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Molecular identification and typing of lactobacilli isolated from kefir grains

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Seventeen heterofermentative lactobacilli isolated from kefir grains were characterized by molecular methods. Bacterial isolates were identified by amplification of 16S rRNA gene and analysis by Amplified Ribosomal DNA Restriction Analysis (ARDRA), using the restriction enzymes *Hae* III, *Dde* I, and *Hinf* I. ARDRA analysis of lactobacilli isolates showed, for each enzyme used, a same banding pattern between the heterofermentative lactobacilli and the reference strains *Lactobacillus kefir* JCM 5818 and *Lb. kefir* ATCC 8007. Other reference lactobacilli and one homofermentative isolate showed differences in at least one of these patterns. The 16S-23S rRNA spacer region was also used to discriminate the bacterial isolates at the species level. The data obtained from the analysis of spacer region confirmed that sequencing of this genome region is a good tool for a reliable identification of members of *Lb. kefir* species. Genotyping of isolates was performed by Random Amplified Polymorphic DNA (RAPD-PCR) analysis using M13, Coc, ERIC-2 and 1254 primers. Patterns obtained allowed the differentiation of isolates in three groups. The three clusters showed by RAPD-PCR analysis could be correlated with at least three different strains of *Lb. kefir* species in the group of heterofermentative lactobacilli isolates obtained from Argentinian kefir grains.

Keywords: kefir grains, heterofermentative lactobacilli, molecular identification, ARDRA, intergenic spacer region, 16S rRNA gene, RAPD-PCR.

Kefir is a sour fermented milk originating from Northern area of Caucasus Mountains that is obtained by incubation of milk with kefir grains (Zourari & Anifantakis, 1988). These grains contain a relatively stable and specific population of microorganisms that exist in a complex symbiotic relationship (Marshall, 1993). Kefir grains are clusters of microorganisms held together by a matrix of polysaccharides and proteins including primarily lactic acid bacteria (lactobacilli, lactococci, leuconostoc), yeasts, acetic acid bacteria (Garrote et al. 2001), and other microorganisms not yet identified. The microbiological composition of grains is still controversial since different reports indicate that microflora strongly depends on the origin of the grains (Lin & Kuo, 1999). Specific characteristics, such as taste, aroma and texture of kefir may be attributed to the presence of a complex microbial population. To understand the relationship between organoleptic features of kefir and responsible microorganisms of the grain population an exhaustive knowledge of this microbial community is required. Characterization of microorganisms at the strain level will be particularly useful in the selection of starter cultures for standardised kefir-like product elaboration.

To analyse and rapidly identify bacteria from microbial communities, classical physiological and biochemical tests are not adequately efficient, since bacterial population involved often has similar nutritional requirements and grows under similar environmental conditions. Therefore, a clear identification within species by simple phenotypic tests may sometimes be difficult. The development of molecular techniques has opened up new perspectives for characterizing strains from fermented dairy foods. Among PCR-based methods, restriction analysis of amplified

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Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5′-3′)	Annealing position	Reference
16-1A	GAATCGCTAGTAATCG	1361 to 1380 from 16S gene sequence of *Lb. casei*	Weisburg et al. 1989
23-1B	GGGTTCCCCCATTCGGA	123 to 113 from 23S gene sequence of *Lb. casei*	Nakagawa et al. 1994
16S-F	GGCGGCGTGCCTAATAC	1 to 17 from 16S gene sequence of *Lb. kefir* NRIC 1693 (AY363303)	This work
16S-R	CACCTTAGACGGCTGGT	1478 to 1462 from 16S gene sequence of *Lb. kefir* NRCI 1693 (AY363303)	This work
ERIC-2	AAGTAAGTGACTGGGGTGAGC		van Belkum et al. 1994
M13	GAGGGTGGCGGTTCT		Stendid et al. 1994
1254	CCGCAGCCAA		Akopyanz et al. 1992
Coc	AGCAGCGTGG		Cocconcelli et al. 1997

rDNA (ARDRA) has been used for differentiation of a variety of microorganisms (Moschetti et al. 1997; Lagase et al. 2004). The 16S-23S rRNA spacer region has also been suggested as a suitable region of bacterial genome from which to derive useful taxonomic information, particularly with regard to identification at the species level (Whiley et al. 1995; Gurtler & Stanisich, 1996). Other techniques are ribotyping (Rondtong & Tannock, 1993; Yansanjav et al. 2003) and randomly amplified polymorphic DNA (RAPD) analysis (Du Plessis & Dicks, 1995; Quere et al. 1997).

