Adventitious dairy Leuconostoc strains with interesting technological and biological properties useful for adjunct starters

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ORIGINAL PAPER

Adventitious dairy *Leuconostoc* strains with interesting technological and biological properties useful for adjunct starters

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Abstract Some species of *Leuconostoc* are very important for fermented dairy products, as they contribute to the organoleptic characteristics of butter and cream, and also contribute to the formation of openings in some soft, semi-hard (Edam and Gouda cheeses), many artisanal or in blue-veined cheeses, such as Roquefort. In this study, 14 *Leuconostoc* strains isolated from cheese and cheese-related products were characterized by genotypic and phenotypic methods, and their technological performance assessed for their potential use as dairy adjunct starters. Phenotypic characterization allowed these strains to be classified to genus level, and genotypic studies (RAPD-PCR and 16S rRNA gene sequencing) identified them to species/subspecies level. Five *Leuconostoc* strains grew well and acidified milk, and most of them grew even at 8 °C. They showed moderate resistance to heat treatments (30 min at 63 °C) and grew well in the presence of 3% and 4% NaCl, and were significantly inhibited at pH ≤ 5. All strains showed resistance against the bacteriophages tested. In general, the antibacterial properties observed were slight and due to acid production, with the exception of *Leuconostoc citreum* MB1, which strongly inhibited *Listeria monocytogenes* ATCC 15313 by the production of a bacteriocin-like compound. All *Leuconostoc* strains studied were susceptible to gentamicin, tetracycline, erythromycin and ampicillin. Some strains also showed interesting technological and antimicrobial properties, thus being potentially appropriate as adjunct starters in fermented dairy products. This study highlights that adventitious lactic acid bacteria can be a great source of novel strains with interesting technological features that could be used for fermented dairy foods.

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摘要

外源性明串珠菌作为附属发酵剂的技术和生物特性。有些明串珠菌能赋予奶油特有的感官特性,有助于一些软质和半硬质干酪(伊顿干酪和高达干酪)、手工干酪或者蓝纹干酪(如 Roquefort 干酪)裂纹的形成,因此这些明串珠菌在发酵乳制品中具有非常重要的作用。本研究从干酪和与干酪相关的产品中分离出14株明串珠菌,采用基因型和表型分析的方法对这14株菌进行了鉴定,并且评价了它们在乳制品中作为附属发酵剂的技术特性。表型分析将这些菌株鉴定到属,而采用基因分析 (RAPD-PCR 16S rRNA 基因测序)将它们鉴定到种或亚种。其中5株明串珠菌在酸化的乳中生长性能良好,在 8 ºC 下这5株菌中的大多数还能够生长。这些菌株的耐热性适中 (63 ºC,30 min), 在 3% 和 4% 的 NaCl 中可以很好地生长,但是在 pH ≤ 5 时这些菌株的生长受到了显著地抑制。所有的菌株对噬菌体具有抗性。总体上来讲,在所有的菌株中除了 *Leuconostoc citreum* MB1外,其他菌株的抗菌特性不是十分明显;由于 *Leuconostoc citreum* MB1 能产生类似细菌素的化合物,所以对李斯特单胞菌 ATCC 15313 具有很强地抑制作用。所有的明串珠菌对庆大霉素、四环素、红霉素和氨苄青霉素敏感。有些菌株表现出有益的技术特性和抗菌特性,因此,这些菌株有可能成为发酵乳制品的附属发酵剂。本研究认为具有特殊性能的外源性乳酸菌可以成为发酵乳制品中有价值的附属发酵剂。

Keywords *Leuconostoc* · Adventitious microflora · Soft cheese · Adjunct starter

关键词 明串珠菌属 · 外源微生物菌群 · 软质干酪 · 附属发酵剂

1 Introduction

Leuconostoc is a Gram-positive lactic acid bacteria (LAB) of great economic importance due to its ability to produce gas (CO_2) from carbohydrates, flavour compounds (diacetyl, acetate and ethanol) in many dairy products, and dextrans in saccharose-containing (dairy) products (Hemme and Foucaud-Sheunemann 2004). Some species of *Leuconostoc* (*Leuconostoc mesenteroides*, *Leuconostoc lactis*) are very important in the fermentative dairy industry, as they have traditionally been used in butter and cream manufacture. *Leuconostoc* metabolises lactose and citrate and produces lactic acid, acetate, CO_2, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol, which contribute to the organoleptic (flavour and texture) characteristics of butter, cream and allows for the creation of openings in some soft and semi-hard cheeses (Edam and Gouda cheeses) (Vedamuthu 1994). The production of CO_2 by *Leuconostoc* is responsible for eye formation in many artisanal cheeses, usually made from raw milk or in blue-veined cheeses, such as Roquefort. In the latter, the openness produced by *Leuconostoc* allows the cheese to be colonized by *Penicillium roqueforti*. However, this phenomenon is not well accepted in other types of cheese, where it results in the deterioration of product quality (Hemme and Foucaud-Sheunemann 2004).

