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Identification of lactic acid bacteria and yeasts, and characterization of food components of sourdoughs used in Japanese bakeries

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Sourdough is a low-pH, fermented product prepared using lactic acid bacteria and yeast mixed with rye flour, wheat flour, and water. It is used and backslopped in bakeries because it enhances texture, flavor, and dough expansion of bread. Various lactic acid bacteria and yeasts have been identified in sourdough, especially in the West. However, microbial and physical characteristics of sourdough from Japan have not been investigated. Here, we characterized the microbial composition and food component characteristics of sourdough from four bakeries in Kansai region, Japan, and performed sensory and quality evaluation of baguettes enriched with 10% sourdough. We detected different species of lactic acid bacteria such as *Lactobacillus brevis*, *Lactobacillus alimentarius*, *Lactobacillus pentosus*, *Lactobacillus vaccinostercus*, *Lactobacillus sanfranciscensis*, and *Lactobacillus sakei*. The identified yeasts primarily included *Saccharomyces cerevisiae*, with *Candida humilis* detected in some samples. Components such as amino acids, lactic acid, acetic acid, ethanol, 3-methyl-1-butanol, ethyl acetate, and phenethyl alcohol differed among samples and distinctively affected flavor, quality, and aroma of sourdough-enriched baguettes. The different species of lactic acid bacteria and the ratio of lactic acid bacteria to yeasts possibly affected food components such as free amino acids, sugars, and organic acids via the Maillard reaction, which influences the savory aromas of bread. Future investigation of the effect of lactic acid bacteria will help to improve the overall quality of bread.

[**Key words:** Lactobacillales; *Saccharomyces cerevisiae*; Lactic acid; Sourdough; Bakery]

Sourdough, a fermented product prepared with lactic acid bacteria and yeast, is used as an ingredient for bread making (1–5). Leaven from France represents a traditionally produced sourdough variety (6). Leaven is produced via natural fermentation by adding flour, such as rye and wheat, and mixing with water. Before use as a bread ingredient, a small amount of the seed starter is mixed with new wheat and water, and the production continues with repeated fermentation (6). Sourdoughs reportedly improve the flavor (7) and mouth-feel (8) of bread and exert beneficial effects, such as suppression of mold growth (9–12). In recent years, sourdoughs have been prepared from flour produced from grains other than rye and wheat to improve the quality of gluten-free bread made for individuals with celiac disease, who cannot eat wheat gluten (12).

Thus, sourdough fermentation impacts the nutritional and functional qualities of bakery products. Further, microbial metabolism of lactic acid bacteria and yeasts acidifies the dough, activates cereal-derived enzyme, and promotes protein degradation (10,13). In particular, extracellular proteases and peptidases increase the peptide and free amino acid content of sourdough during fermentation (10,13). Peptides and free amino acids participate in Maillard (amino-carbonyl) reactions, generating compounds that influence the aroma of bread, depending on baking time (14). Lactic acid bacteria and yeasts from traditionally backslopped sourdough from various regions have been isolated and identified (15–19). Indeed, diverse lactic acid bacteria and yeast species are found in sourdough, particularly in the West (20,21). Culturing behavior and metabolism during fermentation of many lactic acid bacteria have been described, especially those of *Lactobacillus sanfranciscensis* (4,22).

Compared with those in the West, bread and sourdough have only a short history in Japan (6). However, they have become widespread in recent years, with Western model-based sourdough production widely employed in bakeries in Japan (6). Nevertheless, studies of the species of lactic acid bacteria and yeasts used in Japanese bakeries and of food components of the sourdough are limited. To address this, in the current study, we investigated the microbial and sensory characteristics of sourdoughs, and the properties of the resultant bread, backslopped in four bakeries in the Kansai region of Japan.

MATERIALS AND METHODS

Ingredients, storage methods, and preparing conditions of each bakery Sourdough samples (approximately 500 g) from four bakeries in the Kansai region were split into two sterile bags. One bag was refrigerated (4°C) and one frozen (−20°C). The numbers of lactic acid bacteria and yeast in the refrigerated samples were evaluated within 24 h, as described below. The frozen samples were

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stored for two or more days in a freezer. They were thawed under running water before prompt analytical and bread-making evaluation.

The conditions of sourdough backslopping and fermentation in each bakery are shown in Table 1.

Determination of the number of lactic acid bacteria and yeasts Lactic acid bacteria in the sourdoughs were detected on a modified MRS agar (Homohiochii-medium) (23) containing 10 g casein peptone, 2 g meat extract, 7 g yeast extract, 7 g glucose, 7 g maltose, 7 g fructose, 2 g sodium gluconate, 1 g Tween 80, 2.5 g K_2HPO_4, 5 g sodium acetate, 5 g diammonium hydrogen citrate, 200 mg $MgSO_4 \cdot 7H_2O$, 50 mg $MnSO_4 \cdot nH_2O$, 0.5 g cysteine hydrochloride, 20 g agar, 10 mg/L cycloheximide, and 1000 mL distilled water, pH 5.4. Yeasts in the sourdoughs were detected on YPD agar (10 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract, 100 mg/L chloramphenicol, and 20 g agar in 1000 mL of distilled water, pH 6.2). Surface smears of 100-μL sourdough samples, serially diluted in sterile physiological saline (0.8% NaCl), were applied to each culture dish and incubated for 48 h at 30°C. The detection limit for each bacterial count test was 100 colony forming units (CFU)/g, with bacterial counts performed three times for each respective sourdough type. The mean values for each respective bacterial count were calculated.

