Doctoral Thesis

Functionality of lactic acid bacteria in traditional fermented food of Xinjiang, China

中国・新疆ウイグル自治区における伝統的発酵食品中の乳酸菌の機能性

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Kitami Institute of Technology, JAPAN

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

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ABBREVIATIONS

μm	micrometer
ABP	Antibacterial peptide
ADF	Acid detergent fiber
AU	Activity (Arbitrary Unit)
BLM	Blood liver medium
BSA	Bovine Serum Albumin
CFS	Cell free supernatant
Da	Daltons
DNA	Deoxyribonucleic acid
EBFA	Enzymatic Bioanalysis / Food analysis
F6PPK	Fructose-6-P phosphoketolase
GAM	Gifu anaerobic medium
GIT	Gastro intestinal tract
g/L	Gram per liter
JCM	Japan collection of Microorganism
KDa	Kilodalton
LAB	Lactic Acid Bacteria
LB	Lysogeny broth
MRS	De Main Rogasa and Sharpe
mg	Milligram
min	Minute
ml	Milliliter
mmole/L	Millimole per liter

MW	Molecular weight
NCFS	Neutralized Cell Free Supernatant
NDF	Natural detergent fiber
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
UV	Ultraviolet
UF	Ultrafiltration
w/v	Wet volume

GENERAL ABSTRACT

In this study, the author collected traditional fermented dairy products from several pastoral area of Xinjiang, China. Nineteen lactic acid bacteria (LAB) were isolated from kurt, cheeses, fermented camel's milk, koumiss, and other dairy products and identified by 16S rDNA sequencing analysis. Antibacterial activity of cell free supernatants (CFS) of fermented LAB against bacterial pathogens was determined by agar well diffusion method, indicating that the supernatants were found to have potent antibacterial activity for health care.

Eleven isolates of *Lactobacillus* and eight of *Enterococcus* were identified as belonging to 7 validated species, *L. plantarum, E. hirae, E. faecium, E. lactis, L. casei, L. zeae, and E. lactis*. Among them, *L. plantarum, L. zeae,* and *E. hirae* lactic acid bacteria (99% homology) were found to have high antibacterial activity (6-9 mm clean zone) against *E. coli* at neutral pH, suggesting that the antibacterial activity was originated by antibacterial peptides. These lactic acid bacteria were cultured in large scale, and then antibacterial peptides were isolated.

In our laboratory, several lactic acid bacteria were isolated from a traditional Mongolian Airag and identified some lactic acid bacteria. Two lactic acid bacteria, *L. hilgardii and L. diolivorans*, were found to have high antibacterial and proteolytic activities. Therefore, in this thesis, in order to increase the nutritional value of potato pulp, which is discarded without being effectively utilized in starch production, as a livestock feed, a fermentation study using *L. diolivorans* lactic acid bacteria was conducted.

The moisture content of the potato pulp silage was remained 822 g/kg before and after ensiling. After drying for 48 h at 60°C and subsequently for an additional 4 h at

105°C, the moisture content was reduced to 82–84 g/kg. The protein content in the silage increased from an initial concentration of 39 to 57 g/kg and 58 g/kg for *L. lactis* and *L. diolivorans* inoculations, respectively. The NDF content decreased slightly from 363 to 360 g/kg (*L. lactis*) and 354 g/kg (*L. diolivorans*) after inoculations. Similarly, ADF content showed a small decrease from 348 to 345 g/kg and 325 g/kg for *L. lactis* and *L. diolivorans* inoculations, respectively. The soluble sugar content increased roughly by 2 folds in both treatments. The lactic acid content significantly increased from 2 to 52 g/kg (*L. lactis*) and 50 g/kg (*L. diolivorans*) after ensiling, whereas, toxic butyric acid was not detected with either treatment. These results suggest that the inoculation of potato pulp with *L. lactis* or *L. diolivorans* increases the quality and nutrition of potato pulp as silage. In particular, *L. diolivorans* is an efficient inoculant because it produces antibacterial peptides that prevent the increase of saprophyte in silage.

Keywords: Traditional dairy products, lactic acid bacteria, antibacterial activity, potato pulp, *L. diolivorans*

Chapter I

General Introduction

1.1 Background

Fermented foods and drinks have been of beneficial impact on human diet, due to their positive impacts on human health and the importance of food in terms of increased nutrition, lengthy shelf life and improved organoleptic properties (Azam et al., 2017). Several microorganisms are well known in the production of fermented foods. Among them lactic acid bacteria play an important role in fermented food and beverage production (Ahmed et al., 2013; Babu et al., 2009). Lactic acid bacteria (LAB) are an industry-significant group of probiotic organisms that play a major role in human health by inhibiting the development of damaging and pathogenic bacteria, boosting immune function, and improving infection resistance (Amirbozorgi G et al., 2016).

Kazakh nomads use raw milk from cattle, horses, camels, and sheep to make a range of traditional naturally-fermented dairy products. Cow's milk, in particular, is used for this purpose to produce kurt (milk-based semi-dry snack and dry cheese), a unique fermented food that has been consumed since ancient times and is deeply ingrained in the eating habits of Ili Kazakh Autonomous Prefecture in the Xinjiang Uygur Autonomous Region, China. Kurt is made by nomads of the northern Xinjiang with a natural fermentation process from May to October in every year, where the fermentation procedure is completed in approximately three days. A mixed microflora of lactic acid bacteria and yeast strains play roles in the fermentation. Lactic acid bacteria with gram−positive and catalase−negative characteristics are known to have

potent antibacterial activity, due to the production of organic acids such as lactic and acetic acids and other antimicrobial components.

The therapeutic impacts of beneficial microorganisms include, but are not restricted to, improved immune function, maintaining anti-tumor activity, reducing the population of damaging microorganisms, improving microflora equilibrium in the colon, preventing certain intestinal infections, increasing lactose-containing food tolerance, and potentially cancer prevention (Martini et al., 1987; Wang et al., 2010; Amadou et al., 2013).

Ramila AZAT et al. (2016) isolated and identified 6 lactic acid bacteria (*L. rhamnosus, L. helveticus, and E. hirae*) from traditional fermented XinJiang cheese and evaluated for functional and probiotic properties and potentials as starter cultures.

In this study, we aimed to isolate and identify natural lactic acid bacteria in these traditional naturally-fermented dairy products to clarify the antimicrobial properties and evaluate its function as a food.

1.2 Lactic acid bacteria

1.2.1 Classification and physiologic characteristics of LAB

Lactic acid bacteria are acid-tolerant gram-positive bacteria, non-sporulating rods or cocci and are able to produce lactic acid during homo fermentative or hetero fermentative metabolism (Klaenhammer et al., 2002). According to Stiles and Holzapfel LAB include the following genera: *Aerococcus*, *Alliococcus*, *Bifidobacterium*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. However, it is mostly the genera

Carnobacterium, *Enterococcus*, *Lactobacillus* (dairy products, meat, vegetables, cereal), *Lactococcus* (dairy products), *Leuconostoc* (vegetables, dairy products), *Oenococcus* (wine), *Pediococcus* (vegetables, meat), *Streptococcus* (dairy products) and *Weissella* that are subjected to applications in food industries for the development of functionalized foods (Stiles ME et al., 1997; Vandamme P et al., 1996).

Lactic acid bacteria (LAB) have been essential part in food fermentation for human history for its greatly contributed to the nutritional value of end products. Some metabolic properties due to LAB play 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 et al., 2006; Sun et al., 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 et al., 2004).

1.2.2 Lactic acid bacteria and their uses in food

Lactic acid bacteria are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gillian et al., 1990). Species used for food fermentations belong to the genera *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus*, and the newly recognized *Carnobacterium*. These organisms have been isolated from grains, green plants, dairy and meat products, fermenting vegetables, and the mucosal surfaces of animals (Lindgren.S.E et al., 1990). Once used to retard spoilage and preserve foods through natural fermentations, they have found commercial applications as starter cultures in the dairy, baking, meat,

vegetable and alcoholic beverages industries. They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins during lactic fermentations (Lindgren.S.E et al., 1990). These are not only components desirable for their effects on food taste, smell, color and texture, but they also inhibit undesirable microflora. Hence, lactic acid bacteria and their products give fermented foods distinctive flavors, textures, and aromas while preventing spoilage, extending shelf-life, and inhibiting pathogenic organisms.

1.2.3 Antimicrobial compounds produced by lactic acid bacteria

The preservative action of starter culture in food and beverage systems is attributed to the combined action of a range of antimicrobial metabolites produced during the fermentation process (Caplice, E et al., 1999). These include many organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Ross.R.P. et al,2002). They have a very broad mode of action and inhibit both gram-positive and gram-negative bacteria as well as yeast and moulds (Caplice, E et al., 1999). One good example is propionic acid produced by propionic acid bacteria, which has formed the basis for some biopreservative products, given its antimicrobial action against microorganisms including yeast and moulds. Microgard is a Food and Drug Administration (FAD)-approved fermentation produced by *Propionibacterium freudenreichii* subsp. *Shermanii* which contains propionic acid and is used in an

estimated 30% of the cottage cheese manufactured in the United State. In addition to acids, starter strains can produce a range of other antimicrobial metabolites such as ethanol from the hetero fermentative pathway, H_2O_2 produced during aerobic growth which is generated from excess pyruvate coming from citrate (A.M. Daeschel et al., 1989). In particular, H_2O_2 can have a strong oxidizing effect on membrane lipids and cellular proteins and is produced using such enzymes as the flavo protein oxidoreductases NADH peroxidase, NADH oxidase and α-glycerophosphate oxidase (S.Codon et al., 1987). Obviously, each antimicrobial compound produced during fermentation provides an additional hurdle for pathogens and spoilage bacteria to overcome before they can survive and/or proliferate in a food or beverage, from time of manufacture to time of consumption. Since any microorganism may produce a number of inhibitory substances, its antimicrobial potential is defined by the collective action of its metabolic products on undesirable bacteria. Other examples of secondary metabolites produced by lactic acid bacteria which have antagonistic activity include the compound reuterin(Axelsson L et al., 1989) and the recently discovered antibiotic reuterocyclin(M.G. Ganzle et al., 2011), both of which are produced by strains of Lactobacillus reuteri. Reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde. It has broad spectrum of activity and inhibits fungi, protozoa and a wide range of bacteria including both gram-positive and gram-negative bacteria. This compound is produced by stationary phase cultures during anaerobic growth on a mixture of glucose and glycerol or glyceraldehydes. Consequently, in order to use reuterin-producing *L. reuteri* for bio preservation in a food product, it would be beneficial to include glycerol with the strain. This approach was used to extend the shelf-life of herring fillets stored at 5°C and

involved dipping the fish in a solution containing 1×10^9 cfu/ml of *L. reuteri* and 250 mM glycerol (S.E. Lindgren et al., 1990). Results demonstrated that after 6 day of storage, there were approximately 100-fold-less gram-negative bacteria in the *L. reuteri* samples than in the untreated control.