Over the last years, many Argentinean manufacturers producing fermented dairy products have experienced the non-desired blowing in soft and semi-hard cheeses. In particular, Cremoso Argentino cheese, a traditional soft cheese variety largely produced in Argentina, was found to be the most affected. In the majority of the cases, the responsible

spoiling microorganism was found to be *Leuconostoc* (Quiberoni et al. 2008), which was isolated from the raw milk, cheeses or whey protein concentrates (WPC) used during cheese manufacture. The presence of *Leuconostoc* in the final product could be due to the contamination of the milk during collection and/or during manufacture, given the great ability of *Leuconostoc* to survive on surfaces, tools and pasteurizers for long periods of time (Martley and Crow 1993), and their high resistance to both heat treatments and refrigerated storage at 4 °C (Hemme and Foucaud-Sheunemann 2004).

The aim of this work was to genotypic and technologically characterize wild strains of *Leuconostoc* in order to identify novel strains with potential interesting properties which could be satisfactorily used as adjunct starters for the manufacture of fermented dairy foods with novel characteristics and features.

2 Materials and methods

2.1 Isolation and identification of gas-producing lactic acid bacteria

Fourteen dairy samples were analyzed for the presence of gas-producing lactic acid bacteria. Seven of the analyzed samples were derived from cheesemaking ingredients (WPC, pasteurized milk and whey cream), whereas seven samples were soft Cremoso Argentino cheeses with a blown defect. LAB were enumerated on milk plate count agar (Reinheimer et al. 1995) and on de Man, Rogosa and Sharpe (MRS) agar (Biokar, Beauvais, France) and incubated at 32 °C for 48 h in a microaerophilic atmosphere. In addition, nonspecific gas-producing bacteria were enumerated on MRS broth (Biokar, Beauvais, France) tubes (limit dilutions) containing Dürham tubes for gas visualization, after incubation at 32 °C for 5 days. Colonies with visible gas production were isolated, purified and identified according to Bergey's Manual (Hensyl 1994). The preliminary identification of gas-producing strains was based on colony and cell morphology, Gram staining, catalase reaction and growth conditions (temperature, medium). Based on this, 14 strains were presumptively assigned to the genus *Leuconostoc*.

Sugar fermentation patterns of the presumptive *Leuconostoc* strains were determined using the following specific medium: 1.5% *w*/*v* tryptone, 0.6% *w*/*v* yeast extract, 0.5% *w*/*v* NaCl, 0.01% *v*/*v* Tween 80, 0.0025% *w*/*v* bromocresol purple, 1% *w*/*v* sugar, pH 6.8 [Fantuzzi, personal communication]. Sugar (glucose, arabinose, saccharose, trehalose, fructose and mannose) stock solutions (10% *w*/*v*) were prepared in distilled water and sterilized by filtration.

The isolated *Leuconostoc* strains were routinely grown in MRS broth at 32 °C and stored at −80 °C in MRS broth supplemented with glycerol (15% *v*/*v*). For enumeration, MRS agar was used, and the plates were anaerobically incubated at 32 °C for 48 h.

2.2 Genetic analysis

2.2.1 Random amplification of polymorphic DNA-PCR

Bacterial DNA was extracted by the chelex method for Gram-positive (and acid-fast) bacteria according to Giraffa et al. (Giraffa et al. 2000). Genomic DNA of each

Leuconostoc strain was used as template for PCR fingerprinting. Two primers (Biotez, Berlin, Germany) were used for generation of the PCR profiles: the M13 minisatellite core sequence (Huey and Hall 1989) (5′-GAGGGTGGCGGTTCT-3′), and primer R5 (Aymerich et al. 2006) (5′-AACGCGCAAC-3′). A 1 Kb Plus DNA Ladder (Invitrogen, Milan, Italy) was used as a DNA molecular weight marker. Gels were photographed under UV illumination using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Celbio, Milan, Italy) equipped with the EDAS 290 imaging cabinet. Images were saved as TIFF files and analyzed with the pattern analysis software package BioNumerics™ (version 5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium). Calculation of similarity of band profiles was based on the Pearson correlation coefficient *r*. A dendrogram was deduced from the matrix of similarities by the unweighted pair group method using arithmetic average clustering algorithm (Vauterin and Vauterin 1992).