Microbial species identification Microbes were isolated from 50 random colonies from each agar, and colony morphology and microscopic characteristics were evaluated. Species of lactic acid bacteria were identified using a FAST MicroSEQ 500 16S rDNA bacterial polymerase chain reaction (PCR) kit and sequencing kit (Thermo Fisher Scientific, Foster City, CA, USA). The fungal species were identified using a FAST MicroSEQ D2 LSU rDNA fungal PCR kit and sequencing kit (Thermo Fisher Scientific). Microbes from colonies were transferred to 1.5-mL microcentrifuge tubes containing 100 μL of PrepMan ultra sample preparation reagent (Thermo Fisher Scientific). The samples were then heated in a heat block at 100°C for 10 min before centrifugation (8947 × *g*, 5 s). The supernatant was used as the DNA solution for the PCR, 5 μL of which was diluted in 495 μL of nuclease-free water in a 1.5-mL microcentrifuge tube. Next, 15 μL of FAST MicroSEQ 500 PCR master mix (or FAST MicroSEQ D2 LSU rDNA fungal PCR master mix) and 15 μL of the diluted DNA solution were combined and placed in a thermal cycler (Veriti 96-well thermal cycler, Thermo Fisher Scientific). The reaction conditions for the PCR for bacteria were set with an initial step of 95°C for 10 s; followed by 30 cycles of denaturation at 95°C for 0 s, annealing at 64°C for 15 s with a final elongation at 72°C for 1 min, and a hold at 4°C. For fungi, the reaction conditions were the same as for bacteria except with 35 instead of 30 cycles. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Next, 13 μL of MicroSEQ 500 Forward or Reverse sequence mix (in the case of fungi, MicroSEQ D2 LSU rDNA fungal Forward or Reverse sequence mix) (Thermo Fisher Scientific) was added to 7 μL of the purified reaction product, and a sequencing reaction was performed using a thermal cycler (Veriti 96-well thermal cycler, Thermo Fisher Scientific). The reaction conditions were set to an initial step of 96°C for 1 min; 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 1 min 15 s; and a final hold at 4°C. The elongation products were purified using a Dye Ex 2.0 spin kit (Qiagen) and transferred to a capillary electrophoresis device (ABI PRISM, 3130xl Genetic Analyzer, Thermo Fisher Scientific) for DNA sequencing. The DNA sequence was determined using the 3130xl series data collection 4 software (Thermo Fisher Scientific) by electrophoresis on a 3100/3130x 50-cm capillary array (POP-6 polymer, Thermo Fisher Scientific). The obtained sequences were analyzed for homology with microbial sequences using the MicroSEQ ID software version 2 (Thermo Fisher Scientific). Homology of 99.0% or more indicated a microbial species.

pH measurements and organic acid analysis Sourdough samples (5 g) were diluted five-fold in deionized water. The pH of each suspension was then determined using a Seven Easy pH meter (Mettler-Toledo International Inc., Tokyo, Japan). For organic acid analysis, each diluted suspension was centrifuged (8947 × *g*, 5 min, 20°C), and 1 mL of the supernatant was transferred to a new tube. Next, 20 μL of 20% (w/v) sulfosalicylic acid was mixed with 1 mL of the supernatant and filtered through a 0.45-μm membrane filter. Organic acid analysis was performed by high-performance liquid chromatography (HPLC) using an LC10A Series device (Shimadzu, Kyoto, Japan) and an organic acid column (7.8 mm × 300 mm; Waters, Milford, MA, USA), with a column temperature of 40°C. The solvents used were the A buffer phase (9.51 g *p*-toluenesulfonic acid in 100 mL of distilled water) and B buffer phase (9.51 g *p*-toluenesulfonic acid, 41.85 g Bis-Tris, and 0.29 g EDTA-2Na in 100 mL of distilled water) at a flow rate of 0.8 mL/min. The RID-10A (Shimadzu) refractive index (RI) detector was used.

Sugar analysis Sourdough samples (5 g) were homogenized in 25 mL of 50% (v/v) aqueous acetonitrile, placed in a 50-mL test tube, and centrifuged (8947 × *g*, 5 min, 20°C). Next, 20 μL of 20% (w/v) sulfosalicylic acid was mixed with 1 mL of the supernatant and filtered through a 0.45-μm membrane filter. Sugars were analyzed by HPLC using an LC10A Series device (Shimadzu) with an Asahipak NH2P 50-4E (4.6 mm × 250 mm) column (Showa Denko KK, Tokyo, Japan). The column temperature was 40°C; 75% acetonitrile was used as a solvent, with a flow rate of 1 mL/min, and the RID-10A (Shimadzu) RI detector was used. HPLC-grade glucose, fructose, maltose, and sucrose (Kishida Chemical Co., Tokyo, Japan) were dissolved in ultrapure water as standards and analyzed as described above.

Free amino acid analysis For the analysis, 50 mL of 2% (w/v) sulfosalicylic acid solution was added to a sourdough sample (5 g) and homogenized, and the proteins were removed by centrifugation (8947 × *g*, 5 min, 20°C). The supernatant was transferred to a new tube and left to settle overnight. The upper phase was then filtered through a 0.45-μm DISMIC-13CP membrane filter (Advantec, Tokyo, Japan). Free amino acids were quantified using a JCL-500/V fully automated amino acid analyzer (JEOL Ltd., Tokyo, Japan) following the manufacturer's guidelines.

Flavor component analysis by solid-phase microextraction Dynamic-headspace solid-phase microextraction (SPME) was used for flavor component analysis, for which 1 g of sourdough sample, 1 g of table salt, and 100 μL of 0.1% cyclohexanol solution (internal standard) were mixed in a 25-mL glass vial. The vial was then filled with nitrogen gas and hermetically sealed. Each sample was heated to 50°C for 30 min with continuous stirring. With the headspace saturated, a wad of SPME fiber (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane, 2 cm; Superco, Bellefonte, PA, USA) was inserted into the vial. Each sample was heated again at 50°C for 30 min with continuous stirring to adsorb and collect the aroma in the headspace of the vial. Collected flavor components were analyzed by gas chromatography/mass spectrometry (GC/MS) using a 7890A GC system and 5973c inertXL MS detector (Agilent Technologies, Santa Clara, CA, USA). The splitless injection mode was used, and desorption of flavor components was performed for 3 min at 240°C. A TC-WAX column (60 m × 0.25 mm i.d. × 0.25 μm; GL Sciences, Torrance, CA, USA) was used at 40°C for 10 min, and the temperature was then increased to 100°C at a rate of 6°C/min and to 230°C at a rate of 4°C/min, followed by 10 min at 230°C. Helium was used as the carrier gas at a flow rate of 1 mL/min. Electron ionization was employed at an MS ion source temperature of 230°C and ionization voltage of 70 eV. Qualitative analyses of the fragment patterns of each peak were performed using an electron ionization spectrum library (NIST, Wiley). Thereafter, the mass spectra and retention times of putative hits were confirmed using a commercially available reference standard. The relative amounts of individual compounds within each sample were determined using an internal standard method where the peak area of 0.1% cyclohexanol (internal reference) was set as 100%. The mean value of three iterations for each specimen was calculated. Although it was not possible to obtain an absolute value for the area ratio of each aroma compound, relative quantitative comparisons could be made for each identical compound. Variants in each sample were evaluated using Tukey's multiple comparison test (24). The GC column and all SPME fiber plugs were baked for 90 min at 260°C between sample analyses, and the off-gasses were analyzed to confirm that no flavor component residues remained.

