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HEIRLOOM MICROBES: A SURVEY OF LACTIC ACID BACTERIA IN TRADITIONAL MONGOLIAN DAIRY

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HEIRLOOM MICROBES: A SURVEY OF LACTIC ACID BACTERIA IN TRADITIONAL MONGOLIAN DAIRY

A THESIS APPROVED FOR THE
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“Don’t let distractions or circumstances keep you from your future.” – Jim Rohn

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Abstract

Fermentation has sustained human communities for thousands of years. Through most of that time, humans unknowingly used microbes in food production, especially dairy. It wasn't until the late nineteenth century that the first bacterium was isolated from milk. This bacterium is classified in the group of bacteria known today as the lactic acid bacteria (LAB). The microbes in milk, especially LAB, play an essential role in dairy production, contributing to a varied array of products with rich flavors, aromas, and textures. However, the microbes in milk can also cause spoilage and disease, which has resulted in hygiene regulations and microbial standards for modern dairy production. Consequently, traditional methods of dairying and their unique microbial taxa are being lost to industrialized practices, and the microbial diversity remains unknown. Even though dairy production has largely become globalized, the localized production of traditional dairy still exists in certain regions of the world as a major subsistence strategy, one such location is Mongolia. Some of the products from traditional dairy have been investigated to a greater or lesser extent. Therefore, this study aimed to characterize the predominant LAB associated with dairy produced in Khövsgöl, the northern-most province of Mongolia, from cow and yak milk. Using two LAB media, 204 LAB isolates were recovered, constituting 15 different genera and 35 distinct species. The recovered LAB are species commonly found in milk and dairy. Phylogenetic analysis confirmed the classification of each LAB species recovered. Further investigations include mining the genome sequences of each isolate for unique characteristics and maintaining these bacterial cultures in an international culture collection.

Chapter 1: Introduction to lactic acid bacteria and Mongolian dairy

Humans unknowingly used microbes in dairy production for millennia until a series of key scientific discoveries were made starting with the discovery of lactose, the principle sugar in milk, in 1619 (Jodidi, 1913). Forty years later, the presence of bacteria in milk was first described, and then lactic acid was isolated from sour milk in 1780 (Scheele, 1788). However, the link between lactose, bacteria, and lactic acid in milk was not clear until almost a century later. In 1847, Blondeau observed bacteria in milk during his studies on fermentation, and Pasteur proved milk souring was due to the growth of microorganisms (Blondeau, 1847; Pasteur, 1857). Finally in 1873, Joseph Lister linked bacteria to the production of lactic acid in milk through an experiment using a single bacterium isolated in pure culture from milk (Lister, 1873). Lister named this organism *Bacterium lactis,* which was reclassified to *Streptococcus lactis* and later to *Lactococcus lactis* (Schleifer et al., 1985). After these discoveries, several more bacteria capable of producing lactic acid were isolated from dairy and collectively labelled 'the lactic acid bacteria' (Orla-Jensen, 1919). The lactic acid bacteria (LAB), especially those involved in dairy production, have received a tremendous amount of scientific examination over time because of their economic importance.

LAB are broadly defined as Gram-stain-positive, non-sporulating, non-motile, catalase-negative, acid-tolerant, facultatively aerobic rods or cocci. The LAB were originally characterized and grouped together by carbohydrate utilization and growth requirements (Orla-Jensen, 1919). The advent of molecular methods, such as 16S rRNA gene and whole genome sequencing revealed grand taxonomic diversity between the original, classically defined LAB, which spanned two phyla, the *Firmicutes* and *Actinobacteria*. Therefore, sequencing technology advancements eventually led to defining the 'true' LAB as only species contained in the order

Lactobacillales and the description of many novel genera (Table 1), specifically the most resent major revisions in the family *Lactobacillaceae* (Holzapfel and Wood, 2014; Zheng et al., 2020)

Table 1.1. Summary of current LAB families and genera.

Aerococcaceae	***Lactobacillaceae***
Abiotrophia	*Acetilactobacillus*
Aerococcus	*Agrilactobacillus*
Dolosicoccus	*Amylolactobacillus*
Eremococcus	*Apilactobacillus*
Facklamia	*Bombilactobacillus*
Globicatella	*Companilactobacillus*
Ignavigranum	*Convivina*
Suicoccus	*Dellaglioa*
	Fructilactobacillus
	Fructobacillus
Carnobacteriaceae	*Furfurilactobacillus*
Alkalibacterium	*Holzapfelia*
Allfustis	*Lacticaseibacillus*
Alloiococcus	*Lactiplantibacillus*
Atopobacter	*Lactobacillus*
Atopococcus	*Lapidilactobacillus*
Atopostipes	*Latilactobacillus*
Carnobacterium	*Lentilactobacillus*
Desemzia	*Leuconostoc*
Dolosigranulum	*Levilactobacillus*
Granulicatella	*Ligilactobacillus*
Isobaculum	*Limosilactobacillus*
Jeotgalibaca	*Liquorilactobacillus*
Lacticigenium	*Loigolactobacillus*
Lactosphaera	*Oenococcus*
Marinilactibacillus	*Paralactobacillus*
Pisciglobus	*Paucilactobacillus*
Trichococcus	*Pediococcus*
	Schleiferilactobacillus
	Secundilactobacillus
	Weissella
Enterococcaceae	
Bavariicoccus	***Streptococcaceae***
Catellicoccus	*Floricoccus*
Enterococcus	*Lactococcus*
Melissococcus	*Lactovum*
Pilibacter	*Streptococcus*
Tetragenococcus	
Vagococcus	

LAB are found in a variety of habitats and are associated with plants, soil, food, and vertebrate and invertebrate animals. Thus, LAB exhibit a wide range of growth temperatures, physiological tolerances, and biochemical activities adapted to survival in particular ecological niches (George et al., 2018; Holzapfel and Wood, 2014). LAB share some common attributes regarding metabolism despite grand phylogenetic diversity and are metabolically classified as homolactic or heterolactic fermenters (Gänzle, 2015; Zheng et al., 2015). Homofermentative LAB use the Embden-Meyerhof-Parnas (EMP) pathway to metabolize hexoses to pyruvate as the primary intermediate, producing 2 moles of lactic acid from 1 mole of glucose. In contrast, the heterofermentative LAB use the phosphoketolase pathway to metabolize hexoses to the key intermediates pyruvate and acetyl-phosphate, producing 1 mole of each lactic acid, CO_2, and ethanol from 1 mole of glucose. For the present study, LAB associated with milk and dairy products are of most interest. Homofermentative dairy LAB genera include *Streptococcus, Lactococcus, Pediococcus, Tetragenococcus,* and some enterococci and lactobacilli. Heterofermentative dairy LAB genera include *Leuconostoc, Carnobacterium, Weissella,* and some lactobacilli. LAB naturally occur in milk through contamination from a variety of sources during milk collection (Quigley et al., 2013). LAB metabolize the main carbohydrate in milk, lactose, breaking it down to glucose and galactose using the enzyme lactase. The glucose is then used for growth through either homo- or heterolactic fermentation.

LAB are one of the most important and commercially exploited group of microbes used globally for food production and healthcare. For example, LAB are applied industrially as dairy starter cultures to maintain food quality and safety and as probiotic dietary supplements for gut health. Starter cultures consist mainly of dairy LAB such as the lactobacilli and species of *Lactococcus*, *Leuconostoc*, and *Streptococcus*. Starter culture strains generally have well-defined

roles in dairy production and are studied in-depth to determine their pH, temperature, and salt tolerances as well as enzymatic activities and the fermentation characteristics of each strain. Still, novel strains of LAB are sought for their use in the dairy industry and also as probiotics (Quinto et al., 2014). Therefore, microbiologists have travelled the world over seeking unstudied dairy practices and products in search of useful LAB.

Dairy practices have evolved and diversified over time, especially with the globalization of dairy production. Modern day industrial dairy production requires the sterilization of milk before use and, therefore, depends on starter cultures to generate different dairy products. In contrast, traditional style dairying depends on the indigenous microbes found in raw animal milk resulting in many different dairy products. Furthermore, industrialized dairy processing must adhere to strict hygiene and microbial standards. The U.S. federal regulations code states that milk and dairy products must be inspected and tested for quality control purposes throughout each processing operation (7 CFR § 58.335) and that only "harmless cheese cultures" may be used for cheese production (7 CFR § 58.433). Dairy product quality is regularly checked by quantitating the growth of psychotropic bacteria, yeasts, molds, and coliforms (7 CFR § 58.523). Even though the differences are stark between traditional and industrialized dairy practices, some similarities remain such as the use of microorganisms.

As of the early twentieth century, urbanization in Mongolia brought about the industrialization of Mongolian dairy, but traditional practices and nomadic herding still exist in many rural areas of Mongolia. At least seven different animals, including cows, horses, camels, sheep, goats, yaks, and reindeer are used for dairy production throughout Mongolia today. The present study focuses on dairy products made by nomadic herders in Khövsgöl aimag, the northern-most province of Mongolia, using cow and yak milk (Fig 1.1).