2.2.2 16S rRNA gene sequence analysis (DNA sequencing)

Sequencing of the hypervariable regions (V1, V2 and V3 in the first 500 bp) in the 5′ end of the 16S rRNA gene was applied to perform genotypic strain identification. The MicroSeq 500 16S rDNA Bacterial Sequencing and Identification kit System (Applera Italia) was used. A 500-bp 16S ribosomal DNA fragment was amplified from the 5′ end of the gene using the following primers: forward (RIBO I, 5′-CCGAGCTCAACAGAGTTTGATCCTGGCTCAG-3′) (Rodtong and Tannock 1993) and reverse (536 *Escherichia coli* numbering, 5′-GTATTACCGCGGCTGCTGG-3’) according to previously described protocols (Giraffa et al. 2003). Sequence analysis was performed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). The sequence data was analyzed with the MicroSeq software version 1.36. Obtained consensus sequences were compared to the main LAB species entries in the 500 MicroSeq 16S rRNA gene database using the full alignment tool of the software. The identifications were refined following BLAST (www.ncbi.nlm.nih.gov/BLAST) alignment of the 16S rDNA sequences.

2.3 Technological characterization

2.3.1 Acidifying activity

An aliquot of each overnight *Leuconostoc* culture (32 °C, 16–18 h) was washed twice with phosphate buffer (0.1 M, pH 6.5, Cicarelli, San Lorenzo, Santa Fe, Argentina) and resuspended in the same buffer. Washed cells were then inoculated (2% *v*/*v*) into sterile, reconstituted (10% *w*/*v*), commercial dry skim milk (RSM, Milkaut, Santa Fe, Argentina) supplemented or not with casein hydrolysate (0.25%). Milk acidification kinetics were assessed by measuring the pH with a model SA 720 pH meter (Orion, Beverly, MA, USA), at the beginning of the assay and after 3, 5, 7, 8 and 24 h of incubation at 32 °C (Reinheimer et al. 1995). In addition, the acidity developed (24 h, 32 °C) was measured by titration with 0.1 mol L^{-1} NaOH to pH 8.4, and it was expressed as percentage of lactic acid.

2.3.2 Growth at various temperatures

Overnight (32 °C, 16–18 h) cultures were inoculated (2% *v*/*v*) in MRS broth and incubated for 24 h at 8, 32 (optimal growth temperature, used as control) and 45 °C. The optical density at 560 nm (OD_{560}) was measured at pre-established intervals of time.

2.3.3 Thermal resistance

Overnight cultures of the studied *Leuconostoc* strains were inoculated (10^6 cfu mL^{-1}) in RSM and subjected to a thermal treatment of 30 min at 63 °C. Bacterial counts in MRS agar (32 °C, 48 h) were carried out before (initial counts) and after the heat treatment. Survival percentages were calculated from the ratio between initial and final counts obtained on MRS agar.

2.3.4 Salt and acid tolerance

The evaluation of salt and acid tolerance was performed according to Sánchez et al. (Sánchez et al. 2005) with slight modifications. Salt content and pH of tubes containing 3 mL of MRS broth were varied by (1) the addition of 3%, 4%, 5% or 6% *w*/*v* NaCl or (2) pH adjustment by the addition of lactic acid to pH values of 3, 4, 5 and 6. Tubes were inoculated (2% *v*/*v*) with overnight cultures of the strains and incubated at 32 °C for 24 h. Cultures in MRS broth were used as controls. After the incubation period, OD_{560} was measured. Results were expressed as the ratio between OD_{560} in modified MRS and OD_{560} in MRS.

2.3.5 Phage resistance

Leuconostoc strains were tested for cross sensitivity against five *Leuconostoc* bacteriophages using the spot and turbidity tests, as described previously (Svensson and Christiansson 1991). *Leuconostoc* phages (ΦLDG, Φ19A, Φ19B, ΦCyC1 and ΦCyC2) were previously isolated at the Instituto de Lactología Industrial (INLAIN, Santa Fe, Argentina) from cheese manufactures. Four commercial strains sensitive to the phages were used as positive controls: *Leuconostoc mesenteroides* subsp. *mesenteroides* L18 (ΦLDG), L19A (Φ19A), L19B (Φ19B), and CyC (ΦCyC1 and ΦCyC2).

2.4 Antibacterial activity and nature of the pathogen inhibitory compound(s)

The well diffusion agar assay was used as previously described by Vinderola et al. (Vinderola et al. 2002) with slight modifications. Cell-free supernatants (CFS) were obtained by centrifugation of overnight *Leuconostoc* cultures (12,000 × g, 5 min, 5 °C) and further sterilization by filtration (0.45-μm pore diameter) (Millipore, São Paulo, Brazil). For the preparation of plates containing pathogens, nutrient agar (for *Salmonella* sp. OMS-Ca, *Staphylococcus aureus* 76 or *E. coli* V517) or BHI agar (*Listeria monocytogenes* ATCC 15313) was melted and cooled to 45 °C, then vigorously mixed with an overnight culture of each pathogenic strain (OD_{560} of 0.8) and poured onto a Petri dish. Wells of 10 mm in diameter were made in the agar layer, and 180 μL of CFS from each strain were placed in a well. Plates were

incubated overnight at 32 °C and the diameters of the inhibition halos were recorded.

