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Dense tracking of the dynamics of the microbial community and chemicals constituents in spontaneous wheat sourdough during two months of backslopping

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Wheat sourdough is a common traditional fermented food that is produced worldwide. However, product quality of spontaneous sourdough is not easy to control because it depends on natural fermentation and backslopping, about which little is known, notably after ten backslopping steps. To this end, we tracked the spontaneous fermentation of three sourdoughs made from wheat flours during 32 backslopping steps for 60 days. At 24 time points, the microbial community was analyzed by both culture-dependent and culture-independent methods and its chemical constituents were assessed. Dynamic changes were observed in the microbial community, which showed a common succession pattern among the three sourdoughs at the bacterial family level and differences at the species level. The bacterial communities evolved through three phases that were driven by different groups of lactic acid bacteria (LAB) species. The dynamism among the metabolites also differed, depending on the species composition of the LAB and yeast communities. In one sourdough, the growth of *Saccharomyces cerevisiae* was detected along with a concentration of increased ethanol, while in the other two sourdoughs, *Wickerhamomyces anomalus* was detected without ethanol production. Regarding the LAB communities, two sourdoughs were eventually co-dominated by *Lactobacillus plantarum* and *L. brevis*, while the other sourdough was eventually dominated solely by the heterolactic fermentative bacterium *Lactobacillus fermentum*, and ethanol was produced at the same level as lactic acid. Further research is needed to determine the bacterial and yeast species involved in the fermentation of sourdough, to help improve the design and quality control of the final product.

[**Key words:** Sourdough; Microbiota; Yeast; 16S rRNA gene amplicon sequencing; Lactic acid fermentation; Ethanol fermentation; Lactic acid bacteria]

Sourdough is a mixture of flour (wheat and/or rye) and water that is spontaneously fermented with lactic acid bacteria (LAB) and yeast (1). Various sourdoughs have been used worldwide to make wheat and rye sourdough bread, sweet leavened baked goods, crackers, pizza, and Chinese steamed bread (1–4). In recent years, the use of sourdough in Japan has gained popularity due to increased consumer demand for traditional European sourdough breads, such as *pain au levain*, which was introduced from France (5).

To maintain sourdoughs at bakeries, traditionally new flour and water are added to the sourdough and re-fermented, which is referred to as backslopping (1). The number of backslopping steps is considered to be one of the main factors affecting the dynamics of the sourdough microbiota (6,7). Previous studies reported that the LAB community seems to undergo a three-phase evolution in spontaneous sourdough backslopping (8–10). These three phases are respectively dominated by (i) the genera *Enterococcus*, *Lactococcus*, and *Leuconostoc*; (ii) the genera *Lactobacillus*, *Pediococcus*, and *Weissella*; and (iii) specific LAB species, such as *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* (7). More than 70 species of LAB have been identified from bakery and laboratory sourdoughs, whereas the diversity of yeast species is relatively limited, as only six yeast species, including *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* are regularly identified in sourdoughs (11). In addition, *Kazachstania unispora* is sometimes isolated from wheat or rye sourdoughs (12).

During sourdough fermentation, LAB and yeast convert saccharides, such as maltose and glucose, to metabolites. LAB can be grouped into two types based on their lactic acid fermentation pattern: homofermentative LAB, which produce only lactic acid from hexose; heterofermentative LAB, which produce lactic acid, ethanol, and CO_2 as major metabolites from hexose (13–15). In contrast, *S. cerevisiae*, the most familiar yeast for bakers, ferments saccharides to ethanol and CO_2 as the major products (12). *W. anomalus* also ferments flour sugars, and contributes to form flavor compounds such as ethyl acetate (16). Because these chemical constituents are important for the properties of sourdough bread, bakeries would like to develop methods to control the sourdough microbiota to provide consistency throughout repeated backslopping in order to maintain constant quality in the daily production of sourdough breads (12,17). Therefore, we sought to elucidate the relationship between the dynamics of the microbial community and the chemical changes in successively backslopped sourdough. Our hypothesis is that, during two months

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backslopping of spontaneous sourdough, the dynamics of the microbial community, even at the species level, are reflected in the chemical changes in the sourdough.

