Interaction between *Lactococcus lactis* and *Lactococcus raffinolactis* during growth in milk: Development of a new starter culture

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ABSTRACT

Many milk fermentations use mixed cultures of lactic acid bacteria. To select a new mixed starter culture, 100 acid-producing bacterial strains were isolated from raw cow milk. Of these, 13 strains identified as belonging to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, or *Weissella* (based on phenotypic and genotypic tests) were assessed for a symbiotic effect between pairs of isolated strains during growth in milk. Among the strains tested, a mixed culture of *Lactococcus lactis* ssp. *lactis* strain 54 and *Lactococcus raffinolactis* strain 37 stimulated greater acid production during fermentation than occurred with pure fermentation. This stimulatory effect was not observed in milk supplemented with yeast extract or glucose or in constituted medium. Addition of a cell-free filtrate from milk fermented by strain 54 increased acid production by strain 37; however, the converse effect was not observed. The increased acid production by this mixed culture was, therefore, due to stimulation of strain 37 by metabolic products of strain 54, suggesting that the interaction between strains 54 and 37 is commensal. Analysis with a taste-sensing system indicated that fermented milk containing the mixed culture was more acidic, had more anionic bitterness, had greater aftertastes of anionic bitterness and astringency, and was less salty and umami than milk containing the individual cultures. This study identifies a new commensal relationship between 2 lactococcal strains that are commonly used for making dairy products.

Key words: lactic acid bacteria, starter culture, symbiosis

INTRODUCTION

Lactic acid bacteria are widely used as starter bacteria in the manufacture of fermented dairy products, such as cheese and yogurt. Recently, in many countries,

consumers have begun to favor more diverse organoleptic properties and food textures (Chee et al., 2005; Isleten and Karagul-Yuceer, 2006; Thompson et al., 2007). Accordingly, there is great interest in the development of new starter cultures for fermented products. These fermented products are typically produced by using mixed cultures of lactic acid bacteria rather than pure cultures, because pure strains often grow slowly in medium and mixed cultures can improve the flavor (Kothari et al., 1971; Frengova et al., 2000). In mixed starter cultures, it is important that the growth of each lactic acid bacterium is not inhibited by mixing and that the bacteria stimulate each other's growth and acid production. For example, yogurt is made from milk fermented by a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Sieuwerts et al., 2008). *Streptococcus thermophilus* consumes purine (precursors) in milk provided by *L. delbrueckii* ssp. *bulgaricus* and its growth is thus supported (Herve-Jimenez et al., 2009). *Streptococcus thermophilus* provides *L. delbrueckii* ssp. *bulgaricus* with formic acid (Derzelle et al., 2005), folic acid (Crittenden et al., 2003), and carbon dioxide (Driessen et al., 1982). Kefir is a fermented milk beverage that originated in Eastern Europe. The starter is kefir grain, a complex starter culture made up of various kinds of lactic acid bacteria (lactobacilli, streptococci, and lactococci) that are microbial symbionts (Margulis, 1996; Simova et al., 2002). For example, Simova et al. (2006) reported that *Lactobacillus helveticus* MP12 strain produces amino acids, which intensify cell growth activity in lactobacilli and cocci in kefir. An interaction between *Leuconostoc lactis* CNRZ 1091 and *Lactococcus lactis* ssp. *cremoris* AM2 has also been reported (Boquien et al., 1988). In this case, the mixed culture improved the maximum growth rate of *L. lactis* by 50%, whereas that of *L. lactis* ssp. *cremoris* was unaffected. Therefore, the interaction between these strains is commensal: one strain produces or releases compounds that promote the growth of the other (Meers, 1973).

With the aim of developing new industrial starter cultures, we first isolated from raw cow milk lactic acid bacterial strains with useful properties and then identi-

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fied mixed cultures of the isolated strains that demonstrated symbiotic growth. The characteristics of the interactions between these lactic acid bacterial strains during growth in milk were also investigated.

MATERIALS AND METHODS

Raw Milk Sampling

The 7 Jersey dairy cows (cows A, B, C, D, E, F, and G) used in this study were housed on pasture at a farm in Kyotango, Japan. Bramley et al. (1984) reported differences in bacterial flora in raw milk from cows with mastitis and that from healthy cows. The cows used in this study had no subclinical or clinical mastitis. A raw milk sample (about 20 mL) from 1 or 2 mammary glands of each cow was collected following teat disinfection, whereby a towel moistened with cationic surfactant was used to wipe the teat and the sample was collected in a sterile tube on December 14, 2009. The collected samples were kept at 4°C, and the analysis was performed within 24 h of sampling.

