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Research Article

Isolation and Identification of Yeasts from Tibet Kefir

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Abstract: The occurrence and distribution of yeasts in Tibet kefir were investigated in this study. Five samples of Tibetan kefir from Tibet and surrounding areas were collected for yeast isolation. Based on physiological, biochemical characteristics and molecular identification results, eight species of yeast were isolated and identified from Tibet kefir, including *Saccharomyces cerevisiae*, *Pichia fermentans*, *Debaryomyces hansenii*, *Rhodotorula mucilaginosa*, *Candida zeylanoide*, *Candida parapsilosis*, *Kluyveromyces marxianus* and *Kazachstania unispora*. Among the test samples, *K. marxianus*, *Ka. unispora* and *P. fermentans* were the highest three species in frequency of occurrence of yeast isolates. *C. zeylanoides*, *C. parapsilosis* and *R. mucilaginosa* were first found the occurrence in Tibet kefir. The results provided new information of yeast composition and biodiversity of Tibet kefir.

Keywords: Diversity, identification, Tibet kefir, yeast

INTRODUCTION

Tibet kefir is a kind of self-carbonate, traditional natural fermented milk beverage of Tibetan people with long history. Tibetan kefir was considered a probiotic resource and related to a variety of health benefits. It can provide a variety of health benefits in addition to its nutritional status, such as antimicrobial, immune modulating and hypocholesterolemic activities (Diniz *et al*., 2003; Liu *et al*., 2006; Silva *et al*., 2009). The fermented milk is made with Tibet Kefir grains as natural starter. Tibet Kefir grains are a combination of microbe in a matrix of proteins and polysaccharide and this symbiotic matrix, forms "grains" that resemble cauliflower. The microbial composition of Tibet kefir grains has aroused many interest of research. Earlier studies have shown that Lactic Acid Bacteria (LAB), yeasts, in some cases and acetic acid bacteria are the predominant microorganisms in Tibet kefir grains (Zhou *et al*., 2009). Most of related report up to date has focused on LAB occurrence and diversity in the grains. A complex and highly variable community of LAB was isolated and identified (Gao *et al*., 2013; Huang *et al*., 2013; Zheng *et al*., 2013). Yeasts are the major member of the microflora in Tibet kefir grains. Many previous studies have shown that yeasts can ferment lactose and assimilate lactate to promote the growth of LAB in fermented milk and cheese ripening (Corsetti *et al*., 2001; Fleet, 1990). Moreover, dairy yeasts have been reported its probiotic properties and have potential to use as probiotics strains (Chen *et al*., 2010). However, few studies have been conducted on the specific occurrence of yeasts in Tibet kefir grains. The objective of this study is to investigate the diversity and distribution of yeasts in Tibet kefir grains. This study will contribute to the further research of screening beneficial yeast strains for probiotics from traditional fermented milk products.

MATERIALS AND METHODS

Samples collection: Tibet kefir grain samples were collected from private households separately in Tibet and nearby areas. The samples were preserved in sterilized milk at 4°C and activated at 28°C for 48h.

Isolation and purification of yeasts: For yeast isolation, 1g of Tibet Kefir grains was homogenized with sterilized water and diluted with tenfold gradient by sterilized water. The diluents were spread on plates of YPD agar (2%glucose, 0.5% yeast extract, 1% peptone, 1.5% agar) with 0.01% Chloramphenicol. The plates were incubated at 28°C for 2-3d. After incubation, at least one of each representative colony type with different appearance (form or color) was a randomly selected and streaked successively on YPD ager three times for purification. The purified strains were suspended in YPD broth containing 20% (v/v) glycerol at -80°C for preservation.

Phenotypic identification: All the yeast isolates were characterized based on their morphology, spore formation, assimilation of and growth on carbon sources and hydrolysis of urea, using the methods of

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Kurtzman *et al*. (2011). The physiological and biochemical characteristics tests were carried out using HANGWEI trace biochemical reactions tube kits (Hangzhou Microbial Reagent Co., Ltd. Hangzhou, China) according to the manufacturer's instructions. *S. cerevisiae* CGMCC 3262 from China General Microbiological Culture Collection Center (CGMCC) were used as active control.

