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

Irma Caro,^{*1} Javier Mateo,^{*†} María H. Sandoval,^{*} Sergio Soto,[‡] María R. García-Armesto,^{*} and José M. Castro^{†§}

^{*}Department of Food Hygiene and Food Technology, University of León, Campus de Vegazana s/n, C.P. 24071 León, Spain

[†]Instituto de Ciencia y Tecnología de los Alimentos, University of León, La Serna, 56, C.P., 24007 León, Spain

[‡]Research Center of Food Science and Technology, University of Hidalgo State, C.P. 43600 Tulancingo, Hidalgo, México

[§]Department of Molecular Biology, University of León, 24071 León, Spain

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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¹Corresponding author: icarco@unileon.es

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 μ L of broth was then mixed with 200 μ L 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 (H₂O₂, 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'CGGATCCTCTAGACTGCAGT-GCCAGCAGCCGCGGTAA3' and reverse primer 13B: 5'CGGGATCCCAGGCCCGGGAACGTATTAC3', yielding a product of 904 bp, and forward primer 9IE: 5'GGAATTCAAAGGAATTGACGGGGGC3' 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 *EcoRI* at 37°C for 20 min. Fragments once separated by electrophoresis were transferred to a nylon membrane. A DNA probe for the *E. coli* *rrnB* 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 *EcoRI* 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-

eysville, MD). The antibiotics were penicillin G (10 µg), ampicillin (25 µg), cefsulodin (30 µg), cefoxitin (30 µg), and amoxicillin/clavulanic acid (20/10 µg), belonging to group I (β-lactam); bacitracin (10 µg), vancomycin (30 µg), and doxycycline (30 µg) from group II (non-β-lactam cell wall); streptomycin (300 µg), tetracycline (30 µg), clindamycin (10 µg), kanamycin (30 µg), and neomycin (30 µg), from group III (inhibit protein or mRNA synthesis); nalidixic acid (30 µg), norfloxacin (10 µg), metronidazole (80 µg), belonging to group IV (DNA gyrase or DNA synthesis, or both); and finally, trimethoprim (5 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg), and polymyxin B (300 µg) belonging to group V (antimetabolites and cell membrane disruption, respectively). Sensitivity was determined using the agar diffusion method, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) according to the manufacturers. Analyses were carried out on Mueller-Hinton agar (MHA; Oxoid Ltd.). Four antibiotics discs were placed on the plates and after incubation (37°C) the diameter of the inhibition zones was measured.

Antimicrobial activity assays against *Staphylococcus aureus* (strains S13, 5192), *Bacillus cereus* (PI-1), and *Listeria innocua* (125) were carried out. Foodborne pathogens from these genera or species are safety issue concerns in the dairy industry. Strains S13 and 125 were supplied by the Department of Food Hygiene and Food Technology, University of León (León, Spain), and those of PI-1 and 5192 by the Hannah Research Institute (Ayr, UK) and the Colección Española de Cultivos Tipo (CECT; Valencia, Spain), respectively. The tests were carried out in nutrient agar (Merck KGaA, Darmstadt, Germany), using the standard method described by Shahani et al. (1976).

Additional phenotypic characteristics were determined with the Vitek Automated Identification System (bioMérieux, Marcy l'Étoile, France), using a gram-positive identification card. Additional tests were also carried out; these included growth in 6% NaCl, pyruvate fermentation, and motility at 30°C. Moreover, the acidifying activity of strains was measured according to the International Dairy Federation Standard 306 (IDF, 1995b). The strains were subcultured in MRS broth supplemented with 0.006% yeast extract (Oxoid Ltd.) at 30°C for 24 h. The microbial culture was inoculated at 1% (vol/vol) in sterile skim power milk

(reconstituted at 100 g/L) in duplicate and pH values were measured after 6, 12, and 24 h of incubation at 30°C. Furthermore, their ability to grow at 15 and 45°C in MRS broth was tested.