To elucidate the nature of the inhibition of pathogen growth by supernatants of *Leuconostoc* strains, CFS of strains were subjected to heating at 121 °C for 15 min, neutralization (using NaOH) and incubation (1 h, 37 °C) in the presence of 200 mg mL^{-1} proteinase K (Invitrogen Life Technologies, Carlsbad, CA, USA) or 200 mg mL^{-1} pepsin (Merck). CFS of both treated and non-treated (controls) were subsequently filter-sterilized (0.45-μm pore diameter) and assayed for remaining activity by the well diffusion agar assay (Coconnier et al. 1997).

2.5 Antibiotic susceptibility

The minimum inhibitory concentration (MIC, micrograms per millilitre) was tested by broth microdilution using vancomycin, gentamicin, tetracycline, erythromycin, ampicillin, streptomycin and chloramphenicol (Sigma-Aldrich, St. Louis, USA) on all the *Leuconostoc* strains studied. The MIC was defined as the minimal concentration of antibiotic needed to totally inhibit the growth of the bacteria after incubation for 48 h. Vancomycin was used as control of the intrinsic resistance of *Leuconostoc* strains. Antibiotic stock solutions were prepared in distilled water or water to ethanol (6:4 for erythromycin and ampicillin; 5:5 for chloramphenicol) and then filter-sterilized (0.22-μm pore diameter) (Millipore, São Paulo, Brazil). The tested strains were grown overnight at 32 °C in MRS broth and then diluted in order to reach a final inoculum of 10^5 cfu mL^{-1} in the microplates. Strains were diluted in LAB susceptibility test medium (LSM) (Klare et al. 2005), which is a mixture of Iso-Sensitest broth (90% *v*/*v*, Oxoid Ltd., Basingstoke, Hampshire, England) and MRS (10% *v*/*v*, Biokar) adjusted to pH 6.7. Interpretation of the results was based on breakpoints (BP) adopted by the updated technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (European Food Safety Authority 2008).

2.6 Statistical analysis of data

Data were analyzed by the one-way ANOVA procedure of Statgraphics Plus software (version 3.0; Statistical Graphics Corp., Warrenton, VA, USA). Differences among mean values were detected by the least significant difference multiple range test.

3 Results and discussion

3.1 Identification of *Leuconostoc* strains

High levels of gas-producing bacteria were detected (10^6–10^9 cfu mL^{-1}) in all Cremoso Argentino cheeses analyzed, with low levels detected in the ingredients (data not shown). A preliminary identification of the 14 isolates was performed using conventional microbiological methods and all isolates were found to be Gram-positive, catalase-negative, CO_2-producing (from glucose), ovoid-shaped cocci pairs and were presumptively classified into the *Leuconostoc* genus.

Random amplification of polymorphic DNA (RAPD)-PCR analysis was performed by using the primers routinely applied by an Italian research group for LAB genotyping (Giraffa et al. 2000). Bionumeric analysis of the DNA profiles revealed nine patterns at 81.6% of similarity degree (Fig. 1). One strain from of each homology group was further subjected to 16S rDNA sequencing. According to this, six of the studied strains were classified as *Leuconostoc mesenteroides* subsp. *mesenteroides*, five as *Leuconostoc pseudomesenteroides*, two as *Leuconostoc garlicum* and one as *Leuconostoc citreum*. These species have been previously isolated from vegetables, raw milk and dairy fermentative products (Duthoit et al. 2005; Firmesse et al. 2008; Nieto-Arribas et al. 2010), as well as from clinical isolates or human fluids (Bou et al. 2008; Firmesse et al. 2008; Ogier et al. 2008). Strains with similar RAPD-PCR patterns should not be regarded identical, even when they come from the same factory, because they might possess dissimilar technological and/or biological attributes (see Sections 3.2, 3.3 and 3.4).

In a previous study (Cibik et al. 2000), a combination of RAPD-PCR, 16S rDNA sequencing and 16S rDNA fragment amplification with specific primers was applied in order to analyze the molecular diversity of a large collection of strains of *Leuconostoc* mainly isolated from traditional French cheeses. The majority of the strains were classified as *Leuconostoc mesenteroides* (83.7%) or *Leuconostoc citreum* (14%); however, these molecular methods were not useful in discerning the three subspecies (*mesenteroides*, *dextranicum* and *cremoris*).

