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Polyphasic characterization of the lactic acid bacteria in kefir

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Abstract

The lactic acid bacteria of kefir were isolated and characterized using phenotypical, biochemical, and genotypical methods. Polyphasic analyses of results permitted the identification of the microflora to the strain level. The genus *Lactobacillus* was represented by the species *Lb. kefir* and *Lb. kefiranofaciens*. Both subspecies of *Lactococcus lactis* (*lactis* and *cremoris*) were isolated. *Leuconostoc mesenteroides* subsp. *cremoris* was also found.

The kefir studied contained few species of lactic acid bacteria but showed a high number of different strains. We found that the polyphasic analysis approach increases the confidence in strain determination. It helped confirm strain groupings and it showed that it could have an impact on the phylogeny of the strains.

Keywords: Kefir; Lactic acid bacteria; LAB; Complex microflora; PCR; RFLP; 16S rRNA

Introduction

No clear definition of what is kefir exists presently. The FAO/WHO food standards defines kefir starter culture as being kefir grains, *Lactobacillus kefiri*, species of the genera *Leuconostoc*, *Lactococcus*, and *Acetobacter*. It also contains *Kluyveromyces marxianus* and *Saccharomyces unisporus*, *S. cerevisiae* and *S. exiguus* (www.codexalimentarius.net). This definition does not describe what the microflora of kefir grains contains. It also does not include *Lactobacillus kefiranofaciens*, *L. kefirgranum* and *L. parakefir* in the list of *Lactobacillus* species to be present in a kefir starter.

Published reports used phenotypic traits and biochemical tests to identify the species present in kefir [14,17,19,21,25]. Very few studies using molecular techniques for the identification of lactic acid bacteria in specific kefir grains have been published [22,23].

In order to understand the fermentation of kefir, the composition of the final product, and later on be able to make claims about the probiotic properties of such a product, a clear understanding of the microflora has to be attained [6]. Identifying each strain of lactic acid bacteria present in kefir was the aim of this study.

Materials and methods

Bacterial strains

Kefir grains were obtained from the Moscow Dairy Institute (Moscow, Russia) and were maintained by daily transfers in pasteurized cows' milk at 21 °C at the Liberty Company (Brossard, QC, Canada) that produces kefir commercially. Type strains and reference

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Table 1. Bacterial strains used for this work

Species	Strain
Lactobacillus acidophilus	ATCC 4356^T
Lactobacillus helveticus	ATCC 10797
Lactobacillus helveticus	ATCC 12046
Lactobacillus helveticus	ATCC 15009^T
Lactobacillus kefir	ATCC 35411^T
Lactobacillus kefir	ATCC 8007
Lactobacillus brevis	ATCC 14869^T
Lactobacillus brevis	ATCC 13648
Lactobacillus kefirgranum	LMG 15132^T
Lactobacillus parakefir	LMG 15133^T
Lactobacillus kefiranofaciens	ATCC 43761^T
Leuconostoc mesenteroides subsp. *mesenteroides*	ATCC 8293^T
Leuconostoc mesenteroides subsp. *cremoris*	LMG 14531
Leuconostoc mesenteroides subsp. *cremoris*	LMG 6909^T
Leuconostoc pseudomesenteroides	ATCC 12291^T
Lactococcus lactis subsp. *lactis*	LMG 6890^T
Lactococcus lactis subsp. *lactis biovar diacetylactis*	LMG 7931
Lactococcus lactis subsp. *cremoris*	LMG 6897^T

strains (Table 1) were obtained from the American Type Culture Collection (ATCC), USA and the BCCM/LMG Bacteria Collection Laboratory for Microbiology (LMG), Belgium.

Isolation and cultivation

Drained kefir grains (10 g) were recovered from a 20 h fermented mother culture using a sterilized strainer and homogenized with 90 g of sterile saline containing 0.9% NaCl and 0.1% bacto peptone (Difco Laboratories). Serial dilutions were performed and aliquots were plated on M17 agar (BDH) containing 0.5% glucose for the selective growth of lactococci; lactobacilli MRS broth (Difco) supplemented with 1.5% agar and adjusted to pH 5.4 with acetic acid, as well as LAW agar (ATCC) for the growth of lactobacilli. Leuconostocs were isolated using MRS medium containing 5 ml/L of 1% X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) solution. MRS X-gal plates were incubated 7 days at 15 °C under anaerobiosis (5% CO_2, 10% H_2, and 85% N_2). M17 plates were incubated aerobically at 30 °C for 2 days. MRS and LAW plates were incubated under anaerobiosis at 30 °C for 4 days. All colonies with different morphologies or at least 10% of the total number of colonies on the plates counted were transferred to an appropriate growth medium and characterized further.