RESULTS

Microbiological Counts and Basic Characterization of Isolates

Mean counts of microbial groups in the 8 Oaxaca cheeses sampled are given in Table 1. Counts were relatively uniform for aerobic mesophilic, psychrotrophic bacteria, and LAB, but diverse for the other groups, chiefly for molds and yeasts where counts ranged from as low as 0.15 (log₁₀ cfu/g) to as high as 6.25 (data not shown in tables).

Almost 50% (79 out of 160) of the isolates from MRS plates, on the basis of their cell morphology and showing to be gram positive, catalase negative, and oxidase negative, were identified presumptively as lactobacilli. From them, 27 randomly chosen isolates were subject to further identification and characterization studies.

Genotypic Identification

Most of the 27 isolates tested were shown to be *Lactobacillus plantarum* by means of 16S rRNA gene sequencing (21 out of 27; Figure 1). The other 6 were identified as *Lactobacillus brevis* (n = 3), *Lactobacillus sakei* (n = 2), and *Lactobacillus paracasei* (n = 1). The 3 isolates identified as *Lb. brevis* were initially ambiguously identified as *Lb. plantarum*/*Lb. brevis*.

The ribotyping performance is also shown in Figure 1. From the 20 lactobacilli isolates subjected to genetic identification by ribotyping, only 18 showed valid results. The other 2 isolates could not be identified by ribotyping, but were identified as *Lb. plantarum* by sequencing. Most of the ribotyping-identified isolates showed to be *Lb. plantarum* (14 strains). These were grouped in 10 ribogroups (with isolates belonging to the same ribogroup showing similarity coefficients higher than 0.93) by using the RiboPrinter's proprietary software. The other 4 isolates were identified as *Lb. brevis* (n = 3) and *Lb. paracasei* (n = 1). *Lactobacillus brevis* strains were each assigned to a different ribogroup.

Table 1. Counts (mean ± SD) of the different microbial groups (log₁₀ cfu/g) in Oaxaca raw milk cheese¹

AMB	LAB	M and Y	Coliform	<i>Escherichia coli</i>
7.63 ± 0.76	6.52 ± 0.85	4.82 ± 2.52	4.08 ± 1.41	3.61 ± 1.21

¹AMB = aerobic mesophilic bacteria; LAB = lactic acid bacteria; M and Y = molds and yeasts.

Strain number	Identification by 16S rRNA	Identification by ribotyping	DuPont ID Similarity ¹	RiboPrint™ Pattern					Ribogroup
				1 kbp	5	10	15	50	
11	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.91						ECOR1219-47-S-3
19	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.87						ECOR1219-47-S-3
24	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.91						ECOR1219-47-S-3
65	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.89						ECOR1219-47-S-3
20	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.90						ECOR1219-47-S-5
45	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.90						ECOR1219-47-S-7
8	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.74						ECOR1219-48-S-1
29	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.73						ECOR1219-48-S-1
37	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.76						ECOR1219-48-S-3
50	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.95						ECOR1219-48-S-5
39	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.68						ECOR1219-50-S-3
67	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.82						ECOR1219-50-S-5
69	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.87						ECOR1219-50-S-7
74	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	0.81						ECOR1219-50-S-8
27	<i>Lactobacillus plantarum</i>	ND	ND	ND					ND
2	<i>Lactobacillus plantarum</i>	NI	-	-					-
60	<i>Lactobacillus plantarum</i>	NI	-	-					-
57	<i>Lactobacillus plantarum</i>	ND	-	-					-
47	<i>Lactobacillus plantarum</i>	ND	-	-					-
31	<i>Lactobacillus plantarum</i>	ND	-	-					-
43	<i>Lactobacillus plantarum</i>	ND	-	-					-
54	<i>Lactobacillus brevis</i>	<i>Lactobacillus brevis</i>	0.72						ECOR1219-47-S-8
55	<i>Lactobacillus brevis</i>	<i>Lactobacillus brevis</i>	0.63						ECOR1219-50-S-4
13	<i>Lactobacillus brevis</i>	<i>Lactobacillus brevis</i>	0.68						ECOR1219-50-S-1
34	<i>Lactobacillus paracasei</i>	<i>Lactobacillus paracasei</i> sub. <i>paracasei</i>	0.88						ECOR1219-47-S-6
15	<i>Lactobacillus sakei</i>	ND	-	-					-
18	<i>Lactobacillus sakei</i>	ND	-	-					-

