CARIOLATO; ANDRIGHETTO; LOMBARDI, 2008).

Recovered *E. durans* were not resistant to vancomycin, and their haemolytic and gelatinase activities and the presence of genes involved in virulence showed negative results. These

Table 1. Strains identity and characterization of antimicrobial activity against *S. bovis*.

Isolate	Identity	Neutralization	Pepsin	α-quimotripsyn	Proteinasa K	Pronase E	Boiling
8L1A	*L. lactis*	+[a)]	+	–	–	–	+
8L1B	*L. lactis*	+	+	–	–	–	+
9Q11C	*L. casei*	+	-	+	–	+	–
11Q9A	*E. durans*	+	+	–	–	+	–
11Q9D	*E. durans*	+	+	+	–	+	–

[a)] + / - Presence or absence of supernatants antimicrobial effects after the different treatments.

results suggest that *E. durans* strains analyzed in this study may be considered safe to be used for food biopreservation.

In artisan dairy products, enterococci have been used as starter or co-starter cultures and are appropriate due to their bacteriocin production that has activity against foodborne-pathogens (KHAN; FLINT; YU, 2010; FRANZ et al., 2011). The use of enterocin producing strains as starter cultures or co-cultures is a good strategy for reaping food preservation benefits of in-situ bacteriocin production (KHAN; FLINT; YU, 2010).

This is an important and safety feature to be considered in the design of products intended to be used in food processing. There is an increased interest in enterococci by the food industry, particularly for cheese manufacture due to the resistance to high salts and acidic conditions (ALTSHUL et al., 1997; FOULQUIÉ-MORENO et al., 2006; KHAN; FLINT; YU, 2010; FRANZ et al., 2011; ANDRIGHETTO et al., 2001).

3.2 Antimicrobial activity characterization

The antimicrobial activity of the strains appeared unrelated to acidity since their activity was not lost after adjustment of pH to 7. The antagonistic effect may have been related to proteinaceous substances since activity was lost after the treatment with proteases. However, inactivation was heterogeneous and some treatments did not affect antimicrobial activity. The activity of *L. lactis* 8L1A and 8L1B cultures supernatants remained unaltered after the pepsin and heat treatment; heat stability is a characteristic of small peptides, such as nisin, produced by *L. lactis* (YILDRIM; JONHNSON, 2008).

Bacteriocins produced by enterococci are often called enterocins, and many of them are reported in literature (ALTSHUL et al., 1997; FRANZ et al., 2007; ÖZDEMIR et al., 2011). Antimicrobial substances produced by *E. durans* isolates (11Q9A and 11Q9D) were resistant to pronase E and pepsin treatments, while only 11Q9D kept its activity after α-quimotrypsin treatment. Enterococci may harbour several genes that potentially codify for bacteriocins, and not every strain had a visible effect, as recently demonstrated (ÖZDEMIR et al., 2011).

L. casei isolate 9Q11C produced bacteriocins or bacteriocins-like substances resistant to α- quimotrypsin and pronase E treatments. Lactobacilli, and in particular *L. casei,* are known to produce bacteriocins (MOJGANI; AMIMIA, 2007) (Table 1).

A set of five strains out of 509 (*L. lactis* 8L1A and 8L1B isolated from milk, *E. durans* 11Q9A, 11Q9D and *L casei* 9Q11C isolated from cheese) exhibited a promising activity pattern against bacterial pathogens. This analysis indicated that selected LAB strains could be used as starters or for cheese preservation.

LAB antimicrobial products such as bacteriocins are of great importance in bioconservation of various foods. The use of LAB bacteriocins, individually or as biopreservative combinations, may have major applications in improving food safety, especially of traditional products (MOJGANI; AMIMIA, 2007; JAMUNA; JEEVARATNAM, 2004). Autochthonous starters and adjunt cultures composed by bacteriocin-producing strains may further help to reinforce typicity and originality of traditional cheeses (ALEGRÍA et al., 2010).

In addition, some of them were isolated from cheese, suggesting that they can remain viable after the elaboration process. However, expression of the bacteriocins at the different stages of cheese production must be elucidated.