Although numerous studies have reported both the microbial community dynamics and the chemical changes during sourdough backslopping (5,8,10,18–21), most of these studies were focused on the first 10 days of backslopping. To our knowledge, only one study continuously tracked both the dynamics of the microbial community and the chemical changes in backslopped sourdough for more than two months (22). Nevertheless, since the authors only analyzed the backslopped sourdough every 20 days, little is known regarding the dynamics of the microbial community. In addition, the responsiveness of the chemical behaviors to the dynamics of the microbial community after 10 days backslopping of spontaneous sourdough is still unclear.

In this study, we densely tracked spontaneous laboratory wheat sourdoughs during 32 backslopping steps for 60 days with 24 measurement points (average interval, 2.5 days) by combining culture-dependent methods, 16S rRNA gene amplicon sequencing, and quantification of targeted chemicals. The aim of this study was to investigate (i) the microbial community dynamics and the change in the chemicals constituents and (ii) the responsiveness of the chemical concentrations to the dynamics of the microbial community for two months sourdough backslopping.

MATERIALS AND METHODS

Flour Three wheat flours were used to prepare spontaneous laboratory sourdoughs (Table 1). Flour moisture was measured according to the AACC method (AACC 44-15A). Assay kits from Megazyme International (Wicklow, Ireland) were used to determine alpha-amylase activity (K-CERA 02/17) and beta-amylase activity (K-BETA3 10/10) according to the manufacturer's instructions.

Preparation of spontaneous laboratory wheat sourdough Sourdough preparation was based on traditional protocols (23). Sourdough backslopping was carried out in a 1.5-L plastic container covered with a lid. First, 150 g of flour and 150 g of tap water were kneaded manually with a spatula. Next, the mixture was incubated at 30°C for 8 h, and then stored at 4°C for 16 h (24). After storage, 60 g of the sourdough was used to inoculate a mixture of 120 g each of flour and tap water with a dough yield (weight of the dough/weight of the flour × 100) of 200 for the next backslopping. This sourdough was incubated under the same conditions described above and then stored at 4°C for up to 72 h. This 4°C storage corresponds to the actual practice at Japanese bakeries, which maintains sourdough quality without microbial progression. The sourdough was backslopped daily for 10 days. After that, 22 backslopping steps were carried out on days 12, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 37, 39, 42, 44, 46, 49, 51, 53, 56, 58, and 60. Therefore, the sourdough was backslopped 32 times over a time period of 60 days. The condition of the sourdough was semi-liquid. The sourdough was sampled after stirring with a spatula until it reached uniform consistency. Sampling was performed on the un-incubated doughs (backslopping step 0) and immediately before each backslopping step. Samples for chemical analysis and 16S rRNA gene amplicon sequencing were stored at −30°C until use, whereas samples for pH measurement and microbial counts were processed immediately after sampling.

Measurement of pH and determination of carbohydrates, organic acids, and ethanol For pH measurement, samples were diluted ten times with distilled water, and then the pH values were measured with a pH meter (F-73; Horiba, Kyoto, Japan).

The concentrations of maltose, glucose, lactic acid and acetic acid were determined by using an LC-2000Plus series liquid chromatograph (JASCO, Tokyo, Japan) equipped with an RSpak SH-1011 column (Shodex, Tokyo, Japan) and an RI detector at 50°C. The mobile phase was 50 mM H_2SO_4, and the flow rate was 1.0 mL/min. For HPLC determination, water-soluble extractions of sourdough were prepared as follows. A sample of sourdough (1 g) was placed in a 15 mL tube and extracted with 5 mL of water by continuous shaking for 10 min at room temperature. After centrifugation (5800 × g, 1 min), the supernatants were heated at 95°C for 10 min in a water bath to inactivate the microorganisms. After cooling the samples, they were centrifuged again (5800 × g, 5 min), and then a 1.0 mL aliquot of the supernatant was filtered through a 0.2 μm pore size filter.

