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Characterization of natural isolates of *Lactobacillus* strains to be used as starter cultures in dairy fermentation

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Abstract

The technological relevant characteristics of five homofermentative lactobacilli strains, isolated from natural fermented hard cheeses, were studied. Isolates CRL 581 and CRL 654, from Argentinian artesanal hard cheeses, and isolates CRL 1177, CRL 1178, and CRL 1179, from Italian Grana cheeses, were identified as *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus*, respectively, by physiological and biochemical tests, SDS–PAGE of whole-cell proteins and sequencing of the variable (V1) region of the 16S ribosomal DNA. All strains showed high levels of β-galactosidase activity. However, proteolytic activity varied widely among isolates. Strains CRL 581, CRL 654, and CRL 1177 hydrolyzed α- and β-caseins and were able to coagulate reconstituted skim milk in less than 16 h at 42°C. According to the substrate specificity, these proteinases have a caseinolytic activity comparable to that of the P_{III}-type of lactococcal proteinases. No strains produced inhibitor substances (bacteriocin) and all were insensitive to attack by 14 *L. helveticus*- and *L. delbrueckii* subsp. *lactis*-specific bacteriophages. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Lactobacillus helveticus*; *Lactobacillus delbrueckii* subsp. *lactis*; Starter cultures

1. Introduction

There is a continued need to improve existing starter cultures or to select new strains to be used as starters in specific fermentation processes and to develop new products. In Argentina, hard cheese production is mainly based on the use of natural whey starters, which are prepared daily at cheese factories using a technique similar to those used in Italy for Grana cheese (Reinheimer et al., 1996). These starter cultures are complex microbial associations, normally composed of thermophilic lactobacilli like *L. delbrueckii* subsp. *lactis* and *Lactobacillus helveticus* (Quiberoni et al., 1998). Fortina et al. (1998) observed a wide variability within *L. helveticus* strains isolated from natural cheese starters. Therefore, characterizing starter cultures at the strain level could be very useful to the dairy industry.

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In dairy fermentations, a stable predictable rate of acid production is required, so the ability to metabolize lactose by the starter strains is one of the most important properties. Other important metabolic properties of starter cultures are proteolytic activity, bacteriocin production, susceptibility to bacteriophage attack, and extracellular polysaccharide production. It is well known that many of these biotechnologically important properties of lactic acid bacteria, mainly lactose metabolism and proteolytic activity, are associated with plasmids (Kok, 1990), so their presence in starters also needs to be investigated. Therefore, the aim of this work was to evaluate the biotechnologically relevant characteristics of five strains isolated from spontaneous fermented products, identified as *L. delbrueckii* subsp. *lactis* and *L. helveticus*, in order to determine their potential in the development of new starter cultures.

2. Materials and methods

2.1. Microorganisms and growth conditions

The microorganisms used in this study belong to the culture collection of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentine), and are listed in Table 1. Reference strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD); the Centre National de la Recherche Zootechnique (CNRZ, Jouy-en-Josas, France); and the National Collection of Dairy Organisms (NCDO, Aberdeen, Scotland). The microorganisms, stored at −20°C in 10% reconstituted skim milk (RSM) containing 0.5% yeast extract (YE) (Difco Laboratories, Detroit, MI) and 10% glycerol, were grown in MRS broth (De Man et al., 1960) or RSM at 42°C for 16–18 h unless otherwise indicated. When necessary, the medium was supplemented with 1.5% agar (Difco).

2.2. Identification of isolated lactobacilli

2.2.1. Phenotypic and biochemical tests

Selected isolates of lactobacilli were identified by phenotypic and biochemical tests. These included Gram reaction; oxidase reaction; reduction of 1% (w/v) nitrate in MRS broth; bacterial growth, recorded at 15, 37 and 45°C; gas production from glucose; and hydrolysis of arginine (in MRS broth without glucose and meat extract but supplemented with 0.3% arginine (Sigma, St. Louis, MO, USA); NH_3 production was tested by addition of Nessler's reagent. Isomers of lactic acid were evaluated by an enzymatic method (Gawehn and Bergmeyer, 1974; Gutmann and Walhlefeld, 1974). The sugar fermentation pattern was determined by using the API 50 CH as specified by the manufacturer (API-BioMérieux, Marcy l'Etolie, France).

