Chapter 2

The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products

2.1 Introduction

Lactic acid bacteria (LAB) and bifidobacteria are reported to be probiotics with properties beneficial to health (Isolauri *et al*. 2000; Xiao *et al*. 2003; Rosenfeldt *et al*. 2003, 2004; Kato *et al*. 2004; Ishida *et al*. 2005). According to the definition of probiotic bacteria adopted by the joint Food and Agriculture Organization of the United Nation / World Health Organization (WHO) working group (WHO 2001), they are living microorganisms that confer a health benefit on the host when administered in adequate amounts. One of the characteristics required for the selection of candidate probiotic strains is resistance to gastric activity and bile acid. The beneficial functions of probiotics have been reviewed (Sullivan & Nord 2005; Gupta & Garg 2009), and many commercially available yoghurts contain probiotic LAB.

Mongolian food culture, especially concerning dairy products, is different from Western cultures. Historically, Mongolian nomadic people have consumed large amounts of dairy products from their cattle, and have processed milk using natural tools and ingredients. Many of their dairy products contain several types of milk, because their domestic animals include cows, sheep, goats, yaks and horses.

People in Inner Mongolia, an autonomous region of China, have a culture similar to the Mongolian nomadic people, and use the same traditional techniques to prepare dairy products. The microbiota of traditional Inner Mongolian dairy products has been studied (Mitsuhashi *et al*. 1989; Naersong *et al*. 1996; Ishii *et al*. 1997; Watabe *et al*. 1998; Shuangquan *et al*. 2004,

2006), and probiotic LAB isolated from those products (Tsuda *et al*. 2007). Although probiotic LAB from traditional Mongolian dairy products are of interest, few reports have focused on products from Mongolia. Therefore, in the present study the dairy products such as tarag, airag, aaruul, byaslag and eeizgii from three Mongolian provinces were collected and investigated for their LAB microbiota. Moreover, the probiotic potential of LAB isolates from these samples were also evaluated.

2.2 Materials and methods

2.2.1 Materials

A total of 66 samples of traditional Mongolian dairy products (Table 2-1) were collected from the nomads in Tuv, Khuvsugul and Dornogobi provinces starting from July 2005 to July 2006 (Fig. 2-1). Samples after collection were immediately placed in an ice box.

Table 2-1 Samples of traditional Mongolian dairy products were collected

Prefecture	Area	Product								
		Tarag					Aaruul	Airag	Eezgii	Byaslag
		Cow	Goat	Camel	Sheep	Yak	Cow	Mare	Cow	Cow
Tuv	Ulaanbaatar	8	3				5	5	3	
Tuv	Altanbulag	11	6			1	3	2	2	1
Khuvsgul	Murun	8			2					
Dornogobi	Sainshand			6						
Sub-total		27	9	6	2	1	8	7	5	1
Total		45					8	7	5	1

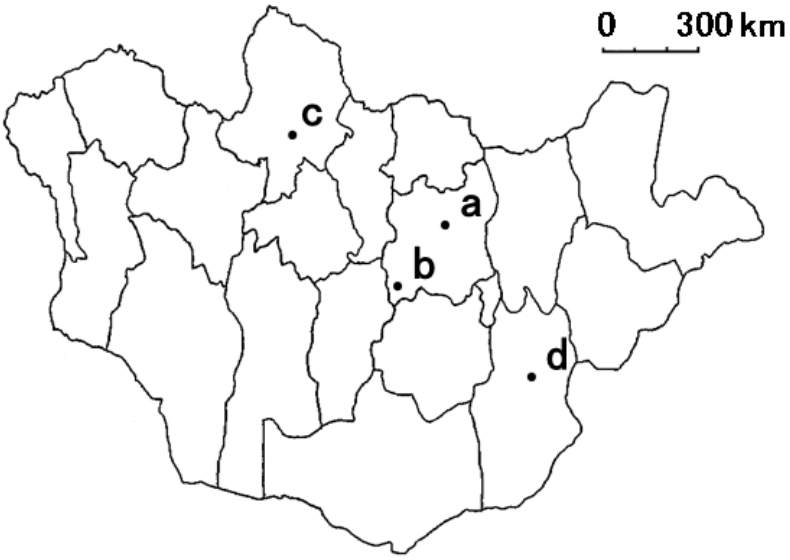

Figure 2-1 Sampling points in Mongolia.

a. Ulaanbaatar area in Tuv province, b. Altanbulag area in Tuv province, c. Murur area in Khuvsgul province, d. Sainshand area in Dornogobi province