Figure 1. Identification and restriction endonuclease *EcoRI* ribogroup of *Lactobacillus* isolated from Oaxaca cheese by ribotyping and 16S rRNA sequences. ND = not determined/not subjected to identification by ribotyping; NI = not identified by ribotyping. ¹DuPont ID similarity = a similarity measure to assign the name of the genus, species, and specific subgroup.

Phenotypic Characterization

Results of antibiotic sensitivity assays (resistance) are shown in Table 2. All isolates showed resistance against at least 3 antibiotics (resistance index of 0.15). The highest resistance index was 0.5 (resistance to 10 antibiotics; strain 57). A total of 63% of isolates showed resistance indexes equal or higher than 0.3. All lactobacilli were found to be resistant to metronidazole (group IV), but, on the other hand, were sensitive to the amoxicillin/clavulanic acid combination (group I); doxycycline (group II); gentamicin, streptomycin, clindamycin, and neomycin (group III); and polymyxin B (group V).

When resistance to antibiotics in group I (β -lactam) was tested for, strains showed less resistance to ampicillin (11% of strains were resistant) and more resistance to cefoxitin and cefsulodin (85 and 81% of the strains, respectively). Remarkably, some strains resistant to ampicillin and penicillin G (4 and 10 out of 27, respectively) were sensitive to the combination of amoxicillin and the β -lactamase inhibitor clavulanic acid. Regarding antimicrobials in group II (non- β -lactam), two-thirds of the strains were resistant to vancomycin and only one strain was resistant to bacitracin, but all were well sensitive to doxycycline.

The highest prevalence of sensitivity was observed for group III antibiotics, with most of them being ami-

Table 2. Antibiotic resistance and antimicrobial activity of *Lactobacillus* spp. isolated from Oaxaca cheese

Strain number (ribogroup)	Antibiotic resistance ²	RI ³	Antimicrobial activity ⁴			
			<i>Staphylococcus aureus</i> S13	<i>Staphylococcus aureus</i> 5192	<i>Bacillus cereus</i>	<i>Listeria innocua</i>
<i>Lactobacillus plantarum</i>						
11 (47-s-3)	Met, Na, Stx	0.15	—	—	—	—
19 (47-s-3)	B, Cfs, Met	0.15	—	+	+	+
24 (47-s-3)	Cfs, Fox, Met, Nor, Tmp, Va	0.30	+	+	+	+
65 (47-s-3)	Cfs, Met, K, Na, Va	0.25	—	+	+	+
20 (47-s-5)	Am, Met, Na	0.15	+	+	—	+
45 (47-s-7)	Fox, Met, Nor, P, Te	0.25	+	+	+	+
8 (48-s-1)	Cfs, Fox, Met, Na, P	0.25	—	—	—	—
29 (48-s-1)	Cfs, Fox, Met, Na, Nor, Va	0.30	+	+	+	+
37 (48-s-3)	Cfs, Fox, Met, Na, Nor, Va	0.30	+	+	+	+
50 (48-s-5)	Cfs, Fox, Met, Na, Nor, Tmp, Va	0.35	+	++	+	+
39 (50-s-3)	Cfs, Fox, Met, Na, Nor	0.25	—	—	—	+
67 (50-s-5)	Cfs, Met, Na, Va	0.20	+	+	+	+
69 (50-s-7)	Cfs, Fox, Met, Na, Nor, Va	0.30	+	+	+	+
74 (50-s-8)	Am, Fox, Met, Na, Nor, P	0.30	—	—	+	+
27 (ND)	Cfs, Fox, Met, Na, Nor, Va	0.35	+	—	+	+
2 (NI)	Cfs, Fox, Met, Nor, Tmp, Va	0.35	—	—	—	—
60 (NI)	Cfs, Fox, Met, Na, Nor, Te, P, Va	0.40	—	—	—	—
57 (ND)	Am, Cfs, Fox, Met, Na, Nor, P, Stx, Tmp, Va	0.50	—	—	—	—
47 (ND)	Am, Met, Te	0.15	—	+	+	+
31 (ND)	Cfs, Fox, Met, Na, Stx, Va	0.30	+	+	—	+
43 (ND)	Cfs, Fox, Met, Na, Nor, P, Va	0.35	—	—	—	—
<i>Lactobacillus brevis</i>						
54 (47-s-8)	Cfs, Fox, Met, Na, Nor, P, Va	0.35	++	++	+	+
55 (50-s-4)	Cfs, Fox, Met, Na, Nor, P, Va	0.35	—	—	—	—
13 (50-s-1)	Cfs, Fox, Met, Na, Nor, P, Va	0.35	—	—	—	—
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i>						
34 (47-s-6)	Cfs, Fox, Met, Na, P	0.25	—	—	+	—
<i>Lactobacillus sakei</i>						
15 (ND)	Cfs, Fox, Met, Na, Stx, Va	0.30	—	—	—	—
18 (ND)	Cfs, Fox, Met, Na, Nor, Stx, Tmp, Va	0.40	—	+	+	++