The aim of this work was the molecular identification and typing of seventeen heterofermentative lactobacilli isolated from different types of Argentinian kefir grains. These isolates were previously characterized and identified as *Lactobacillus kefir* using phenotypic methods including cellular morphology, gas production and sugar fermentation pattern, growth at different temperatures and SDS-PAGE of whole-cell proteins (Garrote et al. 2001).

Materials and Methods

Bacterial isolates

A total of seventeen heterofermentative lactobacilli isolates, phenotypically identified as *Lb. kefir*, and one homofermentative lactobacilli isolate, obtained from four different kefir grains, were analysed. All isolates are from the collection of microorganisms kept at the CIDCA. Also, nine reference strains were included in the analysis (Table 2).

Culture conditions and DNA extraction

Lactobacilli were grown to mid-log phase in 80 ml of MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C, harvested by centrifugation (5000 × ***g***, 15 min, 4 °C), washed twice in physiologic solution and frozen for 1 h at −20 °C. Cells were resuspended in Tris-EDTA buffer (20 mM-Tris, 20 mM-EDTA, 20 mM-NaCl) containing 20 mg/ml lysozyme (Sigma-Aldrich, Missouri, USA) and 100 U mutanolyzyne (Sigma-Aldrich), and incubated overnight at 37 °C. After addition of SDS (Amresco, Ohio, USA) to 1% final concentration, cells were incubated for 1 h at 60 °C. Upon addition of 0·35 mg/ml RNAse (Sigma-Aldrich) and incubation for 10 min at room temperature, 0·6 mg/ml proteinase K (Sigma-Aldrich) were added and incubated at 45 °C for 30 min. After lysis, DNA was extracted using GFX Genomic Blood DNA Purification Kit (Amersham Biosciences Corp, New Jersey, USA) following the manufacturer instructions. Quality of DNA extracted was controlled by electrophoresis on 1% (w/v) agarose (Invitrogen Corp, California, USA) gel using 1 × TAE buffer (40 mM-Tris-acetate, 1 mM-EDTA) and ethidium bromide (0·5 mg/ml).

Amplified ribosomal DNA and restriction analysis (ARDRA)

Primers 16S-F and 16S-R (Table 1) were used for 16S rRNA gene amplification from bacterial isolates and reference strains. These primers were designed using 16S rRNA gene sequence from *Lactobacillus kefir* NRIC 1693 (GenBank accession number ABO24300). DNA amplifications were performed in an Eppendorf Mastercycler gradient (Eppendorf–Netheler–Hinz GmbH, Hamburg, Germany). Mix reaction contained: PCR buffer (20 mM-TRIS-HCl, 50 mM-KCl pH 8·4), 1·5 mM-$MgCl_2$, 0·2 mM-each one dNTPs, 1 U *Taq* DNA polymerase (Invitrogen Corp), 1 μM each primer and 40 ng DNA in a final volume of 10 μl. PCR amplifications were performed under the following conditions: denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, and DNA extension at 72 °C for 30 s. A final extension was added at 72 °C for 5 min. Amplicons were analysed on 1% (w/v) agarose gel with ethidium bromide (0·5 mg/ml) in TAE buffer for 1 h at 90 V and made visible by UV transillumination. After cycling, amplicons from reference strains and lactobacilli isolates were differentiated by a restriction analysis using the enzymes *Dde* I, *Hae* III, *Hinf* I and *Eco* RI (New England BioLabs Inc,

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Table 2. Analysis of 16S-23S ribosomal spacer region

Lactobacilli isolates and reference strains	Base composition (%)				Lenght (nt)	GenBank Accession Number
	T(U)	C	A	G		
CIDCA 8347	37·4	13·5	33·8	15·3	222	AY579581
CIDCA 83116	37·1	13·6	33·9	15·4	221	AY579580
CIDCA 8317	37·2	13·5	34·1	15·2	223	AY579582
CIDCA 8314	36·0	14·4	34·2	15·3	222	AY579570
CIDCA 8315	36·5	14·0	34·2	15·3	222	AY579576
CIDCA 8326	36·5	14·0	34·2	15·3	222	AY579575
CIDCA 83110	36·7	13·6	33·5	16·3	221	AY579578
CIDCA 83111	36·5	14·0	34·2	15·3	222	AY579574
CIDCA 8348	36·5	14·0	34·2	15·3	222	AY579573
CIDCA 8325	36·5	14·0	34·2	15·3	222	AY579571
CIDCA 8321	36·5	14·0	34·7	14·9	222	AY579568
CIDCA 8345	36·8	13·9	33·2	16·1	223	AY579579
CIDCA 8332	36·5	14·0	34·2	15·3	222	AY579577
CIDCA 8343	36·5	14·0	34·2	15·3	222	AY579572
CIDCA 8310	36·9	13·1	34·2	15·8	222	AY558897
CIDCA 8337	35·7	15·9	32·9	15·5	207	AY579583
Lb. kefir JCM 5818	36·5	14·0	33·8	15·8	222	AY579569
Lb. brevis JCM 1059	36·9	15·0	32·7	15·4	214	AY582720
Lb. kefir ATCC 8007	36·5	14·0	34·2	15·3	222	AY821849
Lb. brevis ATCC 8287	36·4	15·4	32·7	15·4	214	AY821851
Lb. buchneri ATCC 4005	37·8	13·5	33·8	14·9	222	AY821850
Lb. rhamnosus ATCC 7469	28·0	19·3	31·2	21·6	218	AF121201
Lb. fructivorans ATCC 8288	39·1	13·6	35·9	11·4	220	AJ616220
Lb. plantarum DSM 20174	36·0	15·3	34·0	14·8	207	AF182722
Lb. hilgardii DSM 20176	34·2	15·5	34·7	15·5	219	AJ616222