2.2.2 Media and isolation of microorganisms

LABs were isolated using Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany), and GYP (1.0% glucose, 1.0% yeast extract, 0.5% peptone, 0.2% Na-acetate, 0.2% $MgSO_4$, 400 ppm $MnSO_4$, 20 ppm $FeSO_4$, 20 ppm NaCl) agar. All medium contained sodium-azide and cycloheximide (10 ppm each). To isolate LAB from tarag and airag, 1 mL was diluted in 9 mL sterilized 0.85% saline and mixed thoroughly. To isolate microorganisms from aaruul, byaslag, and eezgii, 1 g of sample and 9 mL of sterilized 0.85% saline were homogenized in a homogenizer (NIHONSEIKI KAISHA LTD., Tokyo, Japan) at 12,000 rpm for 5 min. Serial dilutions (10^{-1}-10^{-8}) were prepared and 50 μL spread on agar plates, which were incubated anaerobically for 3 days at 37°C with an O_2 absorber (anaerobic packs and jars, Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). After incubation, colonies were purified by streak-plating on new plates of the same agar. Purified strains were suspended into 10% glycerol solution in sterilized tubes and stored at -80°C.

2.2.3 Identification of lactic acid bacteria

For preliminary identification, all strains from MRS and GYP were Gram-stained and tested for catalase activity. Strains that were gram-positive and catalase-negative were classified as LAB and identified based on the 500 base pair (bp) sequence from the 5’-end of 16S ribosomal DNA (16S rDNA). Total DNA was extracted from a colony using a DNA extraction kit (Gen TLE for yeast, TAKARA BIO INC., Otsu, Japan), and 16S rDNA was amplified by PCR, based on a 16S rDNA bacterial identification kit (MicroSeq. 500; Applied Biosystems, Foster city, CA, USA). The PCR reaction was performed in a DNA thermal cycler (Geneamp PCR System 2700, Applied Biosystems, Foster city, CA, USA). Amplified and purified 16S rDNA was sequenced (ABI PRISM 310 genetic analyzer, Applied Biosystems) and a homology search was performed using MicroSeq ID analysis software

version 2.0 (Applied Biosystems). Carbohydrate fermentation patterns were determined using API 50 CH (bioMerieux Japan Ltd., Tokyo, Japan).

2.2.4 Tolerance under condition of bile and gastric acid

To test resistance to bile acid, LABs were cultured at 37°C for 24 h in GYP medium with 0.2% (W/V) oxgall, and viable colonies were counted. Survival in gastric acid was tested as described (Takiguchi & Suzuki 2000) with modifications. Briefly, LABs were cultured for 24 h in the medium from which they were isolated. Fresh cultures were inoculated (1.0%, V/V) into medium with 0.04% pepcin (pH 3.0), and incubated at 37°C for 3 h. Surviving LABs were enumerated by poured-plate cultivation.

2.2.5 Adhesion to Caco-2 cells

Adhesion of LABs to Caco-2 cells was tested according to Maragkoudakis *et al.* (2006) with modifications. Briefly, Caco-2 cells (RIKEN, Tsukuba, Japan) were grown at 37°C in a 5% CO_2, 95% air atmosphere in Dulbecco modified Eagle's medium (DMEM) (Sigma-Aldrich Japan Corporation, Tokyo, Japan) with 10% inactivated (56°C, 30 min) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Caco-2 cells were seeded at 1.0×10^5 cells/mL in six-well tissue culture plates and incubated for 14 days, changing culture medium every 2 days. LABs were cultured in MRS (for *Lactobacilli*) or GYP (for *Lactococci*) at 37°C for 24 h, harvested (centrifuged at 5000 rpm for 10 min at 4°C) and washed twice with phosphate-buffered saline (PBS), and suspended at 1×10^6 cells/mL in DMEM containing 10% fetal bovine serum without antibiotics. LABs were seeded on Caco-2 cells cultured for 14 days, and incubated at 37°C in 5% CO_2, 95% air for 2 h. After incubation, monolayers were washed 3 times with PBS, and 1 mL 0.1% trypsin solution (trypsin / PBS) added to detach Caco-2 cells. The number of LABs adhered to Caco-2 cells was enumerated by

poured-plate cultivation.