Isolation of Lactic Acid Bacteria for Starter Cultures

Each raw milk sample was inoculated on 5 types of agar plates, each containing one of the following: (1) deMan, Rogosa, and Sharpe (MRS) broth (Becton Dickinson and Co., Sparks, MD) to which 1.6% agar and 0.8% CaCO₃ were added; (2) 10% skim milk (Snow Brand Co., Sapporo, Japan) with 0.005% bromocresol purple and 1.6% agar; (3) 10% skim milk with 0.005% bromocresol purple, 0.3% yeast extract (Wako Chemical, Osaka, Japan), 0.5% glucose, and 1.6% agar; (4) acetate agar (Rogosa et al., 1951); and (5) AC agar plates (Nissui Seiyaku, Tokyo, Japan). Milk media were sterilized by autoclaving at 121°C for 10 min. Other media were sterilized by autoclaving at 121°C for 15 min.

Acid-producing bacteria were detected on MRS broth with CaCO₃. Presumptive lactobacilli were detected on acetate agar medium (Rogosa et al., 1951), and enterococci were detected on AC agar plates. The plates were incubated for 2 d at 30°C for MRS agar and milk agar or 37°C for acetate agar and AC agar. Colonies that dissolved in CaCO₃ and formed clear zones around their colonies in MRS agar plates were isolated at random. Yellow colonies on milk agar and all colonies on acetate agar and AC agar were isolated. The broths inoculated with each colony were cultivated at 30°C or 37°C and tested for catalase activity. Briefly, the cultures were centrifuged and 15% (vol/vol) H₂O₂ was added to the pellets. Gas production was indicative of catalase-

positive bacterial strains. The pure, gram-positive, and catalase-negative isolated strains were stored at -80°C in MRS broth supplemented with 15% glycerol.

Working cultures were revitalized by one transfer in MRS or tryptone-yeast extract-glucose (TYG) broth at 30°C for mesophilic lactic acid bacteria and 37°C for thermophilic lactic acid bacteria, respectively. The TYG broth consisted of 0.5% tryptone (Becton Dickinson and Co.), 0.5% yeast extract, and 1.0% glucose supplemented with 1% sodium succinate and 1% sodium chloride and was adjusted to pH 6.86.

To develop new starter cultures, we selected the isolated strains by growth on 3 kinds of liquid milk culture (i.e., 10% skim milk alone, 10% skim milk with 0.3% yeast extract, and 10% skim milk with 0.3% yeast extract plus 0.5% glucose) at 30°C for 1 to 2 d. Strains with the ability to coagulate milk were selected and identified.

Identification of the Strains

To identify lactic acid bacterial strains, the 16S rRNA gene was amplified and sequenced by using the primer pair 27F/1406R (Suzuki and Giovannoni, 1996). In addition, the housekeeping genes *rpoA* (encoding the DNA-dependent RNA polymerase α -subunit) and *pheS* (encoding the phenylalanyl-tRNA synthase α -subunit) were amplified and sequenced by using the primer pairs *rpoA*-21-F/*rpoA*-22-R (Naser et al., 2007) and *pheS*-21-F/*pheS*-21-R (Naser et al., 2005), respectively. The PCR conditions used for *rpoA* and *pheS* were those described by Naser et al. (2005), except for an annealing temperature of 54°C. The DNA sequences were analyzed by the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The BLAST algorithm was used to determine the most closely related sequences in the NCBI nucleotide sequence database (<http://www.ncbi.nlm.nih.gov/BLAST>).

A PCR-based method was also used for specific *Lactococcus lactis* ssp. *lactis* identification (Nomura et al., 2002). Briefly, bacterial cultures (10⁷ cfu) were used as templates for PCR analyses. Primers were designed from the glutamate decarboxylase gene sequence of *L. lactis* ssp. *lactis* bv. diacetylactis 01-7. A 600-bp PCR fragment would be amplified from the strain belonging to *L. lactis* ssp. *lactis*. *Lactococcus* strains other than *L. lactis* were identified by analysis of PCR products (approximately 860 bp) amplified by using primers PiplraR and 1RL (Pu et al., 2002).

Several physiological tests of the strains were carried out: microscopic observation of bacterial shape; growth at 40°C and 45°C in MRS broth for 1 d; and growth in MRS broth with 4% and 6.5% NaCl for 2 d. Sugar

fermentation and hydrolysis of arginine were assessed by using an API 20 STREP kit (BioMerieux, Marcy l'Etoile, France) for cocci strains.

Strains belonging to the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Weissella* were selected as candidates for starter bacteria in this study because *Lactobacillus*, *Leuconostoc*, and *Lactococcus* are generally recognized as safe (Salminen et al., 1998; Ogier et al., 2008), and some strains of *Weissella* have been used as starters for fermented products (Galle et al., 2010).