The results obtained in the present study are in agreement with previous reports (Cibik et al. 2000) which state that the identification of the *Leuconostoc* species has been limited due to a lack of accurate biochemical and physiological tests. It has also been demonstrated that the use of traditional diagnostic test (i.e. sugar fermentation patterns) alone, commonly leads to misidentifications between *Leuconostoc* and other closely related bacteria such as *Lactobacillus*, *Weissella* and *Pediococcus* (Kulwichit et al. 2007). The use of conventional microbiological assays allowed us to correctly identify the isolates at the genus level, and supplementary information from sugar fermentation analysis confirmed that the isolates belonged to the *Leuconostoc* genus. Nonetheless, this approach failed to identify the isolates at species/subspecies level, since they were all misidentified as *Leuconostoc mesenteroides* subsp. *dextranicum* or *Leuconostoc lactis*. This could be due to the fact that this methodology was specifically developed for the

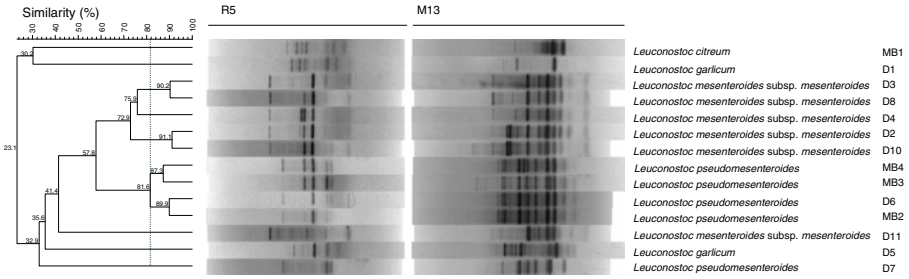

Fig. 1 RAPD-PCR profiles obtained from total DNA from the 14 *Leuconostoc* strains studied using primers R5 and M13, and corresponding dendrogram derived from the unweighted pair group average linkage of Pearson correlation coefficients (expressed as a percentage value). Names of the strains are indicated on the *right hand side*. *Numbers* represent the length of each branch as an indication of genetic distances among strains

most common *Leuconostoc* species used in commercial starters of dairy products, which only include the three subspecies of *Leuconostoc mesenteroides* (*mesenteroides*, *dextranicum* and *cremoris*) and *Leuconostoc lactis*. Consequently, this methodology left out some *Leuconostoc* species that were not traditionally related to dairy plants, although nowadays are found as non-starter lactic acid bacteria.

The general consensus is that molecular methods are useful to characterize the taxonomy and phylogeny of strains; however, the characterization of phenotypic traits remains indispensable as they play a predominant role in food science (Hemme and Foucaud-Sheunemann 2004). In our study, the use of molecular techniques was essential to classify the *Leuconostoc* strains studied at the species/subspecies level.

3.2 Technological characterization

The technological characterization of the isolates was performed in order to evaluate the suitability of individuals to be used as adjunct starter cultures in the manufacture of fermented foods.

The results of the technological characterization of the *Leuconostoc* strains studied are presented in Table 1. In this study, five strains (*Leuconostoc garlicum* D1 and D5, *Leuconostoc mesenteroides* subsp. *mesenteroides* D3, *Leuconostoc citreum* MB1 and *Leuconostoc pseudomesenteroides* MB2) grew and acidified milk to pH values ranging from 4.78 to 4.86 after 24 h of incubation. They also produced a significant amount of lactic acid (0.63–0.70%). Knowledge on the ability of the strains to grow in milk is essential to select those that could be useful as adjunct starters for dairy products. Similar to other LAB, strains of *Leuconostoc* require several amino acids for growth in milk, but as milk is deficient in amino acids lactic acid bacteria need proteinase activity to hydrolyze milk proteins and be able to grow well (Cogan and Jordan 1994). In this study, nine (64%) out of the 14 *Leuconostoc* strains studied were not able to grow well in milk.

Leuconostoc mesenteroides subsp. *mesenteroides* and *Leuconostoc citreum* have previously been reported to be responsible for recurrent spoilage in diverse cooked meat products (Hamasaki et al. 2003). However, these species have not been usually related to the blowing defects in soft and semi-hard cheeses. In cases where *Leuconostoc* have been identified as the contaminating bacteria, their psychrotrophic nature would allow them to grow during cheese ripening, causing early blowing by gas production, hence highlighting the importance of studying their behaviour at refrigeration temperatures. For the genus *Leuconostoc*, the optimal growth temperature ranges from 20 to 30 °C but most strains grow well even at 4 °C (Hamasaki et al. 2003). All *Leuconostoc* strains tested showed maximum growth after 24 h at 32 °C (controls) (Table 1), but delayed growth was observed for all strains when incubated at 8 °C (OD_{560} 0.11–0.67; 3–27% of growth with respect to controls), although after 48 and 72 h they had reached almost maximum growth. The slowest growth was shown by strains D5, D6, D7 and MB2, which reached on OD_{560} 0.62–2.37 only after 6 days of incubation at this temperature. Strains were able to grow, even if slowly, when incubated at 45 °C for 24 h (OD_{560} 0.08–0.25; 3–9% growth) (data not shown).