More recently, the first antibiotic produced by lactic acid bacteria was discovered (M.G. Ganzle et al., 2000). Reuterocyclin is a negatively charged, highly hydrophobic antagonist, and structural elucidation revealed it to be a novel tetramic acid. The spectrum of inhibition of the antibiotic is confined to gram-positive bacteria including *Lactobacillus* spp., *Bacillus subtilis, Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Listeria innocua*. Interestingly, inhibition of *Escherichia coli* and *Salmonella* is observed under conditions that disrupt the outer membrane, including truncated lipopolysaccharides (LPS), low pH and high salt concentrations. Since it is well known that Nisin can kill gram-negative bacteria under conditions which disturb the outer membrane (K.A. Stevens et al., 1992), it is likely that there are similarities in the mode of action of Nisin and this novel antibiotic.

Antibacterial peptides (ABP) are small molecules (<10kDa) with inhibitory activity against some organisms such as bacteria, yeasts, fungi, viruses, and even tumor cells that cause these inhibitory substances as therapeutic agents (João et al., 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 et al., 2004; Rabeeth et al., 2012).

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 et al., 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 et al., 2010). In 2014, the application of ABPs was applied as a combination therapy for treating biofilms if preferred over mono therapy (Guangshun et al., 2015).

Until now, over 2000 ABPs have been described in virtually every eukaryotic organism. 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 inhibit bacteria via oxygen-independent mechanisms (Zeya et al., 1966; Hirsch et al., 1956).

1.3 Traditional fermented dairy products of Kazakh nomads in Xinjiang

Kazakh nomads use raw milk from cattle, horses, camels, and sheep to make a range of traditional naturally-fermented dairy products. Cow's milk, in particular, is used for this purpose to produce kurt (milk-based semi-dry snack and dry cheese), a unique fermented food that has been consumed since ancient times and is deeply ingrained in the eating habits of Ili Kazakh Autonomous Prefecture in the Xinjiang Uygur Autonomous Region, China. Kurt is made by nomads of the northern Xinjiang with a natural fermentation process from May to October in every year where the fermentation procedure is completed in approximately three days. A mixed microflora of lactic acid bacteria and yeast strains play roles in the fermentation. Lactic acid bacteria with gram-positive and catalase-negative characteristics are known to have a potent antibacterial activity, due to the production of organic acids such as lactic and

acetic acids and other antimicrobial components.

1.3.1 Manufacturing process of traditional dairy products of Xinjiang pastoralists in china

(1) Preparation of Ayran

Yogurt is called aryan in Kazakh language. The cow's milk is called sut. The sut was heated to be pasteurized and was cooled to around 40 ºC. About 5% of sut of Uytkhi (sour milk) was added as a starting material, wrapping with cloth to keep warm, and letting it incubate about 5-6 hours.

(2) Preparation of cheese

The households use sour milk and rennet as coagulants in Xinjiang pastoral area. The cheese is called rimshik, and the two types of cheeses (akh rimshik and mayek rimshik) were made from cow's milk. After it reaches to the boiling point, in purpose of pasteurizing sut and add a small amount of ayran that is stirred for a few minutes. The freshly formed curd looks like white jelly, while the whey is a yellow-green liquid and we can always easily differentiate the akh rimshik from the whey.

Rennet is produced from cud-chewing animals such as calf or young goat. The rennet contains an enzyme called rennin, which coagulated livestock milks to form a solid curd several enzymatic reactions. The rennet for cheese was made by households in Xinxiang. Initially the fourth stomach of calf was cleaned, and then the stomach was salted. After drying by hanging up, the stomach was kept at 4 ºC until use. Before cheese making, they broke off a small piece of the dried stomach was finely broken and soaked it in cool and fresh water for a few hour. The soaked water is called mayek.

The sut was pasteurized referring to the same method as above, and then cooled around 35ºC and added about 2% mayek of sut. The mixture was kept for 20-30 minutes and then heated at 60 ºC for 10-15 min. Next, the whey was discarded from rimshik. The rimshik is called mayek rimshik or khizil rimshik.

(3) Processing butter from milk

The cream separation process was mainly performed in the summer and autumn when the temperature was high. Cream separation process covers two processes, the first one is without heating and second is with heating.

***Cream separation process without heating*:** The raw milk sut was left overnight without heating and the cream was separated when it floated to the surface. This cream is called klegey. In recent decades, nomadic herder started to use the cream separator. They use the cream separator to transform raw whole milk into skim milk and cream. The skimmed milk is called kok sut. It is for use process the casein.

***Cream separation process with heating*:** The raw milk sut was boiled and left to stand overnight and the cream was separated when it floated to the surface. This cream is called khaymak. The separated cream was left to incubate for several days and then mixed with natural fermented milk, and churning it in a saba (tub) with a pispek (plunging dasher). The butter is called sarmay and the butter milk produced from this process is called rikit.

(4) Processing khurt from milk

The rikit or ayran can be further processed to make hatik, suzbe or khurt. First the ayran and rikit were dehydrated in white cloth bag for 2days. The leftover inside bag is

called khatik and the the butter milk produced from this process is called rikit. The households of regions around Ili kazakh autonomous prefecture used to make khatik and salted, and forming small 5cm cylindrical shapes before refrigerating or drying them out under the sunshine for about 2days. It is called suzbe or suzbe khurt.

In Aletai and Tacheng regions of Xinjiang, the khurt was made from rikit by heating and dehydrating. Firstly starting with raw milk fermentation, ayran or rikit was heated for 20-30minutes at 60 ºC and dehydrating the whey, after forming it sun drying for 2days after that kept it cool place in the cloth bag.

(5) Preparation of Khimiz and Shubat

Khimiz, fermented mares' milk, is a popular traditional beverage in the northwest pastoralists in china. The mare is milked 3- 6times a day during the summer season. The mares' milk was fermented in bags made from horse-wide or canvas.

Shubat is a traditional beverage that is made by the fermentation of camel’s milk with lactic acid bacteria.

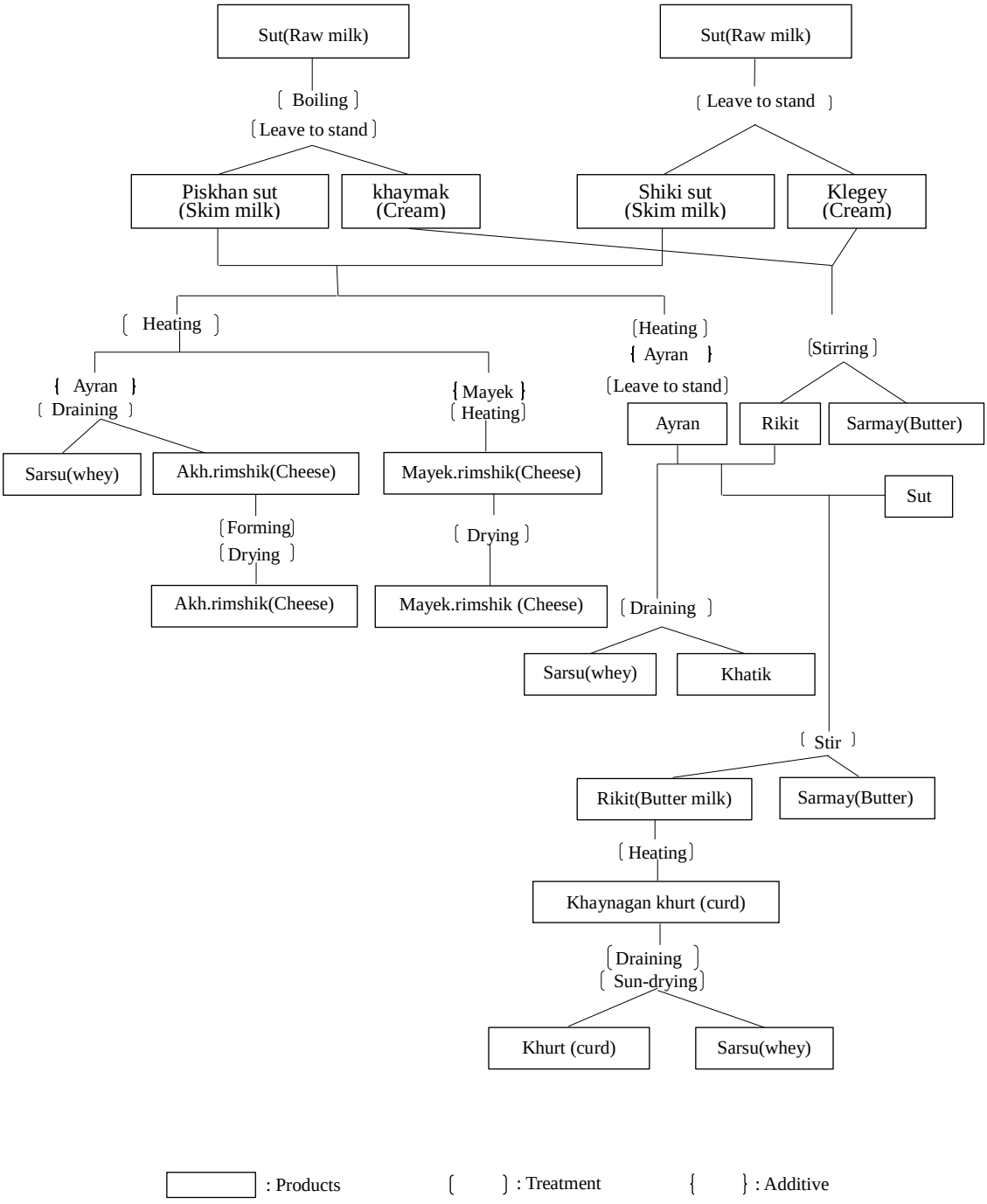

Figure1. Milk processing system of Xinjiang pastoralists in China

1.4 Biochemical components of dairy products

The biochemical component of milk from various animal species presented in Table1 (Massimo Malacarne et al et al., 2001; Gaukhartas Temirova et al et al., 2016). Milk sugar (lactose) is synthesized in the udder of lactating animals. Lactose is an energy source for the biochemical processes in the body, promotes the absorption of calcium, phosphorus, magnesium, barium. Under the influence of lactobacilli, lactose cleaved to form lactic acid, which promotes absorption of calcium and phosphorus needed for the formation of growing animal bones. (Serikbayeva et al., 2004).