Bread making using sourdough To evaluate the characteristics of the products made using sourdough from each bakery, each sourdough sample was used to make baguettes. The ingredients included the following: 2000 g of wheat flour (Merveille, Nippon Flour Mills Co., Ltd., Chiyoda-ku, Tokyo, Japan), 8 g of dry yeast (Lesaffre Instant Dry Yeast Red, Nichifutsu Shoji Co., Ltd., Kobe, Japan), 40 g of salt (Hakata Salt, Hakata Salt Co., Ltd., Matsuyama, Japan), 6.0 g of malt extract (Euromalt, Nichifutsu Shoji Co., Ltd.), 1300 mL of water, and 200 g of sourdough from each bakery. The ingredients were kneaded at a low speed for 9 min in a spiral mixer (AS25C, Aicohsha Manufacturing Co., Ltd., Toda, Japan) to produce the dough. The dough was handled at 23°C until the completion of kneading. Next, the dough was placed in a thermoregulated vessel at 27°C and 75% humidity for

TABLE 1. Fermentation conditions and characteristics of sourdough from each bakery.

	Bakery A	Bakery B	Bakery C	Bakery D
Combination ratio of raw materials (flour:water:seed from the previous day)	100:100:100	100:110:100	100:100:100	100:60:100
Temperature and time of fermentation	4 h at 25°C followed by resting for 20 h at 4°C	16 h at 28°C	24 h at 28°C	16 h at 27°C
Fermentation type	Machine	Manual	Manual	Manual
Sourdough state	Liquid	Liquid	Liquid	Dough
Sourdough origin	Spontaneous fermentation of rye flour and wheat flour	Spontaneous fermentation of rye flour and wheat flour	Spontaneous fermentation of rye flour and wheat flour	Spontaneous fermentation of wheat flour
Period of backslopping	8 years	4 years	4 years	9 years

90 min for fermentation, before dividing into 350-g portions and resting for 30 min at 25°C. The dough was shaped into baguettes using molds to ensure a uniform length of 60 cm, and secondary fermentation was performed in a thermoregulated vessel at 28°C and 75% humidity for 70 min. The baguettes were then baked at 210°C for 25 min. The final products were cooled for 90 min and stored in plastic bags for 24 h before sensory evaluation.

Sensory evaluation The sensory evaluation was based on the quantitative descriptive analysis method (25). Briefly, flavor aspects of "savory," "sour," and "sweet" aromas and "taste" and mouth-feel aspects of "crumb softness," "ease of chewing," "moisture," and "crispness" were set as the sensory evaluation items for a 7-point evaluation system. In addition, to evaluate the savory aromas, 11 types of savory descriptors were provided (germ, coffee, nut, sesame, almond, cookie, malt, popcorn, soy sauce, burnt, and smoked), allowing multiple answers. The sensory evaluation was performed by a panel of 12 specialists from the MC Food Specialties, Inc. Food Research and Development Laboratory (Ami-Machi, Inasiki-Gun, Ibaraki, Japan). The study was conducted in accordance with the Helsinki Declaration of 1964, as revised in 2013, and was approved by the Food Research and Development Department of the company. Informed consent was obtained from subjects after explaining the study to them.

RESULTS

Species of lactic acid bacteria and yeasts identified in sourdoughs Species of lactic acid bacteria and yeast isolated from the sourdough from each bakery are shown in Tables 2 and 3. Sourdough from bakery A harbored 1.7×10^{10} CFU/g of lactobacilli, with three species identified: *L. brevis* (predominant species), *L. alimentarius*, and *L. pentosus*. It also harbored two yeast species, *Saccharomyces cerevisiae* and *Candida humilis*, at a density of 4.7×10^{7} CFU/g. Sourdough from bakery B harbored *Lactobacillus sakei* and *S. cerevisiae*. Yeast CFU counts were lower in sourdoughs from other bakeries. Sourdough from bakery C harbored *L. sanfranciscensis* and *S. cerevisiae*, with the lactic acid bacteria outnumbering yeast cells, 100:1. Finally, sourdough from bakery D harbored two species of lactic acid bacteria, *L. brevis* and *L. vaccinostercus*, and only one yeast species (*S. cerevisiae*). *S. cerevisiae* counts in the sourdough from bakery D were the highest among the sourdoughs examined.

Sourdough food components: pH, organic acids, sugars, and free amino acids The pH and amounts of organic acids and sugars in sourdoughs from each bakery are shown in Table 4. Levels of lactic acid, acetic acid, glucose, and maltose varied greatly between the sourdoughs. Sourdough from bakery A had the highest lactic acid (7149.5 mg/kg) and acetic acid content (1556.4 mg/kg) and markedly low maltose content (278.0 mg/kg), with no glucose detected, which indicated the most-progressed fermentation among the samples tested. Compared with other samples, bakery B sourdough had the lowest lactic acid content (1504.1 mg/kg) with the highest glucose content (2399.2 mg/kg). Sourdough from bakery C had the highest maltose content (23,523.1 mg/kg) and lowest acetic acid content (147.2 mg/kg). Despite the relatively high levels of lactic acid (5265.7 mg/kg) and acetic acid (1027.8 mg/kg), high levels of maltose (14,524.1 mg/kg) were detected in sourdough from bakery D.

