Identification of lactic acid bacteria in Taiwanese ropy fermented milk and evaluation of their microbial ecology in bovine and caprine milk

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ABSTRACT

The purpose of this study was to identify species of lactic acid bacteria in Taiwanese ropy fermented milk and to study their microbial dynamics during the fermentation process through conventional microbiological cultivation and PCR-denaturing gradient gel electrophoresis. Identification results indicated that *Lactococcus lactis* ssp. *cremoris* and *Leuconostoc mesenteroides* ssp. *mesenteroides* were the major lactic acid bacteria in Taiwanese ropy fermented milk. Interestingly, 3 groups were identified as *Lc. lactis* ssp. *cremoris* using 16S rDNA sequencing, but they showed different denaturing gradient gel electrophoresis patterns and assimilation of carbohydrates. In addition, the microbial dynamics study in different fermentation stages demonstrated that *Lc. lactis* ssp. *cremoris* was the most dominant bacterial species in the samples, followed by *Leu. mesenteroides* ssp. *mesenteroides* with no differences among the fermentation stages. Finally, the microbial distribution profiles showed that the microbial ecology was different in bovine, caprine, and reconstituted milk, which might further affect the characteristics of the product.

Key words: lactic acid bacteria, Taiwanese ropy fermented milk, microbial dynamics

INTRODUCTION

Taiwanese ropy fermented milk (TRFM) is a type of domestic fermented milk. Its real origin is unknown, but it has spread from family to family in northern Taiwan; it has a stringy texture, a good diacetyl flavor, and a pleasant taste. The texture and flavor of TRFM is similar to that of traditional Finnish fermented milk viili, and, therefore, we also call this product Taiwanese viili.

The most traditional viili culture is made with the microbial action of mesophilic lactic acid bacteria (LAB) and a surface-growing yeast-like fungus (Tsutsui et al., 1998; Leporanta, 2003; Boutrou and Guéguen, 2005). Various LAB present in viili products have been isolated and identified by physiological and biochemical tests (Kahala et al., 2008), including *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, and *Leuconostoc mesenteroides* ssp. *cremoris*. This ropy fermented milk also contains yeasts. In our previous study (Wang et al., 2008), 3 yeast strains, *Kluyveromyces marxianus*, *Pichia fermentans*, and *Saccharomyces unisporus* were isolated from TRFM and identified using a combination of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rDNA sequencing. Yeasts present in viili could provide the flavor and improve the texture of the viili products (Leporanta, 2003).

A stable and constant starter culture, which is necessary for manufacturing a quality fermented beverage, is difficult to sustain due to the complex microbiological composition of TRFM. In addition, the composition of mixed-strain starter cultures is of utmost importance in the production of uniform and high-quality products. Thus, detecting and identifying the microbial composition of TRFM products with a rapid method is important for quality control. The complete description of TRFM microflora gives a clue to the various bioactive materials produced and in particular those involved in the formation of exopolysaccharides (EPS).

In current microbial determination procedures, many microorganisms present in mixed-strain starters have been detected and identified by selective growth medium and morphological and biochemical characteristics (Naersong et al., 1996; Tsutsui et al., 1998; Lin et al., 1999; Gadaga et al., 2000; Abdelgadir et al., 2001; Gonfa et al., 2001; Mathara et al., 2004; Shuangquan et al., 2004; Lore et al., 2005; Witthuhn et al., 2005; Zhang et al., 2008; Wu et al., 2009). However, phenotypic features are inappropriate to address the taxonomic nomenclature on the basis of polyphasic classification principles. Therefore, it is very important to search for

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reliable and precise methods to obtain the microbial profiles of mixed-strain starters. Molecular techniques have been rapidly developed, including PCR-DGGE, which is based on amplification of ribosomal RNA and electrophoresis of the PCR product in a polyacrylamide gel containing an increasing gradient of denaturants. Analysis using PCR-DGGE has been widely applied in the study of complex microbial communities from food samples or other environments (Zoetendal et al., 1998; Cocolin et al., 2001; van Beek and Priest, 2002; Ercolini et al., 2003; Fasoli et al., 2003; Cocolin et al., 2004; Temmerman et al., 2004; Lee et al., 2005). Our previous studies also illustrated that PCR-DGGE can be used to identify the LAB in different original kefir grains (Chen et al., 2008), to study the microbial dynamics during different ripening stages in traditional Taiwanese fermented hams (Tu et al., 2010), and to investigate yeast distribution in viili and kefir grains (Wang et al., 2008).

Because safety and quality control are important for commercialization of the ropy fermented milk products, investigation of their microbiological profiles is crucial. None of the LAB in TRFM has been determined. Moreover, although bovine and caprine milk are widely used for the manufacture of many different types of fermented milk products, little information is available on the effects of different types of milk on the presence of microorganisms in ropy fermented milk products. Thus, the purpose of this study was to identify the species of LAB in the starters of TRFM and to study their microbial dynamics during the fermentation process, using conventional microbiological cultivation and PCR-DGGE. Concurrently, the effects of different types of milk on the microbial ecological profiles were also investigated in this study.

MATERIALS AND METHODS

Starters

The starters of TRFM were collected in Taipei in northern Taiwan (Lin et al., 1999). In the laboratory, the TRFM starters (5%, wt/vol) were transferred into fresh milk, and incubated at 20°C for 20 h. After this procedure was repeated 3 times, the starters were considered active and used in this study. To study the effect of incubation time on microbial dynamics, the TRFM starters (5%, wt/vol) were sampled every 4 h after being transferred into fresh milk and incubated at 20°C for 20 h.