Characterization of the kefir isolates

Isolates were identified by phenotypic criteria [2]. The identification system API 50CH (bioMérieux, Marcy-l'Etoile, France) was used for assimilation tests of lactic acid bacteria. Produced lactic acid isomers were determined using the D-lactic acid/L-lactic acid UV-test (Boehringer Mannheim). Kefir isolates that gave different biochemical patterns were investigated further. They were characterized by RFLP and/or PCR–RFLP [8,11,12]. Partial sequencing of variable regions of 16S rRNA genes was also performed.

Preparation of genomic DNA from lactobacilli, leuconostocs and lactococci

Extraction of DNA was performed based on a bacterial genomic DNA extraction protocol [27] and modified as follows. A culture (10 ml) was centrifuged at 1430*g* for 10 min. The pellet was resuspended in 1 ml TS buffer (Tris–HCl 25 mM, 12% sucrose, pH 8.0). The suspension was transferred into a 1.5 ml microcentrifuge tube and centrifuged (13,490*g*; 10 min; 25 °C). The pellet was washed twice in TS buffer and resuspended in 400 µl TS together with 100 µg mutanolysin (Sigma) (50 µl of 2 mg/ml TS buffer solution) and 2 mg lysozyme (Sigma) (50 µl of 40 mg/ml TS buffer solution). Tubes were incubated at 37 °C with gentle agitation. After 2 h standing, 100 µl 10% SDS, 200 µl 250 mM EDTA pH 8.0, and 1 mg proteinase K (50 µl of a 20 mg/ml TS buffer solution) were added and incubation was carried on for another 2 h. The contents of each tube were separated into two tubes (~500 µl) and then 120 µl 5 M NaCl and 100 µl 10% CTAB (cetyltrimethyl-ammonium bromide)/0.7 M NaCl were added to each tube. Some strains producing large amounts of polysaccharides were treated with 100 µl 2% PVP (polyvinyl pyrolidone)/10% CTAB/0.7 M NaCl to liberate the residual polysaccharides from the DNA-containing aqueous phase in later stages. Tubes were incubated for 20 min at 65 °C. DNA was purified with three (24:1) chloroform/isoamyl alcohol extractions (550 µl). After centrifugating the tubes (14,300*g*; 10 min; 25 °C), the upper phase was transferred to a fresh tube. Portions (500 µl) of cold isopropanol were added to precipitate the DNA overnight at −20 °C. Tubes were centrifuged (14,300*g*; 10 min; 25 °C) and the pellet was air-dried for 30 min. The DNA was finally resuspended in 100 µl sterile water.

Preparation of plasmid DNA from *Lactobacillus kefir*

The protocol for plasmid DNA preparation was adapted from O'Sullivan and Klaenhammer [16] as follows. Cells were grown in 100 ml MRS broth at 30 °C under aerobic conditions. After log phase cells were centrifuged (1430*g*; 10 min; 20 °C). The pellet was washed twice with 20 ml TES buffer (50 mM Tris–HCl, pH 7.4; 50 mM EDTA, pH 8.0, 12% sucrose). The cells

were then resuspended in 10 ml TES containing 1 mg/ml lysozyme and incubated at 37 °C for 2 h. Mutanolysin (75 µg/ml) was added and the incubation was allowed to continue until most of the cells appeared as protoplasts under the light microscope (2 h). After centrifugation (1430*g*; 10 min; 20 °C), the cells were washed with 20 ml TES, centrifuged and resuspended in 4 ml TE-RNase (10mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mg/ml boiled RNase A). The tubes were incubated at 37 °C for 15 min. Then, 8 ml of freshly prepared alkaline SDS (3% SDS, 0.2 N NaOH) was added and the tubes were incubated at room temperature for 7 min before 6 ml of ice-cold sodium acetate (3 M, pH 4.8) was added. Tubes were gently mixed and put on ice for 15 min. After centrifugation (1430*g*; 35 min; 4 °C), the supernatant was transferred into a new tube containing 13 ml isopropanol and kept at −20 °C overnight. The tubes were then centrifuged and the DNA pellet was air-dried for 15 min and resuspended in 1 ml sterile water containing RNase (Sigma) (0.1 mg/ml boiled RNase A).

RFLP analyses

Chromosomal DNA samples (3–5 µg) from lactobacilli and lactococci were digested with *Hin*dIII or *Eco*RI (10 U/µg of DNA; New England Biolabs). Leuconostocs were digested with *Eco*0109I or *Bso*BI (10 U/µg of DNA; New England Biolabs). Agarose gel electrophoresis was performed. The restriction fragments were transferred to a positively charged nylon membrane (Roche Diagnostics, Laval, QC, Canada). DIG-labelled probes were obtained by PCR. The total genomic DNA from *Lb. brevis* ATCC 14869 (for lactobacilli), *Lc. lactis* subsp. *lactis* LMG 6890 (for lactococci), and *Leuconostoc mesenteroides* LMG 6909 (for leuconostocs) was isolated as previously described. Probes were prepared using a PCR DIG probe synthesis kit (Roche Diagnostics, Laval, QC, Canada) according to the instructions of the manufacturer. Primers used for probe design (located in the 16S rRNA) were synthesized at Bio S&T Inc. (Lachine, QC, Canada). Nucleotide sequences and amplification conditions of the primers are presented in Table 2.