Screening Cultures for Associative Growth in Mixed Culture

To screen strains for associative growth in mixed cultivation, a tube containing 4 mL of 10% skim milk was inoculated with 1% of 2 different strains in pure and mixed culture: the size of the inoculum in the mixed culture was therefore at the 0.5% level for each strain. The cultures were incubated at 30°C or 37°C for 24 h, at which time the pH value of the culture was determined. Any interaction occurring in mixed cultures is reflected by acid production (Kothari et al., 1971; Hugenholtz et al., 1987). The acidification of growth medium in batch culture is a good reflection of bacterial growth, which is why pH measurement is sometimes used to track growth (Cogan, 1978).

To test selected strains 37 and 54 for associative growth, pure and mixed cultivation were conducted in constituted media: 4 mL of TYG, tryptone-yeast extract-lactose (TYL), and TYL with 0.2% ammonium citrate. The TYL broth included 0.5% lactose instead of glucose, and these broths were adjusted to pH 6.86.

Viable Count of the Selected Strains

To distinguish and enumerate the selected strains (37 and 54) from the fermented milk in the pure and mixed cultures, we diluted the fermented milk samples in sterile 0.85% NaCl, plated them on MRS agar medium, and incubated them at 30°C. Because strain 37 grew slowly in MRS agar but strain 54 did not, the small colonies on the MRS medium were strain 37, and the large colonies were strain 54.

In addition, we examined whether this associative growth was stable by inoculating the 2 cultures 3 times in milk and determining the viable count for each.

Preparation of a Cell-Free Culture Capable of Stimulating Acid Production During Associative Growth

Individual cell-free filtrates of milk cultures fermented by the selected strains were prepared by the method of

Kothari et al. (1971). Briefly, 50 mL of milk was inoculated with a fresh overnight culture at the 1% level and incubated at 30°C for 24 h. The cultures were kept at 45 to 50°C for 30 min in a water bath. The whey was then filtered and adjusted to pH 7.0 with NaOH. Control whey was prepared by adding 2.5 mL of 10% lactic acid to 50 mL of fresh sterilized skim milk. The culture filtrates were added to the comparison cultures. The mixed cultures were incubated at 30°C for 24 h and pH value was then determined.

Mechanical Taste Sensing of Fermented Milk

Fifty milliliters of skim milk (10%) was inoculated with a fresh overnight culture at the 1% level and incubated at 30°C for 24 h. The cultures were diluted 5-fold with water and centrifuged at $2,800 \times g$ at 4°C for 20 min. The supernatant was then analyzed by means of the taste-sensing system SA402 (INSENT, Tokyo, Japan). The system comprises 5 taste sensors derived from polymer membranes that fix different lipids. The sensors CT0, CA0, AAE, C00, and AE1 were designated to respond to the individual initial tastes of saltiness, acidity, umami, anionic bitterness, and astringency, respectively (Toko, 1996, 1998), and anionic bitterness, astringency, and umami were measured as aftertastes (Chikuni et al., 2010). Initial taste is the taste a person senses when food, drink, or both are in the mouth, whereas aftertaste is the taste a person senses after swallowing food, drink, or both. The measurement procedure was described by Anjiki et al. (2011). Briefly, zero-adjusted probes were applied to a sample solution, and the sample output was collected as initial tastes. Next, a sensor buffer solution (30 mM KCl plus 0.3 mM L-(+)-tartaric acid solution) was measured before the probe was washed. This output indicated the membrane potential caused by adsorption, which represents an aftertaste. Probes were then washed with the buffer solution and zero-adjusted for measurement of the next sample. All measurement procedures were carried out at room temperature. The sensor signals for each sample are reported as the difference from the sensor buffer solution and converted to values corresponding to the individual tastes with the following formulas: converted value of CT0 output ($cvCT0$) = $-0.25 \times CT0$ signal; converted value of CA0 output ($cvCA0$) = $0.33 \times CA0$ signal; converted value of AAE output ($cvAAE$) = $-0.16 \times AAE$ signal; converted value of C00 output ($cvC00$) = $-0.14 \times C00$ signal + $0.08 \times CT0$ signal; and converted value of AE1 output ($cvAE1$) = $-0.16 \times AE1$ signal + $0.16 \times CT0$ signal. A converted value of 1 corresponds to a 20% difference from the buffer solution in sensory system SA402 (Sasaki et al., 2005; Chikuni et al., 2010).

