Probiotic Properties of Lactic Acid Bacteria Isolated from Mongolian Fermented Mare's Milk*

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Fermented products are an integral part of Mongolian heritage that have developed over a long period of time. They have great social, religious, cultural, economic, and medicinal importance. Among them, there is a wide variety of fermented milk products in Mongolia because of variations in raw materials, and processing methods arising from the different habits and customs of the various regions of the country. The most common fermented milk product of Mongolia is *airag* (*kumiss*), traditionally made from mare's milk. Another kind of fermented milk is *tarag* (yoghurt), which is prepared from cow, goat, and sheep milk. A third indigenous dairy product is *khoormog* (kefir), prepared from camel's milk. Fermented products have probiotic effects as they contain live microorganisms. In particular, lactic acid bacteria (LAB) play a vital role in the fermentations of Mongolian traditional dairyproducts.

Fermented mare's milk (*airag*) prepared with traditional Mongolian technology has played an extremely important role in the Mongolians' diet since ancient times. *Airag* generally contains about 2% alcohol, 0.5-1.5% lactic acid, and 2-4% lactose. It is usually made from mare's milk fermented by its normal microbiota, but the production process is not completely understood.[1] The microflora of the starter cultures of these products is complex. The microbial population mainly consists *of Lactobacillus delbrueckii,* subsp *bulgaricus, Lactococcus lactis, Lactobacillus casei, Kluyveromyces fragilis,* and *Saccharomyces lactis.*

Recently there has been a growing interest in the use of fermented milks in human nutrition and in the treatment of certain human diseases such as hepatitis, chronic ulcer disorder, and tuberculosis. The nutritional and treatment values of fermented milk products depend considerably on the compositions of their microorganisms – those used in starter cultures.

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1 Baldorj and Namsrai 1980,7.

The main purpose of the present study is to determine the probiotic properties of lactic acid bacteria (LAB) isolated from fermented mare's milk. Lactic acid bacteria have been extensively studied for their commercial potentials, in food preservation, and for their health benefits. These are industrially important microorganisms, ones used world-wide, mainly in the dairy industry for manufacturing fermented milk products and cheeses. Lactic acid bacteria and their fermented products are thought to have health-promoting, probiotic effects in humans including inhibition of pathogenic organism, antimutagenic effects, and the reduction of blood cholesterol. Probiotics are defined as "live microorganisms which when administered in abequate amounts confer a health benefit on host" (the Food and Agriculture Organization/ World Health Organization (FAO/WHO). LAB produce different antibacterial substances including organic acids (lactic acid and acetic acid), hydrogen peroxide, and bacteriocins. These substances are used as bioconservants in food preservation, and improve the testing and quality of dairy products. Bacteriocins are ribosomallysynthesized substances of proteinaceous nature produced during the growth of lactic acid bacteria. These bacteriocins protect host organisms through killing or inhibiting the growth of other bacteria. Many bacteriocinogenic lactic acid bacteria have been found in numerous fermented dairy products. Over the past few years, studies focusing on bacteriocins produced by LAB have been the objects of a growing emphasis and many new bacteriocins have been discovered recently. It has been revealed that bacteriocins have not only been used as biopreservatives but also as medicine to prevent different diseases in place of antibiotics. Sometimes bioactive peptides can be released through proteolysis by lactic acid bacterial enzymes. Many industrially-utilized dairy starter cultures are proteolytic to some extent or the other. Bioactive peptides can thus be generated by the proteolytic activities of the strains of starter and non-starter bacteria. The single most effective way to increase the concentration of bioactive peptides in fermented dairy products is to ferment or co-ferment with highly proteolytic strains of LAB. The choice of strains influences the release of effective bioactive peptides.

Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium*. Lactic acid bacteria for use as a probiotic culture must be tolerant to gastric acid and bile which enables selected strains to survive, grow, and have therapeutic benefits in the intestinal tract.[2]

2 Tajabadi-Ebrahimi *et al.* 2011, 25; Bassyouni *et al.* 2012, 2929.

Antioxidants can serve as preventative agents for different types of disease including cancer, atherosclerosis, and diabetes. Therefore consumption of natural antioxidants through food is helpful for human health. The fermentation of milk by lactic acid bacteria releases a large number of peptides and amino acids with biological actions which include angiotensin-converting enzyme inhibitory, immune modulatory, opioid, and antioxidant activities.[3] There are several reports on LAB strains in the traditional fermented milks of Mongolia.[4] However, papers on the probiotic properties of LAB strains isolated from Mongolian traditional fermented milks still remain scarce. There are only a few reports focused on probiotic properties of LAB isolated from the Mongolian fermented milk *airag*.[5] Hence, there is a real need to study the biological activity of lactic acid bacteria isolated from fermented milk and to develop technology for making fermented probiotic products.

