

Short Communication

Isolation and molecular taxonomy of two predominant types of microflora in Kefir

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Kefir is a traditional fermented beverage in the Caucasian Mountains that can be prepared with fresh milk. It is known that Kefir is composed by a symbiotic association of lactic acid bacteria and yeasts living together into gelatinous and irregular materials secreted by them. This association sometimes is mistaken for mushrooms like *Camella assamica* and *Cordyceps sinensis*. Kefir is similar to products that exhibit some anti-bacterial, anti-mycotic, anti-tumor, and anti-inflammatory activity. Although Kefir and its related products (Tibetan mushrooms) are very similar in structure, microbial content, cultivation procedures and fermentation products, Kefir is usually reported to lead to health benefits of a probiotic nature (Cevikbas et al., 1994; Diniz et al., 2003).

Microbial strains belonging to the genera, *Lactobacillus*, and *Streptococcus* are known to exist in fermented products such as yoghurt, cheese, and kimchi, but there is no specific data on those involved in the fermentation of Kefir (Baruzzi et al., 2000; Simova et al., 2002). Therefore, the present study was undertaken to isolate and identify the predominant microflora involved in Kefir fermentation. In this work, we de-

scribe the phylogenetic characteristics of the two major microbes isolated from Kefir.

The Kefir sample was obtained from Dominic Aniteatro, a Kefir producer from Australia. The Kefir was washed twice from the white and gelatinous lump. The lump was added to commercial milk (5–10 fold of volume) every 3 days at room temperature in order to maintain the seed culture. The lump was split by vigorous mixing and suspended in phosphate-buffered saline. Each vial of cell clump (containing a final concentration of 50% glycerol) was stored at –196°C in a liquid nitrogen tank. Three types of media were used for the isolation of microbes. The predominant strains A and B were isolated from colonies cultured on PDA, PCA, and MRS medium (Difco, Detroit, MI, USA). To investigate their morphological and physiological characteristics, strains A and B were mainly cultivated aerobically at 30°C on MRS medium. The cells for DNA extraction were produced from liquid MRS medium. The strains were cultivated aerobically at 30°C on a horizontal shaker at 150 rpm. For fatty acids methyl ester (FAME) analysis, strains A and B were cultivated at 30°C for 3 days on MRS agar. The morphology of cells was examined using a scanning electron microscope (Hitachi S-2500, Tokyo, Japan) as described by the manufacturer's manual. For the isolation of DNA, chromosomal DNA was isolated and purified according to the method previously described (Tamaoka and Komagata, 1984; Yoon et al., 1997), with the exception

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that ribonuclease T1 was used together with ribonuclease A. The 16S rDNA of strain A was amplified by PCR using two universal primers as described previously (Yoon and Park, 2000). The PCR product was purified by using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified 16S rDNA was sequenced using an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. The purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. The 16S rDNA sequences of strains A were aligned with 16S rRNA gene sequences of *Lactobacillus* sp., and the representatives of some related genera by using CLUSTAL W software (Collins et al., 1991; Thompson et al., 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Evolutionary distance matrices were calculated by

using the algorithm with the DNADIST program within the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987) as implemented within the NEIGHBOR program of the same package. The stability of the relationships was assessed by bootstrap analysis of 1,000 data sets using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE of the PHYLIP package. The 26S rDNA of strain B was amplified by PCR using two universal primers as described previously (Boisselier-Dubayle et al., 2002; Vanderpoorten et al., 2002) with slight modification; the PCR product was purified by using a QIAquick PCR purification kit (Qiagen). The purified 26S rDNA was sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as recommended by the manufacturer.