Fig 1.1. Cow, yak, and cow-yak hybrids near Khatgal in the Alag-Erdene sum (district) of Khövsgöl aimag (province), Mongolia.

Fig 1.2. Freshly cut and drying pieces of aaruul outside the home (yurt) of the herders

The dairy production process is very hands on in Khövsgöl. Milk is collected by hand, and food is prepared inside or outside the home (yurt) over a wood burning stove or at room temperature using utensils and equipment made from various materials, such as wood, plastic, and metal. During the dairying season, several fresh and fermented dairy products are made throughout the day after milk collection (Wilkin et al., 2020). Yogurt (tarag) is made by adding culture (khöröngo) to fresh milk or milk left over from making clotted cream (öröm). Clotted cream (öröm) is made by separating the fat from milk through foaming the milk during heating, then cooling the foamy milk until a coagulated cream rests on top. This clotted cream can be scooped off and spread onto bread and enjoyed with hot milk tea (süütei tsai). Milk tea is made through first brewing tea by adding crushed, dried black tea leaves to boiling water over a wood burning stove; salts, minerals, and fresh milk are then added. Fresh cheese (byaslag) is an acid-curdled cheese made by adding fermented milk to foaming milk while being gently heated. After heating, the curdled milk is transferred to a cheese cloth then pressed to remove the whey. Crushed salt and chopped wild onions can be added for extra flavor. A distilled liquor drink called shimiin arkhi is made by boiling fermented milk in a kettle setup over homemade distilling equipment. Pictures of these products are shown in Fig 1.3. The products described here were analyzed in this study and are examples of some of the many dairy products made in Mongolia today.

Fig 1.3. A: clotted cream (öröm); B: milk tea (süütei tsai); C: fresh cheese (byaslag); D: yogurt (tarag); E: vodka made from milk (shimiin arkhi).

Chapter 2: Isolation and identification of lactic acid bacteria in Mongolian dairy from Khövsgöl

Abstract

Milk and fermented dairy products have been a dietary staple in Khövsgöl, Mongolia for thousands of years (Jeong et al., 2018; Wilkin et al., 2020). Today, several fermented milk products with unique and rich flavors, aromas, and textures are made in Khövsgöl. The objective of this study was to isolate and identify, through a cultivation-based approach, the lactic acid bacteria (LAB) associated with 16 samples of cow and yak milk dairy products from Khövsgöl. Using MRS and M17 growth media, 204 lactic acid bacteria isolates, comprising 15 genera and 35 species, were obtained and identified by 16S rRNA gene sequencing. *Enterococcus* and *Leuconostoc* were recovered from the majority of the products analyzed. Fresh milk (süü) from cow was the dairy product that contained the greatest diversity of isolated LAB genera. Using a culture-dependent technique, the predominant LAB associated with traditional dairy from Khövsgöl resembles the results of previous reports on LAB composition of dairy from other provinces of Mongolia. However, the recovered LAB taxa from the products analyzed in this study is more diverse compared to previous reports of LAB in dairy from Khövsgöl (Yu et al., 2011). This is the first comprehensive report describing both the production of specific dairy products made in Khövsgöl, Mongolia and the lactic acid bacteria associated with those products.

Introduction

Dairy pastoralism has an ancient history in Mongolia, and fermented dairy products have been subsistence resources in Mongolia for millennia. The consumption of dairy in Mongolia has been dated to the early Bronze Age (3000 BC) based on ancient proteins found in the calcified tooth tartar (dental calculus) of excavated individuals (Jeong et al., 2018; Wilkin et al., 2020). Although cow, goat, and sheep milk were consumed, the genomic DNA analysis showed that the individuals from the Khövsgöl aimag did not possess the genetic mutation for lactase persistence and could not produce the enzyme necessary for digesting lactose-rich dairy into adulthood. Therefore, the individuals most likely consumed fermented dairy products, such as yogurts and cheeses, containing less lactose than milk to avoid the uncomfortable side effects of lactose indigestion associated with lactase nonpersistence (Bayless et al., 2017).

Milk and fermented dairy products are still a major source of subsistence and nutrition in contemporary Mongolia, and rural Mongolians utilize milk from a diverse set of livestock, including cows, yaks, goats, sheep, camels, horses and reindeer (Wilkin et al., 2020). Traditional Mongolian dairy practices consist of nomadic herding on the steppes, milk collection by hand, and milk processing to create a diverse array of fermented milk products with rich flavors, aromas, and textures. Several different dairy products are made in Khövsgöl, such as tarag (yogurt), öröm (clotted cream), byaslag (fresh cheese), aarts (fresh curd from yogurt), aaruul (dried curd from yogurt), airag (fermented milk alcoholic drink), and many others. Back-slopping is an important technique used in producing all these foods. Back-slopping is the practice of adding a portion of a completed dairy product to fresh milk to make more of the original, or “back-slopped”, product. In Khövsgöl, the back-slopping product used at the start of

each dairying season is called khöröngo (Wilkin et al., 2020). The khöröngo is preserved over the winter and then thawed and revived in fresh milk at the start of the spring dairying season to make fermented dairy products throughout the summer.

The fresh milk from Mongolian dairy livestock may contain indigenous microbes that are important for the development of the specific characteristics of each dairy product. Fresh milk is a nutrient-rich medium, which supports the growth and survival of microorganisms, and the abundance and composition of microbes in milk can largely depend on the animal origin of the milk (Quigley et al., 2013). Lactic acid bacteria (LAB) are microbes naturally found in milk that are beneficial because they help preserve the nutrition in milk by creating products with a longer shelf-life and differing flavors and textures through the process of fermentation. However, some of the indigenous microbes in fresh milk can cause spoilage and disease (Quigley et al., 2013). Consequently, industrial dairy operations must adhere to strict hygiene regulations and microbial standards. For example, industrialized dairy producers are required to pasteurize fresh milk before it can be used for direct consumption or to make subsequent products. Pasteurization destroys the indigenous microbial diversity found in fresh milk, thus requiring the addition of starter cultures containing a limited set of specific bacterial strains. Starter cultures for worldwide dairy production consist of LAB strains because these bacteria ferment the lactose in milk rapidly producing various organic acids, especially lactic acid, which acidifies and coagulates the milk (Johnson, 2013). Through this fermentation process, LAB contribute to the flavor and texture profile of dairy products. Therefore, the indigenous microbes in Mongolian dairy likely work in tandem with physical manipulations, such as heating, cooling, stirring, and drying, to make the vast array of unique, traditional Mongolian dairy products.

Despite the historical importance of dairying in Khövsgöl, the microbiota of contemporary dairy products produced in this province remain unknown. Previous studies have reported the microbial composition and abundance in fermented dairy products from other areas of Mongolia (Liu et al., 2012; Ren et al., 2015; Sun et al., 2010; Wang et al., 2016; Watanabe et al., 2008; Yu et al., 2011). However, a comprehensive study on dairy products produced in Khövsgöl, Mongolia has not been previously published. Therefore, this study aimed to isolate, identify, and catalogue LAB associated with various traditional dairy products made in Khövsgöl. Specifically, this study reports the predominant LAB associated with cow and yak milk-based dairy produced in Khövsgöl, Mongolia.

Materials and Methods

Sampling of dairy products

Sixteen samples were collected from cow and yak dairy producers near the town of Khatgal in the Alag-Erdene sum of the Khövsgöl aimag of Mongolia (Fig. 1). Nine samples were from cow's milk products, and seven samples were from yak's milk products. Approximately 200 ml (liquid) or 100 g (solid) of each product was aseptically collected in sterile bags, maintained at 4ºC during transport to the laboratory in Mongolia, and then frozen at -20ºC until shipment to the Max Planck Institute for the Science of Human History (MPI-SHH) (Jena, Germany). Once the frozen samples arrived at the MPI-SHH, the samples were thawed and aliquots made and then frozen on dry ice for shipment to the University of Oklahoma (Norman, Oklahoma, USA). A production process flowchart shows pictures, as well as the Mongolian names and English translation, for each dairy product analyzed in this study (Fig. 2).