Previously we have isolated bacteriocin-producing *Enterococcus durans* from Mongolian *airag*, purified bacteriocins and characterized them.[6] The resent study is a part of continuing effort to explore the potentials of our indigenous microbial flora in developing fermented milk products with probiotic effect.[7] Recently, there has been no report which has focused on the antioxidant activity of probiotic lactic acid bacteria isolated from Mongolian fermented milk. Therefore, we plan to determine the antioxidant activity of LAB strains isolated from *airag*.

In this study, 42 strains of lactic acid bacteria were isolated from Mongolian *airag*. All isolates were identified using morphological, biochemical, and physiological methods. The isolated bacteria were studied for their antagonistic effects on *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* The results showed that 22 strains had antibacterial activity. When we examined their probiotic properties such as bile acid tolerance and gastric acid tolerance, it was shown that only 6 bacterial strains could survive up to 3 hours in a pH 3.0 acid environment, and up to 8 hours in a 0.3% bile acid environment. Selected probiotic strains were further identified as species with the API 50CHL system. Antioxidant activity of probiotic strains were determined by a 1,1-diphenyl-2 picrylhydrazyl (DPPH) as-

3 Pihlanto *et al.* 2006, 1312; Virtanen *et al.* 2007, 107.
4 Yu *et al.* 2011, 3229; Watanabe *et al.* 2008, 1313; Sun *et al.* 2010, 270.
5 Watanabe *et al.* 2009, 1313; Takeda *et al.* 2011, 571.
6 Batjargal *et al.* 2007, 837.
7 Budragchaa *et al.* 2009, 104; Munkhtsetseg *et al.* 2009, 61.

say. While the antioxidant activity in cell-free supernatant fluctuated between in the range 26.1-38.4%, the antioxidant activity after 72 hours of fermentation in the whey fraction was between 17.23-55.12%.

This study is part of a continuing effort to explore the potentials of our indigenous microbial flora in developing fermented milk products with probiotic effect. We isolated and characterized the bioactive-compound-producing lactic acid bacteria from Mongolian traditional fermented dairy products.

Materials and Methods

Sample Collection

A total of 15 samples of *airag* were collected from nomadic families of Bulgan, Uvurkhangai, and Dundgovi provinces. The samples (250 mL) were collected in sterilized bottles and kept under low temperature using an icebox brought to the laboratory where a procedure for isolation was applied.

Isolation of Lactic Acid Bacteria

All samples (5%, v/v) were propagated twice in sterilized skim milk at 37° C for 16–18 hours under anaerobic conditions. Samples were serially diluted (10-fold) in peptone water from 1:10 to $1:10^8$ (v/v), and 1 mL *aliquot* of the dilution was put into selective medium MRS (Man, Rogosa and Sharpe, Germany) agar. The plates were incubated at 37° C for 24 hours under anaerobic conditions. After incubation, individual colonies were selected and transferred into sterile broth media. The selected colonies were purified with the streak plate technique and stored at -20° C in MRS broth with 20% glycerol, and kept for further investigation.[8]

Probiotic Properties of Isolated LAB Strains

Major selection criteria (antibacterial activity, resistance to low pH and tolerance against bile acid) were chosen for the determination of the probiotic properties of isolated LAB strains.

8 Bassyouni *et al.* 2012,2925.

Antibacterial Activity Assay

The antibacterial activity of cell-free supernatant was determined by the well-diffusion method as described by Batjargal *et al.* (2007).

