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Isolation and Partial Characterization of Halotolerant Lactic Acid Bacteria from Two Mexican Cheeses

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Abstract Isolated strains of halotolerant or halophilic lactic acid bacteria (HALAB) from *Cotija* and *doble crema* cheeses were identified and partially characterized by phenotypic and genotypic methods, and their technological abilities were studied in order to test their potential use as dairy starter components. Humidity, a_w, pH, and salt concentration of cheeses were determined. Genotypic diversity was evaluated by randomly amplified polymorphic DNA-polymerase chain reaction. Molecular identification and phylogenetic reconstructions based on 16S rRNA gene sequences were performed. Additional technological abilities such as salt tolerance, acidifying, and proteolytic and lipolytic activities were also investigated. The differences among strains reflected the biodiversity of HALAB in both types of cheeses. *Lactobacillus acidipiscis*, *Tetragenococcus halophilus*, *Weissella thailandensis*, and *Lactobacillus pentosus* from *Cotija* cheese, and *L. acidipiscis*, *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus farciminis*, and *Lactobacillus rhamnosus* from *doble crema* cheese were identified based on 16S rRNA. Quantitative and qualitative assessments showed strains of *T. halophilus* and *L. plantarum* to be proteolytic, along with *E. faecium*, *L. farciminis*, and *L. pentosus* to a lesser extent. Lipolytic activity could be demonstrated in strains of *E. faecium*, *L. pentosus*, *L. plantarum*, and *T. halophilus*. Strains belonging to the species *L. pentosus*, *L. plantarum*, and *E. faecium* were able to acidify the milk media. This study evidences the presence of HALAB that may play a role in the ripening of cheeses.

Keywords *Cotija* cheese · Lactic acid bacteria · Halotolerant · Isolation · Identification

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Introduction

The lactic acid bacteria (LAB) represent a group of organisms that are functionally related by their ability to produce lactic acid during homo- or heterofermentative metabolism. The acidification and enzymatic processes accompanying the growth of LAB impart the key flavor, texture, and preservative qualities to a variety of fermented foods, predominantly milk, and dairy products. The development of new starter cultures for the manufacture of fermented dairy products usually involves the identification and characterization of lactic acid bacteria from raw milk cheeses manufactured without commercial cultures [1]. The term LAB is not limited to a strictly defined taxonomic group of microorganisms, but it comprises a wide range of phylogenetically related genera of Gram-positive bacteria with several biochemical and ecological features in common [2].

Cheese made from unpasteurized milk and following traditional manufacturing procedures may possess a very diverse and rich microflora; the quality of the cheese depends to a great extent on the composition of that microflora. When cheese is produced following traditional procedures from raw milk, the environmental microflora plays a fundamental role in fermentation and is one of the most important parameters affecting the cheese quality. In addition, the biodiversity of bacteria involved in cheese production can be considered a fundamental factor for the maintenance of the typical features of traditional cheese products [3, 4].

Nonstarter lactic acid bacteria (NSLAB) usually increase from a low number in fresh curd to dominate the microflora of mature cheese [5]. In contrast to starters, NSLAB tolerate the hostile environment of cheese during ripening; this environment typically is characterized by 32% to 39% moisture, 4% to 6% salt in moisture, pH 4.9 to 5.3, 5–13 °C, and a deficiency of nutrients [6, 7]. The role of NSLAB in ripening has not been resolved satisfactorily yet, although inclusion of adjunct cultures of some strains of NSLAB or use of raw milk during cheese manufacturing increases the level of free amino acids, peptides, and free fatty acids, which leads to enhanced flavor intensity and accelerates cheese ripening [8–12].

Traditionally, most knowledge of bacterial diversity in cheese is derived from culturing studies, based on the growth of microorganisms on selective media and their subsequent identification at genus/species level using biochemical and phenotypic characterization. Although these methods may be reasonably sensitive, they do not always allow the discrimination of species or strains, nor the detection of the phylogenetic relationships among certain groups of bacteria. Hence, the application of molecular methods, in particular the PCR-based fingerprinting techniques, is useful in answering ecological questions [13–19].

Randomly amplified polymorphic DNA (RAPD) analysis is a PCR-based method which requires a short time compared with other genetic methods, provides good levels of discrimination, and is applicable to large numbers of strains [19, 20]. Analysis of ribosomal RNA gene sequence is a powerful technique for determining phylogenetic relationships and for rapid species identification [21]. Such information could allow selection of the most suitable strains to introduce as adjunct starters in pasteurized milk cheeses in order to reproduce more closely the flavor of raw milk cheeses or to accelerate cheese ripening [4, 19, 22].

Recently, a group of halophilic or halotolerant lactic acid bacteria (HALAB) present in the rinds of some ripened cheeses (blue, Brie, Camembert, and Tilsiter) was discovered [23]. This has given place to further investigations to try to know the role of these microorganisms for those cheeses. In these studies, cultivation and molecular methods have

been used to enumerate and identify [23–25]. However, saline media for the enumeration or isolation of LAB have not previously been applied to the analysis of the microbiota in cheese.