Massachusetts, USA). Selection of restriction enzymes was made on the basis of recognition site diversity among different species of *Lactobacillus* genus and the number of different size restriction fragments produced. Digestions were carried out overnight at 37 °C, in a final volume of 20 µl containing NEB buffer (100 mM-NaCl, 20 mM-Tris-HCl, 20 mM-$MgCl_2$, 1 mM-DTT) (New England BioLabs Inc), 3 U of enzyme and 5 µl amplicon. Digestions were stopped by incubation for 20 min at 80 °C. Reaction products were electrophoresed on 1·8% agarose gel with ethidium bromide in the conditions previously described.

Sequencing and analysis of PCR-amplified 16S rDNA

Amplification reaction of 16S rRNA gene region, from two randomly selected heterofermentative isolates and the reference strain *Lb. kefir* JCM 5818, was performed under identical conditions to ARDRA analysis. PCR products were purified using QIAquick PCR Purification kit (QIAGEN Corp, California, USA) and sequenced with primers 16S-F and 16S-R (Table 1) by means of an Applied Biosystem Model 3700 DNA Analyser (Perkin-Elmer Corp/Applied Biosystems, California, USA). Multiple alignments of nucleotide sequences were performed by CLUSTAL W (Thompson et al. 1994).

Analysis of the 16S-23S rRNA spacer region

Primers 16-1A and 23-1B (Table 1) were used to amplify the 16S-23S rRNA spacer region and its flanking regions, from seventeen heterofermentative and one homofermentative lactobacilli isolates, and reference strains. The composition of PCR mix was the same as the use for 16S rDNA amplification. PCR reaction was performed under the following conditions: first denaturation at 94 °C for 2 min and 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and DNA extension at 72 °C for 30 s. A final extension was added at 72 °C for 5 min. Amplicons were analysed on 1% (w/v) agarose gel with ethidium bromide (0·5 mg/ml) in TAE buffer for 1 h at 90 V, and made visible by UV transilumination. Minor fragments were excised from agarose gel and purified using QIAquick Gel Extraction kit (QIAGEN Corp). Fragments were cloned in *Escherichia coli* DH5αF′ using pGem-T Easy Vector System I (Promega Corp, Wisconsin, USA), in accordance with the manufacturer instructions. Blue/white colonies were screened on LB agar with ampicillin (Sigma-Aldrich Corp), X-gal and IPTG (Promega Corp). White colonies were screened by PCR with primers used in PCR amplification of 16S-23S rRNA spacer region. Positive clones were grown in 10 ml LB medium with ampicillin and plasmid DNA was extracted with GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech).

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DNA inserts were sequenced using the universal primers T7 and Sp6 (Promega Corp) in an Applied Biosystem Model 377 DNA Analyser (Perkin-Elmer Corp/Applied Biosystems). A multiple sequence alignment using the Clustal X Program (Thompson et al. 1994) was also made.

Random amplified polymorphic DNA (RAPD)

PCR amplifications were performed with four different primers (Table 1). Each reaction mixture was composed of: PCR buffer (20 mM-TRIS-HCl, 50 mM-KCl pH 8·4), 2 mM-$MgCl_2$, 0·2 mM-each of dATP, dCTP, dGTP and dTTP, 1 U of *Taq* DNA polymerase (Invitrogen Corp), 100 nm-primer, and 0·8 µg DNA. Amplifications were performed for 30 cycles using the following temperature profile: denaturing at 94 °C for 1 min, annealing at 30 °C for 30 s and extension at 72 °C for 2 min. Initial denaturation was performed at 94 °C for 4 min and a final extension step of 72 °C for 5 min was employed. PCR products were analysed on 1·8% (w/v) agarose gel using 1 × TAE buffer and ethidium bromide. A 100 bp ladder (Productos Bio-Lógicos, UNQ, Argentina) was used as molecular weight standard. Samples were submitted to electrophoresis for 2 h at 60 V, and DNA was visualized by UV transillumination.

