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Phylogenetic identification of bacteria within kefir by both culture-dependent and culture-independent methods

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A combination of culture-dependent and independent methods was used in an attempt to identify the bacteria present in kefir grains and kefir liquid. Culture-independent methods involved direct extraction of DNA by mechanical means from either grains or liquid, followed by PCR amplification and sequencing of 16S rRNA genes. Culture-dependent methods were performed by inoculating samples from both the kefir grains and the kefir liquid to solid media followed by incubation under either aerobic or anerobic conditions in order to selectively enrich aerobic and anaerobic bacteria. Pure cultures were isolated from enriched bacteria and their DNA was extracted for the amplification and sequencing of 16S rRNA genes. Results indicate that kefir grains had a different bacterial composition compared to the kefir liquid. While *Lactobacillus kefiranofaciens, Lactobacillus kefiri, Enterococcus faecium* and *Acetobacter syzygii* were found only in the kefir grains, *Lactobacillus helveticus* was found only in the kefir liquid. On the other hand, *Lactococcus lactis subsp. lactis, Leuconostoc mesenteroides* and *Acetobacter lovaniensis* were found to be present in both the grains and the liquid. To the best of our knowledge, this work is the first to report the presence of *A.lovaniensis, A. syzygii* and *Enterococcus faecium* in kefir.

Key words: Kefir, 16S rRNA gene analysis, bacterial ecology.

INTRODUCTION

Kefir is a traditional fermented milk beverage with a characteristic viscous, slightly carbonated and acidic taste (Guzel-Seydim et al., 2000). Kefir starter culture, in the form of grains, contains a complex flora of microorganisms composed of bacteria and yeasts. When active kefir grains are continuously cultured in milk, they increase in mass and number. However, a stable microbial population is maintained (Ninane et al., 2005). The association of different microorganisms in kefir was developed by continuous selection over the centuries. These microorganisms not only compete with other potentially pathogenic microorganisms but also produce active substances that inhibit the growth of other bacteria (Lopitz-Otsoa et al., 2006), providing a pathogen-free ecology in kefir.

Kefir is considered to be a probiotic beverage due to its alleged beneficial effects on human health (Zubillaga et al., 2001; Otles and Cagindi, 2003). The word “kefir” is derived from the Turkish word “keyif” which means “good feeling”, reflecting its refreshing taste. Due to such properties of kefir, it is becoming popular and being consumedby people all over the world.

The importance of fermented milk products which have

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health benefits and are safe for human consumption has been increasing. Industrial quality control processes require the knowledge of ecological and biochemical factors and the ability to evaluate the composition of microbial population in such products (Fleet, 1999). Such knowledge promotes the assessment of modern quality assurance, by the creation of predictive fermentation models and risk analysis strategies that are used for the prevention of food borne pathogens and food spoilage and to help in increasing the aroma and taste of certain foods. Also, information about diversity, taxonomic identity, distribution of microorganisms throughout different fermentation stages and relationships between growth and activity of individual species must be known in order to understand the ecology of fermented foods (Ampe et al., 1999).

For decades, the cultivation of microorganisms in pure culture was the only method used for the characterization and identification of microorganisms. However, these methods are generally fastidious, and time-consuming, enabling the identification of only a limited number of strains. Studies showed that the majority of microorganisms that live in a complex environment could not be cultivated by plate culturing techniques (Traut et al., 1995). This is primarily due to the the presence of unknown, novel species that are not culturable by existing methods, and the presence of known species that are metabolically active and viable but have entered a non-culturable state (Fleet, 1999).

In order to overcome the difficulties and limitations related to cultivation techniques, culture-independent methods can be used to determine the bacterial populations in mixed cultures (Handelsman, 2004; Streit and Schmitz, 2004; Allen and Banfield, 2005). DNA from a sample containing mixed populations of microorganisms can be extracted and analyzed by molecular techniques to reveal the microbial diversity. DNA sequences such as 16S rRNA genes which are highly conserved across all domains of life but show minor differences from one species to another can be analyzed by DNA amplification and sequencing techniques without a need for cultivation (Mincer et al., 2005). Such sequences can then be analyzed and compared with the ever growing rRNA gene sequence databases to reveal the identity of the microorganisms in the analyzed sample.