Resistance to Low pH

Resistance to pH 3.0 is often used *in vitro* assays to determine resistance to stomach pH. The LAB strains with antibacterial activity (incubated for 16-18 hours) were used. Cells were harvested by centrifugation for 10 minutes at 5000 rpm and 4° C. Cell pellets were suspended in phosphate saline buffer (pH 3.0) and incubated at 37° C. Viable microorganisms were enumerated at the 3 hour mark with pour plate techniques.[9]

Tolerance against Bile Acid

MRS medium containing 0.3% bile acid was inoculated with the LAB strains with antibacterial activity and incubated for 16-18 hours. For 8 hours during the incubation, viable colonies were enumerated every hour with pour plate technique and also growth was monitored by absorbance at 560 nm.[10]

Determination of Antioxidant Activity

The DPPH radical scavenging activity was evaluated using the method of Son and Lewis.[11] The antioxidant activity of the samples was expressed as a percentage of DPPH inhibition activity, calculated as:

$$\text{DPPH inhibition activity (\%)} = [(A_{control}-A_{test})/A_{control}]\times 100;$$

where $A_{control}$ is the absorbance of the control sample (DPPH solution without whey fraction) and A_{test} is the absorbance of test sample (DPPH solution plus whey fraction).

Preparation of Cell Free Supernatant

Sterile MRS broth was inoculated with 1% (v/v) of the overnight grown culture of the six LAB isolates and incubated at 37° C for 18 hours. The cell free supernatant was obtained by centrifugation of the overnight grown culture at 10 000 rpm for 5 minutes at 4° C.[12]

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9 Pihlanto *et al.* 2006, 1307.
10 Ibid.
11 Son and Lewis 2013, 469.
12 Uchida *et al.* 2007, 652.

Identification of Isolated LAB Strains

Carbohydrate fermentation was determined using the API CH system (Bio Merieux, France), according to the manufacturer's instructions.

Results and Discussion

Isolation of Antibacterial LAB Strains

We have isolated 42 LAB strains from 15 samples of *airag,* and determined their morphological physiological and biochemical characteristics. All isolated strains were Gram positive and catalase negative, long or short-chained rods, and coccus. We tested their antibacterial activity by using test microbial strains such as *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* In all twenty-two LAB strains have antibacterial activity but only eight of them showed significant growth inhibition against indicator strains (Table 1). We have chosen the following LAB strains A-4, A-7, BL-12, BL-13, DU-8, O-9, T-8, and BM8-5 for further investigation.

Table 1: Antibacterial Activity of LAB Strains Isolated from Airag

LAB strains	**Antibacterial activity (Inhibition zone, mm)**									
	Cell-free supernatant					Neutralized cell-free supernatant				
	pH	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	pH	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Lactobacillus paracasei A-4	4.5	6	4	2	3	6.5	5	4	–	3
Lactobacillus plantarum A-7	4.0	6	6	4	5	6.5	6	5	4	5
Lactobacillus paracasei BL-12	5.0	6	4	3	3	6.5	5	2	3	1
Lactobacillus plantarum BL-13	4.0	4	3	6	3	6.5	4	2	5	2
Lactobacillus paracasei DU-8	5.0	4	4	3	5	6.5	4	3	2	3
Lactobacillus brevis O-9	5.0	1	4	2	4	6.5	1	3	2	3
Lactococcus lactis T-8	5.0	6	3	4	4	6.5	5	3	3	3
Lactobacillus lactis BM8-5	4.5	3	1	3	3	6.5	3	–	2	2

Note: The tests were applied twice and the averages of diameters of clear zones are given.

Identification of LAB Strains

The selected LAB strains were identified with the API 50 CHL Carbohydrate Test Kit (Biomerieux Co., France). The tests were done according to

the manufacturer's instructions and the results were interpreted after incubation at 37^0C for 48 hours. Identification of the LAB strains was done by the interpretation of the fermentation profiles using the computerized database program API WEB software.

Table 2: Identification of the LAB Strains Isolated from Airag

Isolate	**API kit (50 CHL)**	**Identification (%)**
A-4	*Lactobacillus paracasei*	94%
A-7	*Lactobacillus plantarum*	97.4%
BL-12	*Lactobacillus paracasei*	99.2%
BL-13	*Lactobacillus plantarum*	91.0%
DU-8	*Lactobacillus paracasei*	94.6%
O-9	*Lactobacillus brevis*	97.6%
T-8	*Lactococcus lactis*	95.9%
BM8-5	*Not determined*	

As for classification, our study results matched different studies that have isolated the probiotic lactic acid bacteria from the fermented milk which primarily consisted of *Lactobacillus plantarum* and *Lactobacillus paracasei*.[13] Takeda *et al.* (2011) reported that 10 homofermented probiotic LAB strains were isolated from Mongolian fermented camel milk, and classified as *L. plantarum* and *L. paracasei.* 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. paracasei* and *L. plantarum* by 16S rRNA.

