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ARTICLE

Identification of Coccoidal Bacteria in Traditional Fermented Milk Products from Mongolia, and the Fermentation Properties of the Predominant Species, *Streptococcus thermophilus*

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Abstract

The objective of this study was to identify the coccoidal bacteria present in 188 samples of fermented yaks', mares' and cows' milk products collected from 12 different regions in Mongolia. Furthermore, we evaluated the fermentation properties of ten selected isolates of the predominant species, *Streptococcus* (*S.*) *thermophiles*, during the process of milk fermentation and subsequent storage of the resulting yoghurt at 4°C. Overall, 159 isolates were obtained from 188 samples using M17 agar. These isolates were presumed to be lactic acid bacteria based on their gram-positive and catalase-negative properties, and were identified to species level using 16S rRNA gene sequence analysis. These coccoid isolates were distributed in four genera and six species: *Enterococcus* (*E.*) *durans*, *Enterococcus* (*E.*) *faecalis*, *Lactococcus* (*Lac.*) subsp. *lactis*, *Leuconostoc* (*Leuc.*) *lactis*, *Leuconostoc* (*Leuc.*) *mesenteroides*. subsp. *mesenteroides* and *S. thermophilus*. Among these *S. thermophilus* was the most common species in most samples. From evaluation of the fermentation characteristics (viable counts, pH, titratable acidity [TA]) of ten selected *S. thermophilus* isolates we could identify four isolates (IMAU 20246, IMAU20764, IMAU20729 and IMAU20738) that were fast acid producers. IMAU20246 produced the highest concentrations of lactic acid and formic acid. These isolates have potential as starter cultures for yoghurt production.

Keywords: coccoidal bacteria, fermentation properties, *Streptococcus thermophilus*, traditional fermented milk products

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Introduction

Fermentation is one of the oldest technologies for processing and preserving food in Mongolia (Liu *et al*., 2011). Spontaneous fermentation of milk into various different products for local consumption has a long history and cultures of the beneficial microorganisms that facilitate this fermentation, are handed down from one generation to the next (Yu *et al*., 2011); many naturally fermented milk products are produced traditionally without any commercial starter cultures. There is a wide bacterial biodiversity associated with each dairy product but by far the most common bacteria in fermented foods are lactic acid bacteria (LAB) (Chao *et al*., 2009; Lu *et al*., 2008). LAB are increasingly finding acceptance as probiotics that stimulate the host's immune system, lower the risk of cancer and prevent infection by pathogenic microorganisms (Castellano *et al*., 2010; Mohd Adnan and Tan, 2010).

The objective of the present study was to isolate and identify the coccoidal bacteria from traditionally fermented milk products from Mongolia, and quantify the acidification properties of *Streptococcus* (*S.*) *thermophilus* during milk fermentation and subsequent storage. This study focus on coccoidal bacteria in order to screen optimal *S. thermophius* strains as starter culture. Due to *S. thermophilus* can produce lactic acid, fomic acid, extracellular polysaccharides and acetaldehyde, diacetyl and other flavoring substances, *S. thermophilus* is considered one of the important starter culture in dairy industry. It is often co-incultured with *Lactobacillus* (*L.*) *delbrueckii* subsp. *bulgaricus* to produce fermented milk and cheese. It is also alone or mixed with some *Lactobacillus* species to product Mozzarella and Cheddar cheese (Yu, 2014). Meanwhile, the rapid production of lactic acid during yoghurt manufacture is correlated with the quality and metabolic activity of the starter culture (Tabasco *et al*., 2014). Moreover variation in acidification activity of dif-

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ferent isolates is related to their ability to assimilate the nutrients available in the growth medium (Badis *et al*., 2004). By evaluating the acidification ability of indigenous isolates of *S. thermophilus* from traditional fermented milk we aimed to select an optimal yoghurt starter culture (Soomro and Masud, 2008).

Materials and Methods

Collection of samples

The 188 samples of dairy products for evaluation were collected from 12 regions in Mongolia in 2009. The majority of samples were from products fermented using traditional processes. Samples were sealed in small, sterile tubes with $CaCO_3$ and amylum (North Day Medical Reagent Company, China), placed on ice and rapidly returned to the laboratory for microbiological analysis.