Table 1
Microorganisms studied[a]

Strain (CRL code)	Synonym and origin
Lactobacillus delbrueckii subsp. *lactis*	
CRL 581	Argentinian hard cheese
CRL 654	Argentinian hard cheese
CRL 960	ATCC 12315[T]; CNRZ 207[T]; Orla-Jensen's *Thermobacterium lactis* 10, Swiss Emmenthal cheese
CRL 934	ATCC 8000
Lactobacillus helveticus	
CRL 974	ATCC 15009[T]; Orla-Jensen's *Thermobacterium* sp. strain 12, Swiss Emmenthal cheese
CRL 1062	NCDO 30; French Gruyère de Comté cheese
CRL 1176	ATCC 15807; CNRZ 328; Emmenthal cheese
CRL 1177	Italian Grana cheese
CRL 1178	Italian Grana cheese
CRL 1179	Italian Grana cheese

[a] ATCC, American Type Culture Collection; CNRZ, Centre National de la Recherche Zootechnique; NCDO, National Collection of Dairy Organisms; CRL, Centro de Referencia para Lactobacilos; [T], type strain.

2.2.2. SDS–PAGE of total soluble cell proteins

Cultures were grown on MRS broth at 42°C for 16 h. Soluble whole-cell proteins were prepared by suspending washed cell pellets ($\approx$100 mg wet weight) in 1 ml of sample treatment buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). Cells were disrupted with glass beads (0.10–0.11 mm, Sigma) on a vortex mixer for 6 min. After lysis, the cell suspensions were heated for 10 min at 100°C and the soluble protein fraction of the supernatant was obtained after centrifugation (10 000 $\times$ *g* for 10 min, 4°C). Protein concentration was determined spectrophotometrically with a Protein Assay (Bio-Rad Laboratories, Richmond, VA, USA) according to the manufacturer's instructions. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) on vertical slab gels (18 $\times$ 13 $\times$ 0.75 cm). Resolving and stacking gel conditions were 12 and 4% acrylamide, respectively (Bio-Rad). Molecular weight markers were purchased from Sigma. Gels were run for 12 h at 7 mA and the proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma). The reproducibility of the SDS–PAGE technique was estimated by including duplicate runs of a single protein extract on separate gels.

2.2.3. Polymerase chain reaction

Total cellular DNA was isolated according to De los Reyes-Gavilán et al. (1992). Oligonucleotide primers (PLB16, 5′-AGAGTTTGATCCTGGCTCAG-3′; and MLB16, 5′-GGCTGCTGGCACGTAGTTAG-3′) were used to amplify the variable (V1) region of the 16S ribosomal RNA gene, as described by Kullen et al. (1999). Primers were synthesized by The Great American Gene Company (Ramona, CA). Briefly, 100 ng of genomic DNA were amplified in a total volume of 50 μl of PCR reaction mixture containing 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$, 1.0 μM of each primer, 50 mM KCl, and 10 mM Tris–HCl, pH 8.3, and 1 U Taq polymerase (Promega, Madison, WI). The PCR tubes were placed in a DNA Thermal cycler 480 (Perkin-Elmer, Norwalk, CT, USA) and the reaction started by denaturation for 5 min at 94°C followed by 30 cycles at 94°C for 1 min, annealing at 48°C for 30 s, and extension at 72°C for 30 s. A final 10 min incubation at 72°C was allowed for the completion of primer extension after the last cycle. PCR products were analyzed by electrophoresis on a 1.2% agarose gel at 70 V for 2 h, followed by a 30 min staining in 0.5 μg/ml ethidium bromide solution and a 15-min de-staining in distilled water, with a final visualization and photography under UV light. Amplicons were excised from the gel and purified using a Prep-A-gene Kit (Bio-Rad). Purified PCR products were resuspended in 20 μl of TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0), and sequenced at the BioResource Center Cornell University (Ithaca, NY). DNA homology searches were performed on line with the BLAST program (Altschul et al., 1990). The sequence of the partial 16S rRNA of *L. delbrueckii* subsp. *lactis* and *L. helveticus* were submitted to the GenBank database (Accession numbers AF213703 and AF213704, respectively).