Table1. Composition of milk from various animal species

Component	Moisture	Total fat	Whole protein	Lactose	Ash	Gross energy
	g/kg	g/kg	g/kg	g/kg	g/kg	kcal/kg
Mare	86-88	5-20	15-28	58-70	3-5	390-550
Cow	86-88	33-54	31-39	44-49	7-8	650-712
Goat	87-88	50-90	45-70	41-59	8-9	-
Sheep	79-82	30-40	30-36	42-50	7-8	660-690
Human	87-88	35-40	9-17	63-70	2-3	650-700

The structural involution of the minor carbohydrate fractions (Kunz et al et al., 1999; Klemen Potočnik et al et al., 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.

1.5 Health benefits of dairy products

Probiotics and prebiotics can both be used to maintain and restore the normal, health promoting intestinal microbiota. They can be administered in different food matrices milk and dairy products being perhaps most common, because of the long association of LAB and milk, which makes these products both familiar to consumers and easy to manufacture (Serikbayeva et al., 2004).

Traditional fermented milk is produced by microorganisms, such as LAB or yeasts, etc., from the natural environment and has a longer shelf life than raw milk. It also has a refreshingly sour taste and unique flavor. As regards Asian traditional fermented dairy products, nomads have long practiced the culture of milk (Sudun et al., 2012).

1.6 Microorganisms of traditional dairy products of Xinjiang

In China, the history of traditional fermented dairy products making goes back hundreds of years, and rich microbial resources have been handed down. As for traditional fermented dairy products, fermentation is of a symbiotic nature and depends on the action of two distinct types of microorganisms, lactic acid bacteria and yeasts. The major microbial composition of traditional fermented dairy products is lactic acid bacteria. It was reported that the indigenous microbiota plays a major fermentation role in affecting the aroma, texture and acidity of the product as well as being of some health benefits to human beings (Montanari et al., 1996; Duan et al., 2008).

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Chapter II

Isolation and identification of Lactic Acid Bacteria from traditional fermented food of Xinjiang, China

2.1. Abstract

In this study, the author collected traditional fermented dairy products from several pastoral area of Xinjiang, China. Nineteen lactic acid bacteria (LAB) were isolated from kurt, cheeses, fermented camel's milk, koumiss, and other dairy products and identified by 16S rDNA sequencing analysis. Eleven isolates of *Lactobacillus* and eight of *Enterococcus* were identified as belonging to 7 validated species, *L. plantarum*, *E. hirae*, *E. faecium*, *E. lactis*, *L. casei*, *L. zeae*, *and E. lactis*. Before the identification of LAB, the isolates were enumerated by the plate count technique and characterized by Gram-staining, catalase activity and microscope observation. The bacteria strains isolated had not catalase activity and were gram-positive, indicating that all bacteria isolated were LAB.

2.2. Introduction

Xinjiang cheese is distinctive, high in nutrients, and always is considered a good staple food. Therefore, in pastoral area of Xinjiang (Alegria et al., 2012), fermented dairy food is consumed daily to improve physical strength, promote digestion, and provide nourishment (Zhong Z, et al., 2016). However, the cheese is only consumed in limited areas in China, such as Xinjiang, Inner Mongolia, and Tibet Regions.

Furthermore, microbial resources, especially the LAB, in various dairy products have not been well characterized, which potentially negatively impacts cheese industries and application in human health promotion.

There are several reports on the identification of LAB in kurt and investigation of the characteristics of kurt as a food (Zhihong Sun,et al., 2010). Ramila AZAT et al. (2016) isolated and identified 6 lactic acid bacteria (*L. rhamnosus, L. helveticus*, and *E. hirae*) from traditional fermented XinJiang cheese and evaluated for functional and probiotic properties and potentials as starter cultures.

In this work, we aimed to isolate and identify natural lactic acid bacteria in these traditional naturally-fermented dairy products to evaluate its function as a food.

2.3 Methods and materials

2.3.1 Materials

De man Rogosa Sharpe (MRS) broth, nonfat dry skim milk were obtained from Kanto Chemical Co.,inc (Tokyo, Japan) and Oxoid Co. Ltd., (Hampshire, England), respectively. Agar powder was purchased from Wako Pure Chemical Ind. Ltd., (Oosaka, Japan). Luria-Bertani (LB) liquid medium was prepared by combining the following 3 reagents in 1 L of distilled water, peptone (100 g, 0.05 mol), sodium chloride (50 g, 0.85 mol) and yeast extract (50 g, 0.18 mol). The mixture was shaking until the solutes have dissolved and adjusted the pH to 6.5 with 5 N of sodium hydroxide, which was sterilized by autoclaving 15 min at 121°C. Agarose L03 and ISOPLANT II were obtained from Takara Biotechnology Co. Ltd., (Tokyo, Japan) and Nippon Gene Co. Ltd., (Tokyo, Japan), respectively. Trypsin and proteinase K were obtained from Sigma-Aldrich Co. Ltd., (Louis, USA). Pathogenic strain, *Escherichia coli*

(ATCC25922), *Bacillus subtilis* (NBRC 13722) were obtained from the National Institute of Technology and Evolution (NITE) of Japan.

2.3.2 Collection of samples

A total of 11 fermented dairy products samples were collected from two pastoral areas of Xinjiang, China, Ili Kazakh Autonomous Prefecture (Ili), Altay area (Alt), in March 2016 and in May 2017. Traditional fermented dairy samples were included kurt, cheeses, fermented camel's milk, koumiss and other dairy products. All solid samples were collected in sterile bags and transferred to the laboratory under aseptic cooled conditions. Fermented camel's milk and koumiss were kept at -80°C in 20% glycerol until use.

2.3.3 Enumeration and isolation of lactic acid bacteria

Ten g of each sample was added to 5 mL of 2.5% of nonfat dry skim milk and incubated at 37°C for 24 h. The samples on the nonfat dry skim milk were then transported on the MRS broth incubated at 37°C for 24 h. Before the isolation of lactic acid bacteria, the lactic acid bacteria were enumerated by the plate count technique (Coventry et al., 1997). Each sample was diluted with sterile water, by serial 10-fold dilutions (10^{-5}, $10^{-6}$and 10^{-8}) and 1 ml sample was plated on agar. After 48h incubation at 37℃, cell counts per milliliter (ml) of the sample was enumerated. Isolated colonies with typical characteristics of LAB were picked from each plate and cultured into new MRS agar plate for 48h days at 37°C. The isolates were further characterized by Gram-staining (Bartholomew et al.,1952), catalase activity and microscope observation (Batdorj et al., 2006).

2.3.4 DNA extraction and identification of lactic acid bacteria

The culture was collected by centrifugation at 8,000 rpm for 5 min and removed the cell free supernatant. The obtained cell was added 30ul of sterile water, 20ul of 20mg/ml lysozyme solution and 10ul of phosphate buffer (pH7) and incubated at 37℃ for 4h. The genomic DNA was extracted by a DNA extraction kit, ISOPLANT II (Nippon Gene, Tokyo). After 4h of incubation, then which was added 300ul of solution I and 150ul of solution II and incubated on the 50℃for 10 minutes. The mixture was added 120ul of solution III-A and 100ul of solution III-B, after mixed well and placed in 4℃for 10minutes. The mixture was centrifuged for 10 min at 10000 rpm at room temperature. Transfer the 200ul of supernatant to a new 1.5mL tube, added 5ul of 3M sodium acetate, 200ul of 2-propanol and 0.5ul of ethachinmate. The mixture was centrifuged for 10 min at 5000 rpm and discarded the supernatant, and then used the tube was washed using 70% of ethanol. The genomic DNA was dissolved in 20ul of deionized water and determined the concentration of genomic DNA and kept it until PCR amplification.

Lactic acid bacteria in traditional dairy products were identified by 16S rDNA analyses, using universal primer 9F (5' -*GAGTTGATCCTGG CTCAG*-3') and 802R (5'-*TACCAGGGTATCTAATCC* - 3'). In a PCR tube, template DNA 1 μL (≥200 ng), each primer solution (0.5 μL), 10×Taq EX buffer (1 μL), Takara EX Taq (0.05 μL), DNTP 1 μL, deionized water (5.95 μL) was added, and the PCR tube was placed in a MJ Research Peltier PTC-200 thermal cycler. The PCR conditions were heating for 5 min at 95℃ as the initial denaturation step and then 35 cycles of 30 sec at 95℃ for denaturation, 1 min at 58℃ for annealing, and 2 min at 72℃ for elongation. A final

extension step for 10 min at 72℃ was performed at the last cycle.

The amplification PCR products were separated in 1.2% of agarose gel by Mupid electrophoresis. After ethidium bromide staining which were subsequently visualized by ultraviolet illumination, and cut the PCR products kept it -10℃ until sequence analysis. PCR products were purified by the NucleoSpin® 8 Plant II kit. In a PCR tube, template DNA 3 μL (≥100 ng), 9F primer solution (1 μL), sequencing buffer (2 μL), pink enzyme (0.2 μL), deionized water (4.8 μL) was added, and the PCR tube was placed in a MJ Research Peltier PTC-200 thermal cycler. Run the following cycling program for 25 cycles: 95°C, 20 seconds 50°C, 15 seconds 60°C, 60 seconds (Cycling was completed in approximately 1 hour). The sequence analysis of the DNA regions was conducted using an Applied Biosystems AB 3130 genetic analyzer with a 36 cm capillary column.