The amount of free amino acids in each sample is shown in Table 5. Sourdough from bakery A had the highest free amino acid content, followed by sourdough from bakery D. The levels of free amino acids aspartate, glutamate, glycine, alanine, arginine, gamma-aminobutyric acid (GABA), and tryptophan varied considerably among the four sourdoughs. In particular, low aspartate levels were noted for samples from bakeries A and D, and low tryptophan levels were noted for samples from bakery D. The proportions of arginine and tryptophan were relatively high in sourdough from bakery B, which was also characterized by relatively low proportions of leucine, phenylalanine, and proline. Sourdough from bakery C had relatively high levels of glutamate and low levels of proline, glycine, and alanine.

Characteristics of the sourdough flavor components Comparison of the flavor components in the SPME headspace of sourdoughs from each bakery is presented in Table 6. Sourdoughs from bakeries A and D contained considerable amounts of alcohols, such as ethanol (sweet aroma), 3-methyl-1-butanol (malt aroma), and phenethyl alcohol (flower-like aroma), as well as esters such as ethyl acetate and 2-hydroxy-propanoic acid, ethyl. Sourdoughs from bakeries B and C had relatively fewer flavor components. Moreover, the proportion of flavor components with a sour aroma, such as acetic acid, was higher in sourdoughs from bakeries A and B than in other samples.

Evaluation of baguettes made using sourdough from each bakery Results of the sensory evaluation of baguettes enriched with 10% sourdough from each bakery are shown in Fig. 1. The savory aromas were most pronounced in baguettes made with sourdough from bakery B, while the use of sourdough from bakery A resulted in baguettes with the strongest sour aroma. Baguettes made with sourdoughs from bakeries C and D had a sweet wheat aroma. In addition, the flavor of baguettes made with sourdoughs from bakeries A and C was strongest during chewing, and the mouth-feel was pleasantly moist. Baguettes made with sourdough from bakery B dissolved well in the mouth. Differences in the attributes of these savory aromas are presented in Fig. 2. The aromas of baguettes made with sourdough from bakery A included germ, nut, and malt, while those of baguettes made with sourdough from bakery B were strongly reminiscent of malt, soy sauce, and smoked food. Further, the aroma of baguettes containing sourdough from bakery C was similar to burnt rice, and the responses to baguettes with sourdough of bakery D suggested a mild, complicated aroma, like that of popcorn.

TABLE 2. Number and species of lactic acid bacteria in sourdough from each bakery.

	Microflora (%)			
	Bakery A	Bakery B	Bakery C	Bakery D
Strain				
Lactobacillus brevis	37	–	–	41
Lactobacillus alimentarius	12	–	–	–
Lactobacillus pentosus	1	–	–	–
Lactobacillus vaccinostercus	–	–	–	9
Lactobacillus sanfranciscensis	–	–	50	–
Lactobacillus sakei	–	50	–	–
Total	50	50	50	50
Number of isolates (CFU/g)[a]	1.7×10^{10}	6.0×10^{7}	3.7×10^{8}	4.1×10^{10}

[a] Number of lactic acid bacteria in sourdough represents the average value of three measurements per sourdough sample.

TABLE 3. Number and species of yeasts in sourdough from each bakery.

	Microflora (%)			
	Bakery A	Bakery B	Bakery C	Bakery D
Strain				
Saccharomyces cerevisiae	39	50	50	50
Candida humilis	11	–	–	–
Total	50	50	50	50
Number of isolates (CFU/g)[a]	4.7×10^{7}	6.0×10^{2}	1.1×10^{6}	1.2×10^{9}

[a] Number of yeasts in sourdough represents the average value of three measurements per sourdough sample.

DISCUSSION

In the current study, we compared the microbial and sensory characteristics of sourdoughs from four Japanese bakeries. When the numbers of yeasts in the four sourdoughs were compared, the proportion of yeasts was relatively highest in sourdoughs from bakeries A and D. That is likely an effect of the short fermentation

TABLE 4. Organic acids and sugars in sourdough from each bakery.

		Amount (mg/kg)			
		Bakery A	Bakery B	Bakery C	Bakery D
pH		3.59 ± 0.01	4.02 ± 0.02	3.78 ± 0.02	3.87 ± 0.01
Organic acid	Citric acid	N.D.	51.2 ± 0.4	154.6 ± 1.2	N.D.
	Malic acid	N.D.	38.1 ± 0.3	N.D.	N.D.
	Succinic acid	60.5 ± 0.5	20.0 ± 0.2	18.6 ± 0.1	N.D.
	Lactic acid	7149.5 ± 57.5	1504.1 ± 12.1	2747.0 ± 22.1	5265.7 ± 42.3
	Fumaric acid	N.D.	18.6 ± 0.1	N.D.	N.D.
	Acetic acid	1556.4 ± 12.5	427.4 ± 3.4	147.2 ± 1.2	1027.8 ± 8.3
Sugar	Fructose	N.D.	359.7 ± 2.9	44.5 ± 0.4	445.5 ± 3.6
	Glucose	N.D.	2399.2 ± 19.3	1752.4 ± 14.1	694.2 ± 5.6
	Sucrose	N.D.	N.D.	460.2 ± 3.7	N.D.
	Maltose	278.0 ± 2.2	13837.8 ± 111.3	23523.1 ± 189.2	14524.1 ± 116.8

The data of pH, organic acids, and sugars show the average value and the standard deviation of three measurements per sourdough sample. N.D., not detected.

time (25°C for 4 h) of sourdough from bakery A and the preparation of sourdough from bakery D in a dough-like state with fermentation at 27°C for 16 h. Short fermentation times allow *S. cerevisiae* to become dominant because its metabolism and growth rate are more rapid than those of lactic acid bacteria. De Vuyst et al. investigated the species of lactic acid bacteria in bakery- and home-made sourdoughs and the fermentation temperatures and fermentation times used (3). They found that at low fermentation temperatures, yeast fermentation is dominant, with ethanol products and flavor components more readily produced (3). In the current study, among the bakeries examined, bakery A employed the lowest fermentation temperature and shortest fermentation time, and this seems to have influenced the number of yeasts, yeast growth, and the prevalence of flavor components. Regarding the wheat flour-to-water ratios, a higher proportion of wheat flour increases the amount of carbon sources available to the microbes and allows for greater buffering capacity, with the acidification taking longer and improving microbial proliferation (3). The wheat flour-to-water ratio employed in bakery D differed from those of the other bakeries. The relatively high lactic acid and acetic acid contents of the resultant sourdough are likely associated with this ratio.

