Doctor Thesis

Study on the microbial interaction between lactic acid bacteria and yeasts isolated from airag, an alcoholic fermented milk

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Background and Introduction

It was approximately one million years ago when humans were born on Earth. Early dietary habits were influenced by local environmental conditions. Initially, humans collected wild plants, hunted mammals and birds, and caught fish for food. Later, they practiced stock farming or agriculture and the selective breeding of animals and plants, which led to great improvement in productivity. The practice of culturing milk developed with stock farming. Milk from animals was collected for human consumption because it contained an abundance of proteins, saccharides, fat, vitamins, and minerals. As regards the processing of milk, fermentation was commonly employed. Known as one of the ancient techniques of food preservation, fermentation has many features: it produces fermented food having a variety of tastes, flavors, and textures and improves digestibility due to predigestion by beneficial microorganisms. Some fermentative organisms synthesize vitamins and amino acids to improve the nutritive value of food, while also reducing the anti-nutritional components in food (Khetarpaul & Chauhan, 1991; Adams, 1999; Joshi & Pandey, 1999).

Since ancient times, raw milk from cow, horse, camel, and sheep, together with unique local starter, has been used to produce various types of fermented milk in Central Asia and Eastern Europe. Considered an important staple, fermented milk is produced by adding lactic acid bacteria (LAB) starter (together with yeast strains) to milk and incubating the mixture. LAB convert milk sugars into lactic acid to reduce the pH of milk, and casein induces both isoelectric point deposition and gelation of milk. During fermentation, milk proteins are broken down to

produce amino acids or peptides, enhancing digestibility and absorption as well as increasing vitamin B content. Approximately 30-40% of lactose, the main sugar in milk, is utilized by LAB, thereby reducing lactose intolerance. Lactic acid produced by fermentation promotes digestive juice secretion and peristalsis of the bowel. Calcium absorption and iron availability are enhanced by lactic acid and a phosphopeptide formed by ß-casein hydrolysis. There are many types of traditional fermented milk in the Middle East, Central Asia, and Mongolia, including kefir, leben, kumiss, and airag.

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, Mongolians have long practiced the culture of milk. Since ancient times, Mongolian nomads have consumed meat and dairy products as a precious protein source. Mongolians are nomads who mainly live in Mongolia and the Inner Mongolia Autonomic Region of China as the center, China's Qinghai Province, China's Xinjiang Uygur Autonomic Region, China's Tibet Autonomic Region, and Buryat Mongolian area of Russia. Mongolians have preserved nomadic culture to this day, producing a variety of traditional dairy products. In this regard, they may provide vital information of the history of the world's nomadic culture.

In recent years, studies of the functionality of fermented milk and LAB have inspired a number of discussions related to the demand of the times (Table 1). At first glance, the effects or mechanisms of action of LAB from various fermented foods seem different. However, they are related mutually; the improvement of intestinal flora has a causal relationship with immunostimulation or the

improvement of nutritive value and absorption. Thus, LAB prolong life through immunostimulation (Perdigon & Alvarez, 1992; Shu *et al.*, 1999), inhibition of cancer, and prevention of cardiovascular diseases.

Many traditional fermented foods originating from different countries are available. The medicinal and nutritional properties of fermented foods have been "experienced" by several generations. However, the scientific community gave notice to fermented foods only after the publication of book "Prolongation of life" by Metchnikoff (1908), in which he ascribed the longevity of Bulgarians to the consumption of large amounts of fermented milk, which has been described to suppress intestinal putrefaction. Most of the LAB used in fermentation are considered GRAS (Generally Regarded As Safe) for human consumption. Fermentation also improves the nutritional status of food by enhancing protein digestibility and increasing amino acid contents, mineral absorption (Paredes-Lopez & Harry, 1988), and vitamin synthesis by specific microflora (Alm, 1982).

The alcoholic fermented milk airag is one of the traditional fermented dairy products of Mongolia. It is deeply rooted in local areas as a unique food and closely related to the eating habits of Mongolians. Airag is produced from cow, mare, and camel milk by a traditional method using an indigenous starter culture containing LAB, yeasts, and other fermentative organisms. It has gained an important position in Mongolian medicine because of its medicinal value. Depending on the type of raw milk used, it is called chigee (kumiss, mare milk liquor), tarag (cow milk liquor), and hoormog (camel milk liquor) in Inner Mongolia of China; and guun airag (kumiss, mare milk liquor), unyen airag (cow milk liquor), and ingin airag (camel milk liquor) in the State of Mongolia.

We describe herein the method for producing airag in the Inner Mongolia Autonomous Region of China. It is made by nomads from June to September of each year. Fresh milk is collected 4-8 times a day and poured into a fermentation vessel supplemented with starter culture called hurunge, and airag is produced by repeated stirring and fermentation for approximately one week. For the fermentation of airag, a leather bag made with cowhide or sheep hide is used. Today, ceramics and a wooden container are used to enable vigorous stirring. Stirring is done with a cross-shaped wooden stick called Buluuru that is moved up and down six or seven 500-1500 times every day. Stirring creates an aerobic condition for yeast growth, suppresses LAB fermentation to regulate acidity, and prevents coagulation and sedimentation of milk protein or floating of milk fat. The optimal acidity of airag is in the range of 0.7-1.0% and alcohol content is 1.5-3.0%.

The beneficial effects of airag became known after long years of use; chigee (guun airag), in particular, has gained an important position in Mongolian medicine as a unique drink. Chigee therapy has been extensively studied at a Mongolian medical research institute in the Xilinguole League of the Inner Mongolia of China. It is said to improve digestion, inhibit the growth of harmful enteric bacteria, suppress the production of enterotoxins, prevent and treat intestinal diseases, reduce serum lipid and cholesterol levels, and treat arteriosclerosis, high blood pressure, pulmonary tuberculosis, scurvy, and trace element deficiency in a technical book called “kumiss therapy.”

Lactic acid fermentation by LAB, alcohol fermentation by yeast, and their by-products play an important role in producing the unique flavor and fresh taste of airag (Wouters *et al.*, 2002). In addition, acids and peptides produced by

fermentation inhibit the growth of putrefactive bacteria to provide the desired environment for LAB and yeast growth. Furthermore, airag produced by proteolytic enzyme contains amino acids, 1.8-2.0% lipids consisting of vitamins A, D, E, and K, and 20 kinds of fatty acids, 1.5-2.0% alcohol, organic acids, such as lactic acid etc., enzymes, lactoglobulin, lactalbumin, immunoglobulin, and serum albumin. In this regard, it is thought that the microorganisms participating in nutrient production and fermentation, and their metabolic products contribute to the physiological function of airag.

Studies of the microbial constitution of airag are few. Airag microflora differs according to fermentation conditions, the place of production, the fermentation time, and the starter culture used. Ishii *et al.* (1997) studied the microflora of kumiss and isolated LAB (*Lactobacillus rhamnosus*, *Lb. paracasei* subsp. *tolerans*, *Lb. curvatus*) and lactose-fermenting yeast (*Kluyveromyces marxianus* var. *lactis*). Montanari *et al.* (1996) isolated and identified 417 yeast strains from 94 samples gathered from different areas. There were 165 strains of non-lactose-fermenting yeasts and 252 strains of lactose-fermenting yeasts. Ham (1999) reported weight increase and significant reduction of fecal microflora in broilers fed *Lactobacillus plantarum* and *Candida kefyr* isolated from Mongolian kumiss.

Airag, a traditional alcoholic fermented milk of Inner Mongolia, is made by using various kinds of LAB and yeast strains from the natural environment to form definite microflora through repeated symbiosis and antagonism among the microorganisms. The microorganisms are responsible for the unique flavor and the beneficial effects of airag. However, there are few studies on the interactions between LAB and yeast strains in airag.

In this study, we isolated and identified yeast strains from chigee (unyen airag) produced by nomads in the Inner Mongolia Autonomous Region of China. Furthermore, the isolated yeast strains and LAB from our laboratory were used to investigate interactions between them, with a view to developing a new type of fermented milk as starter culture.

Table 1 Major research of fermented milk and LAB focusing on nourishment and physiological function

Effect	Mechanism	Factor of LAB	
Nourishment reinforcement	Improvement of the nutritive value, absorption promotion	Preliminary digestion of the protein	Metabolism activity
Life extension		Vitamins generation	Proteolytic enzyme
	Lactose intolerance improvement	Mineral absorption promotion	Vitamin generation ability
		Lactose resolution	Lactic acid generation
			Lactose degrading enzyme
Infection defense	regulation of the function of intestine	Antibacterial substances	Antibacterial substance ability
	Improvement of the intestinal flora	Affinity with cell body and the intestinal tract	
	Inhibition of harmful bacteria		Cell body component
Inhibition of cancer	Inhibition of harmful metabolism activity	Oligosaccharide in fermented milk	Polysaccharides, cell body component
	Antimutagenicity	Adsorption of the variation source substance	Cell surface structure
	Immunostimulation	Macrophage activation	
		Natural killer cell activation	
Prevention of cardiovascular disease	Blood cholesterol reduction	cholesterol	
		Synthetic inhibition	
		Absorption inhibition	
		Excretion promotion	
	Blood pressure descent	Angiotensin-converting enzyme inhibition	

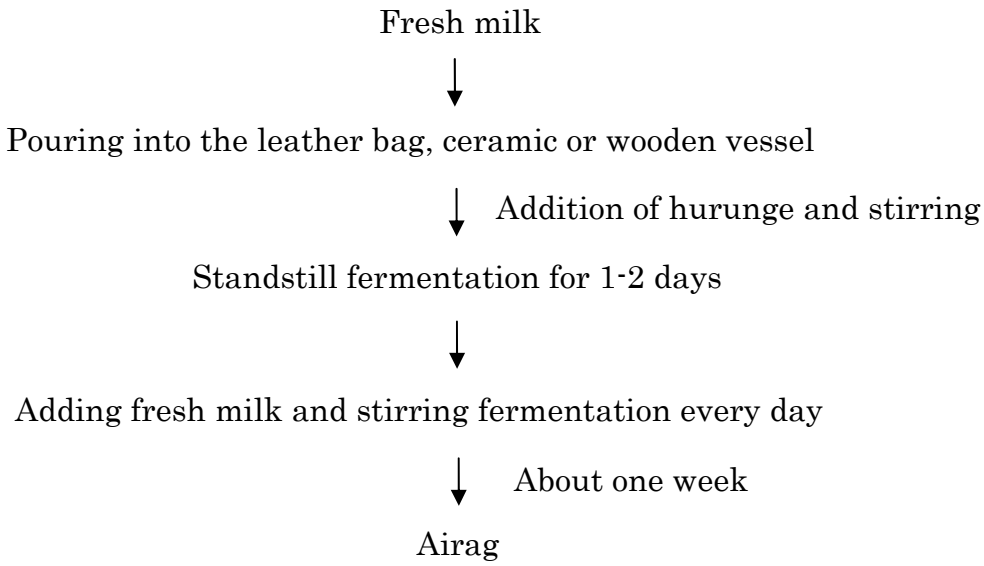

Fig. 1　Method of making airag

Chapter 1

Isolation and identification of yeasts in chigee, traditional fermented mare's milk of Inner Mongolia, China

1. Abstract

"Chigee" is a fermented drink traditionally made from mare's milk in the Inner Mongolia Autonomous Region of China. We isolated and identified the yeasts in five chigee samples collected from the households of Mongolian nomads. Among the lactose-fermenting yeasts, *Candida kefyr* was predominant (21.3%) followed by *Kluyveromyces marxianus* var. *lactis* (11.1%). Of the non-lactose-fermenting yeasts, *C. krusei* was primarily isolated (18.5%) followed by *Saccharomyces cerevisiae* (14.8%) and *S. servazzii* (14.8%). *Pichia cactophila* (12.0%) and *C. valida* (7.4%), non- sugar fermenting yeasts, were also identified.