Molecular identification: Molecular method was used to amplification of the D1/D2 domain of 26s ribosomal gene for yeast species identification. DNA of yeasts was extracted using Axyprep genome DNA extraction kit (Axygen, Corning Incorporated). The PCR was carried out in a 50 μL reaction volume in a 0.2 mL tube. The PCR system consists of 0.2 μL of Ex Taq DNA polymerase (TaKaRa), 4 μL of dNTPs mixture (2.5 mM of each nucleotide) (TaKaRa), 1μl of the yeast genome DNA and 0.5μl of each primer (NL1, NL4). The set of primers NL1 (5'- GCATATCAATAAGCGGAGGAA AAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') was used for amplifying the 26S rDNA region. Amplification was performed for 36 PCR cycles with annealing at 52°C, extension at 72°C for 2 min and denaturation at 94°C for 1 min (Kurtzman and Robnett, 1998). The PCR products were visualized by electrophoresis on 1.0% (w/v) agarose gels stained with Goldview in 0.5×TBE buffer. The PCR products were purified by Axyprep PCR purification kit (Axygen, Corning Incorporated) and sequenced by ABI 3730×l DNA Analyzer (Applied Biosystems). The sequences were compared and analyzed with the BLAST program in the GeneBank database (www.ncbi.nlm.nih.gov).

Frequency analysis: The frequency of appearance of each species in the samples was calculated as a proportion of the number of samples containing the species to the total number of samples.

RESULTS AND DISCUSSION

Identification of yeast isolates: A total of 66 yeast colonies were isolated from 5 Tibet kefir samples. The yeast isolates were identified on the basis of morphology, physiological and biochemical tests. The results (Table 1) showed that yeast isolates were classified into 8 groups belonging to the genera: *Saccharomyces*, *Pichia*, *Debaryomyces*, *Rhodotorula*, *Candida*, *Kluyveromyces* and *Kazachstania*.

The D1/D2 domains of the 26S rDNA genes of isolates were amplified and sequenced. All isolates were identified to species level based on analysis of the D1/D2 domains of the 26S rDNA genes. The amplified sequences of isolate were almost 600bp. Based on comparing with the known sequence of yeasts available in the database of the NCBI, EMBL or DDBJ, the isolate yeasts displaying less than 1% nucleotide difference in the region with type yeasts from database. The sequences information from the 26S rDNA PCR

Table 1: Phenotypic characteristics of yeast isolates

Groups No.	A	B	C	D	E	F	G	H	*S. cerevisiae* CGMCC 3262
Colony color	Milky white	Milky white	Grayish-white	Pink	Off-white	White	Grayish-white	Milky white	Milky white
Surface appearance	Smooth	Rough	Smooth	Smooth	Smooth, shiny	Smooth, butyrous	Smooth, butyrous	Smooth, flat	Smooth
Cell morphology	Oval	Oblong	Spherical	Ovoid	Ovoid to elongate	Ovoid	Oval	Roundness	Oval
Vegetative propagation	Budding	Budding	Budding	Budding	Pseudohypha	Pseudohypha	Budding	Budding	Budding
Pseudohypha	−	−	−	−	+	+	−	−	−
Urea hydrolysis	−	−	−	+	−	−	−	−	−
D-Glucose fermentation	+	+	−	+	+	+	+	+	+
Sucrose fermentation	+	−	−	+	−	−	+	−	+
Lactose fermentation	−	−	−	−	−	−	+	−	−
Maltose fermentation	+	−	−	−	−	−	−	−	+
Glucose assimilation	+	+	+	+	+	+	+	+	+
Sucrose assimilation	+	−	+	+	−	+	+	−	+
Lactose assimilation	−	−	−	−	−	−	+	−	−
Xylose assimilation	−	+	+	+	−	+	+	−	−
Methanol assimilation	−	−	−	−	−	−	−	−	−
Ethanol assimilation	+	+	+	+	−	+	+	+	+
Glycerol assimilation	−	+	+	+	+	+	+	−	−

