Sensory Properties and Taste Compounds of Fermented Milk Produced by *Lactococcus lactis* and *Streptococcus thermophilus*

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The aim of this study was to characterize the sensory properties and taste compounds of the fermented milk (FM) produced by two species of lactic acid bacteria, *Lactococcus lactis* and *Streptococcus thermophilus* for 16 hours, and to compare them with those of yogurt (YG) made from the same reconstituted milk by *Lactobacillus bulgaricus* and *S. thermophilus*. Sensory evaluation showed that the sourness of FM was significantly weaker than that of YG, and that the lactic acid content of FM was significantly smaller than that of YG. The sourness intensity of an organic acid solution reconstituted on the basis of analyses of organic acids in FM was significantly weaker than that of YG. From these results, differences in lactic acid content appeared to be related to differences in sourness intensity between FM and YG.

Keywords: yogurt, fermented milk, sensory evaluation, pH, acidity

Introduction

Fermented foods are of great importance world-wide because their nutritional, organoleptic and shelf-life properties are significantly improved when compared with their raw materials (Oliveira *et al.*, 2002). Yogurt is defined as fermented milk produced by fermentation with two species of lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, which are active in a symbiotic relationship. *S. thermophilus* produces pyruvic acid, formic acid and CO₂ (Higashio *et al.*, 1977b), which stimulate the growth of *L. bulgaricus*. In turn, *L. bulgaricus* produced peptides and amino acids that stimulate the growth of *S. thermophilus* (Bautista *et al.*, 1966; Higashio *et al.*, 1977a). Yogurt has become a very popular food and its consumption has increased in many countries for the past decades (Tamine, 2002). The excellent sensory properties, expanded variety and health benefits of yogurt are major reasons for such popularity.

The flavor characteristics of yogurt are primarily important with reference to the quality of final product (Gallardo-Escamilla *et al.*, 2005; Ulberth *et al.*, 1992). In general, the sourness and aroma of yogurt, comprising more than 60 compounds, are well known to be important to its quality (Ott

et al., 2000a). Flavor compounds are formed by the breakdown of milk protein, fat and lactose in yogurt. Lactose in milk is metabolized by enzyme activities in a phosphotransferase system in lactic acid bacteria starter (De and Gasson, 1989) to produce aroma compounds such as aldehydes, alcohols and carboxylic acids in yogurt, and casein is also metabolized by proteases and amino transferases to produce free amino acids and aroma compounds. Since species of lactic acid bacteria possess different metabolic systems, the fermented milks produced by different lactic acid bacteria starters possess different flavor characteristics.

In yogurt, there are many kinds of taste compounds involved in the formation of the specific taste of yogurt. Sourness was shown to be positively correlated with content of lactic acid and succinic acid in yogurt, but negatively correlated with pH (Yuguchi *et al.*, 1988). Unfortunately, taste compounds other than organic acids are little known.

Recently, it has become well known that consumers prefer sweet and less sour yogurt (Steven *et al.*, 1991; Debbie *et al.*, 1991). In general, mixed strains of starter culture (*S. thermophilus* and *L. bulgaricus*) are used for the production of yogurt. However, some customers do not like this yogurt because of its sourness (Steven *et al.*, 1991; Debbie *et al.*, 1991). Thus, in order to produce less sour yogurt, we manufactured a new type of fermented milk using mixed strains of

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starter culture, *S. thermophilus* and *Lactococcus lactis*, which is one of the most important starters in the manufacture of semi-hard cheeses such as Gouda, Edam, and Cheddar.

In this study, we characterize the sensory properties and taste compounds in the fermented milk (FM) produced by two species lactic acid bacteria, *L. lactis* and *S. thermophilus* for 16 hours, and compared them with those of yogurt (YG) made from the same reconstituted milk by *L. bulgaricus* and *S. thermophilus*.

Materials and Methods

Chemicals Sodium hydroxide, orotic acid, pyruvic acid, formic acid, acetic acid, uric acid, propionic acid, butyric acid and hippuric acid were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Citric acid, DL ($\pm$) lactic acid, lactose and galactose were purchased from Kanto Chemical Co., Inc., Tokyo, Japan. Phenolphthalein was from Nacalai Tesque Ltd., Kyoto, Japan.

Production of the fermented milk and yogurt *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) and *Lactococcus lactis* subsp. *lactis* (*L. lactis*) strains obtained from Nipponluna Co., Kyoto, Japan, were used for the production of fermented milk and yogurt.