Ethanol concentration was determined by using a biosensor (BF-7; Oji Scientific Instrument, Osaka, Japan). Samples (2 g) were mixed with 2 mL of 200 mM Tris-HCl (pH 8.8) by stirring until a uniform consistency was obtained. After centrifuging the samples (13,000 × g, 10 min), an aliquot of the supernatant was used for the determination of ethanol concentration.

Enumeration and isolation of lactic acid bacteria and yeast Sourdough samples (10–15 g) were diluted 10 times with sterile NaCl solution (0.85%), and then homogenized for 2 min with a Stomacher (Pro-media SH-IIM; Elmex, Tokyo, Japan). LAB were counted after incubation on maltose MRS agar medium (25) supplemented with 100 mg/L of cycloheximide at 30°C for 48–72 h. This agar medium gave the highest counts among the agar media used for sourdough lactobacilli analysis (25). Yeasts were counted after incubation on potato dextrose agar medium (Eiken, Tokyo, Japan) containing 4 g/L of potato starch, 20 g/L of glucose, and 15 g/L of agar and supplemented with 100 mg/L of chloramphenicol at 25°C for 48–72 h. Colony isolation was carried out at backslopping steps 2, 5, 10, 15, 19, 24, 28, and 32. Five colonies per sample, except for sourdough A at step 2, of presumptive LAB and yeast were randomly selected. For isolation, selected colonies were streak purified twice onto the same agar medium.

Identification of lactic acid bacteria and yeast The genomic DNA of LAB and yeast was extracted using the alkaline boiling method (8). Briefly, a small portion of one colony was resuspended in 100 μL of 50 mM NaOH, and the mixture was heated at 95°C for 10 min and then cooled immediately under running water. After adding 11 μL of 1 M Tris-HCl (pH 7.5), the sample was centrifuged (13,000 × g, 1 min). Then, the supernatant was used as the genomic DNA template. If the DNA prepared using this method did not produce a high quality PCR amplicon, the strain was cultivated in maltose MRS broth medium (25) at 30°C for up to 3 days, and the DNA was extracted from the medium using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For all isolated LAB, the primers pair 10F/800R (26) was used to amplify the V1–V4 region of the 16S rRNA gene (Table S1). DNA amplification was confirmed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All nucleotide sequences were determined by Fasmac (Kanagawa, Japan) after DNA purification using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). BLAST sequence retrieval (BLASTN, National Center for Biotechnology Information) of the 16S rRNA gene sequences of all isolated LAB showed ≥99% identity to type strains in the database. In addition, multiplex PCR targeting the *recA* gene was performed to discriminate *L. plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus* (27). *Lactobacillus curvatus* was discriminated from *Lactobacillus sakei* by species-specific PCR (28). For *Leuconostoc* species identification, *pheS* gene sequencing was performed (29). To discriminate *Lactococcus lactis* and *Lactococcus taiwanensis*, the *rpoB* gene was sequenced (30). *Weissella cibaria* and *Weissella confusa* were also discriminated by species-specific PCR (31,32). *Pediococcus pentosaceus* was discriminated from *Pediococcus acidilactici* by multiplex PCR (33). To identify yeast strains, the primer pair NL-1/NL-4 was used to amplify the D1/D2 domain of the 26S rRNA gene (34). DNA purification and nucleotide sequencing of yeast were performed using the same procedure as described above. All oligonucleotides primers used in this study are listed in Table S1.

Total genomic DNA extraction Total genomic DNA was extracted from the sourdoughs by the bead beating method as described previously (35,36) using the QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany). A sample of sourdough was mixed well with a dispensing spoon soon after thawing it. Then, 200 mg of the sourdough was washed twice with 1 mL of phosphate-buffered saline (PBS, pH 7.4) and resuspended in 1 mL of PBS. The suspension was transferred to a 2.0 mL screw-capped tube containing 0.3 g of zirconium beads (0.1 mm diameter; Yasuikikai, Osaka, Japan), and the suspension was beaten at 2700 rpm for 1 min with a Multi-beads Shocker MB1300C (Yasuikikai, Osaka, Japan) and then cooled at 4°C for 1 min. This cycle was repeated three times. Subsequently, 300 μL of

TABLE 1. Characteristics of wheat flours used for the preparation of laboratory sourdoughs.