2.4. Results and discussion

2.4.1 Identification of LAB in traditional fermented food

Isolates were isolated by the direct plating method by MRS agar plate. It was found that each sample was contained in several kinds of bacteria. The bacteria strains isolated had not catalase activity and were gram-positive, indicating that all bacteria isolated were LAB. Nineteen LAB were isolated from dairy products and identified by 16S rDNA sequencing analysis. Eleven isolates of *Lactobacillus* and eight of *Enterococcus* were identified as belonging to 7 validated species, *L. plantarum*, *E. hirae*, *E. faecium*, *E. lactis*, *L. casei*, *L. zeae*, and *E. lactis*.

Table2. Isolation and identification of LAB in traditional fermented food samples

Sample[a]	Collected province	primer	Catalase activity[b]	Gram staining[b]	Cell counting[c]	Classification[d]	
						Identified species	Identity(%)
Su-1	Altai region	R	-	+	15×10^{9}	Lactobacillus plantarum	98
Him-2	Altai region	R	-	+	48×10^{10}	*Entero coccus hirae*	99
Rim-3	Altai region	9F	-	+	12×10^{12}	*Lactobacillus plantarum*	99
Ay-4	Altai region	R	-	+	60×10^{8}	Lactobacillus plantarum	99
Rik-5	Altai region	R	-	+	52×10^{9}	*Lactobacillus plantarum*	99
Ku-6	Altai region	R	-	+	11×10^{11}	Enterococcus faecium	99
Rim-7	Yili Prefecture	R	-	+	55×10^{10}	Enterococcus lactis	97
Su-8	Altai region	R	-	+	12×10^{10}	Lactobacillus plantarum	100
Su-9	Yili Prefecture	R	-	+	14×10^{11}	Lactobacillus casei	96
Rim-10	Altai region	R	-	+	73×10^{10}	Enterococcus faecium	96
Su-11	Altai region	R	-	+	10×10^{19}	Enterococcus faecium	98
Shu-12	Altai region	R	-	+	15×10^{15}	Lactobacillus plantarum	98
Shu-13	Altai region	9F	-	+	13×10^{13}	Enterococcus lactis	98
Su-14	Yili Prefecture	9F	-	+	89×10^{10}	*Lactobacillus plantarum*	98
Ha-15	Altai region	9F	-	+	30×10^{8}	Lactobacillus zeae	97
Rim-16	Altai region	R	-	+	71×10^{10}	Lactobacillus plantarum	97
Rik-17	Altai region	R	-	+	39×10^{9}	*Lactobacillus plantarum*	98
Ku-18	Yili Prefecture	9F	-	+	61×10^{10}	*Enterococcus lactis*	99
Ha-19	Yili Prefecture	9F	-	+	51×10^{9}	*Enterococcus faecium*	100

a) Dairy products samples were collected in XinJiang in march, 2016 and in May 2017.

b) Catalase activity and Gram staining were carried out according to the reported methods (Oyundelger Ganzorig et al., 2016; AOAC,1996).

c) Cell counting was used 100 ul volume of sample.

d) Identification of LAB was performed using 16S rDNA sequencing analysis.

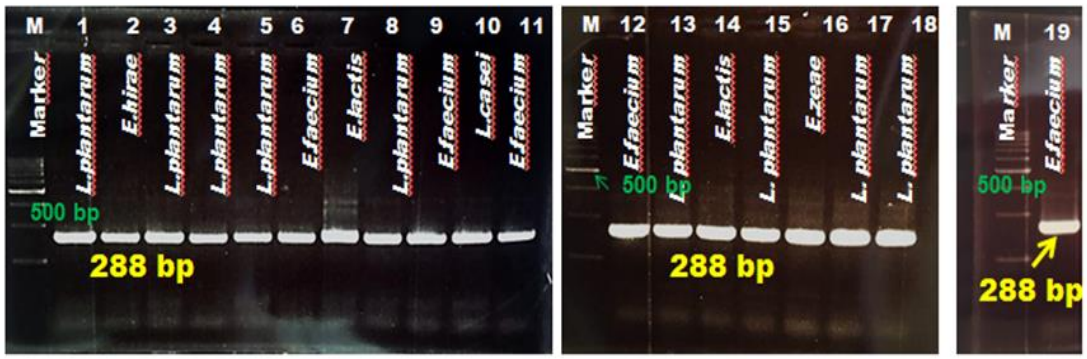

Figure 2. PCR detection of LAB in traditional fermented food samples

Microbial diversity of traditional fermented food samples has been the subjected of a considerable studies by Xiaoji Zheng et al, 2017; Azat, R et al, 2016; F.L.Zuo et al, 2014; Li, W et al, 2014 who traditional fermented food sample were isolated from Xinjiang,China and Akhmetsadykova S.H et al, 2015; Konuspayeva G, 2011; A. Meldebekova et al, 2008 who traditional fermented food samples were isolated from Kazakhstan.

2.4. Conclusion

In this study we collected traditional fermented dairy products from different pastoral area of Xinjiang, China. Nineteen lactic acid bacterial (LAB) strains were isolated and identified from kurt, cheeses, fermented camel's milk, koumiss and other dairy products by 16S rDNA sequencing analysis. The isolates were enumerated by the plate count technique and characterized by Gram-staining, catalase activity and microscope observation. The bacteria strains isolated had not catalase activity and were gram-positive, indicating that all bacteria isolated were LAB.

Eleven isolates of *Lactobacillus* eight isolates of *Enterococcus* were identified as belonging to 7 validated species: *L.plantarum, E.hirae, E.faecium, E.lactis, L.casei, L.zeae,* and *E.lactis*.

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Chapter III

Potent antibacterial activity of peptides produced by lactic acid bacteria in traditional fermented food of Xinjiang, China

3.1. Abstract

We aimed high antibacterial potential probiotic found for the development of a new antibacterial peptide. Nineteen lactic acid bacteria (LAB) were isolated from traditional fermented food of Xinjiang, and the supernatant of LAB were determined by agar well diffusion method, indicating that the supernatants were found to have potent antibacterial activity for health care.

Eleven isolates of *Lactobacillus* and eight of *Enterococcus* were identified as belonging to 7 validated species, *L. plantarum*, *E. hirae*, *E. faecium*, *E. lactis*, *L. casei*, *L. zeae*, *and E. lactis*. Among them, *L. plantarum*, *L. zeae*, and *E. hirae* lactic acid bacteria (99% homology) were found to have high antibacterial activity (6-9 mm clean zone) against *E. coli* at neutral pH, suggesting that the antibacterial activity was originated by antibacterial peptides.

Antibacterial properties were determined by resistance activity of heat, pH and enzymes treatments, respectively. The supernatant of isolates (1.5 mL) was gently stirred with *E. coli* (8×10^6 cells/mL) and LB broth (1.5 mL) 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 antibacterial activity on Bacillus

subtilis was also examined by the same procedure as above. We found that, the time course of antibacterial activity of the supernatant of lactic acid bacteria on *E. coli* and *B.subtilis*, respectively. In both cases, the absorbance was lower than that of the control, and lactic acid bacteria showed high antibacterial properties.

Antibacterial properties were not decreased by heating for 15 min, 30 min, 60 min at100°C. Antibacterial activity decreased by 30% for treatment for 180 min at 100°C. *E. hirae*(Him-2) and *E.faecium*(Ku-6), which LAB had potent antibacterial activity between pH 3 and 9, most activity inhibition of the antibacterial activity was in 6, 7. The antibacterial activity was presumed to be due to the antibacterial peptide produced in the culture supernatant. After dialysis product, by using SDS-PAGE and in one part of gel electrophoresis by used direct antimicrobial activity detection that the molecular weight of the peptide was 2000 Da.

3.2. Introduction

LAB are an industrially important group of probiotic organisms (Chen X, et al., 2010; Liu S, et al., 2004). Probiotics are known as live microorganisms that play an important role in our health by inhibiting growth of harmful and pathogen bacteria and also boosting immune function and increasing resistance to infection (Amara A et al., 2015; Parvez S et al., 2006; Perdigon G et al.,2001). Moreover, the consumption of milk and its derivatives such as yoghurt and cheese is essential to the diet of several millions of people worldwide because these products are source of vitamins and minerals. Information on microbiological composition of traditional dairy products is helpful in choosing LAB strains with useful function and stable fermentation properties from the traditional dairy products, and their usage as dairy starter in the manufacture

(Amirbozorgi G et al., 2016). In addition to traditional food uses LAB are being increasingly used as health promoting or probiotic bacteria in functional food products (Babu et al., 2009).

Several papers appeared on the antibacterial activity of LAB by the production of organic acids. Oyundelger and Zhang Jian Ming were found a strong antibacterial activity of LAB towards Gram-negative bacteria, *E. coli* strains, by the production of lactic and acetic acids and reported the inhibition of growth of Gram−positive bacteria by the production of a bacitracin. In this study, we aimed to evaluate the antibacterial and proteolytic activities of natural LAB in these traditional naturally fermented dairy products to clarify the antimicrobial properties and evaluate its function as a food.

3.3 Methods and materials

3.3.1 Materials

De man Rogosa Sharpe (MRS) broth, nonfat dry skim milk were obtained from Kanto Chemical Co.,inc (Tokyo, Japan) and Oxoid Co. Ltd., (Hampshire, England), respectively. Agar powder was purchased from Wako Pure Chemical Ind. Ltd., (Oosaka, Japan). Luria-Bertani (LB) liquid medium was prepared by combining the following 3 reagents in 1 L of distilled water, peptone (100 g, 0.05 mol), sodium chloride (50 g, 0.85 mol) and yeast extract (50 g, 0.18 mol). The mixture was shaking until the solutes have dissolved and adjusted the pH to 6.5 with 5 N of sodium hydroxide, which was sterilized by autoclaving 15 min at 121°C. Trypsin and proteinase K were obtained from Sigma-Aldrich Co. Ltd., (Louis, USA). Pathogenic strain, Escherichia coli (ATCC25922), Bacillus subtilis (NBRC 13722) were obtained from the National Institute of Technology and Evolution (NITE) of Japan.

