Short communication: Change of naturally occurring benzoic acid during skim milk fermentation by commercial cheese starters

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ABSTRACT

This study sought to investigate the change of naturally occurring benzoic acid (BA) during skim milk fermentation by 4 kinds of commercial cheese starters used in domestic cheese. The culture was incubated at 3-h intervals for 24 h at 30, 35, and 40°C. The BA content during fermentation by *Streptococcus thermophilus* STB-01 was detected after 12 h at all temperatures, sharply increasing at 30°C. In *Lactobacillus paracasei* LC431, BA was detected after 9 h at all temperatures, sharply increasing until 18 h and decreasing after 18 h at 30 and 35°C. In the case of R707 (consisting of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*), BA increased from 6 h to 15 h and decreased after 15 h at 40°C. The BA during STB-01 and CHN-11 (1:1; mixture of *S. thermophilus*, *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *diacetylactis*, *Leuconostoc mesenteroides* ssp. *cremoris*) fermentation was detected after 3 h at 35 and 40°C, sharply increasing up to 12 h and decreasing after 15 h at 35°C, and after 6 h, increasing up to 9 h at 30°C. After 3 h, it steadily decreased at 40°C. The highest amount of BA was found during the fermentation by R707 at 30°C; 15 h with 12.46 mg/kg.

Key words: benzoic acid, skim milk, fermentation, cheese starter

Short Communication

Natural preservatives are produced via the metabolic process of plants and animals as food materials or food processing such as fermentation. Benzoic acid, sorbic acid, and propionic acid, the compounds of food preservatives, are made of natural ingredients and are naturally produced during fermentation. In the case of

preservatives such as benzoic acid, controversy over the food standard steadily increased because there was no exact distinction between added synthetic preservative and natural preservative (Meyer et al., 2002). In addition, judging whether synthetic additions are present in the final product will often be difficult because a preservative such as benzoic acid can be naturally present regardless of raw material characteristics and manufacturing technology (Iammarino et al., 2011).

Benzoic acid is a naturally occurring metabolic product with a free or combined form and found in several plants and animals (Hegnauer, 1992). Benzoic acid is not permitted as an additive in milk but is found in cheese, fermented cream, yogurt, and other dairy products (Urbienė and Leskauskaitė, 2006; Horníčková et al., 2014). It has been reported that benzoic acid content in fermented milk and cheeses was detected up to 50 mg/kg (Sieber et al., 1995). Rukkwamsuk et al. (2012) reported that average concentrations of benzoic acid in composite milk gradually increased from 0.09 ppm at 0 h to 0.13 ppm at 6 h after collection. Also, Toppino et al. (1990) reported that up to 60 mg/kg benzoic acid was detected in Provolone cheese samples. It is affected by the microorganisms used in the production of dairy products (Kisza et al., 1984; Sajko et al., 1984; Hejtmánková et al., 2000). *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* can synthesize benzoic acid in milk (Sajko et al., 1984; García et al., 2003; Garmiene et al., 2010; Yildiz et al., 2012). According to Urbienė and Leskauskaitė (2006), the benzoic acid produced during the fermentation of milk by 3 different commercial dairy starters (La-5, ABT-2, and YC-180) was approximately 14 to 23 mg/kg. During the fermentation of milk with La-5, the benzoic acid content was about 5 times higher than that at the beginning of the fermentation process. The highest concentration of benzoic acid was detected in the sample fermented by La-5.

Mroueh et al. (2008) reported that the benzoic acid content of yogurt using commercial starters MYE95, MY900, CH1, and LYOFASY 4.80F was reportedly 4.7, not detected, 14.7, and 8.5 mg/kg, respectively.