Determination of Probiotic Properties

Resistance to low pH is one of the major selectors for probiotic strains. To reach the small intestine, LAB strains have to pass through the stomach.[14] Selection of strain resistance to low pH 3.0 was used. The time that it takes for digestion in the stomach is around 3 hours, so LAB strains were tested for resistance to pH 3.0 during a 3 hour period. The following are eight LAB strains we identified: acid-tolerant *Lactobacillus paracasei* A-4, *Lactobacillus plantarum* A-7, *Lactobacillus paracasei* BL-12, *Lactobacillus paracasei* DU-8, *Lactobacillus brevis* O-9, and *Lactococcus lactis* T-8. All strains were very stable in pH 3.0 which means that these LAB strains are able to survive this pH value. The strains *Lactobacillus plantarum* BL-13 and *Lactobacillus lactis* BM8-5 were considered acid sensitive and were excluded from further studies.

13 Tajabadi-Ebrahimi *et al.* 2011, 24; Bassyouni *et al.* 2012, 2925; Munkhtsetseg *et al.* 2009, 66f.

14 Tajabadi-Ebrahimi *et al.* 2011, 23; Uchida *et al.* 2007, 656.

Table 3: Acid Tolerance of the LAB Strains Isolated from Airag

LAB strains	Incubation time, hour			
	0	1	2	3
	Log_{10}CFU/ml			
Lactobacillus paracasei, A-4	3.56	3.33	3.43	3.45
Lactobacillus plantarum, A-7	3.60	3.50	3.45	3.38
Lactobacillus paracasei, BL-12	3.70	3.53	3.40	3.60
Lactobacillus paracasei, DU-8	3.61	3.55	3.58	3.65
Lactobacillus brevis, O-9	3.76	3.65	3.64	3.68
Lactococcus lactis, T-8	3.31	3.40	3.43	3.48

The pH resistance quality of our LAB strains yielded slightly lower results than reported by other researchers. Tajabadi-Ebrahimi *et al.* (2011) reported that LAB strains isolated from Iranian traditional fermented milk had an acid tolerance extending from 3.6 to 6.4 Log_{10}CFU/ml. Takeda S. *et al.* (2011) also reported that LAB strains isolated from fermented camel milk had an acid tolerance of between 7.0 and 8.7 Log_{10}CFU/ml. This may be due to the origins of the fermented milks that were used to isolate the strains, the different techniques and cultures used for isolation process, and the geographic locations where the fermented milk was collected. Bile acid tolerance is essential for probiotic strains to colonize the small intestine. A set of six selected LAB strains were tested for their ability to tolerate bile acid. They all could tolerate a bile salt 0.3% concentration during 8 hours.

Table 4: Bile Acid Tolerance of the LAB Strains Isolated from Airag

LAB strains	Medium	Incubation time, hour								
		0	1	2	3	4	5	6	7	8
		Optic density, 560 nm								
Lactobacillus paracasei A-4	MRS	0.06	0.08	0.12	0.37	0.92	1.68	2.25	2.70	2.80
	MRSO-0.3%	0.07	0.05	0.07	0.15	0.18	0.40	0.90	2.00	2.40
Lactobacillus plantarum A-7	MRS	0.06	0.10	0.22	0.78	1.80	2.70	3.00	3.35	3.60
	MRSO-0.3%	0.07	0.05	0.07	0.15	0.39	0.95	2.20	2.90	3.60
Lactobacillus paracasei BL-12	MRS	0.06	0.09	0.17	0.66	1.64	2.70	3.00	3.45	3.60
	MRSO-0.3%	0.08	0.10	0.28	0.75	1.40	2.40	2.80	3.00	3.60
Lactobacillus paracasei DU-8	MRS	0.06	0.09	0.16	0.62	1.62	2.75	3.00	3.45	3.60
	MRSO-0.3%	0.06	0.05	0.11	0.29	0.75	1.40	2.60	3.00	3.00
Lactobacillus brevis ,O-9	MRS	0.06	0.08	0.14	0.54	1.40	2.25	2.85	3.30	3.60
	MRSO-0.3%	0.07	0.06	0.08	0.18	0.38	0.88	1.50	2.80	3.60
Lactococcus lactis, T-8	MRS	0.06	0.06	0.08	0.22	0.58	1.38	2.25	2.85	3.00
	MRSO-0.3%	0.06	0.05	0.06	0.07	0.14	0.30	0.80	2.00	2.90