Enumeration of viable bacterial counts of coccoidal bacteria and their isolation

Enumeration and isolation were performed following the methods of Yu *et al* (2011). 1 mL or 1 g of each product was mixed with 9 mL of 0.85% (w/v) sterile physiological saline (North Day Medical Reagent Company, China). Serially diluted aliquots (10^1-10^8) of milk samples were prepared in sterile physiological saline (0.85% NaCl). The dilutions (10^5-10^8) were plated on appropriate M17 media (Oxoid Ltd., UK) which contain 0.01% (v/v) cycloheximide and polymyxin (North Day Medical Reagent Company, China) in duplicate.

Plates were incubated under anaerobic conditions at 30°C for 48 h. Colonies with distinct morphological differences such as color, shape and size were selected by streaking at least three times on M17 agar (Oxoid Ltd., UK). Positive cocci isolates were indicated by a yellow clear zone around the colonies. These randomly selected colonies were tested for Gram stain, cell morphology, and catalase reaction before further identification. Distinctly Gram-positive, globular and catalase negative isolated colonies were purified and kept in M17 broth at 4°C, and the frozen stock in 10% (w/v) skim milk (Fonterra Ltd., New Zealand) broth were stored at -80°C. Lyophilization of isolates was performed for longer storage.

16S rRNA sequence identification

All the strains grown in 5 mL M17 culture broth at 37°C were used for DNA extraction by a revised CTAB (cetyltrimethylammonium bromide) method. DNA from the LAB isolates was extracted following the methods of Chen *et al.* (2010). Purified DNA was diluted to 100 ng/uL of final concentration for 16S rRNA gene amplification. Approximately 1500 bp of 16S rRNA gene was amplified using a set of universal primers 27F and 1495R. The primer sequences were 27F, 5'-AGAGTTTGATCCTGGCTCAG-3', and 1495R, 5'-CTACGGCTACCTTGTTACGA-3' (Wang *et al*., 2008). The 25 uL reaction mixture contained 100 ng template DNA, 2.5 uL 10× buffer (North Day Medical Reagent Company, China) with 1.5 mM $MgCl_2$, 3.0 unit Taq DNA polymerase (TaKaRa Corporation, Dalian, China), 0.2 mM of dNTP (TaKaRa) , and 10 pmol of each primer. The PCR amplifying procedure was as follows: 5 min at 94°C, 1 min at 72°C and then 10 min at 72°C; It was carried out on an automatic thermal cycler (MJ Research PTC-200). PCR products were electrophoresed in 1.0% agarose gel and visualized by UV transillumination (UVP Company, USA) after ethidium bromide staining.

Positive PCR products were sent to Shanghai Sangni Biosciences Corporation of China for sequencing. Sequences were deposited in the GenBank database. Each sequence was used as a query sequence to search for similar sequences in GenBank using the BLAST software programme (Li *et al*., 2012).

Fermentation and storage characteristics of selected *S. thermophiles* isolates

Manufacture of fermented milk

Whole milk powders (Fonterra Ltd., New Zealand) contained 39.1% lactose and 25.0% proteins (with a casein content above 34%) (North Day Medical Reagent Company, China) were used in this study. The whole milk powders were used in this study. The whole milk powders were hydrated (11.5%,w/w) and supplemented with 6.5% sucrose (w/w) (Zha *et al*., 2015). The hydrated milks were heated to 85°C for 30 min, cooled to 4°C and inoculated with the isolateds at an inoculation level of 5×10^6 CFU/g. After inoculation, the milks were incubated at 37°C. Samples were taken every two hours for the determination of the pH value, titratable acidity (TA), and viable count until the pH value reached 4.5.