PCR–RFLP of *Lactococcus lactis* subspecies

PCR amplification was performed using puRe Taq Ready-To-Go PCR beads (GE Healthcare, NJ, USA) according to the manufacturer's instructions. The primers and amplification conditions used to produce the PCR fragments (PLc1 and PLc2) are shown in Table 2. Primer sequence were chosen based on work by Salama et al. [20] who showed that an area of the 16S rRNA can differentiate the two subspecies. The amplified fragments were digested with *Rsa*I, *Hae*II or *Ear*I restriction endonucleases (New England Biolabs, Mississauga, Ont., Canada) according to the supplier's instructions. The fragments were run on a 2% NuSieve 3:1 agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME, USA) and stained with ethidium bromide (Sigma).

DNA Sequencing

A region of 16S rRNA located near the beginning of the gene [11] of isolates IM002, IM014, IM015, IM017, and IM082 were determined by sequencing the PCR-amplified 16S rRNA gene product (using primers P3Lb and P4i for *Lactobacillus* strains and P3 and P4 for the *Leuconostoc* strain) in both directions by Université Laval sequencing services (Que., QC, Canada). Subsequently, the partial 16S rDNA sequences were aligned and compared with sequences available from the Genbank database using Vector NTI Suite 9 software (Informax Inc., MD, USA).

Results

Morphology of the isolates

All lactic acid bacteria isolated from kefir were gram-positive and non-motile. Lactobacilli strains occurred

Table 2. Primers used for probe design, for PCR-RFKP and/or for sequencing analyses

Primer	Sequence	PCR product (bp)	Amplification conditions
P3	5′GGAATCTTCCACAATGGGCG3′		95 °C 5 min
P4	5′ATCTACGCATTCCACCGCTAC3′	344 bp	94 °C 1 min, 67 °C 40 s, 72 °C 1 min, 40 cycles 72 °C 10 min
P3Lb	5′GGGAATCTTCCACAATGGACG3′		95 °C 5 min
P4i	5′ATGCTTTCGAGCCTCAGCGTC3′	414 bp	94 °C 1 min, 67 °C 40 s, 72 °C 1 min, 40 cycles 72 °C 10 min
PLc1	5′GCGGCGTGCCTAATACATGC3′		95 °C 5 min
PLc2	5′TTCCCCACGCGTTACTCACC3′	90 bp	93 °C 1 min, 54 °C 1.5 min, 72 °C 2.5 min, 30 cycles 72 °C 10 min

singly, in pairs, or occasionally in short chains. Strains which belong to the *Lb. kefir* species formed short rods and did not seem to produce exopolysaccharides (EPS), while those of the *Lb. kefiranofaciens* species were longer and produced EPS or some sort of extra-cellular structure. Our screening method did not allow the isolation of *Lb. parakefir* strains. This species may not be represented or may exist in very low numbers in the kefir studied. Lactococci strains mostly appeared as pairs or short to long chains. *Leuconostoc* strains were also found in pairs or chains and produced a capsular material.

Biochemical and physiological characteristics

As shown in Table 3, *Lactobacillus* sp. IM014, IM015, and IM017 fermented galactose and trehalose but not arabinose, contrary to the other isolates. Neither of these strains grew at 15 °C, nor produced gas from both glucose and gluconate. These strains did not produce ammonia from arginine, while the other isolates did. This homofermentative profile, along with the combination of the other biochemical results suggest that strains IM014, IM015, and IM017 might belong to the *Lb. kefiranofaciens* species, while the other isolates showed similarity to the *Lb. kefir* profile. All isolated lactobacilli strains produced both isomers of lactic acid. Results obtained with the lactococci isolates are shown in Table 4. *Lactococcus* sp. IM103. IM104, and IM105 failed to produce acid from ribose and starch compared to the others isolates. They also did not produce ammonia from arginine. All strains produced L-lactic acid. These results suggested that IM103, IM104, and IM105 belonged to the *cremoris* subsp., while the other isolates belonged to the *lactis* subspecies. The results for the leuconostocs are presented in Table 5. There seemed to be limited diversity within that genus in the kefir studied. Two isolates were studied further. Both kefir isolates could utilize glucose, galactose, and lactose. IM080 produced acid from *N*-acetyl glucosamine but the reaction was weak for IM082. Both strains were able to grow at 10 and 37 °C, and both strains produced D-lactic acid and gas from glucose. Strain IM082 was further investigated and was shown to produce diacetyl and small amounts of mannitol in milk. These results suggested that IM080 and IM082 might belong to the *cremoris* subspecies of *Ln. mesenteroides*.

