**Doctoral Thesis**

# Study on Isolation and Identification of Lactic Acid Bacteria with High Biological Activities in Mongolian Traditional Fermented Beverage, Airag

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This is to certify that we have read this thesisand that in our opinion it is fully adequate, as a thesis for the degree of Doctor of Philosophy.

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## ABSTRACT

Thirty five predominant lactic acid bacteria were isolated from six samples of traditional Mongolian Airag (fermented mare's milk) collected in four provinces near Ulaanbaatar, and the bacteria were classified into ten kinds of lactic acid bacteria by 16s rDNA sequencing after incubation with cycloheximide as an antibiotic. Two kinds of yeast were also isolated from the Airag samples after incubation with an antibiotic, chloramphenicol, and then identified by 26s rDNA sequencing. Antibacterial and proteolytic activities of the supernatant produced by the lactic acid bacteria were examined using *Escherichia coli* (ATCC 25922) or *Bacillus subtilis* as the pathogenic bacterium on agar plates containing 2.5% skim milk at 37°C. The antibacterial activities of two supernatants produced by *Lactobacillus hilgardii* (Uvu-21) and *L. diolivorans* (Tuv-33), which to the best of our knowledge, were first discovered in Airag, were found to have a high thermal range and a wide range of pH stabilities. After treatment of the supernatants with proteases, the antibacterial and proteolytic activities disappeared, suggesting that the biological activity was originated by peptides produced by lactic acid bacteria. In addition, the yeasts isolated were fermented with D-glucose, and comparison of their ability to ferment with that of a standard yeast, the K7 yeast used in sake brewing, indicated that the fermentation ability is weaker than that of K7 yeast.

Finally, following a three step purification protocol provided probably two antibacterial peptides that has been analyzed molecular mass about 1905 and 2350 Da by Tricine SDS PAGE analysis and MALDI TOF MS mass spectrometer. The results suggesting that the antibacterial activity is attributable to the peptides produced by the lactic acid bacteria. The isolation and structure of the peptides are under investigation. Therefore we was intended isolation and purification of antibacterial peptides.

These results provide useful information about Airag as a healthy daily beverage in Mongolia.



**Keyword:** Airag, Lactic acid bacteria, Antibacterial activity, Proteolytic activity, Supernatant, Yeast

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# ABBREVIATIONS

| | |
|---|---|
| µm | micrometer |
| ABP | Antibacterial Peptide |
| AU | activity (Arbitrary Unit) |
| BSA | Bovine Serum Albumin |
| CFS | Cell Free Supernatant |
| Da | Daltons |
| DNA | Deoxyribonucleic acid |
| g | Gram |
| GFC | Gel Filtration Chromatography |
| HPLC | High pressure liquid chromatography |
| ICR | Imprinting Control Region |
| kcal | Kilocalorie |
| kDa | Kilodalton |
| LAB | Lactic Acid Bacteria |
| LDL | Low Density Lipoprotein |
| MALDI TOF MS | Matrix Assisted Laser Desorption Ionization, Time of Flight, Mass spectrometry |
| MF | Microfiltration |
| mg | Milligram |
| min | Minute |
| ml | Milliliter |
| mM | Millimoli |
| MW | Molecular weight |
| NF | Nanofiltration |
| nm | Nanometer |
| OD | Optic density |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| pH | Negative decadal logarithm of the $[H^+]$ ion concentration |
| SDS | Sodium dodecyl sulfate |
| U | Unit |
| UF | Ultrafiltration |
| w/v | Wet volume |

# CHAPTER I

## INTRODUCTION

### Background

Food fermentation is a traditional method for the preservation of food and processing technology, and has many features such as a variety of test, flavors and textures, and importance in general health. Fermented foods improves digestibility due to predigestion of beneficial microorganisms and preventing diseases (Aichi, 2005; Sudun 2012; Aly Savadogo, 2004; N Dönmez, 2014). Milk and fermented milk products are healthy foods and contain a lot of in which calcium and vitamin D for essential factors of bone health as well as protein or other nutriments (Anon., 2011). *Lactobacillus* and *Bifidobacteria* are commonly used as living microorganisms that are defined by The World Health Organization (WHO) in 2001 (Isolauri E, 2000, Takeda Sh, 2013).

In general, lactic acid bacteria (LAB) are non-spore forming bacteria (NSFB) with gram (+), catalase (-) activities, and has low content of guanine citosine. Lactic acid bacteria are fermented carbohydrates to produce (Abed, 2013). Lactic acid bacreria play an important role in the common processing of the food fermentations and are wide variety strains applied for cultures in the manufacturing of dairy, pastry and croissant, vegetables, and meat products. Another functions of these LAB is the elongated shelf life by correlation to the raw materials. The growth of pathogenic and spoilage

microorganisms in fermented foods are inhibited by competition of the presence of starter-derived inhibitor substances and small peptides, lactate other organic acids, and hydrogen peroxide (Boontawan, 2010).

Nowadays, consumers are getting increase the interest of their health maintenance from the influence of taking foods. They have considered to health promotion effect and food security and quality, and more interest on food processing and preservation (Ito et al, 2003; Batdorj et al 2006). Therefore, functional food and probiotics have attracted the attention since 2000.

Traditional fermented milks have been produced by nomads in the world milks from cow, mare, camel, goat, and sheep with wild Lactic acid bacteria and yeasts. For Asian traditional fermented dairy products, Mongolians have practiced fermented milks for a long time (Sudun, 2012). There are many traditional dairy products, yogurt, cheese, airag (koumiss), kefir, leben, khoormog etc. On the other hand, fermented foods, milk, cassava, fish, and cereals are important foods for preserving and introducing variety diet. For example, Ben ~~sa~~alga, a fermented seaweeds, are generally used for people, especially young children, in African countries (Boontawan, 2010).

Fermented dairy products that have great social, religious, cultural, economic, and medical benefits are integral ~~regions~~ foods in ~~of~~ Mongolian tradition and have developed for achieving integration. There ~~is~~ are many variety of fermented dairy milks in Mongolia because of considerable variations in the raw materials and processing methods which come from the habits and customs of the different regions of the country. The most prevalent dairy fermented product of Mongolia is airag that traditionally made from natural starter and mare's milk. Another fermented milk is tarag, which is prepared from cow, yak, goat and sheep's milk. A third indigenous fermented beverage is khoormog that made from camel's milk (Oki K., 2014; Munkhtsetseg B., 2009).

Fermented mare's milk, Airag, is a very popular fermented beverage in Mongolia, which ~~is~~ contains a lot of ferments (lactic acid bacteria and yeast), intestinal trace elements, antibiotics, vitamins A, B1, B2, B12, C, D and E, ethyl alcohol, organic acids (Ahmed M, 2010 and Tamime , 1999). For revealing functionality and useful information about Airag as a healthy daily beverage in Mongolia, the author investigated antibacterial and proteolytic activities of lactic acid bacteria in Airag.

## 1.1. Mare's milk

The around 30 million people consume mare's milk regularly throughout the world where is a vital nutritional source of mammals during the neonatal period. In regions of Central Asia Steppes including Mongolia, Inner Mongolia Autonomic Region of China, China's Qingha Province and Xingjiang Uygur, Turks, Bashkirs, Kazakhs, Kyrygyz, Yakut, Uzbeks, Buryat of Russia to mostly used mare's milk that produce a little bit alcoholic beverage called "Koumiss". It is also called "Airag" by Mongolians which has been apperceived as a wholesome beverage with the widely kind of therapeutic effects such as influences alimentary, blood circulation and immune system.

In past decades, people have been known the health benefit effects of mare's milk when the great interest in their utilization. Drogoul et al reported that some Europian countries as Germany and France studies have enhancing in the utilization of mare's milk for human nutrition concretely in recent years (Massimo et al, 2002; Klemen Potočnik, 2011).

### 1.1.1 *Biochemical components of Mare's milk*

The biochemical component of mare's milk, cow's milk and other milks presented in Table 1 and unique characteristics in conditions of the essential value (Massimo Malacarne, 2001). Based on its composition in fat, proteins, lactose, macro- and oligeoelements (Schryver H.F., 1986) of the mare's milk, which was same to human milk and contrast than cow's milk (Ochirkhuyag., 2000).

Table 1. The chemical components of different types of mammalian's milk

| Component | Value | Mare | Cow | Goat | Sheep | Human |
|---|---|---|---|---|---|---|
| Total fat* | Mean | 12.1 | 36.1 | 75 | 41 | 36.4 |
| | (min-max) | (5-20) | (33-54) | (50-90) | (30-40) | (35-40) |
| Whole protein* | Mean | 21.4 | 32.5 | 54.5 | 34 | 14.2 |
| | (min-max) | (15-28) | (31-39) | (45-70) | (30-36) | (9-17) |
| Lactose* | Mean | 63.7 | 48.8 | 49 | 47 | 67.0 |
| | (min-max) | (58-70) | (44-49) | (41-59) | (42-50) | (63-70) |
| Ash* | Mean | 4.2 | 7.6 | 8.5 | 7.7 | 2.2 |
| | (min-max) | (3-5) | (7-8) | (8-9) | (7-8) | (2-3) |
| Gross energy kcal/kg | Mean | 480 | 674 | - | 670 | 677 |
| | (min-max) | (390-550) | (650-712) | | (660-690) | (650-700) |

+-mean value, minimum to maximum values reviewed in literature.

*-g/kg (1g components containing in 1kg )

The fat content in mare's milk has been containing lower than cows, sheep, goat and human milk. The higher level of unsaturated fatty acids in mare's milk than cow milk. A whole protein in mare's milk compared to other animals shows that the poorest in protein fractions.

The lactose content of mare's milk is homogenous that of human milk and higher than content in other milks indicating that same to between mare's milk and human milk. In additionally, contains galactose is a constitution of the myelinic sheath of the central nervous system cells. The structural involution of the minor carbohydrate fractions (Kunz, 1999; Klemen Potočnik, 2011) of human milk makes a functional comparison with other milks and mare's milk arduous. Sialic acid plays several important roles in the human body for example, these acids containing high concentration of glycosylation of gangloisides in the encephalon and central nervous system that are participating in neural transmission. Also, there are affected in intestinal microflora and known as antiviral activity.

A whole protein and ash content (containing minerals) are compared to mare's milk and other animals milk that cow's milk has higher in minerals, and so lower congruous as a supersession for human milk than other animals milk. Cross energy demand of mare's milk was limpidly lower than cow, sheep, goat milks.

### 1.1.2 *Features of Mare's milk*

The whey globule proteins and non protein nitrogen percentages are compared to the mare and human milks and their protein system is more similar. Besides, cow's milk has higher concentration of casein, which is related phosphoproteins and like thatdetermined as a caséineux milk. These complex proteins are making up total proteins in mare's and cow's milk, respectively 20-45% and 80%. The fraction of whey globule protein in mare's milk is occupied that 40-50%, a little more than 50-60% in human milk, whereas the whey protein in cow's milk is lower than 20%. Also cow milk protein specialies are different as characterised by an acid enzymatic, mixed coagulation from another milks such as sheep, goat.

From this perspective the whey globule protein in the mare's milk is higher supply of nutritional value consisting of essential amino acids that makes more propitious to human nutrition than cow's milk. They could be defined typically as albumineux. (Massimo Malacarne, 2001).

The fat content of mare's milk is lower than other mammalian milks when compared to that of cows and sheep, goat's milk. Lipid contents in milk are dispersed as emulsified globules which are consist of globules about 2-3μm of size in mare's milk (Massimo Malacarne, 2001; Vesna, 2014). The fat globules are assembled three layers when to that an consisting of a mixture of glycoproteins, some enzymes, and phospholipids. For example, inside layer consisting of proteins, an middle layer has polar lipids as phospholipid and the outside layer is surrounding high molecule glycoproteins.

In this outside layer is surrounded glycoprotein structure has branched oligosaccharides that is nearby of fatty acids in the human milk, which is not revealed in cow's milk (Solaroli et al., 1993). In conclusion, the structure of this fatty acid globules is distributed di- and triglycerides its similar to the mare and human milk. Both of milks the portion of unsaturated fatty acids are higher than in cow's milk that owing to a high content in polyunsaturated fatty acid with numerous carbon atoms (Massimo Malacarne, 2001).

### 1.1.3 *Health benefits of mare's milk*

Consider the benefits of drinking milk for healthy mares following:

- Rich in vitamin and minerals
- Overcome inflammatory disorders
- Suitable as a replacement for cow's milk
- Cure ezeme and more durable

Based on study, mare's milk has nutrient than cow's milk. The nutrients include vitamins A, B2, B6, B12, D, C, E and minerals such as iron, calcium, potassium and magnesium. And that is contain a lot of bacteria and virus antibody. There are not many scientific researches made about the benefits of mare's milk. In fact, one the only studies has been conducted in the University of Jena, in Germany 2009, where a group of interviewees suffering from various types of skin conditions were given mare's milk over the period of six months. Over 90% of the participants reported that the condition of their skin had improved. Also 75% of the participants, suffering from different types of problems with respiratory or intestinal systems, liver or circulation, reported that the unwanted symptoms had decreased (Räsänen, 2014).

### 1.1.4 *Usage of Mare's milk*

Mares has been milked for hundreds of years in Russia and in parts of Asia. For example, Mongolia milking mares and producing other products out of the milk is traditional. Kumis or Koumiss (airag) is a traditional dairy fermented milk made out of mare's milk that during fermentation process some alcohol is also produced in the milk. People who have been producing the drink for ages believe that it has health benefits, such as helping with gastrointestinal tract and metabolism; it also has benefits, such as other organs such as kidneys and nervous system. From long times ago, Mongolians has been using raw mare's milk for folk medicine tretment during in summer time.

Adam Salam reported about utilization of mare's milk that has a stronger laxative effect throughout drink raw mare's milk. In some nomadic countries, this effect of raw mare's milk is used for traditional medicine. During fermentation of mare's milk, lactose is converted into lactic acid, ethanol and carbon dioxide, which process has been providing special characteristics of airag. However, different sources present opinions that differ quite a lot. The conflict can be noticed between the opinions of the suitability of mare's milk to people who suffer from lactose intolerance. The high content of lactose is a characteristic feature of mare's milk. For people suffering from intolerance towards lactose, the consummation of mare's milk is advisable. It is due to specific composition of substance, containing all the necessary enzymes for digesting carbohydrates. In some sources suggest that lactose of mare's milk cannot be tolarated by lactose intolerants where one resource suggest mare's milk being a good option for a lactose intolerant person instead of cow's milk includes enzymes that help in digesting the lactose and that would be the reason why also lactose intolerant people would tolerate mare's milk.

Besides the traditional fermented beverage that has been produced in Mongolia and another area of Central Asia, mare's milk has found its way to many other products as well. In the Europian contries, that mare's milk is used for many different purposes varying from drinking it straight to using it in cosmetics (Räsänen, 2014).

## 1.2. Airag

**Figure.1** The skin bag for Airag fermentation, it made by cow's hide (Traditional method)

Mongolian traditional fermented milk airag is made by mare's milk using nomadic method that is also called koumiss in some regions of the world. This beverage has been fermented by the spontaneous starter culture of lactic acid bacteria and yeasts, which has a good effect of health promotion and disease prevention as a probiotic product. Traditionally, Mongolians have unpasteurised raw mare's milk is blended with about thirty percent of old day's airag and aerated in a skin bag (Mongolians say that-huhuur) that made of processed cow also horse hide (Batjargal et al 2006) that is starting from early in June to be continued until the middle of October. The airag commonly contains about 2% alcohol, 0.5-1.5% lactic acid, 2-4% milk sugar and 2% fat (Kerr 2001; Miyamoto, 2010).

Furthermore, airag produced by proteolytic enzymes contains amino acids, small peptides, 1.8-2.0% lipids consisting of vitamin A, D, E, K and 20 kind s of fatty acids, organic acids, small whey proteins such as lactoglobulin, lactoalbumin, immunoglobulin, serum albumin etc. its thought that the microorganisms participating in nutrient production and fermentation, and their metabolic products contribute to the physiological function of airag (Sudun, 2012).