Statistical Analysis

The pH values in media, bacterial counts (log cfu/mL), and relative intensity of taste in fermented milk were expressed as least squares means $\pm$ standard errors of 2 or 3 replicates analyzed by the generalized linear model (GLM) procedure of the SAS system (version 9.1, SAS Institute Inc., Cary, NC). Differences between least squares means were analyzed by the Tukey-Kramer test using the PDIF option of the GLM procedure of the SAS system. Statistical significance was defined as $P < 0.05$.

RESULTS

Isolation and Identification of Lactic Acid Bacteria

One hundred acid-producing bacterial strains were isolated from 6 of the 7 raw milk samples. The milk sample from cow G did not produce any colonies on the agar plates used. Bacterial cell counts from samples on MRS agar, milk agar, or acetate agar ranged from 10^2 to 10^5 cfu/mL. No colonies were found on AC agar plates. Of the 100 isolated strains, 43 strains grew in 10% skim milk with yeast extract and glucose, the medium used to assess milk clotting. These strains were subjected to identification tests as candidates for dairy starters. No strains isolated from cow F's milk could be grown in milk, and thus they were not identified.

Among the 43 strains that grew in milk, 11 were *Enterococcus* spp., 1 was a *Lactobacillus* sp., 14 were *Lactococcus* spp., 1 was a *Leuconostoc* sp., 5 were *Streptococcus* spp., 5 were *Weissella* spp., and 6 were unidentified strains. From 2 to 5 species were detected in each cow's milk. Milk from cow A had 2 strains of *Enterococcus*, 1 strain of *Lactobacillus*, 5 strains of *Lactococcus*, 4 strains of *Streptococcus*, and 1 strain of *Weissella*. Milk from cow B had 1 strain of *Enterococcus* and 2 strains of *Lactococcus*. Milk from cow C had 5 strains of *Enterococcus*, 6 strains of *Lactococcus*, 1 strain of *Streptococcus*, 3 strains of *Weissella*, and 1 unidentified strain. Milk from cow D had 3 strains of *Enterococcus*, 1 strain of *Lactococcus*, and 2 unidentified strains. Milk from cow E had 1 strain of *Leuconostoc*, 1 strain of *Weissella*, and 3 unidentified strains. The 14 isolated lactococcal strains included 7 strains of *L. lactis* ssp. *lactis* and 7 strains of *L. raffinolactis* (data not shown).

Seven strains of *L. lactis* ssp. *lactis* (strains 8, 16, 21, 22, 36, 54, and 95), 3 strains of *L. raffinolactis* (strains 9, 37, and 93), 1 strain of *Lactobacillus plantarum* (strain 19), 1 strain of *Leuconostoc mesenteroides* (strain 89), and 1 strain of *Weissella paramesenteroides* (strain 51) were screened as associative mixed starters. Their physiological and biochemical characteristics and

ability to clot milk for use as dairy starter are shown in Table 1. All strains except 19 and 51 were gram-positive, catalase negative, and cocci in pairs or short chains. Strains 19 and 51 were gram-positive, catalase negative, and rod-shaped in short chains. Among the 13 strains shown in Table 1, *L. lactis* ssp. *lactis* strains 8 and 54 coagulated milk within 1 d with glucose and within 2 d without it (Table 1). In the organoleptic trial, fermented milk with strain 54 had more yogurt flavor than that with strain 8. Thus, strain 54 was selected as a candidate dairy starter bacterium.

Acid Production by Pure and Mixed Cultures of Isolated Lactic Acid Bacteria in Milk

The acid production by pairs of cultures (strain 54 with other strains) grown in milk is shown in Table 2. In cultivation at 30°C and 37°C, a significant decrease of the pH value was not observed in any mixed culture. The pH value of the milk was lowest for pair 54 and 37, although the decrease was not significant. Thus, strain 37 was selected for mixed starter with strain 54.

Acid Production by Pure and Mixed Cultures of Isolated Lactic Acid Bacteria in Different Media

The pH values of pure and mixed cultures of strains 37 and 54 on different media are shown in Table 3. The pH value in milk was significantly lowered by mixed cultivation of strains 37 and 54 compared with that of each pure culture. In pure culture, the pH value of strain 37 or 54 in milk with yeast extract or glucose was significantly lower than that without it, whereas the pH value was not lowered by mixed cultures. On the other hand, the strains could grow in TYG, TYL with citrate, and TYL broth in pure culture. The pH values were not lowered by mixed cultures in TYG, TYL with citrate, or TYL broth.

Potency of Stimulatory Materials

The effect of adding a cell-free filtrate from strain 54 on acid production in a strain 37 culture is shown in Table 4. Skim milk fermented by strain 54 was slightly clotting, whereas that fermented by strain 37 was not, and thus, a cell-free filtrate from strain 37 was not prepared. The control whey was prepared by adding lactic acid to skim milk. Addition of the strain 54 filtrate significantly increased acid production in the strain 37 culture relative to the control samples, to which the control whey was added. No significant progressive increase in acid production was observed with the addition of water to the strain 37 culture.