RFLP analysis and sequencing results of lactobacilli

Results obtained from the Southern blot analysis of total genomic DNA from 10 lactobacilli digested with *Hin*dIII showed different banding patterns for the three different species (Fig. 1). IM014 had the same pattern as

Table 3. Comparison of the carbohydrate metabolism of the lactobacilli strains

Bacterial strain	amygdalin	cellobiose	galactose	lactose	maltose	mannitol	mannose	melibiose	raffinose	salicin	sucrose	trehalose	arabinose	esculin	melizitose	ribose	xylose	gluconate	sorbitol	arbutine	gentiobiose	N-acetyl-glucosamine	fructose
ATCC 14869	-	-	+	-	+	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	+
ATCC 13648	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	+	+
ATCC 35411	-	-	+	+	+	-	-	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	+
ATCC 8007	-	-	+	+	+	-	-	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	+
LMG 15132	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	+
ATCC 43761	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+
IM002	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+
IM005	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+
IM008	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+
IM011	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
IM014	-	-	+	+	+	-	-	+	+	-	+	+	-	+	-	-	-	-	-	-	-	+	+
IM015	-	-	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
IM017	-	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+
IM020	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
IM022	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
IM023	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

Table 4. Comparison of the carbohydrate metabolism of the lactococci strains

Bacterial strain	amygdalin	cellobiose	galactose	lactose	maltose	mannitol	mannose	melibiose	raffinose	salicin	sucrose	trehalose	arabinose	esculin	melizitose	ribose	xylose	gluconate	sorbitol	arbutine	gentiobiose	starch	N-acetyl-glucosamine
LMG 6890	+	+	+	+	+	-	+	-	-	+	-	+	-	+	-	+	+	-	-	+	-	-	+
LMG 7931	+	+	+	+	+	-	+	-	-	+	-	+	+	+	-	+	-	+	-	+	+	+	+
LMG 6897	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
IM101	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	+	+	+
IM102	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	+	+	+
IM103	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	+	-	+
IM104	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	+	-	+
IM105	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	+	-	+
IM106	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	+	+	+
IM107	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	+	+	+
IM109	-	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	+	-	+

Table 5. Comparison of the carbohydrate metabolism of the leuconostocs strains

Bacterial strain	amygdalin	cellobiose	galactose	lactose	maltose	mannitol	mannose	melibiose	raffinose	salicin	sucrose	trehalose	arabinose	esculin	melizitose	ribose	xylose	gluconate	sorbitol	arbutine	gentiobiose	N-acetyl-glucosamine
ATCC 8293	+	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+
ATCC 12291	-	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
LMG 6909	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LMG 14531	-	-	+	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+
IM080	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
IM082	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Lb. kefiranofaciens ATCC 43761, and although it did not seem to produce polysaccharides in LAW broth, it tested positive for esculin hydrolysis, and it produced acid from trehalose. IM015 and IM017 showed unique patterns with 3 or 4 bands in common with *Lb. kefiranofaciens* subsp. *kefirgranum* and *Lb. kefiranofaciens* subsp. *kefiranofaciens*. Partial 16S sequencing results of ATCC 43761, LMG 15132, IM014, IM015, and IM017 (data not shown) revealed a 100% homology within these strains confirming that IM015 and IM017 did belong to either *Lb. kefiranofaciens* subsp. *kefirgranum* or *Lb. kefiranofaciens* subsp. *kefiranofaciens*. Based on the genotypic results, morphologic and phenotypic features, IM015 and IM017 were classed as *Lb. kefiranofaciens* subsp. *kefirgranum*.

Results also showed a homologous RFLP pattern between both reference strains of *Lb. kefir* and IM002, IM008, and IM022. Other restriction enzymes were also used (*Bcl*I, *Sty*I and *Bam*HI) to see if they could further differentiate the strains within that species, but without success (data not shown). A dendrogram corresponding to the consensus matrix from the numerical analysis of the fermentation patterns, based on the Jaccard coefficient, and the numerical analysis of the banding