Prepared sourdough	Characteristics of used wheat flour					
	Moisture[a] (%)	Ash[b] (% of dry matter)	Protein[b] (% of dry matter)	Alpha-amylase activity[a] (mCU/g-flour)	Beta-amylase activity[a] (Betamyl-3 U/g-flour)	Cultivation area of wheat
Sourdough A	12.1	0.63	11.4	81	20	France
Sourdough B	12.5	0.51	12.2	82	41	North America (Canada, USA)
Sourdough C	12.4	0.61	13.7	40	43	Japan

[a] The means of three independent experiments (relative standard deviations are all below 5.0%).

[b] This information was obtained from the producer as standard value.

phenol-chloroform-isoamyl alcohol (25:24:1) was added to the suspension of lysed cells, and the mixture was shaken at 2700 rpm for 40 s. Then, the mixture was centrifuged (20,400 × g, 1 min, 4°C). After removing any PCR inhibitors with an InhibitEX tablet, the upper layer was applied onto a QIAamp Spin column. The column was washed with buffer AW and was then eluted with 100 μL of buffer AE according to the manufacturer's instructions.

Bacterial community analysis by 16S rRNA gene amplicon sequencing The V1–V2 region of the 16S rRNA gene was amplified from total genomic DNA using the universal primers Tru 27F and Tru 354R (37) (Table S1). Both the first and second PCR were performed as described previously (38). The amplicons were purified using FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), and the DNA concentration was measured using the Quant-iT Picogreen dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) in an Infinite M200 microplate reader (Tecan, Zürich, Switzerland). The amplicons were then pooled in equal amounts (20 ng of each amplicon), and the DNA concentration of the library was measured as described above. The amplicon library was mixed with a PhiX control, and then subjected to paired-end sequencing by Illumina MiSeq according to the manufacturer's protocol. The sequencing run was performed with MiSeq reagent v3. The obtained sequences were processed using the Uparse pipeline in Usearch (39) as described previously (38). The merged sequences were clustered into 175 operational taxonomic units (OTUs), each with greater than 97% identity, using the Uparse-OTU algorithm. A total of 3,098,566 reads were obtained from 72 samples (median, 42,560 reads per sample; range, 14,382–81,110 reads/sample).

The taxonomy of each OTU was assigned at the family level by using the Ribosomal Database Project. To gain more information on the LAB community, representative OTU (>5.0% relative abundance) was assigned at closely related species level according to the top-hit species (with ≥99% similarity) to the type strain by BLAST using the OTU as the query. A heat map was generated based on the relative abundance of each LAB species by using Microsoft R open 3.4.3.

Nucleotide sequence accession number The raw data of 16S rRNA gene amplicon sequences were deposited at the DNA Data Bank of Japan (DRA007293) under accession number BioProject PRJDB7396.

RESULTS

Changes in pH and the numbers of lactic acid bacteria and yeast Spontaneous fermentation of laboratory sourdoughs was started with a mixture of wheat flour and water. In all sourdoughs, the pH decreased significantly (from >6.0 to <4.0) during the first eight backslopping steps over an eight day period. The pH then stabilized at 3.5–4.0 until the last step (Fig. 1). After the first three backslopping steps, the presumed LAB counts exceeded 8.5 log cfu/g in all sourdoughs. The maximum counts in sourdoughs A, B, and C were similar at 9.1, 9.2, and 9.6 log cfu/g, respectively. In contrast, the presumed yeast counts differed among the sourdoughs. The counts in sourdough A increased to 7.6 log cfu/g from the fourth to the 12th backslopping steps, and were then maintained at >7 log cfu/g until the last step. In sourdoughs B and C, the presumed yeast counts began to increase after the 10th step, and reached maximums of 6.9 log cfu/g at the 17th step and 6.2 log cfu/g at the 21st step, respectively, and then decreased toward the end of the experiment. Notably, no yeast was detected after the 30th backslopping step in sourdough C.