In this work we focused on the identification of the bacterial content of both the kefir grains and the kefir liquid by using classical cultivation and culture independent methods. The relative effectiveness of those methods was then compared to each other.

MATERIALS AND METHODS

Kefir sample

Kefir grains used in this study were of house hold origin. Fermentation of milk by kefir grains was achieved by adding 50 g of kefir grain into 500 ml ultra high temperature (UHT) treated cow's milk in a sterile jar, followed by incubation without shaking for 3 days at 28°C. The kefir grains from the fermented milk were collected by using a sterile sieve and rinsed with sterile water to be used as a starter for the next cycle of the fermentation process.

Isolation and maintenance of bacterial strains from kefir grains and liquid

Kefir grains inoculated into milk during 3 days were sieved and washed with sterile water. Grains (2 g) were homogenized in 18 ml sterile distilled water for 4 min (IKA Ulltra Turrax Homogenizator, T18 basic, Wilmingtan, USA). Homogenates were used for isolation of single colonies as described below. Fermented kefir grain homogenates and kefir liquid were diluted in MRS (MERCK, Darmstadt, Germany) medium and appropriate dilutions were spread on MRS agar in duplicates. First and second plates of each duplicate were incubated aerobically and anaerobically, respectively, at 28°C. Anaerophilic conditions were created by an anaerobic jar with Anaero*Gen*^{Tm} sachets (OXOID, Wesel, Germany) which produce an anaerobic atmosphere within the jars. After three days of incubation time, unique colonies were selected according to their morphological characters and plated on fresh MRS plates to obtain pure cultures.

Extraction of bacterial DNA from kefir samples and ısolates

For culture-dependent analysis, genomic DNA was extracted from each bacterial isolate by the Genomic DNA Purification Kit (Fermentas, Vilnius, Lithuania). For culture-independent analysis, total bacterial DNA was isolated from 3 days fermented kefir liquid and grains by using Power Soil DNA Kit (MOBIO, Hamburg, Germany) which employs a combination of mechanical and chemical methods for the lysis of bacterial cells. Prior to DNA extraction, 0.25 g kefir grain was sieved and rinsed 5 times with sterilised distilled water. Liquid kefir (500 µl) was not subjected to any treatment prior to extraction.

Amplification of DNA extracted from kefir samples and bacterial isolates for 16S rRNA gene cloning and/or sequencing

The bacterial populations of kefir grain and liquid were identified both by a culture-independent approach based on the construction of a 16S rRNA gene clone library from DNA isolated from kefir samples followed by plasmid insert sequencing and by a culture-dependent approach based on direct sequencing of the amplified 16S rRNA genes of individual isolates recovered from kefir grain and liquid on culture media. To generate the clone library, partial 16S rRNA gene was amplified from total DNA directly obtained from kefir samples. Amplification of partial 16S rRNA gene was carried out with universal bacterial primers E334F (5'-CCAGACTCCTACGGGAGGCAG-3') and E1115R (5'-CAACGAGCGCAACCCT-3'). Each reaction tube contained 10 mM Tris-HCl (pH 8.5), 2 mM $MgCl_2$, 0.1 µM concentrations of each primer, 1 µg of purified DNA, 0.2 mM concentrations of each deoxynucleoside triphosphate, and 1 µ of *Taq* polymerase (Fermentas) and was then adjusted to a total volume of 50 µl. The reaction mixture was placed in a iCycler thermocycler (Bio-Rad, Munich, Germany). After an initial denaturation at 94°C for 2 min, 25 cycles were performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension step of 72°C for 5 min. The products were electrophoresed on a 0.8% agarose gel, viewed by ethidium bromide staining and purified by PCR purification Kit (Invitrogen, Carlsbad, USA). PCR products were ligated to the pTZ57R/T TA cloning vector (Fermentas) and transformed into competent *Escherichia coli* TOP10 OneShot cells (Invitrogen) as specified by the manufacturers. Plasmid inserts were amplified by PCR with