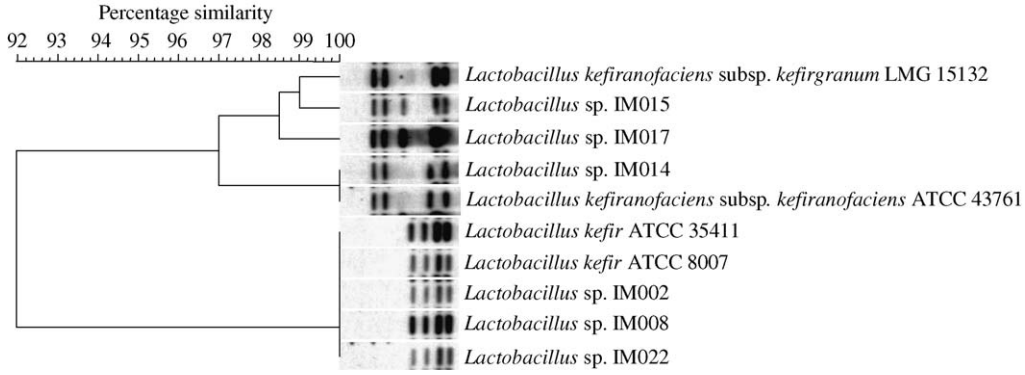

Fig. 1. Dendrogram and ribopatterns of lactobacilli strains.

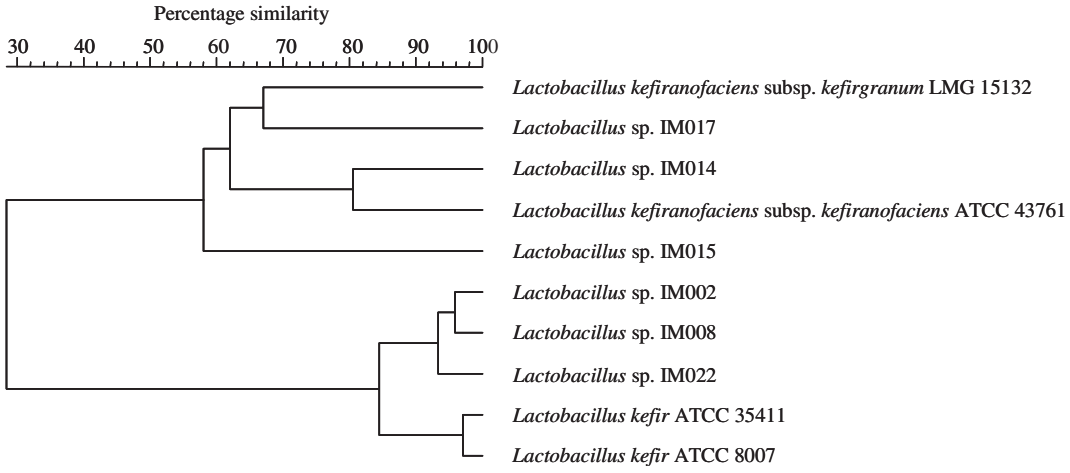

Fig. 2. Polyphasic matrix of lactobacilli strains.

patterns generated by *Hin*dIII restriction, is shown in Fig. 2.

Plasmid profiles of *Lb. kefir*

All strains with a banding pattern corresponding to the *Lb. kefir* species were tested for the presence of plasmid DNA in order to further differentiate the strains. Results are shown in Fig. 3. Reference strains did not show any plasmids while all strains isolated from kefir possessed one or more plasmids. Each isolated strain presented a different plasmid profile.

RFLP analysis of lactococci

Results obtained from the Southern blot analysis of total genomic DNA isolated from 11 strains of lactococci and one strain of *Streptococcus* digested with *Hin*dIII and *Eco*RI are shown in Fig. 4A and B. Dendrograms resulting from the numerical analysis of the banding patterns generated by the two endonucleases grouped IM103, IM104, and IM105 with *L. lactis* subsp. *cremoris* LMG 6897. The level of similarity for LMG 7931 to LMG 6897 was higher than to LMG 6890.

PCR–RFLP of *Lactococcus lactis* subspecies

Digestion of the PCR fragments from the lactococci strains with *Ear*I is shown in Fig. 4C. The endonuclease *Ear*I cuts the PCR fragments from the *cremoris* subspecies. Results indicated that IM103, IM104, and IM105 belong to that subspecies, while isolates IM101, IM102, IM106, IM107, and IM109 belong to the *lactis* subspecies. Interestingly, *L. lactis* subsp. *lactis* LMG 7931 was cut by *Ear*I. Endonucleases *Rsa*I and *Hae*II, which cut the PCR fragments from the *lactis* subspecies confirmed the results obtained with *Ear*I (results not shown). These results are in agreement with the genomic RFLP results. A dendrogram corresponding to the polyphasic consensus matrix from the numerical analysis of the fermentation patterns, based on the Jaccard coefficient, the numerical analysis of the RFLP banding