TABLE 5. Free amino acids in sourdough from each bakery.

	Amount (mg/kg)			
	Bakery A	Bakery B	Bakery C	Bakery D
Alanine	62.1 ± 1.2	46.4 ± 0.9	6.8 ± 0.1	68.1 ± 1.4
Arginine	65.3 ± 1.3	73.4 ± 1.5	34.5 ± 0.6	58.6 ± 1.2
Aspartic acid	37.7 ± 0.8	101.5 ± 2.0	102.4 ± 1.9	38.7 ± 0.8
Citrulline	35.1 ± 0.7	2.5 ± 0.0	3.4 ± 0.1	5.1 ± 0.1
Cysteine	2.6 ± 0.1	4.0 ± 0.1	4.9 ± 0.1	7.0 ± 0.1
GABA	75.1 ± 1.5	66.0 ± 1.3	19.4 ± 0.4	30.6 ± 0.6
Glutamic acid	119.2 ± 2.4	44.6 ± 0.9	117.6 ± 2.1	73.7 ± 1.5
Glycine	55.9 ± 1.1	15.1 ± 0.3	4.5 ± 0.1	47.0 ± 0.9
Histidine	11.2 ± 0.2	7.4 ± 0.1	4.5 ± 0.1	10.4 ± 0.2
Isoleucine	41.0 ± 0.8	5.3 ± 0.1	10.6 ± 0.2	21.5 ± 0.4
Leucine	99.2 ± 2.0	28.9 ± 0.6	56.0 ± 1.0	67.2 ± 1.3
Lysine	17.1 ± 0.3	22.6 ± 0.4	3.0 ± 0.1	41.5 ± 0.8
Methionine	18.7 ± 0.4	10.2 ± 0.2	11.4 ± 0.2	14.3 ± 0.3
Ornithine	3.6 ± 0.1	1.1 ± 0.0	N.D.	8.1 ± 0.2
Phenylalanine	76.1 ± 1.5	16.0 ± 0.3	44.5 ± 0.8	43.8 ± 0.9
Proline	70.0 ± 1.4	15.6 ± 0.3	8.5 ± 0.2	56.8 ± 1.1
Serine	35.0 ± 0.7	18.0 ± 0.4	22.6 ± 0.4	34.1 ± 0.7
Taurine	25.6 ± 0.5	7.1 ± 0.1	3.4 ± 0.1	47.8 ± 1.0
Threonine	25.8 ± 0.5	9.2 ± 0.2	5.2 ± 0.1	22.1 ± 0.4
Tryptophan	50.4 ± 1.0	58.8 ± 1.2	45.9 ± 0.8	14.8 ± 0.3
Tyrosine	34.3 ± 0.7	13.3 ± 0.3	19.9 ± 0.4	20.4 ± 0.4
Valine	63.0 ± 1.3	14.0 ± 0.3	22.6 ± 0.4	32.7 ± 0.7
Total	1024.1 ± 20.4	581.0 ± 11.6	551.5 ± 10.1	764.4 ± 15.2

The data of free amino acids show the average value and the standard deviation of three measurements per sourdough. N.D., not detected.

Further, in the current study, the identified species of lactic acid bacteria were more varied in the sourdoughs from different bakeries than the identified yeasts. Although the raw materials and manufacturing methods of San Francisco sourdough and panettone are different from those of the sourdoughs in this study, they both contain *L. sanfranciscensis* and *Saccharomyces exiguus* (26). In addition, *L. sanfranciscensis* is a common species of lactic acid bacteria identified in sourdoughs made of rye flour and wheat flour from Belgium, Greece, Morocco, and Sweden and rye sourdough from Germany (3,26).

In this study, this species was detected in sourdough from only one of four bakeries (bakery C). The 24-h fermentation time of sourdough from bakery C was the longest compared with those of the other bakeries. The sugar assimilability of *L. sanfranciscensis* is limited to glucose and maltose as compared with that of other lactic acid bacteria (22), and its metabolism is slow. In other words, this method of production involving a long fermentation time may be the reason *L. sanfranciscensis* became dominant.

L. brevis was also identified in wheat sourdough of Belgium, Italy, Iran, Morocco, Russia, and Spain and rye sourdough of Germany. In addition, *L. alimentarius* was identified in sourdough prepared from maize in Mexico and rye sourdough in Germany, and *L. sakei* was identified in sourdough of Naples pizza in Italy. In particular, there are many reports of the detection of *L. brevis*, *L. alimentarius*, *L. pentosus*, and *L. sakei* in sourdoughs, mainly in Europe (3,26–28). However, although *L. vaccinostercus* has been isolated from fermented food products of Thailand (29) and dried cow feces (30), there is no precedence for its detection in sourdough. It is not clear why *L. vaccinostercus* was detected in the sourdough from bakery D in the current study. Further investigation of the role and effects of *L. vaccinostercus* on sourdough and bread should shed light on this aspect. While *L. brevis* was detected in sourdoughs from bakeries A and D, the other species of lactic acid bacteria identified therein were different. Although the ingredients, fermentation conditions (temperature and time), and the period of repeated backslopping were different for sourdoughs evaluated in the current investigation, it is plausible that the selection of lactic acid bacteria suited to these ingredients, fermentation conditions, and backslopping environment resulted in the observed differences in their species.

The sensory evaluation of bread revealed that the mouth-feel and flavor of the sourdoughs differed. We attributed the moist mouth-feel to two causes. Firstly, it is known that exopolysaccharides (EPS) produced by lactic acid bacteria affect moisture content (31). Secondly, structural changes in the gluten film due to the influence of glutathione reductase produced by lactic acid bacteria such as *L. sanfranciscensis* affect moisture retention or chew after baking (32).

In addition, the strengths of savory aromas were different, with bakery B sourdough having the strongest savory aroma,

TABLE 6. Flavor component composition of sourdough from each bakery.