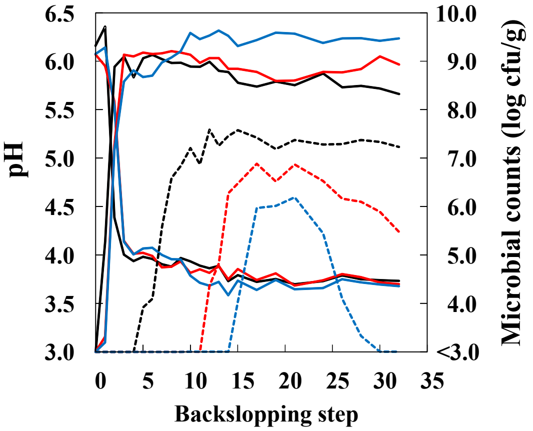

FIG. 1. Changes of pH and microbial counts during 32 backslopping steps of laboratory wheat sourdough A (black), sourdough B (red) and sourdough C (blue). Solid lines indicate pH (left-axis) and presumed lactic acid bacteria (right-axis). Broken lines indicate presumed yeast (right-axis). All measurements were independently carried out in duplicate and mean values are represented.

Identification of lactic acid bacteria and yeast isolates over time Several changes were observed in the LAB community over time in the sourdoughs (Table 2). The identified LAB were classified into three types according to colonizing stage. The early type, which colonized the sourdough within the first five steps, included *Lactococcus lactis*, *Leuconostoc citreum*, *W. confusa*, and *W. cibaria*. The late type, which was observed from the 10th step to the end, included *L. plantarum*, *L. paraplantarum*, *L. brevis*, and *L. fermentum*. *P. pentosaceus*, a unique midterm colonizer, was observed from the 10th to the 19th steps in sourdough B. *L. curvatus* was detected at different stages in sourdough A (2nd step) and sourdough B (19th step), although only one colony was retrieved from each sourdough. From the presumed yeast isolates, three species, *W. anomalus*, *K. unispora*, and *S. cerevisiae* were identified. The detected species and stage varied among the sourdoughs.

Succession of the bacterial community as analyzed by 16S rRNA gene amplicon sequencing In addition to the culture-dependent analysis of isolates, we also performed a culture-independent analysis using 16S rRNA gene amplicon sequencing. All sourdoughs showed a similar succession pattern at the family level, while the closest species varied (Fig. 2). Before fermentation (step 0), the sequenced amplicons were mostly plant chloroplast 16S rRNA genes, suggesting a low number of bacteria, as was also indicated by the bacterial and yeast colony counts shown in Fig. 1. Immediately after the first backslopping,

TABLE 2. Identification of lactic acid bacteria and yeast throughout 32 backslopping steps of three laboratory wheat sourdoughs.

Identified isolates	Backslopping step							
	2	5	10	15	19	24	28	32
Sourdough A								
Lactococcus lactis	1							
Leuconostoc citreum	1	4	4					
Weissella confusa		1						
Lactobacillus curvatus	1							
Lactobacillus plantarum				1	2		2	1
Lactobacillus brevis			1	4	3	5	3	4
Total lactic acid bacteria analyzed	3	5	5	5	5	5	5	5
Wickerhamomyces anomalus		5	1					
Kazachstania unispora			2	1	1	1		
Saccharomyces cerevisiae			2	4	4	4	5	5
Total yeast analyzed		5	5	5	5	5	5	5
Sourdough B								
Lactococcus lactis	1							
Leuconostoc citreum	4	5	1	3	1			
Lactobacillus curvatus					1			
Pediococcus pentosaceus			4	1	2			
Lactobacillus plantarum						1	1	2
Lactobacillus paraplantarum							1	
Lactobacillus brevis				1	1	4	3	3
Total lactic acid bacteria analyzed	5	5	5	5	5	5	5	5
Wickerhamomyces anomalus				5	5	5	5	5
Total yeast analyzed				5	5	5	5	5
Sourdough C								
Leuconostoc citreum		2	2					
Weissella cibaria	5	3						
Lactobacillus fermentum			3	5	5	5	5	5
Total lactic acid bacteria analyzed	5	5	5	5	5	5	5	5
Wickerhamomyces anomalus				5	5	5	5	
Total yeast analyzed				5	5	5	5	

The numbers correspond to the number of colonies identified as the given species at the given backslopping step.