Selection of *S. thermophilus* isolates for evaluation

Ten isolates of *S. thermophilus* were selected from those that were isolated in section 2.2 for their rapid acidification characteristics and short fermentation times as observed in preliminary experiments. The selected isolates were IMAU20764, IMAU20765, IMAU20438, IMAU20728, IMAU20729, IMAU20246, IMAU20738, IMAU20796,

Table 1. Phenotypic characteristics of 10 representative *S. thermophilus* strains from traditional fermented milk in Mongolia

No. of isolates	Dairy product	Isolation location	Fermentation time	Fermentation pH
IMAU20764	Fermented cow milk	Wulaanbatar	6h	4.48
IMAU20765	Fermented cow milk	Wulaanbatar	5h	4.43
IMAU20438	Fermented cow milk	Hovsgol	6h	4.48
IMAU20728	Fermented yak milk	Tov	6h	4.42
IMAU20729	Fermented yak milk	Tov	9h	4.5
IMAU20246	Fermented cow milk	Kentiy	6.5h	4.57
IMAU20738	Fermented yak milk	Tov	10h	4.49
IMAU20796	Fermented cow milk	Wulaanbatar	10.3h	4.33
IMAU20588	Fermented cow milk	Arhangay	10h	4.58
IMAU20713	Fermented yak milk	Tov	11h	4.48

h, hour.

IMAU20588 and IMAU20713. The detailed information of these ten *S. thermophilus* strains show in Table 1.

Measurement of acidification characteristics during fermentation

After inoculation, each sample was incubated at 37ºC. Every two hours samples were taken for evaluation of pH, TA and viable bacterial counts until the pH value fell to 4.5. The pH of samples was measured using a pHFE20 pH meter (Shanghai LTD, China). The TA was determined with 0.1 N NaOH using 0.5% phenolphthalein (North Day Medical Reagent Company, China) as the indicator following the methods of Ali *et al.* (2015). The viable counts of *S. thermophilus* were enumerated on M17 agar according to the methods of Tharmaraj and Shah (2003).

Measurement of acidification characteristics during storage and post-fermentation ripening

Once the pH had fallen to 4.5, that time was recorded, and the samples stored at 4ºC for post-fermentation ripening. At 0 h and every 12 h thereafter for the pH, TA and viable bacterial counts were measured as described in 2.4.2. Furthermore, at each of these time points 1 g samples were taken to determine the organic acid (lactic acid and formic acid) content (Chaves *et al*., 2002). Each 1 g sample was mixed with 3 mL, 1 M HCl in a 10 mL centrifuge tube and centrifuging at 4000 *g* for 10 min; 1 mL samples of the supernatants were filtered through 0.45 μm membranes and stored at -80°C prior to determining the concentration of lactic acid and formic acid using high performance liquid chromatography (HPLC) (Agilent Technologies Inc., USA) following the methods of Bruno *et al* (2002).

Statistical analysis

Statistical analysis of data was carried out using the software package SPSS. Differences between the means of the treatments were compared using ANOVA at the significance level of $p<0.05$.

Results and Discussion

Enumeration of viable coccoidal bacteria counts

The majority of isolates were presumed to be LAB based on characteristics such as gram-positive reactions and absence of catalase. In total, 250 presumptive LAB were isolated from 188 fermented milk samples. There are 159 coccoidal bacteria and 91 rod-shaped bacteria from M17 medium. More coccoidal bacteria were obtained from M17 media (63.6%) than rod-shaped bacteria isolated from M17 media (36.4%). Similar to our results, a prior study of LAB in yogurt demonstrated MRS as the medium of choice for differential counting of lactobacilli and M17 as the prefered medium for counting coccoidal bacteria (Coeuret *et al*., 2003). For our study we focused on coccoidal bacteria; the counts of viable cocci found in each sample, and the sampling locations are shown in Table 2. The average number of viable cocci from the milk products sampled and plated on to M17 agar varied from 8.80×10^6 to 3.47×10^8 CFU/mL; the lowest average viable counts occurred in products from Kentiy. The average number of viable counts in Ovorhangay, Wulaanbatar and Tov varied widely in the range from 1.8×10^8 to 3.47×10^8 CFU/mL. The variable ripening periods of the different products sampled, differences in transportation methods and different sampling regions may have contributed to this variability in the number of viable counts detected in this study.