2.2.6 Random amplified polymorphic DNA PCR analysis

Random amplified polymorphic DNA (RAPD) PCR primers were AT41 (5'-CGGATGTTGT-3') and BT05 (5'-GGAGGAATAC-3') (NIPPON GENE CO., LTD., Tokyo, Japan) for *Lactobacillus* (*L*.) *paracasei* subspecies (ssp.) and p7 (5'-AGCAGCGTGG-3') and p11 (5'-GTTTCGCTCC-3') (Sigma-Aldrich Japan Corporation.) (Angelis *et al*. 2001; Dautle *et al*. 2002) for *L*. *plantarum*. Reactions were performed in 25 μL with 1.0 μL bacterial DNA, 2.0 μL dNTP mixture (2.5 m mol/L dNTP mixture, NIPPON GENE CO., LTD.), 2.5 μL 10 × buffer (10 × Gene *Taq* Universal buffer, NIPPON GENE CO., LTD.), 0.2 μL 5 units/μL *Taq* polymerase (Gene *Taq* FP, NIPPON GENE CO., LTD.), 0.5 μL of each primer (100 μ mol/L), and 18.8 μL pure water. For *L. paracasei* ssp., the reaction condition of PCR was 94°C for 4 min, then 40 cycles of 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min, ending with 72°C for 5 min. The PCR reaction condition for *L*. *plantarum* was 94°C for 4 min, 40 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, ending with 72°C for 5 min. RAPD products were electrophoresed at 100 V on a 2.5% (W/V) agarose gel containing 0.3 μg/mL ethidium bromide. DNA was detected by ultraviolet irradiation (254 nm) and imaged with an AE-6911FXN print graph (ATTO Co., Tokyo, Japan).

2.3 Results

2.3.1 Isolation and identification of lactic acid bacteria

Forty - five tarag, 7 airag, 8 aaruul, 5 eezgii and 1 byaslag samples in the Ulaanbaatar and Altanbulag areas in Tuv province, the Murun area in Khuvsgul province, and the Sainshand

area in Dornogobi province of Mogolia were collected (Table 2-1). Tarag is similar to yoghurt, and is made from cow, goat, sheep, and camel milk (Ishii 2001; Hirata *et al*. 2007). In the current study, approximately half of the tarag samples were from cow milk, and the other were from goat, camel, sheep and yak milks. I also collected airag that was alcoholic fermented horse milk, and the Mongolian cheeses aaruul, eezgii and byaslag (Ishii 2001; Hirata *et al.* 2007). Purification of LABs from the samples yielded 543 isolates, all of which were gram-positive and catalase-negative. Table 2-2 shows the species, numbers of isolates, and the detection rate of LAB species from each sample. In all, 420 strains were isolated from tarag, and classified into 17 species by 16S rDNA sequence. *L*. *delbrueckii* ssp. *bulgaricus*, *L*. *helveticus*, *L*. *fermentum*, *Streptococcus* (*S*.) *thermophilus* were the majority of detected species, and are assumed to be predominant in tarag. *L*. *delbrueckii* ssp. *bulgaricus* was most commonly detected (36.2%) in tarag (Table 2-2a). Sixty - seven strains in airag samples and classified them into 12 species by 16S rDNA were detected. The predominant LAB species from airag was *L*. *helveticus* (46.3%) (Table 2-2b). From aaruul samples, 41 strains were isolated, and classified into 10 species, the most frequently detected of which was *L*. *fermentum* (26.8%) (Table 2-2c). From byaslag, 16 LAB strains were detected and identified as 7 different species, the most frequent of which were *L*. *delbrueckii* ssp. *lactis* and *Lactococcus* (*Lc*.) *lactis* ssp. *lactis*, at 25.0% each (Table 2-2d). However, no LABs strains, isolated from eezgii, were detected.

The tarag samples used in this study were made with the milks derived from various animals as previously mentioned (Table 2-1). I analyzed whether the composition of LAB species in tarag varied by milk source and found that *L*. *delbrueckii* ssp. existed in all types of tarag (Table 2-3). A tendency in the composition of LAB species according to the type of tarag was not distinguished.

Table 2-2 Species and numbers of LAB isolates from (a) tarag, (b) airag, (c) aaruul and (d) byaslag