4 Conclusions

In the present study, 509 LAB isolates were evaluated, and among them, five potentially bacteriocin producers were identified. Two *Lactococcus lactis* were isolated from raw milk and one *Lactobacillus casei* and two *Enterococcus durans* strains were isolated from cheese. No virulence attributes were detected when both *E. durans* strains were characterized. The selected strains showed the production of antimicrobial activity mediated by proteinaceous diffusible substances. These results support the use of native LAB strains in starter cultures or as preservative agents for artisan cheese elaboration.

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References

ALEGRÍA, A. et al. Bacteriocins produced by wild Lactococcus lactis strains isolated from traditional, starter-free cheeses made of raw milk. **International Journal of Food Microbiology**, v. 143, n. 1-2, p. 61-66, 2010.

ALI, A. A. Beneficial role of lactic acid bacteria in food preservation and human health: a review. **Research Journal of Microbiology**, v. 5, n. 12, p. 1213-1221, 2010. http://dx.doi.org/10.3923/jm.2010.1213.1221

ALTSHUL, S. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Research**, v. 25, n. 17, p. 3389-3402, 1997. http://dx.doi.org/10.1093/nar/25.17.3389

ANDRIGHETTO, C. et al. Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. **The Journal of Dairy Research**, v. 68, n. 2, p. 303-316, 2001. PMid:11504393. http://dx.doi.org/10.1017/S0022029901004800

BAGNATO, D. Quesería artesanal: situación actual y desafíos para Uruguay. **Instituto Interamericano de Cooperación para la Agricultura.** 2004. Disponível em: <http://repiica.iica.int/docs/B0899E/B0899E.pdf>. Acesso em 4 jun. 2013.

BAUER, A. et al. Antibiotic susceptibility testing by a standardized single disk method. **American Journal of Clinical Pathology**, v. 45, n. 4, p. 493-496, 1966. PMid:5325707.

CARIOLATO, D.; ANDRIGHETTO, C.; LOMBARDI, A. Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. **Food Control**, v. 19, n. 19, p. 886-892, 2008. http://dx.doi.org/10.1016/j.foodcont.2007.08.019

COGAN, T. M. et al. Characterization of the lactic acid bacteria in artisanal dairy products. **Journal of Dairy Research**, v. 64, n. 3, p. 409-421, 1997. http://dx.doi.org/10.1017/S0022029997002185

DEEGAN, L. H. et al. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. **International Dairy Journal**, v. 16, n. 9, p. 1058-1071, 2006. http://dx.doi.org/10.1016/j.idairyj.2005.10.026

DEVLIEGHERE, F.; VERMEIREN, L.; DEBEVERE, J. New preservation technologies: Possibilities and limitations. **International Dairy Journal**, v. 14, n. 4, p. 273-285, 2004. http://dx.doi.org/10.1016/j.idairyj.2003.07.002

EATON, T. J.; GASSON, M. J. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. **Applied and Environmental Microbiology**, v. 67, p. 1628-1635, 2001. PMid:11282615. PMCid:PMC92779. http://dx.doi.org/10.1128/AEM.67.4.1628-1635.2001

FOULQUIÉ-MORENO, M. R. et al. The role and application of enterococci in food and health. **International Journal of Food Microbiology**, v. 106, p. 1-24, 2006. PMid:16216368. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.06.026

FRAGA, M. et al. Vaginal lactic acid bacteria in the mare: evaluation of the probiotic potential of native *Lactobacillus* spp. and *Enterococcus* spp. strains. **Antonie van Leeuwenhoek**, v. 93, p. 71-78, 2008. PMid:17588124. http://dx.doi.org/10.1007/s10482-007-9180-4

FRANZ, C. M. A. P. et al. Diversity of enterococal bacteriocins and their grouping in a new classification scheme. **FEMS Microbioly Reviews**, v. 31, n. 3, p. 293-310, 2007. PMid:17298586. http://dx.doi.org/10.1111/j.1574-6976.2007.00064.x

FRANZ, C. M. A. P. et al. Review Enterococci as probiotics and their implications in food safety. **International Journal of Food Microbiology**, v. 151, n. 2, p. 125-140, 2011. PMid:21962867. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.08.014

GALVEZ, A. et al. Bacteriocin-based strategies for food biopreservation. **International Journal of Food Microbiology**, v. 120, p. 51-70, 2007. PMid:17614151. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.06.001

GARCÍA, P. et al. Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. **Trends in Food Science & Technology**, v. 21, n. 8, p. 373-382, 2010. http://dx.doi.org/10.1016/j.tifs.2010.04.010

GIRAFFA, G.; CARMINATI, D.; NEVIANI, E. Enterococci isolated from dairy products: a review of risks and potential technological use. **Journal of Food Protection**, v. 60, n. 6, p. 732-738, 1997.