Table 2: Sequences information from the 26S rDNA PCR product of representative strains

Group	Representative strain	Accession No.	Closest relatives	% Identity	Size (bp)
A	K11	JQ686916	*Saccharomyces cerevisiae*	99.8	575
B	K14	JQ665247	*Pichia fermentans*	99.5	559
C	H2	KC534842	*Debaryomyces hansenii*	99.3	580
D	D2	KC160628	*Rhodotorula mucilaginosa*	99.0	586
E	D3	JX441602	*Candida zeylanoides*	99.6	570
F	D4	JX441605	*Candida parapsilosis*	99.6	576
G	Z17	FJ896141	*Kluyveromyces marxianus*	99.6	545
H	K21	HM627101	*Kazachstania unispora*	99.2	593

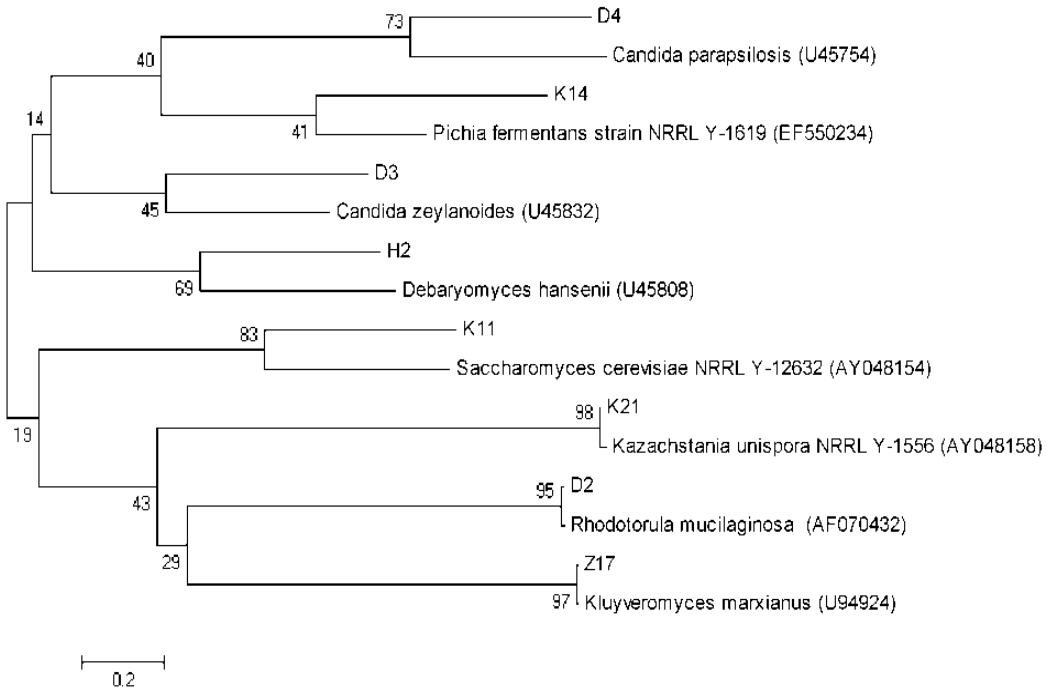

Fig. 1: Phylogenetic tree based on 26S rDNA D1/D2 sequence between isolates and type strains

product of representative strains were showed in Table 2. The representative strains and their related type strains (Kurtzman *et al*., 2011) were chosen to constitute a phylogenetic tree from Neighbor-Joining depicting relationship in the basis of based on 26S rDNA D1/D2 sequence (Fig. 1). As shown in Fig. 1, all representative strains grouped together with the corresponding type strain. The results showed that yeast strains isolated from Tibet kefir grains were identified as belonging to 8 species, including *Saccharomyces cerevisiae* (8), *Pichia fermentans* (11), *Debaryomyces hansenii* (6), *Rhodotorula mucilaginosa* (5), *Candida zeylanoides* (6), *Candida parapsilosis* (7), *Kluyveromyces marxianus* (13) and *Kazachstania unispora* (10).