	RT[a]	Identified compound	Relative amount[b]			
			Bakery A	Bakery B	Bakery C	Bakery D
Alcohol	8.334	Ethanol	235.07 ± 0.81	11.57 ± 0.36	25.58 ± 1.08	127.98 ± 0.62
	16.414	2-Methyl-1-propanol	1.64 ± 0.01	N.D.	N.D.	0.78 ± 0.93
	23.751	3-Methyl-1-butanol	35.14 ± 0.84	0.13 ± 0.01	N.D.	14.63 ± 0.90
	26.372	1-Pentanol	0.56 ± 0.17	0.31 ± 0.02	0.15 ± 0.02	0.47 ± 0.08
	33.42	1-Hexanol	3.82 ± 0.01	2.23 ± 0.07	1.00 ± 0.01	4.18 ± 0.04
	39.654	1-Octen-3-ol	N.D.	0.15 ± 0.01	N.D.	0.61 ± 0.08
	39.906	1-Heptanol	1.50 ± 0.03	0.16 ± 0.03	0.09 ± 0.02	0.68 ± 0.05
	41.835	2-Ethyl-1-hexanol	1.04 ± 0.08	0.27 ± 0.05	0.25 ± 0.03	0.38 ± 0.07
	44.114	2,6-Dimethyl-4-heptanol	0.59 ± 0.06	N.D.	N.D.	N.D.
	45.029	1-Octanol	1.19 ± 0.20	0.06 ± 0.03	N.D.	0.37 ± 0.00
	45.995	*trans*-(2-Ethylcyclopentyl)methanol	0.84 ± 0.12	N.D.	N.D.	N.D.
	50.609	3-Methylthio-1-propanol	0.65 ± 0.12	N.D.	N.D.	0.67 ± 0.03
	57.024	Phenethyl alcohol	71.88 ± 4.09	0.08 ± 0.04	0.12 ± 0.03	9.28 ± 0.54
	59.407	2,4-Decadien-1-ol	0.50 ± 0.05	N.D.	N.D.	N.D.
Ester	6.903	Ethyl acetate	43.53 ± 1.88	0.88 ± 0.10	1.06 ± 0.17	27.22 ± 2.61
	17.315	3-Methyl-1-butanol, acetate	2.79 ± 0.16	N.D.	1.13 ± 0.00	0.81 ± 0.07
	25.05	Hexanoic acid, ethyl	0.51 ± 0.08	N.D.	N.D.	0.39 ± 0.06
	32.305	2-Hydroxy-propanoic acid, ethyl	16.83 ± 0.34	0.57 ± 0.03	0.75 ± 0.15	5.25 ± 0.16
	39.657	Ethyl caprylate	5.10 ± 0.05	N.D.	N.D.	N.D.
	54.466	Acetic acid, 2-phenylethyl	1.65 ± 0.18	N.D.	N.D.	N.D.
	67.618	Isopropyl palmitate	N.D.	N.D.	N.D.	1.15 ± 0.18
	67.92	Hexadecanoic acid, ethyl	0.44 ± 0.15	N.D.	N.D.	0.26 ± 0.06
Acid	38.471	Acetic acid	33.63 ± 3.52	9.42 ± 0.40	4.96 ± 1.05	7.83 ± 0.16
	54.965	Hexanoic acid	1.60 ± 0.21	0.56 ± 0.07	0.22 ± 0.07	0.88 ± 0.03
	61.349	Octanoic acid	2.35 ± 0.75	N.D.	N.D.	N.D.
Aldehyde	15.028	Hexanal	N.D.	0.26 ± 0.05	0.31 ± 0.13	1.53 ± 0.08
	31.558	(*E*)-2-Heptenal	N.D.	N.D.	N.D.	0.21 ± 0.05
	37.593	5-Ethylcyclopent-1-enecarboxaldehyde	N.D.	N.D.	N.D.	0.56 ± 0.10
Ketone	28.5	Cyclohexanone	0.88 ± 0.02	1.12 ± 0.03	1.06 ± 0.03	1.18 ± 0.06
	49.987	(−)-1-Methyl-2-norcaranone	1.23 ± 0.11	0.35 ± 0.42	N.D.	N.D.
	61.098	Dihydro-5-pentyl- 2(3H)-furanone	0.43 ± 0.04	N.D.	N.D.	N.D.

N.D., not detected.
[a] RT, retention time.
[b] Relative peak area of each peak when the value of the internal standard (0.1% cyclohexanol) is set to 100. The values are averages of three measurements, and errors show the standard deviations.

followed by sourdoughs from bakeries C, A, and D. We explain this as follows: although sourdoughs from bakeries B and C contained less free amino acids than other sourdoughs, their glucose and maltose levels were relatively high, and thus, the amino–carbonyl reactions during the baking process may have progressed further, leading to stronger savory aromas. In addition, the presence of few flavor components in the sourdoughs from bakeries B and C may have further contributed to the savory aromas. By contrast, the free amino acid levels in sourdoughs from bakeries A and D were higher than those in sourdoughs from other bakeries; however, glucose in particular was scarce therein, which may have led to weaker savory

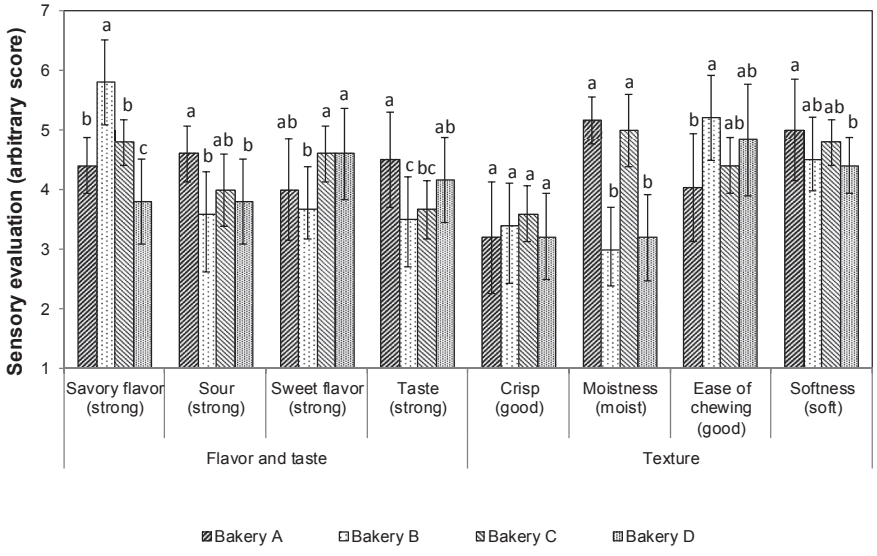

FIG. 1. Sensory evaluation of baguettes with added sourdough from each bakery. Number of panelists: n = 12. Higher scores indicate stronger flavors and better mouth-feel. The data show the average value of the sensory evaluation, and the error bars show the standard deviation. Variations among bakeries were evaluated by Tukey's multiple comparison tests, and the different lowercase letters represent significant differences ($p < 0.05$).