To isolate the major microbes, we crushed the white

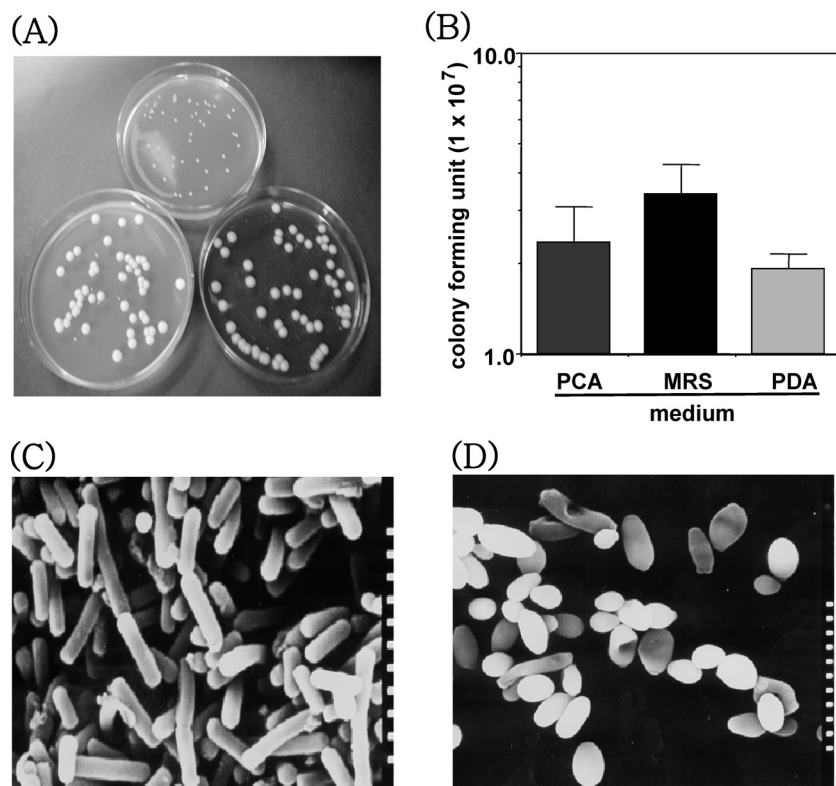


Fig. 1. Isolation and morphology of the major microflora from a traditional Tibetan beverage.

(A) Isolation of microbes from various media. Two types of microbes were grown on three types of medium. PCA medium (upper), PDA medium (bottom left), MRS medium (bottom right). (B) Viable cell numbers in the Kefir sample. PCA medium was used for the isolation of total cell numbers, whereas MRS and PDA were used for specific selection of lactobacilli and fungi, respectively. The beverage was cultured every 2 days by the addition of fresh commercial milk. The cell clump was slightly minced with a homogenizer and suspended by vigorous mixing. (C–D) Morphology of isolated strains A (C) and B (D) by scanning electron microscope. The dotted bars in (C) and (D) indicate 6 µm and 15 µm, respectively.

gelatinous and irregular cell clump and washed the cells twice with phosphate-buffered saline. The cells were split by vigorous mixing and suspended in phosphate-buffered saline. By serial dilution, the cells were seeded on the PCA, PDA, and MRS media. After 3–7 days, the colonies were observed with a phase contrast microscope. We were able to select two types of cells (Figs. 1A–B): strain A, which formed small-sized colonies; and strain B, which formed large-sized glassy colonies.

Strain A was Gram-positive, non-spore-forming, and non-motile. The cells of this strain were short slender rods measuring 0.6–0.8 by 1.5–3.0 µm on MRS

medium at 30°C, and occurred singly, in pairs, or occasionally in short chains. After incubation on MRS agar for 3 days, the colonies appeared white, circular to slightly irregular, convex, smooth, and opaque. Strain A had catalase-negative and oxidase-negative activities. Casein was hydrolyzed by this strain but gelatin, starch, and urea were not hydrolyzed, arginine was not deaminated and indole was not produced. Strain A produced both L(+)-lactic acid and D(–)-lactic acid. Strain A fermented gluconate and did not produce gas from glucose, indicating that this microorganism is facultatively heterofermentative (Vandamme et al., 1996). Strain A grew well in aerobic and strict anaerobic con-