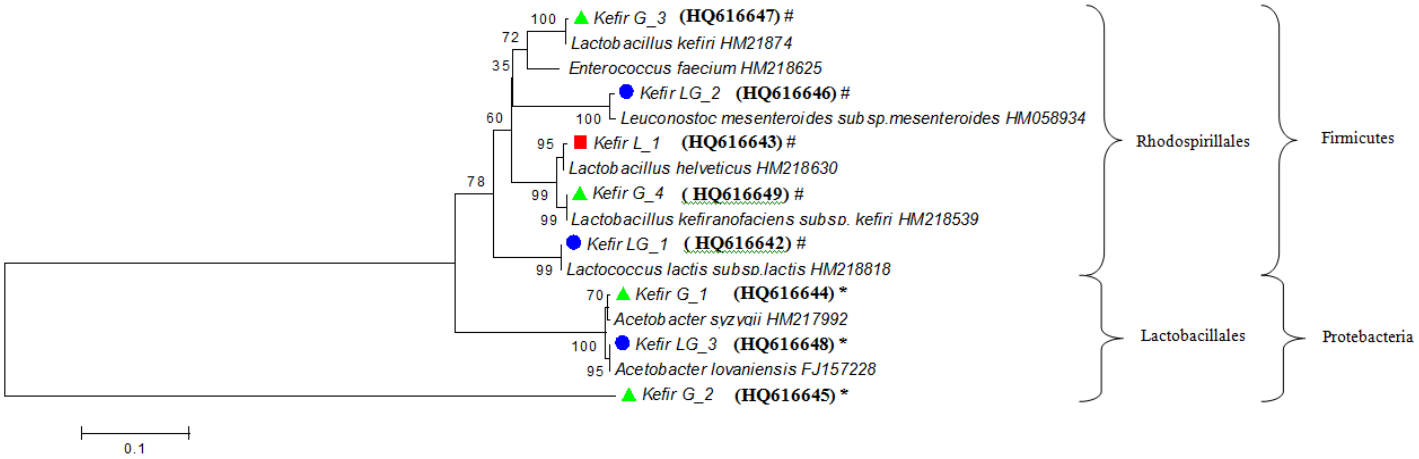

Figure 1. Phylogenetic tree showing relationships of bacterial 16S rRNA gene sequences amplified from kefir grains and kefir liquid by culture-dependent and culture-independent methods. Evolutionary relationships of eight identified bacterial taxa with their reference sequences are shown on the tree. The scale bar represents 0.1 substitutions per base position. Bootstrap values are shown as percentage of 1000 replications and indicated at the branching points. ■, Bacterial species identified only in kefir liquid; ▲, bacterial species identified only in kefir grain; ●, bacterial species identified in both kefir liquid and grains. *, Acetic acid bacteria; #, Lactic acid bacteria.

universal M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers with the same PCR parameters described above. For both amplified inserts and individual isolates, the partial 16S rRNA gene was sequenced with the BigDye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS (Applied Biosystems, Lincoln, USA) and an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Each sequence obtained was identified by comparison with the closest sequences available in nucleic acid databases at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA).

Comparative sequence analysis of 16S rRNA genes

Each clone was sequenced in both directions and chromatograms were evaluated to decide whether a base difference was caused by divergence or ambiguity. Base divergence is a real base difference between the analysed sample and the related sequence present in the database. Base ambiguities are the results of sequencing errors. 16S rRNA gene sequences were submitted to NCBI for similarity searches through the BLAST algorithm (http://www. ncbi.nlm.nih.gov /blast).

Each clone was classified and placed in a taxonomic group according to the results obtained from the Ribosomal Database Project (RDP's) naïve Bayesian rRNA classifier (Wang et al., 2007). Each clone and isolate was identified at species level according to the cut-off similarity levels for species (99%) which were previously described (Drancourt et al., 2000). Phylogenetic tree was constructed from alignments based on the method Minimum Evolution, calculated by the algorithm of Tamura and Nei model (Tamura et al., 2007). Bootstrap confidence values were obtained with 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches. Phylogenetic analyses were conducted in MEGA4 software (Tamura et al., 2007). The evolutionary distances were computed using method Neighbor-Joining (Saitou and Nei, 1987) and the phylogenetic trees were constructed from the alignments based on the model Maximum Composite Likelihood (Saitou and Nei, 1987).