The airag (koumiss) has divided into three types such as strong, moderate and light, there are referred to lactate concentration. The strong airag is engendered by LAB (*lactobacillus bulgaricus, Lfeactobacillus rhamnosus*) which acidity the milk to pH 3.6-

3.3 and its conversion ratio of lactose into lactate is 80-90%. The moderate airag is produced by *lactobacillus* sp. LAB (*Lactobacillus acidophillus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus fermentum*) with limited acidification properties that lower the pH 4.5-3.9 at the last products are conversion ratio approximately 50%.
The light airag is a slightly acidified product pH 5-4.5 its commonly generated by *Streptococcus thermophillus and Streptococcus cremoris*. Hereof the moderate airag has the best aroma, texture and taste (Shigaeva 1983; Baldorj 2000; Danova 2005).

### 1.2.1 *Microorganisms of Airag*

A little information has about bacterial communities in Mongolian traditional fermented mare's milk as airag (Batdorj 2006) what microflora differs according to fermentation conditions, the place of production, the fermentation time and the starter culture used. Two different types of symbiotic microorganisms as LAB and yeasts are depending on fermentation process, their coryphaeus are *Lactobacilli* and *Kluyveromyces*, *Saccharomyces*, *Candida* sp. etc. *Lactobacilli* species are described to play main fermentative role affecting the flavor, texture and acidity of end products furthermore some benefits to human health (Montanari et al 1997; Danova 2005).

***Lactic acid bacteria:*** Lactic acid bacteria (LAB) have been essential part in food fermentation for humani history for its greatly contributed to the nutritional value of end products. Some metabolic properties due to LAB play on an important role in food fermentation, which affect the flavor, texture and acidity of the end products being on some benefits to human health (Ljubisa, 2006; Sun, 2010). Recently, new starter cultures for LAB with an industrially important functionality are being developed. LAB are produces antibacterial peptides, some kind of polysaccharides, hydrogen peroxide, sweeteners, aromatic compounds, vitamins or essential enzymes etc, that has been constituted probiotic properties (Frédéric, 2004).

LAB is divided into two type cocci shape (*Lactococcus, Vagococcus, Leuconostoc, Pediococcus, Aerococcus, Tetragenococcus, Streptococcus*) and rod shape (*Lactobacillus, Carnobacterium, Bifidobacterium*). Morphologically, these LAB has referred members of the Clostridium-Bacillus subdivision of Gram (+) positive eubacteria (Luc De Vuyst., 1994). They produce lactic acid as the principle by product of sugar fermentations that might be relegated as homofermentatives (chemical conversion of glucose into lactate), or heterofermentatives (Glucose is converted to lactate, carbon dioxide, ethanol, and acetate) (Luc De Vuyst., 1994; Dündar, 2006).

Lactobacillus are especially groups of LAB, which are play a useful role in the gastrointestinal track by colonizing there. Some important nutritional and therapeutic effects attributed to these bacteria are follows:

- Enhancement of nutritional value of foods and local humoral immune
- Equalisation of the intestinal microflora and prevention by spoilage bacteria such as *Escherichia coli, Staphylococcus aureus,* and *Salmonella* sp. throughout sticking to the intestinal wall and competition for nutrients
- Protection against free radicals and intestinal or urinary tract infections by producing antibacterial peptides
- Anti-inflammatory properities and improvement of sugar tolerance
- Tumor suppression through stimultion of the immune system to produce macropages and stimulation in a non inflammatory manner. (Gilliland, 1990; Dündar, 2006;).
- Other benefit effects

The main *Lactobacillus* sp. was isolated from Airag of Mongolia, Inner Mongolia and other regions of China is included in predominant species *L.helveticus, L.plantarum, L.casei, L.kefiranofaciens* (Kaihei, 2014). Since 1980s, Mongolian researchers have been started study of morphological and physiological characterizations of traditional fermented mare's milk as Airag that by a culture method based on phenotypic analysis. Baldorj et al (2003) described that, predominant species of LAB such as *L.bulgaricus*

(also known as *L.delbrueckii* subsp. *bulgaricus*), *Streptococcus lactis* (also known as Lactococcuslactis) and alcohol fermenting yeasts such as *Saccharomyces lactis* (also known as *Kluyveromyces lactis*), *Saccharomyces cartiloginosus* (also known as *S.cerevisiae*) and *Saccharomyces torulosus* (also known as *T.delbrueckii*) were identified from Airag which samples collected from different geographic region of Mongolia included in forest-steppe, gobi dessert and Mongolian steppe (Watanabe, 2008; Batdorj 2003).

In 2013, Watanabe et al summarized that evalute the efficiency of Mongolian traditional fermented mare's milk beverage as probiotics it is the more important. Therefore they used the RAPD-PCR technique to the 183 LAB strains isolated from Mongolian Airag samples were classified as according to five different genera (*Enterococcus, Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus*) to species groups, 12 indicated species: (*E.faecium, L.casei, L.diolivorans, L.farciminis, L.helveticus, L.hilgardii, L.kefiranofaciens, L.kefiri, Lparafarranginis, L.plantarum, Lc.lactis* subsp. *lactis, Leuc.mesentroides, Leuc.pseudomesentroides, S.thermophillus, Lactococcus sp.*). Their team also was isolated *Bifidobacterium mongoliense* sp. nov (*mon.go.li.en'se*. N.1.. neut.adj *mongoliense* pertainting to Mongolia) from Mongolian airag which were collected in the province of Umnugovi and Uvurkhangai (Watanabe, 2009).

Many researchers had identified LAB composition from Airag samples of Inner Mongolia in China (Zhishen Mu, 2012; Sun, 2010; Wu, 2009; Takeda, 2011; Uchida, 2011; Shuangquan, 2006; An, 2004). For example, Shuangquan (2006) investigated the LAB in traditional starter for fermented milks, which found *Lactococcus raffinolactis* was the most predominant *Lactococcus* species, and *L.plantarum* and *L.casei* were the most predominant *Lactobacillus* species in airag samples. Wenjun *et al* (2012) and Wu *et al* (2009) described to the predominant *Lactobacillus* species in airag were *L.csei, L.helveticus* and *L.plantarum*. Miyamoto also isolated and characterized many LAB in samples of Inner Mongolian Chigee, a fermented mare's milk from China (Burentegusi *et al*, 2002).

***Yeasts:*** Some yeast species are major constituent of the microbiota of dairy products including fermented milks (traditional fermented milk of mare, camel, cow, yak and goat), sour cream, yogurt and cheese (Zhishen Mu, 2012; Fleet, 1990; Fröhlich-Wyder, 2013). In fermented milks, yeasts share some biochemical and physiological characteristics such as utilization of lactic acid, assimilation of lactose, stimulation of amino acid and vitamins, growth at low temperatures and tolerance to elevated salt concentrations and a high lypolytic and proteolytic activities that can be play considerabley roles in the developments of tastes and flavors (Fröhlich-Wyder, 2013).

In yeast fermentation, the sugar is converted to ethanol and carbon dioxide its generate carbonated mildly alcoholic drink. The commixed microorganisms of these starter cultures in airag seems to improve cell growth and fermentation better than single culture (Suk-Ho 2016).

Most yeast strains isolated from airag have moderate to strong proteolytic and lypolytic activities that may also contribute to the flavor of fermented product (Mu, 2012). Lactose fermenting *Saccharomyces lactis* (also known as *Klyuveromyces lactis*), *S.cartilaginosus* (synonym as *S.cerevisiae*) and *S.torulosus* (synonym as *T.delbrueckii*) plays the principle functions in alcoholic fermentation in Airag (Baldorj R., 2003).

Watanabe et al (2008) reported that phylogenetic analysis based on the sequences of 18S-26S ITS1 and ITS2, the 68 yeast strains isolated from Mongolian traditional airag was identified as eight species: *Candida pararugosa, Saccharomyces cerevisiae, Kluyveromyces marxianus* (also known as *Candida kefyr*), *Kazachstania unisporia, Dekkera anomala, Issatchenkia orientalis, P.mandshurica* and *Torulaspora delbrueckii.* Mu *et al* (2012) isolated and identified 12 different yeast species belonging to 9 genera including *S.cerevisiae, K.marxianus, Ka.unisporia, C.pararugosa, D.anomala, Geotrichum sp., I.orientalis, P.deserticola, P.fermentans, P.membranoefaciens, Peronospora manshurica* and *T.delbrueckii* from the three different regions of China.

In the past decade, the microbiota and diversity of LAB species in Mongolian dairy products have been elucidated (Baldorj *et al*., 2000, Batdorj *et al*., 2003, Takeda *et al*., 2009; 2011; 2014; Miyamoto *et al*., 2010; 2014; Uchida *et al*., 2007; Watanabe *et al*., 2008; 2010;2014; Yu *et al*., 2011). Therefore, further studies elucidating the functions and LAB content of dairy products are anticipated. Several studies demonstrated the functions of LAB in Mongolian dairy products (Takeda *et al*, 2011; Batdorj *et al*, 2006; Kimura *et al*, 2006; Takeda et al, 2013).

Several studies have investigated the population of LAB and yeasts in airag using culture-dependent methods. Special strains as *Lactobacillus plantarum, L.pentosus, and Lactococcus lactis* subsp. *cremoris, Leuconostoc lactis, Enterococcus faecium, Saccharomyces cerevisiae, Candida kefyr, Candida krusei* and *Candida glabrata* were isolated from khoormog which is a traditional camel fermented milk (Shuangquan *et al*., 2004). *Lactobacillus helveticus, L.kefiri, Streptococcus dairensis* and *Klyveromyces wickerhamii* were isolated as the dominant species in airag samples obtained from Mongolia (Uchida et al., 2007).

Watanabe et al reviewed that the predominant LAB species of airag were *Lactobacillus helveticus* and *Lactobacillus kefiranofaciens* that result similar to Takeda et al results but with sligthly differnce that *L.delbrueckii* subsp. *lactis* and *L.fermentum* were the predominant species in Airag rather than *L.kefiranofaciens* (Oki et al., 2013). Among these *Lactobacillus* strains a novel strain with high potential probiotic properties, *Lactobacillus casei* Zhang, was determined and studied greatly for its probiotic properties, health-promoting effect and fermentation characteristics.

***Interaction of LAB and yeasts in Airag:*** Both symbiotic microorganisms of lactic acid fermentation is comprised main part of fermented beverage, the frequently co-occurrence of yeasts and LAB has affect on feature and quality of end products. Obviously, the presence of yeasts is crucial for the desirable properties of carbon dioxide and ethanol production in East European and Asian countries that manufacture to products such as kefyr, koumiss and airag (Judith, 2003). This mechanism of possible interaction between LAB and yeasts has not been complately studied yet. Thus interaction might be inhibition

or stimulation of growth of single or mixed of the co-cultured strains (Marshall, !987 and Judith, 2003). The co-cultured microorganisms could be competing for medium nutrients or their generated metabolites may have been induced for the another growth process (Judith, 2003).

Yeast has a main function in the production of fermented milks being with the ability of produce ethanol production and high concentrated flavor compounds. In particular, LAB is an important part of fermentation by cause of their generate organic acids, aromatic compounds, and small peptides that inhibit the growth of undesirable organisms.

Interactions of symbiotic organism owing in fermentation process have been characterized several properties and that all the substances with proteolytic and lipolytic activity from the yeast fermentation, metabolism of lactic acid that depends on pH, production of carbon dioxide and other metabolites (vitamins, co-enzymes and growth factors etc) (Judith, 2003). Anciently, Mongolians have been widely used airag for traditional medicine because of it has many kind of medicinal values. For example, when some disease included in tuberculosis, hypertension, gastroenteritis etc., and thereof prevention. Previous studies have observed that LAB and yeast are the predominant microorganisms in most fermented foods: several kind of cheese, kefyr and koumiss etc.

Both positive and negative interactions between LAB and yeasts to described what positive interactions like between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from saccharine kefyr grains, add to the stimulation of LAB by yeast strains via production of carbon dioxide, pyruvate, propionate and succinate (Sudun, 2012). Additionally, some strains of LAB release galactose into the growth medium such a product of lactose metabolism that it would favor the growth of lactose negative yeasts (Sudun, 2012). The most lactose negative yeast species found in natural fermented milk which are often able to apply galactose and in some cases lactic acid and citric acids (Judith, 2003). On the other hand, the interaction between LAB and yeast have negative when their major concern the mutual inhibition of growth.

Yeasts are commonly inhibited by LAB producing compounds and so on phenyllactate, 4-hydroxyphenyllactate and several cyclic dipeptides (Sudun, 2012 and Pinar, 2015).Sudun et al, 2012 summarized that interaction between nine LAB and five yeast strains isolated from airag of Inner Mongolia Autonomic Region, China. Both microorganisms demonstrated stimulatory and inhibitory effect on each other in terms of the combinations. *Leuconostoc mesentroides* subsp. *dextranicum* 6B2081 was stimulated in mixed cultures with all yeast excepts for *Saccharomyces servazzii* 5Y01. Also *Leuc.lactis* 420B1 was inhibited in all mixed cultures. The another LAB strains demonstrated stimulatory and inhibitory effects in combined cultures with yeasts (Sudun, 2012b).

### 1.2.2 *Health benefits of Airag*

From the ancient times, Airag has been known as a healthsome beverage. Mongolians consume fermented mare's milk for medical purposes, for recovering strenght, and as a cure for diseases of digestive organs and illnesses such as tuberculosis (Yu et al., 2011). Many researchers have been reviewed that about the health benefits of airag and forth and the like favorable influences on the alimentary canal's activity, the circulatory and nervous systems, blood-forming organs, kidney functions, endocrine glands and the immune systems (Danova 2005; Sukhov et al 1985; Stoianova et al., 1988;). It avails the development of immunity and cardiovascular diseases which has been used to treat weight loss, anemia and decreasing cholesterol level (Solari et al., 1993; Trojannowska 2006; Abdel-Salam et al., 2010; Dönmez et al., 2014).

Physical training is defined by adaptation of cardiovascular actions and permutations in hematological and biochemical characteristics (Dönmez et al., 2014). Dönmez et al evaluated Airag for drink with physical training has several variations and observed their effects on some hematological and biochemical characteristics in sedentary humans. They found no significant permutations in hematological characteristics between their experimental groups.

Leucocytes count, and amount of neutrophils tended to grow in three groups airag and airag with training compared to a just training group to 15 days and a significant decrease in the amount of lymphocytes in training group at the end of the study. In conclusion, they suggesting that Airag together with physical training may promote good health (Dönmez et al., 2014).

Also Dao Dong et al reported that *Lactobacillus fermentum* SM-7 was tested from ten LAB strains which are isolated from Airag sample, a fermented mare's milk that reduced cholestrol by 66.8%. its artificially stimulated hyperlipidaemial ICR mice significantly reduced serum total cholesterol and the total triglyceride levels, low density lipoprotein (LDL) cholestrol level did not increase significantly. In this group were compared to the body mass and the liver mass ratio its less than those mice on a high-cholesterol diet that were not given *lactobacilli* (Dao Dong et al., 2010).

Nowadays, Airag classified to the group of functional foods that related to the health benefits or any disease prevention for basic nutritions.

### 1.2.3 *Possibility of manufacture Airag*

The high cost of mare's milk is the main component for its application and since the availability falls short of the demand even in the traditional home countries of Airag production. Therfore, recent years study of airag created by utilizing cow's milk that has been increasing great interest in scientists and food technologists. In some European regions and the USA, as a koumiss as fermented milk beverage is made by skim and whole milks derived from the cow's milk (Özer and Gülün, 2014)

The difference of chemical composition between mare and cow milk by reason that have a different method to make it suitable for the production of airag. For example, cow's milk is used to produce Airag, even though the fat content and the milk protein composition is different in the both milks. Since the middle part of the last century, scientists have focused on the manufacture of airag by cow milk so as demanded to do some modification.

A few researchers have already been tested under various methods for modification of the cow's milk, such as reducing fat content, increasing amount of water and lactose, proteins changing by ultrafiltration method. However, the success of these approaches has been limited. Lutskova (1957) developed a simple method by adding sucrose in the cow milk, which had been diluted water for modification. Similarly, Seleznev and Artykova (1970) used a method which was adding method into the skim milk derived from cow milk.