2.3. Coagulation tests

Ten milliliters of an overnight culture of cells grown in MRS broth were harvested by centrifugation at 10 000 $\times$ *g* for 15 min, washed twice with 50 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer to the original volume. The cell suspensions were used to inoculate (2%) the following media: RSM; RSM supplemented with 1% glucose (RSM-G); RSM supplemented with 0.25% casein enzymatic hydrolysate (RSM-CH) (Difco); and RSM supplemented with 0.25% yeast extract (RSM-YE). Fast coagulating strains were defined as those able to coagulate RSM at 42°C within 16 h, while slow variants require a longer period of time (more than 36 h). Both fast and slow strains were assayed for their caseinolytic and β-galactosidase activities (see below).

2.4. Proteolytic activity

Proteolytic activity of overnight milk-grown cultures was determined by measuring, at 340 nm, the hydrolyzed milk proteins using the *o*-phthaldialdehyde (OPA) spectrophotometric assay (Church et al., 1983). The results are expressed as μg/ml glycine after comparison with a calibration line. Proteolytic activity was also measured by SDS–PAGE, according to the modification described by Hébert et al. (1997), using as substrates α- and β-caseins (Sigma). The densitograms and the per-

centages of hydrolysis were determined with a Gel Doc 1000 Gel Documentation System (Bio-Rad).

2.5. β-Galactosidase assay

β-Galactosidase activity was determined in cells grown in MRS broth or RSM according to the method of Miller (1972). The release of *o*-nitrophenol (ONP) from the substrate *o*-nitrophenyl-β-D-galactopyranoside (Sigma) was determined at 420 nm. Specific activity was expressed as μmol of ONP released per mg of protein per min.

2.6. Plasmid DNA extraction

The presence of plasmid DNA was evaluated by the procedures described by Anderson and McKay (1983) and Muriana and Klaenhammer (1991) using 10 mg/ml lysozyme. Agarose gel electrophoresis, staining and evaluation of gels were performed as described by Sambrook et al. (1989). Plasmids extracted from *Escherichia coli* V517 (Macrina et al., 1978) were used as size reference markers.

2.7. Bacteriophage resistance

The bacteriophages and their host strains included in this study are listed in Table 2. Standard techniques were used to determine cell sensitivity to bacteriophage attack (Auad et al., 1997). For each strain tested, one drop of sterile 1.0 M $CaCl_2$, 0.3 ml of overnight culture, and 3 ml of MRS top agar (0.6%) were mixed and poured into MRS agar (1.5%). After the plates had hardened, they were spotted with each of the phage lysates (undiluted lysate), incubated for 24 h at 37°C, and examined for zones of clearing.

2.8. Production of inhibitory substances

The production of inhibitory substances was studied by the method of Schillinger and Lucke (1989). All strains were employed both as putative producers and indicators. *Lactobacillus casei* CRL705 (Vignolo et al., 1993), a bacteriocin producer, was used as a positive control.

3. Results

3.1. Characterization of isolated lactobacilli strains by phenotypic methods

The physiological characterization of five randomly selected strains of Gram-positive, catalase-negative lactobacilli, previously isolated from Argentinian (strains CRL 581 and CRL 654) and Italian (strains CRL 1177, CRL 1178, and CRL 1179) artesanal hard cheeses, were assured using the API50 CH system. All strains grew on and fermented glucose, galactose, mannose, lactose and *N*-acetyl-D-glucosamine. Acid was not produced from amygdalin, mannitol, arabinose, cellobiose, melezitose, melibiose, raffinose, rhamnose, ribose, sorbitol or xylose. The fermentation of sucrose, fructose, maltose and trehalose was strain-dependent (Table 3). The isolates did not produce gas or acetoin from glucose. They were thermophilic and grew at 37 and 45°C, but not at 15°C. They were unable to produce ammonia from arginine. Most strains produced DL-

Table 2
Lactobacillus-derived bacteriophages and their host strains used in this study

Host strain	Phage
L. helveticus CRL 1176	328-B1[a]
L. helveticus CNRZ 493[a]	832-B1[a]
L. helveticus CNRZ 892[a]	hv, B2, hw, 835-B11, 1097-B12[a]
L. helveticus CNRZ 35[a]	034[a]
L. delbrueckii subsp. *bulgaricus* CRL 958	c5[c]
L. delbrueckii subsp. *lactis* LKT[b]	mv4[c], lb539[d], LL-H[b], 01444[c], 0235[c]

[a] Received from Dr. M. Desmazeaud, INRA, Jouy-en-Josas, France.
[b] Received from Dr. T. Alatossava, University of Oulu, Finland.
[c] Received from Dr. J.-P. Accolas, INRA, Jouy-en-Josas, France.
[d] Auad et al., 1997.