Table 1. Some characteristics of candidates starter strains isolated from raw milk¹

Species and strain no.	Growth at		Growth in the presence of		Fermentation of			Hydrolysis of arginine	Milk clotting ²				Source of raw milk
	40°C	45°C	4% NaCl	6.5% NaCl	Lactose	Mannitol	Raffinose		Milk	Milk plus Y	Milk plus G	Milk plus YG	
<i>Lactococcus lactis</i> ssp. <i>lactis</i>													
8	+	—	+	—	+	—	—	+	+	++	++	++	Cow A
16	+	—	+	—	+	+	+	+	—	++	—	++	Cow A
21	+	—	+	—	+	—	—	+	—	++	—	++	Cow B
22	+	—	+	—	+	+	—	+	—	++	—	++	Cow B
36	+	—	+	—	—	+	+	+	—	++	—	++	Cow C
54	+	—	+	—	+	—	—	+	+	++	++	++	Cow C
95	+	—	+	—	+	+	+	+	—	++	—	++	Cow C
<i>Leuconostoc mesenteroides</i>													
89	+	—	+	—	—	—	+	—	—	—	—	++	Cow E
<i>Lactococcus raffinolactis</i>													
9	—	—	—	—	+	+	+	—	—	++	—	++	Cow A
37	—	—	—	—	+	+	+	—	—	++	—	++	Cow C
93	—	—	—	—	+	+	+	—	—	++	—	++	Cow A
<i>Lactobacillus plantarum</i>													
19	+	—	+	—	ND ³	ND	ND	ND	—	++	—	++	Cow A
<i>Weissella paramesenteroides</i>													
51	+	—	+	—	ND	ND	ND	ND	—	++	—	++	Cow C

¹*Lactococcus* strains were grown at 30°C; *Lactobacillus* and *Weissella* strains were grown at 37°C.

²Milk = 10% skim milk; milk plus Y = 10% skim milk plus 0.3% yeast extract; milk plus G = 10% skim milk plus 0.5% glucose; milk plus YG = 10% skim milk plus 0.3% yeast extract and 0.5% glucose. Milk clotting: ++ = coagulated within 1 d; + = coagulated within 2 d; — = did not coagulate.

³ND = not done.

Table 2. pH values¹ of milk for pure or mixed cultures of isolated strains² and *Lactococcus lactis* ssp. *lactis* strain 54

Species and strain no.	Cultivation at 30°C		Cultivation at 37°C	
	Pure	Mixed	Pure	Mixed
<i>Lactococcus lactis</i> ssp. <i>lactis</i>				
54	5.03 ± 0.03 ^e	ND ³	5.08 ± 0.02 ^{bc}	ND
8	5.16 ± 0.03 ^{de}	5.11 ± 0.03 ^{de}		
16	5.18 ± 0.03 ^{de}	5.10 ± 0.03 ^{de}		
21	5.21 ± 0.03 ^{de}	5.07 ± 0.03 ^{de}		
22	5.29 ± 0.03 ^d	4.99 ± 0.03 ^e		
36	5.93 ± 0.03 ^a	5.06 ± 0.03 ^e		
95	6.08 ± 0.03 ^a	5.22 ± 0.03 ^{de}		
<i>Leuconostoc mesenteroides</i>				
89	6.11 ± 0.03 ^a	4.94 ± 0.03 ^e		
<i>Lactococcus raffinolactis</i>				
9	5.59 ± 0.03 ^{bc}	5.05 ± 0.03 ^e		
37	5.48 ± 0.03 ^c	4.88 ± 0.03 ^e		
93	5.67 ± 0.03 ^b	5.07 ± 0.03 ^{de}		
<i>Lactobacillus plantarum</i>				
19	5.43 ± 0.03 ^{cd}	5.07 ± 0.03 ^e	5.32 ± 0.02 ^a	4.98 ± 0.02 ^c
<i>Weissella paramesenteroides</i>				
51	5.52 ± 0.03 ^{bc}	5.25 ± 0.03 ^d	5.28 ± 0.02 ^a	5.10 ± 0.02 ^b

^{a-c}Mean pH values within the same cultivation temperature with different superscript letters are significantly different ($P < 0.05$).

¹Results are means ± SE from duplicate samples.

²*Lactococcus lactis* ssp. *lactis* strain 54 was cultivated with other strains in 10% skim milk at 30° or 37°C for 24 h. *Lactococcus* strains were grown at 30°C. *Lactobacillus* and *Weissella* strains were grown at 30° and 37°C.