Aforementioned, studies were focused on sugar content of the cow's milk, but these studies did not change to be protein component. The protein content of the milk is one of the main parts for airag as fermented milk industrial processing. The mixture cow's milk and concentrated whey were considered for modification of cow's milk by Gallman and Puhan (1978). In these modifications, the lactose content of cow's milk was not regarded as a protein content modification of cow's milk that was adjusted to mare's milk.

Membrane technologies are among the most important methods which have reached a matured technical standard and could be applied to airag processing. Kùcùkcetin et al reported that Airag can be made from cow's milk who was modified using three kinds of membrane technology as ultrafiltration, microfiltration and nanofiltration (UF, MF, NF) Fig.2 for modification cow's milk.

They are used, modified cow's milk with a mixed starter culture consisting of *L.delbrueckii* subsp. *bulgaricus*, *L.acidophilus* and *K.lactis* create a Airag, which was similar to the mare's milk with chemical, physical and microbiological characteristics (Kùcùkcetin et al, 2008). Alternatively, cow's milk is diluted with drinking water to a targeted casein level (Malacarne et al, 2002).

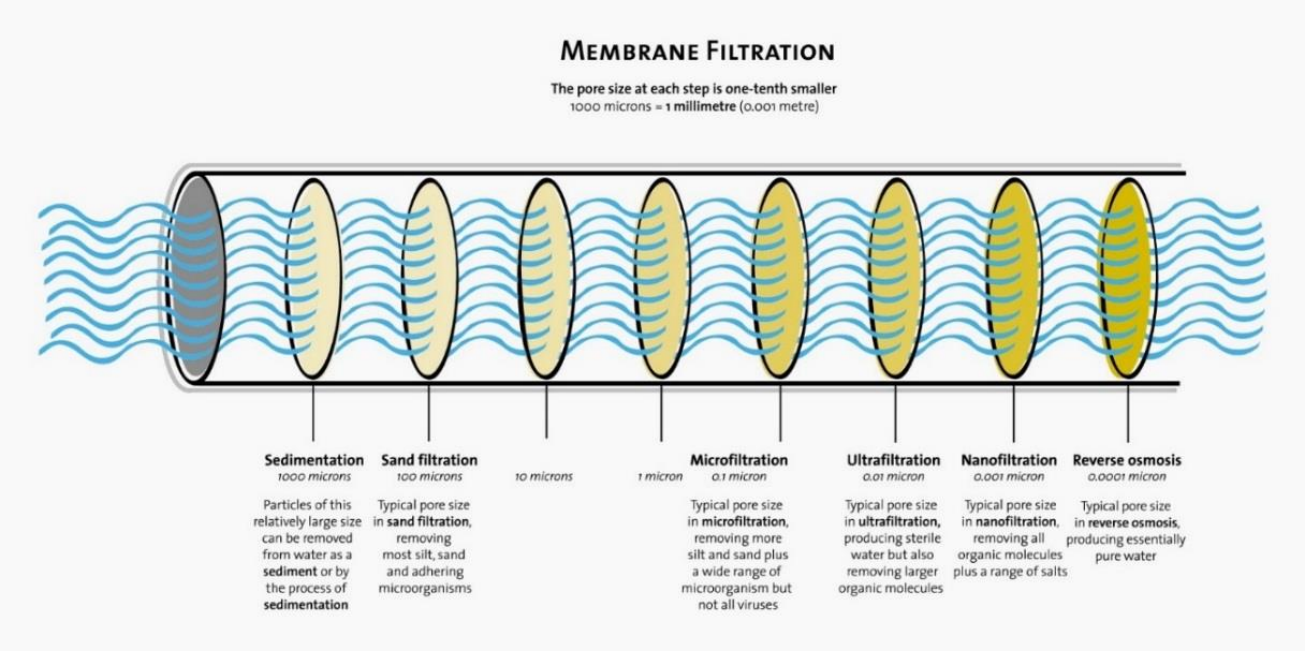


**Figure.2.** System of Membrane filtration. Showing difference of membrane systems. (https://kyocp.wordpress.com/2012/03/14/membranes/)

## 1.3 Antibacterial potential of Lactic Acid Bacteria

Antibacterial peptides (ABP) are small molecules (<10kDa) with inhibitor activity against some organisms such as bacteria, yeasts, fungi, viruses and even tumor cells that cause these inhibitory substances as therapeutic agents (João, 2015). These peptides include two or more positively charged residues provided on aciditic amino acids: arginine (R), lysine (K), histidine (H) and a larger proportion (approximately >50%) of hydrophobic residues (Reddy, 2004; Rabeeth, 2012). Due to the urgent increase of antibacterial resistance, interest in alternative antibacterial sunstances has led to the utilization of ABPs, both synthetic and from natural sources, respectively.

***Features of ABPs:*** Nowadays**,** many peptides-based drug and supplements are commercially available for the treatment of numerous diseases and so on C hepatitis, myeloma, skin infections and diabetes (João, 2015). This demanding interest of the pharmaceutical companies in developing peptide-based drugs has been further intensified by the wide occurrence of protein therapeutics by both physicians and patients (Reichert, 2010). In 2014, the application of ABPs was applied as a combination therapy for treating biofilms if preferred over monotheraphy (Guangshun, 2015).

Until now, over 2000 ABPs have been described in virtually every eukaryotic organisms. Particularly, in the human activity of ABPs was first found out in the 1950s and 1960s. It was presented cationic proteins were responsible for the neutrophils activity against inhibiting bacteria via oxygen-independent mechanisms (Zeya, 1966; Hirsch, 1956).

***Mode action of ABPs:*** Although ABPs configuration a varied group of peptides by their primary structures which are often cationic and amphiphilic, and most of them inhibiting bacterial growth by permeabilizing their cell membranes. The positive charged ABPs are interacted to the negative charged bacterial membrane with phospholipid. They have a high concentration of a specific amino acid, proline that joining into intramolecular disulfide bonds, and those with an amphiphilic part in their molecule if they imagine an α-helical structure (Maria, 2003; Guangshun, 2015).

The mode of action of ABPs initially Figure.3 by membrane depolarization event which is induced by cationic part of the APBs primary structure. Finnally, it has caused to cell death.

Reddy et al (2004) reviewed some structure, function studies on ABPs have been published that chemical and physical characteristics and that all secondary structure, overall charge and hydrophobicity influence the interaction of ABPs with model membranes. These include the formation of pores by the "*Barrel stave pore model*" or the *Toroil-pore* mechanisms as well as the "*The Carpet model*" (Fig 3.) (Reddy, 2004; Nguyen, 2011; João, 2015; Guangshun, 2015).

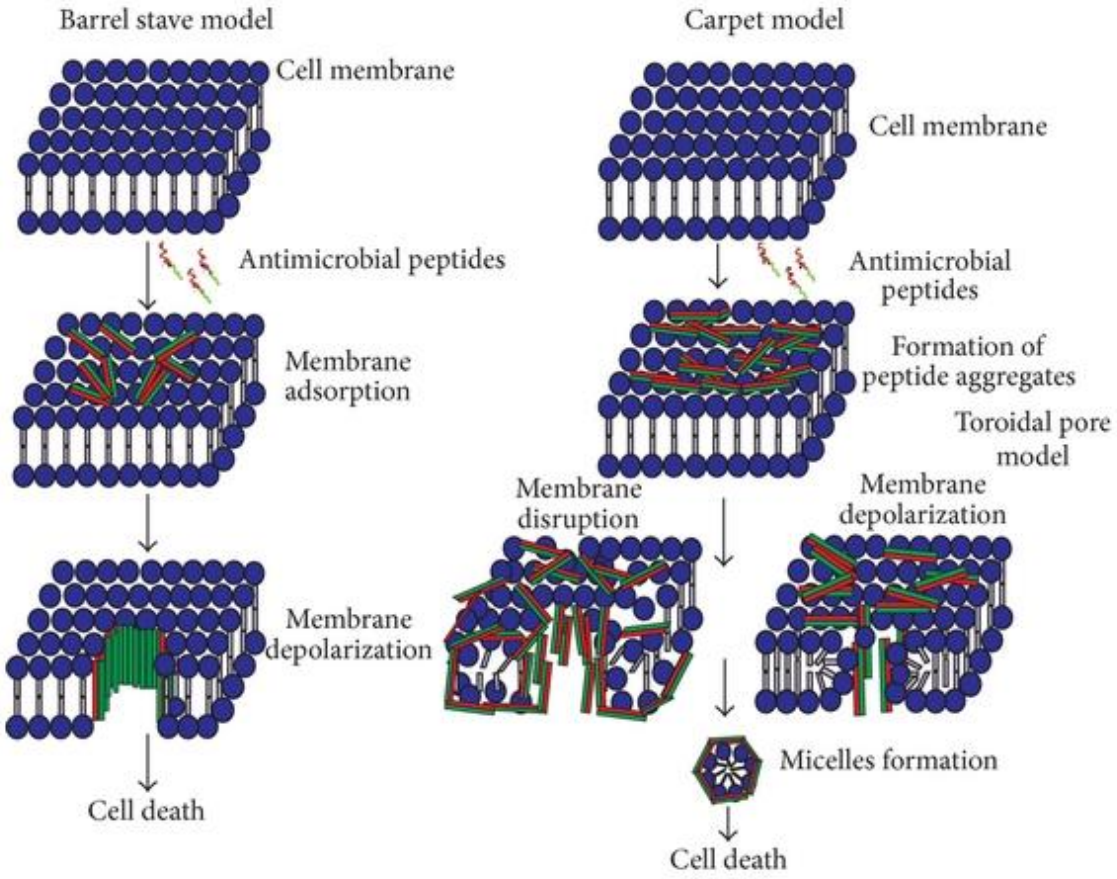


**Figure.3.** Bacterial membrane distruptions mechanisms by initial adsorption of ABPs. (http://www.hindawi.com/journals/ijpep/2013/675391/fig2/)

A wide variety of ABPs were synthesized from the bacterial kingdom (Maria, 2003). Hereof, LAB exerts a strong antagonistic activity against many food-contaminating microorganisms was shown the result of the production of secondary metabolits such as organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Hirsch, 1956; Piard, 1992; Luc De Vuyst, 1994; Hernández, 2009; Kumar, 2009).

Among them, Bacteriocins have the strongest activity that is biosynthesized in the ribosome as a biologically inactive prepeptide, post-translationally modified, and then secreted outside of the producer cell as a mature active peptide (Arakawa, 2016). They falls into three main classes, including in Class I of lantibiotics (containing posttranslationally modified amino acids such as lanthionine and β-methyllanthionine); In Class II regarding to the non-lantibiotics (small heat stable, non-lanthionine containing membrane active peptides), Class III of bacteriocins (including complex bacteriocins) and their classification constantly have been studying extensively in last decade (Dimov, 2005; Zacharof, 2012).

Batdorj et al, 2006 reported that two bacteriocins with molecular weights of 5206 Da and 5218 Da were isolated from Mongolian Airag samples and characterized, revealing similarity with enteriocins L50A, L50B and I, which were isolated from the supernatant of LAB *Enterococcus faecium* and *Ent.faecium* 6T1a.

In 2012-2016, Miyamoto's research team found out that purification and characterization of a bacteriocin-like substance produced by *Leuconostoc mesentroides* strain 406 (Wulijideligen, 2012) and *Leuconostoc mesentroides* subsp. *dextranicum* 213M0 (Arakawa, 2016) strains were isolated from Mongolian airag. These strains were highlighted by their biological and chemical properties and carbohydrate fermentation capabilities as well as.16S rDNA taxonomical analysis. The bacteriocin like substance produced *Leuconostoc mesentroides* strain 406 was characterized by Wulijideligen (2012) where bacteriocin was partially purified gel filtration chromotography. That was detected a molecular weight of approximately 3.3 kDa by SDS PAGE gel electrophoresis. Arakawa (2016) classified that strain 213M0 was fallen down as *Leuconostoc mesentroides* subsp. *dextranicum*, which was corresponded to a typical feature of the Class IIa as pediocin as bacterioncins. That was activated wide range pH and some peptidases, their molecular mass was estimated to be 2.6-3.0 kDa by SDS-PAGE electrophoresis.

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# CHAPTER II

# ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA AND YEASTS FROM AIRAG

## 2.1 Introduction

Mongolian Airag is a traditional beverage that is made by fermentation of mare's milk with lactic acid bacteria and yeast in a big cowhide bag; it contains 2-4w/w% ethanol (Cagno *et al*., 2004; Watanabe, 2011). Mare's milk contains higher proportions of proteins and lower proportions of fatty acids than human and cow's milk, and mare's milk has a remarkably high concentration of unsaturated fatty acids (Malacarne *et al*., 2002; Potocnik *et al*., 2011). Therefore, in Mongolia and Inner Mongolia of China, Airag is drunk daily to improve physical strength, promote digestion, and provide nourishment. There are several reports on the identification of lactic acid bacteria in Airag and investigation of the characteristics of Airag as a beverage.

Two antibacterial peptides with molecular weights of 5206 and 5218 Da were isolated in Mongolian Airag samples and characterized, revealing that the two peptides had similarity with enteriocins L50A, L50B, and I, which were isolated from the supernatant of lactic acid bacteria *Enterococcus faecium* and *Ent. faecium* 6T1a (Batdorj *et al*., 2006). These two peptides had potent antibacterial activity in a wide

pH range on both Gram positive *Lactobacillus*, *Enterococcus*, pathogenic *Listeria*, and *Staphylococcus* strains, and Gram negative *E. coli* bacteria (Batdorj *et al*., 2006). Batdorj also found an antibacterial peptide from *L. delbrueckii subsp. lactis* strain T3 in Tarag, a traditional Mongolian yoghurt produced by fermentation of cow's milk with lactic acid bacteria. That peptide had a potent inhibitory effect on *E. coli* and *Listeria innocua* strains (Batdorj *et al*., 2007).

Watanabe reported that several hundred lactic acid bacteria and yeasts had been isolated from 22 Airag and 31 Tarag samples in Mongolia and identified by culture and molecular biology-based methods (Watanabe *et al*., 2008). In Airag, *L. helveticus* and *L. kefiranofaciens* were the main lactic acid bacteria, and *L. delbrueckii* subsp. *bulgariaus* and *Streptococcus thermophiles* were predominant in Tarag. The lactose-fermenting *Kluyveromyces marxianus* was the major yeast in Airag samples, and *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, and *Kazachstania unispora*, glucose- fermenting yeast were predominant in Tarag samples. These results suggest that the most important factor in the diversity of microbial compositions between livestock animal milks was in the type of Mongolian traditional beverages rather than geographical regions (Watanabe *et al*., 2008; Oki *et al*., 2014).

An antibacterial peptide with the molecular weight of 3.3 kDa produced by the lactic acid bacterium *Leuconostoc mesenteroides* strain 406 in a Mongolian Airag sample was isolated and characterized by Miyamoto and coworkers (Wulijideligen *et al*., 2012). It was found that the supernatant of the lactic acid bacterium inhibited the growth of several lactic acid bacteria, pathogenic organisms, and food spoilage. Although the supernatant was heat- and pH-stable, the inhibitory activity on bacteria was decreased by digestion by several proteases.

Miyamoto also isolated and characterized many lactic acid bacteria in samples of Inner Mongolian Chigee, a fermented mare's milk in China (Burentegusi *et al*., 2002). Many lactic acid bacteria were also isolated from Koumiss, which is a beverage of fermented livestock animal milks produced in Central Asian countries (Danova *et al*., 2005; Mechai *et al*., 2014).

## *Chapter II Isolation and Identification of Lactic Acid Bacteria and Yeasts from Airag*

Research on the classification and functionality of lactic acid bacteria in fermented milks is attracting attention because the fermented beverages and foods are widely consumed in Asian countries to promote health. In the present study, we report the isolation and identification of several lactic acid bacteria in Airag by 16s rDNA sequencing analysis. We found that the supernatants had potent antibacterial and proteolytic activities. We also isolated yeasts in Airag samples and investigated the ability to ferment glucose compared with that of K7 yeast.