Proteolysis is the most important flavor-forming pathway in the ripening of many cheese varieties [26–28]. The complement of proteolytic enzymes in cheese originates from (1) residual indigenous milk proteinases, (2) residual coagulant, (3) proteinases and peptidases of starter LAB, and (4) proteinases and peptidases of adventitious NSLAB [29, 30]. Ripening involves a complex series of biochemical and probably some chemical events, which lead to the characteristic taste, aroma, and texture of each cheese variety. The biochemical changes, which occur during ripening, are caused by the synergistic action of a variety of enzymes, such as coagulant, indigenous milk enzymes, mainly proteinases and perhaps lipases, starter bacteria and their enzymes, and secondary microorganisms and their enzymes [31].

With the purpose to confirm the presence of HALAB in *doble crema* and *Cotija* cheeses, and to gain insight into their role in cheese ripening, isolation of HALAB using saline media, and phylogenetic and taxonomic characterization of these isolates were conducted.

Materials and Methods

Bacterial Strains and Growth Conditions

Two different types of Mexican cheeses, *Cotija* (QC) and *doble crema* (QDC) were used for the isolation of HALAB. The cheeses were made from raw milk and manufactured following traditional techniques [32]. Standard procedures were used to analyze cheeses for humidity content, a_w, and NaCl concentrations. The pH was measured by using a cheese slurry prepared from 2.5 g of cheese in 5 mL of water [19].

For isolation of HALAB, 1 g of the sample was taken from the interior of a cheese portion and homogenized in 9 mL of sterile water; aliquots of serially diluted suspensions were pour-plated with 5% and 7% NaCl glucose–yeast extract–peptone–meat extract (GYPC) isolation agar, and with 5% and 7% NaCl Man–Rogosa–Sharpe (MRS, Difco Laboratories, MI, USA) isolation agar. Plates were incubated at 32 °C for 48 h. The composition of the media GYPC was according to Ishikawa et al. [33] with the following modification: meat extract (Bioxon, México) was used instead of Bonito extract.

After incubation, lenticular colonies were picked up, and the isolates were purified three times with repeated pour-plating with GYPC or MRS agar for each cheese. The isolates were routinely propagated in 5% NaCl GYPC agar and stored in GYPC broth with 50% (*v*/*v*) glycerol as a cryoprotectant at −20 °C. Purity was checked by plating on corresponding agar media and microscopic examination.

Cultural characteristics of colonies were observed directly on the plates. For morphological characteristics, Gram staining was carried out by using a crystal violet/iodine solution (Hycel, México) following the procedure as described by Murray et al. [34].

Strain Identification

DNA Extraction

Cells were cultivated in 5% or 7% NaCl GYPC broth. The total genomic DNA for each isolated strain was extracted according to the Hoffman and Winston method [35].

Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction Typing

The randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis of selected morphotypes isolated from commercial cheeses was performed as described by Seseña et al. [36]. Primer with arbitrary sequence (10-mers) OPE-18 (5'-GGACTGCAGA-3'; Gibco BRL) was used in the amplification. Each RAPD-PCR reaction was performed with the following components: 40 ng of genomic DNA, 0.8 pM of primer, 0.2 mM of each dNTP (Fementas, Ontario, Canada), 2.5 μL 1× *Taq* Mg-free Buffer (Fermentas; 100 mM Tris–HCl, pH 8.8 at 25 °C; 500 mM KCl), 3 mM $MgCl_2$ (Fermentas), 1.5 U of *Taq* DNA polymerase (Fermentas; recombinant, 5 U/μL) and enough double distilled water to bring the volume to 25 μL. The PCR reactions were carried out in a DNA Thermal Cycler (GeneAmp® PCR System 9700, Applied Biosystems, USA) using the following amplification conditions: 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 38 °C, and extension for 1 min at 72 °C; the cycles were preceded by denaturation at 94 °C for 5 min and were followed by extension at 72 °C for 5 min.

The reaction products were analyzed by electrophoresis through 2.5% (*w*/*v*) agarose (Gibco BRL) in 1× TAE gels that were stained with ethidium bromide, and the DNA was detected by UV transillumination. The molecular sizes of the amplified DNA fragments were estimated by comparison with a 100-pb ladder DNA (Gibco BRL). The electrophoretic patterns were analyzed and compared. The RAPD-PCR patterns were grouped, and one representative strain was selected for 16S rRNA sequencing.

16S rRNA Gene Sequence Determination

Representative isolates from each group obtained in the analysis of RAPD-PCR patterns were amplified by PCR. Gene fragments specific for the 16S rRNA-coding regions of the isolates were amplified by PCR with the following two universal bacterial primers: 8 Forward (NVZ-1: 5'GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG3') and 1492 Reverse (NVZ-2: 5'GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T3') [37]. All amplifications were performed with a GeneAmp® PCR System 9700 (Applied Biosystems, CA, USA). Reaction mixtures contained 40 ng of template DNA, 2.5 μL 1× reaction buffer (Fermentas; 500 mM KCl, 100 mM Tris–HCl, pH 8.8 at 25 °C), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 pM of each primer and 1 U of *Taq* polymerase (Fermentas, Canada), adjusted to 25 μL. The following PCR conditions were used: an initial denaturation step at 94 °C for 7 min; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 150 s at 72 °C; and a final extension step at 72 °C for 10 min. The PCR products were electrophoresed and stained as mentioned above.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Sequencing was conducted under BigDye™ terminator cycling conditions and performed with the 3730XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) of Macrogen Inc. (Seoul, Korea), using the same primers.