There was substantial variability in resistance to bile acid among the selected LAB strains and all strains tested showed delayed growth, compared to unsupplemented MRS. After the adaptation time (4-6 hours), they all showed extensive growth. Similar results were reported in other studies on several species of *Lactobacillus*.[15]

Determination of Antioxidant Activity

Lactic acid bacteria are known to have proteolytic activity that hydrolyses protein to produce peptides with bioactivity.[16] The antioxidative potential of LAB has been reported in several other studies.[17]

Table 5: Antioxidant Activity of Probiotic LAB Strains Isolated from Airag

LAB strains	Antioxidant activity in cell-free supernatant, inhibition %	Antioxidant activity in whey fraction, inhibition % Fermentation time, hour		
Lactobacillus paracasei DU-8		24	48	72
Lactobacillus paracasei A-4	35.80	20.89	17.08	45.16
Lactobacillus plantarum A-7	38.40	18.40	21.57	55.12
Lactobacillus paracasei BL-12	26.10	3.98	7.34	19.63
Lactobacillus paracasei DU-8	30.50	10.50	4.02	17.23
Lactobacillus brevis O-9	N/d	27.79	11.52	54.77
Lactococcus lactis T-8	30.20	11.93	12.65	15.87
Control milk	10.60	10.60	10.4	10.20
Ascorbic acid	99.50	99.50	99.05	99.50

Firstly, we determined a probiotic LAB strain's antioxidant activity in a cell free supernatant as described previously. The selected LAB strains demonstrated the DPPH scavenging activity with inhibition rate in the range 26.1-38.4%. Similar results were reported by Osuntoki *et al.* (2010) that the LAB isolated from African fermented foods scavenged between 6.3 and 33.7% DPPH inhibition activity. The *Lactobacillus brevis* O-9 did not show any activity. The LAB strains *Lactobacillus plantarum* A-7 and *Lactobacillus paracasei* A-4 showed higher inhibition rates in the range of 35.8-38.4%. Secondly we have determined our 6 probiotic LAB strains's antioxidant activity during fermentation between 24-72 hours in whey fraction. All LAB strains kept their antioxidant activity in whey fraction and the antioxidant activity in-

15 Bassyouni *et al.* 2012, 2929; Takeda *et al.* 2011, 571.

16 Pihlanto *et al.* 2006, 1306; Virtanen *et al.* 2007, 106.

17 Afify *et al* 2012, 137; Kullisaar *et al.* 2003, 449; Kaizu *et al.* 1993, 2493]; Son and Lewis 2002, 470.

creased in most cases during fermentation as shown in Table 4. The measured DPPH inhibition activity of LAB strains in whey fraction was between 3.98-55.12%. A similar result was reported by Abubakr *et al.* (2012) that the DPPH inhibition activities varied with LAB strain, ranging from 14.7 to 50.8% for 24-72 hours fermentation. The strains *Lactobacillus paracasei* A-4, *Lactobacillus plantarum* A-7 and *Lactobacillus brevis* O-9 showed the highest antioxidant activity in the range 45.16-55.12% after 72 hours fermentation. Milk fermented with *L. brevis* showed DPPH inhibition values of 54.77%, respectively greater than *L. brevis* (33.7±7.8%) and that reported by Osuntoki and Korie (2010). Also Abubakr *et al.* (2012) reported that *L. plantarum* isolated from fruit samples showed the highest DPPH inhibition activity ranging from 50.8±4.5%. DPPH inhibition activity in whey fraction increased with fermentation time. The same results have also been reported by other researchers. Virtanen *et al.* (2007) and Abubakr *et al.* (2012) reported the DPPH values of milk protein hydrolizate from LAB fermented skim milk increased with the fermentation time.

This is the first report of the isolation of lactic acid bacteria with antioxidant activity from Mongolian *airag*. We assume that probiotic lactic acid bacteria *Lactobacillus paracasei* A-4, *Lactobacillus plantarum* A-7, and *Lactobacillus brevis* O-9 can be used for the preparation of probiotic products with antioxidant activity.

Conclusion

In this study, we have isolated six probiotic LAB strains with anti-oxidant activity, capable of surviving the pH of the stomach and the environment of the intestine, making them potential probiotics. The measured anti-oxidant activities differed according to the strain and inhibition in their cell free supernatants varied from 26.1-38.4%. The measured DPPH inhibition activities of LAB strains varied with strains and antioxidant activity ranging from 17.23-55.12% for 72 hour fermentation.

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