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Table 1. Precision and accuracy data for the detection of benzoic acid

Compound	Spiked concentration ($\mu\text{g/mL}$)	Detected concentration ¹ ($\mu\text{g/mL}$)	Recovery ¹ (%)	Intra-day (n = 3, CV%) ²	Inter-day (n = 9, CV%)
Benzoic acid	4.00	3.81 $\pm$ 0.04	95.35 $\pm$ 0.89	2.09	3.19
	2.00	1.97 $\pm$ 0.02	98.60 $\pm$ 0.80	1.82	1.97
	1.00	1.00 $\pm$ 0.02	99.83 $\pm$ 1.78	4.02	2.66

¹Values are shown as the mean $\pm$ SEM.²CV% indicates the coefficient of variation in percentage.

After 25 d of storage, the development of benzoic acid of yogurt samples steadily increased. The benzoic acid content differed according to the kind of starters and fermentation during storage time.

Thus, this study aimed to investigate the change of naturally produced benzoic acid during skim milk fermentation by 4 kinds of commercial cheese starters [STB-01, LC431, R707, and STB-01+CHN11 (1:1)] at different temperature.

Four kinds of cheese starters were used: STB-01 composed of *Streptococcus thermophilus*; LC431 composed of *Lactobacillus paracasei*; R707 composed of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*; and STB01+CHN11 (1:1) composed of *Streptococcus thermophilus*, *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *diacetylactis*, and *Leuconostoc mesenteroides* ssp. *cremoris*. The cheese starters were usually used for making cheese such as Camembert cheese, Brie cheese, string cheese, fresh cheese, and mozzarella cheese in industry. These cheese starters were obtained from Christian Hansen Lab (Hørsholm, Denmark).

The number of viable starter strains was determined by serial 10-fold dilution in 0.1% peptone water. About 10^5 cfu/mL of starter strain was inoculated into 10% reconstituted skim milk; the culture was then incubated at 3-h intervals for 18 h at 30, 35, and 40°C. All 4 plates were incubated aerobically at 37°C for 48 h using a BCP plate count agar (Eiken, Tokyo, Japan). The pH was measured using a pH meter (Inolab 7110 pH BNC, WTW, Weilheim, Germany).

For analysis of benzoic acid production, sample preparation was conducted in accordance with the Korean Food Additives Codex (MFDS, 2014). A 5-g sample was dissolved in 50 mL of distilled water, and 5 mL of this solution was mixed with 0.5 mL of 0.1 N HCl and 0.5 mL of 0.005 M cetyltrimethyl ammonium chloride solution (sample solution). A Sep-Pak C18 cartridge (Waters, Milford, MA) was preconditioned for benzoic acid by consecutively passing 10 mL of methanol and 10 mL of 0.005 M cetyltrimethyl ammonium chloride solution. The sample solutions passed through the Sep-Pak C18 cartridge at a flow rate of 2 mL/min. Afterward the Sep-Pak C18 cartridge was washed with 10 mL of distilled

water and eluted with 10 mL of methanol to fill with 100% methanol. The entire solution was filtered with a 0.45- μm filter before being injected into the HPLC system. Commercial standards of benzoic acid with 99% purity were purchased from Sigma (Saint Louis, MO). The HPLC-grade solvents were purchased from J. T. Baker (Center Valley, PA). Other reagents (analytical grade) were purchased from Wako (Tokyo, Japan). An analysis of benzoic acid was performed using HPLC with a photodiode array detector (PDA; Shiseido Co. Ltd., Tokyo, Japan). The operating conditions were as follows: column temperature of 35°C, flow rate of 1.0 mL/min, injection volume of 10 μL , and PDA detection at 217 nm. The maximum extinction wavelength of benzoic acid was 230 nm. Chromatographic separations were performed on SP Column MF C8 (5.0 μm particle size: 150 $\times$ 4.6 mm i.d.; Shiseido Co. Ltd.), with the mobile phase consisting of 0.1% tetrabutylammonium hydroxide (Phase A) and 100% acetonitrile (Phase B). The following gradient was applied: 0 to 2.5 min, 25% B; 2.5 to 7.0 min, 25 to 35% B; 7.0 to 12.0 min, 35 to 40% B; and 12.0 to 15.0 min, 40 to 30% B. Linearity test was performed by 3 series of analysis on 3 different days by injecting 2 mixture standard solutions at 1.00, 2.00, and 4.00 $\mu\text{g/mL}$. Limits of detection (LOD) and quantification (LOQ) were calculated according to the following equations: $\text{LOD} = 3.3 \times s/S$ and $\text{LOQ} = 10 \times s/S$, where s is the standard deviation of y intercepts of regression analysis and S is the slope of a calibration curve. Recovery was calculated using a standard addition method.