## 2.2 Materials and Methods

### 2.2.1 Materials

Six Airag samples were collected in four provinces of Mongolia near Ulaanbaatar, Arkhangai Khashaat (Ark), Bulgan Saikhan (Bul), Uvurkhangai Bayan-Undur (Uvu), and Tuv Delgerkhaan (Tuv), in July 2014. Each sample (50 mL) was stored at 4°C until use. For bacterial enumeration, Airag samples (2mL) were kept at -80°C in 20% glycerol until use. *Escherichia coli* (*E. coli*) (ATCC25922), *Bacillus subtilis* (NBRC 13722), *L. acidophilus* (NBRC 13951), *L. paracasei* (NBRC 15889), and *L .plantarum* (NBRC 101978) were obtained from the National Institute of Technology and Evolution (NITE) of Japan.


**Figure 3.** Airag samples collcted from 4 different province in Mongolia.
Arkhangai province, Khashaat soum; Bulgan province, Saikhan soum;
Uvurkhangai province, Bayan-Undur soum; Tuv province, Delgerkhaan soum

MRS and M17 broths and fat-free dry skim milk were obtained from Oxoid Co. Ltd., Hampshire, England. α-Chymotrypsin, trypsin, and proteinase K were obtained from Sigma-Aldrich Co., USA. Cycloheximide and chloramphenicol were purchased from Wako Pure Chemical Co. Ltd., Japan, and Anaeropack was purchased from Mitsubishi Gas Chemical Co. Ltd., Japan. YPG (Yeast, peptone, and glucose) medium was prepared from 10 g/L of yeast extract, 20 g/L of peptone and 50 g/L of glucose in 1000 mL of deionized water at pH 5.0, and the medium was autoclaved for 20 min

at 121°C before use. Kyokai No. 7 (K7) yeast (*Saccharomyces cerevisiae*, NBRC 2347) for sake brewing was purchased from NITE and used as the standard yeast for fermentation.

### 2.2.2. Measurement

The concentration of D-lactose, D-glucose, and ethanol in Airag was measured by an aqueous HPLC system using a Tosoh TSK Amide-80 column (particle size: 2 □m, 7.6 mm x 150 mm) eluted with a mixed solution of acetonitrile-water (7:3 v/v) at a flow rate of 0.5 mL/min by a Tosoh RI-8020 detector and by a Shimazu GC-8A gas chromatograph equipped with a capillary column (SE-30, 3.2 mm x 30m) at 60°C for the hydrogen flame ionization detector and at 130°C for column and injection temperatures. The lactic acid and protein concentrations were determined by a titrimetric method according to the Association of Official Analytical Chemists (AOAC) instruction (AOAC, 1996). Amplification of DNA regions was carried out by Bio-Rad Mycycler and MJ Research Peltier PTC-200 thermal cyclers. Electrophoresis of the PCR products was performed by a Shimadzu MCE-202 MultiNA spectrophotometer. The PCR conditions were heating for 5 min at 95°C as an initial denaturation step before performing, 35cycles consisting of 30 sec at 95°C for denaturation, and 1 min at 58°C for annealing, and 2 min at 72°C for elongation. A final extension step for 10 min at 72°C was performed at the last cycle. The sequence analysis of the DNA regions was measured by an Applied Biosystems AB 3130 genetic analyzer with a 36 cm capillary column. Catalase activity (Batdorj *et al*., 2006) and Gram staining (Bartholomew *et al*., 1952) for the identification of lactic acid bacteria in Airag were performed according to the methods described in the literature. Turbidity was detected by the absorbance at 600 nm using a Hitachi U0080D spectrophotometer.

### 2.2.3. Isolation of lactic acid bacteria and yeasts in Airag samples

Lactic acid bacteria (LAB) and yeasts were isolated from Airag samples according to the direct plating method (Coventry *et al*., 1997). For the isolation of lactic acid bacteria, Airag samples (1 mL) were diluted with water (6 mL) and plated on MRS agar plates with cycloheximide (25μg/mL) as the antibiotic and the plate was incubated under anaerobic conditions for 3 d at 37°C using an Anaeropack. After incubation, colonies obtained were purified by restreaking on a new agar plate and then identified by the 16s rDNA sequencing method.

Yeasts in Airag were also isolated by the same method using YPG agar plate with the antibiotic chloramphenicol (50 μg/mL) and incubating the plate for 3 d at 30°C. Yeasts isolated were identified by the 26s rDNA sequencing method. All purified colonies were suspended into 20% glycerol (w/v) in sterilized tubes and kept at -80°C.

### 2.2.4. Identification of lactic acid bacteria and yeasts

Lactic acid bacteria and yeasts in Airag were identified by 16s rDNA and 26s rDNA analyses and then by using BLAST (National Center of Biotechnological Information, USA). The partial sequence of LAB from the universal primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 802R (5'-TACCAGGGTATCTAATCC-3') and identification of yeast using NL4 (5'-GGTCCGTGTTTCAAGACGG-3') and NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3').

Genomic DNA was extracted and purified using IsoPlant II gene extract kit (Nippon Gene, Tokyo, Japan) and the DNA was used as the template for sequencing. About 500-1000 bp of 16S and 26S rDNA was amplified by polymerase chain reaction (PCR). PCR performed with a Bio-Rad Mycycler and MJ Research Peltier PTC-200 thermal cyclers. The cycle sequence was carried out with the Micro SeQ 500 16S rDNA Bacterial Identification Sequencing Kit (Applied Biosystems). The results are shown in Table 2

## 2.3. Results and Discussion

### 2.3.1. Chemical and physical properties of the Airag samples

Airag is a health-promoting beverage that is, in general, prepared by stirring and fermenting fresh mare's milk with a small amount of a previously fermented Airag containing LAB and yeasts. The time for making airag is from start of June of every year till autumn, October. The traditional method of making airag is to bring unpasteurized mare's milk stored in cow's or horse's hide, then one third of old mink is mixed with new milk and churned, beaten with a wooden stick. During fermentation of airag, a continually beaten will be able to develop good flavors and no abnormal fermentation, either (Wu et al, 2009).

There are several papers on separation and identification of LAB in Mongolian fermented milks and characterization as beverages. However, few papers have been reported on the exact functionality or biological activities of lactic acid bacteria in Airag. In this work, six Airag samples were collected in four provinces of Mongolia and we investigated the isolation and characterization of lactic acid bacteria and yeasts in Airag to reveal the functionality. Table 1 shows the results of component analysis of Airag samples, which in main were lactic acid (1.1-1.2 w/w%), lactose (3.5-5.0 w/w%), glucose (0-0.18 w/w%), ethanol (2.7-3.1 w/w%), and proteins. The density was around 1.02 g/cm$^3$. Airag samples collected in Bulgan Saikhan-I (Bul-I) and Tuv Delgerkhaan (Tuv) had higher ethanol and lactose concentrations, respectively.

**Table 2**.Component analysis of Airag samples[a]

| Collected Province of Airag sample | Lactic acid w/w% | Lactose w/w% | Glucose w/w% | Ethanol w/w% | Total protein w/w% | Density g/cm$^3$ |
|---|---|---|---|---|---|---|
| Arkhangai Khashaat | 1.1±0.01 | 4.3±0.01 | 0.13±0.01 | 3.2±0.20 | 3.0±0.01 | 1.02±0.01 |
| Bulgan Saikhan-I | 1.2±0.02 | 3.9±0.01 | nd | 3.5±0.10 | 3.1±0.02 | 1.02±0.02 |
| Bulgan Saikhan-II | 1.2±0.02 | 3.5±0.01 | nd | 2.9±0.01 | 2.8±0.10 | 1.02±0 |
| Bulgan Saikhan-III | 1.2±0.10 | 3.6±0.03 | nd | 3.0±0.02 | 2.7±0.20 | 1.02±0 |
| Uvurkhangai BU | 1.1±0.01 | 4.6±0.01 | 0.17±0.01 | 2.1±0.01 | 2.8±0.30 | 1.02±0.01 |
| Tuv Delgerkhaan | 1.1±0.01 | 5.0±0.02 | 0.18±0.02 | 2.3±0.01 | 2.9±0.10 | 1.02±0.01 |

a) Component analysis was carried out three times and showed the mean value. nd: not detecte

The nourishment value, fragrance and taste of Airag depends on local climate and plant kingdom. We were collected airag samples from four different regions of Mongolia that are making the best airag area. Baldorj et al reported that the best fragrance and taste with airag contains mainly *Lactobacillus* species with restricted acidification properties, lower the pH to 4.5-3.9 at the end of the process and whose conversion ratio of lactose into lactate is about approximately 50% (Baldorj, 2003). The lactic acid concentration of the airag samples lower than those reported by Sun et al, who reported that lactic acids of the airag samples were 1-1.2%. The ethanol content was higher than Miyamoto et al, those are approximately 2.1-3.5%. These results were consistent with previous reports (Baldorj et al., 2000; Watabe et al., 1998; Burentegushi et al., 2002; Watanabe et al., 2009 and Miyamoto et al., 2010).

### 2.3.2. Isolation of lactic acid bacteria in Airag samples

The aim of this section was to isolate LAB with biological potential from airag sample. Microbial diversity of Airag has been the subjected of a considerable studies by Baldorj et al, 2000; Danova et al., 2005; Batdorj et al., 2006; Watanabe et al., 2008; Miyamoto et al., 2010; Sun et al., 2010 and Takeda et al., 2011 who airag sample were isolated from Mongolia and Burentegushi et al., 2002; Ishii et al., 1999 et al., An et al., 2004; Shuanquan et al., 2006; Bilige et al., 2009 and Sun et al., 2010 who airag samples were isolated from Inner Mongolia in China. Both regions are nearby each other in geography (Wu et al., 2009).

In this experiment as shown in Table 2 the isolation and identification of thirty five lactic acid bacteria in Airag samples and tested for their biological potential. Lactic acid bacteria were isolated by the direct plating method using a MRS agar plate with cycloheximide as an antibiotic. It was found that each Airag was contained in several kinds of bacteria. The bacteria strains isolated had not catalase activity (Batdorj *et al*., 2006) and were Gram positive (Bartholomew *et al*., 1952), indicating that all bacteria isolated were lactic acid bacteria (Batdorj *et al*., 2006).

Initially, the isolated strains were named according to their origin. For example all isolated strains from four different regions of Mongolia were named as Arkhangai province (Ark), Bulgan province (Bul), Uvurkhangai province (Uvu) and Tuv province (Tuv), respectively.

All isolates were further tested for morphological and phenotypic characterization. The single colonies of LAB isolates grown on MRS agar plate were showed in Figure 3 which were short and long rod shaped.

Bacterial growth depends on culture medium with its ability to produce a high concentration of cell biomass. The group of complex nutrients such as skim milk, yeast extract (that provides vitamin B complex and dextrose is the fermentable carbohydrate resource) and peptone (to supply nitrogenous and carbonaceous compounds) and polysorbate 80 (fatty acids required for the metabolism of Lactobacilli) were used to satisfy the complex demands of the bacteria. A complex medium, especially MRS is usually employed and supported the growth when cultivating of LAB (Pallin, 0000).

**Table 3**. Isolation and identification of lactic acid bacteria in Airag samples[a]

| Name | Collected province | Catalase activity[b] | Gram staining[b] | Identified species[c] | Identity % |
|---|---|---|---|---|---|
| Ark-1 | Arkhangai Khashaat | - | + | *Enterococcus hirae* | 100 |
| Ark-2 | Arkhangai Khashaat | - | + | *Enterococcus faecalis* | 98 |
| Bul-3 | Bulgan Saikhan-I | - | + | *Enterococcus hirae* | 99 |
| BuI-4 | Bulgan Saikhan-I | - | + | *Enterococcus durans* | 99 |
| BuI-5 | Bulgan Saikhan-I | - | + | *Lactobacillus helveticus* | 100 |
| BuI-6 | Bulgan Saikhan-I | - | + | *Enterococcus faecium* | 100 |
| Bul-7 | Bulgan Saikhan-I | - | + | *Lactobacillus kefiri* | 98 |
| BuI-8 | Bulgan Saikhan-II | - | + | *Enterococcus faecium* | 100 |
| BuI-9 | Bulgan Saikhan-II | - | + | *Lactobacillus sp.* | 98 |
| BuI-10 | Bulgan Saikhan-II | - | + | *Lactobacillus casei* | 99 |
| BuI-11 | Bulgan Saikhan-II | - | + | *Lactobacillus casei* | 100 |
| BuI-12 | Bulgan Saikhan-II | - | + | *Lactobacillus helveticus* | 100 |
| BuI-13 | Bulgan Saikhan-II | - | + | *Lactobacillus kefiri* | 99 |
| BuI-14 | Bulgan Saikhan-III | - | + | *Lactobacillus casei* | 99 |
| Bul-15 | Bulgan Saikhan-III | - | + | *Lactobacillus casei* | 100 |
| BuI-16 | Bulgan Saikhan-III | - | + | *Lactobacillus helveticus* | 99 |
| Bul-17 | Bulgan Saikhan-III | - | + | *Lactobacillus kefiri* | 98 |
| Bul-18 | Bulgan Saikhan-III | - | + | *Lactobacillus paracasei* | 98 |
| Bul-19 | Bulgan Saikhan-III | - | + | *Lactobacillus paracasei* | 99 |
| BuI-20 | Bulgan Saikhan-III | - | + | *Lactobacillus hilgardii* | 100 |
| Uvu-21 | Uvurkhangai BU | - | + | *Lactobacillus hilgardii* | 100 |
| Uvu-22 | Uvurkhangai BU | - | + | *Lactobacillus hilgardii* | 99 |
| Uvu-23 | Uvurkhangai BU | - | + | *Lactobacillus hilgardii* | 98 |
| Uvu-24 | Uvurkhangai BU | - | + | *Lactobacillus diolivorans* | 100 |
| Uvu-25 | Uvurkhangai BU | - | + | *Lactobacillus diolivorans* | 99 |
| Uvu-26 | Uvurkhangai BU | - | + | *Lactobacillus paracasei* | 99 |
| Uvu-27 | Uvurkhangai BU | - | + | *Lactobacillus helveticus* | 98 |
| Uvu-28 | Uvurkhangai BU | - | + | *Lactobacillus helveticus* | 100 |
| Uvu-29 | Uvurkhangai BU | - | + | *Enterococcus durans* | 100 |
| Uvu-30 | Uvurkhangai BU | - | + | *Enterococcus sp.* | 100 |
| Tuv-31 | Tuv Delgerkhaan | - | + | *Lactobacillus casei* | 99 |
| Tuv-32 | Tuv Delgerkhaan | - | + | *Lactobacillus casei* | 100 |
| Tuv-33 | Tuv Delgerkhaan | - | + | *Lactobacillus diolivorans* | 99 |
| Tuv-34 | Tuv Delgerkhaan | - | + | *Lactobacillus paracasei* | 98 |
| Tuv-35 | Tuv Delgerkhaan | - | + | *Lactobacillus kefiri* | 98 |

a) Airag samples were collected in Mongolia on July, 2014.
b) Catalase activity and Gram staining were carried out according to the reported method (Batdorj et al., 2006; AOAC, 1996).
c) Identification of lactic acid bacteria was performed by the 16s rDNA sequencing analysis.

This thirty five isolates were identified based on 98-100 % of percent identity which was obtained by analyzing partial sequences of 16S rDNA gene on BLAST tools. Eight isolates of *Enterococcus* sp, twenty seven isolates of *Lactobacillus* were identified as belonging to 9 validated species: *E.hirae, E.durans, E.Faecalis, L.helveticus, L.kefiri, L.casei, L.paracasei, L.Hilgardii* and *L.diolivorans.*

Previous studies also showed that all the airag samples collected various regions of Mongolia contained *L.helveticus* as predominant LAB (Uchida et al., 2007; Watanabe et al., 2008; Sun et al., 2010; Takeda et al., 2011 and Suk-Ho et al., 2016). *L.helveticus, L.delbrueckii* subsp. *lactis, L.plantarum* and *L.fermentum* were dominant species from seven different airag samples that collected from Ulaanbaatar in Mongolia were identified by Takeda et al and Sun et al. The main species of LAB of airag samples collected from three nomadic families in Dund-Gobi province in Mongolia consist of *L.helveticus* and *L.kefiri* (Uchida et al., 2007).

