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# Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis

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ABSTRACT

The microflora of Tibetan kefir grains was investigated by culture- independent methods. Denaturing gradient gel electrophoresis (DGGE) of partially amplified 16S rRNA for bacteria and 26S rRNA for yeasts, followed by sequencing of the most intense bands, showed that the dominant microorganisms were *Pseudomonas* sp., *Leuconostoc mesenteroides*, *Lactobacillus helveticus*, *Lactobacillus kefiranofaciens*, *Lactococcus lactis*, *Lactobacillus kefiri*, *Lactobacillus casei*, *Kazachstania unispora*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and *Kazachstania exigua*. The bacterial communities between three kinds of Tibetan kefir grains showed 78–84% similarity, and yeasts 80–92%. The microflora is held together in the matrix of fibrillar material composed largely of a water-insoluble polysaccharide.



## 1. Introduction

Kefir is an acid, viscous, slightly carbonated dairy beverage (Garrote et al., 2001), and related to a variety of health benefits (McCue and Shetty, 2005; Rodrigues et al., 2005). Traditionally kefir grains have been used for centuries in many countries, for example, in Tibet, China, as the natural starter in the production of the unique self-carbonated dairy beverage (Saloff-Coste, 1996). Kefir grains contains a complex microbial symbiotic mixture of lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* spp.), and yeasts (*Kluyveromyces*, *Saccharomyces* and *Torula*) included in a polysaccharide–protein matrix (Farnworth, 2005; Witthuhn et al., 2005). Yeasts and lactic acid bacteria co-exist in a symbiotic association and are responsible for lactic-alcoholic fermentation.

For many years, research on the microflora in foods has relied on conventional culture-dependent methods (Nuraida et al., 1995). Culture-dependent methods consist of isolating and culturing microorganisms prior to their identification according to either morphological, biochemical or genetic characteristics. Hence, culture-dependent methods are time-consuming, due to long culture periods and elaborate culture techniques. Moreover, species occurring in low numbers are often out-competed in vitro by numerically more abundant microbial species (Hugenholtz et al., 1998) and some species may be unable to grow in vitro (Head et al., 1998).

In contrast, molecular culture-independent approaches have proven to be powerful tools in providing a more complete inventory of the microbial diversity in food samples. As the development of molecular technology, denaturing gradient gel electrophoresis (DGGE) of PCR generated rRNA gene fragments has recently been shown to be a useful tool for studying community structure at the species level. DGGE takes advantage of the sequence-dependent separation of equally sized PCR fragments amplied from the total DNA extract of a sample (Muyzer and Smalla, 1998). The possibility to extract and sequence bands from the DGGE gels offers an additional valuable tool for identification of predominant ecosystem members. The great potential shown in analyzing samples from natural environments has stimulated food microbiologists to investigate the suitability of PCR-DGGE to study microbial fermentations in food and food-related ecosystems. Now PCR-DGGE has successfully been applied to analyze the microflora in various foods, such as sourdoughs (Vogelmann et al., 2009), kimchi (Chang et al., 2008), sliced vacuum-packed cooked ham (Hu et al., 2009), cheese (Jany and Barbier, 2008), etc.

The microbial community of Tibetan kefir grains depends primarily on their source. It has been reported that Tibetan kefir grains contain Lactobacilli, Lactococci and yeast, and sometimes acetic acid bacteria, depending on the source of

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origin (Yang et al., 2007; Xiao and Dong, 2003). While information is available concerning Irish, Taiwanese, Russian and certain European kefir grains, little is published concerning the microbial intricacies of Tibetan kefir grains. The objectives of this study were to determine the microbial community present in Tibetan kefir grains using DGGE and examine the microbial distribution of Tibetan kefir grains under scanning electron microscopy (SEM).