GOLI'C, N. et al. Evaluation of lactic acid bacteria and yeast diversity in traditional white pickled and fresh soft cheeses from the mountain regions of Serbia and lowland regions of Croatia. **International Journal of Food Microbiology**, 2013.

JAMUNA, M.; JEEVARATNAM, K. Isolation and partial characterization of bacteriocins from *Pediococcus* species. **Applied Microbiology and Biotechnology**, v. 65, n. 4, p. 433-439, 2004. PMid:15205931. http://dx.doi.org/10.1007/s00253-004-1576-8

KHAN, H.; FLINT, S.; YU, P-L. Review Enterocins in food preservation. **International Journal of Food Microbiology**, v. 141, n. 1-2, p. 1-10, 2010. PMid:20399522. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.03.005

LEROY, F.; DE VUYST, L. Lactic acid bacteria as functional starter culture for the food fermentation industry. **Trends in Food Science & Technology**, v. 15, n. 2, p. 67-78, 2004. http://dx.doi.org/10.1016/j.tifs.2003.09.004

MEDINA, P.; BARESI, L. Rapid identification of gelatin and casein hydrolysis using TCA. **Journal of Microbiological Methods**, v. 69, p. 391-393, 2007. PMid:17289191. http://dx.doi.org/10.1016/j.mimet.2007.01.005

MOJGANI, N.; AMIMIA, C. Kinetics of growth and bacteriocin production in *L. casei* RN 78 isolated from dairy sample in IR Iran. **International Journal of Dairy Science**, v. 2, n. 1, p. 1-12, 2007. http://dx.doi.org/10.3923/ijds.2007.1.12

O'BRIEN, M. et al. Occurrence of foodborne pathogens in Irish farmhouse cheese. **Food Microbiology**, v. 26, n. 8, p. 910-914, 2009. PMid:19835780. http://dx.doi.org/10.1016/j.fm.2009.06.009

ORTOLANI, M. B. et al. Microbiological quality and safety of raw milk and soft cheese and detection of autochthonous lactic acid bacteria with antagonistic activity against *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*. **Foodborne pathogens and disease**, v. 7, n. 2, p. 175-180, 2010. PMid:19839761. http://dx.doi.org/10.1089/fpd.2009.0390

ÖZDEMIR, G. B. et al. Phenotypic and genotipic characterization of bacteriocins in enterococcal isolates of different sources. **Indian Journal of Microbiology**, v. 51, n. 2, p. 182-187, 2011. PMid:22654162 PMCid:PMC3209898. http://dx.doi.org/10.1007/s12088-011-0143-0

PERELMUTER, K.; FRAGA, M.; ZUNINO, P. In vitro activity of potential probiotic *Lactobacillus murinus* isolated from the dog. **Journal of Applied Microbiology**, v. 104, n. 6, p. 1718-1725, 2008. PMid:18194243. http://dx.doi.org/10.1111/j.1365-2672.2007.03702.x

RIVAS, F. et al. Antibacterial potential of *Enterococcus faecium* strains isolated from ewe´s milk and cheese. **Food Science and Technology**, v. 46, n. 2, p. 428-436, 2012.

VELJOVIC, K. et al. Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese. **Journal of Applied Microbiology**, v. 103, n. 6, p. 2142-2152, 2007. PMid:18045397. http://dx.doi.org/10.1111/j.1365-2672.2007.03450.x

ZACHAROF, M.; LOVITT, R. W. Bacteriocins produced by lactic acid bacteria: a review article. **APCBEE Procedia**, n. 2, p. 50-56, 2012.

YILDRIM, Z.; JONHNSON, M. G. Detection and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* R isolated from radish. **Letters in Applied Microbiology**, v. 26, n. 4, p. 297-304. 2008. http://dx.doi.org/10.1046/j.1472-765X.1998.00335.x