A similar observation with *L.helveticus, L.paracasei, L.casei* and *L.kefiranofaciens*strains found in airag of Mongolia was published by Watanabe et al. In addition, we was found *L.diolivorans* and *L.hilgardii* as shown in Table 2. Watanabe et al reported the identification of *L.hilgardii* and *L.diolivirans* were isolated in airag of Mongolia. Also these strains were isolated from tarag samles of Inner Mongolia, China observed by Wenjun et al (Wenjun et al., 2012).

A few strains of *Enterococcus* sp. with bioactive potential and satisfying stringent technological characteristics as well, have already been evaluated and used as starters of the production of fermented milk with hypotensive and angiotensin-L-converting enzyme inhibitory activity (Muguerza et al., 2006 and Clemencia et al., 2011). In our study, three different *Enterococcus* species were identified from Airag samples that examined antibacterial and proteolytic activities (Results shown in next chapter).

More specific *L.casei* strains are constantly recognized as the putative candidates of probiotic which may contribute to the health of human being with beneficial and nutritional properties (Rina et al., 2009).

The uniproperties of *L.diolivorans* strain, such as stability in the gastrointestinal tract, the wide spectrum of bactericidal and fungicidal action to the pathogenic species, the relatively high superoxide dismutase and proteolytic activities, and the absence of toxicity, the make it a prime candidate for probiotic culturing (Lidia et al., 2014). Besides, Krooneman et al. demonstrated that the based on the ability of *L.diolivorans* to ferment 1,2-proppanediol to 1-propanol and propionate under conditions that majorityin silage and the observed absence of 1,2-propanediol in experimental and full scale aerobically stable silages, *L.diolivorans* may play an important role in stabilizing maize silages (Krooneman et al., 2002).

### 2.3.3. Isolation and fermentation of yeasts in Airag samples

Yeasts in Airag were isolated by incubation with chloramphenicol as an antibiotic and the identification was performed by 26s rDNA sequencing to give two yeasts, *K. unispora* and *Kazachstania* sp. Among these species were isolated from airag sample from Mongolia and Inner Mongolia of China (Watanabe et al, 2008, Miyamoto et al., 2010 and Mu et al, 2012) which are also found in other traditional fermented dairy products (Lopandic et al, 2006; Nurgul et al, 2009; Wu et al, 2012).

After the two yeasts had proliferated on YPG agar plates, the fermentation ability was compared with that of K7 yeast used for sake brewing. Table 4 shows the results of identification and fermentation. Glucose is ferment to ethanol by Saccharomyces cerevisiae strains that have been selected through a long history of cultivation, ranging from 100 to 400 yeasrs (Shiroma et al, 2014).

The most commonly used sake yeast as *Saccharomyces cerevisiae* sake yeast strain Kyokai no.7 has one of the highest fermentation rates among brewery yeasrts used worldwide (Shiroma et al, 2014) which was isolated from sake mash in 1946 (Tsukahara et al, 1947).

Ethanol fermentation of glucose (2.0 g) was carried out with the yeast ($1x10^8$ cells/mL) in deionized water for 72 h at 30°C and pH 5.0. Ethanol fermentation with a mixture of two yeasts (number of each yeast was $1x10^4$ cells/mL) was also performed.

Although K7 yeast produced ethanol at the concentration of 5.4 g/L, yeasts isolated from Airag produced 2.1-3.0 g/L, respectively. The ethanol concentration by a mixture of two yeasts gave almost the same, 2.3 g/L. Thus, the fermentation ability of yeasts isolated from Airag was lower than that of K7 yeast.

After 72h fermentation, glucose was still detected for *K. unisporia* and *Kazachstania* sp. yeasts isolated from Arkhangai Khashaat and Bulgan Saikhan Airag samples but no glucose remained in the fermented solution obtained using *K. unisporia* isolated from a Tuv Delgerkhaan Airag sample. These results were in agreement with the low ethanol concentration of the Airag used here, as shown in Table 1.

**Table 4**.Identification and fermentation of glucose by yeast in Airag sample.

| No | Collected Province | Classification[a] | Identity[a] % | Ethanol[b] g/L | Glucose remained g/L |
|---|---|---|---|---|---|
| 1 | Arkhangai Khashaat | *Kazachstania unispora* | 97 | 2.0 | 6.2 |
| 2 | Bulgan Saikhan | *Kazachstania unispora* | 99 | 2.1 | 6.0 |
| 3 | Uvurkhangai BU | *Kazachstania* sp | 99 | 2.6 | 2.1 |
| 4 | Tuv Delgerkhaan | *Kazachstania unispora* | 100 | 3.0 | 0 |
| 5[c] | Uvurkhangai BU | *Kazachstania* sp | | 2.3 | 2.9 |
| | Tuv Delgerkhaan | *Kazachstania unispora* | | | |
| | | K7[d] | | 5.4 | 0 |

a) Yeasts in Airag were identified by the 26s rDNA analysis

b)Ethanol fermentation with glucose (2.0 g) by yeast ($1x10^8$ cells/mL) was carried out in deionized water for 72 h at 30°C and pH 5.0.

c) A mixture of two yeasts, *Kazachstania* sp ($1x10^4$ cells/mL) and *Kazachstania unispora* ($1x10^4$ cells/mL), was used for ethanol fermentation.

d) K7 yeast purchased from NITE, Japan, was used as standard yeast.

## 2.4. Conclusion

In conclusion, thirty five lactic acid bacteria were isolated from six Airag samples collected in four different provinces of Mongolia. The six species LAB was identified as *Lactobacillus helveticus* (5 strain) , *L.casei* (6 strain), *L.diolivorans* (3 strain), *L.hilgardii* (4 strain), *L.paracasei* (4 strain) *and L.kefiri* (4 strain) by 16S rDNA sequencing analysis. Two strains of LAB *L. hilgardii* (Uvu-21), *L. diolivorans* (Tuv-33) were discovered high potent proteolytic and antibacterial activities, which LABs were detected on Mongolian traditional tarag as yogurt and airag sample by Watanabe et al, 2009, Oki et al, 2013 and Wenjun et al, 2012. But they were carried out just identification of LAB in traditional dairy products. In our study, this *L. hilgardii* (Uvu-21), *L. diolivorans* (Tuv-33) was examined to biological activities in first time.

In addition, two yeasts were also isolated, and their ability to ferment glucose was compared with that of K7 yeast used for sake brewing. The fermentation ability of the two yeasts was weaker than that of K7 yeast, producing 2.1-3.0 g/L of ethanol. These results were in agreement with the low ethanol concentrations in Airag samples.

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# CHAPTER III

# BIOLOGICAL ACTIVITY OF LACTIC ACID BACTERIA

## 3.1 Introduction

Currently, food industry depends on chemical preservatives to extend the shelf life. The costumers' awareness about the health hazards associated with chemicals have recently increased, and they are demanding for processed foods that are free of preservatives. There are many alternative natural preservatives produced by certain microorganisms, however these microorganisms have to be non-toxic, easy to grow and require simple media for cultivation. Lactic acid bacteria (LAB) are a known potential source for generating a variety of secondary metabolites such as bacteriocines, organic acids and peptides (Lihua *et al*, 2013). There are common bacteria that has been used as a starter culture for the production of fermented foods and beverages and they are friendly bacteria with the status General Recognized as Safe (GRAS). These characteristics have attracted the interest of many researchers for peptides production (Rashid *et* al, 2009; Rodney *et al*, 2014; Belai *et al*., 2016).

Milk protein allergens can be degraded by some proteolytic enzymes produced by LAB during microbial fermentation (Jivka *et al*, 2014) even though they constitute a much unsteady proteolytic system capable of hydrolyzing proteins to low weight peptides and amino acids (Cássia *et al*, 2010). That is why the fermentation with LAB is considered as an effective way to reduce whey protein antigenicity (Jivka *et al*, 2014). Proteolytic systems of LAB have included two kind of proteolytic enzymes as proteinase and peptidase, these are secreted in outside and inside of cell.

In Bulgarian dairy fermented products commonly are used for starter cultures three different genera as *Lactobacillus*, *Lactococcus* and *Streptococcus.* Recently, there are an increasing commercial interest in the production of bioactive peptides from fermented milk (Jivka *et al*, 2014).

The high potential proteins and peptides derived from milk have been provided a non-immune disease defense and control of microbial infections (McCann *et al*, 2006; Isidra *et al*, 2009) which is mostly accepted that the total antibacterial effect on milk is more than the sum of the individual contributions of immunoglobulin, non-immunoglobulin defense proteins like lactoferrin, lactoperoxidase lysozyme and small peptides. This way via synergistic activity of naturally occurring proteins and peptides in addition to peptides produced from inactive protein signals (McCann *et al*, 2006). Typically, antibacterial peptides contains a high portion of basic amino acid residues in an amphipathic structure which have containing hydrophilic and hydrophobic components as in the case of phospholipid molecule (Thomas *et al*, 2014)

The aim of this study was to evaluate the antibacterial and proteolytic activities of LAB from Mongolian Airag, as a traditional fermented mare’s milk.

## 3.2 Material and Methods

In this part, a totally thirty five LAB strains isolated from Airag sample from 6 different regions of Mongolian traditional fermented mare's milk beverage and preliminary identified by morphological and biochemical tests. The microbial cultures were grown and maintained in MRS and M17 as selective broth mediums for LAB that were evaluated for proteolytic and antibacterial activity.

First of all, the frozen LAB strains at -80°C that were reactivated by growing them three times in selective medium at 37°C for 24 h. Then we were screened two kinds of biological activity in all activated LAB strains. Secondly, from screening experiments were detected strains with high potential biological activities, which are determined to protease amount and antibacterial activity by AU unit.

### 3.2.1a. Screening test of Antibacterial activity of supernatant produced by lactic acid bacteria

The antibacterial activity of the supernatant was determined by comparison with that of standard lactic acid bacteria according to the agar well diffusion method (Li *et al*, 2013; Jivka *et al*., 2014). A lactic acid bacterium ($8x10^6$ cells/mL) isolated in Airag was grown in 3 mL MRS broth under anaerobic conditions for 24 h at 37°C. The culture was centrifuged for 10 min at 8000G and then the supernatant was adjusted to pH 6.5 by 0.5 N aqueous NaOH. The supernatant (50 µL) was placed on *E. coli* and *B. subtilis* agar plates and incubated overnight at 37°C. The antibacterial activity was measured by the diameter of each inhibited circle around the wells on the agar plate and expressed as an arbitrary unit (AU) per mL. One AU was defined by the reciprocal of the highest serial 2-fold dilution (Hernández *et al*., 2005; Wulijideligen *et al*, 2012).

### 3.2.1b. Screening test of proteolytic activity of supernatant produced by lactic acid bacteria

The proteolytic activity of the supernatant was assayed using agar plates with 2.5% skim milk and the activity was determined by the diameter of the hydrolyzed clear circle around the lactic acid bacteria (Mechai *et al*., 2014; Guessas *et al*., 2012). The supernatant (50 µL) was placed on an agar plate containing 2.5% skim milk and the plate was incubated for 16 h at 37°C. The proteolytic activity was determined by the diameter of each inhibition circle around the wells and represented as units per mL. One unit of the protease activity was determined by the amount of the supernatant (1 mL) that released 1 µg of tyrosine per 1 min under the above conditions (Jivka *et al*., 2014; Wildeboer *et al*., 2009).

The antibacterial and proteolytic activities were compared with those of three standard lactic acid bacteria, *L. acidophilus*, *L. paracasei*, and *L. plantarum*.

### 3.2.2. Time course of antibacterial activity of supernatant produced by lactic acid bacteria on *E. coli* and *B.subtilus*

A typical procedure for the antibacterial activity of the supernatant produced by lactic acid bacteria on *E. coli* and *B. subtilis* was as follows. A lactic acid bacterium, *L. diolivorans* (Tuv-33) ($1x10^8$ cells/mL) was added to MRS medium (3 mL) and then the mixture was cultured for 24 h at 37°C. After centrifugation, *E. coli* ($8x10^6$ cells/mL) and LB broth (1.5 mL) were added to the supernatant (1.5 mL) and the mixture was gently stirred for 24 h at 37°C. For determination of antibacterial activity, the turbidity of the solution was measured by the absorbance at 600 nm at each prescribed time. The results are presented in Figure 1. The antibacterial activity on *Bacillus subtilis* was also examined by the same procedure as above.

### 3.2.3. Thermal stability of supernatant produced by lactic acid bacteria

The supernatant (1.5 mL) described in 2.6 was heated at 100°C for 15 min, 30 min, 60 min and 180 min, respectively. After cooling, *E. coli* ($8x10^6$ cells/mL) and LB broth (1.5 mL) were added to the solution and then the mixture was cultured for 24 h at 37°C. The turbidity was measured by the absorbance at 600 nm. The results are shown in Figure 3.

### 3.2.4 pH dependence on biological activity of lactic acid bacteria

The pH dependence of the supernatant was performed as follows: After lactic acid bacteria were incubated in MRS broth under anaerobic conditions for 24 h at 37°C and then the pH of the supernatant was adjusted at the desired pH showed in Figure 2. The supernatant was stirred for further 2 h and readjusted to the pH at 6.5. The supernatant was used for the pH dependence on *E. coli* and *B. subtilis*.

The relationship between the pH of the supernatant of lactic acid bacteria and biological activity was elucidated by changing the pH of the supernatant and using the same procedure as described in section 2.5.

### 3.2.5 Enzyme sensitivity of cell free supernatant of LAB

Cell free supernatant were treated with various enzymes such as proteinase K (pH 7.5, 37°C), trypsin (pH 8.0, 25°C), α-chymotrypsin (pH 7.8, 25°C), catalase (pH 7.0, 37°C). All enzymes were obtained from Sigma, St. Louis, MO. USA and used at a final concentration of 1mg/ml. Cell free supernatant without enzymes was used as the control. All preparations were incubated for 1 hour at their respective temperatures and residual antibacterial activity against *E.coli* and *B.subtilus* was examined using the agar well diffusion method. All determinations were repeated in duplicate.

## 3.3 Results and Discussion

### 3.3.1. Antibacterial and proteolytic activities of lactic acid bacteria

All lactic acid bacteria were examined for antibacterial activity against two different bacteria as *E. coli*, *B. subtilis* that are gram negative and positive. Also in all LAB strains screened for their proteolytic potential on 2.5% skim milk agar plate. Inhibitor activity was measured by estimating the diameter of circle zone after incubation hour. We was used to positive control by antibacterial peptide as nisin (Sigma Aldrich, USA) and negative control by MRS broth. The result of screening assays shown as Figure.4.

From 12 different species 35 strains were screened antibacterial and proteolytic activities, which had a broad to observed inhibitory spectrum on 9 different species, 22 LAB strains. This results abbreviated in Table 5.

Both of biological activity in the cell free supernatant was evaluated based on their ability to generate circle zone in medium plate. From the result of a screening assay, six strains of LAB were selected for further studies that were determined to concentration of inhibitor activities, and the biological activities were compared with those of standard lactic acid bacteria, *L. acidophilus*, *L. paracasei*, and *L. plantarum*. The results are shown in Table 3. Among the lactic acid bacteria in Table 2, the supernatants of ten species lactic acid bacteria were found to have one or both biological activities, as shown in Table 6.

**Table 5**. Identification of lactic acid bacteria from Mongolian Airag samples and their screening assays

| No | Name | Catalase activity | Gram strain | Classification | Identity % | Antibacterial activity* | Proteolytic activity* |
|---|---|---|---|---|---|---|---|
| Lactic acid bacteria | | | | | | **A** | **B** |
| 1 | Ar-1 | - | + | *Enterococcus hirae* | 100 | + | - |
| 2 | Ar-2 | - | + | *Enterococcus faecalis* | 98 | + | - |
| 3 | Bul-4 | - | + | *Enterococcus durans* | 99 | - | - |
| 4 | Bul-5 | - | + | *Lactobacillus helveticus* | 100 | ++ | +++ |
| 5 | Bul-6 | - | + | *Enterococcus faecium* | 100 | - | + |
| 6 | Bul-9 | - | + | *Lactobacillus sp.* | 98 | + | ++ |
| 7 | Bul-10 | - | + | *Lactobacillus casei* | 100 | ++ | ++ |
| 8 | Bul-12 | - | + | *Lactobacillus kefiri* | 98 | +++ | + |
| 9 | Tuv-25 | - | + | *Lactobacillus diolivorans* | 99 | +++ | +++ |
| 10 | Tuv-26 | - | + | *Lactobacillus paracasei* | 98 | ++ | - |
| 11 | Uvu-18 | - | + | *Lactobacillus hilgardii* | 100 | +++ | ++ |
| 12 | Uvu-23 | - | + | *Enterococcus sp.* | 100 | - | - |
| Type strains of Lactic acid bacteria | | | | | | | |
| 1 | NBRC 13951 | | | *Lactobacillus acidophilus* | | +++ | + |
| 2 | NBRC 15889 | | | *Lactobacillus paracasei* | | +++ | +++ |
| 3 | NBRC 101978 | | | *Lactobacillus plantarum* | | - | ++ |

a) (**A**)-Inhibition zone of Antibacterial activity; (**B**) Inhibition zone of Proteolytic activity;
b) (+)-Inhibition zone ≤3 mm; (++)- 3-5mm; (+++)- Inhibition zone ≥5.
c) *Antibacterial and proteolytic activity was carried out agar diffusion assays described in Material and methods.