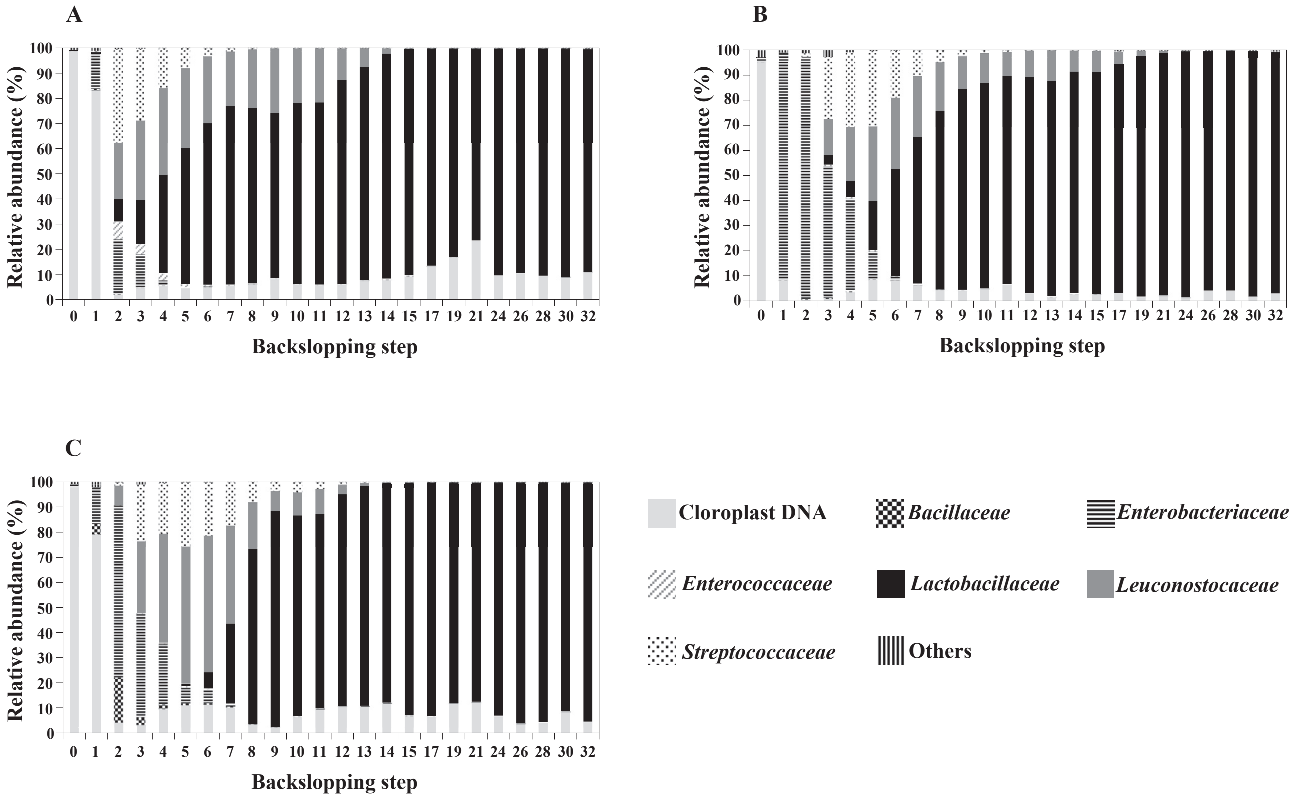

FIG. 2. Bacterial community dynamics at the family level during 32 backslopping steps of laboratory wheat sourdoughs. (A) Sourdough A. (B) Sourdough B. (C) Sourdough C. The relative abundance of each family was determined based on the read counts of each OTU assigned to a family.

non-LAB families belonging to the *Bacillaceae* and *Enterobacteriaceae* appeared; these families were replaced by LAB belonging to the families *Enterococcaceae*, *Leuconostocaceae*, *Streptococcaceae*, and *Lactobacillaceae* within the first eight steps. Eventually, *Lactobacillaceae* dominated all three sourdoughs.

