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Application of culture-dependent and culture-independent methods for the identification of *Lactobacillus kefiranofaciens* in microbial consortia present in kefir grains

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ABSTRACT

The biological and technological characteristics of kefiran as well as its importance in grain integrity led us to analyze the microbial kefir grain consortium with focus on *Lactobacillus kefiranofaciens*. The presence of *L. kefiranofaciens* in the nine kefir grains studied was demonstrated by denaturing gradient gel electrophoresis. By culture dependent methods applying a methodology focused on the search of this species, 22 isolates with typical morphology were obtained and identified applying a combination of SDS-PAGE of whole cell proteins, $(GTG)_5$-PCR and sequence analysis of the housekeeping gene encoding the α-subunit of bacterial phenylalanyl-tRNA synthase (*pheS*). This polyphasic approach allowed the reliable identification of 11 *L. kefiranofaciens*, 5 *Lactobacillus paracasei*, 4 *Lactobacillus kefiri* and 2 *Lactobacillus parakefiri* isolates. Isolated *L. kefiranofaciens* strains produced polysaccharide in strain-dependent concentrations and EPS produced by them also differed in the degree of polymerization. The isolation and accurate identification of *L. kefiranofaciens* is relevant taking into account the important role of this microorganism in the grain ecosystem as well as its potential application as starter in food fermentations.

1. Introduction

Kefir grains are gelatinous irregular masses, white or lightly yellow with a structure similar to tiny florets of cauliflower which vary in size from 0.3 to 3.5 cm diameter. The complex microbiota of kefir grains mainly consists of lactic acid bacteria (LAB) (primarily lactobacilli and lactococci), yeasts and acetic acid bacteria immersed in a protein and polysaccharide matrix (Witthuhn et al., 2005; Garrote et al., 2010). Culture-dependent techniques and traditional molecular methods, as well as denaturing gradient gel electrophoresis (DGGE) have been used to characterize the kefir community (Ninane et al., 2007; Kesmen and Kacmaz, 2011; Leite et al., 2012). The total number of microorganisms and their relative composition in kefir grains is variable, depending on the origin and the method of culturing of the grains and substrates used. It has been established that the microbial diversity of the grains is not uniformly distributed, the interior exhibiting a greater level of diversity compared to the exterior, and differs from the fermented kefir milk (Oberman and Libudzisz, 1998; Londero et al., 2012).

Kefiran, the exopolysaccharide obtained from kefir grains has been reported to have antibacterial and antitumor activities, modulate the gut immune system and protect epithelial cells against *Bacillus cereus* exocellular factors and infection (Medrano et al., 2008, 2011; Chen et al., 2012). So, the production of kefiran by members of the kefir grain ecosystem could be regarded as one of the mechanisms contributing to health benefits for the consumer

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via the consumption of this fermented milk (Abraham et al., 2010). Besides, kefiran also has interesting physicochemical properties and can be used as a thickener, stabilizer and emulsifier, film forming agent, fat substitute or gelling agent (Rimada and Abraham, 2006; Wang et al., 2008; Piermaría et al., 2011). Although kefiran production initially was ascribed to several *Lactobacillus* species isolated from kefir grains (Toba et al., 1987; Yokoi et al., 1991), nowadays, kefiran production is attributed to *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* (Fujisawa et al., 1988; Vancanneyt et al., 2004; Ahmed et al., 2013).

The aforementioned biological and technological characteristics of kefiran as well as its importance in grain integrity (Wang et al., 2012) focused our analysis of the kefir grain consortium towards the search for *L. kefiranofaciens* and the isolation and identification of novel strains of this species. When new strains are isolated, accurate identification is necessary. For this, molecular techniques have emerged complementing traditional phenotypic tests. Amongst these methodologies, sequence-based identification using one or more protein-coding genes has been explored as an alternative to determine the genomic relatedness between LAB strains. In this regard, the phenylalanyl-tRNA synthase gene (*pheS*) has proven to be a valuable tool for the identification of *Lactobacillus* species (Naser et al., 2007). Besides Rep-PCR fingerprinting using the $(GTG)_5$ primer, referred to as $(GTG)_5$-PCR fingerprinting, has proven to be a successful genotypic tool for rapid and reliable speciation and typing of lactobacilli (Gevers et al., 2001).

In this paper strategies to isolate this microorganism from kefir grains were developed and a combination of phenotypic and genotypic methods was applied to identify them.

2. Materials and methods

2.1. Kefir grains

Nine kefir grains from different origins and belonging to the Centro de Investigación y Desarrollo en Criotecnología (CIDCA) were investigated (AGK1, AGK2, AGK3, AGK5, AGK6, AGK7, AGK8, AGK10 and AGK11). The grains were maintained at −20 °C and were subcultured in commercial ultra-high temperature (UHT) low fat milk obtained from Sancor (Santa Fe, Argentina).