Nucleotide sequence accession numbers.

Sequences derived from the analysis of 16S rRNA genes of bacteria in kefir were deposited in GenBank under the acession numbers HQ616642, HQ616643, HQ616644, HQ616645, HQ616646, HQ616647, HQ616648, HQ616649. Reference sequences used were noted in the tree (Figure 1).

RESULTS

Monitoring bacterial communities from kefir by culture-dependent methods

The diversity of the culturable bacteria from kefir on MRS medium under both aerobic and anaerobic conditions was investigated. Table 1 shows the pylogenetic affiliations of distinct isolates obtained from kefir grain homogenate and kefir liquid under aerobic or unaerobic conditions. Seven distinct isolates recovered from kefir were composed of the members of the genera *Acetobacter* (phylum Proteobacteria, order Rhodospirillales), and *Lactobacillus, Leuconostoc, Lactococcus*, and *Enterococcus* (phylum Firmicutes, order Lactobacillales).

Among the seven distinct isolates, *L. kefiri, A. syzygii* and *E. faecium* were only recovered from grains while *L. helveticus* was only recovered from kefir liquid, *L. lactis subsp. lactis*, *L.mesenteroides* and *A. lovaniensis* were recovered from both the grains and the liquid. Among the grain isolates, *L. kefiri* and *A. syzygii* were recovered under aerobic conditions while *E. faecium* was recovered under anaerobic conditions. Kefir liquid isolate *L. helveticus* could be recovered by both aerobic and unaerobic conditions, which was also the case for *L. lactis subsp. lactis* and *L. mesenteroides* which were found in both the grains and the liquid. The other isolate, *A. lovaniensis*, which

Table 1. Taxonomic affiliations of bacterial isolates cultivated from kefir grains and kefir liquid under aerobic or anaerobic conditions.

Presence of isolates				Closest 16S rRNA sequence match in Genbank	Sequence similarity (%)	Taxonomic affiliation
Grain		Liquid				
ae	an	ae	an			
+	+	+	+	*Lactococcus lactis subsp. lactis*	99	Firmicutes
+	+	+	+	*Leuconostoc mesenteroides*	99	Firmicutes
+	-	+	-	*Acetobacter lovaniensis*	100	Proteobacteria
+	-	-	-	*Lactobacillus kefiri*	99	Firmicutes
+	-	-	-	*Acetobacter syzygii*	99	Proteobacteria
-	+	-	-	*Enterococcus faecium*	99	Firmicutes
-	-	+	+	*Lactobacillus helveticus*	99	Firmicutes

ae, aerobic growth; an, anaerobic growth.

Table 2. Taxonomic affiliations of 16S rRNA gene clones analysed from kefir grain and kefir liquid by culture-independent method.

Number of clones analysed		Closest 16S rRNA sequence match in Genbank	Sequence similarity (%)	Taxonomic affiliation
Grain	Liquid			
100		*Lactobacillus kefiranofaciens*	99-100	Firmicutes
	42	*Lactococcus lactis subsp. lactis*	99	Firmicutes
	39	*Acetobacter lovaniensis*	100	Proteobacteria
	19	*Lactobacillus helveticus*	99	Firmicutes

was found in both the grains and the liquid, was only cultivated aerobically.

Monitoring bacterial communities from kefir by culture-independent methods

In order to identify the bacterial populations of kefir, a clone library was constructed from the partial 16S rRNA genes amplified from DNA that was isolated from both the kefir grains and the kefir liquid. Among the 200 clones (100 from each library) analyzed by sequencing the partial 16S rRNA genes, we identified four bacterial species by comparing with sequences from the NCBI database (Table 2). Analysis of the 100 clones from kefir grain library revealed the presence of only one species, *L. kefiranofaciens*. All 100 clones gave 100 or 99.9% sequence similarity with *L. kefiranofaciens*. Clones with 99.9% homology had one or two base divergences.