Phenotypic characteristics tests, such as culturing and physiological tests, were traditionally used to identify yeast from dairy products. However, these tests may lead to a large degree of ambiguity. In some cases, it was hard to identify strains to specie level because of false positive and negative results (Lopandic *et al*., 2006). In this study, the phenotypic test results compared with the characteristics of the type strain revealed the identification to genus level. The D1⁄D2 domain of 26S rDNA, which displayed significant enough differences between yeasts, was consider as the important target gene in yeast identification. Yeasts belong to the same species showed less than 1% nucleotide differences in this region (Kurtzman and Robnett, 1998). In the investigation, the isolate yeasts showed less than 1% nucleotide difference in the region with type yeasts from database. The yeast isolates could be identified to specie level by analysis of sequences in the D1⁄D2 domain.

Frequency and distribution of yeast in Tibet kefir grains: Table 3 showed the frequencies and distribution of yeast species in Tibet kefir grains. Among the test samples, *K. marxianus*, *Ka. unispora* and *P. fermentans* were the highest three species in frequency of occurrence of yeast isolates, which were found in all investigated samples. *S. cerevisiae* was isolated from

Table 3: Frequencies and distribution of yeast species from Tibet kefir

	Number of isolates from different sample					
	1	2	3	4	5	Total frequency (%)
Saccharomyces cerevisiae	2	3	2	0	1	12.1
Pichia fermentans	2	3	2	2	2	16.7
Debaryomyces hansenii	2	0	1	0	3	9.10
Rhodotorula mucilaginosa	0	2	1	2	0	7.60
Candida zeylanoides	2	0	2	2	0	9.10
Candida parapsilosis	2	0	0	3	2	10.6
Kluyveromyces marxianus	2	2	3	3	3	19.7
Kazachstania unispora	2	2	2	2	2	15.2

four out of five samples, while *D. hansenii*, *R. mucilaginosa*, *C. zeylanoides* and *C. parapsilosis* were found in three out of five samples. Yeasts co-cultured with lactic acid bacteria and acetic acid bacteria sometimes composed the complex and dynamic microbial symbiosis of kefir and kefir grains. It is believed that the microflora of kefir and kefir grains depended heavily on geographic, climatic and cultural conditions as well as the diversity of local species of wild yeasts and bacteria (Marsh *et al*., 2013). Tibet kefir (grains) which from dryness climate, low temperatures and scant oxygen Tibetan plateau contained its particular microorganism components. Zhou *et al*. (2009) investigated the microflora of three kinds of Tibetan kefir grains by culture-independent methods, which reported that the dominant microorganisms of yeasts were *Kazachstania unispora*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Kazachstania exigua*. This result was similar to part of our observations. In this study, eight yeast species were isolated and identified from five samples, in which *Kluyveromyces marxianus*, *Kazachstania unispora and Pichia fermentans* were the most frequency percentage of isolates. *K. marxianus* possessed strong ability to utilize lactose and contributed to their growth in dairy product. *P. fermentans* and *D. hansenii* have been reported in kefir from other region (Russia, Turkey, Brazil, etc) (Sarkar, 2008) were the main yeasts species in Tibet kefir as well. *C. zeylanoides*, *C. parapsilosis* and *R. mucilaginosa* were found the occurrence in dairy products (cheese and fermented milk) (Bai *et al*., 2010; Fleet, 2006). In this study, these yeast species were first observed the presence of Tibet kefir grains.

CONCLUSION

The yeast diversity of Tibet kefir was investigated in this study. A total of 66 strains of yeast were isolated and identified by a combination of phenotypic and molecular methods. Eight species of yeast were identified from five Tibet kefir samples. *K. marxianus*, *Ka. unispora* and *P. fermentans* were the most common culturable species. This was the first report the presence of *C. zeylanoides*, *C. parapsilosis* and *R. mucilaginosa* in Tibet kefir. The investigation provided theoretical foundation of yeast diversity of Tibet kefir and could be useful for probiotic strain selection.

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