16S rRNA sequence identification

From their 16S rRNA gene sequences isolates could be divided into six species: *Enterococcus* (*E.*) *faecalis*, *E.*

Table 2. Source location (province and dairy product) of samples and enumeration of viable counts of coccoid bacteria isolated from them onto M17 agar

Province	Counts of cocci (CFU/mL)	Dairy product	Species of coccoid isolates	Number of isolates	Most prevalent species (%)
Arhangay	3.23×10^7	Fermented yaks' milk	*S. thermophilus*	13	*S. thermophiles* (76%)
			Leuc. lactis	2	
			Leuc. mesenteroides. subsp. *mesenteroides*	2	
Bulgan	2.73×10^7	Fermented cows' milk	*S. thermophilus*	11	*S. thermophiles* (92%)
			Leuc. lactis	1	
Dornod	1.50×10^7	Fermented cows' milk	*S. thermophilus*	3	*S. thermophiles* (75%)
			Leuc. lactis	1	
Dzavhan	1.05×10^7	Fermented cows' milk	*S. thermophilus*	17	*S. thermophilus* (81%)
			Lac. lactis subsp. *lactis*	1	
			Leuc. lactis	1	
			Leuc. mesenteroides. subsp. *mesenteroides*	1	
			E. durans	1	
Hovsgol	1.53×10^7	Fermented cows' milk	*S. thermophiles*	22	*S. thermophilus* (92%)
			Leuc. mesenteroides subsp.*mesenteroides*	2	
Kentiy	8.80×10^6	Fermented cows' milk	*S. thermophiles*	7	*S. thermophilus* (70%)
			Leuc. lactis	1	
			E. faecalis	1	
Orhan	1.57×10^7	Fermented cows' milk	*E. durans*	1	*S. thermophilus* (86%)
			S. thermophiles	12	
			Leuc. mesenteroides. subsp. *mesenteroides*	2	
Ovorhangay	1.52×10^8	Fermented cows' milk	*Leuc. mesenteroides*. subsp. *mesenteroides*	3	*Leuc. mesenteroides* subsp. *mesenteroides* (43%)
			Leuc. lactis	2	
			E. durans	1	
			Lac. lactis subsp. *lactis*	1	
Selenge	2.31×10^7	Fermented mares' milk	*S. thermophilus*	1	*Leuc. lactis* (67%)
			Leuc. lactis	2	
Suhbaater	9.66×10^6	Fermented cows' milk	*S. thermophiles*	9	*S. thermophilus* (64%)
			Lac. lactis subsp. *lactis*	5	
Tov	1.80×10^8	Fermented cows' milk	*S. thermophilus*	15	*S. thermophilus* (100%)
Wulaanbatar	3.47×10^8	Fermented cows' milk	*S. thermophilus*	14	*S. thermophilus* (78%)
			Lac. lactis subsp. *lactis*	1	
			Leuc. mesenteroides subsp. *mesenteroides*	3	

S. thermophilus, Streptococcus thermophilus; Leuc. lactis, Leuconostoc lactis; Leuc. mesenteroides. subsp. mesenteroides, Leuconostoc mesenteroides. subsp. mesenteroides; Lac. lactis subsp. lactis, Lactococcus lactis subsp. lactis; E. durans, Enterococcus durans; E. faecalis, Enterococcus faecalis.

durans, Lactococcus (*Lac.*) *lactis* subsp. *lactis*, *Leuconostoc* (*Leuc.*) *mesenteroides* subsp. *mesenteroides*, *Leuc. lactis*, *S. thermophilus* (Table 2). The predominant species was *S. thermophilus*, which was isolated from all sampling sites and represented 29.3% of all isolates collected. Previous studies have shown similar results; *S. thermophilus* was the most frequently isolated bacteria from Columbian dairy products, representing 41.2% of all isolates collected, which was higher than in our study (Vélez *et al*., 2007).

The predominant species present varied depending on the fermented milk product from which it was isolated and the region of Mongolia. For example, 76% (13/17) of isolates from fermented yaks' milk products from Arhangay were *S. thermophilus*. 92% (11/12) of isolates from fermented cow milk products from Bulgan were *S. thermophilus.* 75% (3/4) of isolates from fermented cow milk products from Dornod were *S. thermophilus*. 81% (17/21) of isolates from fermented cow milk products from Dzavhan were *S. thermophilus*. 92% (22/24) of isolates from fermented cow milk products from Hovsgol were *S. thermophilus*. 70% (7/10) of isolates from fermented cow milk from Kentiy were *S. thermophilus*. 86% (12/14) of isolates from fermented cow milk products from Orhan were *S. thermophilus*. 43% (3/7) of isolates from fermented cow milk from Ovorhangay were *Leuc. mesenteroides*

subsp. *mesenteroides*. 67% (2/3) of isolates from fermented mare milk products from selenge were *Leuc. lactis*. 64% (9/14) of isolates from fermented cow milk products from Suhbaater were *S. thermophilus*. 100% (15/15) of isolates from fermented cow milk from Tov were *S. thermophilus*. 78% (14/18) of isolates from fermented cow milk products from Wulaanbatar were *S. thermophilus*.