Phylogenetic Analysis

Selected representative isolates obtained were subjected to phylogenetic analysis. Sequences from isolated bacteria were compared with the nonredundant GenBank library using BLAST search v. 2.2.3 [38] (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A collection of taxonomically

related sequences was obtained from the National Center for Biotechnology Information Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/-taxonomyhome.html/) and Ribosomal Database Project-II Release 9 (http://rdp.cme.msu.edu). Multiple alignment analyses were performed with CLUSTAL_X v. 2.0.10 [39], and edited and confirmed visually in BIOEDIT v. 7.0.9.0 [40].

Maximum likelihood analyses were performed using PhyML v. 3.0. [41] (http://atgc.lirmm.fr/phyml/). jModelTest 0.1.1. [42] was used to select appropriate models of sequence evolution by the AIC model [43]. The GTR+I+G model (α=0.469 for the gamma distribution; A=0.276, C=0.189, G=0.302, T=0.233; p-inv=0.432) was selected for the tree search. The confidence at each node was assessed by 1,000 bootstrap replicates [44]. *Staphylococcus aureus* was used as outgroup. The nucleotide similitude percentages among sequences were calculated using BioEdit v. 7.0.9.0. software [40]. The limits for genus and species were set at 95% and 97%, respectively [45, 46]. Sequences of this study were deposited in the GenBank database.

Physiological and Biochemical Characteristics

Differentiation of Similar Strains

Tests of acid production from carbohydrates and polyols to differentiate between the strains identified as *Lactobacillus pentosus* and *Lactobacillus plantarum* was performed [47]. Filter-sterilized dextrose, glycerol, sorbose, and xylose (1% *w*/*w*) without glucose were added to 4% NaCl GYPC broth.

Determination of the Adequate NaCl Concentration for Growth

To determinate the optimum NaCl concentration and range for growth, tubes with 20 mL of 4% NaCl GYPC broth was inoculated with each isolated strain and incubated 48 h at 32 °C. After the incubation period, 0.5 mL was inoculated into GYPC broths added with NaCl concentrations from 0% to 10% (*w*/*v*) at 1% intervals. The occurrence of growth was judged by optical density (OD_{590}) at 24 and 48 h.

Acidifying Capacity of the Isolates

The acidifying capacity was determined on isolates by recording the pH variations of milk cultures. Activated cultures (in 4% NaCl GYPC) were inoculated at 1% (*v*/*v*) in 10 mL sterile reconstituted skimmed milk (RSM; Difco) incubated at 32 °C, and the pH was measured after 24- and 48-h incubation periods [4].

Quantitative and Qualitative Assessment of Proteolytic Activity

The gelatin (Merck, Germany) liquefaction test was conducted by following Cowan and Steel [48], using 4% NaCl GYPC broth supplemented with 12% (*w*/*v*) gelatin.

Revitalized 4% NaCl GYPC broth cultures were inoculated at 1% (*v*/*v*) in 10-mL volumes of sterile reconstituted skimmed milk (Difco) and incubated at 32 °C for 7 days. Uninoculated milk was also incubated as control. After incubation, an aliquot of 2.5 mL of each lactic culture was removed, proteins were precipitated with 0.75 N trichloroacetic acid (TCA) while agitating the test tube to mix thoroughly the coagulated milk, and the samples were prepared according to the method of Church et al. [49] to determine the

Table 1 Humidity, a_w, NaCl concentrations, and pHs of the cheese samples

Cheese sample	Humidity (%)	a_w	NaCl (%)	pH
Doble crema (QDC)	48.2	0.972	5.34	4.027
Cotija (QC)	39.5	0.862	6.36	6.290

proteolytic activity with *o*-phthaldialdehyde (Sigma-Aldrich). The mixture was filtered. The filtrate (50 μL) was added to 1 mL of OPA reagent, and after 2 min at room temperature (20 °C), absorbance of the solution was measured by a spectrophotometer (Jenway, UK; model 6405UV/Vis) at 340 nm. The proteolytic activity of these bacterial cultures was expressed as the absorbance of free amino groups measured at 340 nm. A relative degree of proteolysis was determined as the difference between proteolytic activities in fermented milk to that of unfermented milk. All the analyses were carried out in triplicate.

Casein hydrolysis test was performed by a qualitative assay using 10% (*v*/*w*) skimmed milk (Difco) added to 4% NaCl GYPC agar. Plates were inoculated for each isolated strain and incubated at 32 °C for 48 h. Clear zones around colonies were recorded as a positive result [50, 51].