¹ND = not determined/not subjected to identification by ribotyping; NI = not identified by ribotyping (ribogroup).

²Antibiotic abbreviations (the number in parentheses indicates the respective group): Am = ampicillin (I); B = bacitracin (II); Cfs = cefsulodin (I); Fox (I) = cefoxitin; K (III) = kanamycin; Met (IV) = metronidazole; Na (IV) = nalidixic acid; Nor (IV) = norfloxacin; P (I) = penicillin; Stx (V) = trimethoprim-sulfamethoxazole; Te (III) = tetracycline; Tmp (V) = trimethoprim; Va (II) = vancomycin. The antibiotic groups are as follows: I = β -lactams; II = non- β -lactams cell wall; III = inhibit protein or mRNA synthesis; IV = DNA gyrase or DNA synthesis, or both; V = antimetabolites or cell membrane disruption.

³Resistance index.

⁴Antimicrobial activity against the pathogens tested is marked as — = no activity detected, + = inhibition zone ≥ 1 mm, and ++ = inhibition zone ≥ 2 mm.

noglycosides. All strains were sensitive to clindamycin, streptomycin, and neomycin. Moreover, most of them were equally sensitive to tetracycline and kanamycin. In the opposite way, the highest prevalence of resistance among the isolates was shown in relation to certain antimicrobial agents in group IV. Strains showed significant resistance to metronidazole (100%), the quinolone nalidixic acid (81%), and to norfloxacin (63%), the new-generation fluoroquinolone. As for group V, 20% of the strains were resistant to trimethoprim alone or in combination with sulfamethoxazole, but, on the other hand, all strains were sensitive to polymyxin B, an effective antibacterial cell membrane disruptor.

The results of the antimicrobial activity test of strains are also shown in Table 2. Two-thirds of the *Lactobacillus* strains showed antimicrobial activity against at least one of the tested strains, especially against *L. innocua* (60%). In addition, 8 out of 27 showed activity against all of them. Lactobacilli strains 50 and 54 showed a relatively strong activity against *Staph. aureus*.

Results on the selected phenotypic characteristics, those of interest in relation to starter culture selection, as determined by using the Vitek gram-positive identification system, are shown in Table 3 (not all the characteristics are shown for brevity). All strains were able to grow in 10 and 40% bile salt solutions. All but 3 strains showed positive growth in 6% NaCl broth. Moreover, only one strain showed arginine decarboxylase activity. Furthermore, all strains tested were able to reduce the pH of milk from 0.3 to 0.5 U after 6 h of incubation (Table 3) and to reduce the pH by approximately 1 U after 24 h. Finally, all strains were capable of growing at 15 and 45°C (data not shown in tables).