TRFM Manufacturing

Both fresh pasteurized bovine and caprine milk were obtained from the National Taiwan University Dairy

farm (Taipei, Taiwan). The reconstituted milk was prepared using 12% (wt/wt) whole milk powder (Anchor Foods, Manurewa, New Zealand). For manufacturing TRFM, the active TRFM starters (5%, wt/vol) were inoculated into different types of milk and incubated at 20°C for 20 h. After fermentation, each TRFM sample was analyzed for its microbial, chemical, and physical properties.

Reference Strains

The reference strains used for this study, including *Lc. lactis* ssp. *cremoris* (BCRC 12586), *Lc. lactis* ssp. *lactis* (BCRC 12312), *Leu. mensenteroides* ssp. *cremoris* (BCRC 14047), *Leu. mensenteroides* ssp. *dextranicum* (BCRC 12260), and *Leu. mensenteroides* ssp. *mensenteroides* (BCRC 12580), were obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute in Hsinchu, Taiwan.

Isolation and Enumeration of Microorganisms in the TRFM Starters and TRFM

Ten grams of each TRFM starter was homogenized in 90 mL of sterile saline solution (0.85% sodium chloride solution, pH 5.5) in a laboratory blender (Stomacher 400, Seward, West Sussex, UK) for 15 min. Concentrations of the viable LAB and yeasts in suspensions were obtained by serial plating dilutions. Yeasts were examined on potato dextrose agar (PDA, Difco, Detroit, MI), with 100 mg/L of chlortetracycline (Sigma, St. Louis, MO) to inhibit the growth of bacteria. The plates were incubated at 25°C for 3 d (Lin et al., 1999) and enumerated. For LAB, 2 different media and fermentation conditions were applied to search the best conditions for the LAB isolation in the TRFM starters. The media used for the microbial enumeration and isolation were de Man, Rogosa and Sharpe (MRS) agar (Acumedia, Lansing, MI) under both aerobic and anaerobic conditions, and LM17 agar [M17 agar (Difco) with 0.5% (wt/vol) of lactose (Sigma)] in an aerobic incubator. In addition, 200 mg/L of cycloheximide (Sigma) was added to both MRS and LM17 agars to inhibit the growth of yeasts. The plates were incubated at 30°C for 7 d and the resulting colonies were counted. After counting, single colony purification was carried out.

For TRFM, 10 mL of each TRFM sample was homogenized in 90 mL of sterile saline solution (0.85% sodium chloride solution, pH 5.5) in a laboratory blender (Stomacher 400, Seward) for 15 min. Concentrations of the viable LAB and yeasts in suspensions were carried out similarly to the method performed in the starters.

The only difference was the LAB medium. The medium used for the microbial enumeration and isolation was MRS agar under aerobic conditions. The procedures of isolation and enumeration were repeated 4 times.

The Harrison Disc Method

The Harrison disc method, adopted from Harrigan (1998), was used to select representative colonies from each plate in a random statistical manner for further purification and identification in this study. This method was also conducted to study the microbial distribution of various microorganisms present in TRFM and the TRFM starters.

The distribution was conducted by 10% of total colonies selected using the Harrison disc method from each plate with 30 to 300 colonies, and transferred to MRS broth for further identification by PCR-DGGE (Witthuhn et al., 2005; Chen et al., 2008; Wang et al., 2008). In order to increase the number of isolates and reveal the real quantitative results, the experiments were repeated independently for 4 times. The microbial distribution was based on the ratio of identified numbers of specific strain to total isolates.

DNA Extraction from Lactic Acid Bacteria and Yeast

This part of the study followed the procedure described by Wang et al. (2008) and Chen et al. (2008). The purified LAB and yeast cultures isolated by the Harrison disc method were inoculated in MRS and potato dextrose broths, respectively. One milliliter of each late exponential phase culture was collected by centrifugation at $7,500 \times g$ (10 min, 4°C). The microbial pellets were subjected to DNA extraction using a blood and tissue genomic DNA extraction system (Viogene-Biotek Corp., Taipei, Taiwan). In culture-independent studies, 0.5 g of homogenized TRFM starter was also directly handled as described above. Extraction of DNA was based on the manufacturer's instructions including protein digestion and isolation of genomic DNA. The pellets were first treated with proteinase K and then the genomic DNA was isolated by silica gel membrane technology and centrifugation. Eventually, the isolated genomic DNA was resuspended in sterilized double-distilled H₂O and stored at -20°C until further analyses.

Carbohydrate Fermentation and Assimilation Test

Lactic acid bacteria isolated from the TRFM starters were examined for fermentation of different carbohydrate using the API 50 CH strip with API 50CHL medium (BioMérieux, Marcy-l'Étoile, France). This

system performs 49 assimilation tests for carbohydrates and includes a database with 52 different species. All LAB identification procedures were conducted in accordance with the manufacturer's instructions. The reactions were examined visually and determined to be positive or negative based on the color change in the carbohydrate wells after 48 h at 30°C. Two replicate samples were tested for each isolated strain.