## 2. Materials and methods

### 2.1. Culture of Tibetan kefir grains

Tibetan kefir grains were collected from common families at Ge'er county, A'li region, located in the western Tibet, China. The grains were cultured in sterile 10% reconstituted skim milk at 20 °C for 20 h. Tibetan kefir grains were then filtered and stored at 4 °C.

### 2.2. DNA extraction from Tibetan kefir grain

The DNA of the microorganisms in Tibetan kefir grains was extracted and purified using the method reported by Tilsala-Timisjarvi and Alatossava (2004). FTA® card (Whatman Inc., USA) are designed for purifying DNA from microorganisms for PCR analysis. Kefir sample of 50 μl was directly applied on the FTA® card, and dried at room temperature. Two Φ1.2 mm punches were taken from the card and placed in a 0.2 ml microcentrifuge tube. The punches were washed three times with 200 μl FTA® purification reagent (Whatman Inc., USA) for 5 min at room temperature and twice with 200 μl TE-buffer. The washed punches were dried at room temperature and stored.

### 2.3. PCR amplification

The bacterial community DNA was amplified with primers 338fgc (5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) spanning the V3 region of the 16S rDNA gene (Muyzer et al., 1993). The yeast community DNA was amplified using the primers NL1GC (5′-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG CGG AGG AAA AG-3′) (the GC clamp is underlined) and a reverse primer LS2 (5′-ATT CCC AAA CAA CTC GAC TC–3′) (Cocolin et al., 2002) spanning the D1 region of the 26S rRNA gene.

A total of 50 μl PCR reaction system containing: 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 μM of the primers, 1.25 UI Taq polymerase (Promega, Milan, Italy) and 2 μl of the extracted DNA. To increase the specificity of amplification and to reduce the formation of spurious by-products, a "touchdown" PCR was performed. The amplification was carried out as follows: template DNA was denatured for 4 min at 94 °C. The initial annealing temperature used was 65 °C, and the temperature was decreased by 0.5 °C every cycle until the touchdown temperature of 55 °C was reached; then 10 additional cycles were carried out at 55 °C. Primer extension was carried out at 72 °C for 30 s. The tubes were then incubated for 10 min at 72 °C for final extension. Aliquots (2 μl) of the amplification products were analyzed by electrophoresis in 1% agarose gels.

### 2.4. DGGE analysis

The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA). Samples were applied to 8% (w/v) polyacrylamide gels in 1× TAE. Optimal separation was achieved with a 30–50% urea-formamide denaturing gradient (100% correspondent to 7 M urea and 40% [v/v] formamide). The gels were electrophoresised for 5–10 min at 200 V and for 13 h at 85 V, and then stained with silver solution as described elsewhere (Ampe et al., 1999). The gel was photographed with the GelDos 2000 system (Bio-Rad) and analyzed with the QuantityOne software package (Bio-Rad).

### 2.5. Sequencing and analysis of DGGE fragments

DNA recovered from each DGGE band was reamplified with the primers 338f (5′ ACT CCT ACG GGA GGC AGC AG 3′) and 518r (5′-ATT ACC GCG GCT GCT GG -3′)for bacteria and NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G -3′)and LS2 (5′-ATT CCC AAA CAA CTC GAC TC -3′) for yeast. DGGE bands were excised with a sterile scalpel and eluted in 30 μl sterile water, overnight at 4 °C to allow diffusion of the DNA. Two microliters of the DNA of each DGGE band was reamplified as described above. PCR products were observed by electrophoresis in 1% agarose gel. Direct sequencing of the fragment was performed on an ABI DNA sequencer.

Sequences were submitted and deposited in GenBank database. Each sequence data was used as a query sequence to search for similar sequences from GenBank by means of blast program. The multiple alignment and phylogenetic tree was made with CLUSTALX 1.81 using neighborhood-joining method replicated 1000 times. MEGA 3.1 was used for the assessment of the phylogenetic tree.