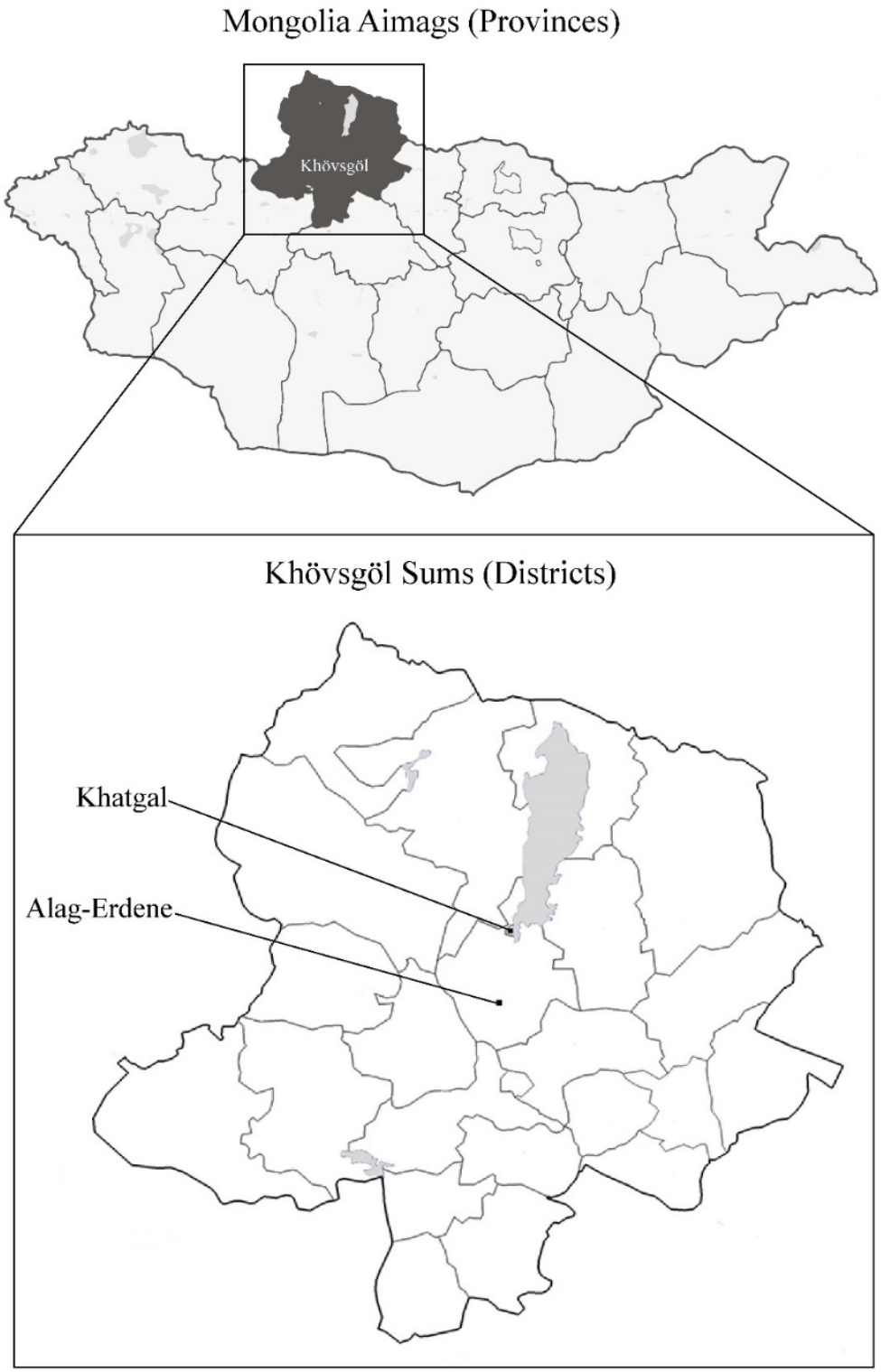

Fig 2.1. The dairy samples analyzed in this study were collected near Khatgal in the Alag-Erdene sum of Khövsgöl aimag, Mongolia.

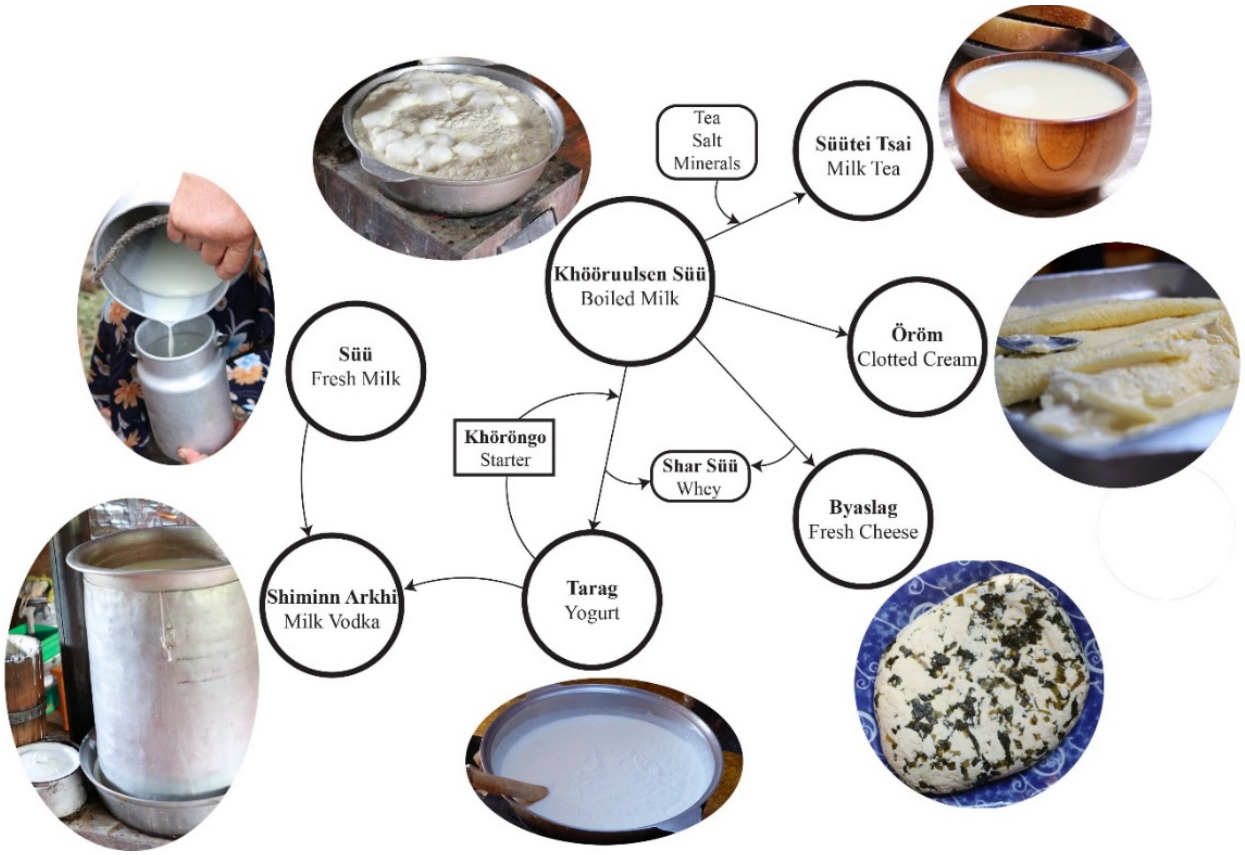

Fig 2.2. Production process of traditional Mongolian dairy products analyzed in this study.

Enrichment and isolation of LAB

The samples shipped on dry ice from Germany to Oklahoma experienced a customs delay and were not properly temperature controlled during the delay. To overcome the possible issue of sample integrity, the samples were enriched with two LAB specific growth media that are routinely used for the selective enrichment of microorganisms found in dairy products: De Man, Rogosa, and Sharpe (MRS) for the selective growth of lactobacilli and M17 for the selective growth of lactic streptococci. In addition, enrichments were incubated at two separate temperatures selective for mesophilic (~27ºC) and thermophilic (>40ºC) LAB (Johnson, 2013). Thus, four enrichments were completed for each sample according to the following method. Briefly, 250 µl (liquid) or 250 mg (solid) of sample was added to a serum-seal glass tube containing 10 ml of sterilize M17 broth (Difco™ Cat. No. 218561, Becton Dickinson, USA) or modified MRS broth (g/l: glucose, 20; beef extract, 10; yeast extract, 5; proteose peptone no. 3, 10; sodium acetate, 5; sodium citrate, 2.5; K_2HPO_4, 2.2; Tween 80, 1.0; $MgSO_4$, 0.1; $MnSO_4$, 0.05). Each tube was aseptically flushed with nitrogen gas after inoculation and then incubated statically at 28ºC or 42ºC for 72 hours. After incubation, the contents of each tube were thoroughly mixed, serially diluted in sterile 1.0% saline, and spread-plated onto the respective media to obtain clearly separated bacterial colonies. Colonies with differing morphologies were selected randomly and sub-cultured onto the respective agar until pure cultures were obtained.

Sequencing and identification of LAB isolates

For each isolate, total genomic DNA was extracted from a several loopfuls of fresh culture using a DNeasy® UltraClean® Microbial kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total DNA was used as a template for partial 16S rRNA gene sequencing (~450 bp) covering variable regions one and two that are very diagnostic for identification purposes. The partial 16S rRNA gene sequence was amplified using the following PCR primers and reaction mixture: 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and pD (5′-GTATTACCGCGGCTGCTG-3′) (Hutson et al., 1993) in a 25 µl reaction mixture containing 0.25 µl of each primer (20 mM), 2.5 µl DreamTaq buffer (10x), 2.5 µl dNTPs (2 mM), and 0.625 µl DreamTaq (1 U/µl). PCR amplification was completed using a Techne TC-512 thermal cycler system as follows: initial denaturation for 5 min at 94ºC, 30 cycles of 30 s at 94ºC, 30 s at 55ºC, 30 s at 72ºC, and a final extension for 5 min at 72ºC. PCR products were quantified by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized using a GD-1000 Axygen® gel documentation system (Corning Life Sciences). Unincorporated primers and dNTPs were removed from the PCR products using ExoSAP-IT (USB Corporation). The purified fragments were sequenced using the Sanger method at the University of Oklahoma Biology Molecular Core Laboratory (Norman, Oklahoma, USA). The presumptive identity of each isolate was determined using the EzBioCloud online identification platform (https://www.ezbiocloud.net/identify) based upon pairwise sequence similarity calculations (Yoon et al., 2017).