³ND = not done.

Viable Cell Counts in Pure and Mixed Cultures

Changes in viable cell count and milk pH value during cultivation of pure and mixed cultures of strains 37 and 54 are shown in Figures 1a and 1b, respectively. At cultivation time 0 h (before starting fermentation), the viable cell count of strain 37 in mixed culture (6.47 ± 0.09 log cfu/mL) was significantly lower ($P = 0.034$) than that in pure culture (7.09 ± 0.12 log cfu/mL). From 0 to 3.5 h, an initial delay in the growth of strain 37 in mixed culture was observed. The viable cell count of strain 37 in mixed culture increased markedly between 3.5 and 7 h, and at the end of the culture period

the counts for this strain in pure culture (9.09 ± 0.09 log cfu/mL) and mixed culture (9.02 ± 0.12 log cfu/mL) were comparable ($P = 1.00$). The growth of strain 54 in pure and mixed cultures was similar, and the viable counts for this strain in pure culture (8.61 ± 0.09 log cfu/mL) and mixed culture (8.84 ± 0.09 log cfu/mL) were comparable ($P = 0.91$) at 24 h. A significant decrease in the pH value of the milk medium in mixed culture compared with each pure culture was observed at 7 and 24 h (Figure 1b).

When inoculating the 2 cultures 3 times in milk, we found that strains 37 and 54 grew in milk at a constant rate (data not shown).

Table 3. pH values¹ of different media for pure or mixed cultures of *Lactococcus raffinolactis* strain 37 and *Lactococcus lactis* ssp. *lactis* strain 54²

Strain no.	Medium ³					
	Milk	Milk plus Y	Milk plus G	TYG	TYL	TYL plus 0.2% citrate ammonium
37	5.56 ± 0.01 ^a	4.64 ± 0.01 ^f	5.51 ± 0.01 ^b	4.86 ± 0.00 ^e	4.91 ± 0.00 ^d	4.95 ± 0.00 ^{bc}
54	5.28 ± 0.01 ^c	4.54 ± 0.01 ^g	4.73 ± 0.01 ^e	4.61 ± 0.00 ^f	4.95 ± 0.00 ^c	5.02 ± 0.00 ^a
Mixed	4.83 ± 0.01 ^d	4.55 ± 0.01 ^g	4.71 ± 0.01 ^e	4.61 ± 0.00 ^f	4.96 ± 0.00 ^b	5.03 ± 0.00 ^a

^{a-f}Mean pH values within each milk-based or constituted medium with different superscript letters are significantly different ($P < 0.05$).

¹Results are means ± SE from triplicate samples.

²*Lactococcus raffinolactis* strain 37 and *Lactococcus lactis* ssp. *lactis* strain 54 in pure and mixed culture were cultivated in different media at 30°C for 24 h.

³Milk = 10% skim milk; milk plus Y = 10% skim milk plus 0.3% yeast extract; milk plus G = 10% skim milk plus 0.5% glucose; milk plus YG = 10% skim milk plus 0.3% yeast extract and 0.5% glucose; TYG = 0.5% tryptone, 0.5% yeast extract, and 1.8% glucose supplemented with 1% sodium succinate and 1% sodium chloride; TYL broth included 0.5% lactose instead of glucose. These broths were adjusted to pH 6.86.

Table 4. Effect of cell-free extract from milk fermented by *Lactococcus lactis* ssp. *lactis* strain 54 on the pH values¹ of milk fermented by *Lactococcus raffinolactis* strain 37²

Sample	pH
Distilled water	5.68 ± 0.01 ^b
Whey from control	5.78 ± 0.01 ^a
Whey from strain 54	5.34 ± 0.01 ^c

^{a-c}Mean pH values with different superscript letters were significantly different ($P < 0.05$).

¹Results are means ± SE from triplicate samples.

²*Lactococcus raffinolactis* strain 37 was cultivated with the cell-free extract in 10% skim milk or distilled water at 30°C for 24 h.

Taste Intensities of Pure and Mixed Cultures in Fermented Milk

The relative intensities of the taste of milk fermented by pure and mixed cultures compared with sensory buffer solution are shown in Table 5. Significant differences in initial tastes of saltiness, acidity, umami, and anionic bitterness and aftertastes of anionic bitterness and astringency were detected between fermented milk samples of strains 37 and 54. In mixed cultures, fermented milk had significantly higher intensities of acidity and anionic bitterness, and aftertastes of bitterness and astringency and significantly lower intensities of saltiness and umami than in either culture alone. Addition of fermented milk of strain 37 to that of strain 54 (1:1) produced significantly different taste intensities (i.e., saltiness, acidity, umami, anionic bitterness, and aftertastes of bitterness, umami, and astringency) compared with the mixed cultivation sample.