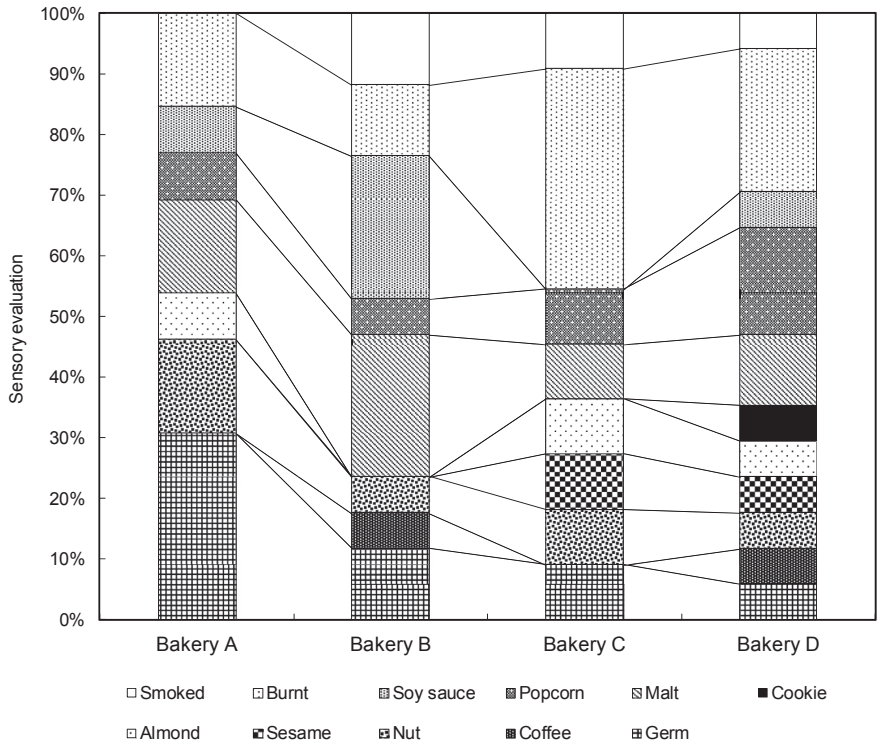

FIG. 2. Savory qualities of baguettes with added sourdough from each bakery. Eleven different descriptors were employed for the panelists to evaluate the savory qualities of the baguettes from the different bakeries (n = 12). Bar charts represent the responses of the panelists.

aromas, resulting in a stronger flavor. Sourdoughs from bakeries A and D were characterized by flavor components such as ethanol, 3-methyl-1-butanol, ethyl acetate, and acetic acid, which may have contributed to the sour and sweet aromas. The bread made with sourdoughs from bakeries B and C, characterized by strong savory aromas, differed with respect to the "soy sauce-like," "malt," and "burnt" aroma attributes. The glucose and maltose contents of these sourdoughs were high; however, sourdough from bakery B contained more free alanine, lysine, arginine, and GABA, while that from bakery C contained more free glutamate, leucine, and phenylalanine. It is possible that these particular free amino acids and their respective amounts influenced the aroma attributes.

With respect to savory aromas, it has been proposed that the amino–carbonyl reactions between sugars and free amino acids alter the profiles of the produced compounds and aromas (14). It has also been proposed that exoproteases of lactic acid bacteria produce free amino acids from proteins and peptides within wheat flour, and yeasts and some lactic acid bacteria utilize these free amino acids (10,13). Further, glucose, maltose, and fructose are utilized or polymerized by both yeasts and lactic acid bacteria, and this affects the sugar content of the sourdough (13). In addition, pH is reduced by lactic acid produced by lactic acid bacteria, which also affects the aromas arising from the amino–carbonyl reactions (14). In the current study, the effect of the combination of free amino acids, sugars, and pH on the aroma qualities of bread was not investigated; however, we propose that these features of the evaluated sourdoughs contribute to the qualities and strength of the aroma.

In conclusion, analysis of sourdoughs from four bakeries in the Kansai region revealed differences in the microbial flora and specific food components. It is possible that the differences in the species and proportions of the cultured microbes influence the food components of the sourdough. Future detailed investigation into the effect of pure cultures of lactic acid bacteria on the aromas and mouth-feel qualities of bread are warranted.

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References

1. **Corsetti, A., Settanni, L., Chaves López, C., Felis, G. E., Mastrangelo, M., and Suzzi, G.:** A taxonomic survey of lactic acid bacteria isolated from wheat (*Triticum durum*) kernels and non-conventional flours, Syst. Appl. Microbiol., **30**, 561–571 (2007).
2. **De Vuyst, L., Vrancken, G., Ravyts, F., Rimaux, T., and Weckx, S.:** Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota, Food Microbiol., **26**, 666–675 (2009).
3. **De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H. M., and Weckx, S.:** Microbial ecology of sourdough fermentations: diverse or uniform? Food Microbiol., **37**, 11–29 (2014).
4. **Minervini, F., De Angelis, M., Di Cagno, R., and Gobbetti, M.:** Ecological parameters influencing microbial diversity and stability of traditional sourdough, Int. J. Food Microbiol., **171**, 136–146 (2014).
5. **Vogelmann, S. A. and Hertel, C.:** Impact of ecological factors on the stability of microbial associations in sourdough fermentation, Food Microbiol., **28**, 583–589 (2011).
6. **Fujimoto, A., Ito, K., Itou, M., Narushima, N., Ito, T., Yamamoto, A., Hirayama, S., Furukawa, S., Morinaga, Y., and Miyamoto, T.:** Microbial behavior and changes in food constituents during fermentation of Japanese sourdoughs with different rye and wheat starting materials, J. Biosci. Bioeng., **125**, 97–104 (2018).