### 2.6. Observation of Tibetan kefir grains using scanning electron microscope (SEM)

Tibetan kefir grains were sliced to produce samples for microscopy (Seydim et al., 2005). Samples were collected from the outer and inner part. For each sampling area, The Tibetan kefir grains were fixed in 30 g/l glutaraldehyde in 0.1M phosphate buffer, pH 7.0, for 4 h at 25 °C. Samples were washed with phosphate buffer for 15 min three times. Then grains were postfixed in 10 g/l osmium tetroxide in phosphate buffer for 1 h at 25 °C. After washing with phosphate buffer, samples were dehydrated in ethanol: 15, 30, 50 and 70% ethanol for 10 min each, 85 and 95% for 15 min each, and 99.5% for 1 h. After dehydrating, samples were critical-point dried and coated with gold using a JFC1100E Ion Coater (Jeol, Tokyo, Japan). The preparations were observed using a JSM-6300 scanning electron microscope (Jeol, Tokyo, Japan).

## 3. Results

### 3.1. DGGE fingerprinting of bacterial and yeast communities

Tibetan kefir grains (Fig. 1) were collected from three families in Tibet, China. V3 region of the 16S rRNA gene of bacteria and D1 region of the 26S rRNA gene of yeast were amplified and the resulting PCR products were analyzed by DGGE (Figs. 2 and 3). Duplicates from all the samples gave identical DGGE patterns thus validating the experimental procedure (data not shown). The fingerprints of the bacterial community in three Tibetan kefir grains (Fig. 2) contained up to eight, eight, and seven visible bands, respectively, and had five bands (B, D, E, F, and I) in common. The similarity between the DGGE patterns of the bacteria community was evaluated to be 78–84% by using the clustering algorithm.

Fig. 3 was the fingerprints of the yeast community in three Tibetan kefir grains, containing three, three and four bands, respectively. All samples had two common bands, M and P. The yeast community was less rich than that of bacteria. Yeast community showed similarity of 80–92% between the three grains.

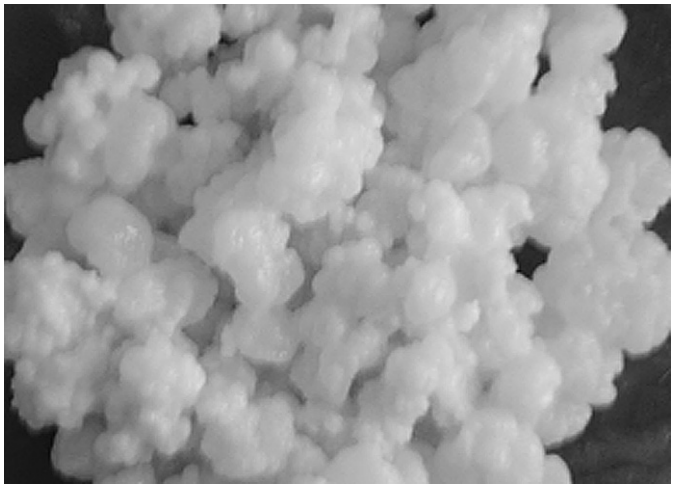

**Fig. 1.** Tibetan kefir grains.

### 3.2. Sequence analysis

To determinate the composition of microflora in Tibetan kefir grains, the presence of the bands in DGGE gel were excised, and DNAs were reclaimed and sequenced. The GenBank accession numbers for the 16S rDNA of bacteria and 26S rDNA of yeast partial