DISCUSSION

Microbiological Counts and Genetic Identification

Aerobic mesophilic and psychrotrophic bacteria as well as LAB counts were found to be consistent with those reported in other studies on fresh raw milk pasta filata cheeses (Çetinkaya and Soyutemiz, 2006; Mucchetti et al., 2008). Coliform, *E. coli*, and mold and yeast counts presented high standard deviations relative to the mean values. There is not a simple and clear explanation for this lack of uniformity. However, at least partially, it reflects variations in the hygienic conditions in the different cheese-making plants used in this study.

As expected, Oaxaca cheese showed high LAB counts, with almost half of them being considered as lactobacilli. During pasta filata cheese making, the LAB microbiota cause a rapid acidification of curd and become predominant; afterward, although the heating of the curd

during kneading and stretching results in a reduction in the microbial population, LAB appears to remain the predominant group (Çetinkaya and Soyutemiz, 2006). A predominance of LAB with a particularly high presence of lactobacilli populations has been found by other authors in pasta filata cheeses (Papademas and Robinson, 2000; Gobetti et al., 2002; Piraino et al., 2005), and other cheeses with a curd acidified to similar pH values (around 5.2), such as Cheddar, Domiati, or Halloumi cheeses (Haddadin, 1986; McSweeney et al., 1993; Papademas and Robinson, 2000).

In this study, good concordance existed between sequencing of the 16S rRNA and ribotyping for the results of genetic identification of the lactobacilli isolates tested. However, the ribotyping method gave additional information on strain discrimination within species by means of the adscription of strains to ribogroups. Several isolates presented identical ribopatterns (i.e., 4 isolates belonged to the 47-s-3 ribogroup and 2 isolates to 48-s-1). However, the isolates in the same ribogroup presented different phenotypic behavior. This leads to the suggestion that isolates in the same ribogroup could be a single strain showing different gene expression.

Phenotypic Characterization

The results of antimicrobial resistance found in this study generally agree with reports indicating that LAB strains are commonly resistant to principal types of antibiotics such as β -lactam, cephalosporins, aminoglycosides, quinolones, imidazole, nitrofurantoin, and fluoroquinolones (Halami et al., 2000). Transfer of resistance to antimicrobial compounds is an essential mechanism of adaptation of bacteria to survive in specific environments (Davies, 1997). Increasing evidence indicates that an overuse of antibiotics in the treatment and prophylaxis of bacterial infections of food-producing animals could be the principal factor regarding the bacterial resistance to such medicines (Tollefson and Karp, 2004). This fact could explain the high levels of resistance shown by the strains examined with respect to many of the antibiotics investigated.

With respect to group I (β -lactam) antibiotics, Zarazaga et al. (1999) found a high portion of *Lb. plantarum* strains to be resistant to penicillin. β -Lactamases, a family of resistance enzymes, which includes penicillinases and cephalosporinases, are involved in resistance to β -lactam antibiotics (Bush et al., 1995). Our results suggest the presence of these enzymes in some of the lactobacilli strains.

With regard to antibiotics in group II (non- β -lactam), the resistance against bacitracin has been related to strains isolated from cattle where this compound was used as a growth-promoting agent (Dutta and Devri-

Table 3. Selected characteristics obtained from the Vitek gram-positive identification (GPI) system (bioMérieux, Marcy l'Étoile, France) and pH decrement at different incubation times (h) of *Lactobacillus* spp. isolated from Oaxaca cheese