		Detected number	Rate (%)
(a) Tarag (n=45)	*Lactobacillus delbrueckii* ssp. *bulgaricus*	152	36.2
	Lactobacillus helveticus	77	18.3
	Lactobacillus fermentum	57	13.6
	Streptococcus thermophilus	43	10.2
	Enterococcus durans	16	3.8
	Lactococcus lactis ssp. *lactis*	15	3.6
	Lactobacillus pentosus	12	2.9
	Weissella confuse	10	2.4
	Lactobacillus kefiri	7	1.7
	Lactobacillus paracasei ssp. *tolerant*	6	1.4
	Lactobacillus plantarum	5	1.2
	Pediococcus parvulus	5	1.2
	Leuconostoc citreum	5	1.2
	Lactobacillus paracasei ssp. *paracasei*	4	1.0
	Enterococcus faecium	3	0.7
	Leuconostoc mesenteroides	2	0.5
	Weissella viridescens	1	0.2
Total		420	100.0
(b) Airag (n=7)	*Lactobacillus helveticus*	31	46.3
	Lactobacillus delbrueckii ssp. *lactis*	15	22.4
	Lactobacillus fermentum	8	11.9
	Weissella viridescens	3	4.5
	Lactobacillus kefiri	2	3.0
	Lactococcus lactis ssp. *lactis*	2	3.0
	Lactobacillus plantarum	1	1.5
	Weissella confusa	1	1.5
	Leuconostoc citreum	1	1.5
	Lactobacillus sakei	1	1.5
	Lactobacillus pentosus	1	1.5
	Leuconostoc garlicum	1	1.5
Total		67	100.0
(c)Aaruul (n=8)	*Lactobacillus fermentum*	11	26.8
	Lactobacillus helveticus	7	17.1
	Lactobacillus buchneri	7	17.1
	Weissella confusa	5	12.2
	Lactobacillus plantarum	3	7.3
	Lactobacillus delbrueckii ssp. *lactis*	2	4.9
	Lactobacillus delbrueckii ssp. *bulgaricus*	2	4.9
	Weissella viridescens	2	4.9
	Lactobacillus kefiri	1	2.4
	Lactobacillus sakei	1	2.4
Total		41	100.0
(d) Byaslag (n=1)	*Lactobacillus delbrueckii* ssp. *lactis*	4	25.0
	Lactococcus lactis ssp. *lactis*	4	25.0
	Lactobacillus delbrueckii ssp. *bulgaricus*	2	12.5
	Enterococcus faecium	2	12.5
	Lactobacillus fermentum	2	12.5
	Lactobacillus plantarum	1	6.3
	Enterococcus durans	1	6.3
Total		16	100.0

Table 2-3 Species and number of LAB isolates from tarag varied by milk source

	Tarag				
	Cow	**Goat**	**Camel**	**Sheep**	**Yak**
Lactobacillus delbrueckii ssp. *bulgaricus*	**91**	**41**	**9**	**7**	**4**
Lactobacillus helveticus	**58**	**8**	**10**		**1**
Lactobacillus fermentum	**27**	**17**	**10**		**3**
Streptococcus thermophilus	**39**	**3**		**1**	
Enterococcus durans	**15**	**1**			
Lactococcus lactis ssp. *lactis*	**4**	**7**	**4**		
Lactobacillus pentosus	**6**	**1**	**5**		
Weissella confusa	**10**				
Lactobacillus kefiri	**6**		**1**		
Lactobacillus paracasei ssp. *tolerans*	**2**		**4**		
Lactobacillus plantarum	**1**		**4**		
Pediococcus parvulus	**5**				
Leuconostoc citreum	**1**	**2**	**2**		
Lactobacillus paracasei ssp. *paracasei*	**1**		**3**		
Enterococcus faecium	**2**		**1**		
Leuconostoc mesenteroides	**1**		**1**		
Weissella viridescens	**1**				
Total	**270**	**80**	**54**	**8**	**8**

2.3.2 Screening of isolates for probiotic lactic acid bacteria

All 543 LAB isolates from Mongolian dairy products were screened for probiotic properties (Table 2-4). Survival of bacteria in an artificial digestive juice was analyzed by subjecting isolates to a bile acid tolerance test of growth in GYP broth with 0.2% oxgall. One hundred and twenty six strains of the 543 isolates grow in nutrient broth with 0.2% oxgall, and were assumed to be tolerant to bile acid. These LAB strains were tested for gastric acid tolerance. I assumed that LAB strain detected at over 7 log colony forming unit (CFU)/mL after exposure to pH 3.0 with 0.04% pepcin for 3 h could tolerate gastric acid. Of the 126 bile acid-tolerant strains, 114 were also viable in low pH with 0.04% pepcin. Homo-fermentative LABs are more frequently used for making yoghurt than hetero-fermentative LABs for its quality. Then the LAB strains have been confirmed to produce gases from glucose. It is suggested that 42 strains from the 114 strains expected to tolerate digestive secretions were homo-fermentative type, which would be considerably contribute to yoghurt production.