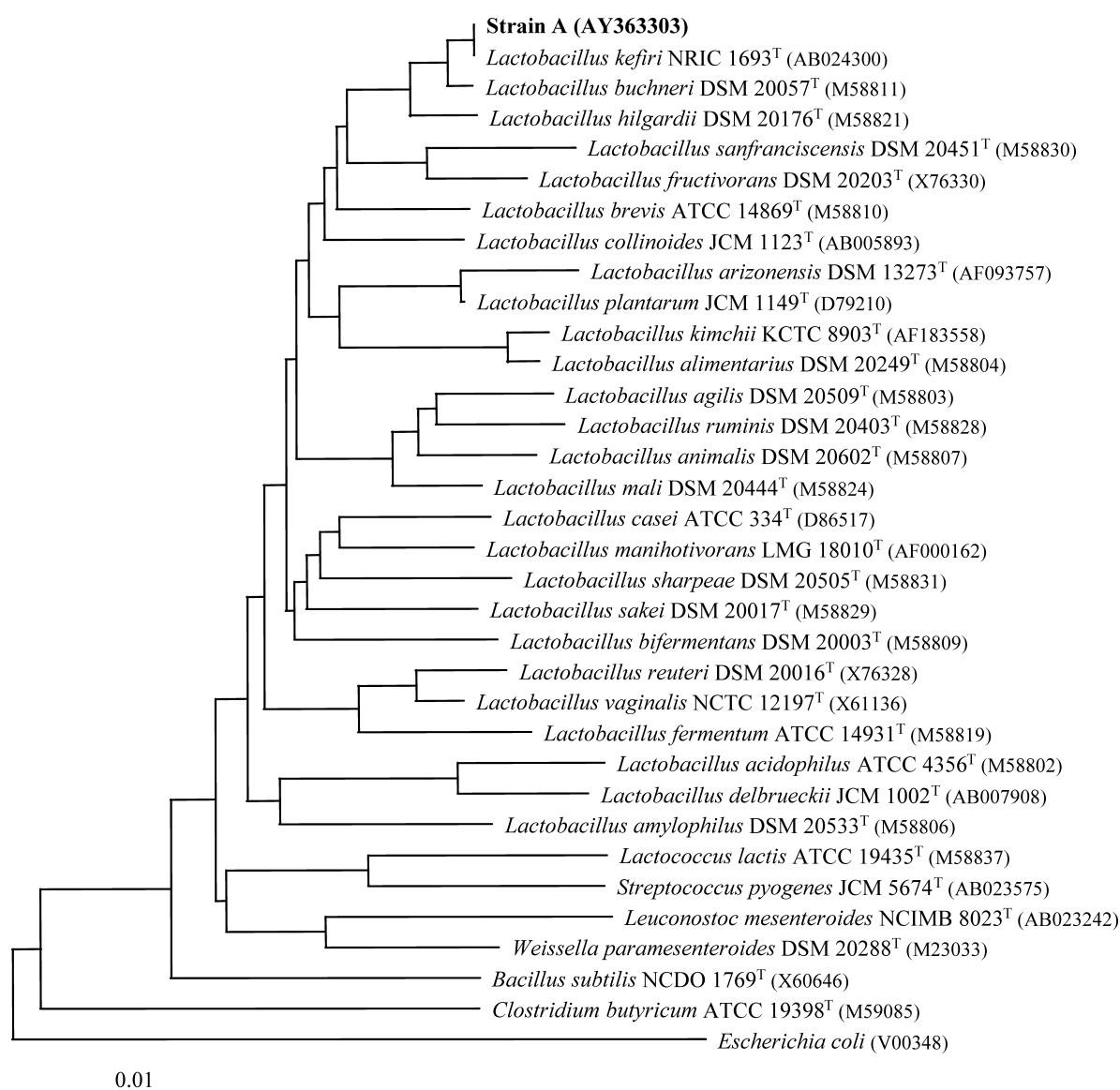


Fig. 2. Phylogenetic tree based on partial 16S rDNA sequences showing the position of the selected strain A.

The strain types of some *Lactobacillus* species and representatives of related taxonomies are shown. Scale bar represents 0.01 substitution per nucleotide position.

ditions on liquid and solid MRS media at 10 and 40°C, but not at 45°C. The optimal temperature for growth of this strain was approximately 30°C. Strain B was a yeast strain that is rod-shaped and opaque. The cells were globulose, ellipsoidal and cylindrical (Hammes et al., 1992; Kandler and Weiss, 1986). Strain B formed a sediment and a ring in glucose-yeast extract broth and assimilated sucrose, raffinose, inulin, and ethanol. The spores ranged in shape from spheroidal to ellipsoidal to reniform. Sporulation occurred after 2–5 days at 17–25°C on 1% malt extract agar (Llorente et al., 2000). We randomly picked up 30 colonies each of strain A and B from the plates, and then carried out the above physiological test for the taxonomic determination of the colonies. The results showed that strain A (30 colonies) and B (30 colonies) are related to *Lactobacillus* sp. and *Kluyveromyces* sp., respectively, with the only two types of bacteria. These results indicate that the major microflora of the strains A and B are *Lactobacillus* sp. and *Kluyveromyces* sp., respectively, suggesting that the two strains are formed by a symbiotic association each other.