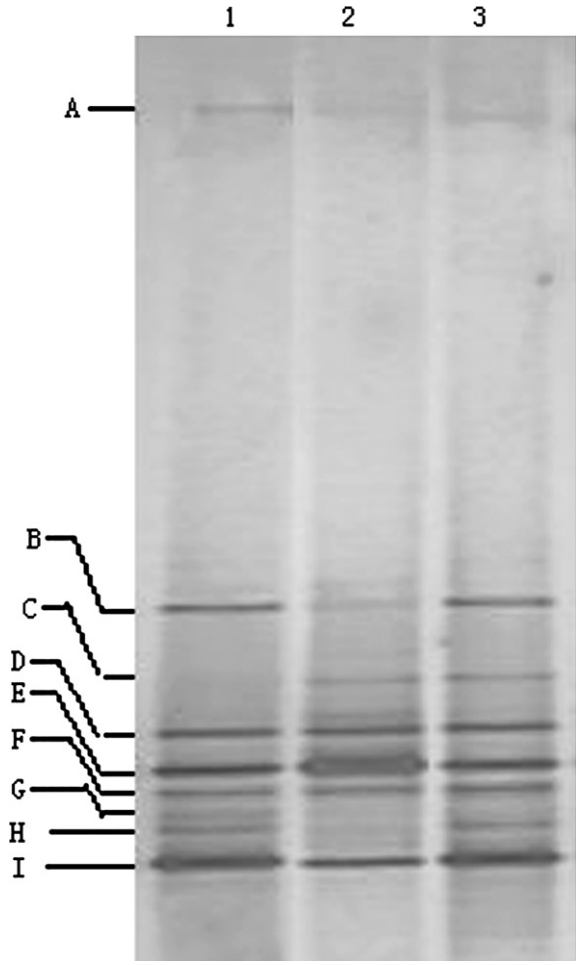


**Fig. 2.** PCR-DGGE fingerprinting of the bacteria community. Lanes 1–3: Tibetan kefir grains 1–3.

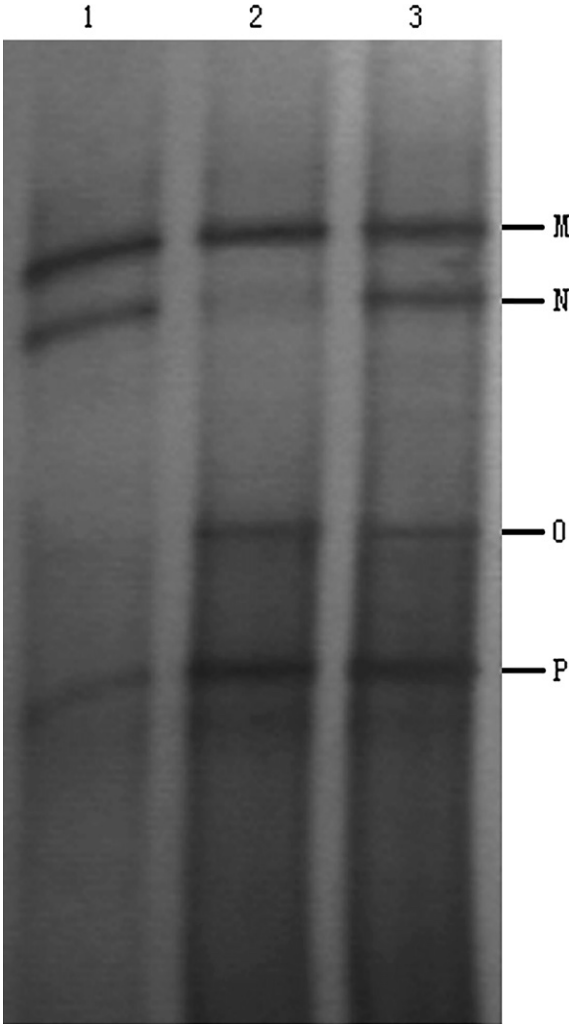


**Fig. 3.** PCR-DGGE fingerprinting of the yeast community. Lanes 1–3: Tibetan kefir grains 1–3.

sequences retrieved from the DGGE bands are obtained. Based on the results of the search for similar sequences in GenBank and EMBL, Figs. 4 and 5 show the phylogenetic relationships based on the sequence results. It was clear that band A was identified as *Pseudomonas* sp., B as *Leuconostoc mesenteroides*, C as *Lactobacillus helveticus*, D as *Lactobacillus kefiranofaciens*, E as *Lactococcus lactis*, F as *Lactobacillus kefiri*, I as *Lactobacillus casei*, M as *Kazachstania unispora*, N as *Kluyveromyces marxianus*, O as *Saccharomyces cerevisiae*, and P as *Kazachstania exigua*.