3.3.2 Detection of antibacterial activity of cell free supernatants

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 sample was grown in 3 mL MRS broth under anaerobic conditions for 24 h at 37°C. The culture was centrifuged for 5 min at 8000G and then the supernatant was adjusted to pH 6.5 by 0.5 N aqueous NaOH. All of them were filter-sterilized through a sterile 0.22-μm-pore-size filter (Whatman Inc, Part of the Healthcare Bio-Sciences Corp, UK). The supernatant (50 μL) was placed on LB agar plates with *E. coli* and *B. subtilis* and incubated overnight at 30°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).

3.3.3 Time course of antibacterial activity of cell free supernatants

A typical procedure for the antibacterial activity of the supernatant produced by LAB toward *E. coli* and *B. subtilis* was as follows. The supernatant of isolates (1.5 mL) was gently stirred with E. coli (8×10^6 cells/ml) and LB broth (1.5 mL) 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 antibacterial activity on Bacillus subtilis was also examined by the same procedure as above.

3.3.4 Sensitivity to heat of cell free supernatants

The cell-free supernatants were heated for 15min, 30 min, 60 min and 180 min, at 100 °C. The supernatant (1.5 mL) described in 3.3.3 was heated at 100°C for 15 min, 30 min, 60 min and 180 min, respectively. After cooling, *E. coli* (8×10^6 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.

3.3.5 pH dependence on biological activity of cell free supernatants

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*. pH dependence on biological activity of cell free supernatants and biological activity was elucidated by changing the pH of the supernatant and using the same procedure as described in section 3.3.2.

3.3.6 Enzyme sensitivity of cell free supernatants

Cell free supernatant was treated with various enzymes such as proteinase K (pH 7.5, 37°C), trypsin (pH 8.0, 25°C), and α-chymotrypsin (pH 7.8, 25°C). All enzymes were obtained from Sigma, St. Louis, MO. USA and used at a final concentration of 1mg/ml (Jivka et al., 2014; Wildeboer et al., 2009). The 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.

3.3.7 Purification of antibacterial peptide from LAB and Molecular weight determination by SDS-PAGE

The culture was collected by centrifugation at 8,000 rpm for 20 min and removed the cell free supernatant. Ammonium sulfate precipitation was according to (Bayar.E., 2019). The obtained precipices were resuspended in five milliliters of sterile distilled water and dialyzed by using molecule weight cut-off 500-1000D of dialysis membrane (Biotech co., Ltd) against sterile distilled water for 24 hours. The total antimicrobial activity was determined against to indicator strain *E.coli* by agar well diffusion assay. The precipitated protein was collected by gel filtration and collected in each fraction. After dialysis substances and cell-free supernatant of molecular weight was determined an SDS-PAGE as described by using vertical gel apparatus (ATTO, Japan) with 14-20% separating gel (Schagger et al.,1987). Antibacterial peptide preparation and a with low molecular weight marker (Precision Plus Protein, BIORAD, U.S.) were run at 30 mA for 60 minutes (Jingping et al, 2016). Positive control and negative control were Boum serum albumin 1mg/ml and sterilized distilled water, respectively.

3.5 Results and discussion

3.5.1 Antibacterial activity of cell free supernatants

Table3 shows the results of the agar well diffusion assay of the isolated lactic acid bacteria toward *E. coli* and *B. subtilis*. Antibacterial activity of *L. plantarum* (Su-1), *E. hirae* (Him-2) and *L.plantarum* (Shu-12) strains were 6400 AU/ml against *E.coli*, and

they have same affected against *B. subtilis*. The antibacterial activity of *E. faecium* (Ku-6), *E. lactis* (Rim-7), *L. casei* (Su-9) and *E. faecium* (Rim-10) strains were 3200 AU/ml against *E.coli*, and they have same affected against *B. subtilis*.

Table3. Antibacterial activity of cell free supernatants produced from LAB

No	Name	Classification	Antibacterial activity			
			E.coli		*B. subtilis*	
			mm*	AU/mL**	mm	AU/mL
1	Su-1	*Lactobacillus plantarum*	9	6400	9	6400
2	Him-2	*Entero coccus hirae*	9	6400	9	6400
3	Rim-3	*Lactobacillus plantarum*	6	1600	6	1600
4	Ay-4	*Lactobacillus plantarum*	7	1600	7	1600
5	Rik-5	*Lactobacillus plantarum*	6	1600	6	1600
6	Ku-6	*Enterococcus faecium*	8	3200	8	3200
7	Rim-7	*Enterococcus lactis*	8	3200	8	3200
8	Su-8	*Lactobacillus plantarum*	4	800	4	800
9	Su-9	*Lactobacillus casei*	9	3200	9	3200
10	Rim-10	*Enterococcus faecium*	6	3200	6	3200
11	Su-11	*Enterococcus faecium*	4	800	4	800
12	Shu-12	*Lactobacillus plantarum*	8	6400	8	6400
13	Shu-13	*Enterococcus lactis*	5	1600	5	1600
14	Su-14	*Lactobacillus plantarum*	3	800	3	800
15	Ha-15	*Lactobacillus zeae*	6	1600	6	1600
16	Rim-16	*Lactobacillus plantarum*	4	1600	4	1600
17	Rik-17	*Lactobacillus plantarum*	4	800	4	800
18	Ku-18	*Enterococcus lactis*	3	800	3	800
19	Ha-19	*Enterococcus faecium*	3	800	3	800

* Inhibitor activity was measured by estimating the diameter of circle zone.

** 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)

3.5.2 Time course of antibacterial activity of supernatants

Figure3 shows the time course of antibacterial activity of the supernatant of LAB on *E. coli* and *B.subtilis*, respectively. *E. coli* is a gram-positive and *B.subtilis* is a gram-negative bacterium, which were selected and used for antibacterial tests. In both cases, the absorbance was lower than that of the control, and lactic acid bacteria showed high antibacterial properties. The antibacterial activity was presumed to be due to the antibacterial peptide produced in the culture supernatant.

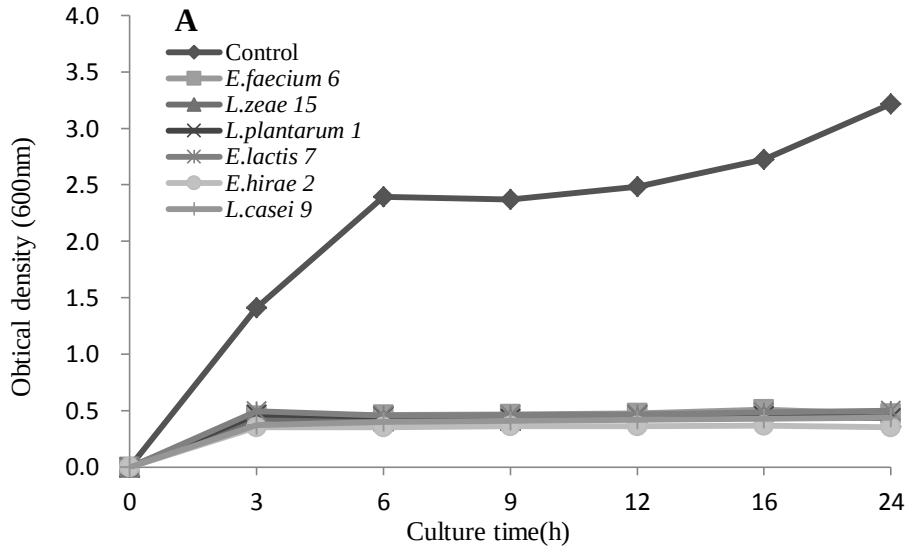

Figure 3. Time course of the antibacterial activity of LAB supernatants toward *E.coli* (A) and *B.subtilis*(B)

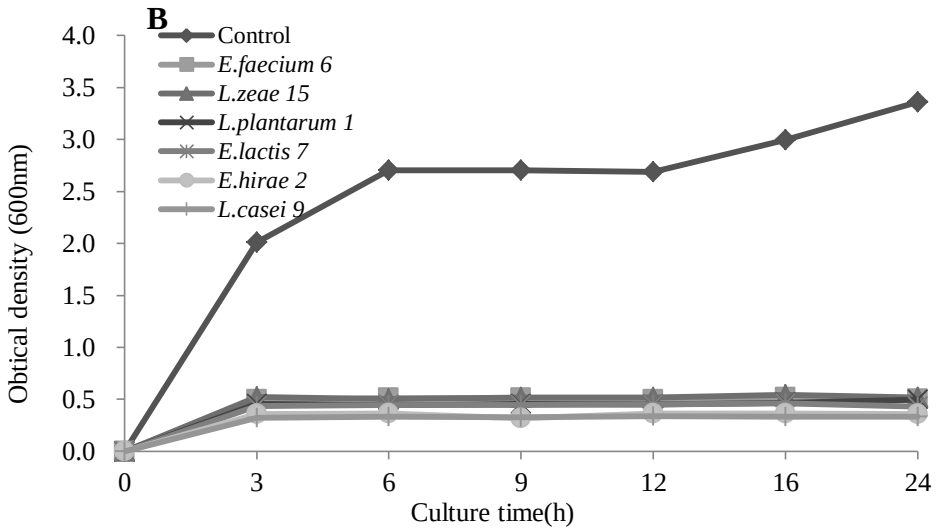

Figure 3. Time course of the antibacterial activity of LAB supernatants toward *E.coli* (A) and *B.subtilis*(B)

3.5.3 Sensitivity to heat of cell free supernatants

Figure 4 shows the sensitivity to heat of cell free supernatants produced from LAB. After heat treatment six strains of LAB with high potent antibacterial activity were incubated with indicator strains for 24h at 30°C and the turbidity was measured by the absorbance at 600 nm. Control was compared with no heated supernatant. The results show that, antibacterial properties were not decreased by heating for 15 min, 30 min, 60 min at100°C. Antibacterial activity decreased by 30% for treatment for 180 min at 100° C.