2. Introduction

Mongolian nomads make a variety of dairy products using raw milk from cattle, horse, camel, and sheep. Mare milk, in particular, is used for this purpose to produce chigee (kumiss, guun airag), a unique drink that has been consumed since ancient times and is deeply ingrained in the eating habits of Mongolians.

Chigee is made by nomads of the Xilinguole League in the Inner Mongolia Autonomous Region from June to September of each year where the fermentation procedure is completed in approximately one week. A mixed microflora of LAB and yeast strains plays roles in the fermentation. The end products of lactic acid and alcoholic fermentation are important contributors to the unique flavor and refreshing taste of kumiss (Wouters *et al.*, 2002). Further, the production of acids and other antimicrobial components during fermentation may promote health (Nout *et al.*, 1989; Mensah *et al.*, 1991; Svanberg *et al.*, 1992; Kingamkomo *et al.*, 1994, 1995). Kumiss is believed to possess health-promoting properties that are primarily related to the ability of the starter to produce B vitamins. In particular, the antimicrobial activity of kumiss against *Mycobacterium tuberculosis* has been reported. These characteristics are attributed to various interactions among yeast strains, LAB, and the secondary microflora such as bacteria and molds (Welthagen & Viljoen, 1998, 1999). The yeast strains, as part of the culture, contribute to the fermentation by supporting the starter (Jakobsen & Narvhus, 1996) and inducing the growth of bacteria whose proteolytic and lipolytic activities are essential for cheese ripening (Fleet, 1990). We studied the microbiological properties of chigee; screened for the fermentative organisms; and enumerated, isolated, and identified the yeast strains from chigee. The objective

of this study was to identify the source microorganisms responsible for the beneficial effects of chigee, which may lead to the development of new methods for the production of healthier fermented milk.

3. Materials and methods

3.1 Sampling

Five samples of chigee produced by nomadic families were collected over a range of approximately 40 km to 100 km from Xilinghot City, Xilingguole League, Inner Mongolia Autonomous Region, China (Fig. 2). The home-made chigee were fermented at 14 °C –20 °C and pH 4.0–5.0 for two to ten days; and brought to the laboratory in a cooler box (4-8 °C) and stored in the refrigerator until analyzed.

3.2 Enumeration and isolation of yeast

Serial dilutions were counted using each of the samples (1 ml) suspended in Ringer's solution (9 ml) and these were poured onto Plate Count YM agar medium containing 100 ppm chloramphenicol. The plates were incubated at 25 °C and 37 °C for 3-5 days. Colonies with distinct morphology features, such as color, shape, and size, were selected and purified by streaking at least three serial times on YM agar medium.

3.3 Identification of yeast

The isolated yeast strains were identified based on their physiologic and morphology characteristics as described in "The Yeasts, A Taxonomic Study" (Kreger-Van Rij NJW, 1984). The identification was performed based on the API ID 32°C test kit (Bio-Merieux) (Gadaga TH, 2000) results using the following tests: fermentation of sugars, assimilation of carbon compounds, assimilation of nitrogen compounds, growth in vitamin-free medium, growth at 37 °C, growth on 50% (w/v) glucose yeast extract agar, resistance to 0.01% cycloheximide, and

carbohydrate assimilation (containing cycloheximide).

4. Results and Discussion

One hundred and eight yeast strains were isolated and identified as *Candida kefyr* (23 strains), *Kluyveromyces marxianus* var. *lactis* (12 strains), *Saccharomyces cerevisiae* (16 strains), *Saccharomyces servazzii* (16 strains), *Candida krusei* (20 strains), *Pichia cactophila* (13 strains), and *Candida valida* (8 strains). Their physiologic and morphology properties are shown in Table 2.

We identified 51 *Candida* species strains that were ovoid, elongated, and cylindrical yeasts that grew to sizes of 2-6 × 2-16 μm after incubation for five days at 25 °C. They formed pseudomycelia that consist of chains of elongated cells; and did not produce ascospores. Among theese strains, we identified 23 strains of *C. kefyr* that were ovoid yeasts at sizes of 2-5 × 2-7 μm and fermented glucose, galactose, lactose, sucrose, and raffinose. *C. kefyr* has been isolated from chigee (Naersong *et al*, 1996; Ishii S *et al*, 1997) and is the predominant important yeast species in a number of traditionally fermented milk products, e.g. laban, a traditional natural fermented milk from Yemen (Arai I *et al*, 2002), amasi from Zimbabwe (Gadaga TH *et al*, 2000), mbanik from Senegal (Gningue PN *et al*, 1991), nono from Nigeria (Okagbue RN & Bankole NO, 1992), and ergo and ititu from Ethiopia (Gonfa A *et al*, 2001). We also identified 20 strains of *C. krusei* that were globose, elongated or cylindrical (2-6 × 2-16 μm); and could ferment only glucose and N-acetylglucosamine and assimilate glycerol and lactic acid using the API ID 32°C test (Table 3). Additionally, they grew in vitamin-free medium, on 50%(w/v) glucose yeast extract agar at 37 °C. *Candida krusei* is a common species in African fermented milk products (Gadaga TH *et al*, 2000). It has been isolated from traditional fermented milk in Inner Mongolia (Burentegusi *et al*, 2002;

Shuangquan *et al*, 2004); and is commonly isolated from yogurt. Another eight strains did not ferment sugars and were identified as *C. valida* that has been previously isolated from surface-ripened cheese (Corsetti A *et al*, 2001). Among the *Candida* species, *C. kefyr* was able to ferment lactose and *C. krusei* that cannot ferment lactose; and were predominantly isolated from the chigees.

We isolated 32 *Saccharomyces* species strains, subglobose and 2-5 × 3-7 μm that were detected after incubation for five days at 25 °C. They produced ascospores but did not form pseudohyphae (Table 2). Among them, 16 strains fermented glucose, galactose, sucrose, maltose, and raffinose, but not lactose. They could not assimilate cellobiose, lactose or inositol, but grew in a vitamin-free medium and not on 50% (w/w) glucose yeast extract agar. *S. cerevisiae* is usually isolated from Mongolian traditional fermented milk products[14] as well as other fermented milk products (Gningue PN *et al*, 1991; Okagbue RN & Bankole NO, 1992; Ishii S *et al*, 1997; Abdelgadir WS *et al*, 2001; Gonfa A *et al*, 2001; Corsetti A *et al*, 2001). It is reported the function of *S. cerevisiae* in the fermentation of foods is the conversion of carbohydrates into alcohols and other aromatic components such as esters, organic acids and carbonyl compounds that play important roles in producing the desired flavors (Torner MJ *et al*, 1992). The other 16 strains that fermented and assimilated glucose and galactose and could grow in 100 ppm cycloheximide but not in vitamin-free medium were identified as *S. servazzii*.

Twelve strains (1-3 × 2-11 μm) were identified as *K. marxianus* var. *lactis*. They fermented glucose, galactose and lactose, and could assimilate glucose, galactose, sucrose, lactose, cellobiose, and trehalose (Table 2). This species has been isolated from Chigee in Inner Mongolia (Ishii S *et al*, 1997) and from several traditional African fermented milk products (Gningue PN *et al*, 1991; Okagbue RN &

Bankole NO, 1992; Gonfa A *et al*, 2001).

Thirteen ovoid and elongated yeasts strains at sizes of 1-4 × 2-8 µm produced ascospores after incubation for five days at 25 °C and formed pseudohyphae. However, they did not ferment or assimilate any sugars except glucose and D-glucosamine HCl (Table 3). They were identified as *P. cactophila* where previously the *Pichia* species have been isolated from cheese (Corsetti A *et al*, 2001).

5. Conclusions

Here, we isolated and identified seven yeast species from "chigee," a fermented drink traditionally made from mare's milk in Inner Mongolia. Among the lactose-fermenting yeasts, *C. kefyr* was the predominant, followed by *Kluyveromyces marxianus* var. *lactis*. Among the non-lactose-fermenting yeasts, *C. krusei* was primarily isolated followed by *S. cerevisiae* and *Saccharomyces servazzii*. *Pichia cactophila* (12.0%) and *C. valida* (7.4%) that do not ferment sugar yeast were also isolated. The results suggest the yeasts are the essential microbial source for fermentation of mare's milk to make chigee. Therefore, we will select these beneficial strains as the starter cultures to improve the technologic properties for the preparation of chigee as well as for the development of other commercial fermented milk products.

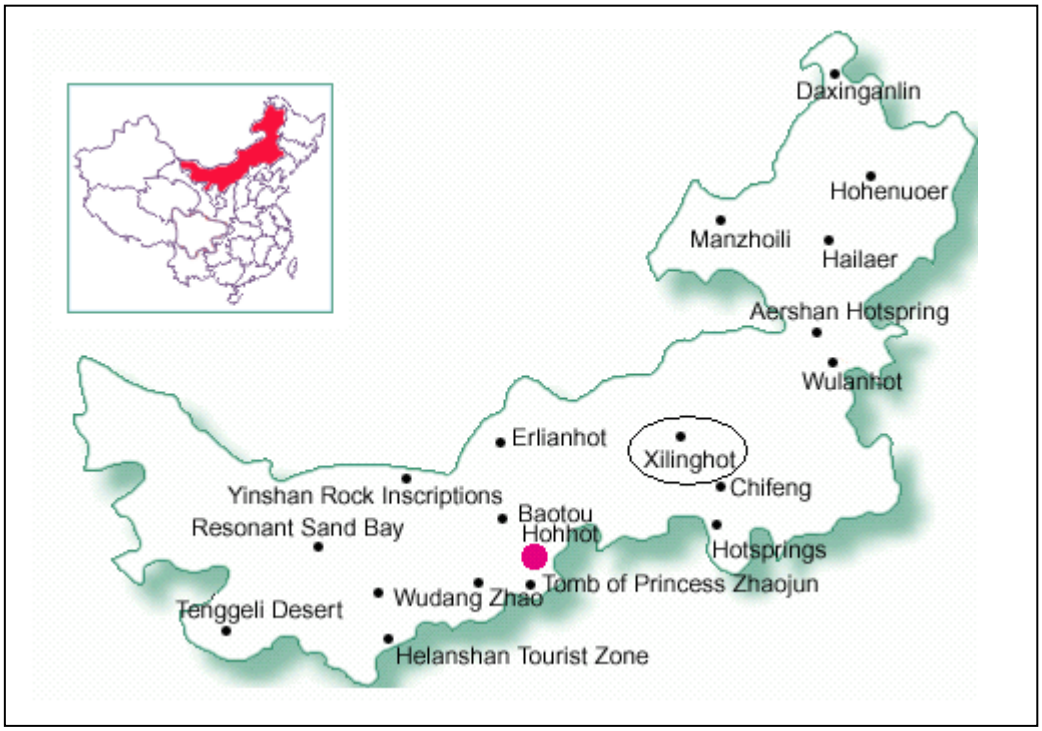

Fig. 2 Map of Inner Mongolia, China

Chigee samples were collected from Xilinghot City (circled on the map).