However, we were not able to identify the minor bands G and H because they could not be excised from the gels due to their low intensity.

### 3.3. Distribution of microorganisms on Tibetan kefir grain

Scanning electron microscopy of Tibetan kefir grains (Fig. 6) indicated that lactobacilli (long and curved), yeasts and fibrillar material were observed on the inner portion of the Tibetan kefir grains, and short lactobacilli and yeast were observed on the outer portion. The density of microbial cell on the inner portion was less than that on the outer portion.

Based on the results of the fingerprints of bacterial community and sequencing, *L. lactis* was dominant microbe, while no lactococcus was found on scanning electron micrographs, which may be

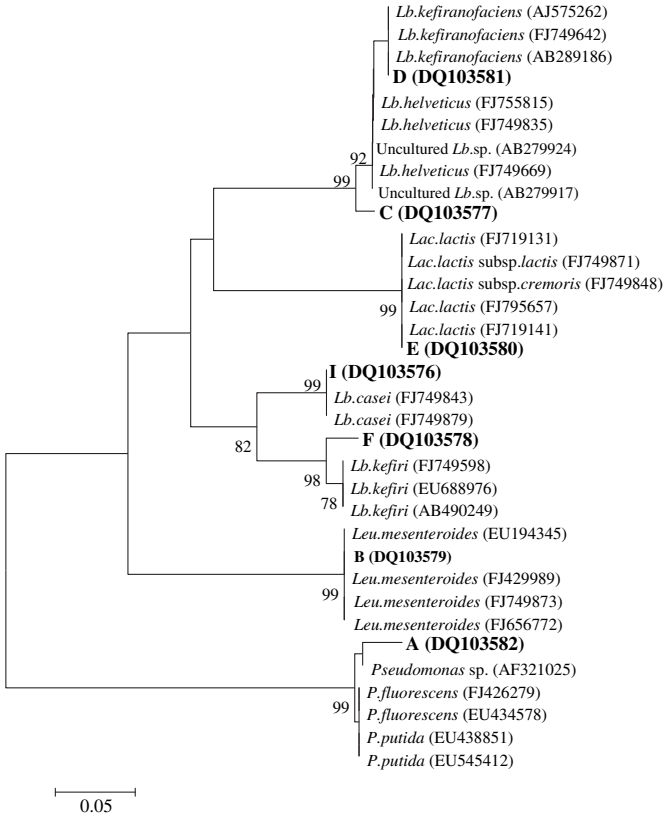


**Fig. 4.** Phylogenetic tree of bacteria retrieved from bands (A–F, and I) in DGGE profile. The number on the branches indicates the support proportion of each branch.