Next, we intentionally selected 10 colonies from each strain and sequenced their rDNA for further molecular taxonomy. First, the 16S rDNA of strain A was directly sequenced following PCR amplification. The almost complete 16S rDNA sequence determined was 1,530 bp long (Accession No. AY363303), and was found to correspond to the region between positions 28 and 1558 by comparison with the 16S rDNA of

Escherichia coli. To determine a possible phylogenetic classification of strain A, the 16S rDNA sequence was subjected to similarity searches with public sequence databases. The results showed that the nucleotide sequence similarity of the two strains is highly conserved, and revealed that all 10 colonies of strain A and B are members of the genus *Lactobacillus*, and *Kluyveromyces* sp. respectively (Figs. 2–3). This relationship became clear from the phylogenetic analysis and nucleotide sequence similarity value. The phylogenetic tree shows that strain A forms an evolutionary lineage within a radiation of a cluster comprising *Lactobacillus* species and is phylogenetically most closely related to *Lact. kefir* NRIC 1693^T (Fig. 2). Levels of 16S rDNA similarity between strain A and *Lact. kefir* NRIC 1693^T and between strain A and *Lact. buchneri* DSM 20057^T were 100% and 98.9%, respectively.

In contrast, the partial 26S rDNA of strain B was sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) as recommended by the manufacturer. The partial 26S rDNA sequence determined was 545 bp (Accession No. AY363304). To determine the possible phylogenetic classification of strain B, the 16S rDNA sequence was subjected to similarity searches with public sequence databases. The results revealed that all 6 colonies of strain B are members of the genus *Kluyveromyces*. The phylogenetic tree shows that strain B forms an evolutionary lineage within a radiation of a cluster comprising *Kluyveromyces* species

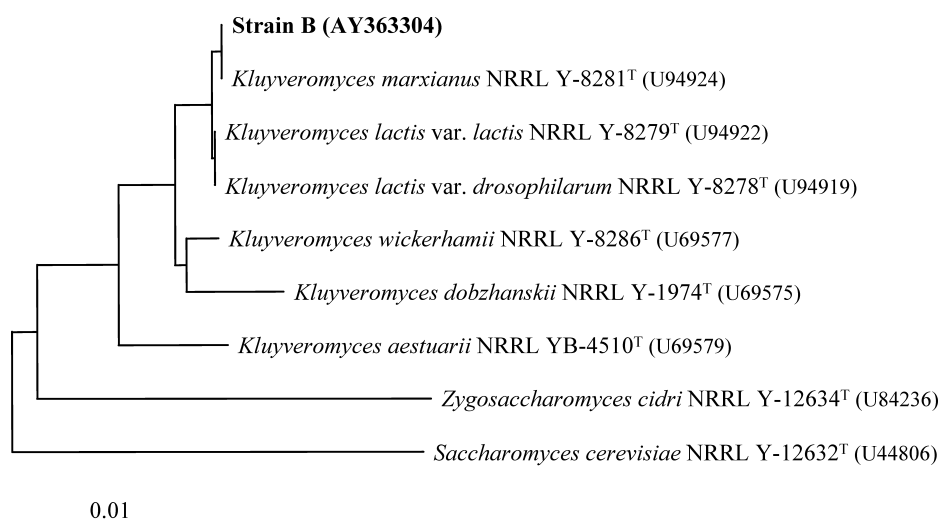


Fig. 3. Phylogenetic tree showing the positions of strain B, some *Kluyveromyces* species and representative strains of other related genera based on 26S rDNA sequences.

Scale bar represents 0.01 substitution per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at the branch points.

and is phylogenetically most closely related to *K. marxianus* (Fig. 3). Levels of partial 26S rDNA similarity between strain B and *K. marxianus* NRRL Y-8281^T, *K. lactis* var. *lactis* NRRL Y-8279^T, and *K. lactis* var. *drosophilae* NRRL Y-8278^T were 100%, 99.8%, 99.8%, respectively (Wayne et al., 1987).

In summary, we isolated and identified two major microflora from Kefir, a traditional Tibetan beverage. The two predominant microbial isolates were related to *Lactobacillus kefir* and *Kluyveromyces marxianus*. The mixed culture of the two strains showed the classical microbial growth of common commercial products such as yoghurt (data not shown). Further in vivo studies into effects such as body weight change, constipation, and cholesterol inhibition will shed additional light on the usefulness of the beverage.

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