Quantitative and Qualitative Assessment of Lipolytic Activity

Lipase activity in cell-free culture supernatants was measured according to Smeltzer et al. [52]. A 0.5% (*v*/*v*) suspension of Tributyrin (Sigma, MO, USA) in 100 mM Tris (pH 8.0; 25 mM $CaCl_2$) was prepared. To stabilize the aqueous emulsion of tributyrin, an emulsification reagent was used, the active agent of which was gum Arabic (Merck, Germany) [53]. The suspensions were emulsified by sonication. Spectrophotometric assays were initiated by adding 1.0 mL of the prewarmed (50 °C) tributyrin emulsion to 100 μL of test solution dispensed into spectrophotometric cuvettes. The reaction was monitored at room temperature (21–23 °C) by measuring the optical density of the emulsion at 450 nm. Additionally, plate assays were performed by adding standard amounts of each test solution into tributyrin agar (Merck, Germany). The plates were incubated at room temperature. Tributyrin degradation by the microorganisms is indicated by clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium. The activity was evaluated by measuring the diameter of these zones.

Table 2 Isolates microorganisms from QC and QDC cheeses samples from GYPC and MRS agar (with 5% and 7% NaCl)

	QC		QDC	
	GYPC	MRS	GYPC	MRS
5% NaCl	24	16	16	8
7% NaCl	18	12	14	18
Total	70		56	

QC Cotija cheese, *QDC doble crema* cheese

Table 3 Isolates for DNA extraction

	QC		QDC	
	GYPC	MRS	GYPC	MRS
5% NaCl	11 r	2 r	14 r	5 r
	2 c		1 c	
7% NaCl	7 r	1 r	8 r	13 r
	1 c	2 c		
Total	21	5	23	18
	26		41	

QC Cotija cheese, *QDC doble crema* cheese, *r* rods, *c* cocci

Results

Growth Conditions and Strains Isolation

The humidity, a_w, NaCl concentration, and pH values for the two cheeses are shown in Table 1. No off-odors or sensorial defects were detected in any of the samples.

Occurrence of HALAB was observed in both cheese samples evaluated by pour-plating. A total of 70 strains from GYPC and MRS plates were isolated from QC, while for QDC, a total of 56 strains from both media were isolated (Table 2). The principal phenotypic and morphological features of strains from each isolation media were determined. For DNA extraction, yeasts were discarded by Gram staining and microscopical observation. All of the isolates were Gram-positive and included 61 rods and 6 cocci (see Table 3 for details).

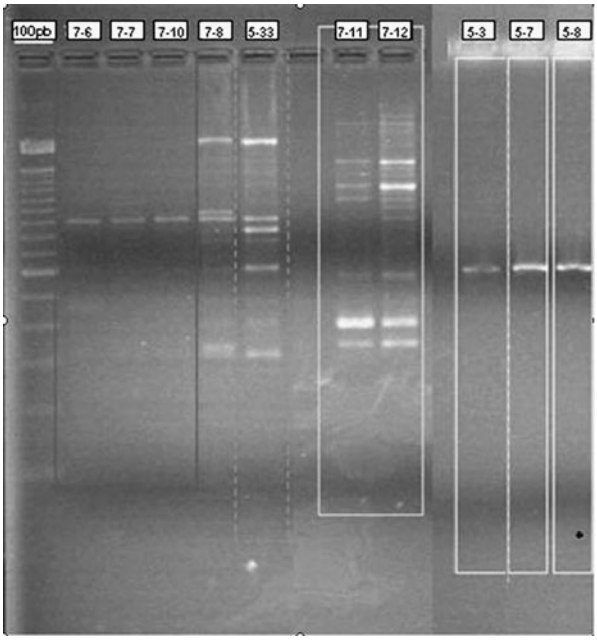

Fig. 1 Major RAPD-PCR patterns of isolated strains obtained with primer OPE-18. The 2.5% (*w*/*v*) agarose gels were stained with ethidium bromide

Table 4 Isolates grouped by RAPD-PCR patterns

Isolates								
Grouped patterns						Single patterns		
a)	5–17	5–20	5–23	5–24	5–28	7–1	7–25	5–19
b)	7–11	7–12	7–33	5–26		7–2	7–26	5–22
c)	5–15	5–27	5–30	5–35		7–3	7–28	5–31
d)	7–14	7–15	7–19	5–21		7–4	7–31	5–32
e)	7–6	7–7	7–10			7–8	7–35	5–33
f)	5–3	5–7	5–8			7–9	5–2	5–34
g)	5–9	5–12	5–13			7–13	5–4	5–36
h)	7–5	5–25				7–16	5–5	
i)	7–29	5–29				7–17	5–10	
j)	7–20	7–22				7–18	5–11	
k)	7–32	7–36				7–21	5–14	
l)	5–1	5–6				7–24	5–18	

DNA Extraction and RAPD-PCR Screening

DNA was isolated from selected colonies from different culture media (Table 3), and the templates were used for RAPD-PCR analysis.

RAPD-PCR patterns of the 67 isolates were grouped and analyzed by observation of the ethidium bromide-stained 2.5% (*w*/*v*) agarose gel. Twenty-four different genotypes (profiles) were observed (Fig. 1). Thirty-one isolates produced a unique RAPD pattern (Table 4).