**Table 6**. Biological activity of lactic acid bacteria on *E. coli* and *Bacillus subtilis* in Mongolian Airag samples.

| Name | Classification | | Antibacterial activity | | Protease activity[a] |
|---|---|---|---|---|---|
| | | | *E. coli* | *B. subtilis* | |
| | | % | AU/mL | AU/mL | unit/mL |
| Ar-1 | *Enterococcus hirae* | 100 | 200 | 200 | 0 |
| Ar-2 | *Enterococcus faecalis* | 98 | 200 | 200 | 0 |
| Bul-5 | *Lactobacillus helveticus* | 100 | 400 | 400 | 2.1 |
| Bul-6 | *Enterococcus faecium* | 100 | 0 | 200 | 0 |
| Bul-9 | *Lactobacillus sp.* | 98 | 200 | 0 | 0.5 |
| Bul-11 | *Lactobacillus casei* | 100 | 800 | 800 | 0.9 |
| Bul-17 | *Lactobacillus kefiri* | 98 | 1600 | 1600 | 0 |
| Uvu-21 | *Lactobacillus hilgardii* | 100 | 1600 | 1600 | 0.8 |
| Tuv-33 | *Lactobacillus diolivorans* | 99 | 1600 | 1600 | 3.9 |
| Tuv-34 | *Lactobacillus paracasei* | 98 | 400 | 400 | 0 |
| NBRC 13951 | *Lactobacillus acidophilus* | | 800 | 800 | 0 |
| NBRC 15889 | *Lactobacillus paracasei* | | 200 | 0 | 3.2 |
| NBRC 101978 | *Lactobacillus plantarum* | | 0 | 0 | 0.4 |

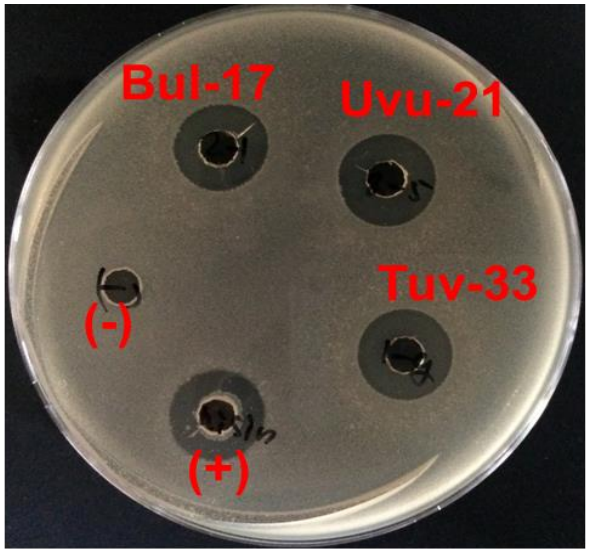
a) One unit of the proteolytic activity was determined by the amount of the supernatant (1 mL) that released 1 □g of tyrosine for 1 min.

It was found that lactic acid bacteria with higher antibacterial activity were contained in each Airag sample. Three lactic acid bacteria, *L. kefiri* (Bul-17), *L. hilgardii* (Uvu-21), and *L. diolivorans* (Tuv-33), produced an inhibition circle zone with a diameter of more than 5 mm on the agar plate containing *E. coli* and were found to have potent antibacterial activity as high as 1600 AU/mL.

Seven lactic acid bacteria in Table 3 had proteolytic activity on the agar plate containing 2.5% skim milk. The skim milk is a source of carbohydrate as lactose and casein or other nutrients demanded for the growth of LAB.

Among them, *L. diolivorans* (Tuv-33) had the highest protease activity more than 3.9 unit/mL. These results suggest that *L. diolivorans* (Tuv- 33) had the most potent biological activity. The results proved that protease was perhaps extracellular and was secreted to culture supernantant (Kaur, 2013)

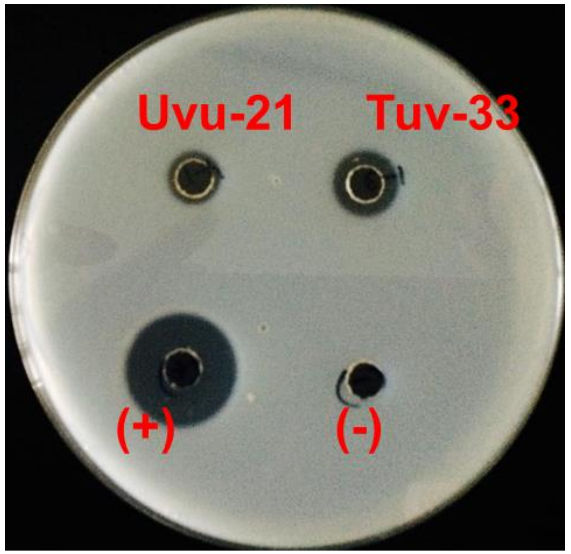
**Figure 4.** Screening assay of Antibacterial and Proteolytic activities on Cell free supernatant by LAB




**Nutrient agar with 100µl *B.subtilis* ($1x10^8$ cell/ml) 24 h at $37^0$C**

**a) Positive- 1mg/ml Nisin from *Lactococcus lactis***

**b) Negative- Pure MRS broth**




**2.5% Skim milk agar, 24h at $37^0$C**

**a) Positive- 1mg/ml Nisin from *Lactococcus lactis***

**b) Negative- Pure MRS broth**

### 3.3.2. Time course of antibacterial activity of supernatant produced by lactic acid bacteria on *E. coli* and *B.subtilus*

To study of antibacterial activity in liquid medium, the viable bacterial cells were inoculated on to the autoclaved Nutrient Broth and incubated for 24 h at 37°C. The time course of the bacterial growth can be observed by turbidity during incubation period. Figure 5 shows the time course of antibacterial activity of the supernatant of lactic acid bacteria on *E. coli*. and *Bacillus subtilis* as Gram positive and negative strains, respectively. In Figures 1A and 1B, the supernatant was incubated with *E. coli* and *B. subtilis* for 24 h at 37°C, respectively, and the turbidity was measured by the absorbance at 600 nm.

The turbidity of the control increased because *E. coli* multiplied without the supernatant of lactic acid bacteria. The turbidity of the two supernatants from *L. casei* (Bul-11) and *L. paracasei* (Tuv-34) increased slightly with increasing incubation time, respectively. However, it was found that the supernatants of *L. helveticus* (Bul-5), *L. kefiri* (Bul-17), *L. hilgadii* (Uvu-21), and *L. diolivorans* (Tuv-33) had potent antibacterial activity on both bacteria because the solutions were still clear after 24 h incubation, indicating that *E. coli* and *B. subtilis* did not multiple.

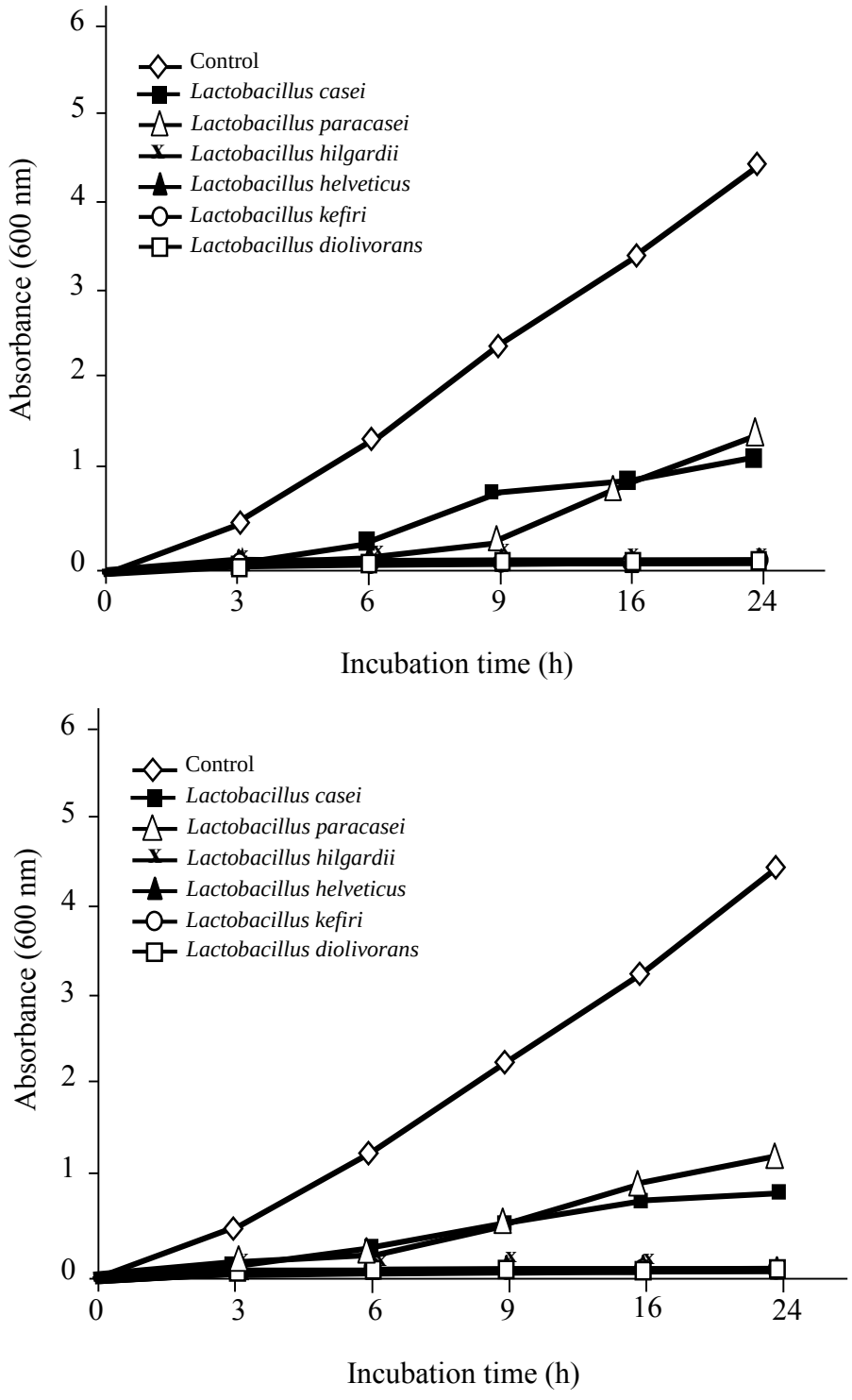



**Fig. 5**. Time course of antibacterial activity of supernatant of lactic acid bacteria. The supernatant was incubated with indicator strains for fixed times at 30°C and the turbidity was measured by the absorbance at 600 nm.

A- *Escherichia coli* (ACCT25922)

B- *Bacillus subtilis* (NBRC13722)

### 3.3.3. pH stability of Antibacterial substance on cell free supernatant

The antibacterial peptides depend on the strong pH and thermal stability. Therefore, we was examined in his experiment in order to indicate to produce bacterial inhibitor activity by antibacterial peptides (Gereltuya *et al*, 2015).

Figure 6 presents the pH dependence of the supernatant produced by the lactic acid bacteria, *L. hilgardii* (Uvu-21) and *L. diolivorans* (Tuv-33), which lactic acid bacteria had potent antibacterial activity between pH 4 and 8 and also showed higher antibacterial activity against *Bacillus subtilis* than *E. coli* in the wide pH range. An increase in the culture pH from 4 to 8 resulted in a raise in maximum inhibitor activity from 800 AU/ml to 1600 AU/ml. At pH 5 to 7 was detected the high inhibitory activity. The activity decreased with decreasing and increasing pH and disappeared at pH 2 and 10.

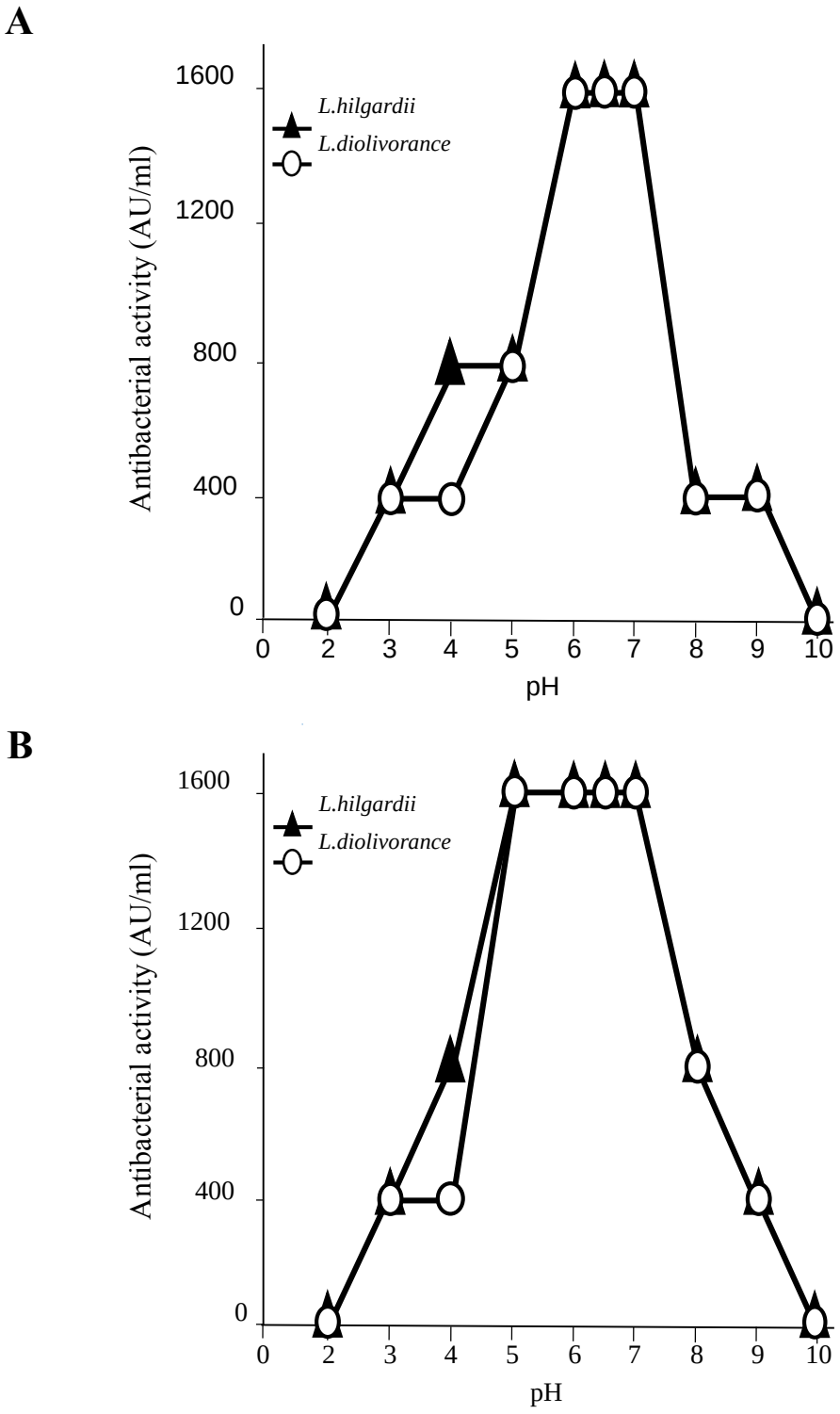



**Fig. 6**. pH Dependence of supernatant of *L. hilgardii* (Uvu-21) and *L. dilolivorans* (Tuv-33) on antibacterial activity of (A) *E. coli* and (B) *B. subtilis*

### 3.3.4. Enzyme sensitivity of cell free supernatant of LAB

In general, the antibacterial activity of LAB originates from peptides. Therefore, the cell-free supernatants of two LAB with potent antibacterial activity, *L. hilgardii* (Uvu-21) and *L. diolivorans* (Tuv-33), were digested by proteases at pH 6.5. After treatment of the supernatant with α-chymotrypsin, trypsin, and proteinase K, respectively, the supernatant was incubated with *E. coli*, and the antibacterial activity of *E. coli* disappeared, suggesting that the antibacterial activity is attributable to the peptides produced by the lactic acid bacteria. The isolation and structure of the peptides are under investigation.