2.2. Reference strains and growth conditions

Type and reference strains used were: *L. kefiranofaciens* subsp. *kefiranofaciens* JCM 6985^T (LMG 19149^T), *L. kefiranofaciens* subsp. *kefirgranum* LMG 15132^T, *Lactobacillus paracasei* LMG 13087^T, LMG 9191^T, LMG 7955, LMG 10744 and R-40076, *Lactobacillus parakefiri* JCM 8573^T (LMG 15133^T), *Lactobacillus casei* DSM 20011^T, and *Lactobacillus kefiri* JCM 5818^T (LMG 9480^T), LMG 11453, LMG 11454 and LMG 11496 (Table 1). *L. kefiranofaciens* strains were cultured in MRS broth pH 5.0 or on MRS agar pH 5.0 (MRS acidified with HCl to reach pH 5.0 with addition of 1.8 g of agar per 100 ml) at 30 °C for 7 days. The other reference strains were grown in MRS broth or on

Table 1
Phenotypic and genotypic characterization of *Lactobacillus* isolated from kefir grains and reference strains.

Isolate		Source	Growth on milk	Gas from glucose	SDS-PAGE cluster (Fig. 2)	(GTG)5-PCR cluster (Fig. 3)	PheS ID (Fig. 4)
CIDCA[a] code	LMG[b] code						
83118	R 45481	AGK1	+	−	B_2	3	i
83119	R 54475	AGK1	+	−	B_2	ND	i
83120	R 45465	AGK1	+	−	C	1	ii
83121	R 45466	AGK1	+	−	C	1	ND
83122	R 45467	AGK1	+	−	B_2	3	i
83123	R 45468	AGK1	+	−	C	1	ii
83124	R 45469	AGK1	+	−	C	1	ND
83125	−	AGK1	−	+	A	2	ND
83211	R 45482	AGK2	+	−	B_2	3	ND
83212	R 45483	AGK2	+	−	B_2	3	i
8339	R 45463	AGK3	+	−	C	1	ii
83310	R 45478	AGK3	+	−	B_2	3	i
83311	R 45477	AGK3	+	−	B_2	ND	i
8352	R 45476	AGK5	+	−	B_2	3	i
8361	R 45480	AGK6	−	−	B_2	3	i
8371	R 45479	AGK7	+	−	B_2	3	i
8381	R 45473	AGK8	−	+	A	4	iii_2
8385	R 45474	AGK8	−	+	A	4	iii_2
83101	R 45470	AGK10	−	+	A	2	iii_1
83102	R 45464	AGK10	−	+	A	4	ND
83103	−	AGK10	−	−	B_2	ND	ND
830111	R 45471	AGK11	−	+	B_2	4	ND
Reference strains							
L. kefiranofaciens subsp. *kefiranofaciens* JCM[c] 6985^T	19149^T		+	−	B_2	3	i
L. kefiranofaciens subsp. *kefirgranum*	15132^T		+	−	B_1	3	i
L. kefiri JCM 5818^T	9480^T		−	+	A	4	iii_2
L. parakefiri JCM 8573^T	15133^T		−	+	A	2	iii_1
L. casei DSMZ[d] 20011^T			+	−	C	ND	ND
L. paracasei	R 40076		+	ND	C	ND	ND
L. paracasei	13087^T		ND	ND	ND	1	ii

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[d] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. ND not determined.

MRS agar at 30 °C for 48 h. All strains were grown under an anaerobic atmosphere obtained with the Anaero Pack-Anaero kit (Mitsubishi Gas Chemical CO Inc., Tokyo, Japan).

2.3. Isolation of bacteria from kefir grains

One gram of each kefir grain was suspended in 5 ml of sterile distilled water, incubated for 60 min at 40 °C and mechanically homogenized with an Ultra-turrax T25 (Janke& Kunkel, IKA-Labortechnik, Staufen, Germany) at 24,000 min^{-1} until complete disintegration. A 100 μl aliquot from this suspension was inoculated in 5 ml of MRS broth pH 5.0, incubated at 30 °C for 7 days in an anaerobic atmosphere and then plated on MRS agar pH 5.0. Selection of ropy strains was based on colony morphology. After at least three passages of subculturing of isolated colonies, 22 pure isolates were obtained and stored in sterile milk at −80 °C.

2.4. DNA isolation from kefir grains

One gram of dried kefir grains with 20 ml of sterile water was heated in a boiling water bath for 20 min in order to dissolve the polysaccharide attached to the cells. Cells were collected by centrifugation at 15,000 *g* for 15 min at 20 °C in a Sorvall RC-5B plus centrifuge (Sorvall Products, L.P. Newtown, CT, USA). Cells were subsequently resuspended in 10 ml of sterile water, heated and centrifuged as described above. The resulting pellet was suspended in 100 μl of lyticase buffer (0.9 M sorbitol, 0.1 M Tris–HCl pH 8.0, 0.1 M EDTA) and 10 μl of lyticase 2.5 mg/ml (Sigma Chemical, St. Louis, USA) and incubated for 1 h at 37 °C. Subsequently, 40 μl of TES buffer (50 mM Tris–HCl, 5 mM EDTA, 50 g sucrose/l, pH 8.0) and 40 μl of a 10 mg/ml lysozyme solution (Sigma Chemical, St. Louis, USA) were added. After 15 min of incubation at 37 °C, DNA was extracted and purified using the AccuPrep Genomic DNA Extraction kit (BIONEER, Korea) according to the manufacturer's protocol.

