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Alcohol-brewing properties of acid- and bile-tolerant yeasts co-cultured with lactic acid bacteria isolated from traditional handmade domestic dairy products from Inner Mongolia

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ABSTRACT

In this study, acid- and bile-tolerant yeast and lactic acid bacteria (LAB) were isolated from Inner Mongolian cheeses (*khuruud, zöökhii, tsur*) and an alcoholic drink (*saaliin arhi*) and its starter (*khörönggö*), and the alcohol-brewing capacities of the isolates were tested. Among 69 yeast and 89 LAB isolates, 6 typical yeast (5 *Saccharomyces cerevisiae* and 1 *Naumovozyma castellii*) and 6 LAB (*Lactobacillus plantarum*) strains were selected on the basis of acid tolerance (pH 3.5), bile tolerance, and/or lactose fermentation properties. The selected yeasts, particularly the *S. cerevisiae* SY1 strain isolated from *khörönggö*, produced high levels of ethanol in both a small Japanese sake brewing model and in grape juice fermentation. Among the selected *L. plantarum* strains, ZL1 and KL1, isolated from *zöökhii* and *Saaliin arhi*, respectively, showed weak and strong lactose fermentation in milk. Although a monoculture of *S. cerevisiae* SY1 could not produce ethanol during milk fermentation, it produced high levels of ethanol in a co-culture with *L. plantarum* strain ZL1. In contrast, in co-culture with a more vigorous lactose fermenter, strain KL1, *S. cerevisiae* SY1 was not able to produce a comparable level of ethanol. These results demonstrate co-fermentation roles for yeast and LAB in traditional fermented dairy foods.

1. Introduction

Since ancient times, dairy products have been an important food material in Mongolia and Inner Mongolia (FAO, 2013). Mongolian herders have a long history of producing various kinds of dairy products using different production processes (Yu et al., 2011). Raw milk for traditional fermented products is mainly obtained from the goat, sheep, cow, mare, and camel (Degen, 2007; Konuspayeva, Faye, & Loiseau, 2009; Salimei & Francesco, 2012). The processes traditionally used for milk fermentation in Mongolia, particularly for cheese products, involve spontaneous fermentation by lactic acid bacteria (LAB) and yeasts (Liu et al., 2012; Uchida, Hirata, Motoshima, Urashima, & Arai, 2007; Yu et al., 2011). Mongolia, particularly in Inner Mongolia, widely expands and has various steppe (climate) areas. Inner Mongolia is located in the north-eastern plains of China and spans approximately 1,200,000 km^2. It mainly consists of meadow steppes, typical steppes, desert steppes, and alpine steppes (Han, Owens, Wu, & Huang, 2009; Kang, Han, Zhang, & Sun, 2007). Therefore, it is considered that the microbiota in the traditional fermented milk varies according to the area.

Since the 1990s, many researchers have reported the isolation, identification (Fuller, 1991; Ouwehand & Röytiö, 2015), and probiotic roles of microorganisms in various fermented foods, including traditional spontaneous fermented foods (Kawahara et al., 2015; Kuda, Kanno, Kawahara, Takahashi, & Kimura, 2014). In the case of LAB isolated from Inner Mongolian dairy products, various probiotic properties, such as antimicrobial activity, antioxidant capacity, cholesterol assimilation, and regulation of intestinal microbiota have been reported (Wu et al., 2009; Zhang et al., 2014). In contrast, the probiotic and starter properties of spontaneous yeast are not well studied. Furthermore, the fermentation properties of yeast and LAB isolates are not clear.

In this study, we isolated acid- and bile-tolerant yeast and LAB from three kinds of fermented cheese (*khuruud, zöökhii, tsur*), an alcoholic drink (*saaliin arhi*), and their handmade starter (*khörönggö*). The fermentation capacity of the isolates was tested in a small Japanese sake brewing model, in the fermentation of grape juice, and in the fermentation of commercial pasteurized cow milk.

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2. Materials and methods

2.1. Chemical compounds and viable counts in samples

A total of nine traditional handmade dairy products were used in this study (Table 1 and Fig. 1). The products originated from seven regions in Inner Mongolia, China, and were made domestically from cow milk. A range of products were selected, including four types of cheese (3 *khuruud* and 1 *tsur*), three types of yogurt (*zöökhii*), a starter for milk liquor (*khörönggö*), and a milk liquor (*saaliin arhi*). The pH of each product was measured directly using a pH meter (pH Spear, Eutech Instruments, Singapore). The lactose organic acid and ethanol contents were measured as previously reported (An, Kuda, Yazaki, Takahashi, & Kimura, 2014; Kuda, Matsuda, Yasunaka, & Yano, 2011). Briefly, the samples were diluted (1:10) with nine volumes of distilled water. After centrifugation (15,000 × *g* for 3 min) at 4 °C, the supernatant was passed through a filter (0.45 μm pore size) and injected into the HPLC instrument under the following conditions: column, ICSep ICE-ORH-801 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); operating temperature, 35 °C; elution, 0.005 mol/L of H_2SO_4; flow rate, 0.8 ml/min. Eluted compounds were detected by a refractive index (RI) detector.