**Table 7**. Antibacterial activity of cell free supernatant of Uvu-21 (*Lactobacillus hilgardii*) and Tuv-33 (*Lactobacillus dilolivorans*) lactic acid bacteria after enzymatic digestion and changing of pH

| Treatment | Residual activity (AU $ml^{-1}$) | |
|---|---|---|
| | Uvu-21 | Tuv-33 |
| Control | 1600 | 1600 |
| Enzymes | | |
| α-Chymotrypsin | 0 | 0 |
| Trypsin | 0 | 0 |
| Proteinase K | 0 | 0 |

* AU $ml^{-1}$ - Arbitrary Unit (One AU was defined as the reciprocal of the highest serial 2-fold dilution that resulted in inhibition of the indicator lawn)

### 3.3.5. Thermal stability of Antibacterial substance on cell free supernatant

The temperature is a major parameter which required to be controlled as it was tested to stability of antibacterial substances.

As shown in Figure 7. Four strains of LAB with high potent antibacterial activity on previous experiments was tested thermal stabilization at 100°C on several time variations.

*L.helveticus* (Bul-5) and *L. kefiri* (Bul-17) disappeared after heating for 30 min at 100°C, the supernatants produced by *L. hilgardii* (Uvu-21) and *L. diolivorans* (Tuv-33) was found to have high thermal stability. After heating for30 min at 100°C, the supernatants had antibacterial activity as potent as that before heating. However, the antibacterial activity of all supernatants decreased after heating for more than 60 min, suggesting that the peptides in the supernatants were denatured by heating. Therefore, the biological activity should originate from peptides produced by the lactic acid bacteria.

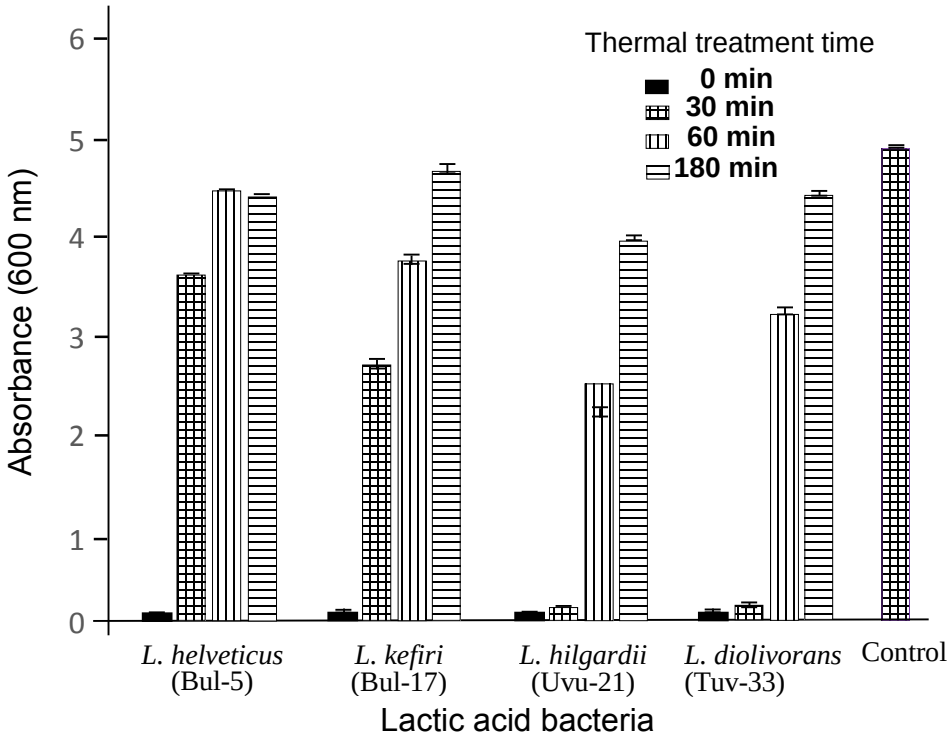



**Fig. 7**. Thermal stability of supernatant of lactic acid bacteria. Supernatant was heated for fixed times at 100°C and then incubated with *E. coli* for 24 h at 37°C. Turbidity was measured by the absorbance at 600 nm.

## 3.4 Conclusion

In this part of experiment, studies on the biological activity as proteolytic and antibacterial activities that we was screened six different species LAB with potent activities. Among the lactic acid bacteria, the supernatants of the ten lactic acid bacteria were found to have potent antibacterial activity against both *E. coli* and *Bacillus subtilis*, and proteolytic activity on 2.5% skim milk. The supernatants of the four lactic acid bacteria, *L. helveticus* (Bul-5), *L. kefiri* (Bul-17), *L. hilgardii* (Uvu-21), and *L. diolivorans* (Tuv-33), completely inhibited the increase of pathogenic *E. coli* and/or *Bacillus subtilis* for 24 h at 37°C because the turbidity measured by the absorbance at 600 nm was near 0 and the solution was clear.

Furthermore, we were focused on antibacterial peptide produced by LAB in cell free supernatant where was studied effect of different parameters as the time course of antibacterial substances in liquid medium, their enzymatic effects and pH, thermal stabilities on CFS against *E.coli* and *B.subtilis.* These parameter depends on a feature of antibacterial peptides (Agraval, 2012). In our study, the antibacterial substances on CFSN by LAB isolated from Mongolian airag sample have probably indicated as peptides that has notable results such as inactivated by proteases, a wide range pH stability and intolerance a high temperature.

The results suggesting that the antibacterial activity is attributable to the peptides produced by the lactic acid bacteria. The isolation and structure of the peptides are under investigation. Therefore we was intended isolation and purification of antibacterial peptides.

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# CHAPTER IV

# ISOLATION AND PURIFICATION OF ANTIBACTERIAL PEPTIDES FROM CELL FREE SUPERNATANT

## 4.1 Introduction

Antibacterial peptides are small proteins occurring in living organisms and are produced as defense molecules against pathogens such as bacteria. Therefore, it is considered the first line of defense in invaded eukaryotic and prokaryotic cells (Papagianni., 2003). Their mode of action varies between peptides. Three factor play a major role in determining the mode of action: the net positive charge on the surface, its 3-D amphipathic structure and the selective disruption points on the target cell membrane (Lazarev., 2010). These actions were studied by Hallock et al. based on solid-state NMR, which showed an interaction between antibacterial peptides and membranes, leading to membrane disruption. However, in terms of medical and food applications, antibacterial peptides have no toxic effects on mammalian cells (Matsuzaki et al., 2009), making them a promising candidate for further studies.

In 1925, the first antibacterial peptides was discovered from Gram-negative bacteria. *Escherichia coli,* known as colicin (Oscáriz *et al*, 2001). Three years after, in England discovery of nisin in 1928, which was an antibacterial peptides produced by the Gram-positive *Streptococcus lactis* (formerly known as *Lactococcus lactis*) (Haely et al., 2010). Since then, several Gram-positive bacteria, in particular soil bacteria bacilli, have been reported to produce antibacterial agent bacitracin, lichenin and cerein, which are produced by *Bacillus subtilus*, *Bacillus lichrniformis* and *Bacillus cereus*, respectively (Oscariz et al., 1999; Martin et al., 2003; Cladera-Olivera et al., 2004;). Production of antibacterial peptides is considered an unstable bacterial characteristic because it changes according to growth conditions. A tradeoff between metabolism and gene expression affect antibacterial peptide biosynthesis during bacterial growth (Riley et al., 2002).

*Paenibacilus alvei* are Gram-positive, rod shaped, motile, spore forming and catalase positive bacteria. The first report of antibacterial peptide production from this bacteria was by Anandaraj et al, who isolated a strain from fermented tomato fruit and detected two antibacterial peptides, Paenibacilin P and Paenibacilin N. other species of *Paenibacillus* have been reported as antibacterial peptide producers too. For example, polymixins, LI-F complex, saltavalin and gatavalin are produced by different strains of *Paenibacillus polymyxa* (Bassam et al., 2013).

The aim of in this study to isolate of antibacterial peptide from cell free supernatant of *Lactobacillus diolivorans* (Tuv-33) strain, screen its antibacterial activity and purification.

## 4.2 Material and Methods

### 4.2.1. Bacterial enumeration

*Lactobacillus diolivorans* (Tuv-33) was isolated from Airag, Mongolian traditional fermented mare's milk beverage, which was identified using 16S rDNA sequencing analysis. It was grown in MRS Broth medium (Oxoid limited, England) at 37°C for 3 d. The test strains used in this section *Escherichia coli* (*E. coli*) (ATCC25922), *Bacillus subtilis* (NBRC 13722), and Nutrient Broth medium at 30°C for 24h.

### 4.2.2 Production of Antibacterial peptides on Cell cultivation

*Lactobacillus diolivorans* (Tuv-33) was grown in MRS Broth at 37°C for 24 h. The resulting culture was inoculated a 1 L flask containing 750ml MRS broth, and incubated 37°C for 3d. The cell free supernatant was obtained 6 h intervals by culture centrifugation at 8000G for 20 min followed by 0.22μm membrane filtration. The cell free supernatant was heated 70°C for 20 min for protease inactivation (Sambrook *et al*, 1989; Pingitore *et al*, 2007). The antibacterial substance production during the *L.diolivorans* (Tuv-33) growth cycle was determined by measuring total activity in Arbitrary Unit (AU), defined as the reciprocal of the highest dilution of supernatant yielding clear zones against indicator strains.

### 4.2.3 Isolation and Purification of Antibacterial peptides from Lactobacillus diolivorans Tuv-33 strain

In this part, Antibacterial peptides were purified by Sephadex for gel filtration, SP Sepharose Fast Flow column for cation exchange column and HPLC. The cell free supernatant (CFS) was loaded at 5 ml crude protein as previously precipitated by ammonium sulfate into a Sephadex G-25 gel filtration column (20 mm x 800mm, Sigma Aldrich, USA), which was equilibrated with 50 mM, pH 7.0 PBS (Sodium Phosphate Buffer) (Yamamoto *et al*, 2003; Rabeeth *et al*, 2012). Three ml fractions were collected from the column at the flow rate 0.5 ml/min, upto 50 fractions and the antibacterial activity of fractions were screened by agar diffusion method by Fleming (Piddock, 1990; Philip, 2001). The fractions were pooled and applied for further step.

The active fractions obtained from gel filtration chromatography (GFC) were concentrated and reloaded into SP Sepharose Fast Flow column (1.6 x 250 mm, GE, HEALTHCARE Sweden). The cation exchange column was equilibrated with 50 mM PBS with salt gradient (0.01M-0.1M NaCl) at 0.5 ml/min, upto 50 fractions, which active fractions concentrated by vacuum evaporation. In this step, partially purified antibacterial substance from SP Sepharose (Ru *et al*, 2012; Bassam *et al*, 2014; Jingping *et al*, 2016) was purified by Sephadex G-10 (Sigma, USA) column 12 x 120 mm with the same buffer.In this step, antibacterial screening was same to purification of gel filtration.

Finally, the active fractions of 2 step column chromatographic purification were used to further purified in a size exclusion HPLC system (TOSOH) consisted of CCPS pump and UV8020 detector that is equipped with Agilent Bio SEC-3 column (3µm, 150A, 4.6 x 300 mm). The protein fractions were eluted in 50 mM PBS, pH 6.8 and the flow rate was 0.35 ml/min.

### 4.2.4 Determination of protein concentration

Protein concentration was determined by using the BCA (Bovine Serum Albumin) Protein assay kit by Bradford's method (Quick Start TM Bradford Protein Assay, Bio RAD, U.S.), as recommended by the supplier. It involves the addition of an acidic dye to protein solution and subsequent measurement at 595 nm with a standard curve provides a relative measurement of protein concentration (Bradford, 1976).

### 4.2.5 Molecular weight determination by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Purity and molecular weight of the active fractions were estimated in a tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (SDS-PAGE) as described by Schägger *et al* (1987) , using vertical gel apparatus (ATTO, Japan) with 14-20% separating gel. Antibacterial peptide preparation and a low molecular weight marker (Precision Plus Protein, Dual Xtra Standards, BIORAD, U.S.) were run at 30 mA for 3h (Hernandéz *et al*, 2005; Jingping *et al*, 2016).

After electrophoresis, the gel was cut into two parts. The half of the gel was stained with Coomassie Brilliant Blue G-250 (ATTO, Japan) and shake slowly for 3 h, and other half part was examined for antibacterial activity, according to Bhunia et al, (1987) a slightly modified. Second part of gel was overlaid with 1% (w/w) nutrient soft agar inoculated with *B.subtilis* and incubated 37°C for overnight (Batdorj et al, 2006).

### 4.2.6 Mass spectrometry

The molecular mass of purified peptides were determined with MALDI-TOF MS (Ultraflex II instrument, Bruker Daltonics Inc. Bruker BioSciences Corporation, Massachusetts). The peptide sample was dissolved in Milli Q water and was mixed 1:4

ratio with a CCA matrix (10 mg/ml, α-cyano-4-hydroxycinnamic acid in 30% acetonitrile containing 0.1% TFA. A volume of 1µl from the mixture was dropped on a stainless steel target. The MALDI-TOF MS was run in the positive refractor mode and the mass range was scanned 500 to 2500 Da (Yun et al, 2015).

## 4.3 Results and discussion

### 4.3.1 Production of Antibacterial peptides produced by Lactobacillus diolivorans (Tuv-33) strain

*Lactobacillus diolivorans* (Tuv-33) strain was grown on MRS broth medium for 24 h at 37°C. The cell free supernatant (CFS) contains antibacterial peptides that was precipitated by 80% $(NH_4)_2SO_4$ (ammonium sulfate). After precipitation, the antibacterial activity was screened on the crude protein by agar diffusion method where it with increasing diameter of inhibition zone was obtained a maximum 8 mm. The total protein was determined for the crude protein, according as Bradford, (1987). Result shown in Table.8.

### 4.3.2 Isolation and Purification of antibacterial peptides on CFS

Many lactic acid bacteria has been produced antibacterial peptides that are generally divided in to two different types as ribosomally synthetized biopeptides and non ribosomally synthetized peptides (Luis, 2002, Jingping et al, 2016). The ribosomally synthetized peptides are shown the most effective action against pathogenic or food born bacteria, which are usually depends on bacteriocins (Cheikhyoussef et al, 2009).
In this study, the antibacterial activity produced by *Lactobacillus diolivorans* (Tuv-33) was isolated and purified by a three-step purification protocol.

First and second step, the antibacterial peptide was partial purified by Sephadex G-25 gel filtration chromatography and SP Sepharose Fast Flow ion exchange chromatography. There were one peak (shown Figure.7), which were examined for antibacterial activity.