2.5. DNA isolation from pure cultures

Two procedures for total DNA preparation from LAB were performed. DNA for Rep-PCR fingerprinting and PheS gene sequencing was isolated and purified according to Gevers et al. (2001). The purity and concentration of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDropTechnologies Inc, Wilmington, DE, USA). DNA work solutions were prepared at a concentration of 50 ng/μl in MilliQ water and stored at −20 °C. DNA for PCR-DGGE was extracted by the use of the AccuPrep Genomic DNA Extraction kit.

2.6. PCR-DGGE and identification of DGGE bands

The V3 region of the 16S rRNA gene was amplified by using primers 518R (5′-ATTACCGCGGCTGCTGG-3′) and 338f (5′-ACTCCTACGGGAGGCAGCAG-3′) coupled to a 5′-GC clamp (Muyzer et al., 1993). PCR was performed in 0.2 ml tubes by using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Each reaction mixture contained 0.2 μM of each primer, 1.25 U of Taq free DNA polymerase (Inbio-Highway, Tandil, Argentina), 1.5 μl of PCR reaction buffer supplied with the enzyme (100 mM Tris–HCl, 500 mM KCl, pH 9.0), 2.5 mM $MgCl_2$, and 0.2 mM of each dNTP. To 19 μl of premix, 1 μl of the template DNA was added. The following amplification program was used: an initial denaturing step at 94 °C for 5 min, 30 cycles consisting of 94 °C 30 s, 60 °C 60 s, 72 °C 30 s, and a final elongation for 5 min at 72 °C. The PCR products were analyzed by DGGE using a DGGE-2401 device (C.B.S. Scientific Co., Del Mar, CA, USA). Samples were applied to 8.0% w/v polyacrylamide gels in 1× TAE buffer. Optimal separation was achieved with a 40–60% urea and formamide denaturing gradient (100% corresponds with 7 M urea and 40% v/v of formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h at 60 °C. Gels were stained with SYBR Gold 0.2 μl/ml in TAE buffer (INVITROGEN, Oregon, USA) and visualized under UV light.

For sequencing, bands of interest were excised from the gels, resuspended in 50 μl 1× TE buffer (50 mM Tris–HCl, 5 mM EDTA, pH 8.0), and stored at 4 °C overnight. The DNA was then amplified with the original primers but without the GC clamp.

The direct sequencing of PCR products was performed on a 3730XL 2 DNA analyzer (Applied Biosystems) and the resulting sequences were finally compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) through the BLAST program in order to determine the most similar sequence relatives.

2.7. Phenotypic characterization of LAB isolated from kefir

Lactic acid bacteria were classified on the basis of their cellular morphology, Gram staining, spore formation, motility, catalase reaction and gas production from 10 g/l glucose. To determine the whole cell protein pattern of the isolates, cell extracts were prepared as described by Gomez-Zavaglia et al. (1999). Protein profiles were analyzed by SDS-PAGE in 125 g/l acrylamide gels according to the protocol of Laemmli (1973) using a Miniprotean II cell (Bio-Rad Lab, Richmond, CA 94804, USA). Electrophoresis was done at 120 V. A band-based numerical analysis was done using the Systat software (version 12).

2.8. Molecular identification of LAB isolated from kefir

2.8.1. Rep PCR fingerprinting

DNA from all 22 isolates was subjected to rep-PCR fingerprinting with the single oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′). PCR was performed according to Versalovic et al. (1994) with a Perkin Elmer 9600 DNA thermal cycler (Perkin Elmer, Massachusetts, USA) using Goldstar DNA polymerase (Eurogentec, Belgium). PCR products were separated under strictly standardized conditions as described by Gevers et al. (2001). The resulting fingerprints were analyzed by the BioNumerics software package 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity among digitized profiles was calculated using Pearson correlation, and an average linkage (UPGMA) dendrogram was constructed from the resulting similarity matrix.

2.8.2. Phenylalanyl-tRNA synthase (PheS) gene sequencing

For those strains that could not be identified unambiguously by $(GTG)_5$-PCR fingerprinting and SDS-PAGE, *pheS* amplification and sequencing was performed. Primer combinations used were pheS-21-F/pheS-22-R and pheS-21-F/pheS-23-R. PCR conditions and thermal cycles were performed according to Naser et al. (2005) in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, CA, USA). To perform the sequence analysis, data were transferred to Assembler (Applied Maths) to determine the consensus sequences. Consensus sequences were subsequently imported into BioNumerics and aligned and compared with a user-generated database containing the *pheS* sequences of type and reference strains used.