In addition to the ability of many *Leuconostoc* species to grow at low temperatures, some strains of *Leuconostoc* have been described as thermoduric (Martley and Crow 1993). In this work, the strains of *Leuconostoc* showed a

Table 1 Technological characterization of *Leuconostoc* strains (values given are the means of three determinations±standard deviation)

Strain		Final pH after growth in milk[a]	Growth at 8 °C after[b]			Resistance to heat treatment (log cfu· mL^{-1}) [c]	Growth (%)[d] in the presence of NaCl (%, *w/v*)				Growth (%)[d] at different pH values			
			Time (h)											
			24	72	144		3	4	5	6	3	4	5	6
Leuconostoc mesenteroides subsp. *mesenteroides*	D2	5.43±0.03f	0.70±0.02g	1.99±0.05i	2.21±0.13g	4.9±0.4g	79.2±2.0de	73.0±1.7f	35.3±2.2f	4.7±0.2cde	4.9±1.2ab	3.0±0.5ab	22.5±2.7d	61.3±3.6cd
	D3	4.78±0.07ab	0.39±0.02f	1.87±0.12i	2.23±0.08g	4.2±0.2fg	64.4±3.1b	66.2±2.3e	36.0±1.8f	9.2±0.7f	3.9±1.5a	2.4±0.3a	15.1±3.2bc	50.0±2.5b
	D4	5.01±0.09de	0.06±0.01b	0.79±0.06c	1.37±0.10c	4.2±0.5fg	65.2±2.3b	67.4±3.5e	56.0±2.5h	13.0±0.4g	4.5±0.9ab	2.7±1.1ab	13.2±3.5b	43.1±3.2a
	D8	5.00±0.06d	0.31±0.02e	1.72±0.10h	1.95±0.06f	3.6±0.1def	72.1±3.5c	57.1±2.6d	49.0±1.3g	23.3±2.6h	4.2±0.8ab	3.0±0.3ab	23.6±1.9d	57.5±2.7c
	D10	5.08±0.02e	0.28±0.05e	2.45±0.15j	2.74±0.12i	3.0±0.4bcd	86.4±2.2gh	78.2±1.2g	11.0±0.9bc	3.6±0.4bc	4.6±0.4ab	3.3±1.2ab	23.0±2.6d	65.3±3.2de
	D11	6.26±0.01i	0.06±0.01b	1.40±0.07g	1.55±0.07d	3.2±0.7cde	85.2±2.8fg	25.1±1.9b	0.5±0.1a	0.1±0.0a	5.2±1.3ab	3.5±1.0ab	17.6±1.8c	82.5±4.5gh
Leuconostoc citreum	MB1	4.82±0.02bc	0.04±0.01ab	1.23±0.05f	1.63±0.04d	2.3±0.4b	81.0±1.5ef	33.0±2.0c	13.1±0.3c	5.3±1.2de	4.6±0.2ab	3.5±0.5ab	24.5±3.1de	69.0±3.5ef
Leuconostoc garlicum	D1	4.73±0.03a	0.13±0.01c	0.63±0.03b	1.07±0.05b	0.8±0.1a	63.2±2.5b	24.2±2.5b	1.6±0.2a	3.9±0.6bcd	5.6±0.8b	3.2±0.2ab	4.0±0.3a	57.1±3.3c
	D5	4.78±0.05ab	0.01±0.01a	0.22±0.01a	0.62±0.02a	2.7±0.5bc	62.0±1.8b	30.5±3.1c	10.0±0.5b	4.4±0.3cd	7.6±1.4c	4.0±0.7b	3.0±0.1a	77.0±2.6g
Leuconostoc pseudomesenteroides	D6	5.47±0.04f	0.13±0.03c	1.28±0.11fg	2.37±0.09h	3.4±0.3cde	7.0±0.4a	2.4±0.4a	0.8±0.1a	0.4±0.1a	4.9±0.5ab	3.5±0.5ab	25.2±2.2de	58.5±3.6c
	D7	5.59±0.01g	0.17±0.02d	1.10±0.08e	1.91±0.06f	2.9±0.3bcd	90.1±3.6hi	57.3±3.5d	37.4±1.5f	6.0±1.1e	5.4±0.3b	3.3±1.2ab	36.6±3.1f	71.0±4.2f
	MB2	4.86±0.03c	0.12±0.01c	0.98±0.04de	1.97±0.07f	3.3±0.2cde	94.3±4.1i	76.0±3.2fg	26.0±2.0d	5.3±0.8de	5.1±0.1ab	3.9±0.4b	48.2±2.8g	100.0±3.5i
	MB3	6.10±0.03h	0.11±0.01c	0.96±0.03d	1.98±0.04f	3.9±0.6ef	75.0±2.3cd	54.4±2.6d	0.2±0.1a	2.7±0.7b	4.4±0.6ab	3.3±1.4ab	28.1±1.7e	69.6 ±2.5ef
	MB4	5.59±0.04g	0.13±0.02c	0.67±0.01bc	1.77±0.03e	2.9±0.8bcd	72.2±2.1c	55.1±1.9d	28.3±1.1e	3.2±0.5bc	4.7±0.3ab	3.5±0.5ab	3.4±0.4a	84.1±3.7h