Reconstituted milk containing 12 % (wt/vol) skim milk powder (Snowbrand, Tokyo, Japan) was sterilized at 90°C for 10 minutes and cooled to approximately 40°C. It was incubated with 1 % of *S. thermophilus* and 1 % of *L. lactis* at 38°C for 16 hours. The incubation was stopped at 0.85 ± 0.05 % of acidity as lactic acid. This product was defined as FM in this study. To produce YG, we incubated the same reconstituted milk with 2 % *S. thermophilus* and 1 % *L. bulgaricus* at 42°C for 4 to 8 hours. This incubation was also stopped at 0.85 ± 0.05 % of acidity.

Measurement of acidity and pH Acidity was measured by titration with 0.1 N NaOH, and expressed as percentage of lactic acid. The pH was measured with a pH meter (Beckman, California, state, USA).

Analyses of organic acids and lactose in FM and YG Organic acids were analyzed according to the method described by Garcia and McGregor (1994). The fermented milk and yogurt were homogenized at 3,000 rpm for 5 minutes. Next, 6.2 volumes (v/w) of 0.01 N H_2SO_4 solution were added to homogenate and mixed. This mix was centrifuged at $10,000 \times g$ for 10 minutes and the supernatant was obtained. The organic acids in this supernatant were analyzed by high-pressure liquid chromatography (HPLC) with an AMINEX HPX-87H ion-exchange column ($\phi 300 \text{ mm} \times 7.8 \text{ mm}$) at 65°C. A 7.5 mM H_2SO_4 solution was used as mobile phase, and the flow rate was 0.7 ml/min. Each organic acid was detected at 210

nm.

Lactose was measured using enzymatic kits obtained from J.K. International, Tokyo, Japan. In this kit, lactose was cleaved to beta-D-galactose and D-glucose by beta-galactosidase. Beta-D-Galactose was oxidized to D-galactono-gamma-lactone by beta-galactose dehydrogenase in the presence of nicotinamide adenine dinucleotide (NAD). The content of NADH formed was measured by the change in absorption at 340 nm and was directly proportional to the content of lactose.

Analyses of free amino acids and peptides in FM and YG FM and YG were homogenized at 3,000 rpm for 5 minutes. One volume (v/w) of 10 % trichloro-acetic acid solution was added to this homogenate in order to remove proteins. This mixture was centrifuged at $10,000 \times g$ for 30 minutes, and the supernatant was used as a sample for the analysis of free amino acids and peptides.

The free amino acids in this supernatant were analyzed by an amino acid analyzer (Shimadzu Co., Kyoto, Japan) using the method of *o*-phthalaldehyde derivatives. The contents of peptides in FM and YG were analyzed by the following method. The supernatant was hydrolyzed in 6 N HCl at 110°C for 24 hours. Amino acid in the hydrolyzed supernatant was also analyzed with an amino acid analyzer (Shimadzu) by the method of *o*-phthalaldehyde derivatives. The amino acid composition of peptides was determined by subtraction of free amino acids from total amino acids in the hydrolyzed supernatant.

Preparation of peptide fraction for sensory evaluation FM was homogenized and centrifuged at $10,000 \times g$ for 20 minutes and the supernatant was collected. Ethanol was added (final concentration, 80 %) to this supernatant, followed by centrifugation and filtration. The supernatant containing peptides was ultrafiltrated through a 1,000 molecular weight cut-off membrane (Amicon Co., Beverly, California, USA), and then the solution containing peptides with molecular weight over 1,000 was freeze-dried as peptide fraction for sensory evaluation.

Sensory evaluation The intensity of each basic taste was evaluated between FM and YG by 13 trained panelists, using a modification of the paired preference test. The contribution of organic acids to the sourness intensity was examined by the reconstituted solution on the basis of the analyses of the organic acids contents in both FM and YG. The 0.71 % and 0.78 % lactic acid solutions (pH 4.5) were prepared and evaluated. The 0.71 % lactic acid solution (pH 4.5) containing 0.20 % orotic acid and 0.78 % lactic acid solution (pH 4.5) containing 0.16 % orotic acid were also prepared and evaluated. Sensory evaluation was performed by 11 trained panelists. The pH of all solutions was adjusted to 4.5 with 1

N NaOH and 1 N HCl.

The contribution of lactose to the sweetness intensity was examined by the reconstituted solution on the basis of the analyses of the lactose contents in both FM and YG. The 3.84 % and 3.34 % lactose solutions were prepared and evaluated. Sensory evaluation was performed by 13 trained panelists. The effect of the addition of peptide fraction on taste intensity of each basic taste solution was examined by sensory evaluation with trained panels, using a modification of the paired preference test. Solutions of 0.2 % lactic acid (sourness), 0.04 % monosodium glutamate (umami), 0.001 % quinine hydrochloride (bitterness), 0.5 % sodium chloride (saltiness), and 1.0 % sucrose (sweetness) were used as basic taste solution. The pH of all solutions was adjusted to 4.5 with 1 N NaOH and 1 N HCl.