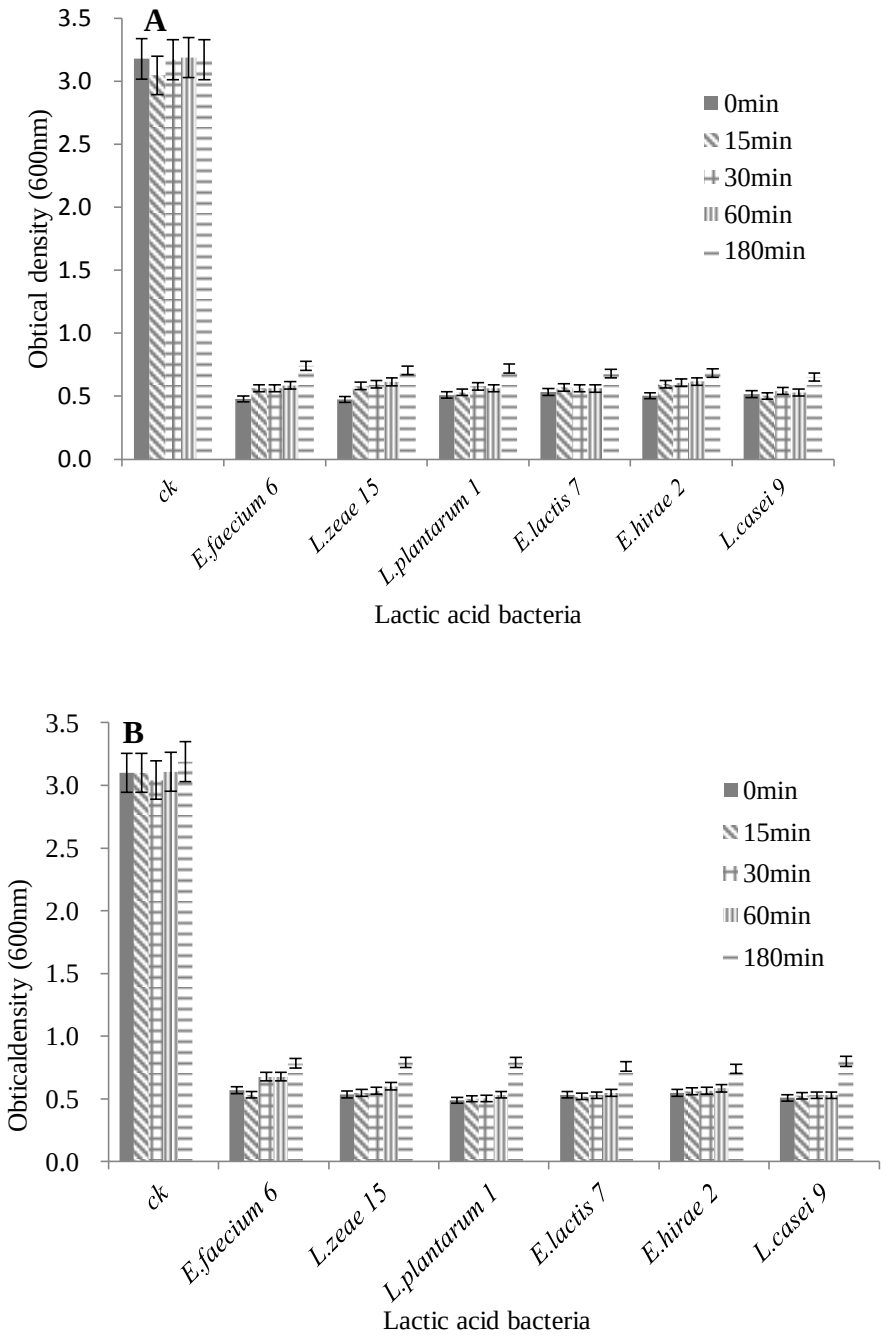

Figure 4. Sensitivity to heat of cell free supernatants produced from LAB supernatants were heated at 100°C for 15,30,60,180 min, respectively, after cooling incubated with *E.coli* (A) and *B.subtilis*(B) for 24h at 37 °C.

3.5.5 pH dependence on biological activity of LAB

Figure 5 presents the pH dependence on the cell-free supernatant produce from LAB. *E.hirae* (Him-2) and *E.faecium* (Ku-6),which lactic acid bacteria had potent antibacterial activity between pH 3 and 9, most activity inhibition of the antibacterial activity was in 6, 7. The antibacterial activity was presumed to be due to the antibacterial peptide produced in the culture supernatant.

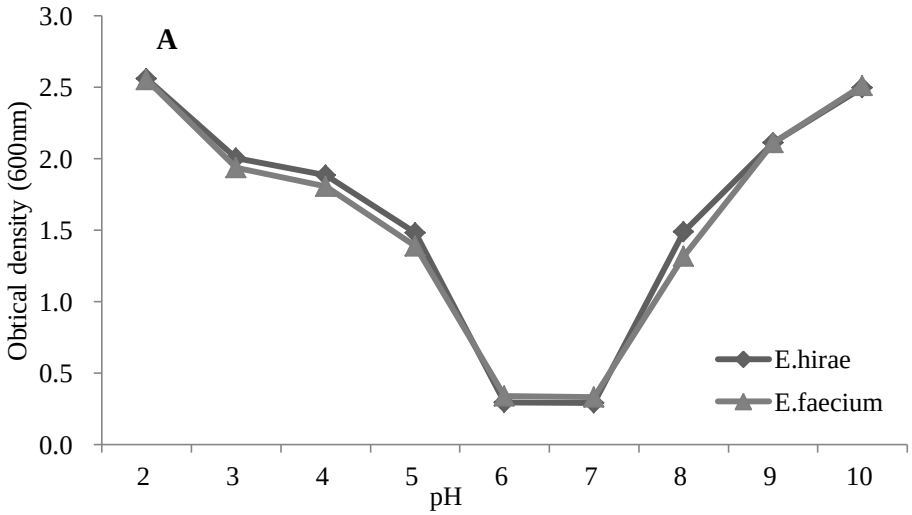

Figure 5. pH dependence of supernatant of *E.hirae*(Him-2) and *E.faecium* (Ku-6) on antibacterial activity of *E. coli* (A) and *B. subtilis*(B)

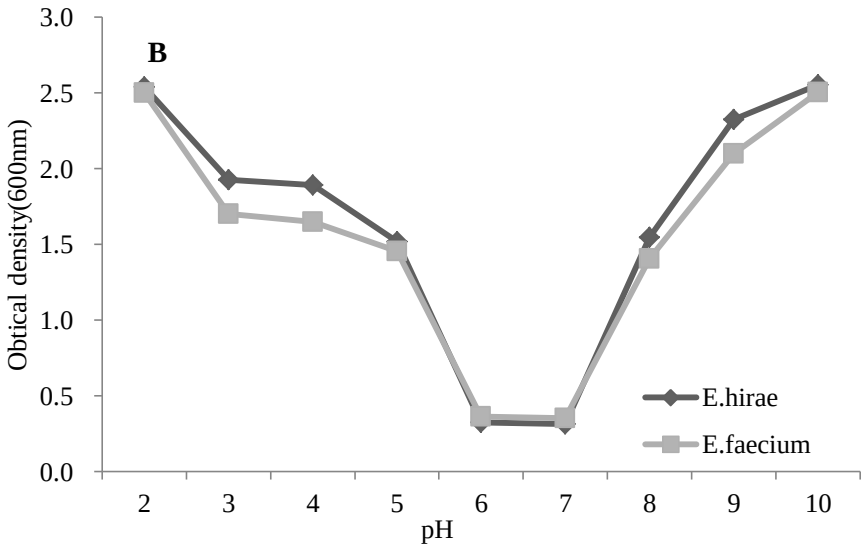

Figure 5. pH dependence of supernatant of *E.hirae*(Him-2) and *E.faecium* (Ku-6) on antibacterial activity of *E. coli* (A) and *B. subtilis*(B)

3.5.6 Enzyme sensitivity of cell free supernatant

Table 4 presents the effect of three protease enzymes treatment on the antibacterial peptide of *L. plantarum* (Su-1), *E. hirae* (Him-2) and *L. zeae* (Ha-15) in the cell free supernatant. The effect of three protease activity enzymes treatment antibacterial activity of cell free supernatant of produce by *L.plantarum* (Su-1), *E. hirae*(Him-2),*L. zeae* (Ha-15). It was sensitive to α-Chymotrypsin, proteinase K and trypsin

Table 4. Effect of enzymes treatment on the antibacterial peptide of cell free supernatant of lactic acid bacteria

Treatment	Antibacterial activity of peptide (AU/ml)		
	L.plantarum (Su-1)	*E. hirae*(Him-2)	*L. zeae*(Ha-15)
Control	6400	6400	1600
Enzymes			
α-Chymotrypsin	0	0	0
Trypsin	0	0	0
Proteinase K	0	0	0

3.5.7 Purification of antibacterial peptide from LAB and molecular weight determination by SDS-PAGE

Figure6 shows that results of the purification of antibacterial peptide from LAB and Molecular weight determination by SDS-PAGE. After dialysis product, by using SDS-PAGE and in one part of gel electrophoresis by used direct antimicrobial activity detection that the molecular weight of the peptide was 2000 Da. That peptide part of SDS PAGE (2000Da) became a clear zone, revealing the presence of high antibacterial and antibacterial peptides.