Table 3
Carbohydrate fermentation of isolated lactobacilli

Fermentation	Strain[a]				
pattern	CRL 581	CRL 654	CRL 1177	CRL 1178	CRL 1179
Fructose	+[b]	−[b]	−	+	−
Maltose	+	+	−	−	−
Trehalose	+	+	+	−	+
Sucrose	−	+	−	−	−

[a] For explanation of strain abbreviations see Table 1.
[b] +, positive reaction; −, negative reaction.

lactic acid while strains CRL 581 and CRL 654 only produced D-lactic acid.

3.2. Identification of strains by SDS–PAGE and 16S rRNA gene sequence analysis

The isolates were further characterized by SDS–PAGE and sequencing of the variable (V1) region of the 16S ribosomal DNA. The SDS–PAGE electrophoretic patterns of whole-cell proteins from natural isolates were compared with those of the respective thermophilic lactobacilli type strains (Table 1 and Fig. 1). Strains CRL 581 and CRL 654 displayed a protein profile distinct from all the other natural lactobacilli examined, particularly on the range of 20–45 kDa (Fig. 1, lanes 6 and 7). These electrophoretic patterns were similar with those of the reference strains of *L. delbrueckii* subsp. *lactis* (strains CRL 960 and CRL 934). On the other hand, strains CRL 1177, CRL 1178, and CRL 1179 showed protein patterns similar to neotype *L. helveticus* strain (CRL 974) and to strain CRL 1062 (Table 1; Fig. 1), as well as to strain CRL 1176 (data not shown). These strains could clearly be distinguished from CRL 581 and CRL 654 by the presence of a major protein band of approximately 36 kDa.

On the basis of their biochemical and physiological characteristics as well as the SDS–PAGE profiles of their total soluble cell proteins, strains CRL 581 and CRL 654 were classified as *L. delbrueckii* subsp. *lactis* and strains CRL 1177, CRL 1178 and CRL 1179 as *L. helveticus*. These data were confirmed by 16S rRNA gene sequences analysis. The amplified 16S rRNA genes of the strains CRL 974, CRL 1062, CRL 1176, CRL 1177, CRL 1178, and CRL 1179 showed a sequence similarity of 99% with the 16S rRNA DNA sequence of *L. helveticus* NCDO 2712 (databank accession number X61141). On the other hand, the amplified 16S rRNA gene of strains CRL 581 and CRL 654 showed 99 and 88% sequence similarity with the 16S rRNA gene of *L. delbrueckii* subsp. *lactis* DSM 20072 (accession number M58823) and *L. helveticus* NCDO 2712 (accession number X61141), respectively.

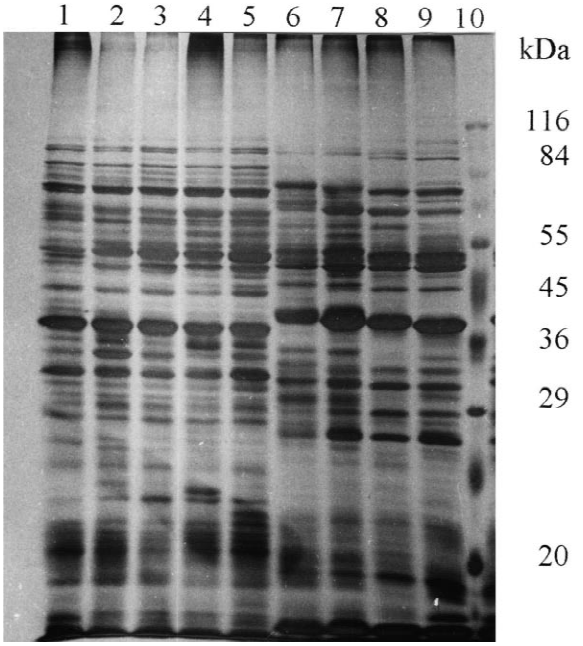

Fig. 1. SDS–PAGE of cell-free protein extracts of the isolated lactobacilli: lane 1, *L. helveticus* CRL 974^T; lane 2, *L. helveticus* CRL 1062; lane 3, *L. helveticus* CRL 1177; lane 4, *L. helveticus* CRL 1178; lane 5, *L. helveticus* CRL 1179; lane 6, *L. delbrueckii* subsp. *lactis* CRL 581; lane 7, *L. delbrueckii* subsp. *lactis* CRL 654; lane 8, *L. delbrueckii* subsp. *lactis* CRL 960^T; lane 9, *L. delbrueckii* subsp. *lactis* CRL 934; lane 10, molecular weight marker.