Table 2. Taxonomic properties of yeast strains isolated from traditional fermented milk chigee in Inner Mongolia, China.

Species	*Candida*(C) *kefir*	*C. krusei*	*C. valida*	*Pichia*(P) *cactophila*	*Saccharomyces*(S) *cerevisiae*	*S. servazzii*	*Kluyveromyces*(K) *marxianus* var. *lactis*
Strain	23	20	8	13	16	16	12
Colony color [1)]	White	White	Yellowish	Yellowish	White	White	Yellowish
Vegetative cell							
Shape	Ovoid	Ovoid, Long-ovoid or Cylindrical	Ovoid or Cylindrical	Ovoid or Long-ovoid	Ovoid	Ovoid or Spheroidal	Spheroidal, Ovoi or Cylindrical
Size (μ m) [2)]	(2～5)×(2～7)	(2～6)×(2～16)	(2～6)×(3～11)	(1～4)×(2～8)	(2～5)×(3～7)	(2～4)×(2～	(1～3)×(2～11)
Pseudomycelium	+ [3)]	+	+	+	−	−	−
Mycelium	−	−	−	−	−	−	−
Ascospore	−	−	−	+	+	+	+
Assimilation of KNO_3	−	−	−	−	−	−	−
Vitamin-free medium	−	+	V [4)]	−	+	−	−
50% Glucose	+	−	+	+	−	+	−
Growth at 37℃	+	+	−	+	+	−	−
Cycloheximide	+	−	−	−	−	+	+
Fermentation of :							
Glucose	+	+	−	−	+	+	+
Galactose	+	−	−	−	+	+	+
Sucrose	+	−	−	−	+	−	V
Lactose	V	−	−	−	−	−	+
Maltose	−	−	−	−	+	−	−
Raffinose	V	−	−	−	+	−	−
Assimilation of:							
Glucose	+	+	+	+	+	+	+
Galactose	+	−	−	−	+	+	+
Sucrose	+	−	−	−	+	−	+
Lactose	+	−	−	−	−	−	+
Maltose	−	−	−	−	+	−	−
Cellobiose	V	−	−	−	−	−	+
Melibiose	−	−	−	−	−	−	−
Trehalose	V	−	−	−	+	V	+
Raffinose	+	−	−	−	+	−	−
Inositol	−	−	−	−	−	−	−
Soluble Starch	−	−	−	−	−	−	−

[1)] After incubation for two days on YM agar. [2)] After incubation for five days on YM agar. [3)] + indicates positive. − indicates negative. [4)] V indicates some are positive .

Table 3. Results of API ID 32℃ test[1] for yeast strains isolated from the traditional fermented milk chigee in Inner Mongolia, China.

Species	*C. kefyr*	*C. krusei*	*C. valida*	*P. cactophila*	*S.cerevisiae*	*S.servazzii*	*K. marxianus* var. *lactis*
Strain	5	4	3	3	3	4	2
Galactose	+[2]	−	−	−	+[2]	+	+
Cycloheximide	+	−	−	−	−	+	+
Sucrose	+	−	−	−	+	−	+
N-acetylglucosamine	−/+	+	+/−	−/+	−	−	−
Lactic acid	+	+	−	−	+/−	−	−
L-Arabinose	+/−	−	−	−	−	−	−
D-Cellobiose	−/+	−	−	−	−	−	+
Raffinose	+	−	−	−	+	−	−
D-Maltose	−	−	−	−	+	−	+/−
Trehalose	−/+	−	−	−	−/+	+	+
2-Ketogluconic calcium	−	−	−	−	−	−	−
α -Methyl-D-glucoside	−	−	−	−	−/+	−	−
Mannitol	+/−	−	−	−	−	−	+
Lactose	+	−	−	−	−	−	+
Inositol	−	−	−	−	−	−	−
D-Sorbitol	+	−	−	−	−	−	−
D-Xylose	+	−	−	−	−	−	−
D-Ribose	−/+	−	−	−	−	−/+	−
Glycerol	−/+	+	+	−/+	−	−	−
L-Rhamnose	−	−	−	−	−	−	−
Parathinose	−	−	−	−	−/+	−	−
Erythritol	−	−	−	−	−	−	−
D-Melibiose	−	−	−	−	−	−	−
Glucuronic acid	−	−	−	−	−	−	−
D-Melezitose	−	−	−	−	−	−	−
Potassium gluconate	−	−	−	−	−	−	−
Levulinic acid	−	−	−	−	−	−	−
Glucose	+	+	+	+	+	+	+
L-Sorbose	−	−/+	−	−	−	−	−
D-glucosamine·HCI	−	−	+	+	−	−	−
Aesculin	+	−	−	−	+	−	+

[1] After incubation for two days on the medium for API ID 32℃ test. [2] + indicates positive. − indicates negative.

Chapter 2

Interaction between lactic acid bacteria and yeasts in airag, an alcoholic fermented milk

1. Abstract

The interaction between nine lactic acid bacteria (LAB) and five yeast strains isolated from airag of Inner Mongolia Autonomic Region, China was investigated. Three representative LAB and two yeasts showed symbioses were selected and incubated in 10% (w/v) reconstituted skim milk as single and mixed cultures to measure viable count, titratable acidity, ethanol and sugar content every 24 h for 1 week. LAB and yeasts showed high viable counts in the mixed cultures compared to the single cultures. Titratable acidity of the mixed cultures was obviously enhanced compared with that of the single cultures, except for the combinations of *Lactobacillus reuteri* 940B3 with *Saccharomyces cerevisiae* 4C and *Lactobacillus helveticus* 130B4 with *Candida kefyr* 2Y305. *C. kefyr* 2Y305 produced large amounts of ethanol (maximum 1.35 g/L), whereas non-lactose-fermenting *S. cerevisiae* 4C produced large amounts of ethanol only in the mixed cultures. Total glucose and galactose content increased while lactose content decreased in the single cultures of *Leuconostoc mesenteroides* 6B2081 and *Lb. helveticus* 130B4. However, both glucose and galactose were completely consumed and lactose was markedly reduced in the mixed cultures with yeasts. The result suggests that yeasts utilize glucose and galactose produced by LAB lactase to promote cell growth.

2. Introduction

Airag, a traditional fermented milk well loved by Mongolians, has been drunk habitually since ancient times as a unique drink. It is produced from cow, mare, and camel milk by a traditional method using an indigenous starter culture (previously made airag) containing LAB, yeast, and other fermentative organisms. Airag has gained an important position in Mongolian medicine because of its medicinal value. The beneficial effects of traditional fermented milk in Inner Mongolia, which include the prevention of hypertension, gastroenteritis, and tuberculosis, have been experimentally demonstrated, and the relationship of the distinct flavor of airag with its microbiota has been reported (Burentegusi *et al.*, 2002; Shuangquan *et al.*, 2004, 2006). Earlier works have shown that LAB and yeast strains are the predominant microorganisms in most fermented foods (Steinkraus, 1996; Olasupo *et al.*, 1997; Nago *et al.*, 1998; Kunene *et al.*, 2000; Gadaga *et al.*, 2001). Marshall (1987) reviewed the possible interactions between LAB and yeasts in fermented milk, but did not elucidate the mechanisms underlying those interactions. Several microbial interactions between LAB and yeasts have been found in fermented products, such as blue cheese, white mould cheese, bacterial surface ripened cheese, kefir, and koumiss (Subramanian & Shankar, 1985; Fleet, 1990; Jakobsen & Narvhus, 1996).

Both positive and negative interactions between LAB and yeasts have been reported, although the mechanisms underlying the interactions are not well understood. Positive interactions, such as those between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from sugary kefir grains, include the stimulation of LAB by yeast strains through the production of carbon dioxide, pyruvate, propionate, and succinate (Leroi & Pidoux, 1993a). In addition, some LAB strains release galactose into the growth medium as a by-product of lactose metabolism (Davidson & Hillier, 1995; Marshall & Tamime, 1997), which would favor the growth of lactose-negative yeasts. On the other hand, the negative interactions mainly concern the mutual inhibition of growth. Yeasts are generally inhibited by LAB-producing compounds, such as phenyllactic acid and

4-hydroxyphenyllactic and cyclic peptides (Nielsen *et al.*, 1998). Conversely, LAB growth is partly inhibited by fatty acids produced during the metabolism of lipolytic yeasts (Broome *et al.*, 1979). The positive and negative interactions that influence the growth and metabolism of either LAB or yeasts would change fermentation time and/or the production of essential flavor compounds (Gadaga *et al.*, 2001).

The aims of this work were to elucidate the interactions between LAB and yeasts in airag by examining changes of the metabolites in 10% (w/v) reconstituted skim (RS) milk and comparing growth, acidity, and ethanol and sugar contents in mixed cultures with those in single cultures; and to clarify the role of microorganisms in airag production.

3. Materials and Methods

3.1 LAB and yeast strains

The following nine strains of LAB and five strains of yeast used in this study were isolated from traditional fermented milk, airag, at our laboratory and stored at –80°C in RS skim milk supplemented with 0.1% glutamic acid monosodium (Nacalai Tesque Co., Kyoto, Japan) for later use. The LAB and yeast strains were cultured three times in MRS (10g Peptone, 8g Lab-Lemco Powder, 4g Yeast Extract, 20g Glucose, 1mL Tween 80, 2g Di-potassium Hydrogen Phosphate, 5g Sodium Acetate $3H_2O$, 2g Tri-ammonium Citrate, 0.2g Magnesium Sulphate $7H_2O$, 0.05g Manganese Sulphate $4H_2O$/L) broth (Oxoid, Basingstoke, Hampshire, England) and YM broth (5g Bacto™ Tryptone, 3g Bacto™ yeast extract, 3g extract malt, 10g glucose/L) medium at 30 and 25°C for 24 h, respectively. *Leuconostoc mesenteroides* subsp. *dextranicum* 6B2081 and *Lactobacillus reuteri* 940B3 were identified in this study. *Lactobacillus helveticus* 130B4 (Shuangquan *et al.* 2004), *Streptococcus thermophilus* 1230B2, *Leuconostoc lactis* 420B1, *Enterococcus faecium* 3B3083, *Lactobacillus plantarum* 440 M6, *Lactobacillus casei* 6B4084, *Lactobacillus paracasei* 6B3073 (Burentegusi *et al.* 2002), *Saccharomyces cerevisiae* 4C, *Saccharomyces servazzii* 5Y201, *Candida kefyr* 2Y305, *Candida krusei* 3Y201 and *Kluyveromyces marxianus* var. *lactis* 5Y305 (Sudun *et al.* 2010) were isolated from traditional fermented milk in Inner Mongolia, China.

3.2 Identification of two LAB strains

Two strains, 6B2081 and 940B3, were identified on the basis of their biochemical and physiological characteristics, sugar fermentation profiles in the API 50 CH test (BioMerieux, Marcy l'Etoile, France) and partial 16S rDNA sequences; as described by de Vos *et al.* (2009) and Wood and Holzapfel (1995).

The growth of strain 6B2081 at 10, 15 and 45°C and that of strain 940B3 at 10, 15 and 45°C were investigated. Then, gas production from glucose and NH_3 production from arginine and lactic acid isomer (Otsuka *et al.* 1994) were examined.