Cluster analysis using RAPD-PCR patterns

High-performance photographs of RAPD-PCR gells were obtained by Kodak Electrophoresis Documentation and Analysis System 120, and analysed by Kodak Digital Science V.3.0.2 (Kodak, Connecticut, USA). From the four RAPD PCR profiles, a unique dendrogram was obtained by pair-wise comparison of profiles. Pattern evaluation was made by calculation of genetic similarity index, using simple matching coefficient (Apostol et al. 1993). Cluster analysis was carried out similarly with estimates by the Unweighted Pair Group Method using Arithmetic Averages (UPGMA), Dendrograms obtained showed the relationships between lactobacilli isolates and reference strains. Analysis was performed by using PAUP* 4.0b10 (Sinauer Associates, MS, USA).

Results

Identification of lactobacilli isolates by ARDRA analysis

ARDRA analysis was first applied on reference strains to verify the capacity of the method to provide enough discrimination among species of *Lactobacillus* genus. Results obtained from reference strains showed that this method was able to differentiate each of the strains used from the remaining ones at the species level (Fig. 1A). ARDRA analysis of lactobacilli isolates showed, for each enzyme used, the same banding pattern between the heterofermentative lactobacilli and the reference strains *Lb. kefir* JCM 5818 and *Lb. kefir* ATCC 8007 (Fig. 1B). Remaining reference strains and the homofermentative isolate CIDCA 8337 showed differences in at least one of these patterns (Fig. 1B). Digestion of 16S rDNA amplicons with *Eco* RI enzyme did not produce fragments under the conditions used (data not shown).

Identification by sequencing of 16S rRNA gene

A fragment of approximately 1500 bp of the 16S rRNA gene from heterofermentative isolates CIDCA 8325 and CIDCA 8348, and the reference strain *Lb. kefir* JCM 5818 was sequenced (GenBank Accession Numbers AY579585, AY579586, and AY579584, respectively). Comparison of sequences obtained with sequences deposited in the Ribosomal Database Project (RPDII) (Maidak et al. 1994, 2001) (Center for Microbial Ecology at Michigan State University, www.cme.msu.edu/RDP/analyses.html) and in the GenBank Database, showed the highest identity value (above 97%) with the 16S rRNA gene sequence of *Lb. kefir* strains NRCI 1693 and JCM 5818.

Comparative sequence analysis of 16S-23S rRNA spacer region

A gene region including the 16S-23S rRNA spacer, was amplified by PCR from lactobacilli isolates and reference strains. Amplification patterns obtained showed four fragments of different length between homofermentative and heterofermentative lactobacilli, but the same length among the lactobacilli of each group (data not shown). From fifteen heterofermentative and one homofermentative lactobacilli isolates, and five reference strains (*Lb. brevis* JCM 1059, *Lb. brevis* ATCC 8287, *Lb. buchneri* ATCC 4005, *Lb. kefir* ATCC 8007, *Lb. kefir* JCM 5818), the minor length fragment was cloned and sequenced. Depuration of flanking regions was made using conserved sequences of 3′ 16S rRNA and 5′ 23S rRNA genes, identified by comparison with sequences of *Lb. brevis* strains deposited in the GenBank Database. Nucleotide size of ribosomal spacer region and base composition of each isolate and reference strain are shown in Table 2.

Analysis of 16S-23S rRNA spacer region sequences with tRNAscan (SE Search Server, Washington University, St. Louis, Missouri) was developed, and tRNA genes were not found. The absence of tRNA genes and the size of the minor length fragment of 16S-23S rRNA spacer region, are in good correlation with data reported by Nour (1998) for members of the *Lactobacillus* genus.

A multiple sequence alignment including 16S-23S rRNA spacer region sequences obtained from the lactobacilli isolates and several sequences from the GenBank database (*Lb. plantarum* DSM 20174, *Lb. hilgardii* DSM 20176, *Lb. rhamnosus* ATCC 7469 and *Lb. fructivorans* ATCC 8288) was made. The resulting unrooted phylogenetic tree (Fig. 2) showed a high similarity among the