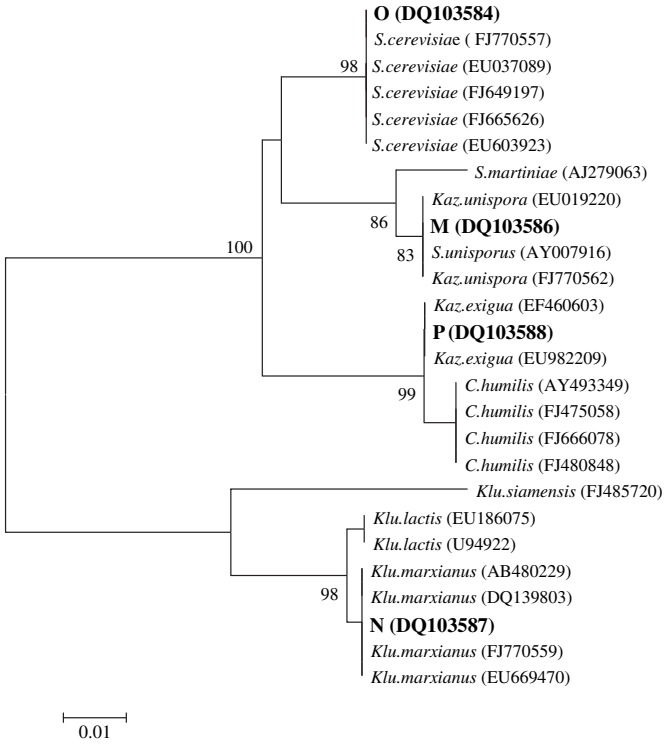


**Fig. 5.** Phylogenetic tree of yeast retrieved from bands (M–P) in DGGE profile. The number on the branches indicates the support proportion of each branch.

due to the bad attachment of lactococcus, this is coincided with the results that Seydim et al. (2005) obtained on Turkey kefir using scanning electron microscope.

## 4. Discussion

Identification and detection of different organisms from food samples is very important in the food industry for hygiene and monitoring purposes. Various DNA-based methods have been established as routine identification methods in many laboratories. Yet, in order to use these DNA methods it is necessary to obtain the DNA of microbial origin from food samples. Many extraction methods have been described (Rossiv et al., 1999; Straub et al., 1999), most of which include various steps of conventional DNA isolation. FTA®-method has previously been used for obtaining DNA from other kinds of food samples (Orlandi and Lampel, 2001; Lampel et al., 2000; Tilsala-Timisjarvi and Alatossava, 2004). Tilsala-Timisjarvi and Alatossava (2004) found that the results from FTA®-based method and conventional DNA isolation of milk and dairy samples are very similar, except in the case where a strain-specific detection of *L. rhamnosus* GG was attempted in a yoghurt sample. The species-specific detection was achieved from this sample with both pretreatment methods. In addition, the FTA® method is easier to perform and contrary to the conventional DNA isolation protocols it does not include the use of volatile solvents or other toxic reagents. So a rapid FTA® membrane card-based method could be used as the pretreatment of milk and dairy samples for DNA-based identification.

Genetic fingerprinting techniques are able to provide a profile representing the genetic diversity of a microbial community from a specific environment. Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used among the culture-independent fingerprinting techniques. The possibility to identify the bacterial species by sequencing the DGGE bands in profiles from food products represents an important step forward to the innovation of the methods of analysis in food microbiology. The technique may be considered a new tool in food microbiology (Ercolini, 2004).

V3 region of 16S rDNA fragments of bacteria and D1–D2 domains of 26S rRNA gene based on differences in the GC content and distribution in each fragment have been developed and widely applied to evaluate the microbial diversity of various samples (Lopandic et al., 2006; Baradei et al., 2007). Previous results show that two groups of microorganisms exist in Tibetan kefir grains: lactic acid bacteria and yeast (Xiao and Dong, 2003; Liu et al., 2004). The present study investigated the microbial diversity of Tibetan kefir grains using PCR-DGGE. We found that the bacterial community in Tibetan kefir grains was more complex than that of yeast. There was some difference between Tibetan kefir grains from different origins, which is coincided with other researches (Angulo et al., 1993; Liu et al., 2004; Witthuhn et al., 2004).

Simova et al. (2002) isolated and identified LAB and yeast in kefir, results indicated that *L. lactis* was a dominant microbe, 58–70% of the total microflora. Liu et al. (2004) isolated two strains of *L. lactis* from Tibetan kefir grains. *L. lactis* cannot be seen on the SEM graph in our study, suggesting the weak adherence between *L. lactis* and Tibetan kefir grains, which resulted in falling into the milk, formed primary microflora. It is coincided with the result of plate counting using culture media (Zhou et al., 2006).