DISCUSSION

Raw milk is a source of new strains of lactic acid bacteria (Badis et al., 2004; Nomura et al., 2006). The indigenous bacterial community in milk can be influenced by cow feeding conditions, such as feeding systems and season (Lopez-Benavides et al., 2007; Hagi et al., 2010). Further, to apply lactic acid bacterial strains as dairy starters, we selectively identified strains that grow well in milk with yeast extract and glucose. Thus, although our results for lactic acid bacterial species and populations isolated from raw milk may not be directly extrapolated to other experimental conditions (e.g., different countries, animals, or feeding systems), bacterial species isolated from raw cow milk in this study, except *Weissella* spp., were found in other studies (Giannino et al., 2009; Hagi et al., 2010). Although there are no reports of *Weissella* isolated from raw milk, this genus has been isolated from traditional fermented milk products (Sengun et al., 2009).

Associative growth between *L. lactis* ssp. *lactis* strain 54 and *L. raffinolactis* strain 37 was observed in milk

Figure 1. Viable cell count (a) and pH value (b) in pure and mixed cultures of *Lactococcus raffinolactis* strain 37 and *Lactococcus lactis* ssp. *lactis* strain 54 in 10% skim milk. (a) Viable cell counts of strain 37 in pure culture (○) and mixed culture (●) and strain 54 in pure culture (□) and mixed culture (■); (b) pH values of strain 37 (○) and strain No. 54 (□) in pure culture and mixed culture (▲). Data are the means of 2 or 3 replicate samples. The pH values of mixed culture versus that of each pure culture: ** $P < 0.01$; *** $P < 0.001$.

but not in milk with yeast extract or glucose and in constituted medium. This was because proteinaceous compounds, amino acids, or other available carbon sources or minerals derived from these media stimulate bacterial growth in pure culture, suggesting that these 2 strains need minerals and amino acids for growth. *Lactococcus lactis* strains have limited capacity to synthesize amino acids (Chopin, 1993) and depend on nitrogen sources, such as casein in milk, for growth. These 2 strains behave like nonproteolytic strains, although strain 54 coagulated milk when glucose was

Table 5. Relative intensity of taste of milk fermented by pure and mixed cultures of *Lactococcus raffinolactis* strain 37 and *Lactococcus lactis* ssp. *lactis* strain 54 by taste-sensing system

Sample ¹	Relative intensity of initial taste ²				Relative intensity of aftertaste ²			
	Saltiness	Acidity	Umami	Anionic bitterness	Astringency	Anionic bitterness	Umami	Astringency
Strain 37	20.4 ± 0.39 ^a	-28.2 ± 0.28 ^c	10.2 ± 0.08 ^a	0.65 ± 0.00 ^d	0.34 ± 0.01	2.03 ± 0.07 ^c	1.23 ± 0.07 ^{ab}	-15.4 ± 0.21 ^c
Strain 54	15.2 ± 0.39 ^c	-24.5 ± 0.28 ^b	9.10 ± 0.08 ^b	0.75 ± 0.00 ^b	0.35 ± 0.01	3.02 ± 0.07 ^b	0.88 ± 0.07 ^b	-12.7 ± 0.21 ^b
Mixed	11.6 ± 0.39 ^d	-21.5 ± 0.28 ^a	8.30 ± 0.08 ^c	0.78 ± 0.00 ^a	0.34 ± 0.01	3.67 ± 0.07 ^a	0.80 ± 0.07 ^b	-10.7 ± 0.21 ^a
Add	18.0 ± 0.39 ^b	-26.8 ± 0.28 ^c	9.78 ± 0.28 ^a	0.70 ± 0.00 ^c	0.33 ± 0.01	2.42 ± 0.07 ^c	1.33 ± 0.07 ^a	-14.3 ± 0.21 ^c

^{a-d}Mean values within a column with different superscript letters are significantly different ($P < 0.05$).

¹Strain 37 (or 54) = milk fermented by strain 37 (or 54); Mixed = milk fermented by mixed culture of strains 37 and 54; Add = equivalent mixture of milk fermented by strains 37 and 54.

²Values are shown as the difference from the sensory buffer solution (30 mM KCl plus 0.3 mM L-(+)- tartaric acid solution) converted to values corresponding to the individual tastes; values represent means ± SE from duplicate samples.

added. This strain is protease-positive, but its activity may be low.