As observed in the colony analysis, the closest LAB species (as assigned by the 16S rRNA amplicon analysis) were also divided into three types according to growth stage (Fig. 3). *Lactococcus lactis* (Otu4), *Lactococcus garvieae* (Otu14), *Leuconostoc lactis* (Otu155), *Leuconostoc* sp. (Otu9), *W. confusa* (Otu7), and *W. cibaria* (Otu43) were early colonizers, while *L. plantarum* group LAB (*L. plantarum*, *L. paraplantarum*, and *L. pentosus*; Otu10), *Lactobacillus paralimentarius* (Otu8), *L. brevis* (Otu11), *Lactobacillus rossiae* (Otu18), and *L. fermentum* (Otu3) were late colonizers. *L. curvatus* (Otu2), *L. sakei* (Otu96), and *P. pentosaceus* (Otu5), which were present between the early and late stages, were categorized as middle colonizers. These findings mostly coincided with the results of the culture-dependent analysis. Although Otu9 showed 99% similarity to three *Leuconostoc* species (*Leuconostoc kimchii*, *L. citreum*, and *Leuconostoc lactis*), it appeared to be *L. citreum*, which was identified in the colony analysis. *L. paraplantarum* detected in the colony analysis was assigned to the *L. plantarum* group (Otu10) in the amplicon analysis. *L. garvieae* (Otu14), *Leuconostoc lactis* (Otu155), *L. sakei* (Otu96), *L. paralimentarius* (Otu8), and *L. rossiae* (Otu18) were not detected in the colony analysis. These LAB species might not grow well on maltose MRS agar medium.

Sourdough A and B showed succession similar patterns in the 16S rRNA profiles, in which *Lactococcus lactis* (Otu4) and *Leuconostoc* sp. (Otu9) dominated in the early stage, *L. curvatus* (Otu2) and *P. pentosaceus* (Otu5) dominated in the middle stage, and *L. brevis* (Otu11) and *L. plantarum* group (Otu10) and/or *L. paralimentarius* (Otu8) dominated in the late stage. In sourdoughs A and C, *W. confusa* (Otu7), *W. cibaria* (Otu43), *Lactococcus lactis* (Otu4), and *Leuconostoc* sp. (Otu9) colonized in the early stage. The middle-stage colonizer *P. pentosaceus* (Otu5) was also observed in sourdough C, while *L. fermentum* (Otu3) uniquely dominated the late stage of sourdough C, instead of *L. plantarum* group (Otu10) and *L. brevis* (Otu11), which was present in the other sourdoughs.

Changes in chemical constituents of the sourdoughs during backslopping At the beginning of the experiment (backslopping step 0), maltose was abundant in all sourdoughs (41, 43, and 41 mM in sourdoughs A, B, and C, respectively; Fig. 4). Then, in sourdough A at the 11th step, maltose decreased to $\leq$3 mM and glucose decreased to $\leq$4 mM. In contrast, in sourdough B, the concentration of maltose dramatically increased to 81 mM during the first four backslopping steps, and was then maintained at $\geq$66 mM until the last backslopping step.