3.3. Coagulation tests, β-galactosidase and proteolytic activity

The natural isolates were further analyzed for their technological features. In these studies, *L. helveticus* CRL 974 (neotype), CRL 1062, and CRL 1176, were also included. Strain CRL 1176 has been described to have low proteolytic activity (Reinheimer et al., 1996). Most of the microorganisms coagulated RSM in 16 h at 42°C, except *L. helveticus* CRL 1176 (control), CRL 1178 and CRL 1179, and so they were considered as 'slow variants' (Table 4). However, these slow variants were able to coagulate RSM supplemented with yeast extract (medium RSM-YE) or casein enzymatic hydrolysate (medium RSM-CH) but not RSM supplemented with glucose (medium RSM-G) (Table 4). All microorganisms showed high levels of β-galactosidase activity (Table 4). The proteolytic activity was strain dependent. *L. delbrueckii* subsp. *lactis* CRL 581 showed the highest proteolysis while *L. helveticus* CRL 1178 and CRL 1179 produced undetectable levels of free amino groups (Table 4).

3.4. Caseinolytic activity

SDS–PAGE electrophoretograms of the products of hydrolysis of α- and β-caseins by the microorganisms are shown in Fig. 2. Caseinolytic activity of whole cells in phosphate buffer (pH 7.0) varied considerably among isolated lactobacilli. β-Casein was the preferred substrate for *L. delbrueckii* subsp. *lactis* strains, *L. delbrueckii* subsp. *lactis* CRL 654 being the most efficient microorganism. This strain completely degraded β-casein after 4 h of incubation at 42°C (Fig. 2B, lane 3). On the other hand, *L. helveticus* strains exhibited activity against α- and β-caseins, although α-casein was the preferred substrate (Fig. 2). No detectable hydrolysis of α- and β-caseins was observed with *L. helveticus* CRL 1178 and CRL 1179 cells (Fig. 2, lanes 8 and 9).

3.5. Other characteristics

All microorganisms were screened for the production of inhibitory substances, phage sensitivity, and plasmid DNA content. None of the strains produced detectable levels of an inhibitor. Phage susceptibility, examined by standard methods, showed that *L. delbrueckii* subsp. *lactis* strains and *L. helveticus* CRL 1177, CRL 1178 and CRL 1179 were insensitive to all *L. helveticus* and *L. delbrueckii* subsp. *lactis*-specific bacteriophages used in this study (Table 2). Plasmid DNA analysis showed that *L. helveticus* CRL 1177 and CRL 1179 possess a plasmid of 10.0 kb and three plasmids of 16.0, 10.0 and 6.0 kb, respectively (data not shown). The presence of three plasmids (22.0, 6.0 and 3.5

Table 4
Growth characteristics, β-galactosidase and proteolytic activities of *L. helveticus* and *L. delbrueckii* subsp. *lactis* strains[a]

Strain	Growth[b]				β-Galactosidase activity (μmol/min · mg)[c]	Proteolytic activity (μg/ml)[d]
	RSM	RSM-G	RSM-CH	RSM-YE		
L. delbrueckii subsp. *lactis* CRL 581	+[e]				5.7±0.2	522±25
L. delbrueckii subsp. *lactis* CRL 654	+				5.6±0.2	392±19
L. helveticus CRL 974	+				5.7±0.2	260±12
L. helveticus CRL 1062	+				6.6±0.3	284±13
L. helveticus CRL 1176	−	−	+	+	5.8±0.2	34±2
L. helveticus CRL 1177	+				7.1±0.3	299±14
L. helveticus CRL 1178	−	−	+	+	6.0±0.2	36±2
L. helveticus CRL 1179	−	−	+	+	6.2±0.3	18±1

[a] The results are averages of four independent trials.