2.9. Exopolysaccharide characterization

2.9.1. Exopolysaccharide production in milk and determination of molecular mass

Polysaccharide was obtained from 100 ml of fermented milk heated in boiling water bath for 30 min with discontinuous stirring and centrifuged at 10,000 *g* for 20 min at 20 °C (Avanti J25 Beckman

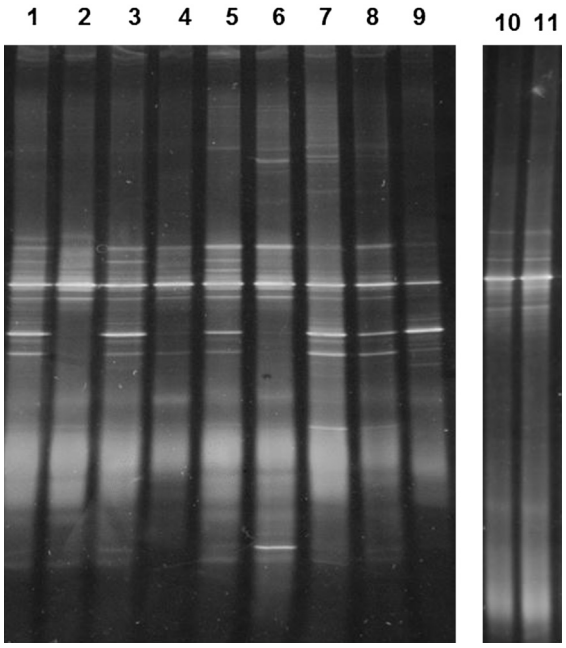

Fig. 1. V3 DGGE fingerprints of nine different CIDCA kefir grains and reference strains. Lane 1, AGK1; lane 2, AGK2; lane 3, AGK3; lane 4, AGK5; lane 5, AGK6; lane 6, AGK7; lane 7, AGK8; lane 8, AGK10; lane 9, AGK11; lane 10, *L. kefiranofaciens* subsp. *kefirgranum* LMG 15132^T; lane 11, *L. kefiranofaciens* subsp. *kefiranofaciens* JCM 6985^T.

Coulter Inc. centrifuge, Palo Alto, California). Two volume of cold ethanol was added to supernatant and left at −20 °C overnight. The mixture was centrifuged at 10,000 *g* for 20 min at 4 °C. Pellets were dissolved in hot distilled water and dialyzed (molecular weight cut-off (WMCO) for the dialysis membranes: 1000 (Spectra/Por, The Spectrum Companies, Gardena, CA, USA)) for 48 h at 4 °C against four changes of twice-distilled water (Rimada and Abraham, 2003). The molecular mass of purified polysaccharide was determined by gel filtration using an OH-PAK SB-805HQ gel filtration chromatography column (SHODEX, Kawasaki, Japan) in a HPLC system (Waters, Milford) associated to a RI (refractive index) detection system (Waters, Milford). Samples, dissolved in water, were eluted at room temperature, using $NaNO_3$ 0.1 M. The flow rate was kept constant at 0.95 ml/min (pressure 120–130 psi) and 50 μl of filtered through 0.45 μm filters polysaccharide solutions (0.5 g/l) were injected for each run. Dextrans with MW range from 97,000 to 3,800,000 ALO-2770 (Phenomenex, Torrance, CA) were used as standard.

3. Results and discussion

3.1. Application of a culture-independent method for detecting *L. kefiranofaciens* into kefir grain associated microbial consortia

Kefir grains harbor a diverse spectrum of microbial species. Different reports point out that the kefir grain microbiota strongly depends on the origin of the grains, the local conditions of culturing and the storage and elaboration processes (Garrote et al., 2010). Previous attempts to isolate *L. kefiranofaciens* in our lab by using conventional culture techniques were unsuccessful, so in order to determine the presence of *L. kefiranofaciens* in nine kefir grains of the CIDCA collection, total microbial DNA was extracted from the grains and analyzed by PCR-DGGE. DGGE profiles of the PCR amplicons belonging to the V3 regions of bacterial 16S rDNA are shown in Fig. 1. The analysis of the band patterns showed heterogeneity between the grains confirming the varied bacterial