Statistical analysis All data in "Results" were expressed as means (standard deviation of values obtained from at least three independent experiments. Evaluation of statistical significance was determined by Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Changes in acidity and pH of FM and YG In the production of both FM produced by *L. lactis* and *S. thermophilus* and YG produced by *L. bulgaricus* and *S. thermophilus*, fermentation was stopped at 0.85 (0.05 % acidity as lactic acid). The changes in acidity and pH of FM and YG during fermentation are shown in Figs. 1 and 2. The acidity of YG increased linearly to 0.57 % at 4 hours and 0.85 % at 6.7 hours after the start of fermentation. Conversely, the acidity of FM rose linearly to 0.70 % at 8 hours after the start of fermentation, and then rose gradually to 0.79 % at 12 hours and 0.85 % at 16 hours. The fermentation time for the production

of FM was about twice that for the production of YG.

The pH value of YG decreased linearly to 5.2 at 4 hours and 4.5 at 6.7 hours after the start of fermentation. In contrast, the pH value of FM decreased slowly to 5.8 at 4 hours, 4.8 at 8 hours, 4.6 at 12 hours, and 4.5 at 16 hours after the start of fermentation. The changes in pH of both FM and YG negatively corresponded to changes in acidity.

Sensory evaluation of FM and YG Each taste of FM was compared with that of YG by sensory evaluation (Table 1). Although there was no difference in acidity (0.85 %) between both products, the sourness of FM was significantly weaker than that of YG, and the sweetness of FM was significantly stronger than that of YG. There were no significant differences in saltiness and umami between both FM and YG. Therefore, the weak sourness and strong sweetness resulted in the mildness of the taste of FM. Next, taste compounds of FM were analyzed and compared with those of YG.

Analyses of organic acids and lactose in FM and YG The contents of organic acids in FM and YG were analyzed

Table 1. Comparison of taste of FM and YG.

Sensory evaluation was performed by 13 trained panelists. Numbers in table indicated the number of panelists who judged the sample as possessing the stronger taste.

Taste	FM	YG
Sweetness	12 *	1
Sourness	2 *	11
Bitterness	10	3
Saltiness	5	8
Umami	9	4

Fig. 1. Changes in acidity FM and YG.

Acidity was measured by titration with 0.1 N NaOH and expressed by lactic acid equivalent (%). Vertical lines indicate standard deviation ($n=3$).

Fig. 2. Changes in pH of FM and YG.

Vertical lines indicate standard deviation ($n=3$).

with HPLC. In both products, lactic, orotic, uric and citric acids were detected, among which lactic and orotic acids had the highest contents. Lactic and orotic acid contents in FM were 710 mg/100 g and 202 mg/100 g, respectively, while the contents of lactic and orotic acids in YG were 780 mg/100 g and 164 mg/100 g, respectively (Table 2). There were no differences in the contents of uric and citric acids between FM and YG. The content of lactic acid in FM was significantly smaller than that in YG, while the content of orotic acid in FM was significantly larger than that in YG.

The contribution of major organic acids to sourness intensity in both FM and YG was evaluated by sensory evaluation (Fig. 3). The sourness intensity of 0.71 % lactic acid solution

at pH 4.5 was significantly weaker than that of 0.78 % lactic acid solution at pH 4.5. Additionally, the sourness intensity of 0.71 % lactic acid solution containing 0.20 % orotic acid was also significantly weaker than that of 0.78 % lactic acid solution containing 0.16 % orotic acid at pH 4.5. These results indicated that sourness intensity was related to the lactic acid content in both products and was not influenced by the content of orotic acid.

The lactose content was analyzed with kits using the enzyme method. The content of lactose in FM and YG was 3.84 g/100 g and 3.34 g/100 g, respectively (Fig. 4). This difference seemed to be caused by the difference in the conversion rate of lactose to lactic acid.

Table 2. Content of organic acids in FM and YG.

All values are mean±standard deviation for three samples. Details in the measurement of organic acids are given in "Materials and Methods".

	Organic acids (mg/100 g)				
	Lactic acid	Orotic acid	Uric acid	Citric acid	Total
FM	710±0.029 *	202±8.2 *	4.03±0.150	1.97±0.081	918.0±8.46
YG	780±0.031	164±6.3	4.08±0.059	2.00±0.082	950.1±10.49

*Significantly different ($p<0.05$) from YG.