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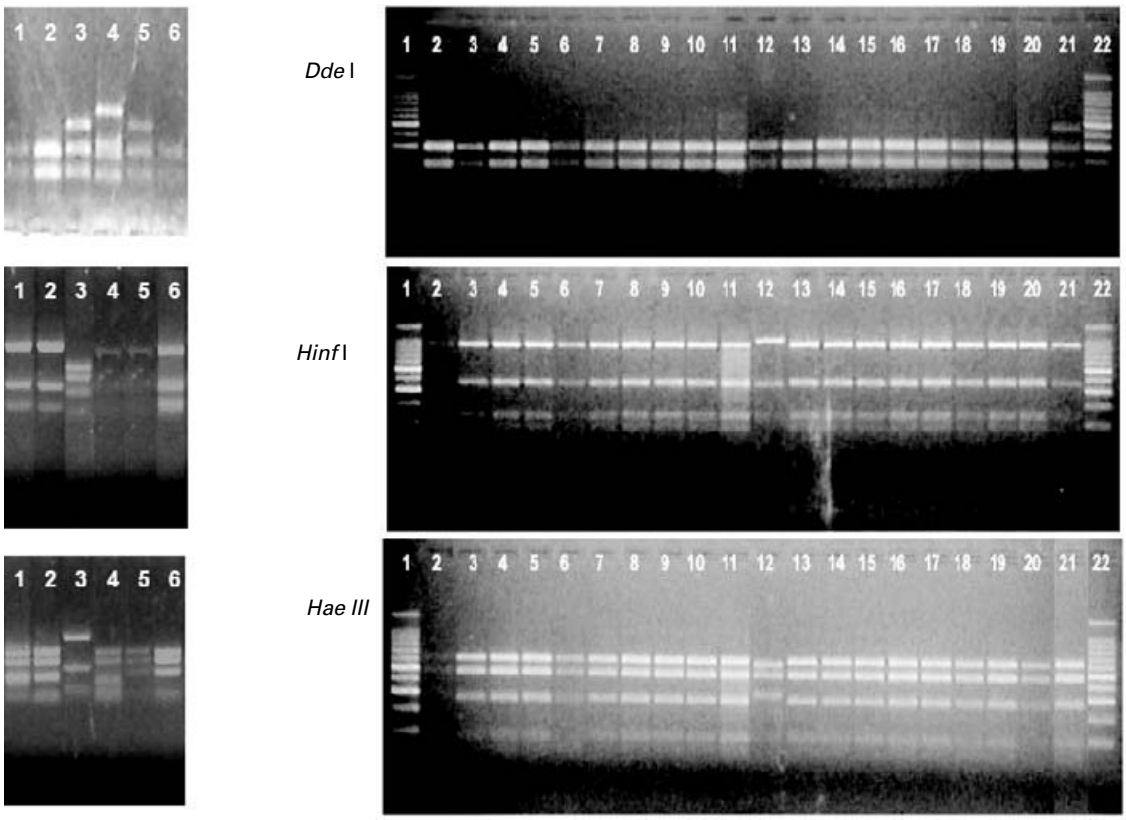

Fig. 1. Electrophoresis of *Dde* I, *Hinf* I, and *Hae* III ARDRA patterns on 1·8% agarose gel. Fig. 1A: Lanes: 1, *Lb. kefir* JCM 5818; 2, *Lb. kefir* ATCC 8007; 3, *Lb. buchneri* ATCC 4005; 4, *Lb. hilgardii* DSM 20176; 5, *Lb. plantarum* DSM 20174; 6, *Lb. rhamnosus* ATCC 7469. Fig. 1B: lanes 1, MW ladder (100 bp); 2, CIDCA 8317; 3, CIDCA 8321; 4, CIDCA 8325; 5, CIDCA 8326; 6, CIDCA 8335; 7, CIDCA 8348; 8, CIDCA 83115; 9, CIDCA 8310; 10, CIDCA 83116; 11, CIDCA 8315; 12, CIDCA 8337; 13, CIDCA 8346; 14, CIDCA 8347; 15, CIDCA 83110; 16, CIDCA 8314; 17, CIDCA 83111; 18, CIDCA 8332; 19, CIDCA 8345; 20, *Lb. kefir* JCM 5818; 21, *Lb. brevis* JCM 1059; 22, MW ladder (100 bp).

heterofermentative isolates and both *Lb. kefir* reference strains. In this cluster, only the heterofermentative isolate CIDCA 8345 showed a similarity value lower than 99% (98·5%). On the other hand, all homofermentative lactobacilli grouped in a cluster placed in the most distant branch of the tree, showing similarity values lower than 80%.

Genotypic diversity of lactobacilli isolates by RAPD-PCR analysis

RAPD-PCR analysis was performed with M13, ERIC-2, Coc and 1254 primers and patterns obtained are shown in Fig. 3 (right). Primer M13 yielded a few different RAPD patterns. The most common profile was exhibited by twelve isolates (60%), and it was similar to pattern shown by *Lb. kefir* JCM 5818.