Finally, these 42 strains were tested for adhesion to Caco-2 cells, using CFU/mL greater than 5.0×10^3 as the threshold for adhesion. Ten strains adhered to Caco-2 cells by this criterion, with colony numbers ranging from 7.0×10^3 to 7.5×10^4 CFU/mL, and *L. plantarum* 05AM23 showing the highest values (Table 2-5). Briefly, with a slight modification I referred to the method described by Maragkoudakis *et al.* (2006). They assayed the adhesion of LABs to Caco-2 cells by the unit of percent and they set the threshold as 4%. Yet, instead of unit of percent method I thought it was better to estimate the adhesion of LABs to washed Caco-2 cells by CFU. Although it might be lower the threshold 5.0×10^3 CFU/mL than reference data, I considered that this value was appropriate in this study. The 10 strains from the screens were analyzed by carbohydrate fermentation and the results compared to identification by 16S rDNA and carbohydrate utilization profiles. All LAB species were confirmed, except for strain 06TCa39. This strain was identified as *L. paracasei* ssp. *tolerans* by 16S rDNA

sequencing analysis, but was classified as *L. paracasei* ssp. *paracasei* by carbohydrate utilization profile (Table 2-6). Thus, the identification of strain 06TCa39 requires more detailed analysis.

Table 2-4 Screening of all LABs isolated from Mongolian dairy products for probiotic properties

LAB species	Detected number	Viability in bile	Tolerance in low pH	Gas procuct(-)	Adheasion on Caco-2
Lactobacillus delbrueckii ssp. *bulgaricus*	**155**	**8**	**2**	**2**	**0**
Lactobacillus helveticus	**115**	**0**			
Lactobacillus fermentum	**78**	**50**	**50**	**0**	
Streptococcus thermophilus	**43**	**5**	**0**		
Enterococcus durans	**17**	**0**			
Weissella confusa	**16**	**16**	**16**	**0**	
Lactococcus lactis ssp. *lactis*	**20**	**2**	**2**	**2**	**0**
Lactobacillus buchneri	**7**	**1**	**1**	**0**	
Lactobacillus kefiri	**10**	**0**			
Lactobacillus plantarum	**11**	**11**	**11**	**11**	**5**
Lactobacillus pentosus	**13**	**9**	**9**	**9**	**0**
Lactobacillus delbrueckii ssp. *lactis*	**21**	**2**	**2**	**2**	**1**
Pediococcus parvulus	**5**	**5**	**5**	**5**	**0**
Enterococcus faecium	**5**	**0**			
Weissella viridescens	**6**	**6**	**6**	**0**	
Lactobacillus paracasei ssp. *torelans*	**6**	**5**	**5**	**5**	**2**
Lactobacillus paracasei ssp. *paracasei*	**4**	**4**	**4**	**4**	**2**
Lactobacillus sakei	**2**	**2**	**2**	**2**	**0**
Leuconostoc mesenteroides	**2**	**0**			
Leuconostoc citreum	**6**	**0**			
Leuconostoc garlicum	**1**	**0**			
Total	**543**	**125**	**115**	**42**	**10**

All LAB isolates were classified in each species and subjected to each tests. The number shows LAB strains which was confirmed for positive in each tests.

Table 2-5 Profile of LABs screened for probiotics and utilizing as yoghurt production

No	Species	Bile acid tolerance (%)†	Viability in Low pH (Log CFU/mL)‡	Adheasion on Caco-2 (x10^{5}CFU/mL)¶	Products	source	Prefecture	Area
05AM23	*L. plantarum*	97.0	8.1	72.0	Airag	Mare	Tuv	Ulaanbaatar
06TCa8	*L. plantarum*	88.9	8.2	6.7	Tarag	Camel	Dornogobi	Sainshand
06TCa19	*L. paracasei* ssp. *paracasei*	87.9	8.0	10.6	Tarag	Camel	Dornogobi	Sainshand
06TCa22	*L. paracasei* ssp. *paracasei*	92.6	8.0	11.5	Tarag	Camel	Dornogobi	Sainshand
06TCa39	*L. paracasei* ssp. *tolerance*	87.4	7.3	17.3	Tarag	Camel	Dornogobi	Sainshand
06TCa40	*L. plantarum*	85.4	7.7	12.5	Tarag	Camel	Dornogobi	Sainshand
06TCa43	*L. paracasei* ssp. *paracasei*	85.6	7.0	15.4	Tarag	Camel	Dornogobi	Sainshand
06CC2	*L. plantarum*	97.0	8.7	12.5	Aaruul	Cow	Tuv	Altanbulag
06TC3	*L. delbrueckii* ssp. *lactis*	83.0	8.5	25.9	Tarag	Cow	Tuv	Altanbulag
06CC9	*L. plantarum*	92.6	8.0	7.7	Aaruul	Cow	Tuv	Altanbulag

† : The percent of viable colonies in GYP broth including 0.2 % oxygall compared to the control. ‡ : The viable colonies of LAB after a treatment in 0.04 % pepcin at pH 3.0 for 3 h. ¶ : The viable colonies LAB adhered on Caco-2 cells after incubation.