DNA Amplification

The DNA amplification for LAB and yeast was adopted from Chen et al. (2008) and Wang et al. (2008), respectively. Three primers were used in this study. For the LAB, 2 bacterial-specific primer sets targeting 2 different regions of 16S rDNA gene primers were selected. The V3 region of the 16S rDNA gene was amplified by PCR using a forward primer GC338f (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGA GGG G ACT CCT ACG GGA GGC AGC AG-3'; the GC clamp is underlined) and a reverse primer 518r (5'-ATT ACC GCG GCT GCT GG-3'; Cocolin, et al., 2001). The second primer set, GCP1V1 (5'-GCC AGC GGC CCG GCG CGG GCC CGG CGG CGG GGG CCG CGG C GCG GCG TGC CTA ATA CAT GC-3') and P2V1 (5'-TTC CCC ACG CGT TAC TCA CC-3'; Cocolin, et al., 2004), spanned the V1 region of the 16S rDNA. For yeast, the D1 region of the 26S rRNA gene was amplified by PCR using the primers NL1GC (5'-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG GGG AGG AAA AG-3') and a reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3'; Cocolin et al., 2000, 2002).

PCR-DGGE

The PCR fragments were separated by DGGE using the DCode universal mutation detection system (Bio-Rad, Hercules, CA) with gels of 16 × 16 × 0.01 cm. The procedures were modified from Chen et al. (2008) and Wang et al. (2008). Separation of the PCR amplicons was obtained by direct application of 20 µL of PCR products onto the denaturant gradient polyacrylamide gel, which ranged from 35 to 55% for lactic acid bacteria and from 30 to 55% for yeasts (100% corresponds to 7 M urea, J. T. Baker, Philipsburg, NJ; and 40% wt/vol of formamide, J. T. Baker). The DGGE reference markers were amplicons obtained from 4 lactic acid bacteria species and 3 yeast strains (Wang et al., 2008) in equal amounts.

DNA Sequencing

To verify the PCR-DGGE results and ensure that all isolates within each group belonged to just one species,

randomly selected isolates from each group were further identified by 16S rDNA gene sequencing. The full length of the 16S rDNA was amplified by forward primer 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1512r (5'-AAG GAG GTG ATC CAG CCG CA-3'; Coenye et al., 1999). The PCR amplicons were purified with the Concert Rapid PCR Purification system (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA) and sent for sequencing. Sequencing was carried out at the Genomics Co. (Taipei, Taiwan) using an ABI 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA). The DNA sequence identities were determined using the National Center for Biotechnology Information BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

In order to compare the similarity of V1 variable region sequences of 16S rRNA genes, DGGE bands were excised from the acrylamide gels. The DNA fragments were purified using a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA) and then reamplified by the primer P1V1 (without a GC clamp) and P2V1. The PCR products were submitted to sequencing after purification using PCR-M clean up system (Viogene). The DNA sequence similarities were determined by the BLAST program.

Determination of Exopolysaccharide

The EPS isolation was carried out using the modified method according to the procedure described by Rimada and Abraham (2003). The TRFM samples were heated in a boiling water bath for 30 min. The precipitated bacteria and other milk compounds were removed by centrifugation at $10,000 \times g$ at 20°C for 30 min. The supernatant was then directly dialyzed (molecular weight cut-off for the dialysis membranes: 3,500; Membrane Filtration Products Inc., Seguin, TX) at 4°C for 48 h against 4 changes of double-distilled water. The EPS contents were determined by the modified phenol-sulfuric acid method (Dubois et al., 1956), measuring absorbance at 485 nm. A mixture of L-rhamnose, D-galactose, and D-glucose was used as standard.

Water-Holding Capacity

The water-holding capacity (WHC) was determined using the centrifuge method (Kalab et al. 1976). A 30-g TRFM sample was centrifuged at $13,500 \times g$ at 10°C for 30 min. After centrifugation, the supernatant was discarded. The weight of the pellet was determined and WHC was calculated as

$$\text{WHC (\%)} = \frac{\text{Precipitated curd weight}}{\text{initial sample weight}} \times 100.$$

Apparent Viscosity

The apparent viscosities of TRFM were determined with a digital Brookfield Viscometer Model DV-II (Brookfield, MA). All samples were kept at 4°C during determination. The measurement was performed with a No. 2 spindle at a steady speed of 0.3 rpm. Viscosity values were expressed in Hertz.

Statistical Analysis

Data were analyzed using ANOVA. Statistical significance was judged at the level of $P < 0.05$ and differences between treatments were tested using Duncan's multiple range test. All statistical analyses of data were performed based on 4 replications using Statistical Analysis Systems software (SAS Institute Inc., 2001).