Analysis of 100 clones from liquid Kefir library revealed the presence of three different bacterial species with high sequence similarity scores. Their ratios were as follows; 39/100 (39%) *A. lovaniensis*, 42/100 (42%) *L. lactis subsp. lactis,* and 19/100 (19%) *Lb. helveticus* (Table 2).

Comparison of culture-dependent and independent methods.

The distribution of the different bacterial species recovered from the clone libraries obtained from direct isolation of DNA from kefir liquid and grains, and from the individual colonies obtained from kefir grains and liquid is shown in Table 3. While *A. lovaniensis, L. lactis subsp. lactis* and. *L. helveticus* were identified by both the culture-dependent and culture-independent methods from kefir samples, *L. mesenteroides, L. kefiri*, *E. faecium* and *A. syzygii* were only identified by culture-dependent methods. *L. kefiranofaciens* was only identified by culture - independent methods.

Phylogenetic analysis was used to place all the gene sequences from culture-dependent and culture-independent methods to major taxonomic groups. Analyzed 16S bacterial rRNA gene sequence libraries of kefir were dominated by sequences belonging to Firmicutes of which the majority were related to *L. mesenteroides, L. kefiri*, *E. faecium, L. lactis subsp. lactis, L. kefiranofaciens* and. *L. helveticus.* The most second abundant phylotype in the libraries were assigned to the Proteobacteria represented by *A. lovaniensis* and *A. syzygii* (Figure 1).

DISCUSSION

Kefir is a traditional fermented dairy product which is known as a probiotic beverage, produced and consumed all over the world. Determination of the kefir microbial population is important for food quality control and studies on bioactive products produced by kefir microbial population. Most studies on the bacterial communities of kefir generally focused on identification of bacteria by

Table 3. The distribution of the bacterial species identified from the clone libraries obtained from direct isolation of DNA from kefir grains and liquid (culture independent method) and from the individual colonies obtained from culture media (culture dependent method).

Bacterial species	Culture-dependent Method		Culture-independent method	
	Kefir grains	Kefir liquid	Kefir grains	Kefir liquid
Lactobacillus kefiranofaciens	-	-	+	-
Lactobacillus kefiri	+	-	-	-
Lactobacillus helveticus	-	+	-	+
Leuconostoc mesenteroides	+	+	-	-
Lactococcus lactis subsp. lactis	+	+	-	+
E. faecium	+	-	-	-
A.lovaniensis	+	+	-	+
A. syzygii	+	-	-	-

traditional culture-dependent methods from only grain-samples (Delfederico et al., 2006; Heo and Lee, 2006; Mainville et al., 2006). However, such methods may have limited ability for the identification of diverse bacterial populations in mixed samples. Therefore, in this study, we used a complementary culture-independent method in addition to the isolation of bacterial population on culture medium to study the microbial communities present in kefir grains and kefir liquid seperately.

A diverse spectrum of bacterial genera were identified in kefir samples including; lactococci (1 species); lactobacilli (3 species); leuconostoc (1 species); acetobacteria (2 species); and enterococcus (1 species). Table 3 reports on the identified bacterial species in this study from both kefir grain and kefir liquid samples by culture-dependent and culture-independent methods.

Pyhlogenetic analysis was used to assign all gene sequences in bacterial 16S rRNA clone libraries to major taxonomic groups. Analysis of the kefir grains by culture-dependent approaches revealed the presence of *L. lactis subsp. lactis, L. mesentorides, L. kefiri*, *A. lovaniensis, A. syzygii,* and *E. faecium.* However, these bacteria were not identified by the culture independent method, most likely due to their relative low abundance in grains as compared to other bacterial species. Garrote et al. (1997) showed that the number of lactobacilli (10^9cfu/g) was higher than lactococci (10^7cfu/g) in kefir grains. Due to this difference in numbers, the amplification probability of *L. kefiranofaciens* DNA would be expected to be several orders of magnitude higher than lactococcal DNA during a PCR reaction. Because of this, rare bacterial genome as a template has little chance of competing with bacterial genomes that are in higher amounts in samples (Schabereiter-Gurtner et al., 2001). Indeed, all the clones screened from kefir grains by the culture-independent method were identified as *L. kefiranofaciens*, which we could not isolate by the culture-dependent method.