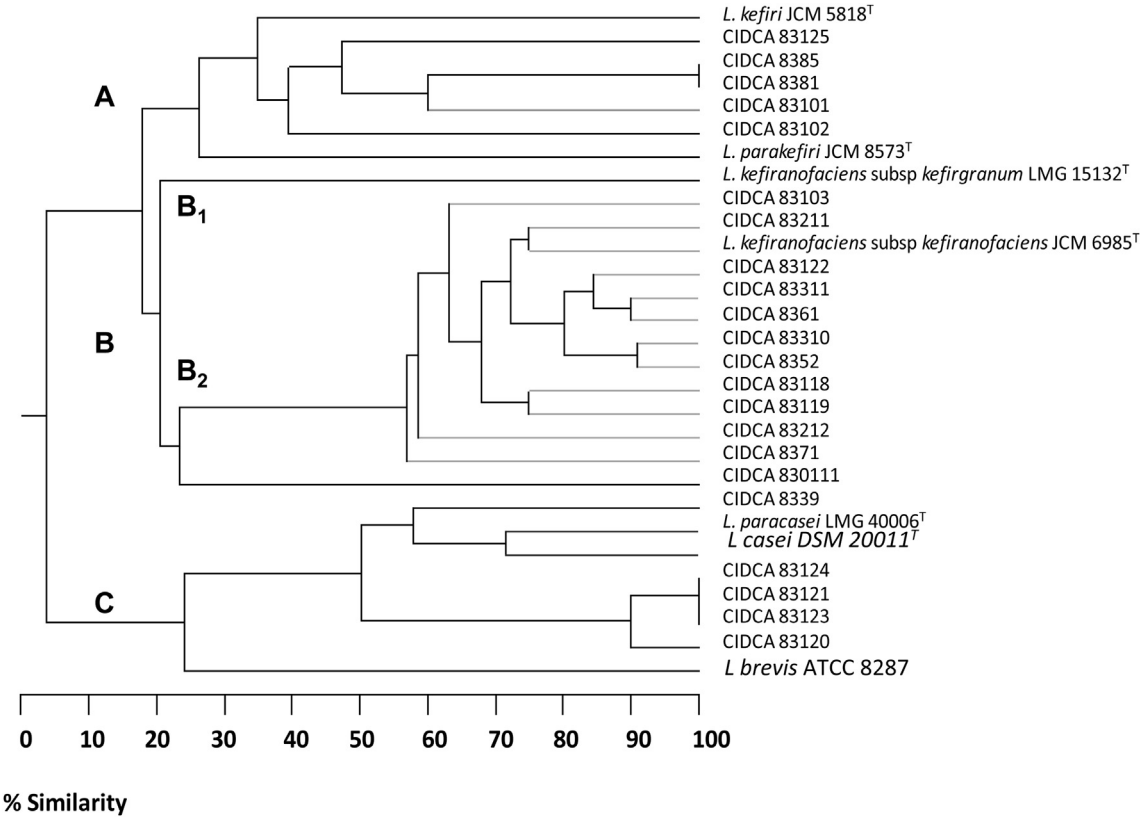

Fig. 2. UPGMA tree of SDS-PAGE whole cell protein profiles of the 22 strains isolated in this study and reference strains. Data were compared using Pearson correlation coefficient.

diversity as was previously reported (Witthuhn et al., 2005; Garrote et al., 2010). Yet, similarity existed between the profiles of kefir grains CIDCA AGK1, AGK3 and AGK10, as was the case for grains AGK2 and AGK5 (Fig. 1). Despite differences in the DGGE patterns between different kefir grains, it was observed in all grains that the most intense band had a migration equivalent to that of the reference strains *L. kefiranofaciens* subsp. *kefiranofaciens* JCM 6985^T and *L. kefiranofaciens* subsp. *kefirgranum* LMG 15132^T. The band, sequenced and compared to GenBank database, exhibited 100% homology to both subspecies of *L. kefiranofaciens*, suggesting the possibility that this microorganism would be present in all kefir grains studied. These results are in concordance with previous findings for Belgian kefir grains (Ninane et al., 2007), Tibetan kefir grains (Zhou et al., 2009), Turkish kefir grains (Kesmen and Kacmaz, 2011), Brazilian kefir grains (Leite et al., 2012) and Taiwanese kefir grains (Chen et al., 2008). Conversely, when applying PCR-DGGE analysis, Garbers et al. (2004) did not find this microorganism, neither in Irish kefir grains nor in traditionally cultured South-African kefir grains where the only species identified were *L. kefiri*, *L. parakefiri*, *Lactobacillus crispatus* and *Lactobacillus gallinarum*. Likewise, using a conventional culturing approach for the identification of 270 isolates in combination with DGGE and sequence analysis, Da Cruz Pedrozo Miguel et al. (2010) reported that the dominant bacterium in kefir grains from different Brazilian states, Canada and the United States of America, was *L. kefiri* whereas they did not describe *L. kefiranofaciens*.

3.2. Application of culture-dependent methods for the identification of *L. kefiranofaciens* strains isolated from microbial consortia present in kefir grains

Up till now, only few reports have described the isolation of *L. kefiranofaciens* from kefir grains by conventional culturing techniques (Vancanneyt et al., 2004; Mainville et al., 2006; Chen et al., 2008, 2012). In a previous report, more than 120 isolates were obtained from kefir grains from different household in Argentina. These isolates were characterized and identified as *Lactococcus lactis*, *Leuconostoc mesenteroides*, *L. kefiri*, *L. parakefiri*, *L. plantarum*, *Acetobacter* sp., *Saccharomyces* sp. and *Kluyveromyces marxianus* (Garrote et al., 2001; Bosch et al., 2006) but no isolates of *L. kefiranofaciens* were obtained. The strictly anaerobic character and particular growth requirements of this microorganism (Wang et al., 2008) whether or not in combination with their high affinity for the grain matrix components might account for their unsuccessful recovery. Besides, the symbiotic nature of the kefir microbiota can complicate the specific isolation and subsequent identification of *L. kefiranofaciens*.

In our approach, kefir grains were disrupted, grown in MRS broth pH 5.0 under anaerobic conditions at 30 °C and then plated on MRS agar pH 5.0 for one week. Twenty two isolates were selected according to the typical colony morphology of *L. kefiranofaciens* subsp. *kefiranofaciens*, as was previously described by Vancanneyt et al. (2004), and were subsequently characterized by Gram staining, catalase production, growth in