Bacterial genomic DNA of the two strains was extracted and purified according to the lysozyme-SDS (sodium dodecylsulfate) method as described in Miyamoto *et al.* (2010). Their 16S ribosomal RNA genes (16S rDNA) were partly amplified from the extracted DNA solutions by PCR using the following different primer pairs. In the case of 6B2081, *Leuconostoc* species-specific primers, Lmes-f and Lmes-r, were used (Lee *et al.* 2000), whereas universal primers, 8F and 1492R, were used for 940B3 (Cibik *et al.* 2000). PCR was performed using the GeneAmp PCR system 9700 (Perkin-Elmer, Boston, MA, USA) and a PCR kit (GE Healthcare, Buckinghamshire, UK). The reaction mixture components and the PCR conditions were almost the same as those in Cibik *et al.* (2000) and Lee *et al.* (2000). The PCR products were sequenced using an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA). Homology search was accomplished by using a BLAST (Basic Local Alignment Search tool) search of the DNA Databank of Japan (DDBJ).

3.3 Inoculation of RS milk with single and mixed cultures

LAB and yeasts were transferred to RS milk medium by 2% and 1% (v/v) inocula in single and mixed cultures from MRS and YM broth, respectively and incubated to measure titratable acidity and ethanol at 30°C for 7 days. During the entire fermentation time of selected strains, the samples were analyzed for titratable acidity, viable LAB counts, viable yeast counts, and ethanol and sugar contents every 24 h.

3.4 Selection of representative LAB and yeasts

Three LAB and two yeast strains were representatively selected from all strains, based on their ability to produce lower and highest levels of acids for LAB, and to ferment lactose or not for yeasts at 30°C for a week. The following strains were selected and investigated in detail about their interaction by measuring viable count, titratable acidity, ethanol and sugar content every 24 h for 1 week: *Leuconostoc mesenteroides* subsp. *dextranicum* 6B2081, *Lactobacillus reuteri*

940B3, *Lactobacillus helveticus* 130B4, *Saccharomyces cerevisiae* 4C and *Candida kefyr* 2Y305.

3.5 Viable microorganisms

For enumeration of viable LAB and yeast counts, the diluted samples were spread on BCP (5g Peptone, 2.5g Yeast Extract, 1g Glucose, 1g polysorbate 80, 0.1g L-cysteine, 0.06g, 15g agar/L) agar (Nissui, Tokyo, Japan) and YM agar (containing 1.5% agar purified powder in YM broth) and incubated at 30 and 25°C for 3 and 4 days, respectively. Ten parts per million of cycloheximide was used for the growth-inhibition of yeast for bacterial counts, whereas 100 ppm chloramphenicol was used for the growth-inhibition of LAB for yeast counts in mixed cultures.

3.6 Chemical analyses

Titratable acidity was determined by acid-base titration with 0.1 mol/L NaOH, and the value was calculated as equivalent percent (w/w) of lactic acid.

Ethanol was analyzed with the F-kit (Roche Diagnostics GmbH, Mannheim, Germany). Each sample (5 g) was mixed with 25 mL of distilled water and kept under slight agitation at 50°C for 15 min. For protein precipitation, 2.5 mL of Carrez solution I (3.6 g of $K_4(Fe(CN)_6) \cdot 3H_2O$ in 100 mL), 2.5 mL of Carrez solution II (7.2 g of $ZnSO_4 \cdot 7\ H_2O$ in 100 mL) and 5 mL of 0.1 mol/L NaOH were added and the volume was adjusted to 50 mL by adding distilled water. After the reaction mixture was completely mixed and filtrated, the absorbance of the supernatant was measured at 340 nm.

Sugar content was quantified by high-powered liquid chromatography as described in Richmond *et al.* (1982). Each fermented milk sample (5 mL) was adjusted to 80% (v/v) ethanol solution by adding 99.8% (v/v) ethanol, and then centrifuged at 10 000 × *g* for 5 min. Sugar was extracted from the supernatant and mixed with distilled water, and filtered with Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA).

4. Results

4.1 Identification of two LAB strains

Two LAB strains, 6B2081 and 940B3, were Gram-positive and catalase-negative, and produced gas from glucose. Strain 6B2081 grew at 10 and 15°C but not at 45°C, and produced D-lactic acid isomer. Ammonia production from arginine was not confirmed on 6B2081. In the API 50 CH test, 6B2081 fermented arabinose, ribose, galactose, glucose, fructose, mannose, acetyl glucosamine, maltose, lactose, sucrose and trehalose, but not the other sugars (Table 4). These characteristics are similar to those of the type strain, NCDO 529, of *Leu. mesenteroides* subsp. *dextranicum* (Table 4), and 6B2081 was therefore identified as *Leu. mesenteroides* subsp. *dextranicum*. Furthermore, this identification was supported by the attainment of the PCR amplification of the *Leuconostoc* species-specific fragment (data not shown).

Strain 940B3 grew at 45°C but not at 10 and 15°C, and produced DL-lactic acid isomers. 940B3 also produced ammonia from arginine; and fermented arabinose, ribose, galactose, glucose, maltose, lactose, melibiose, sucrose, raffinose and gluconate, but not the other sugars. These profiles almost correspond to those of *Lb. reuteri* DSM 20016 type strain (Table 4). In addition, the partial 16S rDNA sequence of 940B3 (accession No. AB693939) had 99.7% homology to that of DSM 20016. These results identify strain 940B3 as *Lb. reuteri*. *Lb. reuteri* has been isolated from Zabady, a traditional fermented milk from Egypt (El-Baradei *et al.* 2008).

In this study, 54 dominant strains (25.2%) of *Leuc. mesenteroides* subsp. *dextranicum* (Watanabe *et al.* 2008) and three strains of *Lb. reuteri* (1.4%) were identified from 214 isolated strains of LAB. The biochemical and physiological characteristics of two type strains, *Leu. mesenteroides* subsp. *dextranicum* NCDO 529 and *Lb. reuteri* DSM 20016, are shown in Table 1 as described by Sneath *et al.* (1986).

4.2 Selection of representative LAB and yeasts

LAB fermented the RS milk to increase in titratable acidity from 0.17% to 0.28–2.57% and to 0.23–2.69% in single and mixed cultures at 30°C for 7 days, respectively. *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 and *Lb. reuteri* 940B3 produced low levels of acids in the single culture and were drastically enhanced in mixed cultures with yeasts or nearly not changed in combination with *Lb. reuteri* 940B3 with non-lactose fermenting yeasts. *Lactobacillus helveticus* 130B4 showed the highest acidity in the single culture and decreased in mixed cultures except with *S. cerevisiae* 4C. There was no significant changes of acidity for the other LAB strains in mixed cultures compare to the single culture (Table 5).

In the lactose-fermenting yeasts, ethanol of *C. kefyr* 2Y305 was obviously promoted in mixed cultures with LAB apart from *Lb. paracasei* 6B3073. However, *K. marxianus* var. *lactis* 5Y305 had almost no appreciable different for ethanol between single and mixed cultures. In the non-lactose fermenting yeasts, *S. cerevisiae* 4C produced large numbers of ethanol in mixed cultures with LAB, but the other yeast strains showed no significant increase (Table 6).

4.3 Viable microorganisms in single and mixed cultures

The viable counts for *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in RS milk peaked at 8.75 $\log_{10}$ colony-forming units (CFU)/mL on the second day and afterward decreased to 6.65 $\log_{10}$ CFU/mL on the seventh day in the single culture. The final viable count for *Leuc. mesenteroides* 6B2081 was higher in the mixed cultures with *S. cerevisiae* 4C and *C. kefyr* 2Y305 (8.17 and 7.65 $\log_{10}$ CFU/mL) than in the single culture (Table 7). *Lb. reuteri* 940B3 grew over one log cycle and the viable count was between 7.18 and 8.66 $\log_{10}$ CFU/mL in both single and mixed cultures. *Lb. helveticus* 130B4 showed high viable count in the mixed cultures with *S. cerevisiae* 4C, but final counts were reduced in combination with *C. kefyr* 2Y305 (Table 7).

The ability of yeast culture to grow in RS milk also differed. The final counts

were in the range of 6.40–7.28 $\log_{10}$ CFU/mL. *S. cerevisiae* 4C cells increased in number when grown in the mixed culture with two *Lactobacillus* strains, but showed poor growth ability in the single culture. *S. cerevisiae* 4C cell growth was slightly enhanced in the mixed culture with *Leuc. mesenteroides* after the third day. *C. kefyr* 2Y305 had higher viable count in the mixed culture with *Leuc. mesenteroides* 6B2081 and *Lb. reuteri* 940B3 than in the single culture and in mixed culture with *Lb. helveticus* 130B4 (Table 8).

4.4 Titratable acidity and ethanol contents

Leuc. mesenteroides subsp. *dextranicum* 6B2081 showed a significant increase in acidity from 0.17% to 0.70–0.87% when cultured with the two yeast strains than when cultured alone (0.28%). *Lb. reuteri* 940B3 acidified the RS milk in both single and mixed cultures with *C. kefyr* 2Y305, reaching a final acidity of 0.31 and 1.10%, respectively. When grown in the mixed culture with *S. cerevisiae* 4C, the acidity was slightly low compared to that in the single culture. *Lb. helveticus* 130B4 fermented the RS milk and produced almost the same acidity values (2.57 and 2.69%, respectively) in the single and the mixed culture with *S. cerevisiae* 4C. On the other hand, the acidity decreased when *Lb. helveticus* 130B4 was grown in the mixed culture with *C. kefyr* 2Y305 (Table 7). In the single cultures of yeasts, *S. cerevisiae* 4C did not cause any notable changes in the acidity of RS milk, whereas *C. kefyr* 2Y305 increased the acidity from 0.17 to 0.35% (data not shown).

C. kefyr 2Y305 in both single and mixed cultures, and *S. cerevisiae* 4C in the mixed culture with *Leuc. mesenteroides* produced large amounts of ethanol (up to 1.35 and 1.24 g/L). In contrast, *S. cerevisiae* 4C in the single culture and in the mixed culture with *Lb. reuteri* 940B3 produced very little ethanol (0.2 and 0.36 g/L, respectively) (Table 8). From the results, it was concluded that *C. kefyr* 2Y305 could assimilate lactose, whereas *S. cerevisiae* 4C almost could not do so.

4.5 Sugar contents

In the 7 days of fermentation, total glucose and galactose content increased to

16.17 g/L while lactose content decreased to 24.32 g/L from 51.19 g/L in the single culture of *Leuc. mesenteroides* 6B2081. Lactose content was obviously reduced to 21.11 g/L in the mixed culture of *Leuc. mesenteroides* 6B2081 with *S. cerevisiae* 4C and to 0 g/L with *C. kefyr* 2Y305. The decrease in lactose content was faster in the mixed cultures than in the single culture, particularly after the fourth day of fermentation. Meanwhile, glucose and galactose were completely consumed in the mixed cultures (Fig. 3). Compared with the rapid decrease of the lactose content in the single culture of *C. kefyr* 2Y305, the decrease of the lactose content was slow in the mixed culture of *Lb. reuteri* 940B3 with the two yeast strains. Final lactose content was significantly reduced to 14.19 g/L in the mixed culture of *Lb. helveticus* 130B4 with *S. cerevisiae* 4C and to 0 g/L with *C. kefyr* 2Y305 on the fourth day of fermentation while glucose and galactose were all utilized.

5. Discussion

The co-existence of LAB, yeasts, and molds in naturally fermented milk has led to the suggestion of possible interactions among these groups of microorganisms. In the process of making airag, lactic acid fermentation by LAB, alcohol fermentation by yeast and their by-product, play an important role in flavor formation.