Results and Discussion

The cultivation technique used in this study recovered a diverse range of LAB from the 16 Mongolian dairy samples. A total of 204 LAB isolates were identified, representing 15 genera and 35 species (Table 1). Overall, the distribution of recovered LAB genera was: 38.2% *Enterococcus*, 21.6% *Leuconostoc*, 13.2% *Lacticaseibacillus*, 4.9% *Lactococcus,* 4.4% *Latilactobacillus,* 3.4% *Levilactobacillus,* and <3.0% each of *Lactiplantibacillus, Lactobacillus, Limosilactobacillus, Lentilactobacillus, Companilactobacillus, Pediococcus, Streptococcus*, *Carnobacterium*, and *Weissella*. The predominant LAB species isolated were *Enterococcus durans* (63 isolates), *Leuconostoc mesenteroides* subsp. *dextranicum* (31 isolates), and *Lacticaseibacillus paracasei* subsp. *tolerans* (24 isolates).

Table 2.1. Summary of LAB genera and species isolated from all Mongolian dairy products analyzed in this study.

LAB isolate	No. of strains isolated	LAB isolate	No. of strains isolated
Enterococcus	**78**	***Lactiplantibacillus***	**6**
E. durans	63	*L. pentosus*	6
E. thailandicus	10		
E. faecium	2	***Pediococcus***	**5**
E. hermanniensis	1	*P. acidilactici*	5
E. lactis	1		
E. mundtii	1		
		Lactobacillus	**4**
Leuconostoc	**44**	*L. delbrueckii* subsp. *bulgaricus*	2
L. mesenteroides subsp. *dextranicum*	31	*L. bombicola*	1
L. mesenteroides subsp. *mesenteroides*	5	*L. delbrueckii* subsp. *lactis*	1
L. mesenteroides subsp. *jonggajibkimchii*	3		
L. suionicum	3		
L. mesenteroides subsp. *cremoris*	1	***Limosilactobacillus***	**4**
L. gelidum subsp. *aenigmaticum*	1	*L. fermentum*	4
Lacticaseibacillus	**27**		
L. paracasei subsp. *tolerans*	24	***Streptococcus***	**4**
L. paracasei subsp. *paracasei*	2	*S. thermophilus*	2
L. rhamnosus	1	*S. cristatus*	1
		S. vestibularis	1
Lactococcus	**10**		
L. lactis subsp. *lactis*	4	***Carnobacterium***	**2**
L. raffinolactis	3	*C. divergens*	2
L. garvieae subsp. *garvieae*	2		
L. lactis subsp. *hordniae*	1	***Lentilactobacillus***	**2**
		L. parabuchneri	2
Latilactobacillus	**9**		
L. curvatus	7	***Companilactobacillus***	**1**
L. sakei subsp. *sakei*	2	*C. metriopterae*	1
Levilactobacillus	**7**	***Weissella***	**1**
L. brevis	7	*W. viridescens*	1

Although most of the LAB isolates belonged to the genus *Enterococcus*, *Enterococcus* species were isolated from 11 of 16 samples, while *Leuconostoc* species were isolated from 12 of 16 samples (Fig. 3). In general, the distribution of culturable LAB genera differed between dairy products and by milk source. *Lacticaseibacillus* and *Lactobacillus* were not isolated from boiled yak milk but were isolated from yak milk-based yogurt (tarag) and whey (shar süü) separated from that same yogurt (Fig. 3). Fresh cow milk (süü) contained nine LAB genera, the most of any product analyzed in this study, and the number and diversity of culturable LAB genera decreased between fresh and boiled cow milk. Furthermore, members of the LAB genera recovered differed between boiled yak milk and boiled cow milk. Some of the dairy products contained similar LAB genera regardless of the source of the milk used to make the product. For example, milk tea (süütei tsai) had the exact same two LAB genera recovered, *Leuconostoc* and *Lactococcus*, possibly because the tea, salt, and minerals make the milk tea more biochemically selective for these LAB genera. *Lactococcus* species are used primarily as mesophilic, homofermentative cheese starter cultures in industrialized dairy production, but their natural occurence in fresh cow's milk is well documented (Quigley et al., 2013). Specifically, the species *Lactococcus lactis* can grow quickly and produce large amounts of lactic acid making it an important starter strain for the manufacture of cheeses (Johnson, 2013). This species was mostly recovered from the cow's milk dairy products analyzed here and was identified in five different products, including the fresh cheese (byaslag).

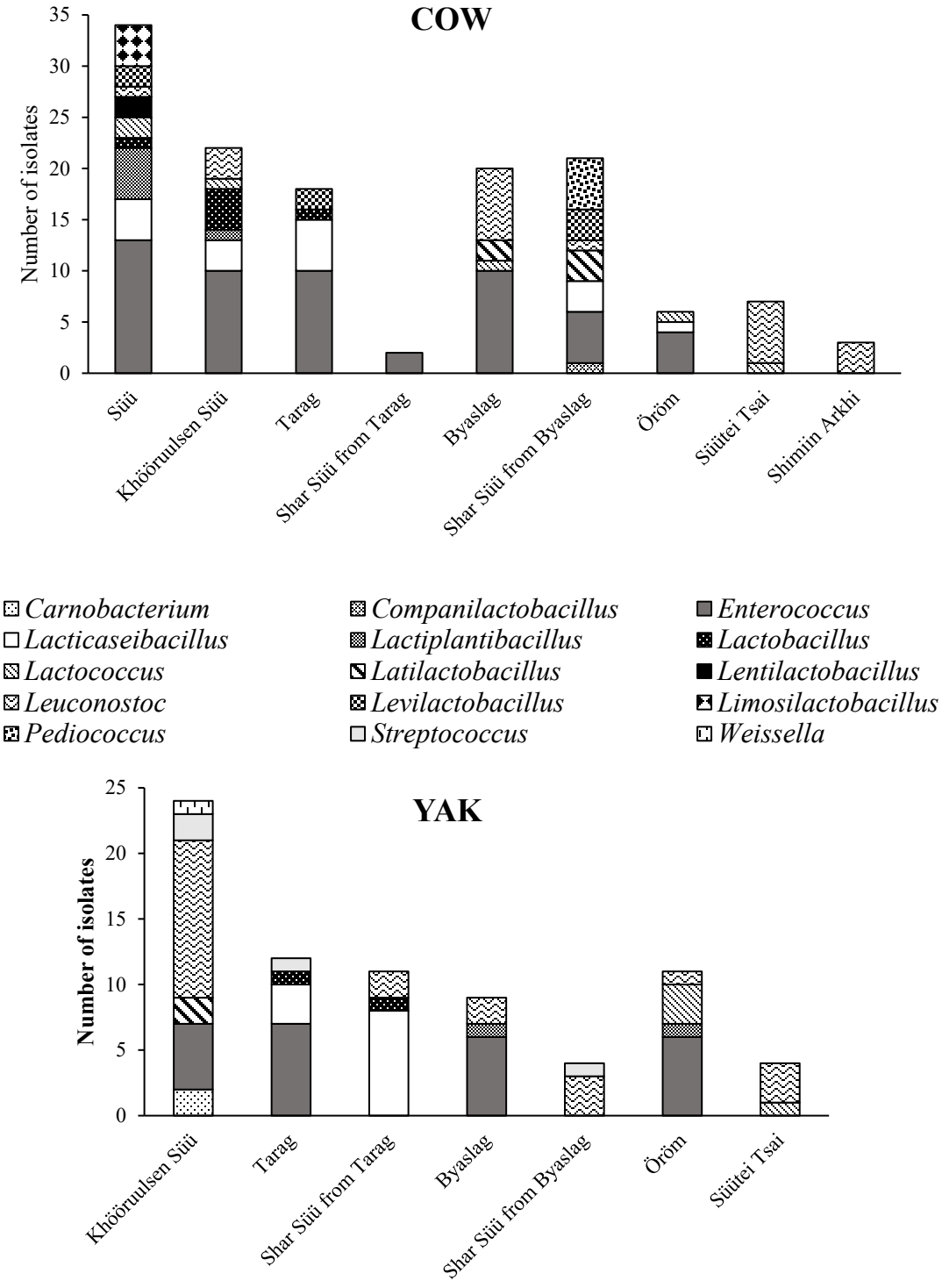

Fig 2.3. Distribution of total number LAB isolates for each genus by milk source and dairy sample.