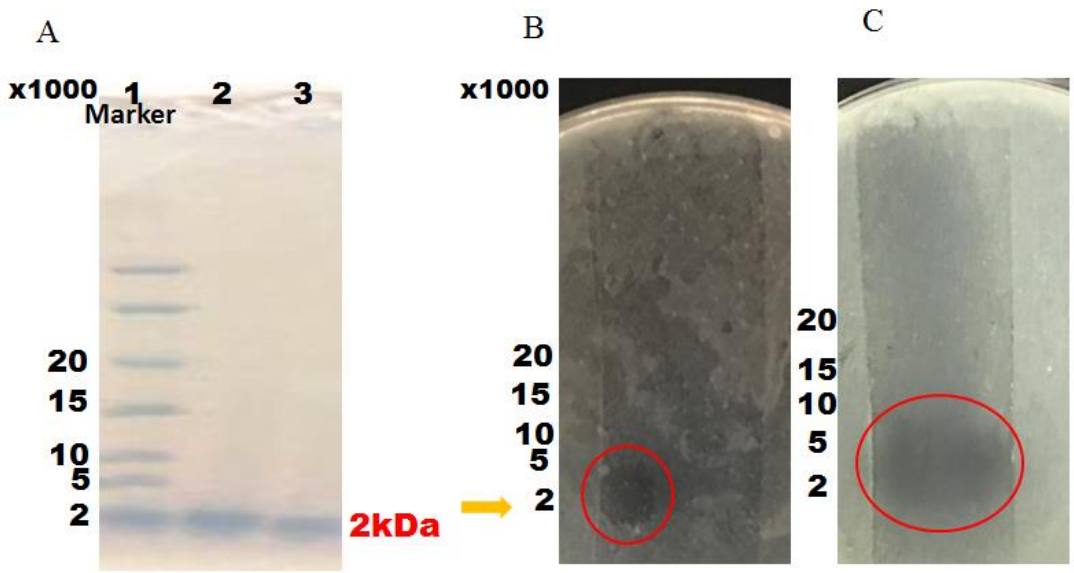

Figure 6. Tricine SDS PAGE analysis of peptide of *L. plantarum*(Su-1) and *L.zeae* (Ha-15) strain. A: Coomassie blue stained gel, lane 1: marker of 2-250 kDa, lane 2: L. plantarum(Su-1),lane 3: L.zeae (Ha-15). B and C: The gel was overlaid with *E. coli* to identify the band corresponding antibacterial activity of *L. plantarum*(Su-1) and *L.zeae*(Ha-15), respectively.

3.6 Conclusion

Nineteen lactic acid bacteria were isolated and identified from 11 dairy products. Among them, *L. plantarum*, L. zeae, *E. hirae* lactic acid bacteria (99% homology) is found to show high antibacterial activity (6-9mm clean zone) against *E. coli*.The antibacterial properties are determined by the results of electrophoresis and antibacterial tests. These lactic acid bacteria were cultured in large quantities, and antibacterial peptides were isolated.

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Chapter IV

Effective use of *L. diolivorans* with high antibacterial and proteolytic activity for fermentation of potato pulp

4.1 Abstract

Potato pulp was inoculated either with the antibacterial lactic acid bacteria *Lactococcus lactis* and *L. diolivorans* and the inoculated potato pulp was ensiled under anaerobic conditions for 30 d at room temperature in a mini-silo. We have previously reported that *L. diolivorans* produces antimicrobial peptides with potent antibacterial activity. therefore, the bacterium is expected to increase the fermentation quality of the potato pulp. The quality of the potato pulp silage was evaluated. The moisture content of the potato pulp silage was remained 822 g/kg before and after ensiling. The protein content in the silage increased from an initial concentration of 39 to 57 g/kg and 58 g/kg for *L. lactis* and *L. diolivorans* inoculations, respectively. The lactic acid content significantly increased from 2 to 52 g/kg (*L. lactis*) and 50 g/kg (*L. diolivorans*) after ensiling, whereas, toxic butyric acid was not detected with either treatment. These results suggest that the inoculation of potato pulp with *L. lactis* or *L. diolivorans* increases the quality and nutrition of potato pulp as silage. In particular, *L. diolivorans* is an efficient inoculant because it produces antibacterial peptides that prevent the increase of saprophyte in silage.

4.2 Introduction

Potato pulp is a by-product of the starch industry, where processing can generate approximately 10% waste pulp (Oda et al., 2002). Potato production in China is the largest in the world, producing >20% of the global yields (Tian et al., 2017). Although potato pulp is suitable as a livestock feed because it comprises starch, peptic substances, and minerals (Mayer et al., 1997; Okine et al., 2005), the product decays easily when exposed to several pathogenic bacteria during preservation for high moisture content (Charmley et al., 2006; Tawila et al., 2008). Therefore, most of the potato pulp (5 million tons/year) is discarded as waste without effective utilization (NBSC 2010). The high energy costs associated with drying and processing the pulp are deterrent to its production and use. Therefore, effective and energy efficient means of processing are necessary for economic and environmental benefits (Tian et al., 2017). Ensiling is an efficient method to prevent potato pulp with high moisture content (McDonald 1991; Kayouli 1999) if the following three requirements are met: (1) adequate levels of fermentable substrate, (2) low buffering capacity, and (3) dry matter contents within 250–400 kg (Wilkinson et al., 2005). Sugimoto reported that the addition of beet pulp, which is an agricultural by-product with very low moisture content, to potato pulp helped reduce the moisture content in the potato pulp silage (Sugimoto et al., 2010).

As an added benefit, inoculation of the pulp with lactic acid bacteria or other bacteria under anaerobic conditions improves the quality of silage and exerts probiotic effects that can enhance livestock performance (Daniel et al., 2018). The production of lactic acid reduces the pH of silage by approximately 4.0 (MacDonald et al., 2002). It has been previously reported that potato pulp ensiled with bacterial or fungal inoculants

L. rhamnosus or *Rhizopus oryzae*, respectively, for 50 d showed increased levels of lactic acid and carbohydrates, whereas starch and pectin content decreased, suggesting that the modified potato pulp silage is a suitable energy source of ruminant feed (Aibibula et al., 2007). Moreover, Oda reported that *R. oryzae* IFO04707 decreased the pH of potato pulp within 1 d and then produced lactic acid under aerobic conditions (Oda et al., 2002). The highest concentration of lactic acid was observed after 6-d of fermentation. Amylases secreted from the fungus also hydrolyzed starch to produce a water-soluble carbohydrate. Therefore, fungus is an effective inoculant for use in ensiling of potato pulp.

L. lactis inoculation of grass silage reportedly increases lactic acid content and produces good quality livestock feed (Charmly et al., 1966), and the relationship between inoculation and livestock performance has been reported by several authors (Gallo et al., 2018; Li et al., 2018; Xu et al., 2018). Ellis also reported that the fermentation quality and nutritive value of grass silage was improved by the addition of *L. lactis* (Ellis et al., 2016). Previously, we have found that a lactic acid bacterium, *L. diolivorans*, isolated from Mongolian fermented mare milk, "airag", produces peptides with molecular weights of approximately 2000 Da and has potent antibacterial activity within a wide range of temperatures and pH (Oyundelger et al., 2016). In this paper, we report the protein and lactic and acetic acid production in potato pulp inoculated with *L. lactis* and *L. diolivorans* to demonstrate the quality of the resultant potato silages.

4.3 Materials and methods

4.3.1 Material

The experiment was conducted at the Zhaosu horse ranch of Yili Kazakh

Autonomous Prefecture in Xinjiang Uygur Autonomous Region, China. For analysis, potato pulp was dried for 48 h at 60°C and then for 4 h at 105°C. The dried potato pulp was ground to powder by passing through a 1-mm screen and then stored at room temperature until use. Lactic acid bacteria, *L. lactis* NCIMB 30160 and L. diolivorans SBS 0007 for inoculation were purchased from the Institute of Microbiology, Xinjang Academy of Sciences, Urumqi, China, and Snow Brand Seed Co. Ltd., Sapporo, Japan, respectively. Neutral detergent solution was prepared by mixing the following 4 reagents in 1 L of distilled water, ethylenediamine tereacetic acid disodium salt (18.6 g, 0.05 mol), sodium borate (6.8 g, 0.02 mol), sodium dodecyl sulfate (3.0 g, 0.01 mol), sodium phosphate (4.6 g, 0.03 mol), and triethylene glycol (6.8 g, 0.05 mol). The mixture was stirred for 1 h at 100°C to produce a clear solution. Ethyl trimethyl ammonium bromide (20 g, 0.05 mol) solution in 1N sulfuric acid (1 L) was used as the acid detergent solution.

4.3.2 Inoculation and ensiling

Inoculation with *L. lactis* was as follows. Dry *L. lactis* (0.15 g, 1×10^8 cell/g) was dispersed in water (30 mL), and the mixture was then sprayed onto the fresh potato pulp (30 kg). The inoculated potato pulp was ensiled in a mini-silo under anaerobic conditions for 30 d at room temperature between 22°C and 25°C. Ensiling with *L. diolivorans* inoculation was performed in the same manner and conditions. Each experiment was performed three times, and the average values of the replicates were used in the analyses.

4.3.3 Component analysis

The content of organic matter and ash was measured by heating ensiled pulp at 550°C for 4 h according to the method of AOAC (AOAC 1990). Protein and fat contents were determined by a Soxhlet extraction with hexane for 24 h, respectively, according to the AOAC method (AOAC 1990). Soluble sugar content was obtained from the dried potato pulp (2 g) by extraction with a mixture of ethanol (800 mL) and water (200 mL) at 80°C. This was repeated three times and the extracts were then hydrolyzed by 5% aqueous sulfuric acid. Starch in the dried potato pulp (2 g) was extracted for 2 h by boiling water with 60% of $HClO_4$ aqueous solution (600 mL), and the extracts were measured for starch content by a spectrophotometric method. Acetic, lactic, propionic, and butyric acid contents were measured by a HPLC according to the method of AOAC (AOAC1990). The neutral and acid detergent fiber (NDF and ADF, respectively) contents were analyzed according to the methods reported by Van Soest (Van Soest et al., 1991). The powdery, dried potato pulp (1 g) was added to 100 mL of the neutral detergent solution. This mixture was then boiled for 1 h, and the precipitate was collected by vacuum filtration using a glass filter (Whatman grade 4). The filtrate was washed with acetone (20 mL) and dried for 2 h at 105°C to yield 0.917 g of NDF. The ADF (0.650 g) was obtained by boiling the dried potato pulp (1.0 g) with the acid detergent solution (100 mL) for 1 h in the above-mentioned manner. Starch content in the dried potato pulp (1.0 g) was determined by boiling the mister with 60% HClO4 aqueous solution (100 mL) for 2 h. Hemicellulose content was calculated from the difference between the NDF and ADF contents. The results of the component analysis were performed three times, and the average values were used.

4.4 Results and discussion

4.4.1 Inoculation of potato pulp with lactic acid bacterium

Potato pulp itself is not suitable as a livestock feed because of its high moisture content (>800 g/kg) and low amount of protein. Ensiling with inoculant is an accepted method to increase nutritional value of crops. However, inoculation of potato pulp with *L. rhamnosus* or *R. oryzae* strains did not increase protein content (Okine et al., 2005). However, *L. diolivorans* showed potent antibacterial activity against *E. coli* and *B. subtilis* across a wide range of pH and temperature that appeared to originate from the produced peptides (Oyundelger et al., 2016). Therefore, *L. diolivorans* is a suitable lactic acid bacterium for use as an inoculant in potato pulp ensiling; it also reportedly prevents saprophyte propagation by the production of antibacterial peptides during fermentation. The produced peptides are also important for nutrition. *L. lactis*, a lactic acid bacterium produced in the manufacture of fermentation foods has also been successfully used for the inoculation of potato pulp (Li et al., 2017).