For each column considered, values with the same letters are not statistically different ($P>0.05$)

[a] Growth in RSM for 24 h at 32 °C

[b] Optical density at 560 nm (OD_{560}) after incubation at 8 °C in MRS broth

[c] Decrease in viable cell counts (log orders cfu·mL^{-1}) after exposure to heat treatment (30 min at 63 °C)

[d] Results are expressed as the ratio (OD_{560} in modified MRS / OD_{560} in standard MRS)×100

moderate resistance to heat treatments (63 °C for 30 min), with a reduction of 2.3 to 4.9 logs being observed (Table 1). *Leuconostoc* strain D1 proved to be highly thermo-resistant, as a reduction of only one log was observed. However, even if a large reduction in cell viability was detected, the low level of remaining active cells could be enough to be responsible for spoilage in fermented food products. Strains of *Leuconostoc* which are capable of resisting pasteurization, as well as their presence on the surface of equipment could favour the cross-contamination of the milk which is further pasteurized (Martley and Crow 1993).

During cheese manufacture, lactic acid bacteria are exposed to an acidic and high salt environment. Starter bacteria produce acid during the fermentation, whereas salt is added to the curd before the ripening stage (Sánchez et al. 2005). Assessing the ability of strains to tolerate salt and acid stresses would facilitate the selection strains which would be suitable for high salt or acidic fermented food manufacture. Most *Leuconostoc* strains tested proved to grow well (between 63% and 94%) in the presence of 3% and 4% NaCl, with the exception of strain D6, which was very sensitive, even at the lowest salt concentration assayed. In the presence of 5% and 6% NaCl, they showed a marked reduction in their growth. Similar results have been reported for strains of *Leuconostoc* isolated from artisanal Afuega'l Pitu (Sánchez et al. 2005) and Manchego cheeses (Nieto-Arribas et al. 2010). Almost all *Leuconostoc* strains used in this study (13 out of 14) were isolated from products related to soft cheese manufacture. The low salt concentration in this type of cheese could explain their limited adaptation to this stress factor. The normal pH of Cremoso Argentino cheese is around 5.3. In the present work, the growth of the strains was influenced more by the pH of the medium than the salt concentration. At pH 6, strains grew from moderately well to normal (43–100%) with respect to the controls. A clear reduction in cell growth was observed at pH 5, showing a wide range of growth rates (4–48%) for all strains, whereas growth was almost non-existent at pH 3 and 4 (growth rates of 3–7.6%). The low resistance to acidic conditions of the strains tested may be related to the low acidification ability of this genus, and is in agreement with previous results (Nieto-Arribas et al. 2010; Sánchez et al. 2005).

As it is known, phage infection can lead from partial to total inhibition of the acidifying activity required for the correct manufacture of diverse fermented food products, with subsequent large economic losses (Moineau and Lévesque 2005). As a consequence, the ability of strains to be able to resist phage attack is a desired technological feature of starter cultures. All 14 *Leuconostoc* strains showed resistance to the five bacteriophages tested (ΦLDG, Φ19A, Φ19B, ΦCyC1 and ΦCyC2), as shown by a normal growth during three consecutive subcultures in their presence. Even though *Leuconostoc* is used mostly as an adjunct starter in cheese manufacture, phage resistance of these strains is essential, since their diminished activity can lead to the lack of holes in blue-veined cheeses, which could result in difficulties for the subsequent colonization by *Penicillium*.

3.3 Antibacterial activity and nature of the pathogen inhibitory compound(s)

Similar to other LAB, *Leuconostoc* can inhibit the growth of either unrelated LAB, spoilage or pathogen microorganisms. The mechanism of inhibition has been attributed to the degradation products of carbohydrate and citrate metabolism. Lactic

and acetic acids produced during fermentation by LAB affect the integrity of cell membranes, compromising cell viability and leading in many cases to their lysis (Hemme and Foucaud-Sheunemann 2004). Additionally, several bacteriocins produced by *Leuconostoc* strains have been identified and reported (Blom et al. 1999; Ennahar et al. 2000; Stiles 1994), all showing anti-*Listeria* activity (Schillinger et al. 1995; Trias et al. 2008). An advantage of bacteriocins over classical antibiotics is that digestive enzymes are capable of destroying them (Caplice and Fitzgerald 1999).