16S rRNA Gene Sequence Determination and Phylogenetic Analysis

Representative isolates of each RAPD-PCR group were subjected to direct sequencing of amplified 16S rRNA gene fragments (Table 5) and compared with those of other related bacteria from public databases (NCBI) in the phylogenetic tree (Fig. 2). Fourteen isolates identified as *L. plantarum* and *L. pentosus* using 16S rRNA sequence identification were discriminated further by sugar fermentation tests. The results of these fermentation assays are shown in Table 6.

The results of molecular identification of the QC and QDC isolates are presented in Tables 7 and 8, respectively.

Table 5 Isolated strains selected for PCR amplification and sequencing of 16S rRNA gene fragment

	QC		QDC	
	GYPC	MRS	GYPC	MRS
5% NaCl	9	1	7	4
7% NaCl	5	3	3	12
Total	18		26	

QC Cotija cheese, *QDC doble crema* cheese

Fig. 2 Phylogenetic relationships between the cheeses isolates and other related bacteria, according to 16S rRNA gene sequences. The tree, constructed using maximum likelihood (-lnL=10439), is based on comparison of approximately 1,500 bps. Bootstrap values, expressed as percentage of 1,000 replications, are given at *branching points*; only values >50% are shown

Physiological and Biochemical Characterization of the Isolates

Further characterization of the isolates, which included acidifying, proteolytic and lipolytic activities, was carried out in order to evaluate their potential to be used as indigenous starter cultures. The values obtained for the first test (acidifying activity) are shown in Table 9. All isolates have this capacity with the exception of the strains of *Weissella thailandensis* and

Table 6 Acid production from carbohydrates of the bacterial strains isolated from *Cotija* and *doble crema* cheeses

Isolated strain	Carbohydrate source				Identification
	D(+)-dextrose	Glycerol	L(−)-sorbose	D(+)-xylose	
5–10	+	+	−	+	*L. pentosus*
5–20	+	−	−	−	*L. plantarum*
5–22	+	−	−	−	*L. plantarum*
5–30	+	−	−	−	*L. plantarum*
5–31	+	−	−	−	*L. plantarum*
5–36	+	+	−	+	*L. pentosus*
7–2	+	+	−	+	*L. pentosus*
7–9	+	−	−	−	*L. plantarum*
7–11	+	−	−	−	*L. plantarum*
7–13	+	−	−	−	*L. plantarum*
7–18	+	+	−	+	*L. pentosus*
7–21	+	+	−	+	*L. pentosus*
7–31	+	+	−	+	*L. pentosus*
7–32	+	−	−	−	*L. plantarum*

Lactobacillus acidipiscis. The most acidifying microbial species was *L. plantarum* and *L. pentosus*, which were able to reduce the pH of skim milk from its normal value of about 6.2 to less than 3.8 and 4.4 in 24 h at 32 °C, respectively. These species are known for their good technological properties. The isolates tested in this study could be classified as moderate acidifying strains. *Enterococcus faecium* tested in this study was a slow acidifying strain. The results of a quick sensory evaluation of milks inoculated and incubated with the different isolates are shown in Table 9. The yogurt-like odor was the most frequently perceived.

The OD_{590} of broth cultures incubated at 32 °C for 24 and 48 h indicated that the strains of *L. acidipiscis* and *Tetragenococcus halophilus* were slightly halophilic, and their optimal NaCl concentrations for growth were between 2.0% (*w*/*v*) to 4.0%, and 4.0% to 8.0%, respectively, while *E. faecium*, *L. pentosus*, and *L. plantarum* were slightly halotolerant (Fig. 3).

Proteolytic Activity

Table 10 shows the qualitative proteolytic activity of the identified isolates from both cheeses. All the isolates grew in 10% skimmed milk–4% NaCl GYPC agar, but the ability to hydrolyze casein was confirmed for *E. faecium*, *L. farciminis*, *L. pentosus*, *L. plantarum*, *T. halophilus*, and slightly *W. thailandensis*.

The most proteolytic strains at 32 °C were *T. halophilus* and *L. plantarum* (Fig. 4). The other tested isolates demonstrated poor proteolytic activity. These two species have been classified as NSLAB, and it has been demonstrated that these bacteria contributed to development of the cheese flavor [54]. The extent of proteolysis varied among strains and appeared to be time-dependent. As depicted in Fig. 4, the amount of liberated amino groups increased during fermentation from 3 to 21 days for some strains.

