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Genotypic and technological characterization of *Leuconostoc* isolates to be used as adjunct starters in Manchego cheese manufacture

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ABSTRACT

Twenty-seven *Leuconostoc (Ln.)* isolates from Manchego cheese were characterized by phenotypic and genotypic methods, and their technological abilities studied in order to test their potential use as dairy starter components. While phenotypic diversity was evaluated by studying the biochemical characteristics of technological interest (i.e. acidifying and aminopeptidase activities), genotypic diversity was evidenced by using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR). Additional technological abilities such as lipolytic, proteolytic and autolytic activities, salt and pH tolerance and production of dextran, flavour compounds and biogenic amines, were investigated. The marked differences among strains reflected the existing biodiversity in naturally fermented products. After statistically evaluating their performance, strains C0W2, belonging to *Ln. lactis*, and C16W5 and N2W5, belonging to *Ln. mesenteroides* subsp. *dextranicum*, revealed the best properties to be used in mixed dairy starter cultures. This study evidences the fact that natural environments can be considered as a proper source of useful strains, for the dairy industry.

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1. Introduction

Manchego cheese is the most popular and widely sold cheese variety in Spain. It is a cured, hard, enzymatically coagulated cheese, manufactured in the La Mancha region (Spain) from the milk of Manchega breed ewes. Since 1985 it is protected by the Designation of Origin (RDO) "Manchego" cheese. The RDO Regulatory Board authorizes two manufacturing processes: artisanal, when raw milk is used, and industrial, when pasteurized milk is used. In the last case, milk is generally inoculated with commercial mixed-strain starter cultures, which comprise strains of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Núñez et al., 1989; Gómez et al., 1999). The pasteurization of milk ensures a higher uniformity of the product and improves the sanitary conditions of cheesemaking, but eliminates some of the indigenous microbiota of milk, which is partly responsible for the development of typical cheese flavour. The observation that raw milk cheeses develop more intense flavour than pasteurized milk cheeses (Gaya et al., 1990; Lau et al., 1991) stimulated interest in adding non-starter lactic acid bacteria (NSLAB) as adjunct cultures in the manufacture of industrial cheeses (Peterson and Marshall, 1990; Lane and Fox, 1996; Gómez et al., 1996; Lynch et al., 1997; Sánchez et al., 2006).

For distinct varieties of cheese, a predominance of NSLAB during ripening has been reported. Mesophilic lactobacilli are among the most common groups in this microbiota (Demarigny et al., 1996; Bouton et al., 1998), although pediococci, micrococci and leuconostoc are also present in low proportions (Williams and Banks, 1997). An important role in ripening has been attributed to these minority genera (Manolopoulo et al., 2003).

The presence of *Leuconostoc* species in different varieties of raw milk cheeses has been reported, with *Ln. mesenteroides* subsp. *mesenteroides* and *Ln. mesenteroides* subsp. *dextranicum* being the most frequently isolated subspecies in raw ewe's milk cheeses (Fernández-del-Pozo et al., 1988; Arizcun et al., 1997).

Leuconostoc spp. grow poorly in milk, but they produce compounds such as acetaldehyde, diacetyl and acetoin (McSweeney and Sousa, 2000) from lactate and citrate, which contribute to the organoleptic properties of dairy products. *Leuconostoc* spp. show other valuable technological properties, such as synthesis of dextrans from sucrose, which allows their use as thickeners or texturizers in fermented milks (Vedamuthu, 1994), and the presence of proteolytic, lipolytic and aminopeptidase activities (Macedo and Malcata, 1997).

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However, it is important to highlight that some *Leuconostoc* species can also induce spoilage in cheese by producing biogenic amines (González de Llano et al., 1998; Bover-Cid and Holzapfel, 1999; Fernández-García et al., 2000). The use of *Leuconostoc* for dairy fermentations, together with acid-producing lactococci, in mesophilic starter cultures, has been extensively reported (Vedamuthu, 1994; Server-Busson et al., 1999).

There are several studies that reports the presence of *Leuconostoc* in raw milk Manchego cheese during ripening (Núñez, 1976; García-Ruíz et al., 1995; Ballesteros et al., 2006) and also the use of *Leuconostoc* strains in Manchego cheese manufacture (Barneto and Ordóñez, 1979; Ramos et al., 1981; Núñez et al., 1982; Poveda et al., 2003) have been reported.

This paper reports on the genotyping and technological characterization of wild *Leuconostoc* strains isolated from artisanal Manchego cheeses as a first step in selecting strains with excellent properties to be introduced as adjunct cultures in pasteurized milk cheeses. Their use in the manufacture of these cheeses will make it possible to more closely reproduce the flavour of raw milk cheeses and will contribute to accelerate cheese ripening.

In addition, this study will contribute to the preservation of the indigenous microbial population responsible for the typical features of this important variety of cheese.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 27 isolates obtained from Manchego cheese manufactured in two dairies, as described by Sánchez et al. (2006), were analyzed. They had been presumptively assigned to *Leuconostoc* genus attending to the cell morphology, Gram staining and results for production of CO_2 from glucose and ammonia through arginine hydrolysis. The isolates were routinely propagated in MRS broth (Scharlab, Barcelona, Spain) and stored at −80 °C with 20% (v/v) glycerol as a cryoprotector. The cultures were activated by two successive transfers in the same broth before use.