In this work, most strains of *Leuconostoc* tested showed low to moderate antibacterial effects against *Salmonella* sp. OMS-Ca, *E. coli* V517 and *S. aureus* 76, revealing partial growth inhibition halos. On the other hand, the majority of *Leuconostoc* strains exhibited good antibacterial effects against *Listeria monocytogenes* ATCC 15313, as shown by total growth inhibition halos. The production of organic acids was the mechanism of inhibition most frequently observed among the tested strains. However, *Leuconostoc citreum* MB1 showed strong inhibition of *Listeria monocytogenes* ATCC 15313 by a non-acidic, heat-resistant, proteinaceous compound, indicative of a bacteriocin or bacteriocin-like substance. This suggests that *Leuconostoc citreum* MB1 could be an interesting candidate for potential use either as part of or as an

Table 2 MIC (micrograms per millilitre) values of diverse antibiotics for the 14 *Leuconostoc* strains tested

Strain	Antibiotic						
	VAN	GEN	TET	ERY	AMP	STR	CMP
D1	>256	8	4	0.5	0.25	64	8
D2	>256	8	8	0.5	1	64	8
D3	>256	4	8	0.5	1	64	8
D4	>256	8	4	0.5	1	32	16
D5	>256	8	4	0.5	0.25	32	8
D6	>256	8	4	0.5	2	128	8
D7	>256	8	2	0.5	0.5	64	8
D8	>256	8	8	0.5	1	32	16
D10	>256	2	8	0.5	1	64	8
D11	>256	2	4	0.25	0.5	32	8
MB1	>256	2	8	0.5	0.25	16	8
MB2	>256	8	4	0.5	1	32	8
MB3	>256	4	4	<0.125	0.25	64	8
MB4	>256	4	8	0.25	0.5	32	8
BP[a]	n.r.[b]	16	8	1	2	64	4

VAN vancomycin, *GEN* gentamicin, *TET* tetracycline, *ERY* erythromycin, *AMP* ampicillin, *STR* streptomycin, *CMP* chloramphenicol, *n.r.* not required

[a] Breakpoints suggested by FEEDAP Panel (Coconnier et al. 1997) for *Leuconostoc* genus

[b] Not required

adjunct to starter cultures used in manufacture of fermented foods in order to improve safety and quality.

3.4 Antibiotic susceptibility

It is highly recommended that LAB used as starters do not carry antibiotic resistance genes and to avoid their possible transfer to other spoilage or pathogenic microorganisms present in the same environment. MIC values obtained for the antibiotics tested are shown in Table 2. They were compared to BP defined by the FEEDAP Panel (European Food Safety Authority 2008). According to this, a microorganism is defined as susceptible when it is inhibited at the breakpoint level of a specific antimicrobial in a defined phenotypic test system. All *Leuconostoc* strains tested demonstrated the intrinsic resistance to vancomycin, but showed susceptibility to gentamicin and erythromycin. A similar behaviour was observed for ampicillin, with the exception of *Leuconostoc pseudomesenteroides* D6, which showed an MIC identical to the BP value. For streptomycin and chloramphenicol, some strains presented MIC values similar or even higher than the BP levels. The high MIC values observed in this study could be due to the limitations of the methodology, as there is a lack of standardized methods of microdilution for antimicrobial susceptibility testing for *Leuconostoc* genus, and the absence of quality control strains for susceptibility testing (European Food Safety Authority 2008). On the other hand, the BP values suggested by the FEEDAP Panel refer to *Leuconostoc* genus, without considering that the diversity within *Leuconostoc* species/subspecies which could encompass very different susceptibility to the antimicrobials tested. To confirm antibiotic resistance on those *Leuconostoc* strains with higher MIC than BP values, it would be worth searching for the genes involved in antimicrobial resistance (Rojo-Bezares et al. 2006) and the type thereof (intrinsic or acquired) carried by them.

4 Conclusions

Studying adventitious *Leuconostoc* strains isolated from Cremoso Argentino cheese and related products, led us to find out some interesting features. From a strict point of view, strains of *Leuconostoc* with similar RAPD profiles and isolated from the same dairy plants, could be replicates. Despite this, many strains of *Leuconostoc* with similar patterns showed diverse technological and/or biological performance, which may exclude that possibility. Strains that grew well in milk could be used as adjunct starters in dairy products. Alternatively, as most of the studied strains grew well in the presence of NaCl, they could be used for high salt foods. *Leuconostoc citreum* MB1 was shown to produce an inhibitory substance against *Listeria monocytogenes* ATCC 15313 as well as having a good ability to grow in milk and salt-resistance. This study has shown that adventitious lactic acid bacteria can be a source of new strains with interesting technological and antibacterial properties that could be used for fermented dairy foods.

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