The diversity of species present appears to vary by region and the fermented milk product. This diversity is not surprising because many factors may influence the presence or absence of particular species. These include, different production methods, recipes and raw materials. Furthermore, the variability in environmental conditions at each location could also contribute to this variation, as has been observed in other studies (Yu *et al*., 2012).

Changes in pH, TA and bacterial viability during fermentation of milk by ten *S. thermophilus* isolates

As expected, an increase in TA and a decrease in pH of milk, were observed in milk during fermentation by all isolates of *S. thermophilus* (Fig. 1). It took less than 10 h for milk to reach a pH of 4.5 when fermented by all *S. thermophilus* isolates, due to their rapid acidification capacity; amongst the *S. thermophilus* isolates, IMAU20765 acheived the fastest pH reduction (within 4 h) and IMAU 20713 was the slowest (10 h). Two isolates (IMAU20765 and IMAU20246) would be good candidates for use as yoghurt starter cultures because they reduce the pH to approximately 4.5 within 6h, which is considered an optimal time from previous studies (Erkus *et al*., 2013). By the end of fermentation, three isolates (IMAU20246, IMAU20713 and IMAU20765) had the highest TA values of 59.03, 57.72 and 57.16°T, respectively. The remaining seven isolates (IMAU20764, IMAU20438, IMAU 20728, IMAU20729, IMAU20738, IMAU20796 and IMAU20588) had a relatively lower acidification capacity. All ten isolates achieved TA values in excess of 50.00 °T within 10 h of initiating fermentation. This would