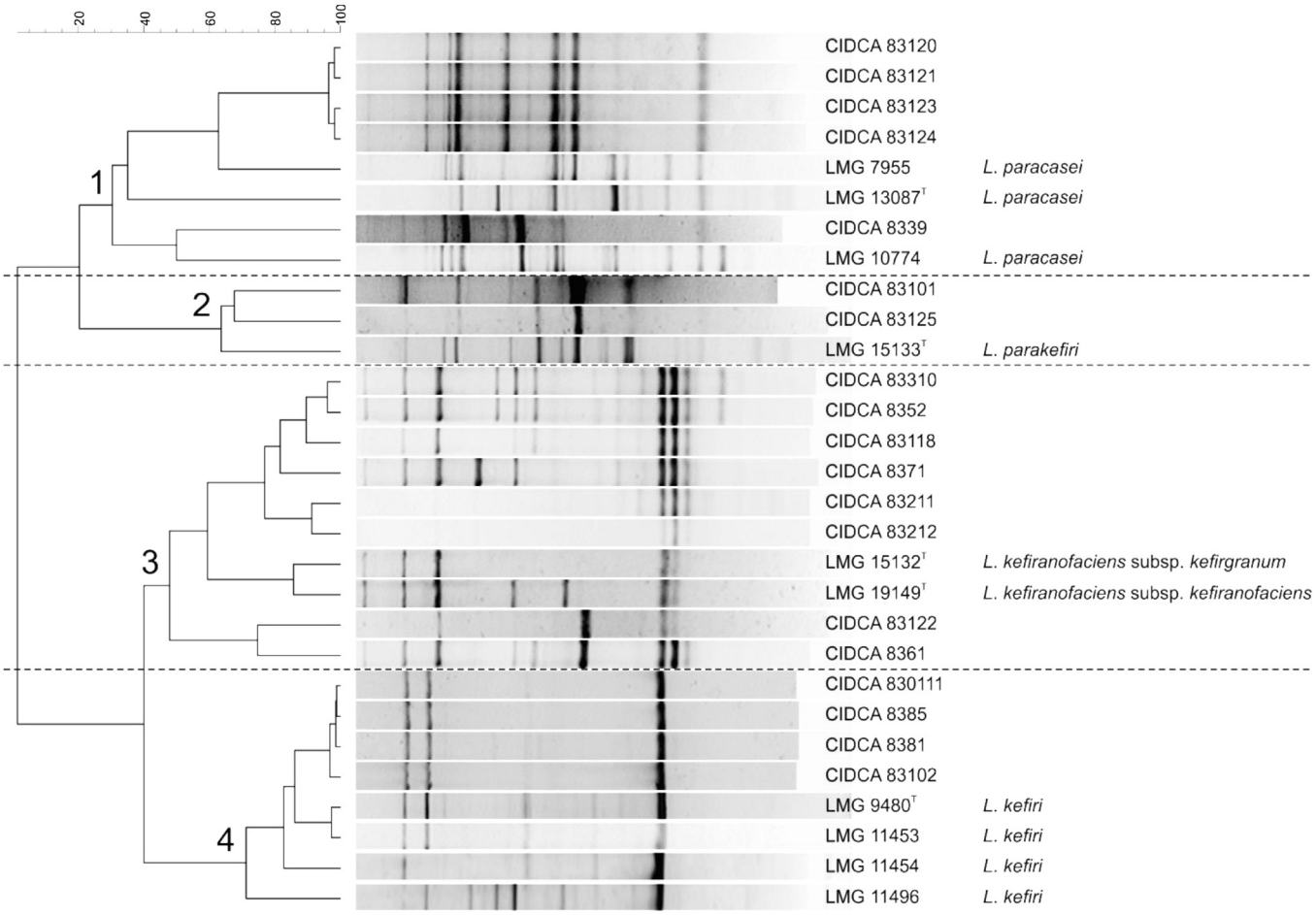

Fig. 3. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of strains isolated in this study and reference strains.

milk and gas production from glucose (Table 1). In the same table, characteristics of the type strains of *L. kefiranofaciens* subsp. *kefiranofaciens* JCM 6985^T and *L. kefiranofaciens* subsp. *kefirgranum* LMG 15132^T are shown. All isolates were Gram positive and catalase negative. Fifteen of 22 isolates were able to grow in milk and six were able to produce gas from glucose. All isolates were subsequently identified using a polyphasic approach combining phenotypic and genotypic validated methods: SDS-PAGE of whole cell proteins, $(GTG)_5$-PCR and sequence analysis of the housekeeping gene encoding the α-subunit of the bacterial phenylalanyl-tRNA synthase (*pheS*).

A UPGMA based dendrogram of SDS-PAGE protein profiles of isolates and reference strains is shown in Fig. 2. SDS-PAGE profiles are organized in three clusters A, B and C. In cluster A, five isolates are grouped together with the obligate heterofermentative species *L. kefiri* and *L. parakefiri* (Kandler and Kunath, 1983; Takizawa et al., 1994). Cluster B is composed of the profiles of 12 isolates and the two subspecies of *L. kefiranofaciens*. Yet, the profile of the *kefirgranum* subspecies is separated (B_1) from a group (B_2) composed of the isolates and *L. kefiranofaciens* subsp. *kefiranofaciens*. The third cluster, C, encompasses the profiles of five isolates and reference strains of the *L. casei*/*paracasei* group.