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL). The differences were analyzed using one-way ANOVA with Duncan's multiple range tests. Values of $P < 0.05$ and $P < 0.01$ were considered statistically significant. All data are expressed as the mean $\pm$ standard error of the mean.

To evaluate the HPLC-PDA method, linearity, limits of detection, limits of quantification, recovery, and reproducibility were calculated. The LOD is defined as the smallest peak detected with signal height that is 3 times that of the baseline; LOQ refers to the lowest level of analysis that can be determined with an accept-

Table 2. Viable cell count, benzoic acid content, and pH of skim milk fermented by cheese starters at 30, 35, and 40°C

Starter	Incubation temperature (°C)	Incubation time (h)						
		0	3	6	9	12	15	18
STB-01	Viable cell count ¹ (log cfu/mL)	5.2 ± 0.0	5.4 ± 0.1	6.3 ± 0.0	7.2 ± 0.0	8.0 ± 0.1	8.2 ± 0.0	8.6 ± 0.0
		5.2 ± 0.0	6.2 ± 0.0	8.1 ± 0.0	8.6 ± 0.1	8.7 ± 0.0	9.5 ± 0.2	9.9 ± 0.0
	Benzoic acid ¹ (mg/kg)	5.2 ± 0.0	6.9 ± 0.0	8.7 ± 0.0	8.8 ± 0.1	9.0 ± 0.0	9.6 ± 0.2	10.7 ± 0.0
		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.1	3.1 ± 0.0**	3.3 ± 0.3**
LC431		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.1	1.8 ± 0.1	1.9 ± 0.4
	pH	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.1*	1.6 ± 0.1	1.5 ± 0.1	1.9 ± 0.2	1.7 ± 0.2
		6.4	5.95	5.96	5.92	5.64	5.2	5
		6.4	6.29	5.91	5.1	4.8	4.67	4.59
R707	Viable cell count (log cfu/mL)	6.4	6.24	5.15	4.74	4.57	4.48	4.39
		5.5 ± 0.0	5.8 ± 0.0	6.0 ± 0.0	6.5 ± 0.0	7.0 ± 0.0	7.9 ± 0.0	8.6 ± 0.1
	Benzoic acid (mg/kg)	5.5 ± 0.0	5.8 ± 0.0	6.2 ± 0.0	7.0 ± 0.0	8.1 ± 0.0	9.7 ± 0.0	9.9 ± 0.2
		5.5 ± 0.0	5.8 ± 0.0	6.3 ± 0.0	7.3 ± 0.0	8.3 ± 0.1	8.7 ± 0.0	9.8 ± 0.1
STB-01 + CHN11 (1:1)		0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.1	2.6 ± 0.1	8.1 ± 0.3	9.7 ± 0.2*	9.7 ± 0.3*
	pH	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.5 ± 0.1	9.3 ± 0.2*	9.0 ± 0.5*	11.1 ± 0.1**
		0.0 ± 0.0	0.0 ± 0.0	2.4 ± 0.3	4.0 ± 0.3	2.8 ± 0.1	3.6 ± 0.2	3.3 ± 0.1
		6.2	6.2	6.2	6.2	6.2	6.1	5.4
R707	Viable cell count (log cfu/mL)	6.2	6.1	6.1	6.1	6.0	5.3	4.7