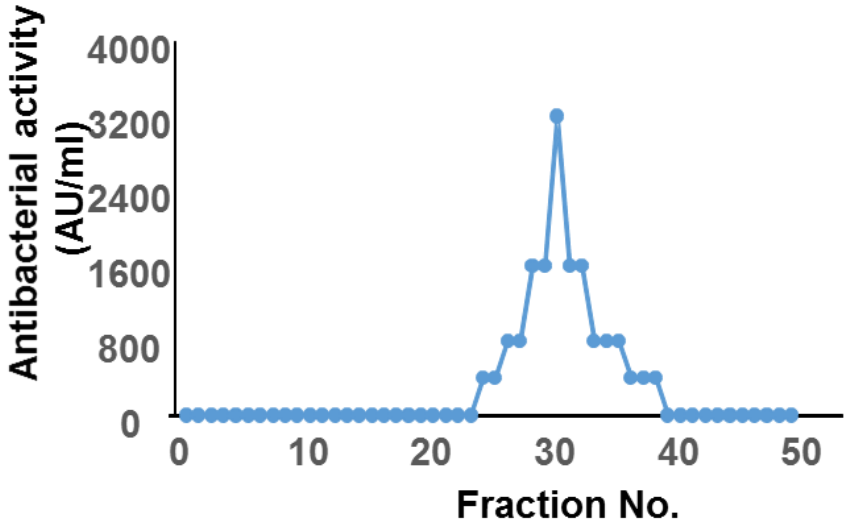


**Figure.8.** Purification of antibacterial peptides from *Lactobacillus diolivorans* (Tuv-33) culture supernatant by Gel Filtration Chromatography (Sephadex G-25 column (200 x 800 mm) and 50 mM PBS, pH 7; Flow rate-0.5 ml/min)

As a shown Figure.8. The fifty active fractions were collected from gel filtration chromatography. Their fifteen fractions were obtained from the crude protein of Tuv-33 strain with retention time between 144 to 234 min. The inhibitor activity for the fractions 26-27, 28-29, 30, 31-32, 33-35 and 36-38 were detected 400, 800, 1600, 3200, 1600, 800 and 400 AU/ml, respectively. These active fractions were concentrated and used to further step of purification.

The crude protein that contains peptides was fractioned by size exclusion chromatography using Sephadex, which is with high molecular weight are eluted in the earlier fractions and the low molecular peptides are eluted in the last fractions (Belai et al, 2016).

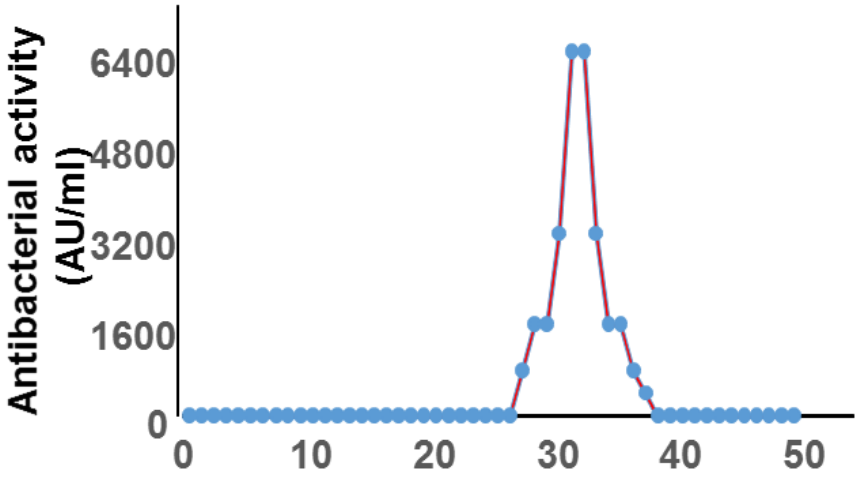


**Figure.9**. Purification of antibacterial peptides from *Lactobacillus diolivorans* (Tuv-33) culture supernatant by Cation Exchange Chromatography
(SP Sepharose Fast Flow (10 x 250 mm) and Flow rate-0.5 ml/min;
50 mM PBS with 0.01-0.1 M NaCl, pH7)

In second step, collected active fractions from the Sephadex G-25 column were applied on a SP Sepharose Fast Flow. Totally fifty fractions were collected and examined antibacterial activity that revealed the presence one main peak eluted at 29 to 40 min (Presented in Figure 9). Thirty two and thirty three fractions were detected high inhibitor activity, 6400 AU/ml which activity was increased than previous step (Table 8.).

A final purification of antibacterial peptides by size exclusion HPLC detected two peaks at 18 and 21 min and their inhibitor activity was equal 1600 AU/ml. In that result obtained to probably two different active peptides in CFS produced by *L.diolivorans* (Tuv-33) strain, Purified antibacterial peptides were further determined molecular weight by mass spectory.

**Table 8.** Purification and activity of Antibacterial peptide

| Purification stage | Volume (ml) | Total protein (mg)* | Total activity (AU) | Specipic activity (AU/mg) | Purification fold |
|---|---|---|---|---|---|
| Supernatant | 750 | 1350 | $120 \times 10^4$ | 888.9 | 1 |
| Ammonium Sulfate precipitation | 50 | 150 | $16 \times 10^4$ | 1066.7 | 1.2 |
| Sephadex G-25 | 50 | 140 | $32 \times 10^4$ | 2285.7 | 2.14 |
| SP Sepharose FF | 1 | 2.5 | $128 \times 10^2$ | 5120 | 2.24 |

*Total protein determined by Bradford method (Bradford, 1987, Batdorj, 2006)

Initially, antibacterial peptides in 750ml CFS was determined total protein with 1350mg and 888.9 AU/mg with specific activity. After SP Sepharose Fas Flow column the specific activity of antibacterial peptides increased to 5120 AU/mg and the final purification fold was 2.24. The antibacterial activity, fold purification and purification way of antibacterial substance are concluded in Table 8.

Batdorj et al, (2006) reported that two kind of bacteriocins named as A5-11A and A5-11B with high antibacterial activity were purified from *Enterococcus durans* A5-11 strains of Mongolian airag. Who was determined total protein in CFS, specific activity of purified peptides that specific activity were increased 256 to 581 88 AU/mg.

Analysis of the antibacterial peptide isolated after chromatography by SDS-PAGE,

### 4.3.3 Molecular weight determination

The analysis of the purified peptides isolated further two step chromatograph by Tricine SDS-PAGE analysis, and direct detection of antibacterial activity on the electrophoresis gel indicated that molecular mass of the substances is approximately between 1800-2500Da . The overlaid part of the gel shows that the fractions are active against indicator strains (Figure.9).

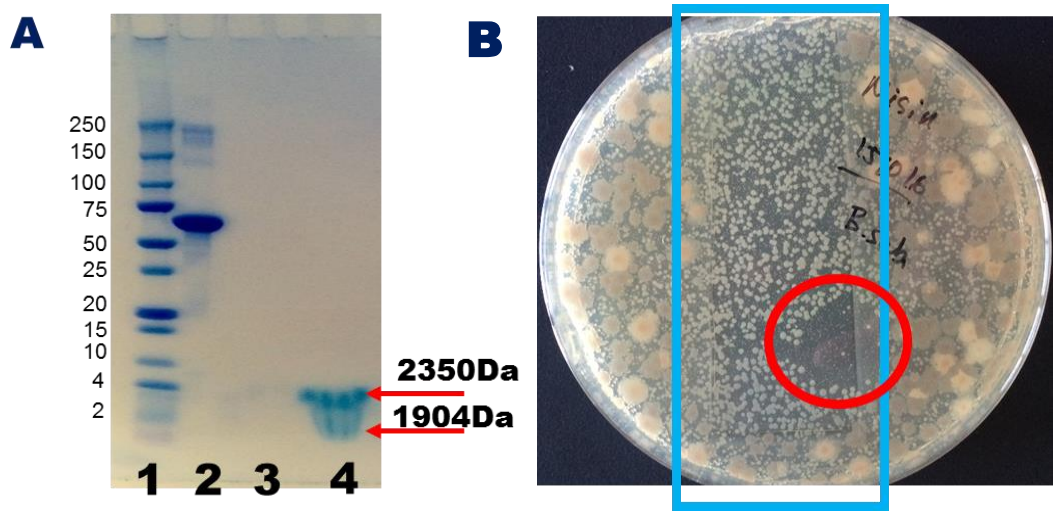


**Figure 10**. Tricine SDS PAGE analysis of purified peptides of L.diolivorans (Tuv-33) strain

A- Coomasie blue stained gel
1-marker, 2-BSA (positive control), 3-negative control, 4-active peak
B- The gel was overlaid with *B.subtilis* to identify the band corresponding antibacterial activity.

### 4.3.4 Mass spectrometry

The antibacterial peptides of *L.diolivorans* (Tuv-33) strain purified by SP Sepharose Fast Flow column that peptides molecular weight was analyzed by MALDI/TOF MS spectrometer. The mass spectrum presented that obtained two different peak with 1905 and 2350 Da shown in Figure 11. Miyamoto et al, (2012, 2016) reported that *Leuconostoc mesentroides* subsp. *dextranicum* 213M0 and *Leuconostoc mesentroides* 406 with bacteriocins activity were isolated from Mongolian airag, which are purified two different bacterion-like substance and bacteriocins about molecular mass were 2.2-3kDa and 3.3kDa, respectively.

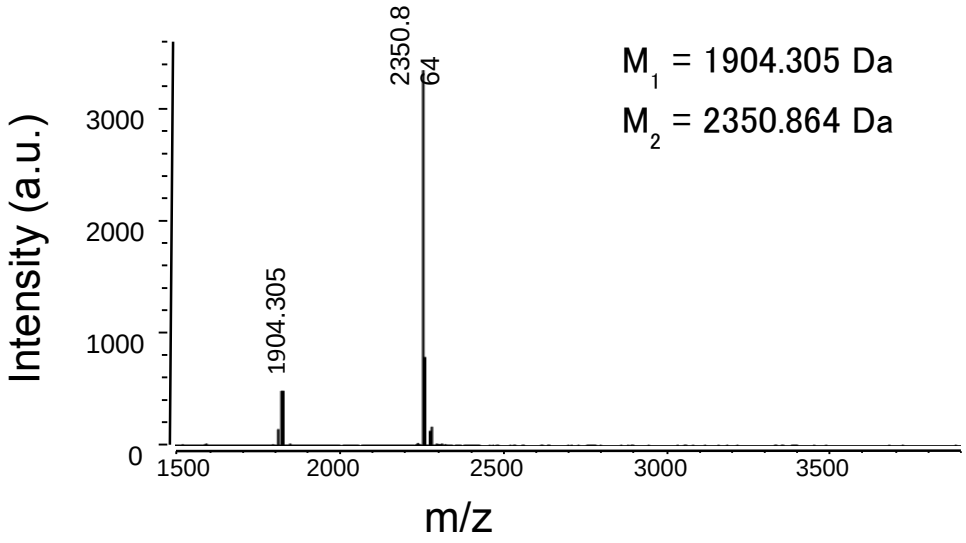


**Figure 11**. Mass spectrum of antibacterial peptides produced by *L.diolivorans* (Tuv-33) strain isolated from Mongolian airag

## 4.4 Conclusion

In conclusion, *Lactobacillus diolivorans* (Tuv-33) strain with high potential biological activity as antibacterial and proteolytic activity was isolated from Mongolian traditional fermented mare's milk as airag. Previously this strain was isolated and identified from Mongolian traditional dairy products, described by Oki and Watanabe et al, 2013; and Wenjun et al, 2012, who was isolated from Tarag sample in Inner Mongolia of China. Furthermore, Vasiee et al, (2014) studied that LAB was isolated from Tarkihineh as fermented cereal of Iranian traditional fermented food, who characterized to probiotic properties. Also, *L.diolivorans* species were previously isolated from some dairy products and plants such as OHL cheese, traditional pickles, aerobically stable maize silage ((Krooneman et al (2002); Coton et al, (2008) and Simel et al, (2015)). However, studies of biological activity as ability of bacterial inhibitory from *L.diolivorans* has not yet. To our knowledge, this is first time that *L.diolivorans* of airag sample has been examined to their biological activities and purified antibacterial peptides.

Following a three step purification protocol provided probably two antibacterial peptides that has been analyzed molecular mass about 1905 and 2350 Da by Tricine SDS PAGE analysis and MALDI TOF MS mass spectrometer. Now this study undergoing in our laboratory are focusing on the amino acid sequencing and their structure analysis.

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# GENERAL CONCLUSION

Airag as fermented mare's milk is a very popular fermented beverage in Mongolia, which ~~is~~ contains a lot of ferments (lactic acid bacteria and yeast), intestinal trace elements, antibiotics, vitamins A, B1, B2, B12, C, D and E, ethyl alcohol, organic acids (Ahmed M, 2010 and Tamime , 1999). For revealing functionality and useful information about Airag as a healthy daily beverage in Mongolia.

This thesis consists of study on isolation and identification of lactic acid bacteria and yeasts from Mongolian traditional fermented mare's milk as Airag, determination of their biological activity and purification of antibacterial substance as peptide from cell free supernatant.

In chapter 2, thirty five lactic acid bacteria were isolated from six Airag samples collected in four different provinces of Mongolia. The six species LAB was identified as *Lactobacillus helveticus* (5 strain) *, L.casei* (6 strain), *L.diolivorans* (3 strain), *L.hilgardii* (4 strain), *L.paracasei* (4 strain) *and L.kefiri* (4 strain) by 16S rDNA sequencing analysis. Two strains of LAB *L. hilgardii* (Uvu-21), *L. diolivorans* (Tuv-33) were discovered high potent proteolytic and antibacterial activities, which LABs were detected on Mongolian traditional tarag as yogurt and airag sample by Watanabe et al, 2009, Oki et al, 2013 and Wenjun et al, 2012. But they were carried out just identification of LAB in traditional dairy products. In our study, this *L. hilgardii* (Uvu-21), *L. diolivorans* (Tuv-33) was examined to biological activities in first time. In addition, two yeasts were also isolated, and identified as *Kazachstania* sp and *Kazachstania unispora* by 26S rDNA analysis.

Their ability to ferment glucose was compared with that of K7 yeast used for sake brewing. The fermentation ability of the two yeasts was weaker than that of K7 yeast, producing 2.1-3.0 g/L of ethanol. These results were in agreement with the low ethanol concentrations in Airag samples.

In chapter 3, we studied on the biological activity as proteolytic and antibacterial activities that was screened six different species LAB with potent activities. Among the lactic acid bacteria, the supernatants of the ten lactic acid bacteria were found to have potent antibacterial activity against both *E. coli* and *Bacillus subtilis*, and proteolytic activity on 2.5% skim milk. The supernatants of the four lactic acid bacteria, *L. helveticus* (Bul-5), *L. kefiri* (Bul-17), *L. hilgardii* (Uvu-21), and *L. diolivorans* (Tuv-33), completely inhibited the increase of pathogenic *E. coli* and/or *Bacillus subtilis* for 24 h at 37°C because the turbidity measured by the absorbance at 600 nm was near 0 and the solution was clear.

Furthermore, we were focused on antibacterial peptide produced by LAB in cell free supernatant where was studied effect of different parameters as the time course of antibacterial substances in liquid medium, their enzymatic effects and pH, thermal stabilities on CFS against *E.coli* and *B.subtilis.* These parameter depends on a feature of antibacterial peptides (Agraval, 2012). In our study, the antibacterial substances on CFSN by LAB isolated from Mongolian airag sample have probably indicated as peptides that has notable results such as inactivated by proteases, a wide range pH stability and intolerance a high temperature. The results suggesting that the antibacterial activity is attributable to the peptides produced by the lactic acid bacteria. The isolation and structure of the peptides are under investigation. Therefore we was intended isolation and purification of antibacterial peptides.

In chapter 4, *Lactobacillus diolivorans* (Tuv-33) strain with high potential biological activity as antibacterial and proteolytic activity was noticed previous study (in thesis part, chapter 2 and 3). Previously this strain was isolated and identified from Mongolian traditional dairy products, described by Oki and Watanabe et al, 2013; and Wenjun et al, 2012, who was isolated from Tarag sample in Inner Mongolia of China. However, studies of biological activity as ability of bacterial inhibitory from *L.diolivorans* has not yet. To our knowledge, this is first time that *L.diolivorans* of airag sample has been examined to their biological activities and purified antibacterial peptides.

Following a three step purification protocol provided probably two antibacterial peptides that has been analyzed molecular mass about 1905 and 2350 Da by Tricine SDS PAGE analysis and MALDI TOF MS mass spectrometer. Now this study undergoing in our laboratory are focusing on the amino acid sequencing and their structure analysis.

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