Methods based on PCR product analysis for typing purposes have been reported to be simple and rapid to perform, and to provide good levels of discrimination up to strain level; they are therefore the method of choice when a large number of strains must be analyzed (Seseña et al., 2004). Repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting, that represents an easy-to-perform fingerprinting technique, was one of the methods selected in this paper for identifying the 22 isolates. $(GTG)_5$-PCR fingerprinting has been applied for the classification and identification of several groups of bacteria, including lactobacilli (Gevers et al., 2001; Van Hoorde et al., 2008; Švec et al., 2011). Digitized $(GTG)_5$-PCR profiles of the isolated strains were compared to the profiles of reference strains (Fig. 3). For this, fingerprints were clustered (UPGMA algorithm) using the curve-based Pearson correlation similarity coefficient, and four clusters could be discriminated: five isolates clustered together with reference strains of *L. paracasei* (cluster 1); in cluster 2, two strains exhibited similarity to the type strain of *L. parakefiri*. Cluster 3 contains eight isolates which clustered together with both subspecies of *L. kefiranofaciens*; a fourth cluster (cluster 4) grouped four isolates with *L. kefiri* reference strains.

Comparing protein patterns and $(GTG)_5$-profiles (See Figs. 2 and 3 and Table 1) revealed discrepancies between both techniques. Two isolates, CIDCA 83125 and CIDCA 83101, were assigned to cluster A together with the *L. kefiri* and *L. parakefiri* type strains using SDS-PAGE, yet rep-PCR was able to tentatively identify both *Lactobacillus* isolates as being *L. parakefiri*. An inconsistency was found for isolate CIDCA 830111 which clustered together with *L. kefiranofaciens* or *L. kefiri*, whether SDS-PAGE or $(GTG)_5$-fingerprinting was used, the latter being more in concordance to the ability of this isolate to produce gas from glucose (Kandler and Kunath, 1983).

To confirm identification results and to identify the isolates for which conflicting results were obtained, the PheS gene sequence of

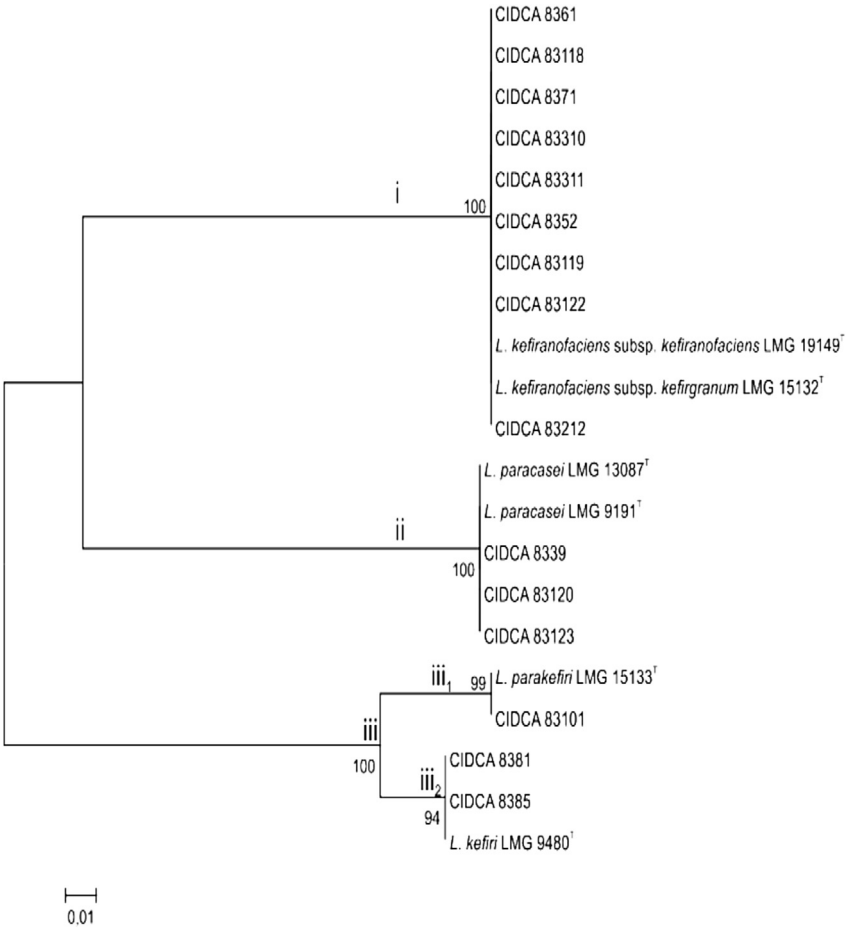

Fig. 4. Neighbor-joining tree based on the PheS gene sequences of 15 strains isolated in this study and reference strains.

Table 2
Exopolysaccharide production by *L. kefiranofaciens* during growth in milk at 30 °C during 7 days and EPS molecular weight distribution.