[b] Strains were inoculated (2%) into reconstituted skim milk (RSM); RSM supplemented with 1% glucose (RSM-G); RSM supplemented with 0.25% casein enzymatic hydrolysate (RSM-CH) and RSM supplemented with 0.25% yeast extract (RSM-YE), and incubated for 16 h at 42°C.

[c] Specific β-galactosidase activity is expressed as μmol of ONP liberated per μg of protein per minute at 42°C.

[d] Proteolytic activity is expressed as μg/ml glycine.

[e] +, Denotes milk coagulation.

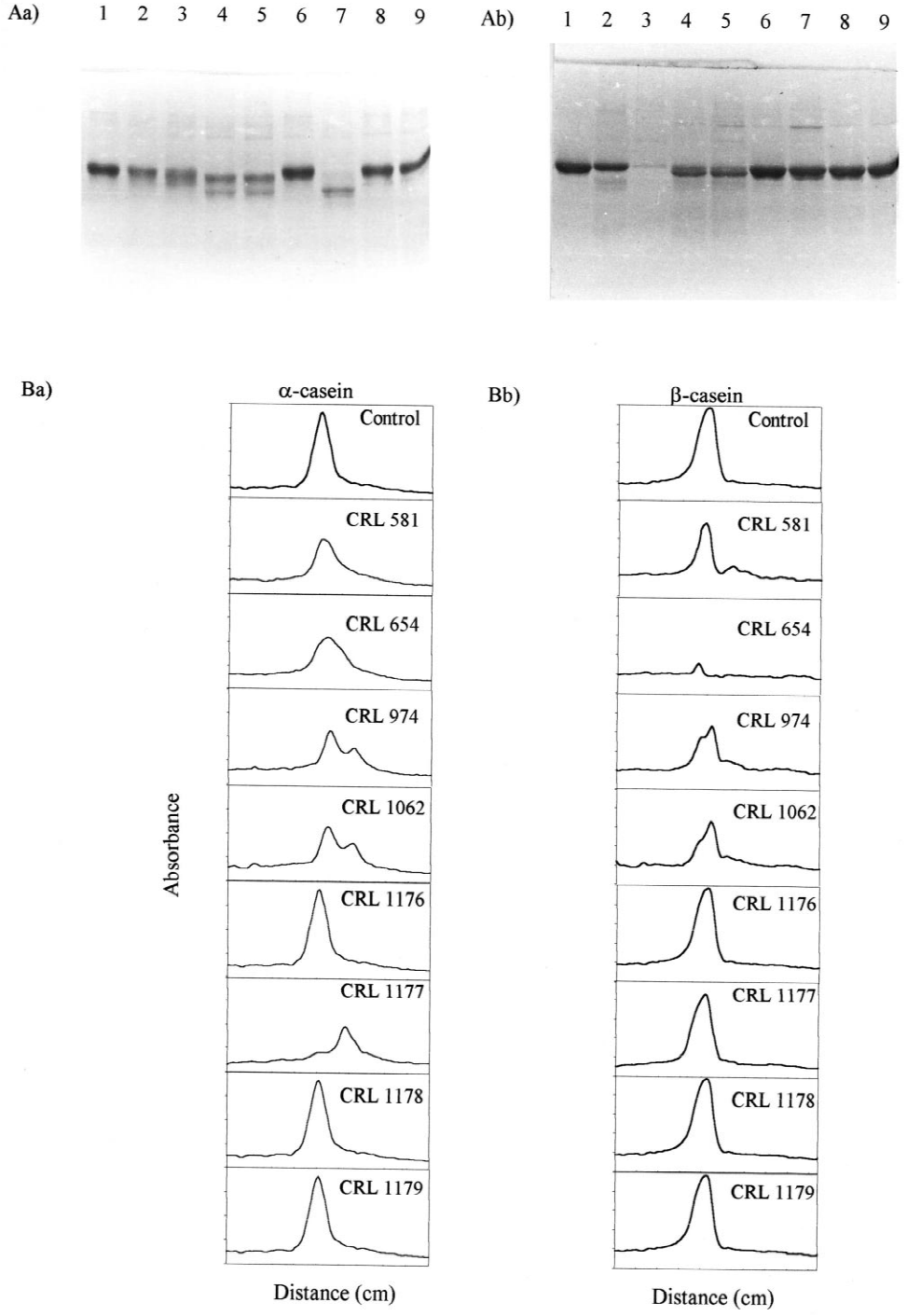

Fig. 2. (A) Hydrolysis of (a) α-casein and (b) β-casein by whole cells for 4 h at 42°C. Lane 1, control; lane 2, *L. delbrueckii* subsp. *lactis* CRL 581; lane 3, *L. delbrueckii* subsp. *lactis* CRL 654; lane 4, *L. helveticus* CRL 974; lane 5, *L. helveticus* CRL 1062; lane 6, *L. helveticus* CRL 1176; lane 7, *L.helveticus* CRL 1177; lane 8, *L. helveticus* CRL 1178; lane 9, *L. helveticus* CRL 1179. (B) Densitograms of the hydrolysis of α- and β-caseins by lactobacilli strains.

kb), already described in strain CRL 974, was confirmed (Pridmore et al., 1994). No plasmid DNA was observed in *L. delbrueckii* subsp. *lactis* CRL 581 and CRL 654 and *L. helveticus* CRL 1178.

4. Discussion

Five isolates of homofermentative, thermophilic lactobacilli (three *L. helveticus* and two *L. delbrueckii* subsp. *lactis* strains), isolated from artesanal hard cheeses, were identified and characterized. Isolates CRL 1177, CRL 1178 and CRL 1179 could be classified as *L. helveticus* by API 50CH fermentation patterns, while CRL 654 and CRL 581 were identified as *L. delbrueckii* dubsp. *lactis*. Strain CRL 581 was sucrose-negative, like typical *L. helveticus* strains, but showed D(−)-lactate dehydrogenase activity instead of lactic acid racemase. The analysis of protein profiles by SDS–PAGE and 16S rRNA gene sequence analysis successfully confirmed the taxonomic characterization of these strains. As reported by Fortina et al. (1998), *L. helveticus* strains isolated from natural habitats often shown phenotypic variability, due to different environmental pressures. Phenotypic variability in the fermentation of fructose, maltose, sucrose and trehalose, was also observed in our strains.