RESULTS AND DISCUSSION

Identification of LAB by the Culture-Dependent Method in the TRFM Starters

Classification by PCR-DGGE. Because many plating procedures are only partially selective and exclude parts of the microbial community, 3 different enrichment treatments (MRS agar with aerobic cultivation, MRS agar with anaerobic cultivation, and LM17 agar with aerobic cultivation) were tested to count and isolate LAB colonies to determine all LAB in starters of TRFM. Two different PCR-DGGE primers, 338fGC/518r and P1V1GC/P2V1, were also evaluated. Results indicated that 84, 74, and 68 colonies were isolated by the Harrison disc method from MRS agar with aerobic cultivation, MRS agar with anaerobic cultivation, and LM17 agar with aerobic cultivation, respectively. A comparison of 2 different PCR-DGGE primers showed that 3 groups (TL1, TL2, and TL3) were identified by P1V1GC/P2V1 primers (Figure 1D, 1E and 1F), but only 2 (TL1 and TL2) were recognized using 338fGC/518r as primers (Figure 1A, 1B, and 1C). When further evaluating the effects of different cultivation treatments on the profiles of LAB strains, TL1 grew on all 3 media using 338fGC/518r primers (Figure 1A, 1B, and 1C), whereas TL2 could only be isolated from MRS agar (Figure 1A and 1B). Similarly, 2 groups (TL1 and TL3) were found on all 3 media (Figure 1D, 1E, and 1F), whereas TL2 could only be isolated from MRS agar using P1V1GC/P2V1 primers. Additionally, the distributions of TL1, TL2, and TL3 in different enrichment treatments using P1V1GC/P2V1 primers were also studied. For the MRS agar with aerobic cultivation, the proportions of TL1, TL2, and TL3 to total colonies were 64/84, 15/84, and 5/84,

respectively. Similarly, the prevalent group present in the MRS agar with anaerobic cultivation was TL1 (56/74), followed by TL3 (13/74) and TL2 (5/74). For the LM17 agar, only TL1 and TL3 groups were found with ratios of 55/68 and 13/68, respectively. The above findings revealed that TL1, occupying more than 75% of total LAB isolates, was the most dominant group in the TRFM starters. No differences existed among the 3 cultivation treatments. Furthermore, the MRS medium was the most suitable for the isolation and classification of LAB. Thus, MRS agar under aerobic cultivation was selected as the isolation medium for the subsequent culture-dependent studies.

Identification Using Morphological and Physiological Characteristics and API 50 CHL. The results in Table 1 indicate that all 3 groups (TL1, TL2, and TL3) isolated from the TRFM starters were cocci, gram-positive, and catalase-negative. They could grow at 15°C but not at 40°C. Only TL2 could produce gas and survive in the presence of 4% of NaCl. Axelsson (1998) indicated that homofermentative, gram-positive, and catalase-negative cocci, capable of growing at 10 and 40°C, but not in the presence of 4.5% of NaCl, were considered lactococci, whereas, the leuconostoc were characterized as heterofermentative, gram-positive, catalase-negative cocci, which were capable of growing in the presence of 4.5% of NaCl (Garvie, 1984). Accordingly, TL1 and TL3 were lactococci, and TL2 was leuconostoc. It is worth noting that TL1 exhibited 2 different phenotypes. One could produce EPS but the other did not. Thus, TL1 was further divided into 2 different groups (TL1 and TL4) based on their ability to make EPS.

With API 50 CHL, all 4 groups fermented D-galactose, D-glucose, N-acetylglucosamine, esculin, and lactose. Only TL2 could metabolize D-xylose, D-maltose, and sucrose. Except for utilization of salicin, TL1, TL3, and TL4 all showed a very similar assimilation of carbohydrates, and all were identified as *Lc. lactis* ssp. *cremoris* using the API system with 99.9, 99.7, and 99.7% identities, respectively (Table 1). Among the 3 *Lc. lactis* ssp. *cremoris*, only TL1 could metabolize salicin (C₁₃H₁₈O₇) which is an alcoholic β-glycoside that contains D-glucose. Normally, *Lactococcus lactis* could use salicin as a carbon source for fermentation. However, the *bglR* mutant strain (*Lactococcus lactis* IL1403) is unable to grow on salicin (Kowalczyk et al., 2008). Consequently, TL3 and TL4 might be genetic mutants in utilization of salicin. In addition, the genetic loci for EPS expression have been reported to be associated with plasmids (Knoshaug et al., 2007). The presence of the EPS genes on plasmids has been implicated as the cause of EPS expression instability with higher temperatures and frequent transfers (Vedamuthu and

Neville, 1986; Cerning et al., 1992). This could explain the difference between TL1 and TL4 in the production of EPS.

The API system failed to recognize TL2. Our results are also in agreement with the study of Kulwichit et al. (2007) in which the API 50 CHL also failed to reliably provide accurate identification of *Leuconostoc*. Although this method is effective for identifying relatively common LAB, its application is more limited for accurate identification of less frequently recovered taxa (Ramani et al., 1998).