Strain number	Ribogroup ¹	Vitek GPI system ²				pH decrement ³		
		6NaCl	10B	40B	ARG	6 h	12 h	24 h
<i>Lactobacillus plantarum</i>								
11	47-s-3	+	+	+	—	0.37	0.68	0.99
19	47-s-3	+	+	+	—	0.40	0.64	0.96
24	47-s-3	+	+	+	—	0.38	0.71	0.98
65	47-s-3	+	+	+	—	0.35	0.69	1.04
20	47-s-5	+	+	+	—	0.45	0.79	1.22
45	47-s-7	+	+	+	—	0.36	0.60	0.99
8	48-s-1	+	+	+	—	0.40	0.63	0.99
29	48-s-1	+	+	+	—	0.39	0.64	0.98
37	48-s-3	+	+	+	—	0.52	0.71	0.98
50	48-s-5	+	+	+	—	0.50	0.69	0.96
39	50-s-3	+	+	+	—	0.39	0.69	0.97
67	50-s-5	+	+	+	—	0.50	0.75	1.20
69	50-s-7	+	+	+	—	0.46	0.70	1.15
74	50-s-8	—	+	+	—	0.45	0.79	1.18
27	ND	+	+	+	—	0.36	0.60	0.99
2	NI	+	+	+	—	0.40	0.58	0.95
60	NI	—	+	+	—	0.37	0.69	0.97
57	ND	+	+	+	—	0.37	0.66	1.05
47	ND	+	+	+	—	0.37	0.61	0.90
31	ND	+	+	+	—	0.30	0.77	1.45
43	ND	—	+	+	—	0.38	0.60	0.90
<i>Lactobacillus brevis</i>								
54	47-s-8	+	+	+	—	0.36	0.65	0.93
55	50-s-4	+	+	+	—	0.40	0.70	0.99
13	50-s-1	+	+	+	+	0.48	0.80	1.23
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i>								
34	(47-s-6)	+	+	+	—	0.28	0.34	0.46
<i>Lactobacillus sakei</i>								
15	ND	+	+	+	—	0.42	0.71	0.96
18	ND	+	+	+	—	0.40	0.70	0.97

¹ND = not determined/not subjected to identification by ribotyping; NI = not identified by ribotyping (ribogroup).

²6NaCl = ability to grow in 6% NaCl solution; 10B and 40B = ability to grow in 10 and 40% bile solution, respectively; ARG = arginine decarboxylase activity.

³The value expressed is the decrease in pH with respect to the initial pH of milk (6.05); the standard deviations of replicates were always lower than 0.05.

ese, 1981). In this study, lactobacilli generally showed resistance to the glycopeptide vancomycin, which is an antibiotic used in clinical situations when all others fail. This is certainly not new and agrees with the results reported by De Fabrizio et al. (1994). Resistance to vancomycin seems to be transferable; for example, the curing of vancomycin resistance from a *Lactobacillus acidophilus* strain by removal of plasmids has been reported (Vescovo et al., 1982).

A significantly high sensitivity (sensitivity of 100% or near to) was observed in antimicrobials belonging to group III (protein synthesis inhibitors), such as neomycin, clindamycin, kanamycin, streptomycin, and gentamicin, which bind to small or large ribosomal subunits. High resistance to aminoglycoside antibiotics seems to be considered to be intrinsic in the *Lactobacillus* genus and is attributed to the absence of cytochrome-mediated electron transport, which mediates drug uptake (Monteagudo-Mera et al., 2012). However, these results do not agree with those obtained by Curragh and Col-

lins (1992), who observed a high spontaneous mutation rate to streptomycin and kanamycin resistance.

The observed resistance among lactobacilli to the synthetic and wide-spectrum quinolones (group IV) nalidixic acid and the new-generation fluoroquinolone norfloxacin has already been mentioned (Halami et al., 2000). On the other hand, the observed resistance to metronidazole agrees with other studies (Charteris et al., 1998; Katla et al., 2001). This resistance could be due to the absence of hydrogenase activity (Church et al., 1996).

Lactobacillus spp. have decreased sensitivity to trimethoprim (group V) because of a trimethoprim-insensitive dihydrofolate reductase (Huovinen, 1987). In concordance with this, 26 *Lactococcus lactis* strains isolated from food by Teuber et al. (1999) were resistant to trimethoprim.

Cheeses produced from raw milk or with a starter including LAB resistant to antimicrobials may act as vectors in generating resistance to certain antibiotics in

bacteria infecting humans (Herrero et al., 1996; Teuber et al., 1999). The use of suitable starter cultures and appropriate substrates, such as pasteurized milk, for food fermentation are among the measures that could hinder the distribution of bacteria spreading resistance. In this context, among the isolates tested, those showing a low resistance index (i.e., ≤ 0.2) could be considered as candidates to be used in starter cultures.