Table 2-6 Profiles of carbohydrate utilization on LAB strains selected for probiotics

No	05AM23	06TCa8	06TCa19	06TCa22	06TCa39	06TCa40	06TCa43	06CC2	06TC3	06CC9
Species	1	1	2	2	2	1	2	1	3	1
D arabinose	−	−	−	−	−	−	−	−	−	−
L arabinose	−	−	−	−	−	−	−	−	−	−
D ribose	+	+	+	+	-	+	+	+	-	+
D xylose	−	−	−	−	−	−	−	−	−	−
L xylose	−	−	−	−	−	−	−	−	−	−
D galactose	+	+	+	+	+	+	+	+	−	+
D glucose	+	+	+	+	+	+	+	+	+	+
D fructose	+	+	+	+	+	+	+	+	+	+
D mannose	+	+	+	+	+	+	+	+	+	+
L rhamnose	−	−	−	−	−	−	−	−	−	−
D mannitol	+	+	+	+	−	+	+	+	−	+
D sorbitol	+	+	+	+	−	+	+	+	−	+
Amygdalin	+	+	+	+	−	+	+	+	−	+
Esculin	+	+	+	+	+	+	+	+	−	+
Salicin	+	+	+	+	+	+	+	+	−	+
D celibiose	+	+	+	+	−	+	+	+	−	+
D maltose	+	+	+	+	−	+	+	+	−	+
D lactose	+	+	+	+	+	+	+	+	+	+
D melibiose	+	+	−	−	−	+	−	+	−	+
D sucrose	+	+	+	+	−	+	+	+	−	+
D trehalose	+	+	+	+	+	+	+	+	+	+
D melezitose	+	+	+	+	+	−	+	−	−	+
D raffinose	−	−	−	−	−	+	−	+	−	+
Gluconate	+	+	+	+	+	+	+	+	−	+
Starch	−	−	−	−	−	−	−	−	−	−

1: *L . plantarum*, 2: *L. paracasei* ssp. *paracasei*, 3: *L. delbrueckii* ssp. *lactis*, +: Positive and -: Negative

2.3.3 Differentiation of lactic acid bacteria strains selected as probiotics

In this study, RAPD-PCR analysis were conducted to determine homology between the LAB strains originating from the same products, province and sampling area, specifically between *L. plantarum* 06TCa8 - *L. plantarum* 06TCa40, *L. plantarum* 06CC2 - *L. plantarum* 06CC9, and *L. paracasei* ssp. *paracasei* 06TCa19 - *L. paracasei* ssp. *paracasei* 06TCa22 - *L. paracasei* ssp. *paracasei* 06TCa43. Based on genotypes determined by this method, strains 06TCa8 - 06TCa40, 06CC2 - 06CC9, and 06TCa19 - 06TCa22 - 06TCa43 might represent individual isolates, respectively (Fig. 2-2).