Identification Using PCR-DGGE and DNA Sequencing Methods. The above 4 groups with 5 reference strains that have been recognized in traditional viili starters were further analyzed by PCR-DGGE using a 30 to 55% DGGE gel (Figure 2). For reference strains, *Leu. mesenteroides* ssp. *mesenteroides* (Figure 2, lane 5) gave a specific electrophoretic pattern that could easily be used for identification purposes using 338fGC/518r as the PCR-DGGE primers. However, *Lc. lactis* ssp. *cremoris* (Figure 2, lane 1) and *Lc. lactis* ssp. *lactis* (Figure 2, lane 2) had similar patterns in the gel that could not be identified. Similar results occurred with *Leu. mesenteroides* ssp. *cremoris* (lane 3) and *Leu. mesenteroides* ssp. *dextranicum* (lane 4). For P1V1GC/P2V1, *Lc. lactis* ssp. *cremoris* (lane 10) provided a unique electrophoretic pattern that could easily be used for identification purposes. Conversely, *Lc. lactis* ssp. *lactis* (lane 11) and *Leu. mesenteroides* ssp. *cremoris* (lane 12), as well as *Leu. mesenteroides* ssp. *dextranicum* (lane 13) and *Leu. mesenteroides* ssp. *mesenteroides* (lane 14) showed analogous patterns in the gel that could not be recognized. Chen et al. (2008) reported that some LAB reference strains were undifferentiated by migration of their V3 regions of 16S rDNA in DGGE analysis. In those cases, the species or subspecies distinction might be achieved by analyzing other variable genetic domains or integrating with additional methods of differentiation. It was worth noting that 2 *Lc. lactis* subspecies, *lactis* and *cremoris*, could be identified using the V1 region from 16S rDNA rather than the V3 region in this study. The separation of 2 *Lc. lactis* subspecies has often been based on differences in the 16S rRNA gene sequence (Ward et al., 1998; Ercolini et al., 2001). Ward et al. (1998) revealed that the 16S rRNA of *Lc. lactis* ssp. *lactis* and ssp. *cremoris* differ by 9 to 10 bp within the first 200 bp of the sequence. Targeting of P1V1GC/P2V1 in the V1 region could differentiate these 2 subspecies (Figure 2). Figure 2 also indicate that a combination of P1V1GC/P2V1 and 338fGC/518r primers was necessary to identify the 5 reference strains.

The results of LAB identification by PCR-DGGE are also shown in Figure 2. Compared with the band posi-

Figure 1. Denaturing gradient gel electrophoresis (DGGE) profiles of the amplified V3 (A, B, and C) and V1 (D, E, and F) regions of 16S rDNA obtained from lactic acid bacteria isolates from the Taiwanese ropy fermented milk (TRFM) starters under different culture conditions: (A, D) de Man, Rogosa, and Sharpe (MRS) agar, aerobic cultivation; (B, E) MRS agar, anaerobic cultivation; and (C, F) LM17 agar, aerobic cultivation.

tions of reference strains, the TRFM starters contained *Lc. lactis* ssp. *cremoris* (TL1 and TL4, lanes 6, 9, 15, 18) and *Leu. mesenteroides* ssp. *mesenteroides* (TL2, lanes 7, 16). Strain T3 (lanes 8 and 17) remained unidentified due to lack of pattern similar to that of the reference strains. Two additional DGGE bands (TL3a and TL3c) were found in the T3 group. Further comparison of the V1 regions retrieved from the DGGE gel (Table 2) showed that the DNA sequences of TL1-V1, TL3b-V1, and TL4-V1 were identical. They were recognized as *Lc. lactis* ssp. *cremoris*. On the other hand, TL3a-V1 and TL3c-V1 demonstrated only 96 and 86% similar-

ties with TL1-V1, TL3b-V1, and TL4-V1, respectively. Surprisingly, the TL3c-V1 sequence was identified as *Lc. lactis* ssp. *lactis* BCRC12267(data not shown). Inconsistency between phenotypic and genotypic methods in classifying the subspecies of *Lactococcus lactis* has been shown in several studies. Kahala et al. (2008) indicated that the strains classified as *Lc. lactis* ssp. *cremoris*, based on 16S rRNA gene sequences, exhibit 2 phenotypes. One is a typical subspecies, the *cremoris* phenotype and another is a subspecies *lactis*-like phenotype. Comparison of the complete genome sequences of *Lc. lactis* ssp. *cremoris* and *Lc. lactis* ssp. *lactis* revealed

Table 1. Identification of lactic acid bacteria species isolated from the Taiwanese ropy fermented milk (TRFM) starters by biochemical methods and sequence information from the 16S rDNA PCR product

Item	Strain isolated from the TRFM starters			
	TL1	TL2	TL3	TL4
Cell shape	Cocci	Cocci	Cocci	Cocci
Gram staining	+	+	+	+
Catalase reaction	—	—	—	—
Slime	+	—	—	—
Gas	—	+	—	—
Growth at				
15°C	+	+	+	+
40°C	—	—	—	—
4% of NaCl	—	+	—	—
D-Xylose	—	+	—	—
L-Xylose	—	—	—	—
D-Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	—	+	+
D-Mannose	+	—	+	+
N-Acetylglucosamine	+	+	+	+
Amygdalin	—	—	—	—
Arbutin	—	—	—	—
Esculin	+	+	+	+
Salicin	+	—	—	—
D-Celibiose	—	—	—	—
D-Maltose	—	+	—	—
D-Lactose	+	+	+	+
D-Melibiose	—	—	—	—
Sucrose	—	+	—	—
Identification: API kit				
Closest relative	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	<i>Leuconostoc lactis</i>	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>
Identity (%)	99.9	40.7	99.7	99.7
Identification: sequencing				
Closest relative	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	<i>Leuconostoc</i> <i>mesenteroides</i> ssp. <i>mesenteroides</i>	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>
Accession number	CP000425	CP000414	CP000425	CP000425
Identity ¹ (%)	100	99	100	100

¹Identical nucleotide percentage in the sequence obtained from the agarose band and in the sequence found in the National Center for Biotechnology Information (NCBI) database.

evidence of horizontal gene transfer and recombination between related genomes, leading to the uncharacteristic genotypic and phenotypic traits.