RAPD-PCR analysis performed with primer ERIC-2 gave several different patterns. The most common pattern was shared by five isolates (25%) and the second one was shared by four isolates (20%). *Lb. kefir* JCM 5818, *Lb. kefir* ATCC 8007, *Lb. brevis* JCM 1059, and the remaining isolates showed a different ERIC-2 profile between them and from profiles above mentioned (Fig. 3, right).

The highest number of different RAPD-PCR profiles was obtained using Coc primer. Only two isolates, CIDCA 8345 and 8332 (10%), showed the same pattern (Fig. 3, right). The remaining isolates and reference strains gave each one a different and specific RAPD-PCR profile.

Primer 1254, like primer M13, was not able to differentiate isolates, since patterns obtained with this primer were almost the same for all heterofermentative lactobacilli (Fig. 3, right).

The UPGMA dendrogram was obtained by combination of Coc, ERIC-2, 1254, and M13 profiles (Fig. 3, left). The similarity value between the homofermentative isolate and the remaining lactobacilli was 75%, meanwhile this value was 79% between *Lb. brevis* JCM 1059, the heterofermentative isolates and both *Lb. kefir* reference strains. The similarity value between the heterofermentative isolates and both *Lb. kefir* reference strains was higher than 80%.

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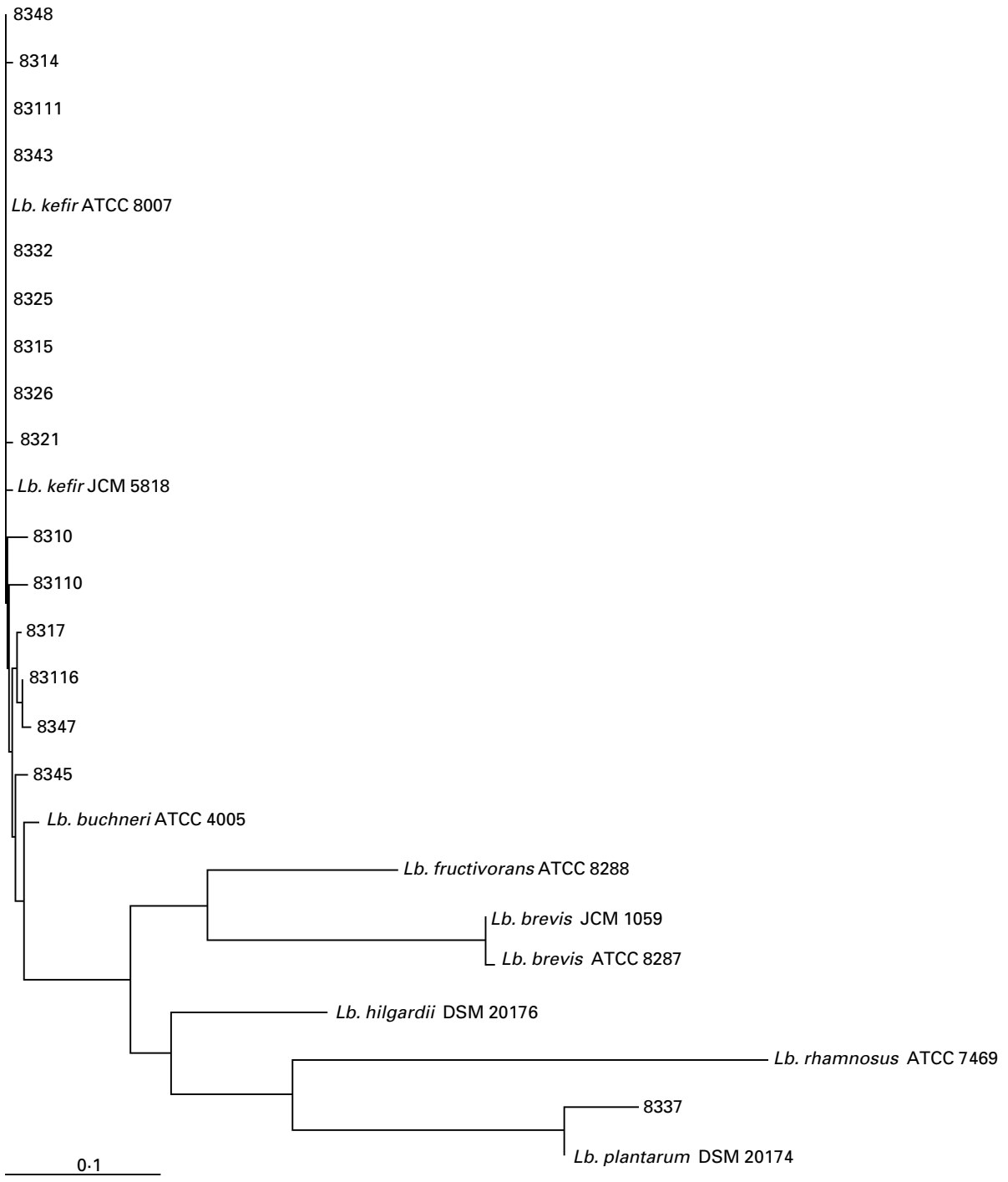

Fig. 2. Unrooted phylogenetic tree generated on the basis of 16S-23S gene spacer region sequences from fifteen heterofermentative, and one homofermentative lactobacilli isolates, and the nine reference strains indicated in the figure.