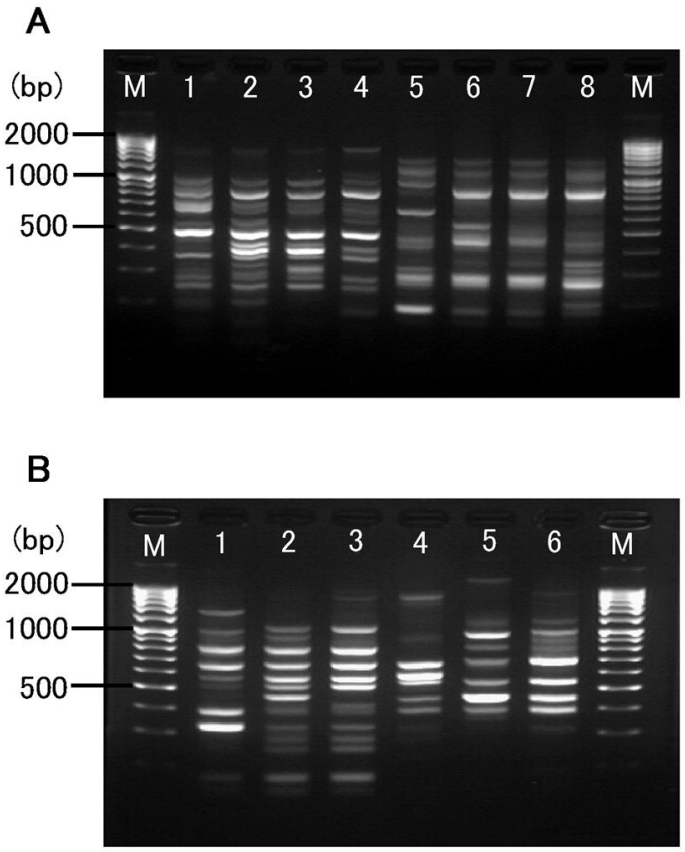

Figure 2-2 RAPD-PCR profiles obtained from 7 LAB isolates.

A. Lane 1, 2, 5 and 6 were used primer p7. Lane 3, 4, 7 and 8 were used primer p11. Lane M, DNA size marker; lane 1 and 3, 06TCa8; lane 2 and 4, 06TCa40; lane 5 and 7, 06CC2; lane 6 and 8; 06CC9, respectively. 06TCa8 and 06TCa40 were isolated from a fermented camel's milk in Sainshand area, Dornogobi province. 06CC2 and 06CC9 were isolated from a fermented cow's milk in Altanbulag area, Tuv province. All of these isolates were *L. plantarum*.

B. Lane 1, 2, and 3 were used primer AT41. Lane 4, 5 and 6 were used primer BT05. Lane M, DNA size marker; lane 1 and 4, 06TCa19; lane 2 and 5, 06TCa22; lane 3 and 6, 06TCa43, respectively.

06TCa19, 06TCa22 and 06TCa43 were isolated from a fermented camel's milk in Sainshand area, Dornogobi prefecture. All of these isolates were *L. paracasei* ssp. *paracasei*.

2.4 Discussion

Because the culture and production of Mongolian dairy products is distinct, they might involve potentially unique microorganisms. However, to the best of my knowledge, few studies have been performed on the diversity of the microorganisms in Mongolian dairy products. Therefore, in this study, LABs from traditional Mongolian dairy products were isolated and investigated their diversity. Moreover, the probiotic potential of the isolated LAB was estimated. This study surveyed tarag, which is similar to yoghurt, airag which is alcoholic fermented horse milk; and aaruul, eezgii, and byasulag which are similar to cheese. The predominant species of LABs in tarag were *L. delbrueckii* ssp. *bulgaricus*, *L. helveticus*, *L. fermentum*, and *S. thermophilus*. The major LAB species in yoghurt are usually *L. delbrueckii* ssp. *bulgaricus*, and *S. thermophilus*. Ishii (2001) reported that Tarag processing is similar to yoghurt processing, therefore I consider that tarag and yoghurt were the same type of dairy product, in addition they had similar LAB species content.

The predominant species of LAB in airag were *L. helveticus*, *L. delbrueckii* ssp. *lactis*, *L. fermentum* and *Weissella* (*W.*) *viridescens*, which differs slightly from reports from Uchida *et al*. (2007) and Watanabe *et al*. (2008). Using molecular biological methods, Watanabe *et al*. (2008) identified mainly *L. kefiranofaciens* from airag, whereas this specie was not detected from the airag used in the current study. Although *L. delbrueckii* ssp. *lactis*, *L. fermentum* and *W. viridescens* were detected from airag in this study, these species were not detected in a previous report of Watanabe *et al*. (2008). Therefore, further investigations of LAB in Mongolian tarag and airag are needed to isolate additional LAB species.

The predominant species of LAB in aaruul were *L. fermentum*, *L. helveticus*, *L. buchneri* and *W. confusa* and the predominant species of LAB in byasulag were *L. delbrueckii* ssp. *lactis* and *Lc. lactis* ssp. *lactis*. In spite of that there were no LAB strains isolated from eezgii.