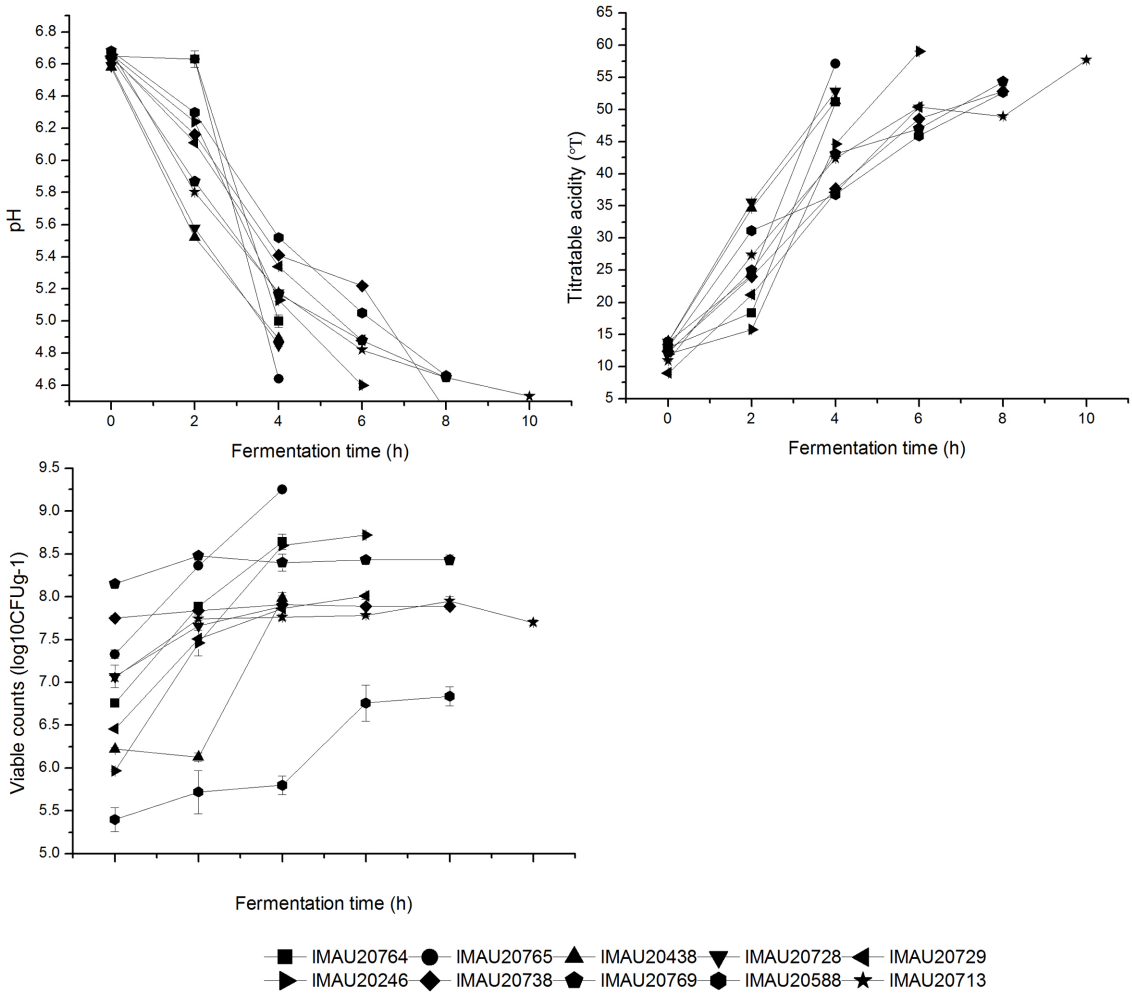

Fig. 1. Effect of fermentation time on pH, TA and viable counts in milk inoculated with ten *S. thermophiles* isolates.

allow them all to be considered as 'fast acid-producing isolates' for yoghurt production according to the catagories described by Raquib *et al.* (2003). Soomro and Masud (2008) found that the majority of isolates of *S. thermophilus* derived from dahi yoghurt were also fast acidifiers. In contrast, all *S. thermophilus* isolates from fermented milk laban had low acid-producing capabilities (Chammas *et al*., 2006), suggesting that *S. thermophilus* from different dairy products vary in their acidification capabilities.

Rapid growth of *S. thermophilus* in milk is a reflection of their high proteolytic activity (Letort and Juillard, 2001). Previously, cheese-derived *S. thermophilus* were thought to have the highest proteolytic activity (Donkor *et al*., 2006). However, in our study the final bacterial counts achieved in yoghurt production were greater than 6.84 $\log_{10}$ CFUg^{-1}, which is also high; IMAU20765 achieved the highest count of viable bacteria (9.25 $\log_{10}$ CFUg^{-1}) (Fig. 1). Our results were similar to those reported by Güler-Akn and Akn (2007), confirming *S. thermophilus* as the predominant and most rapidly acidifying species among LAB.

Changes in pH, TA and bacterial viability during storage of fermented milk produced by ten *S. thermophilus* isolates

During storage, the yoghurts produced by the ten isolates could be separated into two groups with respect to their changing pH and TA (Fig. 2). Yoghurts produced by Group I isolates (IMAU20729, IMAU20738, IMAU20796, IMAU20713, IMAU20246, IMAU20765 and IMAU20764) continued to decrease in pH for 36-48 h in storage but with only minor changes in overall TA. In contrast, in yoghurts produced by Group 2 isolates (IMAU20728, IMAU20438 and IMAU20588) the pH decreased rapidly with large increases in TA. Purwandari and Vasiljevic (2009) reported storage time and variability amongst isolates significantly affected TA but that the storage temperature did not. They also observed that three *S. thermophilus* isolates had distinct acid-producing phases during storage. Post acidification is one of main causes of reductions in yoghurt sensory quality during storage (Han *et al*., 2014). Consequently, *S. thermophilus* isolates in Group I with greater TA stability and weak post-acidification during storage are