Fig. 3. Comparison of sourness intensity of the major organic acids solution reconstituted on the basis of the analysis of FM and YG.

The 0.71 % (FM) and 0.78 % (YG) lactic acid solutions (pH 4.5) were prepared and evaluated (A). The 0.71 % lactic acid solution (pH 4.5) containing 0.20 % orotic acid (FM) and 0.78 % lactic acid solution (pH 4.5) containing 0.16 % orotic acid (YG) were also prepared and evaluated (B). Sensory evaluation was performed by 11 trained panelists. Numbers in figure indicate the number of panelists who judged the sample as possessing stronger sourness.

*Significantly different ($p<0.05$).

Fig. 4. Lactose content of FM and YG.

Vertical lines indicate standard deviation (n=3).

*Significantly different ($p < 0.05$). Details of the measurement of lactose were given in "Materials and Methods".

Fig. 5. Peptide contents of FM and YG.

Vertical lines indicate standard deviation (n=3). Details of the measurement of peptides were given in "Materials and Methods".

Table 3. Content of free amino acids in FM and YG.

All values are mean ± standard deviation for three samples. Details in the measurement of organic acids are given in "Materials and Methods".

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Cys
FM	0.250 ±0.023 *	0.145 ±0.048 *	0.242 ±0.114 *	1.934 ±0.403 *	1.368 ±0.110 *	0.125 ±0.053 *	1.068 ±0.063 *	0.053 ±0.032 *	ND ^a
YG	1.263 ±0.117	0.304 ±0.018	1.305 ±0.110	3.243 ±0.354	5.072 ±0.315	0.554 ±0.163	1.991 ±0.215	0.412 ±0.103	ND
Threshold value ^b	3	260	150	5	300	110	60	150	— ^c

	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
FM	ND	0.008 ±0.007 *	0.011 ±0.011 *	0.663 ±0.078	0.106 ±0.003 *	0.071 ±0.007 *	0.116 ±0.012 *	0.375 ±0.056 *	6.536 ±0.262 *
YG	ND	0.219 ±0.045	0.655 ±0.128	0.611 ±0.018	0.349 ±0.002	1.187 ±0.071	0.347 ±0.012	0.772 ±0.028	18.285 ±1.030
Threshold value	30	90	380	—	150	20	50	10	—

^a Not detected; ^b threshold value was quoted from Amino Acids Handbook (Ajinomoto Co. Inc., 2003); ^c data not shown; *significantly different ($p < 0.05$) from YG.

The contribution of lactose to sweetness intensity in both FM and YG was evaluated by sensory evaluation (date not shown). The sweetness intensity of 3.84 % lactose solution was not significantly weaker than that of 3.34 % lactose solution, indicating that sweetness intensity was not influenced by lactose.

Analyses of free amino acids and peptides in FM and YG
The contents of free amino acids in FM and YG were measured, and are shown in Table 3. Total free amino acids in FM were smaller than those in YG. The major free amino acids in both products were glutamic acid, proline, and alanine.

The contents of glutamic acid, proline and alanine were 1.93 mg/100 g, 1.37 mg/100 g and 1.07 mg/100 g in FM, respectively, and 3.23 mg/100 g, 5.07 mg/100 g and 1.99 mg/100 g in YG, respectively. The concentration of these amino acids was lower than the threshold level (glutamic acid and proline, 5 mg/100 g and 300 mg/100g, respectively), indicating that free amino acids had little contribution to the taste of FM and YG.

The contents of peptides in FM and YG were measured, as shown in Fig. 5. Peptide content of FM was smaller than that of YG, at 46.7 mg/100 g and 52.7 mg/100 g, respec-

tively. The effect of peptide fraction in FM on basic taste was evaluated by sensory evaluation, because it has been recently reported that sourness-suppressing peptides played an important role in suppressing sourness in meat (Okumura *et al.*, 2004). The sensory evaluation showed that the addition of peptide fraction prepared from FM had no effect on the basic taste of solution (data not shown).