Cultures that grow symbiotically supply each other with needed nutrients and thus can grow well in deficient media, as observed for yogurt starters. The acid production of strain 37 was stimulated by metabolites from milk fermented by strain 54. In terms of the bacterial growth curve, although we observed an initial delay in the growth of strain 37 in mixed culture because of its lower inoculum, its growth increased markedly thereafter, which may correspond to the exponential growth period in strain 54. In contrast, the growth curve of strain 54 was unaffected by mixing with strain 37. These findings indicate that strain 37 may not provide milk metabolites for strain 54 in mixed culture. In other words, the stimulatory action was one-sided; that is, this interaction between strains 37 and 54 represents commensalism (Meers, 1973). The stimulatory materials in milk fermented by strain 54 have not yet been identified. In addition, by inoculating 3 times, we found that strains 37 and 54 grew in milk at a constant rate, indicating that each strain may not be eliminated from mixed culture because of its sensitivity to bacteriocins produced by the other strain, if the strains produce them.

Although few reports exist on the interactions among *L. lactis* strains (Kothari et al., 1971; Juillard et al., 1996; Flambard et al., 1997), there are no reports on commensalism between *L. lactis* and *L. raffinolactis*. Significant acid production in mixed culture between *L. raffinolactis* strain 37 and *L. lactis* ssp. *lactis* strains 22, 36, or 95 was observed (data not shown), similar to that occurring in mixed cultures of strains 37 and 54. Further investigation of the stimulatory materials from strains 22, 36, or 95 on strain 37 are needed. When a *L. lactis* ssp. *lactis* strain has beneficial industrial traits, such as production of a desirable flavor, but shows poor growth in milk, the time required for acid production might be shortened by mixing strain 37 with slow-growing strains of *L. lactis* ssp. *lactis* in milk, which may be useful for developing a new dairy starter.

Lactococcus raffinolactis strains are not currently used by the dairy industry, mainly because of their lack of caseinolytic activity (Holler and Steele, 1995), although some strains of *L. raffinolactis* have been detected in traditionally fermented milk products such as Zabady in Egypt (El-Baradei et al., 2008) and Kajmak in the Balkan Peninsula (Jokovic et al., 2008). Is there merit in utilizing *L. raffinolactis* as an industrial dairy starter? We investigated the symbiosis between *L. raffinolactis* strain 37 and dairy starter *L. lactis* ssp. *cremoris* strain H61 (Kimoto-Nira et al., 2007) in this study. Although mixed cultivation of strains 37 and H61 did not promote acid production compared with

that of each pure culture, strain 37 was not eliminated by mixed cultivation with strain H61 (data not shown). Strain 37 fermented lactose and citrate to lactate, formate, acetate, ethanol, and acetoin (data not shown). Flavor compounds such as acetoin have been reported in dairy products (Callon et al., 2005; Chammas et al., 2006). For example, diacetyl and its reduced form, acetoin, are of interest for their organoleptic properties and are responsible for notes of a fermented buttery flavor. Because *L. lactis* ssp. *cremoris* strains, such as strain H61, are not able to utilize citrate, strain 37 might add a different flavor to fermented milk products made by dairy starters of *L. lactis* ssp. *cremoris*.

On the other hand, *L. raffinolactis* strains are able to ferment α -galactosides, such as melibiose and raffinose, that are not utilized by *L. lactis* strains (Boucher et al., 2003). These sugars are dominant in plant-derived foods, such as soymilk, and are known to cause flatulence. Recent trials of their removal by fermentation with lactic acid bacteria have been reported (LeBlanc et al., 2004; Machielsen et al., 2011). To develop dairy products with new organoleptic properties, it would be useful to mix milk with plant-derived materials (i.e., soymilk, fruits, and vegetables) and ferment it with lactic acid bacteria, such as a mixture of *L. raffinolactis* strain 37 and *L. lactis* ssp. *lactis* strain 54 or other dairy starters.

Several natural isolated strains are presently used as starter bacteria. For example, *L. lactis* ssp. *lactis* 527 (MAFF 400103, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) was isolated from raw cow milk and has been used as starter bacteria for more than 50 yr (Kimoto et al., 1999). Trials have also been conducted to apply lactic acid bacteria isolated from the natural environment, such as raw milk (Fortina et al., 2007; Bravo et al., 2009) and plant material (Nomura et al., 2006), to making dairy products. However, if natural isolated strains are to be developed as new starter organisms, their safety must be assessed, even if they are generally recognized as safe, because the incidence of virulence factors such as antibiotic resistance and production of undesirable substances seem to be strain-specific properties (Fortina et al., 2007).

CONCLUSIONS

Mixed culture of *L. lactis* ssp. *lactis* strain 54 and *L. raffinolactis* strain 37 isolated from raw cow milk may be useful for developing new dairy products. In addition to assessing their safety, further evaluation of traits such as their resistance to bacteriophages and organoleptic evaluation by panelist will be required.

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