Table 7 Identification of bacteria isolated in GYPC and MRS media from DCC according to 16S rRNA gene sequence

Isolated strain	Best match database[a] (GenBank accession number)	Similarity[b] (%)
5–1 QC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.5
5–2 QC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.1
5–3 QC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	98.2
5–4 QC/GYPC 5%	*Tetragenococcus halophilus* T3 (EU522085)	99.8
5–5 QC/GYPC 5%	*Weissella thailandensis* FS45-1 (AB023839)	95.8
5–9 QC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.1
5–10 QC/GYPC 5%	*Lactobacillus pentosus* NRIC1555 (AB362712)	100.0
5–11 QC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.2
5–13 QC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.1
5–14 QC/MRS 5%	*Weissella thailandensis* FS45-1 (AB023839)	99.6
7–1 QC/GYPC 7%	*Lactobacillus acidipiscis* NBRC102163 (AB326356)	98.6
7–2 QC/GYPC 7%	*Lactobacillus pentosus* NRIC1555 (AB362712)	100.0
7–3 QC/GYPC 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.5
7–4 QC/GYPC 7%	*Lactobacillus acidipiscis* NBRC102163 (AB326356)	98.1
7–7 QC/GYPC 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.8
7–8 QC/GYPC 7%	*Tetragenococcus halophilus* T3 (EU522085)	99.8
7–25 QC/MRS 7%	*Weissella thailandensis* FS45-1 (AB023839)	98.3
7–26 QC/MRS 7%	*Weissella thailandensis* FS45-1 (AB023839)	99.0
7–28 QC/MRS 7%	*Tetragenococcus halophilus* T3 (EU522085)	99.8

[a] The best match was selected using the closest sequence from the phylogenetic tree

[b] Similarity percentage was estimated by considering the number of nucleotide substitutions between a pair of sequences divided by the total number of compared bases × 100% [71]

Lipolytic Activity

The assay for tributyrin hydrolysis using agar showed that isolates *E. faecium*, *L. pentosus*, *L. plantarum*, and *T. halophilus* exhibited lipolytic activity. Strains that showed enzymatic activities using the qualitative assay were subjected to quantitative determination of substrate hydrolysis.

Lipase of *Candida rugosa* caused a gradual clearing of the tributyrin emulsion that could be monitored spectrophotometrically at 450 nm. Figure 5 shows the construction of a standard curve by using lipase (from *C. rugosa*); the substrate was 8.5 mM tributyrin. Figure 6 shows tributyrin degradation as a function of time for each isolated strain. Table 11 shows the results of the quantitative assays for lipolytic activity.

Discussion

Most of the studies on cheeses microbiota analysis have been performed with non-salt media [14, 55]. The knowledge of HALAB communities in cheeses and their proteolytic, lipolytic, and acidifying capacities provides an insight of the role of these species on the maturation process. There are no many studies about the presence of HALAB in cheeses and their potential role as new starter cultures for high salt cheeses [23, 24].

Table 8 Identification of bacteria isolated in GYPC and MRS media from DCC according to 16S rRNA gene sequence

Isolated strain	Best match database[a] (GenBank accession number)	Similarity[b] (%)
5–18 QDC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	90.4
5–19 QDC/GYPC 5%	*Enterococcus faecium* IMAU10052 (FJ915708)	100.0
5–20 QDC/GYPC 5%	*Lactobacillus plantarum* EW-p (EU096230)	76.4
5–22 QDC/GYPC 5%	*Lactobacillus plantarum* EW-p (EU096230)	99.3
5–25QDC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	100.0
5–29 QDC/GYPC 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	100.0
5–30 QDC/GYPC 5%	*Lactobacillus plantarum* EW-p (EU096230)	100.0
5–31 QDC/GYPC 5%	*Lactobacillus plantarum* EW-p (EU096230)	100.0
5–32 QDC/MRS 5%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	100.0
5–33 QDC/MRS 5%	*Lactobacillus farciminis* Al2-3 (DQ056421)	99.6
5–34 QDC/MRS 5%	*Lactobacillus rhamnosus* MH22 (FJ409226)	100.0
5–36 QDC/MRS 5%	*Lactobacillus pentosus* NRIC1555 (AB362712)	100.0
7–9 QDC/MRS 7%	*Lactobacillus plantarum* EW-p (EU096230)	99.2
7–11 QDC/MRS 7%	*Lactobacillus plantarum* EW-p (EU096230)	99.1
7–13 QDC/MRS 7%	*Lactobacillus plantarum* EW-p (EU096230)	99.6
7–16 QDC/MRS 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	100.0
7–17 QDC/MRS 7%	*Lactobacillus farciminis* Al2-3 (DQ056421)	99.6
7–18 QDC/MRS 7%	*Lactobacillus pentosus* NRIC1555 (AB362712)	100.0
7–19 QDC/MRS 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.7
7–20 QDC/MRS 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.0
7–21 QDC/MRS 7%	*Lactobacillus pentosus* NRIC1555 (AB362712)	100.0
7–24 QDC/MRS 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	99.4
7–31 QDC/GYPC 7%	*Lactobacillus pentosus* NRIC1555 (AB362712)	100.0
7–32 QDC/GYPC 7%	*Lactobacillus plantarum* EW-p (EU096230)	99.1
7–35 QDC/GYPC 7%	*Lactobacillus acidipiscis* NBRC102164 (AB326357)	97.7

[a] The best match was selected using the closest sequence from the phylogenetic tree

[b] Similarity percentage was estimated by considering the number of nucleotide substitutions between a pair of sequences divided by the total number of compared bases × 100% [71]

Table 9 Milk acidifying activity of identified strains

Isolated strain	pH		Odor
	24 h	48 h	
L. pentosus	4.405	4.586	Yogurt
L. plantarum	3.856	3.723	Yogurt
W. thailandensis	5.846	5.424	Milk
E. faecium	5.198	5.012	Yogurt
L. acidipiscis	5.615	5.539	Milk
T. halophilus	5.137	5.067	Yogurt
Sterile skimmed milk	6.213		