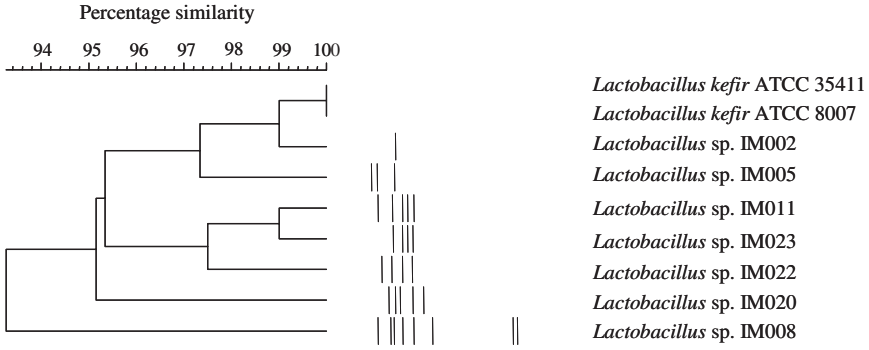

Fig. 3. Dendrogram and plasmid patterns of *Lb. kefir* strains.

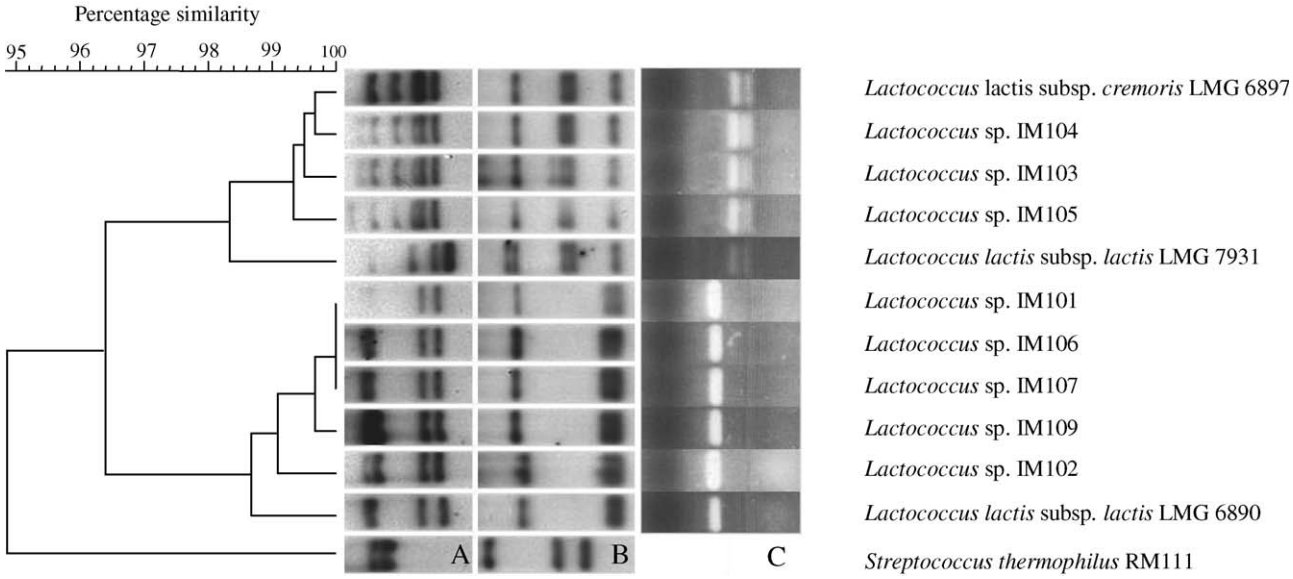

Fig. 4. Polymorphic matrix of genotypic results for lactococci strains. (A) Ribopatterns of lactococci digested with *Hin*dIII. (B) Ribopatterns of lactococci digested with *Eco*RI. (C) Digestion with *Ear*I of the PCR product of lactococci strains.

patterns generated by *Hin*dIII and *Eco*RI restriction and the PCR–RFLP results, is shown in Fig. 5.

RFLP analysis and sequencing results of the *Leuconostoc* strains

Results obtained from the Southern blot analysis of total genomic DNA isolated from 6 strains of *Leuconostoc* digested with *Eco*0109I and *Bso*BI are expressed in a dendrogram (Fig. 6). Results from the numerical analysis of the banding patterns generated by the two endonucleases showed that the two isolates have an identical banding pattern to *Ln. mesenteroides* subsp. *cremoris* LMG 6909. A dendrogram corresponding to the consensus matrix from the numerical analysis of the fermentation patterns, based on the Jaccard coefficient, the numerical analysis of the RFLP banding patterns generated by *Eco*0109I and *Bso*BI restriction is shown in Fig. 7. Both isolates show a high similarity to LMG 6909. Partial 16S sequencing results (data not shown) of IM082 showed homology to strains of *Ln. mesenteroides* when compared to the Genbank database.