Growth of the most viable LAB in RS milk was promoted by the mixed culture rather than the single culture. This suggests that yeast stimulated the growth of LAB by providing such essential metabolites as pyruvate, amino acids and vitamins (Roostita & Fleet 1996; Gadaga *et al.* 2001). In contrast, some LAB strains were inhibited by some yeast strains, such as *Leuc. lactis* 420B1 which was inhibited in all mixed cultures. This may be due to the fact that yeast may produce free fatty acids and other metabolites that inhibit LAB (Venugopal 2000).

C. kefyr 2Y305 was able to reach a maximum count of 7.18 $\log_{10}$ CFU/mL from an initial inoculum count of 5.79 $\log_{10}$ CFU/mL. Ethanol production reached 1.35 g/L and titratable acidity increased to 0.35% from 0.17% in RS milk. The above results can be explained by the ability of *C. kefyr* 2Y305 to utilize lactose. Meanwhile, *S. cerevisiae* 4C showed poor growth in RS milk. This can be explained by the fact that *S. cerevisiae* 4C does not utilize lactose because it lacks a transport mechanism for lactose into the yeast cell (Walker 1998). Furthermore, there are suggestions that some strains of *Lactococcus* and *Lactobacillus* metabolize not the galactose moiety but the glucose moiety of lactose, and galactose is then secreted into the medium (Marshall 1987; Montanari *et al.* 1996). This may partly explain the growth of non-lactose-fermenting yeasts in the milk. In support of our results, other researchers have related the growth of *S. cerevisiae* in cultured milk to its ability to metabolize lactic acid (Fleet 1992; Sarais *et al.* 1996).

The LAB strains were found to be responsible for acidifying the milk whereas the yeast strains caused little change in the titratable acidity. There was a slight increase in titratable acidity in the RS milk cultured with *C. kefyr* 2Y305. This

may be partly attributed to the production of acidic compounds, such as acetic acid (not determined), or to proteolysis and lipolysis. *Kluyveromyces marxianus* (perfect state of *C. kefyr*) excretes proteases and lipases that hydrolyze milk proteins and fat (Roostita & Fleet 1996). However, the proteolytic and lipolytic properties of *C. kefyr* 2Y305 have thus far not been studied.

Ethanol is the major volatile compound produced by yeasts and is very important for determining the properties of fermented milk. In the mixed culture of non-lactose-fermenting *S. cerevisiae* 4C with LAB, ethanol production was obviously enhanced compared to the single culture. From taxonomic studies, this strain could assimilate lactate and galactose (Gadaga *et al.* 2000). However, lactose-fermenting *C. kefyr* 2Y305 produced large amounts of ethanol and some other fermented products in both single and mixed cultures in kefir (Tamime & Marshall 1997) and koumiss (Mann 1989). This may suggest similarities in our LAB–yeast products with kefir and kumiss.

In the present study, non-lactose-fermenting *S. cerevisiae* 4C in the mixed cultures (except with *Lb. reuteri* 940B3) could utilize glucose and galactose produced by LAB lactase in RS milk, enhancing cell growth and ethanol production. A similar result has been reported (Cheirsilp *et al.*, 2003). For lactose-fermenting *C. kefyr* 2Y305, the transient appearance of glucose and galactose in the single culture may be due to lactose transport protein (LacS) activity when lactose concentration was high at the initial stage of culture (Cheirsilp *et al.*, 2001). *Lb. helveticus* 130B4 assimilated glucose and galactose to produce much lactic acid and competes for sugars with the yeast strains at the early stage of fermentation. However, after 2–3 days a significant reduction of lactose and the disappearance of glucose and galactose were observed in the mixed culture. This suggests that in the interaction of LAB and yeasts in airag, LAB end products, such as glucose and galactose, could be used by the yeasts as an energy source. On the other hand, the yeasts can provide vitamins, amino acids, products of proteolysis, and growth factors to LAB (Leroi & Pidoux 1993b). The stimulatory substances from yeast should be investigated in future studies.

6. Conclusions

Both LAB and yeasts showed stimulatory and inhibitory effects on each other depending on the combination. *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 was stimulated in mixed cultures with all yeasts except for *S. servazzii* 5Y201. In contrast, *Leuc. lactis* 420B1 was inhibited in all mixed cultures. The other LAB strains showed stimulatory or inhibitory effects in mixed cultures with yeasts. *C. kefyr* 2Y305 and *S. cerevisiae* 4C were evidently enhanced in mixed cultures compared to their single culture except for with *Lb. paracasei* 6B3073 or/and *Lb. plantarum* 440 M6. There was no clear positive or negative effects observed for *S. servazzii* 5Y201 and *C. krusei* 3Y201 with LAB apart from *C. krusei* 3Y201 with *Leuc. mesenteroides* subsp. *dextranicum* 6B2081. During fermentation, LAB was mostly responsible for milk acidification and the yeast strains were almost solely responsible for ethanol production. Growth of yeasts in the mixed culture was enhanced by consuming glucose and galactose produced by LAB lactase. Meanwhile, the high viable counts of some LAB in mixed cultures could be an indication of the beneficial effect of the yeasts on the LAB. However, further examination is needed to confirm this hypothesis. These possible positive interactions may be important for the development of LAB–yeast strains as starter cultures for the production of new and beneficial alcoholic fermented milk. To our knowledge, this is the first report on the microbial interaction between LAB and yeasts in airag, a traditional fermented milk in Mongolia.

Table 4 Biochemical and physiological characteristics of two type strains and two isolated LAB strains

Characteristics	NCDO 529[a]	6B2081[b]	DSM 20016[c]	940B3[d]	Carbohydrate fermentation	NCDO 529	6B2081	DSM 20016	940B3
Morphology	cocci	cocci	rods	rods	Methy-α D-Glucopyranoside	ND	-	ND	-
Growth at					N Acetyl Glucosamine	ND	+	ND	-
10℃	+	+	-	-	Amygdalin	d	-	o	-
15℃	+	+	-	-	Arbutin	-	-	ND	-
45℃	-	-	+	+	Esculin ferric citrate	ND	-	o	-
Gas from glucose	+	+	+	+	Salicin	d	-	-	-
NH3 from arginine	-	-	+	+	Cellobiose	d	-	-	-
Lactic acid isomer	D	D	DL	DL	Maltose	+	+	+	+
Carbohydrate fermentation					Lactose	+	+	+	+
Glycerol	ND	-	ND	-	Melibiose	d	-	+	+
Erythritol	ND	-	ND	-	Sucrose	+	+	+	+
D-Arabinose	ND	+	+	+	Trehalose	+	+	-	-
L-Arabinose	-	-	ND	-	Inulin	ND	-	ND	-
Ribose	ND	+	+	+	Melezitose	ND	-	-	-
D-Xylose	d	+	-	-	Raffinose	d	-	+	+
L-Xylose	ND	-	ND	-	Starch	ND	-	ND	-
Adonitol	ND	-	ND	-	Glycogen	ND	-	ND	-
Methy-β	ND	-	ND	-	Xylitol	ND	-	ND	-
Galactose	d	+	+	+	Gentiobiose	ND	-	ND	-
Glucose	+	+	+	+	D-Turanose	ND	-	ND	-
Fructose	+	+	+	-	D-lyxose	ND	-	ND	-
Mannose E	d	+	-	-	D-Tagatose	ND	-	ND	-
Sorbose	ND	-	ND	-	D-Fucose	ND	-	ND	-
Rhamnose	ND	-	-	-	L- Fucose	ND	-	ND	-
Dulcitol	ND	-	ND	-	D-Arabitol	ND	-	ND	-
Inositol	ND	-	ND	-	L-Arabitol	ND	-	ND	-
Mannitol	d	-	-	-	Gluconate	ND	-	+	+
Sorbitol	ND	-	-	-	2-Keto-Gluconate	ND	-	ND	-
Methy-α D-Mannopyranoside	ND	-	ND	-	5-Keto-Gluconate	ND	-	ND	-

[a] *Leuconostoc mesenteroides* subsp. *dextranicum* type strain NCDO 529 (Data from Sneath *et al*); [b] *Leuconostoc mesenteroides* subsp. *dextranicum* 6B2081; [c] *Lactobacillus reuteri* type strain DSM 20016 (Data from Sneath *et al*);
[d] *Lactobacillus reuteri* 940B3; +, positive; -, negative; ND, no data; d, 11-98% of strains positive; o, reaction not determined.

Table 5 Titratable acidity for LAB strains in single culture and mixed cultures with yeast strains for 7 days

LAB strains	Titratable acidity[a] in single culture	Titratable acidity in mixed cultures with				
		Candida kefyr 2Y305	*Kluyveromyces marxianus* var. *lactis* 5Y305	*Saccharomyces cerevisiae* 4C	*Saccharomyces servazzii* 5Y201	*Candida krusei* 3Y208
Control[b]	0.17	0.17	0.17	0.17	0.17	0.17
Leuconostoc mesenteroides subsp. *dextranicum* 6B2081	0.28	0.87	0.94	0.70	0.27	0.65
Lactobacillus reuteri 940B3	0.31	1.10	0.51	0.28	0.26	0.23
Lactobacillus helveticus 130B4	2.57	1.63	1.80	2.69	2.10	2.00
Streptococcus thermophilus 1230B2	0.87	0.77	1.08	0.86	0.87	0.74
Leuconostoc lactis 420B1	0.99	0.75	0.86	0.84	0.95	0.77
Enterococcus faecium 3B3083	0.95	0.87	1.21	1.00	1.03	0.84
Lactobacillus plantarum 440M6	1.41	1.15	1.63	1.38	1.40	1.17
Lactobacillus casei 6B4084	1.25	1.47	1.44	1.26	1.23	1.03
Lactobacillus paracasei 6B3073	1.26	1.05	1.60	1.20	1.22	1.00

[a] Unit for titratable acidity is %. [b] Control is 10% reconstituted skim milk.

Table 6 Ethanol content of yeast strains in single culture and mixed cultures with LAB for 7 days

Yeast strains	Ethanol in[a] single culture	Ethanol in mixed cultures with								
		Leuc. mesenteroides subsp. *dextranicum* 6B2081	*Lb. reuteri* 940B3	*Lb. helveticus* 130B4	*Sc. thermophilus* 1230B2	*Leuc. lactis* 420B1	*Ec. faecium* 3B3083	*Lb. plantarum* 440M6	*Lb. casei* 6B4084	*Lb. paracasei* 6B3073
Control[b]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Candida kefyr 2Y305	0.78	0.91	0.90	1.09	0.80	0.81	0.78	0.79	0.77	0.09
Kluyveromyces marxianus var. *lactis* 5Y305	1.58	1.63	1.61	1.59	1.52	1.59	1.55	1.60	1.57	1.58
Saccharomyces cerevisiae 4C	0.20	1.24	0.36	1.08	0.79	0.66	0.81	0.07	0.22	0.03
Saccharomyces servazzii 5Y201	0.02	0.02	0.04	0.38	0.00	0.01	0.00	0.00	0.00	0.00
Candida krusei 3Y208	0.02	0.15	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00

[a] Unit for ethanol is g/L. [b] Control is 10% reconstituted skim milk.