*L. mesenteroides* is another dominant microbe. It's a hetero-fermentative lactic acid bacteria producing aroma, can degrade lactose to lactic acid, acetic acid, ethanol and carbon dioxide, and degrade citric acid into diacetyl, endowing good flavor. In some countries, *L. mesenteroides*, as well as *L. lactis*, is often used to ferment dairy product, for example, buttermilk.

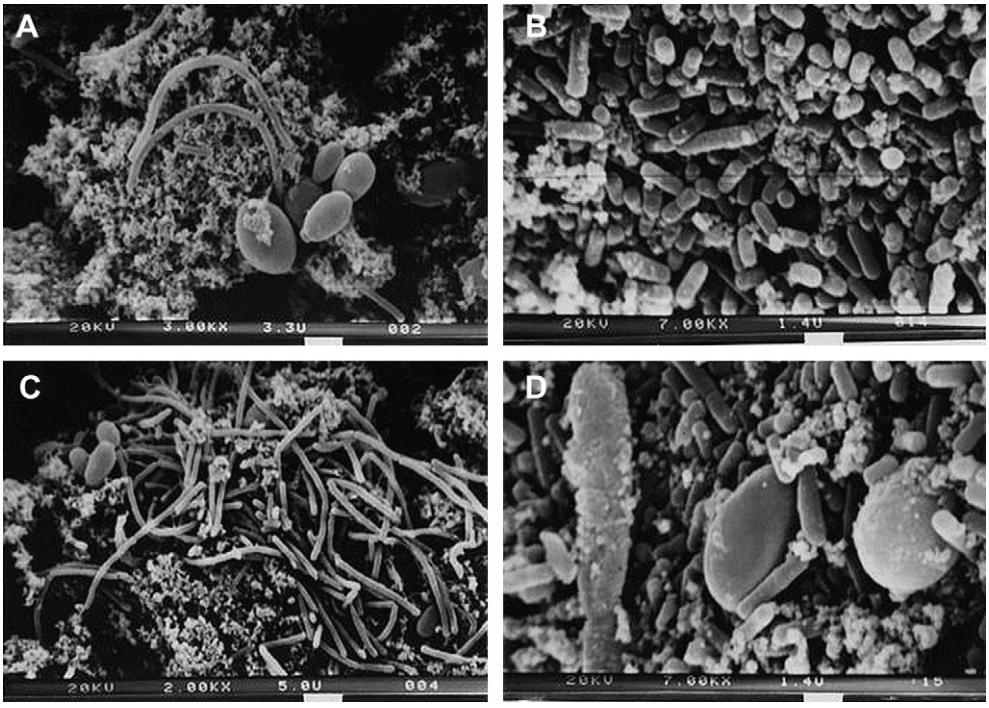


**Fig. 6.** Scanning electron micrographs of Tibetan kefir grains. (A, C) The inside surface of Tibetan kefir grain. (B, D) The outside surface of Tibetan kefir grain.

*L. casei* and *Lactobacillus* sp. group (homologous to *Lb.acidophilus*) were another dominant homofermentative lactobacillus, producing lactic acid to make milk acid. They are probiotics, good at improving the intestinal environment. Our lab obtained a strain of *L. casei*, having effective activity of degrading cholesterol (Xiao and Dong, 2003).

Based on the DGGE profiles of yeast, *K. marxianus* presents in Tibetan kefir grains. It uses lactose as carbonic source, so showing dominance at first, and produces ethanol and carbon dioxide endowing Tibetan kefir grains good flavor. Yeast showed strong activity under high acid condition, consumed part of the lactic acid during fermentation and storage, thus the acidity differed little during storage, avoiding the problem of excess acidity.

In general, DGGE, combined with sequence analysis of 16S rDNA (bacteria) and 26S rDNA (yeast), is efficient in the analysis of microflora diversity in Tibetan kefir grains. PCR-DGGE can monitor the fermentation, providing real-time information and theory basis for the development of the starter.

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