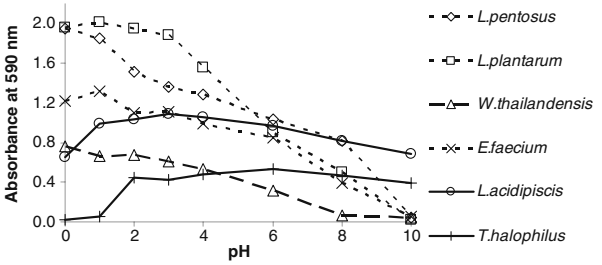

Fig. 3 Growth behavior of strains isolated from CC and DCC cheeses in GYPC medium supplemented with NaCl

HALAB were isolated from two Mexican cheeses (*Cotija* and *doble crema*) by cultivation on 5% and 7% NaCl GYPC and MRS isolation agar. The diversity of indigenous strains was explored by RAPD-PCR, and representative isolates were identified by sequence analyses of 16S rRNA genes.

The NaCl concentrations of the cheese samples ranged from 5.34% (*w*/*w*) for QDC and 6.36% for QC (Table 1). Considering the water content and a_w of cheeses, the actual NaCl concentrations in the aqueous phases of the cheese samples might have been sufficiently high to yield favorable osmotic conditions for growth of HALAB [23]. In particular, the concentrations of NaCl present at some levels in cheeses might have permitted *L. acidipiscis* and *T. halophilus* to grow, as they were absolutely halophilic. The stability of foods is inversely related to moisture content. Cheese is a medium-moisture food, containing about 30–50% moisture. The water activity (a_w) of cheese varies from 0.98 to 0.87, and these values are highly correlated with the total nitrogen and ash content (mainly NaCl). Biochemical reactions that occur during the ripening of cheese contribute to the depression of a_w by increasing the number of dissolved low molecular weight compounds and ions [56].

The pH values of the cheese samples were 4.027 for QDC and 6.290 for QC. In general, the pH of cheeses increases during ripening from an initial value of about 5.0 to a final level of >7.5 (hard cheeses) or of 7.0 (mold-ripened soft cheeses). This increase of pH during the ripening process likely provides pH conditions under which HALAB can initiate growth [23].

RAPD-PCR was used as a typing molecular tool to distinguish between different isolated strains, on the assumption that each strain displays a characteristic profile. In the present study, the genetic diversity of 26 isolates from *Cotija* cheese and 41 from *doble crema* cheese manufactured following traditional techniques was investigated using RAPD-PCR. This method turned out to be rapid and sensitive, as has been widely reported by other authors [57–59].

Table 10 Qualitative proteolytic activity of the bacteria isolated from CC and DCC cheeses

Isolate	Casein hydrolysis	Gelatin liquefaction
E. faecium	+	−
L. acidipiscis	−	−
L. farciminis	+	+
L. pentosus	+	+
L. plantarum	+	+
L. rhamnosus	−	+
T. halophilus	+	−
W. thailandensis	+	−

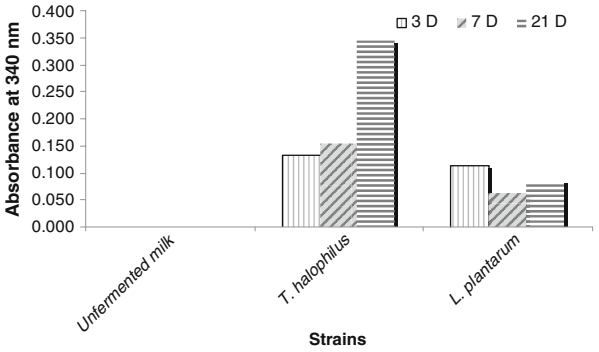

Fig. 4 Changes in extent of proteolysis (A_{340}) of TCA filtrates prepared from RSM fermented by isolated bacteria at 32 °C. *3D* 3 days, *7D* 7 days, *21D* 21 days

In this work, *L. acidipiscis*, *T. halophilus*, *W. thailandensis*, and *L. pentosus* were detected from *Cotija* cheese, while from *doble crema* cheese, *L. acidipiscis*, *E. faecium*, *L. plantarum*, *L. farciminis*, *Lactobacillus rhamnosus*, and *L. pentosus* were isolated. The relative abundance of certain species and, especially, the heterogeneity of NSLAB strains in cheese may determine the relationships between NSLAB and cheese flavor [19, 60].

L. acidipiscis was isolated from both cheeses; this strain has the capacity to grow in the saline conditions of the used culture media and was present at a high proportion in the phylogenetic tree.