Cluster analysis from RAPD-PCR patterns of heterofermentative isolates

The dendrogram obtained from the RAPD-PCR patterns of heterofermentative isolates and *Lb. kefir* reference strains is shown in Fig. 4. Based on an arbitrary similarity value of 87% to define clusters, this unrooted tree has three clusters. Cluster 1 includes the largest number of isolates, and it is composed by four sub-clusters: 1A (six isolates), 1B (two isolates and *Lb. kefir* ATCC 8007), 1C (two isolates), and 1D (four isolates). Clusters 2 (CIDCA 8326) and 3 (CIDCA 8343 and 8317) have a more distant relationship with respect to the remaining heterofermentative isolates, being the similarity values of 80·1% and 86%, respectively.

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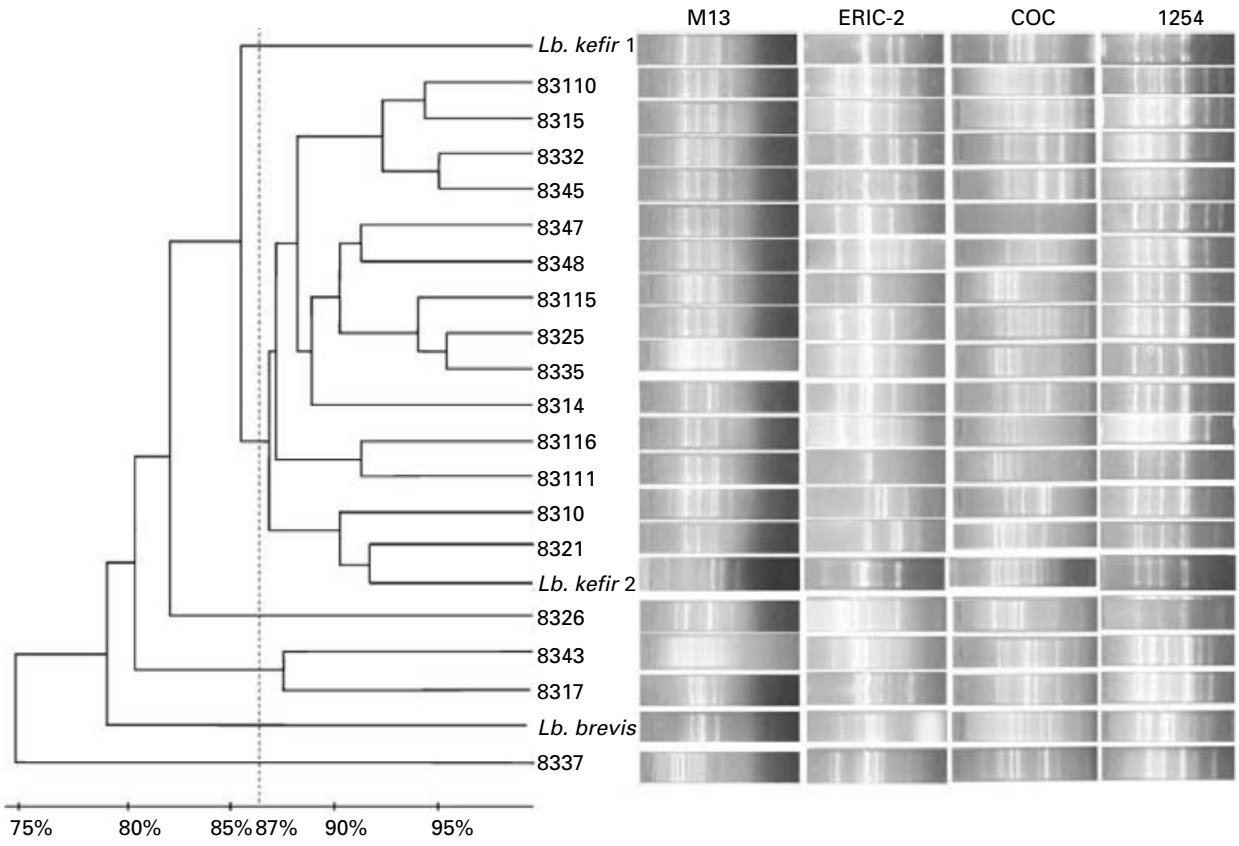

Fig. 3. Right: RAPD-PCR patterns obtained with Coc, ERIC-2, 1254, and M13 primers. Left: Combined dendrogram obtained from Coc, Eric-2, 1254, and M13 RAPD-PCR patterns. *Lb. kefir* 1 correspond to *Lb. kefir* JCM 5818 and *Lb. kefir* 2 with *Lb. kefir* ATCC 8007.