Inhibition of foodborne pathogens by the LAB strains was clearly seen through the tests carried out (Table 2), but the nature of the inhibitory substances was not studied. Lactic acid bacteria are well known to produce antibacterial substances (i.e., organic acids, H_2O_2 , or bacteriocin-like substances), which can be beneficial for cheese preservation and the prevention of growth of foodborne pathogens (Stiles, 1996; Dunne et al., 2001). No apparent correlation was observed between antibiotic resistance and antimicrobial activity of the isolates tested. However, among the isolates studied, out of the strains showing the lowest antibiotic resistance (index ≤ 0.2), 4 also showed high antimicrobial activity (strain numbers 19, 20, 47, and 67).

Adaptation of LAB to NaCl allows these microorganisms to grow and survive under the restrictive conditions found in cheese. Studies of salt tolerance of lactobacilli have given contradictory results. Durlu-Ozkaya et al. (2001) and Siezen et al. (2010) found that most *Lb. plantarum* and (or) *paracasei* strains isolated from food, cheese included, were not able to grow in 6 to 6.5% NaCl solutions. However, Petrovic et al. (2006) reported that all the 43 lactobacilli strains isolated from several fermented foods (mostly cheeses) were tolerant of NaCl at 6%. In the present study, most of the *Lactobacillus* spp. isolated from Oaxaca cheese (89%) and, specifically, all the 4 above-mentioned valuable strains, were able to grow in the presence of NaCl at 6%.

The totality of lactobacilli isolated in the present study showed bile-salt resistance (at concentrations of 10 and 40%). In agreement with this, a considerable number of LAB strains have shown bile-salt resistance in other studies (Erkkilä and Petäjä, 2000; Moser and Savage, 2001). This property would confer LAB, together with acid resistance, the ability to reach the human intestine, which is a critical requirement for potential probiotic strains for use as cultures in fermented food (Ammor and Mayo, 2007). Furthermore, LAB cultures should be composed of amine-negative carboxylase strains. In this context, the activity of arginine decarboxylase appears to be a good indicator of the ability of LAB for generating biogenic amines in cheese (Schneller et al., 1997). Advantageously, all the strains tested in the present study except one [*Lb. brevis* (strain number 13)] were negative for arginine decarboxylase activity.

Finally, the good ability of LAB to produce lactic acid is one of most important characteristic for LAB strains to be used in starter cultures for the manufacture of unripened pasta filata cheeses (Coppola et al., 1990; Bernardeau et al., 2008). However, a potential culture should not only include LAB strains with high acid-producing activity, responsible for rapid curd acidification. Appropriate lactobacilli strains could, thus, be used as adjunct cultures (those added to cheese for purposes other than acid formation), to contribute to the elimination of defects by adventitious nonstarter LAB and improvement in cheese flavor (Wouters et al., 2002). In the present study, all the lactobacilli strains tested showed poor acid-producing ability in milk for the first 12 h of incubation. After 24 h, the strains were able to reduce the pH by approximately 1 U. These results agree with the low acidification ability found for lactobacilli by Cogan et al. (1997) and Morea et al. (1998). Moreover, Herreros et al. (2003) found that the acidification developed by strains of *Lb. plantarum* and *Lb. brevis* isolated from cheese was even lower.

CONCLUSIONS

Lactobacilli are an important part of the microbiota of raw milk Oaxaca cheese. Significant divergences in relevant characteristics among the lactobacilli isolated were observed, suggesting considerable intraspecies variability. According to the strains' characteristics studied, 4 strains could be selected to be used in an adjunct culture, together with a starter culture with high acidifying power, for the manufacture of pasteurized-milk Oaxaca cheese: *Lb. plantarum*, strain numbers 19, 20, 47, and 67. All 4 strains showed an antibiotic resistance index equal to or lower than 0.20, antimicrobial activity against the enterotoxigenic *Staph. aureus* strain, and *L. innocua*, amine-negative decarboxylase activity, and resistance to NaCl and bile salt solutions.

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