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Characterization of Oaxaca raw milk cheese microbiota with particular interest in *Lactobacillus* strains

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ABSTRACT

The aim of this work was to identify and characterize lactobacilli strains from Mexican Oaxaca cheese. Twenty-seven lactobacilli isolated from Oaxaca cheese were identified at species level by 16S rRNA sequencing. Selected isolates were further characterized by ribotyping. Isolates were screened, among others, by acidifying capacity, antibiotic resistance, and activity against pathogens. *Lactobacillus plantarum* was predominant in Oaxaca cheese. The intraspecies variability of *Lb. plantarum* isolates was great. Multiple antibiotic resistances were observed. Eight isolates showed antimicrobial activity against the pathogenic species tested. Four *Lb. plantarum* strains showing low antibiotic resistance index, antimicrobial activity against enterotoxigenic *Staphylococcus aureus* and *Listeria innocua* stains, amine-negative decarboxylase activity, and resistance to NaCl and bile salt solutions, could be preselected to complete studies focused on designing a culture for use in pasteurized-milk Oaxaca cheese manufacturing.

Key words: lactobacilli, antibiotic resistance, Mexican cheese, starter culture

INTRODUCTION

Oaxaca cheese is one of the most popular cheeses in Mexico (Villanueva-Carvajal et al., 2012). It is a pasta filata cheese normally produced from raw milk when manufactured at small dairy plants (Domínguez-López et al., 2011). The acidification process of the curd is a key point of the Oaxaca cheese-making process. It usually takes from 4 to 8 h and a strict control of the pH decline of the curd at 35 to 38°C (to a pH of approximately 5.3) is required, in order to obtain the characteristic fibrous curd straps by kneading and stretching of the acidified curd in hot water (Aguilar-Uscanga et al., 2006).

The presence of lactobacilli in cheese is common. Lactobacilli are an important part of the nonstarter lactic acid bacteria (**LAB**) and can contribute to cheese flavor and texture (Khalid and Marth, 1990). The nonstarter lactobacilli most frequently isolated from cheese are facultatively heterofermentative (Beresford et al., 2001). These microorganisms appear to be predominant in the microbiota of the intensively acidified cheese curds due to their ability to grow at low pH (Sánchez et al., 2005). Lactobacilli species from cheeses have been identified on the basis of phenotypic and genotypic characteristics (Kongo et al., 2007).

The presence of antibiotic resistance genes in many LAB and the transfer of genetic resistance determinants to and from other LAB is a well-known fact (Mathur and Singh, 2005). Starter cultures can serve as a means of transmission of such genes. Knowledge on natural resistance for particular *Lactobacillus* species increases the safety of the products via implementation of an increased monitoring of antibiotic resistance of starter cultures.

To our knowledge, several aspects of Oaxaca cheese quality have been previously studied (De Oca-Flores et al., 2009; Caro et al., 2011; de los Ángeles Colín-Cruz et al., 2012). However, no scientific information is available on its microbiology. The knowledge of the LAB, mainly lactobacilli, involved in the elaboration of this cheese would be of great interest to obtain not only a real picture of its quality but also a proper and safe starter culture to be used for pasteurized milk Oaxaca cheese making. The development of standardized procedures for the elaboration of this cheese using well-defined and appropriate starters is of obvious importance. This study, therefore, was aimed at the identification and the characterization of lactobacilli isolates present in Oaxaca cheese made from raw milk, which could be taken into consideration to select the proper strains for a suitable starter culture for the manufacturing of this cheese.

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MATERIALS AND METHODS

Cheese Samples and Microbiological Analyses

Eight samples of traditionally made Oaxaca cheese made with raw milk were purchased directly from the correspondent (n = 8) local cheese plants in Valle de Tulancingo (Hidalgo, Mexico). Cheese samples weighing 200 to 500 g were transported for up to 1 h from the cheese plant to the laboratory inside their original packages, where they remained at 4°C for up to 6 h before analysis. From each sample, a 10-g aliquot was aseptically obtained by means of radial cuts (IDF, 1995a) and homogenized in 90 mL of buffered peptone water (CM 509; Oxoid Ltd., Basingstoke, UK), according to methods of the International Dairy Federation (IDF, 1996) with a Stomacher 400 circulator (Seward Ltd., London, UK) for 2 min. Duplicate serial dilutions were prepared in buffered peptone water and then 1 mL of each dilution was plated on specific media. Aerobic mesophilic bacteria were grown on plate count agar (Difco Laboratories Inc., Detroit, MI) at 32°C for 48 h; molds and yeast on potato dextrose agar (Oxoid Ltd.), acidified using 10% tartaric acid to pH 3.5 at 22°C for 7 d; LAB on de Man, Rogosa, and Sharpe (**MRS**) agar (Oxoid Ltd.), adjusted to pH 5.5 with lactic acid, under anaerobiosis at 37°C for 48 h; and *Escherichia coli* and coliforms were enumerated on 3M Petrifilm *E. coli*/coliform (3M Microbiology, St. Paul, MN), at 37°C for 24 to 48 h.