Some of the products did not have a high incidence of unique culturable LAB, with members of only one or two genera being recovered. Additionally, different numbers of isolates were obtained from each dairy product analyzed in this study, ranging from 2 and 34 isolates per dairy product (Fig. 3). The LAB isolate selection method used in this study contributes to the wide range in number of isolates recovered per product since only colonies with differing morphologies were selected for purification and identification, which is subjective. For example, only one distinguishable colony morphology grew on the dilution plates from all the enrichments of cow milk-based whey from tarag. Therefore, only one LAB genus, *Enterococcus,* was identified for this sample. Similarly, *Leuconostoc* was the only genus isolated from shiminn arkhi. *Streptococcus* species are associated with modern-day dairy practices, but only a few *Streptococcus* species were recovered from the yak dairy products and none were isolated from the cow dairy products. *Pediococcus acidilacti* was only isolated from whey of the fresh cheese (shar süü from biyaslag).

Enterococcus was abundant and widespread across the dairy products investigated. Enterococci are found in many artisanal dairy products and are claimed to contribute to the diversity of flavors in these products (Donnelly, 2013). Therefore, we expected to find *Enterococcus* species in the traditional Mongolian dairy products we analyzed. However, the presence and use of enterococci in dairy production has a lengthy, complicated history regarding health and safety, partly because *Enterococcus* species freely acquire and transfer plasmids, many of which contain antibiotic resistance genes, conferring this resistance to other bacteria in the human gut (Lerminiaux and Cameron, 2019). Enterococci are therefore banned from use in the industrial manufacture of dairy in the European Union (Hanchi et al., 2018). Our results indicate *Enterococcus* species are present in traditional Mongolian dairy and studying the

biochemical attributes of these isolates could provide insights as to how they contribute to product characteristics.

Streptococcus species are commonly found in dairy products worldwide, used as starter culture strains, and have been reported in dairy from other provinces of Mongolia. Therefore, more *Streptococcus* isolates were expected to be found but only a few were recovered from the yak milk products and none were recovered from the cow milk products. *Weissella* and *Carnobacterium* species were isolated from the boiled yak milk (khööruulsen süü). These genera are commonly associated with raw animal milk and artisanal dairy products but are typically not used as starter culture strains for industrialized dairy practices (Fessard and Remize, 2017). Lactobacilli are a particularly important group of dairy LAB used as starter culture strains and probiotics, and several lactobacilli strains were isolated in this study. The genus *Lactobacillus* was recently split into 25 genera, and the results reported here reflect the revised taxonomy and nomenclature (Zheng et al., 2020). Seven of those novel lactobacilli genera were recovered from the dairy products analyzed in this study, including *Companilactobacillus, Lacticaseibacillus, Lactiplantibacillus, Latilactobacillus, Lentilactobacillus, Levilactobacillus, Limosilactobacillus*.

On examination of the literature, most published studies reporting the microbial diversity of Mongolian dairy products focused on airag and tarag or did not specifically name the products analyzed (Liu et al., 2012; Ren et al., 2015; Sun et al., 2010; Watanabe et al., 2008; Yu et al., 2011). One published study reported the microbial diversity in öröm which could be used for comparison; however, no published reports were found describing the microbial diversity of Mongolian milk tea (süütei tsai), milk vodka (shimiin arkhi), whey (shar süü) separated from curdled milk, or boiled cow and yak milk (khööruulsen süü). Watanabe et al. (2008) reported *Lactobacillus, Limosilactobacillus, Lentilactobacillus, Lactiplantibacillus, Pediococcus* and

Streptococcus species associated with tarag made from cow and yak milk produced in four provinces (not including Khövsgöl), but the tarag produced in Khövsgöl contained mostly different LAB genera (*Enterococcus, Lactobacillus, Lacticaseibacillus*, *Levilactobacillus,* and *Streptococcus*). The difference in the results from comparable studies could be attributed to the inherent differences of the analyzed products or could be due in part to performing a prior enrichment on the samples rather than directly plating the samples. Despite the difference in methodology, some results were consistent between comparable reports. For example, Wang et al. (2016) reported *Lactobacillus, Lacticaseibacillus, Lactiplantibacillus, Levilactobacillus, Lactococcus,* and *Leuconostoc* in clotted cream (öröm) made in Inner Mongolia, China, and the clotted cream (öröm) produced in Khövsgöl contained all of these same genera, except for *Lactobacillus* and *Levilactobacillus*, plus *Enterococcus*.

The culturable LAB associated with specific dairy products made in Khövsgöl are reported here for the first time. Yu et al. (2011) reported LAB associated with 24 samples of "fermented" cow and yak milk from Khövsgöl using a cultivation-based approach, but the specific dairy products were not named, inhibiting direct comparison by product. However, the products analyzed here appear to have greater culturable diversity of LAB in them than what Yu et al. (2011) reported. Yu et al. (2011) obtained 66 LAB isolates, consisting of only six distinct species, from the 24 fermented milk samples from Khövsgöl examined, while we recovered 204 LAB isolates, consisting of 35 species, from 16 dairy samples. The results reported here that could be compared to previously published studies show both differences and similarities, depending on the product, with dairy produced in other areas of Mongolia.

Conclusions and Future Directions

This study reports the LAB species found in 16 samples of various traditional Mongolian dairy products from Khövsgöl. Altogether, 15 LAB genera, consisting of 35 unique species, were recovered from the dairy products analyzed in this study. The predominant LAB species isolated belonged to the genera *Enterococcus, Leuconostoc*, and *Lacticaseibacillus.* Future studies could focus on the biochemical properties of the LAB recovered in this study to better understand what they contribute to the flavors, textures, and aromas unique to dairy produced in Khövsgöl. Additionally, metagenomic sequence analysis could be performed on the whole dairy products used for cultivation in Oklahoma to determine how successful the cultivation efforts were, and the recovered *Enterococcus* strains should be screened for phenotypic expression of antibiotic resistance genes coded for on their genomes and plasmids.

Chapter 3: Phylogenetic analysis and taxonomy of lactic acid bacteria isolated from Khövsgöl dairy

Abstract

Taxonomy is generally in a state of flux and changes constantly due to advancements in DNA sequencing technologies and polyphasic taxonomy approaches. Presented here is the current taxonomic classification and description of all the lactic acid bacteria genera isolated from the analyzed dairy samples from Khövsgöl. Zheng et al (2020) recently split the genus *Lactobacillus* into 23 new genera and combined the families *Lactobacillaceae* and *Leuconostocaceae.* Of the 15 LAB genera recovered in this study, 7 of the genera were the newly defined lactobacilli. Eighty-eight of the recovered isolates were sent to Germany for full genome sequencing to explore their genotypic potential.

Introduction

In the 1980s and 1990s the use of 16S rRNA sequence analysis, pioneered by Carl Woese (1987) became pivotal in the reorganization of bacterial taxonomy leading to revisions of many classification schemes. The LAB especially became a focus of interest due to their economic importance, the application of molecular methods led to a plethora of novel taxa being described (Holzapel and Wood, 2014). However, taxonomy is never static and taxonomic revisions continue to this day. In 2014, new minimal standards for description of new taxa of the *Bifidobacterium, Lactobacillus* and related genera were proposed (Mattarelli et al., 2014). More recently, a major revision of the genus Lactobacillus was undertaken by Zheng et al (2020) who proposed the reclassification of *Lactobacillus* into 25 genera and the description of the family *Lactobacillaceae* to include all genera that were previously included in families *Lactobacillaceae* and *Leuconostocaceae* (Zheng, 2020).

Presently, the LAB group consists of all the species classified in the order *Lactobacillales*. This order contains five families, *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae,* and *Streptococcaceae*, consisting of 68 genera and a plethora of species and subspecies. Species from four of the five LAB families were isolated from the analyzed Khövsgöl dairy products. Of the 204 isolates recovered in this study, 94 isolates belonged to the families *Carnobacteriaceae, Enterococcaceae,* and *Streptococcaceae*, and 110 isolates belonged to the *Lactobacillaceae* family. None of the recovered isolates belonged to the *Aerococcaceae* family, which is not surprising because species within this bacterial family are generally not found in dairy or able to utilize lactose for growth (Holzapfel and Wood, 2014).

Chapter 2 included the identification using 16S rRNA of the isolates recovered. However, pairwise analysis only goes so far in providing a preliminary identification. Additional phylogenetic analysis and the construction of evolutionary trees provides information on specific relationships between taxa and the diversity present. Furthermore, it is important to demonstrate the recent taxonomic revisions of the LAB especially with respect to the genus *Lactobacillus* (Zheng et al., 2020).