Table 7 Viable count and titratable acidity of LAB strains in single culture and mixed cultures with yeasts strains

LAB strains	Incubation time(day)	Viable counts[a] and (titratable acidity[c]) in single culture	Viable counts[a] and (titratable acidity[c]) in mixed cultures with	
			Saccharomyces cerevisiae 4C	*Candida kefyr* 2Y305
Leuconostoc mesenteroides subsp. *dextranicum* 6B2081				
	0[b]	7.52(0.17)	7.23(0.17)	6.49(0.17)
	1	8.63(0.20)	8.67(0.21)	8.72(0.35)
	2	8.75(0.27)	8.45(0.40)	8.28(0.48)
	3	8.36(0.33)	8.42(0.51)	7.11(0.65)
	4	8.11(0.31)	8.18(0.58)	8.15(0.83)
	5	7.74(0.31)	8.30(0.60)	8.93(0.81)
	6	6.81(0.28)	8.15(0.65)	8.15(0.92)
	7	6.65(0.28)	8.17(0.70)	7.65(0.87)
Lactobacillus reuteri 940B3				
	0 [b]	5.86(0.17)	6.00(0.17)	6.79(0.17)
	1	7.36(0.19)	7.28(0.18)	8.66(0.31)
	2	7.45(0.21)	7.48(0.22)	8.56(0.57)
	3	7.49(0.24)	7.34(0.24)	8.49(0.78)
	4	7.40(0.26)	7.36(0.25)	8.59(0.86)
	5	7.34(0.28)	7.18(0.26)	8.60(1.10)
	6	7.28(0.31)	7.28(0.27)	7.97(1.00)
	7	7.18(0.31)	7.48(0.28)	8.04(1.10)
Lactobacillus helveticus 130B4				
	0[b]	7.20(0.17)	6.81(0.17)	6.81(0.17)
	1	8.18(0.48)	8.20(0.46)	8.34(0.58)
	2	8.66(1.17)	9.30(1.18)	9.18(1.37)
	3	8.77(1.79)	9.11(1.67)	8.92(1.57)
	4	8.85(2.02)	9.15(2.16)	8.79(1.63)
	5	8.94(2.24)	9.08(2.17)	7.40(1.63)
	6	8.92(2.36)	9.11(2.33)	6.65(1.44)
	7	8.70(2.57)	9.18(2.69)	6.18(1.63)

[a] Viable count in single and mixed cultures is expressed as $\log_{10}$ CFU/mL. [b] Inoculum count for LAB in single and mixed cultures. [c] Unit for titratable acidity is %.

Table 8 Viable count and ethanol content of yeast strains in single culture and mixed cultures with LAB

Yeast strains	Incubation time(day)	Viable counts[a] and (ethanol[c]) in single culture	Viable counts[a] and (ethanol[c]) in co-culture with		
			Leuc. mesenteroides subsp. *dextranicum* 6B2081	*Lactobacillus reuteri* 940B3	*Lactobacillus helveticus* 130B4
Saccharomyces cerevisiae 4C					
	0[b]	6.04(0.00)	5.85(0.00)	5.40(0.00)	5.53(0.00)
	1	6.56(0.26)	6.42(0.45)	5.26(0.03)	6.83(0.31)
	2	6.63(0.20)	6.30(0.76)	6.08(0.20)	7.11(0.64)
	3	6.46(0.25)	6.61(1.13)	6.34(0.23)	7.32(0.87)
	4	6.40(0.17)	6.52(1.24)	6.70(0.25)	7.20(0.83)
	5	6.32(0.18)	6.71(1.24)	6.53(0.30)	7.15(0.87)
	6	6.54(0.19)	6.75(1.24)	6.78(0.35)	7.11(1.30)
	7	6.58(0.20)	6.65(1.24)	6.95(0.36)	7.08(1.08)
Candida kefyr 2Y305					
	0[b]	5.79(0.00)	5.04(0.00)	5.04(0.00)	5.52(0.00)
	1	7.18(1.30)	6.62(0.79)	6.64(0.77)	6.08(0.82)
	2	7.11(1.30)	6.11(0.91)	5.40(0.90)	7.49(0.77)
	3	6.88(1.28)	6.65(0.91)	6.18(0.91)	6.61(0.85)
	4	6.86(1.35)	7.11(0.92)	7.26(0.88)	6.36(0.83)
	5	6.51(1.13)	7.08(0.90)	7.11(0.86)	6.46(0.85)
	6	6.60(1.18)	7.26(0.91)	7.00(0.90)	6.52(1.30)
	7	6.40(0.78)	7.28(0.91)	7.20(0.90)	6.61(1.09)

[a] Viable count in single and mixed cultures is expressed as the $\log_{10}$ CFU/mL. [b] Inoculum count for yeasts in single and co-cultures. [c] Unit for ethanol is g/L.

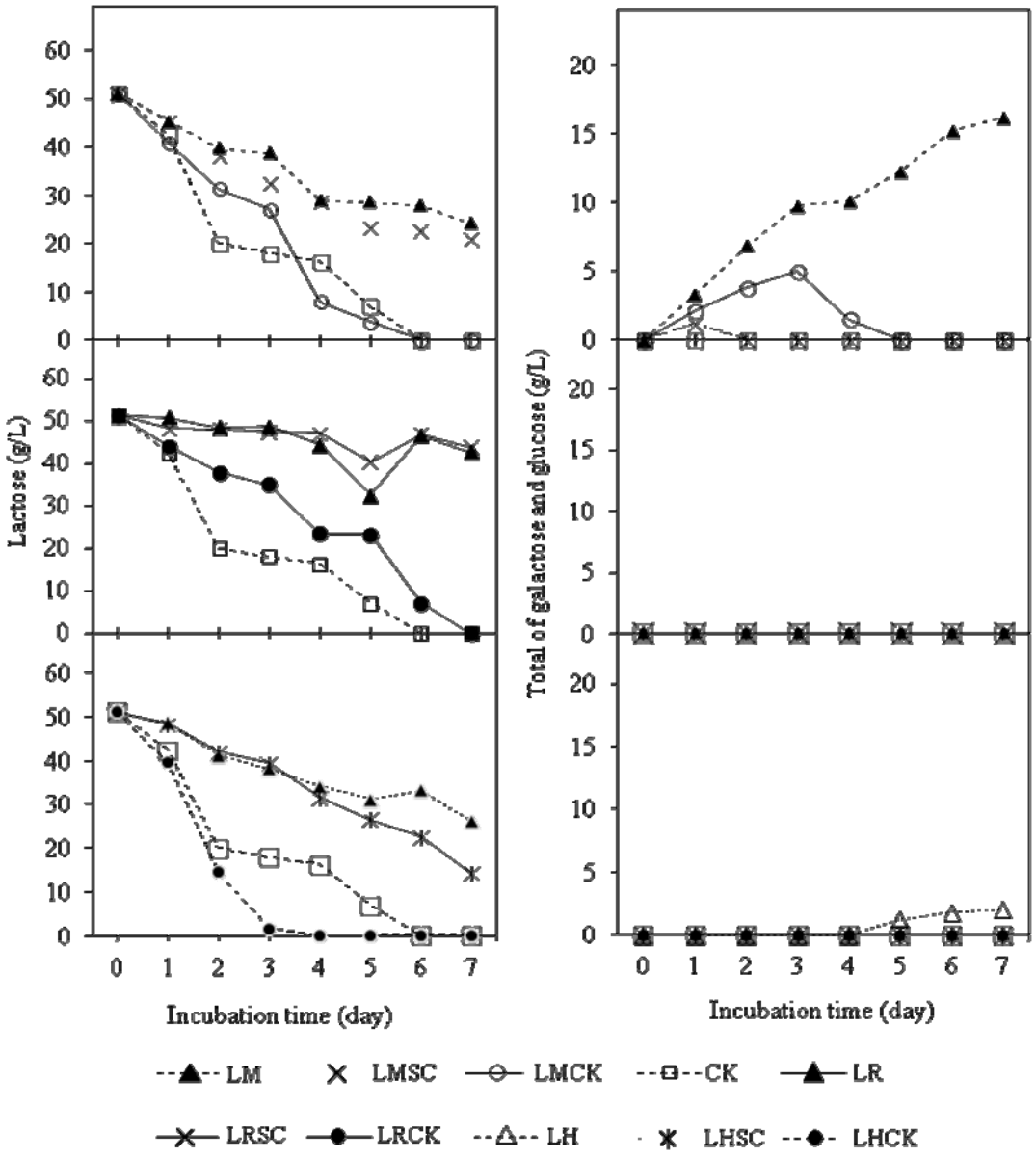

Fig. 3 Sugar contents in single and mixed cultures of LAB and yeasts during one week fermentation. LM, *Leuc. mesenteroides* 6B2081; LMSC, *Leuc. mesenteroides* 6B2081+*S. cerevisiae* 4C; LMCK, *Leuc. mesenteroides* 6B2081+*C. kefyr* 2Y305; CK, *C. kefyr* 2Y305; LR, *Lb. reuteri* 940B3; LRSC, *Lb. reuteri* 940B3+*S. cerevisiae* 4C; LRCK, *Lb. reuteri* 940B3+*C. kefyr* 2Y305; LH, *Lb. helveticus* 130B4; LHSC, *Lb. helveticus* 130B4 + *S. cerevisiae* 4C; LHCK, *Lb. helveticus* 130B4+*C. kefyr* 2Y305.

Chapter 3

Growth stimulatory effect on lactic acid bacteria in skim milk incubated with lactose-fermenting yeast from airag in Inner Mongolia, China

1. Abstract

The lactose-fermenting yeast was used to investigate the growth stimulation effect for lactic acid bacteria (LAB) in skim milk and they were isolated from airag in Inner Mongolia, China. Titratable acidity and viable counts of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 were enhanced in 10% (w/v) reconstituted skim (RS) milk containing *Candida kefyr* 2Y305 whey compare with the supernatant of RS milk whey as a control. In the TG liquid medium supernatant ethanol insoluble whey, the 6B2081 strain showed higher cell density value than in containing ethanol insoluble whey. The peptide composition of ethanol insoluble whey from *Candida kefyr* 2Y305 was analyzed by using gel filter and HPLC, and the peptide fractions decreased were observed in the mixed culture with LAB compare to the single culture. Each fraction was collected from chromatogram of the HPLC to add in TG liquid medium for measuring the growth stimulation effect and the highest cell growth was detected containing of fraction 9. The result suggests that the LAB utilize peptide ingredients from yeast whey to promote cell growth.

2. Introduction

LAB and yeasts are the predominant microorganisms in most types of fermented milk (Ishii *et al.,* 1997; Gadaga *et al.,* 2001; Shuangquan *et al.,* 2004; Watanabe *et al.,* 2008; Mari *et al.,* 2010). They form definite microflora through repeated symbiosis and antagonism between microorganisms. Several microbial interactions between LAB and yeasts are found in fermented products, such as blue cheese, white mould cheese, bacterial surface ripened cheese, kefir, and kumiss (Subramanian & Shankar, 1985; Fleet, 1990; Jakobsen & Narvhus, 1996; Pablo *et al.,* 2008; Marina *et al.,* 2009). Marshall (1987) reviewed the possible interactions between LAB and yeasts in fermented milk, but did not elucidate the mechanisms underlying those interactions. Adachi *et al.* (1990) proposed that the interaction of microflora proceeded as follows: The microorganisms are classified into four groups: homofermentative LAB, heterofermentative LAB, lactose-assimilating yeast, and non-lactose-assimilating yeast. The non-lactose-assimilating yeast should survive by consuming galactose, which is a product of lactose-assimilating microorganisms. Homofermentative LAB were thought to be stimulated by CO_2, ethanol, or unknown metabolites produced by the other three groups of microorganisms.