To verify the PCR-DGGE results, the full lengths of 16S rDNA of the 4 groups were sequenced for further confirmation. After alignment was carried out in BLAST, sequence results showed 99 to 100% identity with the sequences that were retrieved from the GenBank accession numbers (Table 1). Strains TL1, TL3, and TL4 were identified as *Lc. lactis* ssp. *cremoris* with 100% identification. Strain TL2 was *Leu. mesenteroides* ssp. *mesenteroides*. The results of full-length 16S rDNA were identical with those of PCR-DGGE.

Identification results indicated that ropy fermented milk from Taiwan contained 2 LAB, *Lc. lactis* ssp. *cremoris* and *Leu. mesenteroides* ssp. *mesenteroides*. Interestingly, comparison with other studies showed the ropy fermented milk products all contain similar LAB

strains (Tsutsui et al., 1998; Leporanta, 2003; Boutrou and Guéguen, 2005; Kahala et al., 2008; Uchida et al., 2009). It might be concluded that the list of LAB in ropy fermented milk products is not very lengthy, even when these products are obtained from different parts of the world.

Identification of LAB in the TRFM Starters by the Culture-Independent Method

The total DNA of bacterial strains in the TRFM starters were extracted and directly identified by PCR-DGGE to eliminate the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment. In culture-independent studies, 3 independent TRFM starter samples with 2 primer sets were used. Results (Figure 3) indicated that the varieties of the LAB strains identified by PCR-DGGE were

Table 2. The identities of V1 variable region sequences of 16S rRNA genes obtained from the PCR-denaturing gradient gel electrophoresis gel

Item	Variable region sequence identity						
	TL1-V1 ¹	TL2-V1 ²	TL3a-V1 ³	TL3b-V1 ⁴	TL3c-V1 ⁵	TL4-V1 ⁶	CP000425 ⁷
TL2-V1	0.52						
TL3a-V1	0.96	0.49					
TL3b-V1	1.00	0.56	0.96				
TL3c-V1	0.86	0.62	0.90	0.86			
TL4-V1	1.00	0.56	0.96	1.00	0.86		
CP000425	1.00	0.52	0.96	1.00	0.86	1.00	
CP000414 ⁸	0.52	1.00	0.49	0.56	0.62	0.56	0.52

¹TL1-V1: AAGTTGAGCGATGAAGATTGGTGCTTGCACCAATTTGAAGAGCAGCGAACG.
²TL2-V1: AAGTCGAACGCACAGCGAAAGGTGCTTGCACCTTTCAAGTGAGTGGCGAACG.
³TL3a-V1: AAGTTGAGCGATGAAGATTGGTGCTTGCACCAACTTGATGAGCAGCGAACG.
⁴TL3b-V1: AAGTTGAGCGATGAAGATTGGTGCTTGCACCAATTTGAAGAGCAGCGAACG.
⁵TL3c-V1: AAGTTGAGCGCTGAAGGTTGGTACTTGTACCAACTGGATGAGCAGCGAACG.
⁶TL4-V1: AAGTTGAGCGATGAAGATTGGTGCTTGCACCAATTTGAAGAGCAGCGAACG.
⁷CP000425: AAGTTGAGCGATGAAGATTGGTGCTTGCACCAATTTGAAGAGCAGCGAACG.
⁸CP000414: AAGTCGAACGCACAGCGAAAGGTGCTTGCACCTTTCAAGTGAGTGGCGAACG.

Figure 2. Denaturing gradient gel electrophoresis (DGGE) profiles obtained from 5 reference lactic acid bacteria (LAB) strains and 4 isolates from the Taiwanese ropy fermented milk (TRFM) starters after amplification by 338fGC/518r (lanes 1–9) and P1V1GC/P2V1 (lane 10–18) primers. Lanes 1 and 10, *Lactococcus lactis* ssp. *cremoris* BCRC 12586; lanes 2 and 11, *Lactococcus lactis* ssp. *lactis* BCRC 12312; lanes 3 and 12, *Leuconostoc mensenteroides* ssp. *cremoris* BCRC 14047; lanes 4 and 13, *Leuconostoc mensenteroides* ssp. *dextranicum* BCRC 12260; lanes 5 and 14, *Leuconostoc mensenteroides* ssp. *mensenteroides* BCRC 12580; lanes 6 and 15, TL1; lanes 7 and 16, TL2; lanes 8 and 17, TL3; lanes 9 and 18, TL4.

Figure 3. Denaturing gradient gel electrophoresis (DGGE) profiles obtained from 4 isolates and DNA extracted directly from the Taiwanese ropy fermented milk (TRFM) starters after amplification by 338fGC/518r (lanes 1–7) and P1V1GC/P2V1 (lanes 8–14) primers. Lanes 1 and 8, TL1; lanes 2 and 9, TL2; lanes 3 and 10, TL3; lanes 4 and 11, TL4; lanes 5, 6, 7, 12, 13, and 14, the TRFM starters.

less than those identified with the use of the initial enrichment stage on nutritive media. Only *Lc. lactis* ssp. *cremoris* was found in the TRFM starters for all of the replicates using the 2 different primer sets (Lane 5, 6, 7, 12, 13, and 14). One possible explanation is that *Lc. lactis* ssp. *cremoris* has a large population in the mixture. Prakitchaiwattana et al. (2004) indicated that species with a large population in the mixture might give greater amounts of template DNA, and, therefore, have a higher probability of detection. Various culture ages and cell proteins in the mixture may also interact with the genomic DNA, thus disturbing primer annealing to the template or the activity of the DNA polymerase (de Barros Lopes et al., 1996; Beh et al., 2006). Cocolin et al. (2001) defined the sensitivity of DGGE for the detection of the V1 region as 10^4 cfu/g.