Discussion

Current literature on the identification of the microflora of kefir is difficult to interpret, particularly for the lactic acid bacteria. Many articles written on the subject use old methodology to characterize the microflora [9,15,19]. Even more recent publications identify the strains isolated based uniquely on phenotypic traits [1,21]. Garrote et al. [7] and Pintado et al. [18] included whole-cell protein profiles with their phenotypic results. Takizawa et al. [23] added to the cell protein determination, GC% and DNA/DNA hybridization. RFLP,

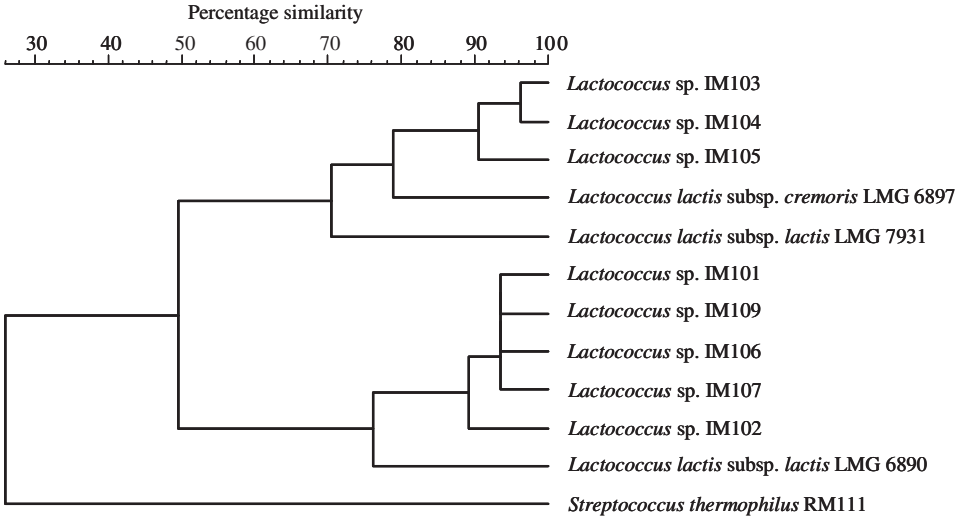

Fig. 5. Polyphasic matrix for lactococci strains.

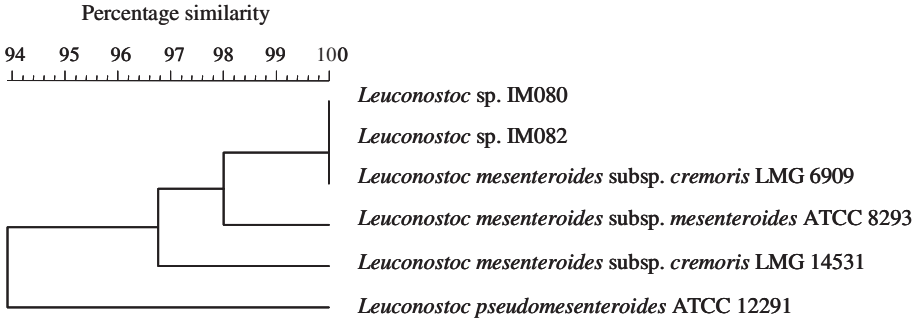

Fig. 6. Dendrogram of RFLP results for leuconostocs.

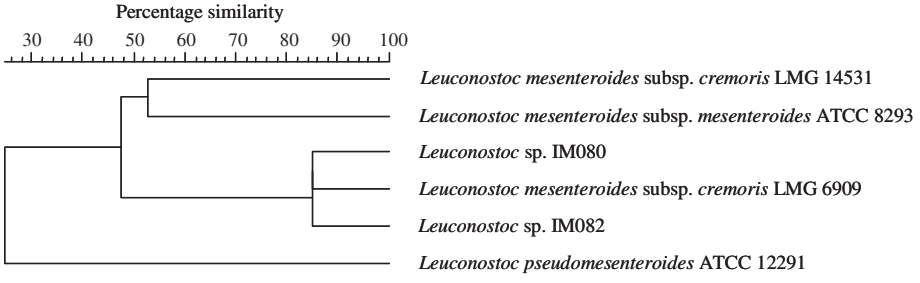

Fig. 7. Polyphasic matrix of leuconostocs strains.

which is a very discriminating method, was never used, to our knowledge, to identify the LAB of kefir grains.