Stable co-metabolism between LAB and yeasts is common in many foods, enabling the utilization of substances that are otherwise nonfermentable (for example, starch) and thus increasing microbial adaptability to complex food ecosystems (Gobbetti *et al.*, 1994; Stolz *et al.*, 1995). It has been suggested that the proliferation of yeasts in foods is favored by the acidic environment created by LAB while the growth of bacteria is stimulated by the presence of yeasts, which

may provide such growth factors as vitamins and soluble nitrogen compounds (Nout, 1991). The association of LAB and yeasts during fermentation may also yield metabolites that may impart taste and flavor to foods (Halm *et al.*, 1993; Brauman *et al.*, 1996; Hansen and Hansen, 1996). Proteolytic yeasts, such as *Yarrowia lipolytica* and *C. catenulata,* grow in milk and produce free amino acids, such as leucine, phenylalanine, lysine, arginine, glutamic acid, and valine (Roostita and Fleet, 1996), which can be a source of metabolizable substrates for other microorganisms, resulting in the production of secondary metabolites, including flavor compounds. The release of free amino acids may also promote the growth of LAB with a poor proteolytic system.

Our previous study has suggested that in the interaction of LAB and yeasts in airag, the growth of yeasts was enhanced by consuming glucose and galactose produced by LAB lactase as an energy source. Meanwhile, the growth of LAB was also promoted by the beneficial effects of the yeasts, although the growth-promoting substances remain unclear (Susdun *et al.*, 2012). The aims of present work were to clarify the growth-promoting substances of LAB in whey preparations from skim milk incubated with yeast strains and to apply symbiotic LAB and yeast strains to the development of a new type of fermented milk as a starter culture to explore its benefits to human health.

3. Materials and methods

3.1 Bacterial strains and culture conditions

Leuconostoc mesenteroides subsp. *dextranicum* 6B2081 and *Candida kefyr* 2Y305 isolated from traditional fermented milk, airag in Inner Mongolia, China were identified and maintained in our laboratory ,and stored at -80°C in 10% RS skim milk (Snow Brand Milk Products Co., Ltd., Tokyo, Japan) supplemented 0.1% glutamic acid monosodium (Nacalai Tesque Co., Kyoto, Japan) for later use. The LAB and yeast strain were cultured three times in MRS broth (Oxoid, Basingstoke, Hampshire, England) and YM broth (5g Bacto™ Tryptone, 3g Bacto™ Yeast Extract, 3g Extract Malt, 10g Glucose/L) medium at 30 and 25°C for 24 h, respectively. Then LAB and/or yeasts were transferred to RS milk medium by 2% and 1%(v/v) inocula in single and mixed cultures from MRS and YM broth, respectively and incubated at 30°C for seven days.

3.2 Preparation of fermented milk

The 10% (w/v) reconstituted skim (RS) milk was pasteurized, inoculated with the starter culture containing *Candida kefyr* 2Y305 and/or *Leuc. mesenteroides* subsp. *dextranicum* 6B2081, and fermented at 30 ◦C for a week. The whey fraction of the fermented milk was used to examine the growth stimulation effect on lactic acid bacterium and to analyze an active fraction. The whey fraction was obtained as follows. The fermented milk was adjusted to pH 4.6 with 5mol L^{-1} HCl or 5 mol L^{-1} NaOH, and then was centrifuged at 10 000 × *g* for 20min. The supernatant was adjusted to original pH of the fermented milk with 5mol L−1 NaOH, and then was percolated by filter. The final supernatant was used as the

whey fraction.

3.3 Changes in titratable acidity and viable counts for *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in RS milk containing whey

Leuc. mesenteroides subsp. *dextranicum* 6B2081 was incubated in RS milk containing filter-sterilized threefold concentrate RS milk whey (as control) or yeast whey to measure titratable acidity and viable counts at 30°C every 24h for a week.

Titratable acidity was determined by acid-base titration with 0.1 mol/L NaOH, and the value was calculated as equivalent percent (w/w) of lactic acid. For enumeration of viable LAB counts, the diluted samples were spread on BCP agar (Nissui, Tokyo, Japan) and incubated at 30°C for three days.

3.4 Assay for growth stimulation fraction

Fermented milk whey was fractionated by adding 9 times quantity of 99.8% (v/v) ethanol in refrigerator at 8°C, and then centrifuged at 10,000 × *g* for 20 min. The ethanol soluble fraction and insoluble fraction were divided to evaporate ethanol with Rotaty Vacuum Evaporator N-N Series, respectively. And then distilled water was added and the solution was sterilized by filter. Cell density was assayed using the TG liquid medium (10g Tryptone, 5g Glucose, 1g Tween 80, 0.1g L-cysteine hydrochloride/L) containing ethanol soluble fraction or insoluble fraction by *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 at 30°C every 2h for 20h after inoculation. The absorbance was measured with U-1800 spectrophotometer at 600 nm.

3.5 Active fraction of the peptide composition by using ethanol insoluble whey

The ethanol insoluble whey fraction was prepared and subjected to gel filtration with pH7 of distilled water (Sephadex G25, 1.6 cm diameter × 90cm length; Pharmacia, Uppsala, Sweden). After the sample collected, the elution was evaporated to adjust original quantity of the sample by adding distilled water and filtered to assay peptide composition. In the reverse-phase High Performance Liquid Chromatography (HPLC), 5C18-AR-300 column (4.6mm diameter × 250mm length; Waters, Kyoto, Japan) was used. The elution was performed with a linear gradient of acetonitrile from 0% to 80% in 0.05% trifluoroacetic acid for 50min at a flow rate of 0.8mL/min. The chromatography was monitored at a wavelength of 220 nm.

3.6 Growth promoting effect of each peptide fraction

Each peptide fraction was collected and purified, and sterilized through a filter of 0.20 μm membrane (Toyo Roshi Kaisya, Tokyo, Japan). The culture supernatant of each peptide fraction in TG liquid medium (10g Tryptone, 5g Glucose) by *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 was measured cell density (at 600 nm) at 30°C every 2h for 20h after inoculation. The fraction of the highest growth promoting effect was rechromatographed in the same column with a moderate acetonitrile gradient from 0% to 80% for 40 min.

4. Results and discussion

4.1 Changes in titratable acidity and viable counts for *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in RS milk containing whey

Leuc. mesenteroides subsp. *dextranicum* 6B2081 fermented the RS milk supernatant of RS milk whey (control) to reach 0.74% of titratable acidity from initial of 0.34% at 30°C every 24h for a week. In the RS milk containing of yeast whey, the acidity was significantly increased to 1.11% of final value from 0.32% in the same culture condition (Fig. 4).

The viable counts for *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 peaked at 8.64 $\log_{10}$ CFU/mL on the third day and afterward decreased to 6.12 $\log_{10}$ CFU/mL on the seventh day in control. The cell growth was obviously enhanced in the RS milk supernatant of yeast whey compare with control after the third day and the final counts were reached to 8.03 $\log_{10}$ CFU/mL for a week (Fig. 4). These results can explain the effective substance to promote the growth of lactic acid bacteria was contained in the skim milk whey of the yeast due to the milk ingredient is occurred big changes with the fermentation of lactose-fermenting yeast and their by-product become easy for utilization by living body to improve the digestion and absorption. It had been reported the *Kluyveromyces marxianus* (perfect state of *C. kefyr*) excretes proteases and lipases that hydrolyze milk proteins and fat. *C. kefyr* fermented the milk to produce the acidic compounds, such as acetic acid, or to proteolysis and lipolysis (Roostita & Fleet 1996).

4.2 Assay for growth stimulation whey fraction

Figure 5 shows the growth of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in TG liquid medium as a control, TG liquid medium supplement the whey fraction of ethanol soluble or insoluble ingredient by measuring the cell density. The cell density was obviously enhanced in TG liquid medium containing ethanol insoluble whey ingredient at 30°C every 2h for 20h after inoculation. It may be due to the growth stimulation substance of LAB was contained in ethanol insoluble whey more than in ethanol soluble whey. This suggest that those between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from sugary kefir grains, the growth of LAB was promoted by yeast through the production of carbon dioxide, pyruvate, propionate and succinate (Leroi & Pidoux 1993a).

4.3 Active fraction of the peptide composition by using ethanol insoluble whey

The ethanol insoluble whey ingredient was concentrated after the collected from gel filtration. The concentrate was eluted on a 5C18-AR-300 reverse-phase column with a linear gradient of acetonitrile solution, and the peptide fraction divided into nine parts was detected in the single culture by yeast. The most peptide fraction was obviously reduced in mixed culture contrast with the single culture of yeast (Fig. 6). These peptide fractions were reputed the effective substance for the growth of the lactic acid bacteria. It suggests that the yeast stimulated the growth of LAB by providing such essential metabolites as peptides, amino acids and vitamins.

4.4 Growth promoting effect of each peptide fraction

In the TG liquid medium containing fraction 9, the growth of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 showed highest value comparing to the TG liquid medium as a control and TG liquid medium supplement the other peptide fraction by the cell density at 30°C every 2h for 20h after inoculation (Fig. 7). Fraction 9 was estimated the most effective peptide fraction for the growth of LAB from yeast whey.

5. Conclusion

The growth stimulation effect of LAB was observed in skim milk containing whey of the yeast according to the increase of titratable acidity and viable counts. The growth stimulation substance of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 was contained in ethanol insoluble ingredient of *Candida kefyr* 2Y305 whey because the cell density was insignificantly enhanced in the TG liquid medium supernatant ethanol insoluble whey. For the analysis of peptide composition by using the ethanol insoluble whey of *Candida kefyr* 2Y305, the peptide fractions decreased were observed in the mixed culture with LAB compare to the single culture with HPLC measurement. The cell growth was showed highest value in TG liquid medium containing fraction 9, which was collected from chromatogram of the HPLC. These results suggest the LAB utilize peptide ingredients from yeast whey to promote cell growth. The present work is the first report about the growth stimulation substance of LAB from yeast for interaction in airag. It contributes to clarify the relations among LAB and yeast co-exist in a complex microbial ecosystem as the starter culture in fermented milk.

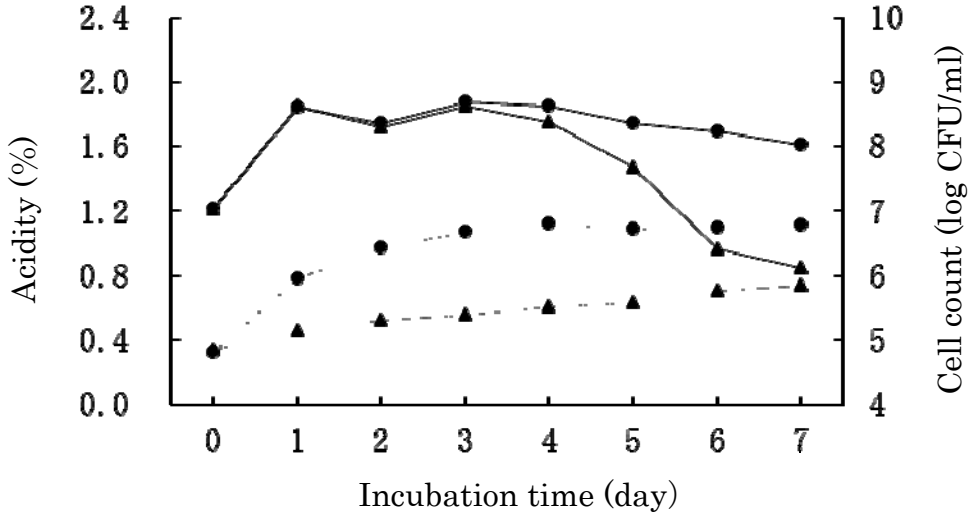

Fig. 4 Changes in titratable acidity and viable counts of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in RS milk supplemented with yeast whey (●) and RS milk whey (▲).
Line: cell count, dotted line: acidity.