Although the cheese-like aruul, eezgii and byasulag are common foods for Mongolian nomadic people, fewer reports have described microbiota from these products than from tarag and airag. I found that homo-fermentative LABs including *L. helveticus* and *L. delbrueckii* ssp. *lactis* and hetero-fermentative LABs including *L. fermentum*, *L. buchneri* and *W. confusa* coexisted in aaruul. However, the hetero-fermentative LABs in aaruul might be from an environmental source, similar to the isolation of *L. buchneri* and *Weisella* species from silage (Cai *et al*. 1998; Holzer *et al*. 2003). According to process of the production of aaruul, it is made by boiling tarag for approximately an hour, followed by sun-drying. Therefore it is suggested that these processes might kill the LAB from tarag. This supports the hypothesis that almost all LABs in aaruul were environmental origin. Estimate of bacterial diversity in byaslug were difficult, because of lack of enough samples for an investigation. Therefore, further investigation of LAB diversity in byaslug is required. Eezgii is made by a complete evaporation of highly fermented milk, followed by crushing and sun-drying. Since I did not detect LAB strains in eezgii, I propose that evaporation and sun-dry processes had an impact on the moisture content of eezgii, which reduces the total survival strains of LAB. As a result, 10 homo-fermented probiotic LAB strains were isolated, classified as *L. plantarum* or *L. paracasei* ssp. Further study is needed to estimate whether these strains are beneficial in producing yoghurt. While, a lot of *L. fermentum* strains that tolerated bile salts and gastric acids were isolated from samples in this study. *L. fermentum* strains which much strongly tolerated to gastric acid and bile salts were also found in traditional Kenya fermented milk (Mathara *et al*. 2008). Many *L. fermentum* strains that tolerated bile salts and gastric acids were isolated. They were hetero-fermentive LAB, and generally this LAB is not suitable for yoghurt production. Thus, *L. fermentum* strains were not subjected to further screening process such as the test of adhesion to Caco-2 cells. But, the functional characteristics of hetero-fermenting strains such as *L. fermentum* should be investigated further.

In this study, it was found that 6 of 10 probiotic LAB strains were isolated from camel milk tarag from the Dornogobi province. Of 54 LABs from tarag made with camel milk, 11 strains were identified as *L. plantarum* or *L. paracasei* ssp. Although these LAB species might be indigenous in Dornogobi province, they appeared to be more frequent in tarag made with camel milk than that with other animal milks. Khedid *et al*. (2009) characterized LAB isolated from one-humped camel milk from Morocco and found that one of the most frequently isolated LAB was *L. casei* ssp. *casei* in the same cluster as both *L. paracasei* ssp. and *L. plantarum* by 16S rRNA (Collins *et al.* 1991). Therefore, *L. paracasei* ssp. and *L. plantarum* could appear to be more frequently isolated strains from camel milk tarag than other milk. Other probiotic LABs have been isolated from foods, and functionally characterized (Gotcheva *et al*. 2002; Arana *et al*. 2005; Ortu *et al*. 2007; Mathara *et al*. 2008; Lim *et al*. 2009). Most probiotic homo-fermentative LABs from fermented dairy products are classified as *L. plantarum* or *L. paracasei* ssp., this is consistent with the present results. Tarag from camel milk might be superior to tarag from other milk for detecting probiotic homo-fermented LABs. Several reports found that *L. paracasei* and *L. plantarum* as probiotic LAB. Nishida *et al.* (2008) reported that *L. paracasei* ssp. *paracasei* KW 3110 has probiotic potential, and that KW 3110 tolerates 2.0 % bile salts and pH 3.0 gastric acid. Feeding and monitoring tests in humans showed that KW 3110 had the potential to survive in and colonize the human gut, and improve the human intestinal microflora. Vries *et al*. (2005) also showed that *L. plantarum* proved to have the ability as probiotics for human. Further study is required to confirm the survival in, and colonization of the human intestine of the strains isolated in this study, in particular *L. plantarum* 05AM23, which may be a desirable probiotic strain.

The importance of probiotic LABs to the host has been demonstrated by many reports that focus on probiotic effects on human health and disease (Isolauri *et al*. 2000; Xiao *et al*. 2003; Rosenfeldt *et al*. 2003, 2004; Kato *et al*. 2004; Ishida *et al*. 2005). Therefore, studying the

function of LAB from traditional Mongolian fermented dairy products, and determining the quality of yoghurt prepared with 10 probiotic LAB strains described here, are worthy of further analysis.

2.5 Conclusion

In this study, I collected total 66 samples classified into 5 kinds of the traditional Mongolian dairy products, from which 543 LAB strains were isolated and identified. In the identified LABs, it was observed that the species not only frequently described in various dairy products but also the species from the environment such as often isolated in the silage. Furthermore, all LAB isolates were screened for tolerance to low pH and to bile acid, gas production from glucose, and adherence to Caco-2 cells. *In vitro*, it was found that 10 strains possessed probiotic properties, and were almost identified as *L. plantarum* or *L. paracasei* subspecies, based on 16S ribosomal DNA and carbohydrate fermentation pattern. These strains were differentiated from each other individually. Further study is needed to investigate about the effects of those LAB strains on human health and disease.