		4.3 ± 0.0	6.1 ± 0.0	7.3 ± 0.0	8.6 ± 0.0	8.9 ± 0.0	9.5 ± 0.0	10.5 ± 0.1
	Benzoic acid (mg/kg)	4.3 ± 0.0	6.0 ± 0.0	7.5 ± 0.0	8.6 ± 0.0	8.8 ± 0.0	8.9 ± 0.1	10.1 ± 0.1
		4.3 ± 0.0	6.2 ± 0.0	7.3 ± 0.0	7.7 ± 0.0	8.0 ± 0.0	8.4 ± 0.0	9.5 ± 0.1
STB-01 + CHN11 (1:1)		0.0 ± 0.0	0.0 ± 0.0	9.3 ± 0.2	12.1 ± 0.6**	10.4 ± 0.2*	12.5 ± 0.2**	10.5 ± 0.3*
	pH	0.0 ± 0.0	2.2 ± 0.0	8.8 ± 0.3	8.4 ± 0.1	10.5 ± 0.8*	7.4 ± 0.2	7.6 ± 0.3
		0.0 ± 0.0	5.7 ± 0.2	5.3 ± 0.1	6.3 ± 0.1	8.3 ± 0.2	9.3 ± 0.3	6.1 ± 0.3
		6.2	6.2	6.1	5.8	4.8	4.5	4.4
R707	Viable cell count (log cfu/mL)	6.2	6.1	6.0	5.3	4.7	4.6	4.5
		4.2 ± 0.1	4.8 ± 0.1	5.7 ± 0.1	7.3 ± 0.0	7.9 ± 0.1	8.6 ± 0.1	8.2 ± 0.0
	Benzoic acid (mg/kg)	4.2 ± 0.1	5.3 ± 0.0	7.4 ± 0.0	8.4 ± 0.1	8.6 ± 0.1	9.1 ± 0.1	9.1 ± 0.0
		4.2 ± 0.1	6.1 ± 0.0	8.6 ± 0.0	8.7 ± 0.0	8.8 ± 0.0	8.9 ± 0.0	8.9 ± 0.0
STB-01 + CHN11 (1:1)		0.0 ± 0.0	0.0 ± 0.0	5.1 ± 0.1	9.5 ± 0.2**	8.5 ± 0.2*	5.5 ± 0.2	4.7 ± 0.1
	pH	0.0 ± 0.0	4.8 ± 0.1	5.7 ± 0.1	6.3 ± 0.1	8.1 ± 0.2	7.6 ± 0.2	6.3 ± 0.1
		0.0 ± 0.0	4.9 ± 0.2	1.1 ± 0.0	1.2 ± 0.0	1.0 ± 0.1	1.9 ± 0.0	1.9 ± 0.1
		6.4	6.3	6.3	6.1	5.5	4.9	4.6
R707	Viable cell count (log cfu/mL)	6.4	6.4	6.3	5.3	4.9	4.8	4.4
		4.2 ± 0.1	4.8 ± 0.1	5.7 ± 0.1	7.3 ± 0.0	7.9 ± 0.1	8.6 ± 0.1	8.2 ± 0.0
	Benzoic acid (mg/kg)	4.2 ± 0.1	5.3 ± 0.0	7.4 ± 0.0	8.4 ± 0.1	8.6 ± 0.1	9.1 ± 0.1	9.1 ± 0.0
		4.2 ± 0.1	6.1 ± 0.0	8.6 ± 0.0	8.7 ± 0.0	8.8 ± 0.0	8.9 ± 0.0	8.9 ± 0.0

¹Values are shown as the mean ± SEM.* $P < 0.05$, ** $P < 0.01$.

able degree of confidence. At the same time, recovery was determined by 3 sets of samples with benzoic acid at concentrations of 1.0, 2.0, and 4.0 µg/mL. Recoveries were obtained at levels 1.00, 1.97, and 3.81 µg/mL in samples, varying from 99.83, 98.60, and 95.35%, respectively (Table 1). The linearity range was 0.20 to 8.00 µg/mL. In the regression equation $y = ax + b$, where y is the peak area, x refers to the concentration of compounds (µg/mL), a is the slope, and b is the intercept, the slope, intercept, and correlation coefficient were calculated to be 8.63×10^3 , -2.34×10^3 , and 1.00, respectively. The LOD and LOQ values were calculated to be 0.08 and 0.23 µg/mL, respectively.