Materials and Methods

16S rRNA gene sequence phylogenetic analysis

Phylogenetic analysis of the isolates was completed using 16S rRNA gene sequence analysis to demonstrate the relatedness of each recovered genera and species. Briefly, pairwise similarity values between the recovered strains and closely related type strains were determined using the EZBioCloud's 16S-based identification service (https://www.ezbiocloud.net/identify). Full 16S rRNA gene sequences were obtained from NCBI database (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed with the full 16S rRNA gene sequences using MEGA version 7 (Kumar et al., 2016) after multiple sequence alignments with the Clustal W program (Thompson et al., 1994). Phylogenetic trees based on the neighbor-joining (NJ) method (Saitou and Nei, 1987) were reconstructed and genetic distances were calculated using the Kimura two- parameter model (Kimura, 1980). Bootstrap values were calculated based on 1000 replications (Felsenstein, 1985).

Results and Discussion

Enterococcaceae, Streptococcaceae, and *Carnobacteriaceae*

In 1984, *Enterococcus* was separated from *Streptococcus* based on DNA-DNA and DNA-rDNA hybridization studies, and *Enterococcus* is the type genus for the family *Enterococcaceae* (Schleifer, 2009). The genus *Enterococcus* contains 67 species and 2 subspecies, and *Enterococcus faecalis* is the type strain for the genus. *Enterococcus faecalis* was not isolated in this study and is not a close relative to the *Enterococcus* species recovered from the analyzed Khövsgöl dairy. All but one of the *Enterococcus* isolates grouped together phylogenetically in the *E. faecium* group (Fig 3.1). This group consists of the following eight species: *E. canis, E. mundtii, E. durans, E. thailandicus, E. hirae, E. ratti, E. faecium* and *E. lactis.* As depicted in the phylogenetic tree (Fig 3.1), *E. hermanniensis* forms a separate branch because it groups with *E. pallens* (Zhang and Cai, 2014). The recovered *Enterococcus* species are associated with food, especially meat and dairy, and animal microbiomes. *Enterococcus* species have complex nutritional requirements, are homofermentative LAB, and grow optimally at 35-37ºC but are also able to grow at 42-45ºC. *Enterococcus* species were abundantly isolated from the Khövsgöl dairy products using the enrichment and isolation method presented here most likely because of these species ability to grow quickly at this higher temperature range.

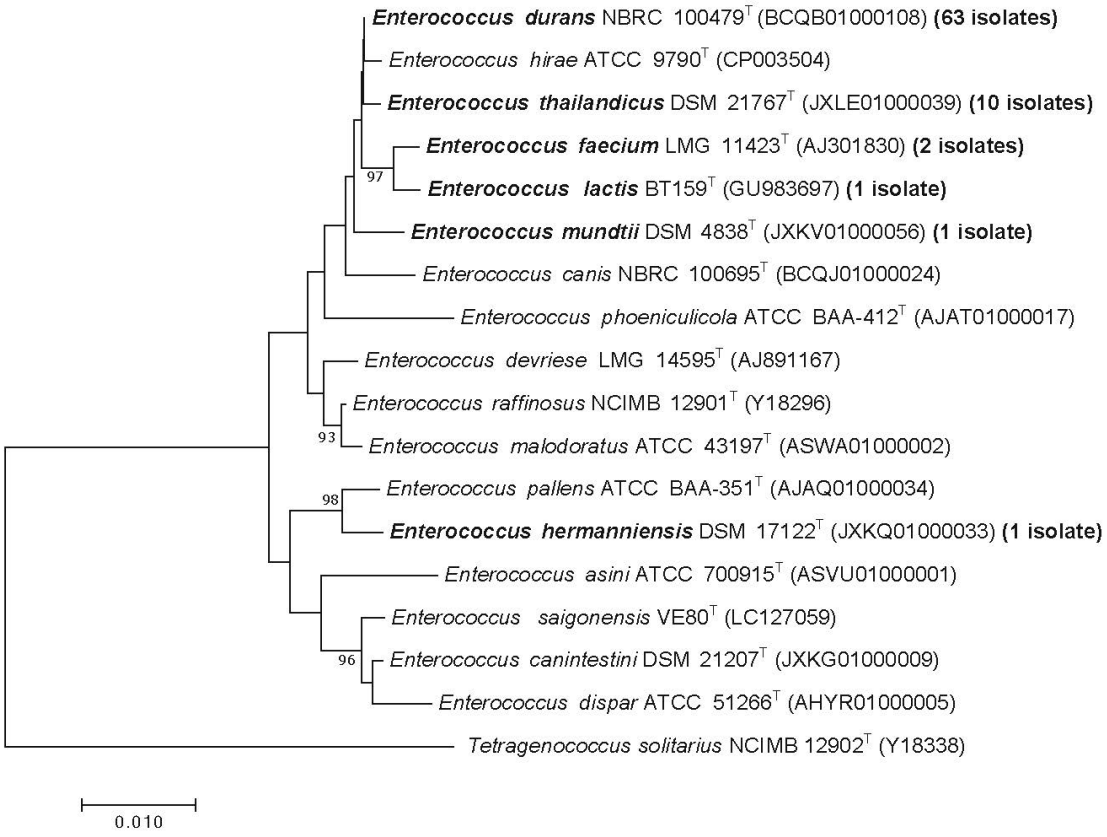

Fig 3.1. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence analysis of closely related *Enterococcus* species isolated from Khövsgöl dairy (shown in bold). *Tetragenococcus solitarius* NCIMB 12902^T was used as the outgroup. Bootstrap values at nodes based on 1000 replications. Scale bar represents 1% sequence divergence.

Species of the family *Carnobacteriaceae* are more closely related to the *Enterococcaceae* rather than to the *Strepotococcaceae* species as depicted by the phylogenetic tree (Fig 3.2). *Carnobacterium divergens* was the only species from the *Carnobacteriaceae* family isolated in this study. This species is the type strain for the genus *Carnobacterium,* which contains 12 species and 2 subspecies and is the type genus for the family *Carnobacteriaceae*. Carnobacteria are facultative heterolactic fermenters, and these species, especially *C. divergens*, are often found in dairy products and on the surface of some cheeses (Schleifer, 2009).

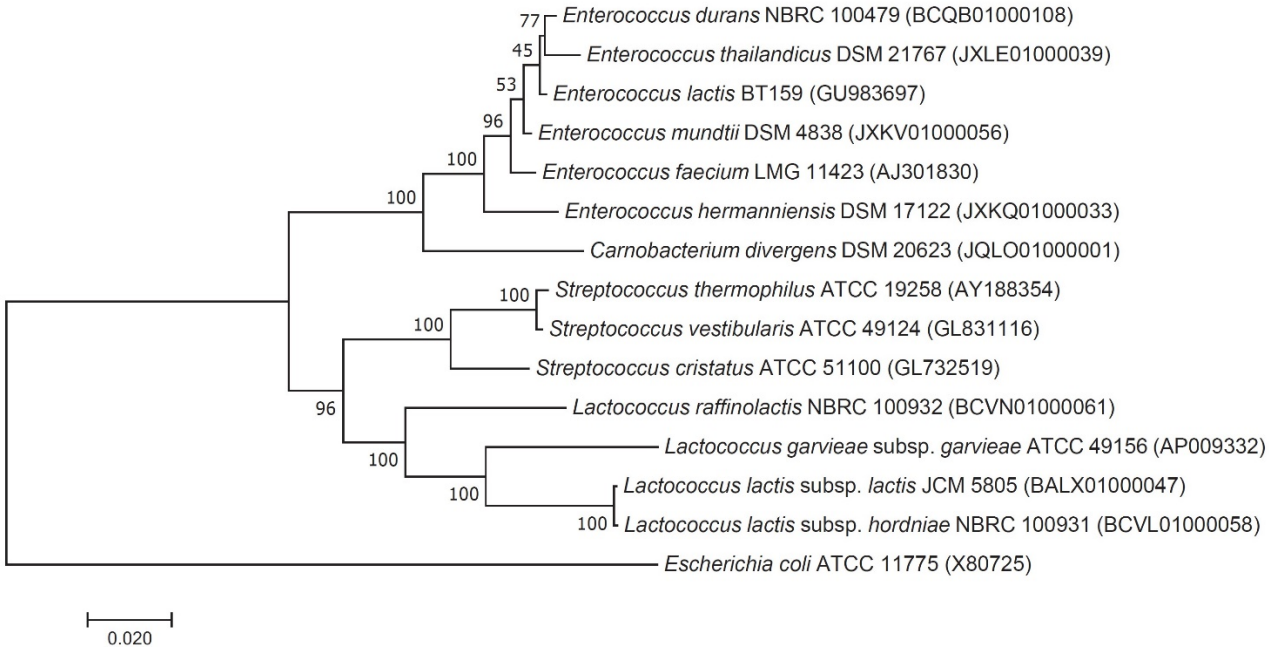

Fig 3.2. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence analysis of representative type strains of the *Enterococcaceae*, *Streptococcaceae*, and *Carnobacteriaceae* species isolated from Khövsgöl dairy. *Escherchia coli* ATCC 11775^{T} was used as the outgroup. Bootstrap values at nodes based on 1000 replications. Scale bar represents 2% sequence divergence.