	Strain	Polysaccharide concentration (mg L^{-1})	Molecular weight distribution (%)			
			$<10^4$ Da	10^4–10^5 Da	10^5–10^6 Da	$>10^6$ Da
L. kefiranofaciens subsp. *kefiranofaciens*	JCM 6985	110.1 ± 7.6	0	74.7	0.3	25.0
	CIDCA 83118	202.4 ± 12.9	100	0	0	0
	CIDCA 83119	301.9 ± 15.1	78.2	21.8	0	0
	CIDCA 83122	198.2 ± 8.2	78.3	21.7	0	0
	CIDCA 83211	112.9 ± 3.1	77.5	22.6	0	0
	CIDCA 83212	125.9 ± 7.9	97.7	2.3	0	0
	CIDCA 83310	178.9 ± 9.1	100	0	0	0
	CIDCA 83311	183.2 ± 11.7	100	0	0	0
	CIDCA 8351	111.9 ± 5.6	100	0	0	0
	CIDCA 8371	85.1 ± 3.2	79.7	20.4	0	0

representative isolates selected from $(GTG)_5$-PCR and SDS-PAGE clusters was determined (Fig. 4). As an alternative to sequence analysis of the 16S rRNA gene, of which it is known that its taxonomic resolution can be too low for closely related species and as a result that several closely related LAB species share nearly identical 16S rRNA gene sequences, the use of housekeeping genes was introduced. The use of *pheS* as a taxonomic marker has proven its potential to identify LAB isolates belonging to a broad spectrum of genera including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Weissella* (Naser et al., 2005, 2007; Van Hoorde et al., 2008). Here, the analysis of PheS genes confirmed the results obtained by whole cell protein profiles and by $(GTG)_5$-fingerprinting: strains CIDCA 83120, CIDCA 83121, CIDCA 83123, CIDCA 83124 and CIDCA 8339 were identified as *L. paracasei*. Nasser and co-workers were also able to discriminate *L. kefiri* from *L. parakefiri* based on their respective *pheS* sequences (Naser et al., 2007). In this study strains CIDCA 8381, CIDCA 8385, CIDCA 83102 were identified as *L. kefiri* being coincident with the results obtained with all the methodologies used. Moreover, also strains CIDCA 83125 and CIDCA 83101 which could not be discriminated from *L. kefiri* by whole cell protein profile analysis, were identified as *L. parakefiri*, confirming genotypic identification by $(GTG)_5$-PCR.

Comparison of the obtained sequences with sequences available for *Lactobacillus* type and reference strains allowed to assign strains CIDCA 83118, CIDCA 83212, CIDCA 83310, CIDCA 8352, CIDCA 8361, CIDCA 8371, CIDCA 83119, CIDCA 83122 and CIDCA 83311 to *L. kefiranofaciens*, also confirming the results obtained by whole cell protein profile analysis and, at least for those fingerprinted, $(GTG)_5$-PCR profiling. In this regard, the phenylalanyl-tRNA synthase gene (*pheS*) has proven to be a valuable tool for the identification of *Lactobacillus* species from kefir grains. However, molecular typing of *L. kefiranofaciens* at the subspecies level appeared not to be possible with this method, neither was $(GTG)_5$-fingerprinting. Thus, our polyphasic approach combining SDS-PAGE, $(GTG)_5$-PCR, and sequence analysis of *pheS* allowed to classify 11 isolates as *L. kefiranofaciens* but not to differentiate the two subspecies. Possibly, whole cell protein profile analysis could be used to discriminate *kefiranofaciens* from *kefirgranum* as both subspecies clustered separately. These results are in agreement with previous work of Vancanneyt et al. (2004). They demonstrated that strains isolated from various kefir grains in France, that were assigned phenotypically to *L. kefiranofaciens*, and which showed a very similar profile to that of the type strain, could be distinguished from strains with the *L. kefirgranum* phenotype. To substantiate this, additional *L. kefiranofaciens* subsp. *kefiranofaciens* and *kefirgranum* strains should be included.

Isolated *L. kefiranofaciens* strains produced polysaccharide in strain-dependent concentrations that varied between 85.1 and 301.9 mg l^{-1} during growth in milk (Table 2). Three of them produced higher amounts of polysaccharide than *L. kefiranofaciens* JCM 6985 (110.1 mg l^{-1}) under the same growth conditions. Polysaccharides produced by these strains also differed in the degree of polymerization; whereas *L. kefiranofaciens* subsp. *kefiranofaciens* JCM 6985 produced an EPS that contains a fraction of molecular weight higher than 10^6 Da, isolated strains of *L. kefiranofaciens* produced EPS of low molecular weight (below 10^4 Da), and polysaccharides produced by strains CIDCA 83211, CIDCA 8371, CIDCA 83122 and CIDCA 83119 also presented a fraction with molecular weight between 10^4 and 10^5 Da.

4. Conclusion

The accurate species identity of kefir isolates is relevant taking into account the important role of each microorganism in the grain ecosystem as well as their potential application as starter in food-fermentation. Our data emphasize the usefulness of combining SDS-PAGE whole cell protein profiling and/or $(GTG)_5$ PCR with *pheS* gene sequence analysis for reliable identification of different species of the genus *Lactobacillus* isolated from kefir. Whereas $(GTG)_5$-PCR fingerprinting allowed to discriminate *L. parakefiri* from *L. kefiri* isolates, SDS-PAGE profiling did not. The latter, however, was the only approach that allowed identifying *L. kefiranofaciens* at subspecies level.

This study contributes to improve the knowledge about isolation and identification of *L. kefiranofaciens* for future applications in foods with defined physicochemical and health promoting properties.

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