Additionally, in the present study, *Leu. mesenteroides* was not found in the TRFM starters using the culture-independent method directly identified by PCR-DGGE.

A similar finding was observed for *Leu. mesenteroides* with amplification of the V3 region of 16S rDNA, when Ercolini et al. (2003) studied the bacterial community in Stilton cheese and Chen et al. (2008) studied the LAB distribution in kefir grains. It might be due to the preferential amplification as a mixed bacterial template DNA which was subjected to PCR.

Microbial Dynamic Study at Different Fermentation Stages in the TRFM Starters

The microbial counts of LAB during fermentation in the starters are shown in Table 3. The LAB counts in the TRFM starters were significantly lower ($P < 0.05$) at the beginning of fermentation with mean values 6.94 ± 0.06 log cfu/g. The fermentation of the starters was characterized by a rapid increase in the number of LAB, which increased from an initial value of 10^6 cfu/g to 10^9 cfu/g ($P < 0.05$) during the first 20 h of

Table 3. Microbial dynamics in the Taiwanese ropy fermented milk (TRFM) starters during different fermented stages (results are expressed in %; identified number/total isolates in parentheses)

Item	Fermentation time (h)						
	0	4	8	12	16	20	24
LAB count (log cfu/g) ¹	6.94 ± 0.06 ^f	7.33 ± 0.02 ^e	7.91 ± 0.02 ^d	8.18 ± 0.04 ^c	8.51 ± 0.10 ^b	8.99 ± 0.01 ^a	9.00 ± 0.15 ^a
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> TL1	71 (17/24)	59 (16/27)	65 (13/20)	50 (15/30)	67 (18/27)	80 (16/21)	71 (15/21)
<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i> TL2	21 (5/24)	11 (3/27)	15 (3/20)	13 (4/30)	11 (3/27)	15 (3/21)	5 (1/21)
<i>Lc. lactis</i> ssp. <i>cremoris</i> TL3	4 (1/24)	4 (1/27)	—	17 (5/30)	7 (2/27)	10 (2/21)	19 (4/21)
<i>Lc. lactis</i> ssp. <i>cremoris</i> TL4	4 (1/24)	26 (7/27)	20 (4/20)	20 (6/30)	15 (4/27)	—	5 (1/21)

^{a-f}Values with different letters are significantly different ($P < 0.05$).

¹Values of lactic acid bacteria (LAB) counts are means ± standard deviation for 4 independent experiments.

fermentation and then remained stable for the rest of processing. LAB levels of 10^8 to 10^9 cfu/g were also found by Uchida et al. (2009) when they analyzed the microbiota of domestic ropy fermented milk (Caspian Sea yogurt).

The Harrison disc method used for random statistical selection of representative colonies allowed for the calculation of the percentage distribution of the bacteria found in the TRFM starters. Table 3 also depicts the percentage of the prevalent LAB populations present at different stages during the sample fermentation. At the beginning of fermentation (0 h), *Lc. lactis* ssp. *cremoris* TL1 accounted for 71% of the total isolates, which made it the most dominant bacterial species found in the samples. This was followed by *Leu. mesenteroides* ssp. *mesenteroides* (21%) and *Lc. lactis* ssp. *cremoris* TL3 and TL4 (4%). Similar ecological profiles were detected in the samples at different stages, which were dominated by *Lc. lactis* ssp. *cremoris* TL1 (50–80%), followed by *Leu. mesenteroides* ssp. *mesenteroides* (5–21%) and *Lc. lactis* ssp. *cremoris* TL3 and TL4 (4–26%). Uchida et al. (2009) reported that *Lc. lactis* ssp. *cremoris* was the dominant strain in all samples of Japanese domestic

ropy fermented milk. *Lactococcus lactis* ssp. *cremoris* has been described as a ropy strain in the fermented milk in many reports (Nakajima et al., 1992; Knoshaug et al., 2007; Uchida et al., 2009). The high population of *Lc. lactis* ssp. *cremoris*, which produces a high level of EPS, is necessary for a ropy fermented milk product.

The Microbial Dynamics in Different Types of Milk

The effects of different types of milk on the microbial cell count are indicated in Table 4. The LAB count in TRFM samples ranged from 8.92 to 9.02 log cfu/g with no significant difference ($P > 0.05$) among different types of milk. The yeast count, on the other hand, showed a significant difference. The TRFM samples made from caprine milk had the highest ($P < 0.05$) cell count (6.89 log cfu/g), followed by bovine milk (6.63 log cfu/g), and reconstituted milk (6.16 log cfu/g). Wszolek et al. (2001) also reported that the yeast count in bovine kefir was lower than that in caprine kefir.