The reference strains *Leuconostoc mesenteroides* 394 and *L. mesenteroides* subsp. *dextranicum* 912, from the Spanish Type Culture Collection (CECT), were used for identification purposes.

2.2. DNA isolation

The total genomic DNA was extracted according to the procedure described by Rodas et al. (2003).

2.3. Randomly amplified polymorphic DNA-Polymerase chain reaction (RAPD-PCR) typing

RAPD-PCR analysis was performed as described by Seseña et al. (2005). Primers with arbitrary sequences OPL-05 (5′-ACGCAGGCAC-3′) and Lp1 (5′-ACGCGCCCT-3′), purchased from Genotek (Sabadell, Spain), were used in two separate amplifications.

Each RAPD-PCR reaction was performed in 50 μL with the following components: 5 μL of crude DNA extract, 150 pmol of primer, 200 μM of each dNTP (Biotools B&M Labs., Madrid, Spain), 5 μL 10× *Taq* Mg-free Buffer [Biotools; 1×: 75 mM Tris–HCl, pH 9 at 25 °C; 50 mM KCl; 20 mM $(NH_4)_2SO_4$], 2 mM $MgCl_2$ (Biotools), 2.0 U *Taq* polymerase (Ultratools DNA Polymerase, Biotools) and enough double distilled water to bring the volume to 50 μl. The PCR reactions were carried out in a GeneAmp 2400 Thermal Cycler (PCR System, Applied Biosystems, Foster City, CA, USA) using the amplification conditions described by Sánchez et al. (2006). Each set of reactions included a negative control without DNA.

The amplification products were analyzed in 1% (w/v) agarose in 1× TBE gels that were stained with ethidium bromide (Sigma) and photographed under UV light.

The electrophoretic patterns were analyzed using the GelCompar (version 4.0) pattern analysis software package (Applied-Maths, Kortrijk, Belgium) (Vauterin and Vauterin, 1992). The RAPD-PCR patterns were grouped attending to the Pearson product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis (Sneath and Sokal, 1973).

In order to determine the minimum percentage similarity necessary for strain discrimination, reproducibility studies were carried out according to Sánchez et al. (2004). The level of similarity (r) obtained between repeats, when included within the dendrogram for all strains, established a discrimination threshold below which patterns were considered to be different.

2.4. Strain identification

2.4.1. Amplification and restriction analysis of 16S-rRNA gene (16S-ARDRA)

Representative isolates from clusters obtained in the numerical analysis of RAPD-PCR patterns and the reference strains were analyzed by 16S-ARDRA as described by Rodas et al. (2003). Restriction of the amplified fragment was carried out overnight at 37 °C in 20 μL of incubation buffer containing 5 U of the restriction enzyme MseI (New England BioLabs, Ipswich, UK) and enough PCR product to give 500 ng DNA (2–10 μL). The restriction fragments patterns were analyzed by electrophoresis at a constant voltage of 90V in 2% (w/v) agarose in 1× TBE gels using a 100 bp ladder (Biotools) as a molecular size marker.

2.4.2. Phenotypic identification

Isolates showing 16S-ARDRA patterns different from those corresponding to the reference strains were phenotypically identified using API 50CH galleries (API System, BioMerioux, Marcy l'Étoile, France) following the manufacturer's instructions and the Apilab Plus computer-aided identification program (version 4.0).

2.5. Technological characterization of the isolates

2.5.1. Acidifying activity

Tubes containing 10 mL of sterile skim milk (RSM 10% w/v) (Oxoid, Barcelona, Spain) were inoculated (1% v/v) with revitalized strains and incubated at 37 °C pH was measured using a Crison pH-meter (Crison, Barcelona, Spain) after 24 h of incubation, and the values were expressed as ΔpH (Garriga et al., 1996).

2.5.2. Proteolytic activity

Strains were grown in milk as described for the acidifying activity. After incubation, 0.5 mL of the culture was removed and the proteins precipitated using trichloroacetic acid. Subsequently, the samples were tested following the o-phthaldehyde (oPA) method of Church et al. (1983). Spectrophotometric determinations were performed using a Beckman DU-530 spectrophotometer at 340 nm. A calibration curve with glycine concentrations between 0.1 mM and 10 mM was used. The results were expressed as mM glycine/L.

2.5.3. Autolytic activity

The autolytic activity was measured according to the method described by Lansgrud et al. (1987). Cells from exponentially growing cultures (Optical Density (OD) at 650 nm = 0.7–0.8) in MRS broth were harvested by centrifugation at 10,000× g for 10 min at 4 °C. The pellets were washed and resuspended in 20 mM sodium phosphate buffer (pH 6.8). The lysis was monitored during 4 h of incubation at 30 °C by recording the decrease in OD_{650} using

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