Lactobacilli Isolation and Basic Characterization

Ten colonies per sample were randomly picked from MRS agar plates, reaching a total of 160. These isolates were then grown in MRS broth (Oxoid Ltd.) at 37°C overnight. A total of 800 μL of broth was then mixed with 200 μL of glycerol and the mixture was stored at −80°C until further use. Afterward, the stored isolates were grown at 30°C on MRS broth (Oxoid Ltd.) and then a loopful of growth was subcultured in MRS agar. From this, basic characterization of the isolates was performed through Gram reaction, morphology, and catalase (H_2O_2, 3% vol/vol) and cytochrome-oxidase activities according to Harrigan (1998).

Genotypic Identification

Polymerase chain reaction amplification and DNA sequence analysis were performed on 27 isolates randomly chosen from the MRS isolates identified as lactobacilli (n = 79) according to a basic characterization test. Isolates were grown on MRS plates for 48 h at 37°C before analysis. Methods and conditions of the identification were those described by González et al. (2007). The oligonucleotide primers used were as follows: forward primer 515 FPL: 5′CGGATCCTCTAGACTGCAGTGCCAGCAGCCGCGGTAA3′ and reverse primer 13B: 5′CGGGATCCCAGGCCCGGGAACGTATTCAC3′, yielding a product of 904 bp, and forward primer 9IE: 5′GGAATTCAAAKGAATTGACGGGGGC3′ and reverse primer 13B, giving a product of 475 bp. These were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ) and described by Relman (1993). The PCR fragments were purified with Montage PCR centrifugal filter devices from Millipore Corp. (Billerica, MA) and then sequenced directly. Sequencing was performed using the DYEnamic ET Dye Terminator Kit and MEGABACE 500 DNA Analysis System (GE Healthcare Life Sciences, Piscataway Township, NJ). Fluorograms were analyzed with the sequencing software Chromas 2.23 (Technelysium Pty Ltd., Helensvale, Australia), which opens chromatogram files produced by Applied Biosystems Inc. (Foster City, CA) equipment and exports sequences in plain text, formatted with base numbering, FASTA format, or EMBL format. The sequences obtained were then sent to the National Centre for Biotechnology Information (NCBI) to be analyzed for the nucleotide-nucleotide BLAST database (http://blast.ncbi.nlm.nih.gov/) in FASTA format (http://www.ebi.ac.uk/Tools/fasta33/genomes.html) and 16S ribosomal database (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Furthermore, an automated DuPont RiboPrinter Microbial Characterization System (Qualicon Inc., Wilmington, DE) was used to provide additional information on the identifications obtained by 16S rRNA sequencing. Briefly, 20 out of the 27 MRS lactobacilli isolates were grown on MRS plates for 48 h at 37°C. For each plate, a loopful of the growth was suspended in the sample buffer recommended by the manufacturer, inactivated by a heat kill step, treated with lytic enzymes to release the DNA, and then cut with the restriction endonuclease *Eco*RI at 37°C for 20 min. Fragments once separated by electrophoresis were transferred to a nylon membrane. A DNA probe for the *E. coli rrn*B operon was then hybridized to the genomic DNA on the membrane. The genetic fingerprint was visualized and captured using a chemiluminescent detection module and a digitizing camera provided with the aforementioned RiboPrinter System. The analysis software automatically characterized and identified the digitalized image. Each clone was identified using the comparison of the RiboPrint pattern with an identification database of *Eco*RI RiboPrint patterns created by E. I. DuPont de Nemours and Company (Qualicon, Wilmington, DE).

Phenotypic Characterization

The 27 isolates identified were tested for resistance to antibiotics (BBL Sensi-Disc; Becton Dickinson, Cock-

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