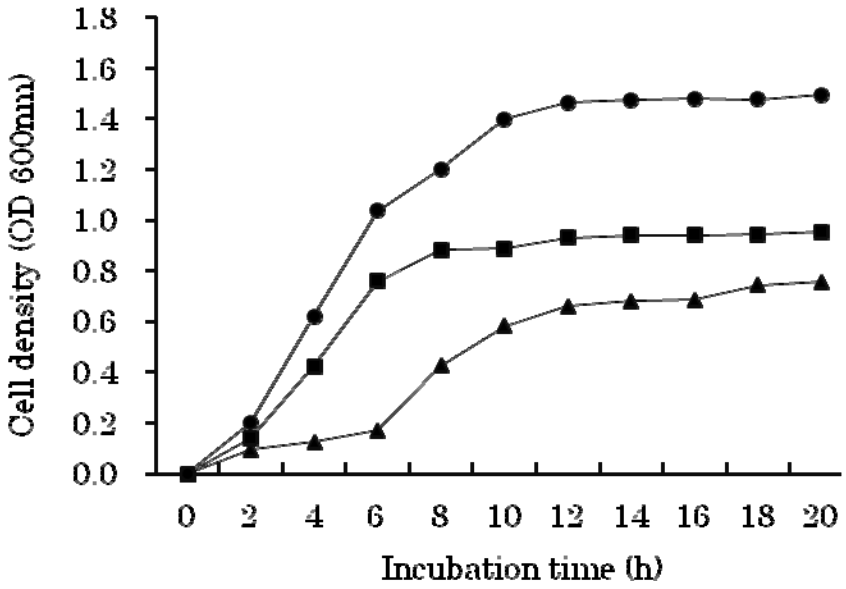

Fig. 5 Growth stimulation effect of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in TG liquid medium (▲), TG liquid medium supplemented with ethanol-soluble ingredient (■) or ethanol-insoluble ingredient of whey (●).

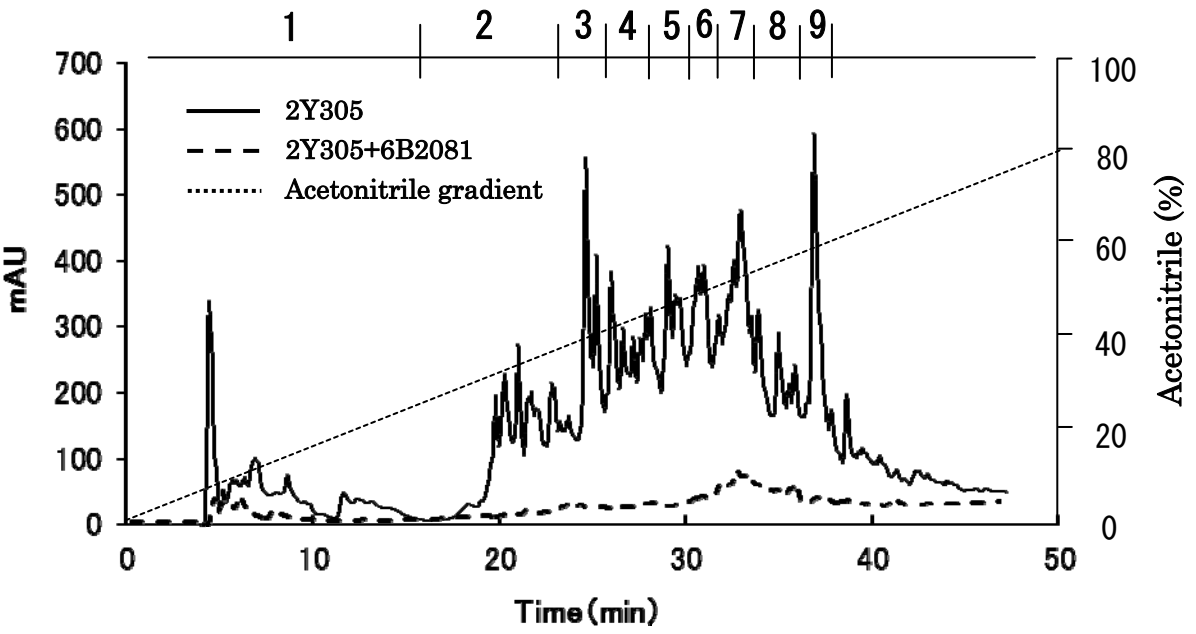

Fig. 6 Reverse-phase HPLC chromatogram of peptide composition by using the ethanol-insoluble ingredient of whey from single culture of *Candida kefyr* 2Y305 (line) and the mixed culture with *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 (dashed line). The elution was performed with a linear gradient of acetonitrile (dotted line) from 0% to 80% in 0.05% trifluoroacetic acid for 50min at a flow rate of 0.8mL/min by using Cosmosil 5C18-AR-300 column at the wavelength of 220nm.

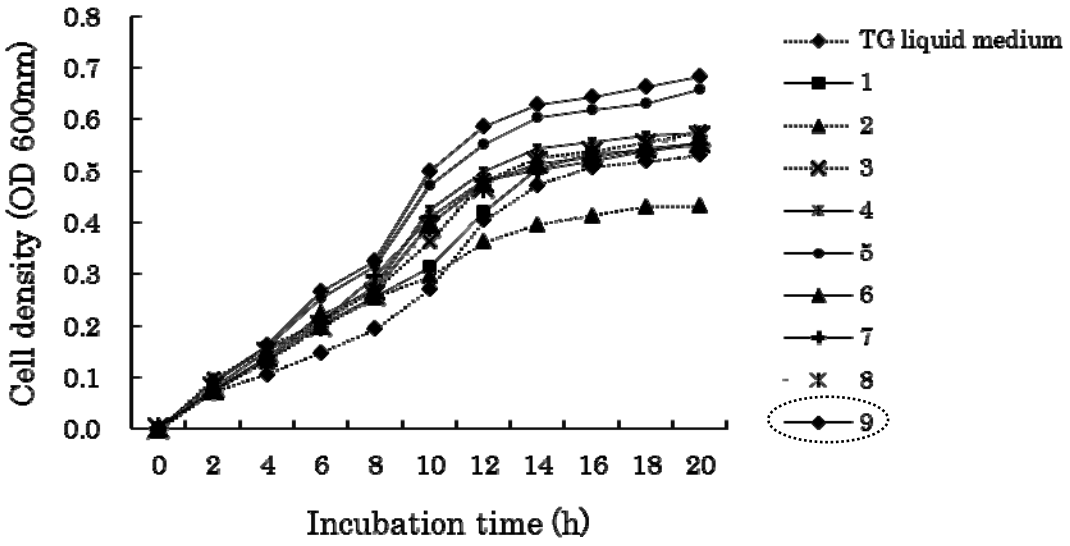

Fig. 7 Growth of *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 in TG liquid medium containing each peptide fraction collected from HPLC chromatogram.

Summary

Airag, a traditional alcoholic fermented milk from Mongolia, has been habitually drunk by Mongolian nomads since ancient times. It is produced from cow, mare, and camel milk by a natural fermentation method using an indigenous starter culture containing LAB and yeast. Various kinds of LAB and yeast strains from the natural environment form definite microflora through repeated symbiosis and antagonism and thus, it is important to clarify the co-existence of LAB and yeast strains in the study of airag production. In this study, airag samples produced by nomads from the Inner Mongolia Autonomous Region of China were used and the interaction between LAB and yeast strains isolated from the airag samples was investigated. Furthermore, growth stimulation effects were examined in various combinations of LAB and yeast strains for the development of fermented milk starter.

In Chapter 1, we isolated and identified yeast strains from five airag samples collected from households of Mongolian nomads. Among the lactose-fermenting yeasts, *Candida kefyr* was the most predominant (21.3%), followed by *Kluyveromyces marxianus* var. *lactis* (11.1%). Of the non-lactose-fermenting yeasts, *C. krusei* was primarily isolated (18.5%), followed by *Saccharomyces cerevisiae* (14.8%) and *S. servazzii* (14.8%). *Pichia cactophila* (12.0%) and *C. valida* (7.4%), which are non-sugar fermenting yeasts, were also identified.

In Chapter 2, nine LAB and five yeast strains isolated from airag were incubated in 10% (w/v) RS milk as single and mixed cultures to investigate microbial interaction by changing titratable acidity and ethanol content every 24

h for one week. Among them, three LAB and two yeast strains were selected as representative and their interactions examined in detail. As a result, a symbiotic relationship was found between *Leuconostoc mesenteroides* subsp. *dextranicum* 6B2081 and *Saccharomyces cerevisiae* 4C or *Candida kefyr* 2Y305. As regards sugar content, total glucose and galactose contents increased whereas lactose content decreased in single cultures of *Leuconostoc mesenteroides* 6B2081 and *Lb. helveticus* 130B4 incubated for one week. However, both glucose and galactose were completely consumed and lactose was markedly reduced in the mixed cultures with yeast strains. The result suggests that the yeast strains utilize glucose and galactose produced by LAB lactase to promote cell growth.

In Chapter 3, to identify the substance produced by yeast that stimulates LAB growth, RS milk whey from the lactose-fermenting yeast *Candida kefyr* 2Y305 was used to investigate the growth stimulation effect. Titratable acidity was obviously enhanced and viable count yielded a high value after the fourth day of culture in RS milk whey from *Candida kefyr* 2Y305 compared with the supernatant of RS milk whey from *Leuc. mesenteroides* subsp. *dextranicum* 6B2081 as control. After incubating strain 2Y305 in RS milk for one week, the fermented milk was adjusted to pH 4.6 and centrifuged at 10 000 × *g* for 20 min. The supernatant was fractionated to ethanol-soluble and -insoluble whey by adding ninefold volume of ethanol and added to TG liquid medium to examine the growth stimulation effect on LAB. Strain 6B2081 showed higher cell density value in the ethanol-soluble whey fraction than ethanol-insoluble whey fraction. For the purification of the growth-stimulating substance, the peptide composition of ethanol-insoluble whey from *Candida kefyr* 2Y305 was analyzed by gel filtration (Sephadex G25, 1.6 × 90cm) and HPLC (5C18-AR-300, 4.6 × 250mm),

and the decrease of the peptide fractions was observed in the mixed culture with LAB compare to the single culture. Each fraction was recovered from the HPLC chromatogram to add in TG liquid medium and the highest cell growth was detected in fraction 9.

In conclusion, the results obtained in this study clarified the symbiotic relationship between LAB and yeast strains in airag, a traditional alcoholic fermented milk of Inner Mongolia, for the first time. In the process of making airag, lactic acid fermentation by LAB, alcoholic fermentation by yeast, and their by-products, play an important role in flavor formation. A symbiotic relationship was observed: the yeast strains utilized glucose and galactose produced by LAB lactase and LAB utilized the peptides produced by protease from the yeast strains. Our findings provide meaningful information for the improvement of airag production technology. In the future, we expect to develop a new and beneficial alcoholic fermented milk product by using LAB–yeast strains as the starter culture.

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