To count viable LAB and yeasts, 1 g of each sample and 9 ml of phosphate buffered saline solution (PBS, Nissui Pharmaceuticals, Tokyo, Japan) containing 1 g/L agar were homogenized (Kanno, Kuda, An, Takahashi, & Kimura, 2012). Ten-fold serial dilutions from 10^{-1} to 10^{-5} (0.05 ml) were inoculated on de Man Rogosa Sharpe medium agar (MRS agar, Oxoid, Hampshire, UK) to detect LAB and potato dextrose agar (PDA, Tokyo, Japan) containing 100 mg/L of chloramphenicol (Wako Pure Chemical, Osaka, Japan) to detect yeasts. Plates were incubated at 25 °C for 48–72 h under aerobic conditions (for PDA) or anaerobic conditions (for MRS) with an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan). Viable counts were calculated as log colony forming units (CFU) per gram of sample.

2.2. Screening for acid and bile tolerance and lactose utilization

A total of 69 colonies, including 9–10 colonies from the each sample, were isolated after culturing on PDA. On MRS agar, a total of 89 colonies, including 8–11 colonies from each sample, were isolated. Yeast growth was tested in glucose yeast peptone agar (GYP) formulated with 50 g of glucose, 10 g of yeast extract, and 20 g of peptone per litre. The acid tolerance of each isolate was determined by inoculating 3 ml of test broth (GYP for yeast or MRS for LAB) adjusted to pH 3.5 using HCl. The bile tolerance of each isolate was determined using the same culture medium (GYP or MRS) supplemented with 10 g/L bile (Oxgall, Wako Pure Chemical) (Kuda, Noguchi, et al., 2014a). Lactose utilization was indicated by a decrease in pH (production of acidic fermentation products) in Gifu Anaerobic Medium (GAM) semi-solid medium (Nissui Pharmaceuticals) containing 0.5% (w/v) of lactose (Kuda, Kawahara, Nemoto, Takahashi, & Kimura, 2014).

Carbohydrate utilizations for selected 20 yeast isolates and eight LAB isolates were determined using commercial kits (Yeast: API®/ID32c and LAB: API® 50 CHL, bioMérieux, Marcy-l'Étoile, France) (Kuda, Kaneko, Yano, & Mori, 2010; Kuda et al., 2011). Based on the carbohydrate utilization patterns and sample origin, 6 strains of yeasts and LABs were selected for the subsequent experiments.

2.3. Food fermentation tests

2.3.1. One-step small model of sake brewing test

The sake (Japanese rice wine) brewing capacity of the six selected yeast strains and *Saccharomyces cerevisiae Kyokai*-No.7 was determined using the one-step small sake brewing model as previously reported (Kuda et al., 2011), with slight modifications. The yeast strains were incubated in GYP broth at 30 °C for 24 h. Cells were then washed and suspended in spring water (*Minami-Alps no Tennensui*, Suntory, Osaka, Japan) to an optical density (OD_{660nm}) of 2.0. Cell suspensions (0.15 ml), 2 g of *Aspergillus oryzae* malted rice (*koji*, Nagano, Japan) and 4 g of alpha-processed rice (Alpha Food, Izumo, Japan) were mixed and diluted to 17.5 ml with spring water containing 0.025% (v/v) lactic acid in a polypropylene (PP) tube. After 14-day incubation at 15 °C, the supernatants were obtained by centrifugation (2200 × *g* for 10 min). Ethanol content, organic acid content, and pH were determined as described above.

2.3.2. Grape juice fermentation test

The six selected yeast strains were tested in grape juice fermentation according to the method of Amparo, Eladio, Tomas, and Daniel (1992) with slight modification. Commercial grape juice (Koshin Milk Products, Tokyo, Japan) was amended with 0.1 g/L potassium sulphite and pasteurized at 65 °C for 10 min (pH 3.2, Brix%, 11.3). Yeast cell suspension (0.1 ml) was inoculated into 10 ml of the prepared grape juice and incubated at 20 °C for 19 days. Ethanol content, organic acid content, and pH were determined as previously described.

2.3.3. Soymilk fermentation test

The soymilk fermentation capacity for production of yogurt or cheese type products was tested for six selected yeast and LAB strains, *S. cerevisiae Kyokai* No.7, and the type strain of *Lactobacillus plantarum* NBRC15891^T as reported previously (Kawahara et al., 2015). Briefly, each of the cell suspensions (0.1 ml) was

Table 1
Chemical compounds and viable cell counts of Inner Mongolian fermented dairy products used in present study.

Sample No.	Name	Region	Fermentation time (days)	pH	Sugar and organic acid (mg/g)			Ethanol (μL/g)	Log CFU/g	
					Lactose	Lactic acid	Acetic acid		LAB	Yeasts
1	*Khuruud*	Huhhot	3	4.7	11.3	9.8	1.7	7.4	7.9	7.9
2		Ernhot	14	5.1	7.8	8.1	2.5	7.6	7.0	ND
3		Zhenglan	7	4.4	7.0	1.2	7.0	t	7.7	7.4
4	*Zöökhii*	Huhbot	3	5.1	18.6	0.8	t	t	3.2	3.3
5		Xilingol	14	3.8	10.5	28.8	4.1	26.0	6.4	6.3
6		Banner	7	4.4	6.4	3.3	4.3	11.6	7.7	7.5
7	*Tsur*	League	7	4.5	8.8	11.9	2.6	8.8	8.0	ND
8	*Khörönggö*	Xilingol		3.3	9.6	12.2	18.6	t	5.7	5.5
9	*Saaliin arhii*	League		3.3	t	t	0.5	92.1	3.4	3.3

Values are mean of measurement duplicate.
CFU: colony forming unit. MRS and PDA: for lactic acid bacteria and fungi, respectively.
LAB: lactic acid bacteria. ND: not detected (<2 log CFU/g). T: trace.