RFLP was shown to be a useful method to differentiate between the different species of lactobacilli present in kefir grains. However, strain variations within the *Lb. kefir* species could not be demonstrated by the RFLP analysis. Morphologic differences (colony shape and size) were evident between strains ATCC 35411 and ATCC 8007 but our genotypic results, including plasmid profiles, could not differentiate the two. This suggests that a very small difference in a genetic locus, not detectable by RFLP, or a low abundance plasmid, responsible for colony aspect, could not be detected. The strains isolated from kefir were differentiated by their plasmid profiles. RFLP results showed that there seems to be a single pattern for the *Lb. kefir* species, suggesting low genome diversity. Plasmid profiles showed many different patterns, suggesting their importance in strain

diversity. The strains obtained from the ATCC may have lost their plasmids through frequent sub-culturing in a non-milk-based medium [3,4].

Strain variations within the *Lb. kefiranofaciens* species were shown with the RFLP method using *Hin*dIII as the restriction endonuclease, although 16S partial sequencing results were not able to differentiate between both subspecies. IM014 gave a RFLP profile identical to ATCC 43761 but due to its morphologic features, (mainly growth characteristics in broth and apparent lack of EPS production) it was classified within the *kefirgranum* subspecies. It is possible that the main difference between *Lb. kefiranofaciens* subsp. *kefiranofaciens* and *Lb. kefiranofaciens* subsp. *kefirgranum*, which consist mainly of EPS production by the subspecies *kefiranofaciens*, might be caused by the loss of a plasmid coding for the slime-producing trait by the *kefirgranum* subspecies [13,26]. RFLP profiles showed four different patterns for the *Lb. kefiranofaciens* species. It was not possible to attribute one particular pattern to the *kefiranofaciens* or the *kefirgranum* subspecies. Phenotypic attributes justifies that two subspecies should be present in the *Lb. kefiranofaciens* species [24]. Genotypic results based on RFLP analysis did not create 2 distinct groups for the subspecies. Two strains with the same RFLP pattern were classed in different subspecies (ATCC 43761 and IM014) while strains with different patterns could also be classed in a same subspecies (LMG 15132, IM014, IM015, and IM017). Takizawa et al. [23] created subgroups within the *kefirgranum* and *kefiranofaciens* subspecies based on phenotypic and biochemical characteristics but they did not demonstrate genotypic differences. RFLP analysis suggested that there might be a wide variety of genotypically different strains within the *Lb. kefiranofaciens* species. These results also demonstrated that even though the genotypic identification is essential for the proper classification of a strain, phenotypic characteristics also are essential for the proper typing. The polyphasic approach proved to be a valuable tool for the typing of these strains.

Lactococci strains isolated from kefir formed four different genomic RFLP patterns with both *Hin*dIII and *Eco*RI showing that at least four different strains were isolated for the kefir grains. Strains from both *lactis* and *cremoris* subspecies were found. This was further demonstrated through the PCR–RFLP results. Genomic RFLP of lactococci enabled strain differentiation and the groups formed did coincide to the PCR–RFLP subspecies grouping indicating that the genomic RFLP might be used for subspecies differentiation as well. More strains should be tested to validate this observation. It is worth noting that the 16S PCR fragment of *Lb. lactis* subsp. *lactis* LMG 7931, a strain that produces diacetyl, was cut by *Ear*I endonuclease, suggesting that this strain belongs to the *cremoris* subspecies. Genomic RFLP groupings also clustered this strain closer to the *cremoris* group. In the past, this strain was probably classified into the *lactis* subspecies based on its sugar utilization profile that more closely resembled that of a *lactis* subspecies.

Leuconostocs were also isolated from the studied kefir grains. Because of their ability to grow at 15 °C and produce ß-galactosidase, they were isolated using MRS-X-Gal since they were not able to grow selectively on other differential media tested. The detected heterogeneity of the strains in the studied kefir grains may have been limited by the culturing method used. New screening methods could also be useful for this genus. Strains isolated were identified as *Ln. mesenteroides* subsp. *cremoris*. Their limited sugar utilization profile probably makes them very dependant on other bacteria for their maintenance and growth in the grains.

When one studies more closely the list of organisms isolated from kefir grains from various parts of the globe, it becomes evident that the earlier lack of molecular tools for the proper identification of the species probably inflated the list of species found in kefir grains. For example, some claimed to have isolated *Lb. acidophilus* and *Lb. brevis* from kefir [1,10,17] but these species of lactobacilli are so phenotypically and biochemically closely related to the *Lb. kefiranofaciens* and *Lb. kefir* species, respectively, that they may have been misidentified. More rigorous methods of characterization will demonstrate that kefir grains from different parts of the world are not as different as once thought. Takizawa et al. [23] studied different sources of kefir grains and isolated only three species of lactobacilli. Our study using a kefir grain from Russia contained two species of lactobacilli, the same species isolated by the Japanese team. Molecular biology and bioinformatics are technologies now at our disposal [5]. Polyphasic characterization combining phenotypic, biochemical, genotypic, and, ideally, sequencing results should become a requirement for the classification of strains.

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