The presence of *E. faecium* during the maturation suggests that these bacteria may play an important role in the ripening of this type of cheeses. Enterococci have been reported to

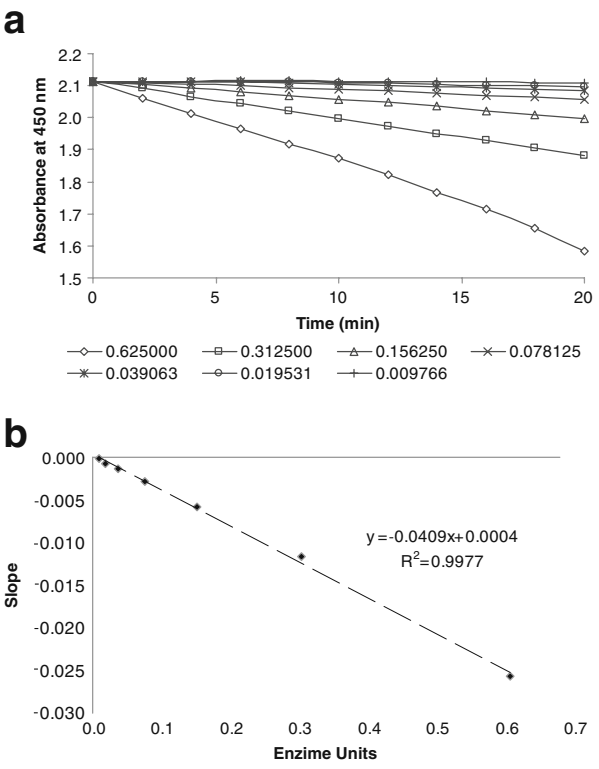

Fig. 5 Kinetics of the tributyrin degradation of cheese bacterial strains supernatants. The substrate was 8.5 mM tributyrin. **a** Decrease in absorbance over time with lipase concentrations of 0.625 (◊), 0.3125 (□), 0.1562 (▲), 0.0781 (□), 0.0390 (ж), 0.0195 (o), and 0.0097 U/mL (|). **b** Linear relationship between reaction rate and enzyme concentration

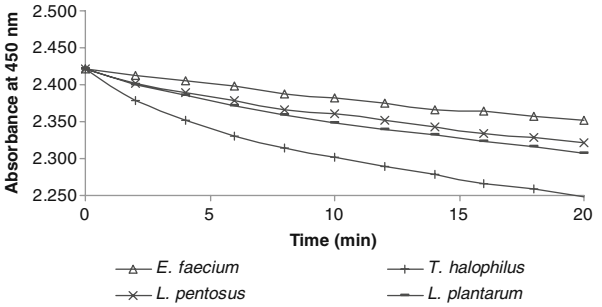

Fig. 6 Kinetics of the tributyrin degradation of bacterial strains isolated from cheeses

be one of the most resistant microbiological groups to adverse conditions such as salt and acidity, which explains their predominance in several cheeses [1]. The role of enterococci as a relevant component of natural cultures involved in the fermentation of artisanal cheeses has been described in detail [61], and *E. faecium* has been included in the list of the LAB starters by the International Dairy Federation [4].

We assessed the proteolytic activities of selected LAB in RSM as presented in Fig. 4. During fermentation, milk proteins were hydrolyzed by LAB proteinases and peptidases resulting in an enhanced amount of free amino groups and peptides. These findings were consistent with those reported by Nielsen et al. [62]. Juillard et al. [63] reported that the level of free amino acids and peptides in milk is low; therefore, LAB depend on a proteolytic system that allows for an efficient degradation of milk proteins. The presence of aminopeptidases is important for the release of amino acids for growth by microorganisms through the hydrolysis of peptides in the growth medium.

Similar to our findings, those of Shihata and Shah [64], also showed that the amount of free amino groups formed in the medium during fermentation was strain-dependent. Thus, the differences in the amounts of amino groups released during fermentation of milk observed for the microorganisms could probably relate to the different proteinases of the strains.

The proteolytic activity seems to be essential for LAB growth in milk, and in addition, it is involved in the development of some sensorial characteristics in different fermented milk products [65, 66].

The production of high quality fermented dairy products is dependent on the proteolytic system of the starter bacteria used, since peptides and the amino acids formed have a direct impact on flavor or serve as flavor precursors [67, 68]. The impact of cell lysis on the proteolysis rate, and its possible contribution to the decrease in bitterness through the hydrolysis of peptides, has been reported [69].

A few of the HALAB strains tested showed lipolytic activity when assayed on tributyrin agar. This low lypolitic activity of LAB may represent an important advantage when they are used as starter cultures, as only a slight breaking of the milk fat during maturation is enough to induce aroma production without giving a rancid flavor in cheese [70].

Table 11 Lipolytic activity of the identified isolates

Isolate	U/mL
E. faecium	95.35
L. pentosus	126.25
L. plantarum	141.59
T. halophilus	203.16

Results from this study allow us to conclude that HALAB isolates *E. faecium*, *L. pentosus*, *L. plantarum*, *W. thailandensis*, *T. halophilus*, and *L. farciminis* could be selected to be included as adjunct starter cultures, since they fulfilled the customary requirements for cheesemaking. These isolates must be further evaluated as a part of several mixed starter cultures, in order to determine their growth dynamics and the influence on flavor formation during ripening. The use of selected strains obtained from raw milk cheeses as starters or adjunct cultures in the manufacture of pasteurized milk cheeses would be advisable.

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