Both the genera *Streptococcus* and *Lactococcus* are both homofermentative LAB and are classified in the family *Streptococcaceae*, but *Streptococcus* is the type genus for the family (Skerman et al., 1980). The genus *Streptococcus* contains 106 species, and the type species is *Streptococcus pyogenes*. Three *Streptococcus* species were recovered from the Khövsgöl dairy: *S. thermophilus*, *S. vestibularis,* and *S. cristatus. Streptococcus thermophilus* and *S. vestibularis* belong to the *S. salivarius* group, and *S. thermophilus* is a synonym still commonly used for the reclassified subspecies *S. salivarius* subsp. *thermophilus* (Farrow and Collins, 1984; Holzapfel and Wood, 2014). *Streptococcus cristatus* is part of the *S. mitis* group, which is depicted by the separate branch in the phylogenetic tree (Fig 3.1). A *Streptococcus* species with a 98.64% similarity to *S. cristatus*, based on the partial 16S rRNA gene sequence, was isolated in this study. A species percentage similarity less than 98.7% constitutes a novel bacterial species (Yarza et al., 2014). Therefore, to determine if the isolate is a true novel species, the full 16S rRNA gene along with the genome of the isolate should be sequenced and analyzed using the suite of tools available on the EzBioCloud website (http://www.ezbiocloud.net/).

As previously mentioned, the genus *Lactococcus* is part of the family *Streptococcaceae* (Ludwig et al., 2009; Holzapfel and Wood, 2014), and the phylogenetic tree shows their relatedness but also divergence (Fig 3.1). The genus *Lactococcus* is considerably smaller than *Streptococcus*, being made up of only 17 species and 6 subspecies. In this study, four species of *Lactococcus* were isolated: *L. lactis* subsp. *lactis, L. lactis* subsp. *hordniae*, *L. garvieae* subsp. *garvieae*, and *L. raffinolactis*. Lactococci are commercially important for the production of fermented dairy and cheeses because of their homolactic fermentation and ability to simultaneously metabolize glucose and galactose, the two saccharides that constitute lactose (Holzapfel and Wood, 2014).

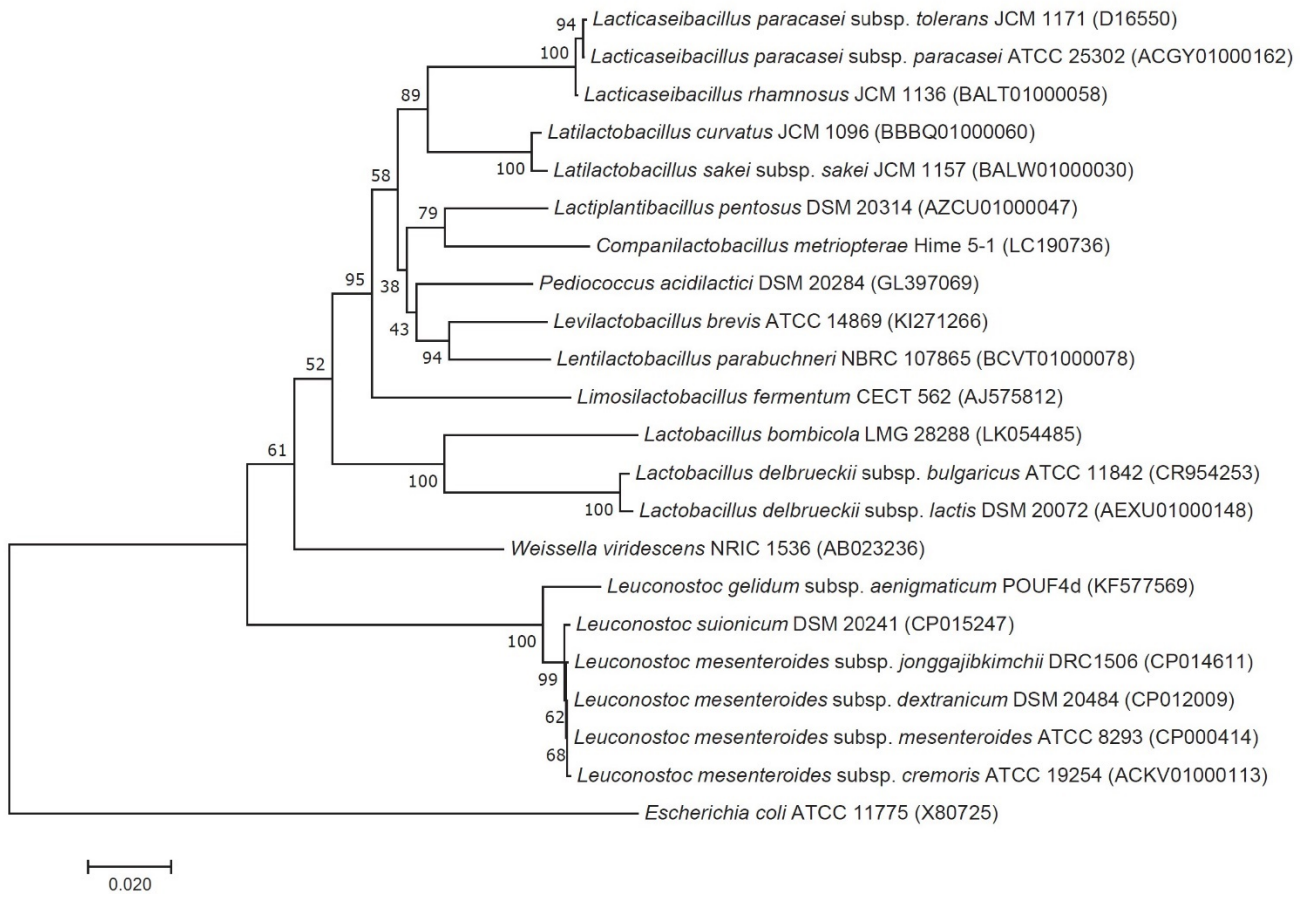

Fig 3.3. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence analysis of the representative type strains of the *Lactobacillaceae* species isolated from the Khövsgöl dairy products analyzed in this study. *Escherchia coli* ATCC 11775^{T} was used as the outgroup. Bootstrap values based on 1000 replications are given at the nodes, and the scale bar represents 2% sequence divergence.

Lactobacillaceae

All of the species within the families *Lactobacillaceae* and *Leuconostocaceae* were recently combined into one family, *Lactobacillaceae,* based on phylogenetic analysis using full genome sequences (Zheng et al., 2020). The genus *Lactobacillus* is still the type genus for the *Lactobacillaceae* family, and the type species of the genus *Lactobacillus* is *L. delbrueckii*. However, the genus *Lactobacillus*, which originally contained 261 species, was split into 23 novel genera, and now consists of only 46 species. The phylogenetic relationship of the recovered lactobacilli genera, including the revised nomenclature, is shown (Fig 3.2). Species from seven of the newly described genera were recovered from the Khövsgöl dairy products.

The lactobacilli were also phylogenetically assessed based on metabolic pathways. The heterofermentative lactobacilli were determined to be more closely related to *Leuconostoc, Weissella,* and *Pediococcus*, with *Pediococcus* and the *L. plantaram* group being the evolutionary link between homo- and heterofermentative LAB (Zheng et al., 2020). The genus *Pediococcus* clusters within the lactobacilli, and only one *Pediococcus* species, *P. acidilactici*, was cultivated in this study. Similarly, only one species of the genus *Weissella* was recovered, which was the *Weissella* type strain *W. viridescens*. The genus *Leuconostoc* contains 26 species and 8 subspecies, and the type species is *L. mesenteroides.* Six species of *Leuconostoc* were isolated in this study, majority of which are subspecies of *L. mesenteroides*: *L. mesenteroides* subsp. *dextranicum,* subsp. *mesenteroides*, subsp. *jonggajibkimchii,* subsp. *cremoris*, *L. suionicum,* and *L. gelidum* subsp. *aenigmaticum*. Several lactobacilli and *Leuconostoc* species are commonly used as primary or secondary starter cultures in dairy production.

Concluding Remarks and Future Investigations

The LAB presented here associated with dairy products from Khövsgöl, Mongolia are frequently found in dairy products throughout the world. However, the individual recovered strains may have some unique features about them that are not yet explored. A goal of this study was to isolate the strains in pure culture so their full genomes could be sequenced as well at their physiological tolerances and biochemical characteristics could be analyzed. Several strains (>80) have already been grown in bulk, freeze-dried, and sent for full genome sequencing at the Warinner lab at the Max Planck Institute for the Science of Human History in Jena, Germany. Additionally, cultures of each strain will be lyophilized and sent to Germany and Mongolia to create an international culture collection.

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Appendix A: Scanning electron microscopy of tarag and öröm

Materials and Methods

A method was developed to examine fluid and semi-solid dairy products using scanning electron microscopy. This method was developed based on methods described by Allan-Wojtas and Kalab (1984), and Alleyne et al (1993).