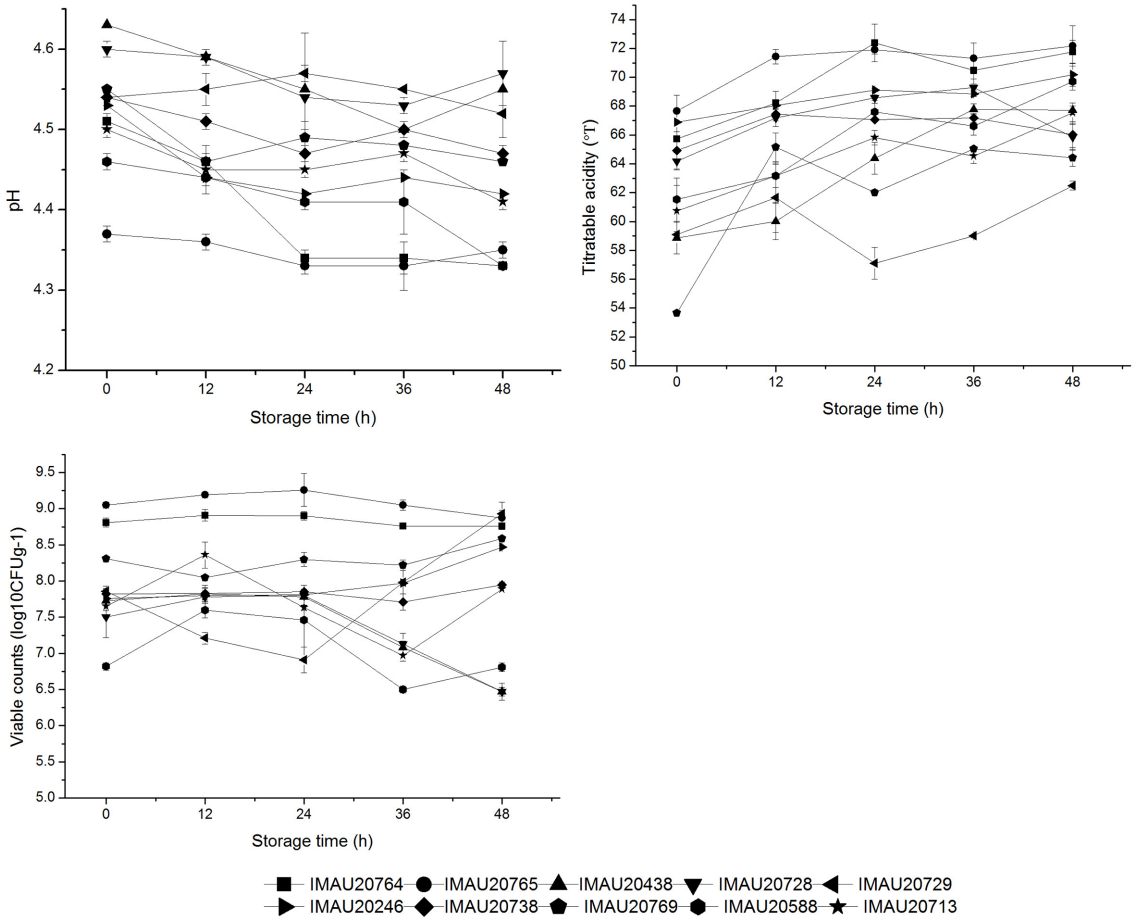

Fig. 2. Effect of storage time on pH, TA and viable counts in yoghurt made using ten *S. thermophilus* isolates.

potentially more suitable for yoghurt production than the Group 2 isolates. There were no dramatic differences in the viable counts of *S. thermophilus* during storage (Fig. 2). Generally, slight decreases in the number of viable bacteria were observed as storage time increased.

Changes in lactic acid and formic acid concentration during storage of fermented milk produced by ten *S. thermophiles*

Generally, changes in lactic acid and formic acid concentration, was highly dependent on isolate (Fig. 3). Lactic acid is the main flavouring agent in yoghurt, contributing to its sour, refreshing taste. During storage, the concentration of lactic acid can change by varying degrees. In our study, the acidification ability of *S. thermophilus* was closely related to the final concentration of lactic acid. Formic acid is also an important metabolite of lactic acid fermentation and its production was isolate-dependent. Many researchers reported that *S. thermophilus* and *L. bulgaricus* had a good symbiotic role. With the growth of *S. thermophius*, the urease in *S. thermophilus* decomposes urea to produce CO_2, and combining with the formic acid produced during growth to provide the conditions for the growth of *L. bulgaricus*. (Yu *et al.,* 2014) During storage, yoghurt produced by isolate IMAU20246 had the highest concentration of both lactic acid and formic acid.