The changes in viable cell count, benzoic acid content, and pH during the fermentation of skim milk with 4 kinds of cheese starters were in Table 2. The optimum growth temperature of *S. thermophilus* STB-01 culture was 40°C. The pH continuously decreased in accordance with the fermentation time. In particular, pH steadily decreased during fermentation time at 40°C from pH 6.4 to pH 4.4. In terms of benzoic acid content, the highest value was recorded after 15 h incubation from 30°C with 3.3 mg/kg. Lactic acid bacteria could convert hippuric acid of phenylalanine, which is naturally present in milk, into benzoic acid (Nishimoto et al., 1969; Sieber et al., 1995; Hejtmánková et al., 2000; Tavaría et al., 2002). Oxidation of benzaldehyde may also contribute to benzoic acid production (Sieber et al., 1995). Another possible mechanism for the production of benzoic acid could be the anaerobic metabolism of phenol (Ping et al., 2009). It could be considered a natural component of milk and milk products.

The number of viable *Lactobacillus paracasei* LC431 cells was sharply increased at 35 and 40°C. The optimum growth temperature of *L. paracasei* LC431 was 35°C. During fermentation time, the growth of LC431 reached 10.2 log cfu/mL. Moreover, the production of benzoic acid constantly increased at 35°C from 12 to 18 h up to 11.13 mg/kg. The changes in pH showed a similar pattern with changes in benzoic acid production. The pH values rapidly decreased after 9 h up to pH 4.23 at 40°C. In case of R707 culture, the optimum growth temperature was 30°C, and the growth of R707 was up to 10.5 log cfu/mL at the end of fermentation. Skim milk inoculated with R707 contained up to 12.5 mg/kg of benzoic acid after 15 h fermentation at 30°C. Such was the highest production of benzoic acid among other commercial starters including STB-01, LC431, and STB01+CHN11. The benzoic acid content decreased after 18 h. Benzoic acid considered to have decomposed through denitrifying (Ziegler et al., 1987). Braun and Gibson (1984) investigated the aerobic and anaerobic growth on the benzoate of a denitrifying *Pseudomonas* sp. strain, KB740. Evans (1977) found

that bacteria can degrade benzoic acid in the absence of molecular O₂ through nitrate respiration.

The mixed STB-01 and CHN11 culture tended to lead the strain's growth by *Streptococcus thermophilus*. At 40°C, during the next 6 h, the mixed STB-01 and CHN11 strain cultures and pH changed markedly. In the case of 30 and 35°C, the strain cultures steadily grew up to about 9 log cfu/mL for 15 h. During 6 h fermentation, the strain cultures showed a lag phase, with the pH reaching 5.5. The amounts of benzoic acid were detected after 3 h at 35 and 40°C sharply increasing up to 12 h and decreasing after 15 h at 35°C and after 6 h, increasing up to 9 h at 30°C. Benzoic acid is considered to decrease with the lactic acid bacteria reaching the death phase after a stationary phase during lactic acid bacteria's metabolic process (Yildiz et al., 2012). The benzoic acid was naturally produced and the content of benzoic acid was different depending on the incubation time, temperature, and the kinds of starters. The range of benzoic acid content was 0.0–12.5 mg/kg, and the highest concentration of benzoic acid was detected in the skim milk fermented by R707 composed of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*.

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