Briefly, an agar capsule was created to insulate and preserve the dairy product during SEM preparation. A 5% solution of Oxoid purified agar was melted then cooled to 50ºC and dispensed into a sphere-shaped mold using a Pasteur pipette. The dairy product was then aspirated into the center of the sphere and the agar allowed to solidify. The capsule was then transferred to an Eppendorf tube and submerged in buffered 2% glutaraldehyde (0.1 M phosphate buffer, pH 6.6) for 24 hours at 4ºC.

After fixation, the capsule was washed with 0.1 M phosphate buffer (pH 6.6) for 10 minutes. After 3 consecutive washes, the capsule was immersed in buffered 1% OsO_4 for 1 hour at 4ºC. The capsule was then dehydrated in an ethanol series, air-dried in HMDS (hexamethyldisilazane), and mounted on an aluminum stud using carbon tape. Once mounted, the capsule was sliced in half using a razor blade in order to expose the sample and then sputter coated with gold-palladium for 4 minutes at 10 mA immediately prior to examination using a Zeiss Neon 40 EsB dual beam scanning electron microscope.

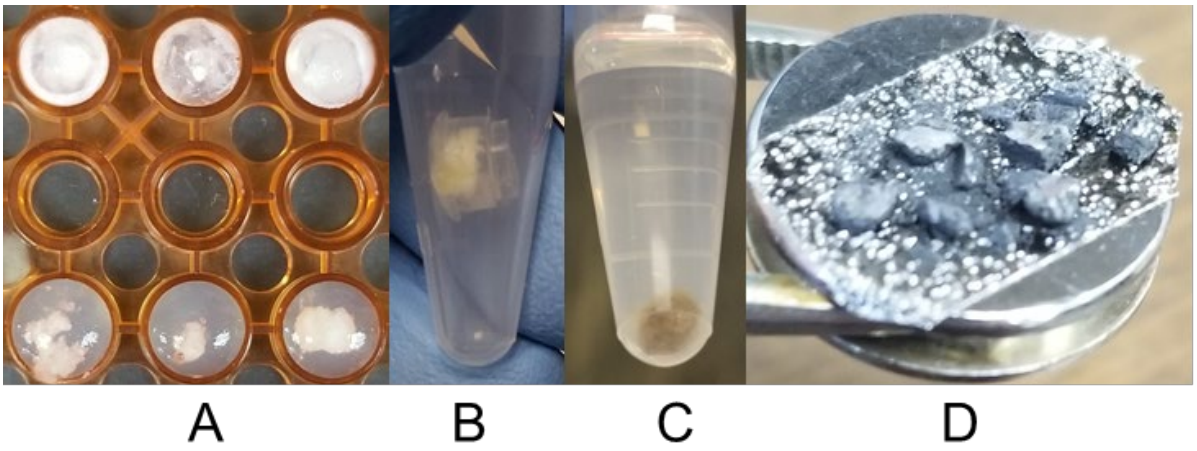

Fig A1. A: agar capsule spheres containing tarag (top row) and öröm (bottom row). B: capsule before fixation. C: capsule after OsO_4 treatment and dehydration. D: the final mounted product to be analyzed.

Results

There was an observable difference in structure and presence of microrganisms between the tarag and öröm. Yeast and bacteria were observed in the tarag samples, but only bacteria were observed in the öröm samples. The tarag SEM images (Fig A2.A-B) depict budding yeast and rod- and coccus-shaped bacteria. The öröm SEM images (Fig A2.C-D) show rod- and coccus-shaped bacteria, but no yeasts.

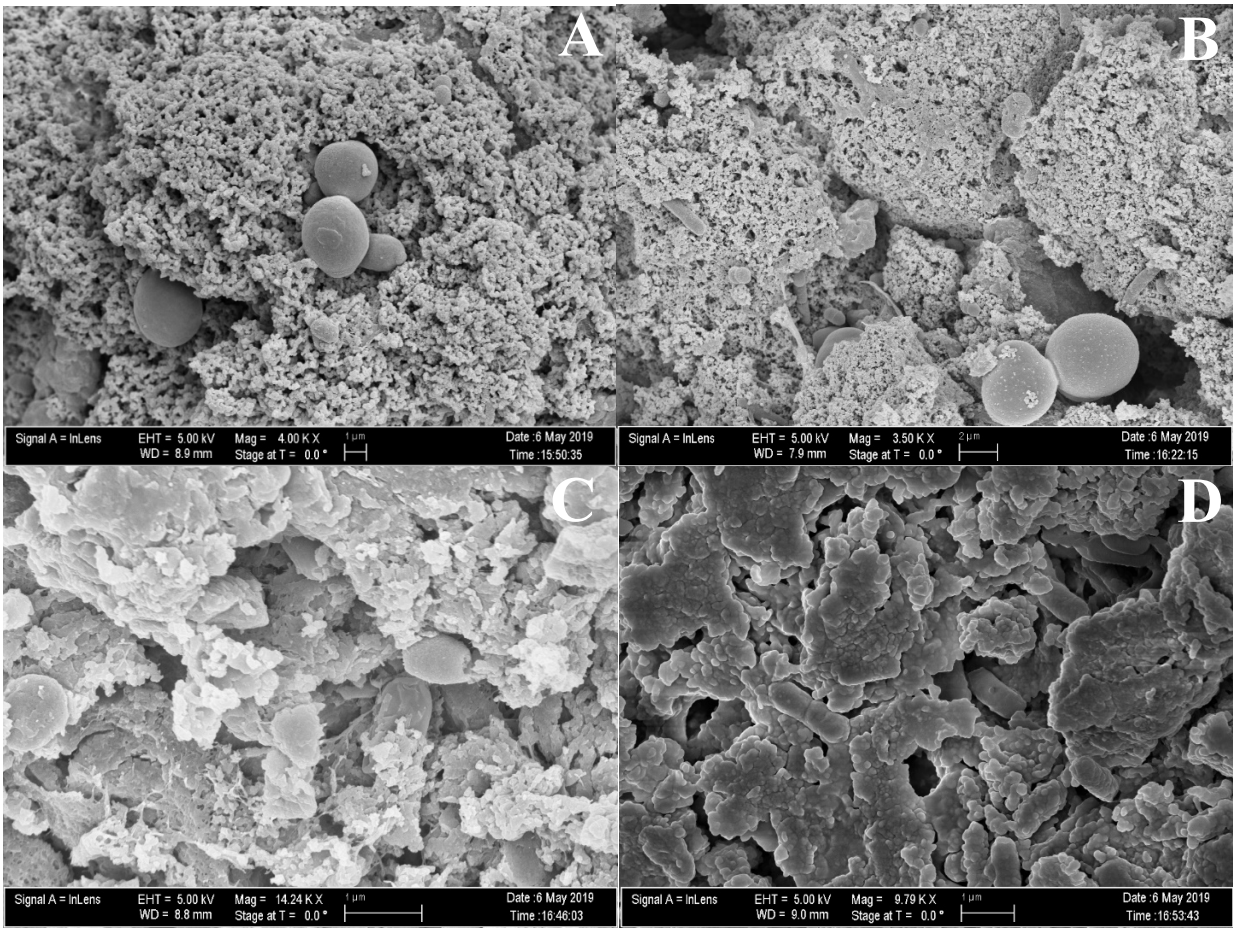

Fig A2. SEM images of tarag made with milk from: cow (A) and yak (B); SEM images of öröm made with milk from: cow (C) and yak (D).

Conclusions

This SEM sample preparation method works best with semi-solid or viscous samples. The tarag was more fluid than the öröm which made it difficult to control during aspiration into the agar capsule. However, this did not seem to disturb the product quality for imaging purposes. The yak-milk based öröm had issues with charging because the sample was not well grounded on the aluminum stub. The SEM images show filament structures which are dehydrated exopolysaccharides (EPSs) produced by the bacteria (Bintsis, 2018). The EPSs make the tarag and öröm thicker, or more viscous, than milk. As expected, the öröm images contain more of the filamentous structures. In addition to bacterial cells, yeast was also observed in the tarag, but not in the öröm. Based on the morphology of cells observed in the four products analyzed, the yak tarag contained the most abundant and diverse bacteria. In the future, it would be useful to use scanning electron cryomicroscopy to view these products and compare the results to this method. The SEM images were used on banners and in presentations for outreach purposes.

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Appendix B: Lyophilization of cells for full genome sequencing

Lyophilized cells of 88 strains were prepared and shipped to Germany for full genome sequencing. Using the stored glycerol stock, fresh cultures of each strain were grown on agar plates of the growth media the strain was originally isolated on (modified MRS or M17). After incubation at the original isolation temperature (27 or 42ºC), 500 or 1000 ml of sterile growth media was inoculated with several colonies of the strain and incubated with shaking for 24-48 hours at the appropriate temperature. Once the growth media became turbid, the cells were centrifuged, separated from the growth media, and pelleted in a 50 ml Corning tube. The pelleted cells were washed with 10 mM phosphate buffer (pH 8.0) twice and then frozen at -80ºC overnight before being placed on the freeze-drier for several hours until the cells were completely dried.