The primary function of a starter culture is the fermentative conversion of the milk sugar, lactose, into acidic products that contribute to the preservation, flavour and texture of the fermented dairy product. All strains tested showed high levels of β-galactosidase activity. However, only *L. delbrueckii* subsp. *lactis* strains (CRL 654 and CRL 581) and *L. helveticus* CRL 1177 were able to coagulate RSM in 16 h at 42°C (strains CRL 1178 and CRL 1179 coagulated RSM only when the medium was supplemented with yeast extract or casein enzymatic hydrolysate). *L. delbrueckii* subsp. *lactis* CRL 581 and CRL 654, considered as 'fast coagulating' variants, are plasmid-free. Furthermore, regardless the presence of plasmid carried by *L. helveticus* CRL 1178 and CRL 1179, these strains showed a 'low coagulating' phenotype. Also, slow variants of strain CRL 974 (isolated in our laboratory; unpublished) showed similar plasmid profiles to the wild type strain (data not shown). These results would indicate that the proteolytic activity in these thermophilic lactobacilli strains are not plasmid DNA encoded.

L. delbrueckii subsp. *lactis* strains hydrolyzed both α- and β-caseins. As reported for lactococci (Exterkate and Veer, 1985; Bockelmann et al., 1989), β-casein was the preferred substrate for *L. delbrueckii* subsp. *lactis* proteinases. On the other hand, *L. helveticus* strains degraded α-casein more efficiently, strain CRL 1177 being particularly active on this substrate. According to the substrate specificity these proteinases have a caseinolytic activity comparable to that of the P_{III}-type of lactococcal proteinases (Kok, 1990)

Our study shows that three strains, *L. delbrueckii* subsp. *lactis* CRL 581 and CRL 654, and *L. helveticus* CRL 1177, exhibited important metabolic features for their development as dairy starter cultures. In addition, these strains proved to be insensitive to the attack by selected phages, which have been shown to be active against all the indicator strains tested so far (Séchaud et al., 1992). These strains could be used in single- or multiple-strain starter cultures, since they do not produce inhibitory substances. This is important, since inhibitor production by one strain could rapidly result in that strain dominating the culture. The careful choice of natural isolates will be essential for successful development of new starters.

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