The effect of different types of milk on the microbial ecology is also shown in Table 4. For bovine TRFM, *Lc. lactis* ssp. *cremoris* TL1 and *Kluyveromyces marxianus*

Table 4. The effects of type of milk on microbial counts and distribution of lactic acid bacteria (LAB) and yeast in Taiwanese ropy fermented milk (TRFM; results are expressed in %; identified number/total isolates in parentheses)

Item	Culturing milk type		
	Bovine TRFM	Reconstituted TRFM	Caprine TRFM
LAB count (log cfu/g) ¹	8.97 ± 0.07 ^{ab}	8.92 ± 0.05 ^b	9.02 ± 0.04 ^a
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> TL1	46 (13/28)	64 (16/25)	18 (6/33)
<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i> TL2	4 (1/28)	4 (1/25)	3 (1/33)
<i>Lc. lactis</i> ssp. <i>cremoris</i> TL3	18 (5/28)	16 (4/25)	0 (0/33)
<i>Lc. lactis</i> ssp. <i>cremoris</i> TL4	32 (9/28)	16 (4/25)	79 (26/33)
Yeast count (log cfu/g)	6.63 ± 0.05 ^b	6.16 ± 0.03 ^c	6.89 ± 0.04 ^a
<i>Kluyveromyces marxianus</i>	68 (107/157)	59 (196/331)	93 (65/70)
<i>Pichia fermentans</i>	23 (36/157)	41 (135/331)	7 (5/70)
<i>Saccharomyces unisporus</i>	9 (14/157)	0 (0/331)	0 (0/70)

^{a-c}Values with different letters within the same row are significantly different ($P < 0.05$).

¹Values of LAB and yeast counts are means ± standard deviation for 4 independent experiments.

Table 5. The effects of type of milk on chemical and physical properties of Taiwanese ropy fermented milk (TRFM)¹

Property	Bovine TRFM	Caprine TRFM	Reconstituted TRFM
pH value	4.29 ± 0.03 ^a	4.27 ± 0.04 ^a	4.28 ± 0.09 ^a
Water-holding capacity (%)	47.93 ± 4.52 ^a	52.17 ± 4.64 ^a	29.08 ± 3.25 ^b
Exopolysaccharide count (µg/mL)	138.29 ± 2.62 ^b	192.38 ± 8.80 ^a	120.55 ± 10.69 ^b
Viscosity (cps)	7,048 ± 187 ^a	645 ± 114 ^c	3,118 ± 499 ^b

^{a-c}Values with different letters within the same row are significantly different ($P < 0.05$).

¹Values are means ± standard deviation for 4 independent experiments.

accounted for 46 and 68% of the total isolates, respectively, which made them the most dominant species found in the samples. A similar ecological profile was detected in the reconstituted milk sample, which was dominated by *Lc. lactis* ssp. *cremoris* TL1 (64%) and *K. marxianus* (59%). On the contrary, *Lc. lactis* ssp. *cremoris* TL4 (79%) was the most prevalent LAB species in caprine TRFM. In addition, although *K. marxianus* was the most abundant yeast for all samples, this strain occupied more than 90% of the total isolates in the caprine samples. This microbial ecological profile revealed that the type of milk significantly affected the microbial distribution in the TRFM products.

The physical and chemical properties of TRFM were also significantly influenced by the type of milk (Table 5). The caprine sample showed a lower viscosity than the other samples ($P < 0.05$). This finding was paralleled with previous studies. Wszolek et al. (2001) indicated that product properties, such as the viscosity of kefir, were mainly influenced by the type of milk used (bovine > caprine) rather than by the starter cultures. Many studies also reported that caprine milk, with a very low proportion of α_{S1} -CN and a higher degree of CN micelle dispersion, influences rheological properties.

However, the EPS result was contradictory to the viscosity in this study. The caprine samples showed the highest EPS content ($P < 0.05$) with the highest water-holding capacity ($P < 0.05$) compared with that of the other samples, followed by bovine milk and reconstituted milk (Table 5). Although some authors supposed a direct correlation between viscosity of fermented milk and EPS concentration, no clear relation has been demonstrated. In addition, the molecular characteristics of the EPS also play an important role in viscosity. Several studies demonstrated that the stiffness of the polymer chains (Tuinier et al., 2001) and the molecular mass and radius of gyration (Faber et al., 1998; Tuinier et al., 1999; Ruas-Madiedo et al., 2002) were related to the thickening effect and viscosity of the EPS in aqueous solution. Besides the differences in chemical and physical compositions, our microbial distribution profiles also showed that the type of milk had a great

influence on the microbial ecology, which might further affect product characteristics.

Taiwanese ropy fermented milk made from reconstituted milk had lower viscosity ($P < 0.05$) and water-holding capacity ($P < 0.05$) compared with that made from bovine milk. Martin et al. (2007) reported that the process of heat treatment and water removal causes whey protein and calcium in the serum to become increasingly associated with the micelles. This results in much larger and denser micelles, decreasing the viscosity of milk.

CONCLUSIONS

In this study, we demonstrated that *Lc. lactis* ssp. *cremoris* and *Leu. mesenteroides* ssp. *mesenteroides* were the major LAB in TRFM. The percentages of the prevalent LAB populations present at different stages during the sample fermentation were similar. *Lactococcus lactis* ssp. *cremoris* TL1 was the most dominant bacterial species found in the samples and this species was followed by *Leu. mesenteroides* ssp. *mesenteroides* and *Lc. lactis* ssp. *cremoris* TL3 and TL4. Furthermore, we also showed that the type of milk had a great influence on the microbial ecology, which might further affect the product characteristics. To the best of our knowledge, this is the first report identifying the LAB in the TRFM starters and studying their microbial dynamics during different fermentation stages. This is also the first study demonstrating the effects of the type of milk on the microbial ecological profiles.

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