Discussion

In this study, we characterized the sensory properties and taste compounds of FM produced by *L. lactis* and *S. thermophilus* and compared them with those of YG produced by *L. bulgaricus* and *S. thermophilus*. In taste quality, the sourness of FM was significantly weaker than that of YG, although the contents of all organic acids, acidity and pH of FM were almost the same as those of YG. It was concluded that the content of lactic acid in both products affected the sourness intensity from the following results: (1) lactic acid content in YG was larger than that in FM; (2) orotic acid had no influence on the difference in sourness intensity of FM and YG; and (3) the contents of other taste compounds such as citric acid, uric acid, lactose, free amino acids and peptides were too low to contribute to the taste of FM. In yogurt, the content of lactic acid plays an important role in its taste (Yuguchi *et al.*, 1989). In this study, lactic acid has also been shown to play an important role in the sourness intensity of FM. Thus, the intensity of sourness in fermented milk did not appear to be necessarily decided by organic acid content, acidity, or pH.

The lower content of lactic acid in FM than YG resulted in the weaker sourness and stronger sweetness of FM (Table 1), indicating that FM seemed to be milder than YG. Fermented milk with low acidity has been reported to show relatively stronger sweetness, and it has been shown that sourness and astringency increase and pH and sweetness decrease as acidity gradually increases (Debbie *et al.*, 1991; Steven *et al.*, 1991). From these reports, acidity was shown to be an important factor in taste quality. In this study, however, the sourness intensity of FM was weaker than that of YG, even though acidity was almost equivalent. Therefore, lactic acid content was also clarified to play an important role in the taste quality of the fermented milk in this study.

Furthermore, it is possible that the ratio of L/D lactic acid as well as lactic acid content also influenced the difference in sourness intensity between FM and YG. The sourness of yogurt containing large amounts of L-lactic acid has been shown to be lower than that of yogurt containing large amounts of D-lactic acid (Tamime and Robinson, 1985). Although, the ratio of L/D lactic acid was not examined in this study, FM was thought to contain more L-lactic acid than

YG, due to the fact that the *L. lactis* and *S. thermophilus* used for FM production were previously shown to produce mainly the L form of lactic acid (Thomas, 1976; Garvie, 1978; Benner, 1976). In contrast, *L. bulgaricus* used for YG production was previously shown to produce mainly the D form of lactic acid (Gasser, 1970; Gasser and Gasser, 1971). From these reports, it was considered that the higher ratio of L-lactic acid in FM, as well as lactic acid content, also caused the weaker sourness of FM. The clarification of the ratio of L and D lactic acids in FM is the next issue to be addressed.

It took 16 hours and 6.7 hours for FM and YG, respectively, to reach to 0.85 % of acidity (Fig. 1). This difference in fermentation times of both products was caused by the differences in temperature of fermentation and the combination of lactic acid bacterium between FM and YG. In the production of FM, *L. lactis* in mesophilic bacterium (Miyoshi *et al.*, 2003) and *S. thermophilus* in thermophilic one (Tamime and Robinson, 1985) were used as lactic acid bacterium of starter. However, *L. bulgaricus* and *S. thermophilus*, which were both thermophilic bacteria (Tamime and Robinson, 1985), were used in YG production. The difference in starter led to the different condition of the fermentation temperature. That is, FM and YG were fermented at 38°C and 42°C, respectively. As a result, the production rate of organic acids at 38°C in FM seemed to be smaller than that at 42°C in YG.

The symbiosis relationship between two types of lactic acid bacteria was thought to give influence to the difference in the fermentation time. It is reported that *L. bulgaricus* and *S. thermophilus* have a good symbiosis relationship in the production of essential amino acids during fermentation (Bautista *et al.*, 1966; Higashio *et al.*, 1977a). In particular, the production of histidine, leucine and isoleucine brings about growth of lactic acid bacteria in YG production. However, there does not seem to be a strong symbiotic relationship between *L. lactis* and *S. thermophilus* in this study, as the content of free amino acids, such as valine, leucine, isoleucine and histidine in FM was significantly smaller than that in YG. Thus, the combination of lactic acid bacteria was clarified to contribute to the formation of the different tastes of fermented milk in this study.

The contents and composition of free amino acids in FM were also clarified and compared with that in YG. The contents of free amino acids in FM (6.5 mg/100 g) were about one-third of that in YG (18.3 mg/100 g). It was thought that this difference was caused by the strong activity of proteases of *L. bulgaricus* in YG. The major amino acids in FM were glutamic acid, proline and alanine, while those in YG were proline, glutamic acid, alanine, serine, asparagine acid, and histidine. In fermented milk, free amino acid, as well as lactose, is known to be materials as precursor of aroma com-

pounds (Ott *et al.*, 2000b). In this study, the aroma characteristic of FM was different from that of YG (date not shown). This difference in aroma between products might be caused by the difference in free amino acid contents as precursor. The clarification of the relationship between aroma and free amino acid composition is the next issue to be addressed.

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