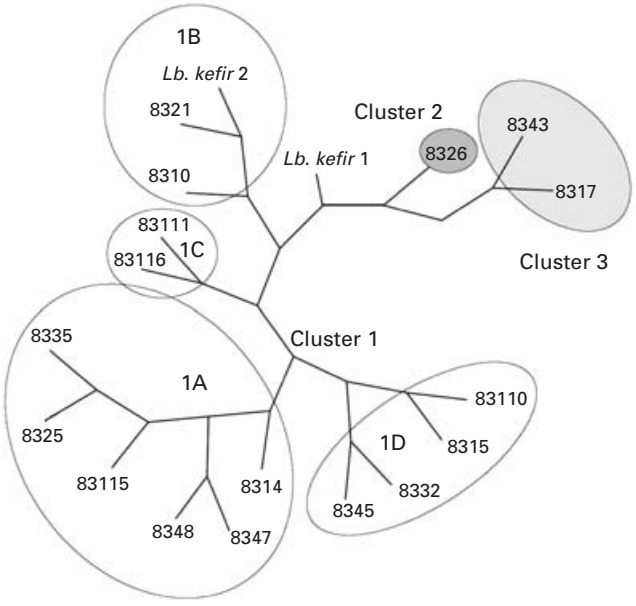

Fig. 4. Dendrogram generated on the basis of combinated RAPD-PCR patterns. Clusters are indicated by numbers, subclusters by a number and a letter.

Discussion

In the present work, the identity of seventeen heterofermentative lactobacilli isolates obtained from kefir grains was confirmed, by molecular methods. Results of ARDRA analysis from the closely related reference strains studied, including *Lb. kefir*, *Lb. brevis*, *Lb. buchneri*, and *Lb. hilgardii*, established the value of this technique for the species differentiation of *Lactobacillus* genus. Results are in good agreement with previous data where ARDRA analysis was applied to discrimination of species belonging to *Lb. acidophilus* and *Lb. casei* groups (Roy et al. 2001). The identical *Dde* I ARDRA patterns exhibited by all heterofermentative lactobacilli isolates and both *Lb. kefir* reference strains confirmed the that the isolates belonged to the species *Lb. kefir* (Garrote et al. 2001).

Sequencing of 16S rRNA gene applied on two randomly selected lactobacilli isolates, reach the cut-off value proposed by Stackebrandt & Goebel (1994) at the species level. Although sequencing of 16S rRNA gene is the most widely used approach for species identification, there are some methodological limitations due to the size of the generated amplicon. This problem could be highly simplified by sequencing of the spacer region between 16S

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and 23S rRNA genes. To analyse the reliability of this molecular tool, several reference lactobacilli strains were used. The heterofermentative species *Lb. kefir*, *Lb. buchneri*, *Lb. brevis*, *Lb. fructivorans* and *Lb. hilgardii*, belonging to the same phylogenetic group (*Lb. casei–Pediococcus group*) (Vandamme et al. 1996), were chosen. Some less related lactobacilli, the homofermentative species *Lb. plantarum*, *Lb. rhamnosus* and the isolate CIDCA 8337 were also included. The fact that all isolates and reference strains identified as *Lb. kefir* were grouped in the same branch of the tree and showed high similarity values, demonstrated that this molecular tool is reliable to identify members of this species. Our data show that close genetically related species as *Lb. kefir*, *Lb. buchneri*, *Lb. hilgardii* and *Lb. brevis* can be well differentiated by 16S-23S rRNA spacer region sequence analysis.

Reproducible RAPD patterns were obtained with primers selected. Differences in discriminatory power of these primers became apparent after the analysis of several lactobacilli isolates. RAPD-PCR analysis with Coc primer, specifically designed for lactic acid bacteria discrimination (Cocconcelli et al. 1997), gave the best differentiation among lactobacilli isolates. For isolates belonging to the species *Lb. kefir*, RAPD analysis allowed discrimination of lactobacilli in three clusters, sharing a similarity value higher than 80% with both *Lb. kefir* strains. The three clusters showed by RAPD-PCR analysis could be correlated with at least three different strains of *Lb. kefir* species in the group of heterofermentative lactobacilli isolates obtained from Argentinian kefir grains.

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