These results clearly demonstrate that *L. kefiranofaciens* is the dominant organism in grains. *L. kefiranofaciens* was shown to populate the center of the grains (Arihara et al., 1990), thus indicating the anareobic nature of this organism. Studies involving the cultivation of *L. kefiranofaciens* report the use of anerobic conditions (Chen et al., 2008). Our inability to cultivate this bacteria could be due to the insufficiency of the anerobic environment created by the method we employed. On the other hand, this inability to cultuvate *L. kefiranofaciens* could have also been an advantage for us, since such a dominant organism would have certainly lead us to miss out other less abundant bacteria during isolation from the culture medium. *L. lactis subsp. lactis, L. mesentorides, L. kefiri* and *L. kefiranofaciens* were also recovered from kefir grains in a previous study (Chen et al., 2008) by a DGGE based culture-independent method rather than the sequencing of 16S rRNA genes or gene libraries. In addition to those four bacteria we also identified the presence of *A. syzygii, A. lovaniensis* and *E. faecium* species in kefir grains by applying both culture-dependent and culture-independent methods.

Although our results indicate that *L. kefiranofaciens* is the predominant species found in kefir grains, kefir liquid contains an almost equivalent population of *A. lovaniensis, L. lactis subsp. lactis,* and *Lb. helveticus* because these could be identified by both culture-dependent and culture-independent methods. The identification of *Leu. mesenteroides* in kefir liquid by only culture-dependent methods is probably due to the lower population number of this species compared to the other three species.

Acetobacter species are aerobic bacteria (De Ley et al., 1984) which explains the recovery of *A. syzgii* and *A. lovaniensis* under aerobic conditions. Previously, immunoflorescence microscopic studies demonstrated that *L. kefiri* was populated only at the surface layers of the kefir grains(Arihara et al., 1990). This indicates that *L. kefiri* prefers aerobic conditions in agreement with our recovery of this bacteria under aerobic conditions and only from kefir grains (Table 1).

Our study is the first to report the presence of *A. lovaniensis, A. syzygii*, and *E. faecium* in kefir. Chen and his colleagues were the first to study kefir grains by both culture-dependent and culture-independent methods. However, only four species were identified in their studies

(*L. kefiranofaciens, L. kefiri, L. mesenteroides* and *L. lactis subsp. lactis*) (Chen et al., 2008).

Among the identified species in kefir, *Lactococcus sp., Leuconostoc sp., Lactobacillus sp*. and *Enterococcus sp.,* are the lactic acid bacteria. Lactic acid bacteria are the major acid producers in kefir fermentation. *L. kefiri* is a heterofermentative bacterium that produces ethanol, acetic acid and carbon dioxide as well as diacetyl, acetoin, 2-3-butanediol and formate in addition to lactic acid(Liu, 2003). These products determine the flavor characteristics of kefir drink. *Lb. helveticus* is a homofermentative species and produces lactate from sugar as a major product of the glycolytic pathway. The identified *Lb. kefiranofaciens* (homofermentative) and *L. kefiri* have been reported as kefiran producers in kefir(Yokoi et al., 1991). *L. mesenteroides* is a heterofermentative bacterium which increases the viscosity and enhances the flavor of kefir. *Acetobacter* species are acetic acid bacteria and they use ethanol as a carbon source and they can survive during the fermentation process. Additionally, *E. faecium* can be present in natural flora of many fermented foods contributing to the flavor of different fermented milk products (Saavedra et al., 2003).

Our study shows that both conventional cultivation methods and PCR based molecular strategies (culture-independent) have drawbacks; none of which is sufficient alone or in combination to identify the bacterial community in complex environmental samples. The DNA extraction method of choice in culture-independent approaches may affect the efficiency of capturing bacterial diversity from kefir grains because a given method may be better suited for some bacteria and increase their representations. To overcome such biases, several different DNA isolation methods can be chosen (physical, chemical DNA extraction or a combination of both).

In conclusion, both culture-dependent and culture-independent methods should be used as complementary approaches while studying a mix microbiological sample.

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