7. **Hansen, A. and Schieberle, P.:** Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects, Trends Food Sci. Technol., **16**, 85–94 (2005).
8. **Arendt, E. K., Ryan, L. A., and Dal Bello, F.:** Impact of sourdough on the texture of bread, Food Microbiol., **24**, 165–174 (2007).
9. **Chavan, R. S. and Chavan, S. R.:** Sourdough technology-a traditional way for wholesome foods: a review, Compr. Rev. Food Sci. Food Saf., **10**, 170–183 (2011).
10. **Gänzle, M. G.:** Enzymatic and bacterial conversions during sourdough fermentation, Food Microbiol., **37**, 2–10 (2014).
11. **Gobbetti, M., Rizzello, C. G., Di Cagno, R., and De Angelis, M.:** How the sourdough may affect the functional features of leavened baked goods, Food Microbiol., **37**, 30–40 (2014).
12. **Rühmkorf, C., Jungkunz, S., Wagner, M., and Vogel, R. F.:** Optimization of homoexopolysaccharide formation by lactobacilli in gluten-free sourdoughs, Food Microbiol., **32**, 286–294 (2012).
13. **Gänzle, M. G., Loponen, J., and Gobbetti, M.:** Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality, Trends Food Sci. Technol., **19**, 513–521 (2008).
14. **Pico, J., Bernal, J., and Gómez, M.:** Wheat bread aroma compounds in crumb and crust: a review, Food Res. Int., **75**, 200–215 (2015).
15. **Harth, H., Van Kerrebroeck, S., and De Vuyst, L.:** Community dynamics and metabolite target analysis of spontaneous, backslopped barley sourdough fermentations under laboratory and bakery conditions, Int. J. Food Microbiol., **228**, 22–32 (2016).
16. **Lhomme, E., Lattanzi, A., Dousset, X., Minervini, F., De Angelis, M., Lacaze, G., Onno, B., and Gobbetti, M.:** Lactic acid bacterium and yeast microbiotas of sixteen French traditional sourdoughs, Int. J. Food Microbiol., **215**, 161–170 (2015).
17. **Ercolini, D., Pontonio, E., De Filippis, F., Minervini, F., La Storia, A., Gobbetti, M., and Di Cagno, R.:** Microbial ecology dynamics during rye and wheat sourdough preparation, Appl. Environ. Microbiol., **79**, 7827–7836 (2013).
18. **Corona, O., Alfonzo, A., Ventimiglia, G., Nasca, A., Francesca, N., Martorana, A., Moschetti, G., and Settanni, L.:** Industrial application of selected lactic acid bacteria isolated from local semolinas for typical sourdough bread production, Food Microbiol., **59**, 43–56 (2016).
19. **Ripari, V., Cecchi, T., and Berardi, E.:** Microbiological characterisation and volatiles profile of model, ex-novo, and traditional Italian white wheat sourdoughs, Food Chem., **205**, 297–307 (2016).
20. **Gobbetti, M., Minervini, F., Pontonio, E., Di Cagno, R., and De Angelis, M.:** Drivers for the establishment and composition of the sourdough lactic acid bacteria biota, Int. J. Food Microbiol., **239**, 3–18 (2016).
21. **De Vuyst, L., Harth, H., Van Kerrebroeck, S., and Leroy, F.:** Yeast diversity of sourdoughs and associated metabolic properties and functionalities, Int. J. Food Microbiol., **239**, 26–34 (2016).
22. **Stolz, P., Böcker, G., Vogel, F. R., and Hammes, P. W.:** Utilisation of maltose and glucose by lactobacilli isolated from sourdough, FEMS Microbiol. Lett., **109**, 237–242 (1993).
23. **Kitahara, K., Kaneko, T., and Goto, O.:** Taxonomic studies on the hiochi-bacteria, specific saprophytes of sake. 1. Isolation and grouping of bacterial strains, J. Gen. Appl. Microbiol., **3**, 102–110 (1957).
24. **Tukey, J. W.:** Comparing individual means in the analysis of variance, Biometrics, **5**, 99–114 (1949).
25. **Stone, H. and Sidel, J.:** Sensory evaluation practices, 3rd ed. Elsevier Academic Press, Redwood City (2004).
26. **De Vuyst, L. and Neysens, P.:** The sourdough microflora: biodiversity and metabolic interactions, Trends Food Sci. Technol., **16**, 43–56 (2005).
27. **Minervini, F., Di Cagno, R., Lattanzi, A., De Angelis, M., Antonielli, L., Cardinali, G., Cappelle, S., and Gobbetti, M.:** Lactic acid bacterium and yeast microbiotas of 19 sourdoughs used for traditional/typical Italian breads: interactions between ingredients and microbial species diversity, Appl. Environ. Microbiol., **78**, 1251–1264 (2012).
28. **Corsetti, A., Lavermicocca, P., Morea, M., Baruzzi, F., Tosti, N., and Gobbetti, M.:** Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy, Int. J. Food Microbiol., **64**, 95–104 (2001).
29. **Tanasupawat, S. and Komagata, K.:** Lactic acid bacteria in fermented foods in Thailand, World J. Microbiol. Biotechnol., **11**, 253–256 (1995).
30. **Okada, S., Suzuki, Y., and Kozaki, M.:** A new heterofermentative lactobacillus species with meso-diaminopimelic acid in peptidoglycan, *Lactobacillus vaccinostercus* KOZAKI and OKADA sp. nov., J. Gen. Appl. Microbiol., **25**, 215–221 (1979).
31. **Corsetti, A. and Settanni, L.:** Lactobacilli in sourdough fermentation, Food Res. Int., **40**, 539–558 (2007).
32. **Tang, K. X., Zhao, C. J., and Gänzle, M. G.:** Effect of glutathione on the taste and texture of type I sourdough bread, J. Agric. Food Chem., **65**, 4321–4328 (2017).