Table 5. Analysis of potato pulp after inoculation for 30 d at room temperature.[a, b]

Inoculant	Organic substance							Ash
	Total	NDF[c]	ADF[d]	Starch	Soluble sugar	Protein	Fat	
	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
Potato pulp[e]	967	363	348	206	5	39	6	33
Control[f]	974	364	348	195	15	46	6	26
L.lactis[g]	975	360	345	196	10	57	7	25
L.diolivorans[g]	964	354	325	208	13	58	6	36

[a]Original potato pulp had 822 g/kg of moisture.

[b]Before analysis, potato pulp was dried for 48 h at 60°C and then for 4 h at 105°C.

[c]NDF: Neutral detergent fiber consisting mainly of cellulose, hemicellulose, and lignin.

[d]ADF: Acid detergent fiber consisting mainly of cellulose and lignin.

Table 5 summarizes the results of the analysis of potato pulp ensiled in mini-silos for 30 d at room temperature under anaerobic conditions with *L. lactis* and *L. diolivorans* and then dried for 48 h at 60°C and then for 4 h at 105°C. The moisture content in the potato pulp remained unchanged at 822 g/kg before and after ensiling. Similarly, ensiling did not drastically affect the content of organic substances. The NDF obtained by treatment with the neutral detergent solution decreased only slightly from 363 g/kg before ensiling to 360 and 354 g/kg for L. lactis and L. diolivorans inoculations, respectively. The ADF obtained by treatment with the acidic detergent solution also only decreased to 345 and 325 g/kg after ensiling with *L. diolivorans* inoculation responsible for the decrease of the greatest decrease in ADF contents. Hemicellulose and starch were somewhat increased and decreased, respectively, before ensiling. The soluble sugar content increased after ensiling, the digestion of starch and

hemicellulose. The protein content also increased to 57 and 58 g/kg due to peptide production by *L. lactis* and *L. diolivorans* lactic acid bacteria, which worked more effective for the production of proteins than the previous reported *L. rhamnosus* and *R. oryzae* bacteria (Okine et al., 2005). The fat content in the potato pulp remained low even after ensiling. These results suggest that potato pulp ensiled with lactic acid bacteria improves the quality of potato pulp as a livestock feed.

4.4.2 Acid content in potato pulp after ensiling

Figure 7 presents the relationship between pH and lactic acid production during ensiling. The pH of the potato pulp silage decreased rapidly to approximately 4.0 after 10 d and then gradually decreased below 4.0 as the production of lactic acid increased. After ensiling for 30 d, lactic acid content was 52 and 50 g/kg for *L. lactis* and *L. diolivorans* inoculated silage, respectively. Moreover, *L. diolivorans* reportedly increases the quality of potato pulp silage by the production of antibacterial peptides (Oyundelger et al., 2016).

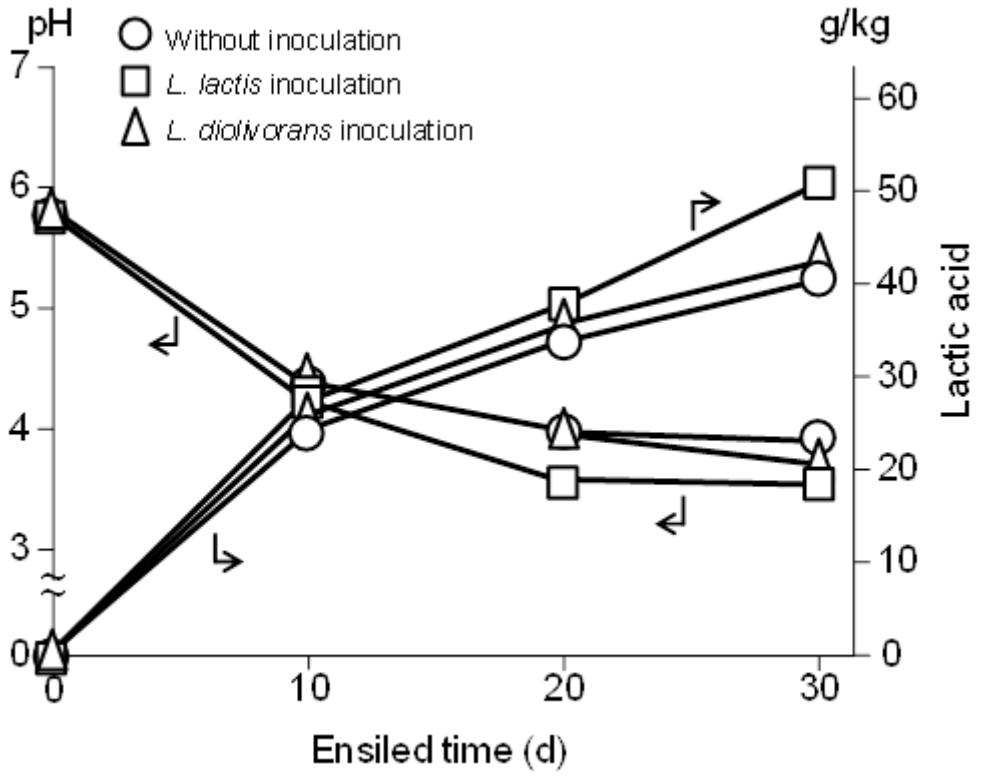

Figure 7. Relationship between pH (left) and production of lactic acid (right) on potato pulp fermentation with lactic acid bacteria.

Table 6. Acid content in potato pulp silage.[a]

Inoculant	Moisture	pH	Lactic acid	Acetic acid	Prop ionic acid	Butyric acid
	g/kg		g/kg	g/kg	g/kg	g/kg
Potato pulp[b]	822	5.8	2	0	0	nd
Control[c]	822	3.9	41	10	9	nd
L.lactis[d]	838	3.6	52	9	9	nd
L.diolivorans[d]	816	3.8	50	8	7	nd

[a]Acid content was measured after drying for 48 h at 60°C and subsequent for 4 h at 105°C.

[b]Original potato pulp had 822g/kg of moisture before ensiling.

[c]Without inoculation.

[d]Inoculation with *L. lactis* and *L .diolivorans*, respectively.

Table 6 summarizes the acid content in the potato pulp after ensiling. The acid content in the potato pulp before ensiling was low. After ensiling, lactic, acetic, and propionic acid contents increased, whereas toxic butyric acid was not detected. The control silage also produced similar concentration of organic acids possibly due to lactic acid bacteria contained in the pre-inoculated potato pulp or present in the mini-silo used for storage. However, inoculation with L. lactis and L. diolivorans yielded large amounts of lactic acid and peptides and decreased the pH below 4.0. These results indicate that the quality of potato pulp was improved by inoculation with L. lactis and L. diolivorans.

4.5 Conclusion

Ensiling of potato pulp with lactic acid bacteria L. lactis and L. diolivorans reduced the pH below 4.0, whereas lactic, acetic, and propionic acids were produced; however, no toxic butyric acid was detected. The protein content of inoculated silage increased due to the production of peptides by the lactic acid bacteria. The NDF content after ensiling decreased with a decrease in the ADF content. These results demonstrated that the inoculation of potato pulp with L. lactis or L. diolivorans is effective in improving the quality of the potato pulp silage. Notably, L. diolivorans lactic acid bacterium produced a large increase in peptide and lactic acid content; therefore, L. diolivorans is expected to be an efficient inoculant. The efficacy of L. diolivorans and other lactic acid bacteria in improving the yield of potato pulp ensiling needs further exploration to establish the usefulness of the pulp as livestock feed. In addition, we plan to further investigate the moisture content in potato pulp before and after ensiling with lactic acid bacteria.

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Chapter V

Conclusion

The author isolated and identified several lactic acid bacteria from traditional fermented dairy products of Kazakh nomads in Xinjiang by using the universal primers and the antibacterial activity of the isolated lactic acid bacteria was investigated. Nineteen lactic acid bacterial (LAB) were isolated and identified by 16S rDNA sequencing analysis. Eleven isolates of Lactobacillus and eight isolates of Enterococcus were identified as belonging to 7 validated species, *L. plantarum, E. hirae, E. faecium, E. lactis, L. casei, Lactobacillus zeae, and E. lactis.*

Before the isolation of lactic acid bacteria, the lactic acid bacteria were enumerated by the plate count technique. Each sample was diluted with sterile water by serial 10-fold dilutions (10^{-5}, 10^{-6}, and 10^{-8}) and 1 ml sample was plated on agar. After 48h incubation at 37℃, cell counts per milliliter (ml) of the sample was enumerated. Isolated colonies with typical characteristics of LAB were picked from each plate and cultured into new MRS agar plate for 48h days at 37°C. The isolates were further characterized by Gram−staining, catalase activity and microscope observation. The bacteria strains isolated had not catalase activity and were Gram positive, indicating that all bacteria isolated were lactic acid bacteria, and these lactic acid bacteria were cultured in large quantities, and antibacterial peptides were isolated. The author found that the time course of antibacterial activity of the supernatant of lactic acid bacteria on *E. coli* and *B. subtilis*, respectively. In both cases, the absorbance was lower than that of the control, and lactic acid bacteria showed high antibacterial properties. Antibacterial properties were not decreased by heating for 15 min, 30 min, 60 min at100°C.

Antibacterial activity decreased by 70% for treatment for 180 min at 100° C. *E. hirae* (Him-2) and *E. faecium* (Ku-6),which lactic acid bacteria had potent antibacterial activity between pH 3 and 9, the most activity was shown at pH 6 and 7. The antibacterial activity was presumed to be due to the antibacterial peptide produced in the culture supernatant.

After dialysis product, by using SDS-PAGE and in one part of gel electrophoresis by used direct antimicrobial activity detection that the molecular weight of the peptide was 2000 Da.

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