Overall there were some significant correlations between the acid-related compounds and other parameters (Table 3). In yoghurts produced by *S. thermophilus* isolates IMAU20764, IMAU20729 and IMAU20738 lactic acid concentration was significantly positively correlated with viable counts (r=0.976, p=0.019), (r=0.937, p=0.019), (r=0.952, p=0.037), respectively. It seems that the increase in viable counts also leads to greater production of lactic acid. For isolate IMAU20764, TA was significantly positively correlated with lactic acid concentration (r=0.983, p=0.003). These relationships suggest that lactic acid is the main constituent of the overall acid content. For *S. thermophilus* isolate IMAU20246, there was a significant positive correlation between pH and formic acid concentration.

Table 3. Correlation analysis between different fermentation characteristics (n=5)

Strains	Correlation	*p* value	r value
IMAU20246	P vs F	0.014	0.948
IMAU20764	TA vs L	0.003	0.983
	V vs L	0.019	0.976
IMAU20729	V vs L	0.019	0.937
IMAU20738	V vs L	0.037	0.952

TA, titratable acidity; P, pH; V, viable counts; F, formic acid; L, lactic acid.

Conclusions

Here we describe the diversity of coccoidal bacterial species from traditionally fermented dairy products in Mongolia. Six species of coccoidal LAB were recorded, of which the predominant species was *S. thermophilus*. The acidification properties of ten selected *S. thermophilus* isolates were analyzed during fermention of milk and subsequent storage of the yoghurt produced. The production of organic acids varied considerably amongst the iso-

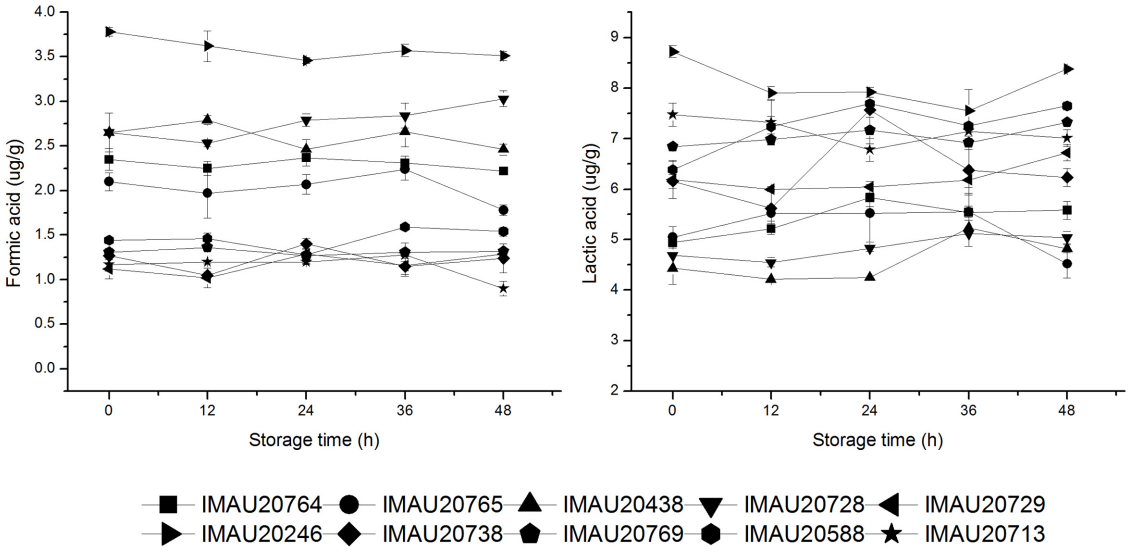

Fig. 3. Effect of storage time on the content of lactic acid and formic acid in yoghurt produced using ten *S. thermophilus* isolates.

lates and there were significant differences in the pH, TA and viable counts of bacteria in the yoghurt produced by the ten isolates evaluated. Lactic acid was the major acid-produced component and formic acid was less predominant. These results provide the baseline data for selecting a good starter culture for traditional fermented dairy products. Some of the naturally occurring isolates we found have great potential as starter cultures for industrial production of traditional dairy products.

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