Lactic acid production was observed in all sourdoughs. The concentration of lactic acid increased to 42 mM in sourdough A and to 38 mM in sourdoughs B and C during the first five backslopping steps. Then, the lactic acid concentration in sourdoughs A and B gradually increased to maximums of 70 mM and 66 mM, respectively, by the last backslopping step. Conversely, lactic acid production in sourdough C showed a biphasic pattern, in which a second LAB fermentation occurred during backslopping steps 8–11, as the concentration reached 68 mM by the 11th step. Then, the lactic acid concentration was maintained until the last step. The ethanol fermentation profile also differed among sourdoughs. In sourdough A, the ethanol concentration increased from 13 mM at backslopping step five to 224 mM at backslopping step 11, and then gradually decreased to 133 mM at the last step. The maximum concentration of ethanol in sourdough B (25 mM) was observed at the third step, which was the lowest maximum ethanol concentration among the three sourdoughs. In sourdough C, the ethanol

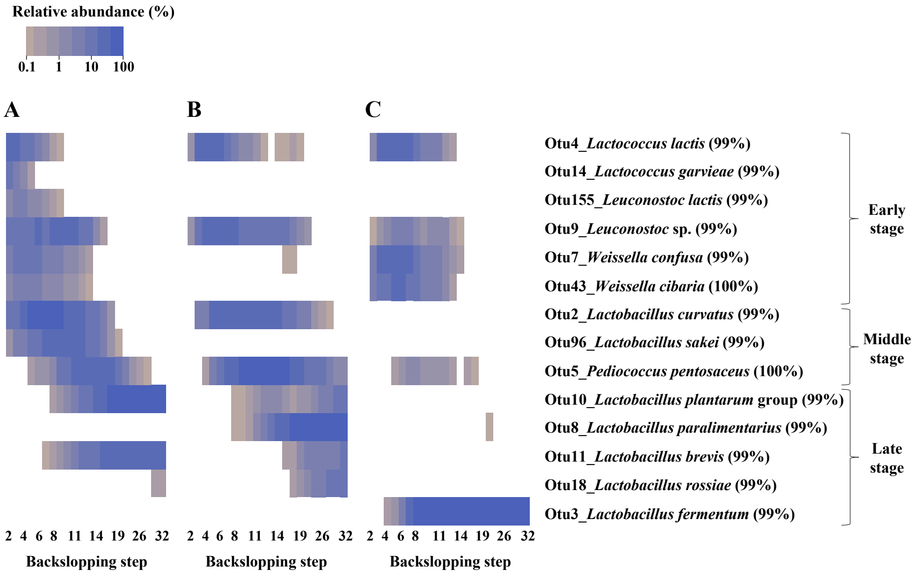

FIG. 3. Heat map of OTUs of lactic acid bacteria with relative abundance higher than 5.0% for any sample. Closely related species in each OTU was assigned by BLAST. The percentage of identity is shown within parenthesis. Otu10_*Lactobacillus plantarum* group consisted of *Lactobacillus plantarum*, *L. paraplantarum* and *L. pentosus*.

concentration increased in two phases, similar to lactic acid, although the lactic acid concentration was approximately two-fold higher than the ethanol concentration in the first phase, whereas the lactic acid and ethanol concentrations were approximately the same in the second phase. The first phase occurred during the first three backslopping steps, and second phase was from backslopping steps 8–11. No significant increase in acetic acid was observed, although it was continuously detected at a low level.

DISCUSSION

A number of studies on sourdoughs have demonstrated the dynamics of the LAB and yeast communities and the changes in the chemical constituents, most of which were mainly focused on the first 10 backslopping steps (5,8,10,18–21). In contrast to these studies, we aimed to elucidate the relationship between the microbial community and chemical constituents after the first 10 backslopping steps. To this end, we evaluated three spontaneous wheat sourdoughs during 32 backslopping steps for 60 days, with 24 measurement points.

To determine the LAB community dynamics in the two months backslopped wheat sourdoughs, we performed deep sequencing of the 16S rRNA gene amplicons as well as colony counts of cultured LAB and yeast species. Both culture-dependent and culture-independent analysis showed that three different LAB groups sequentially dominated the three backslopped sourdoughs. Previous reports also suggested that the LAB community in sourdough evolves through three phases (8–10).

The early-stage elevations in lactic acid and ethanol concentrations, which occurred within the first five backslopping steps, were observed in association with the growth of homofermentative LAB species, such as *Lactococcus lactis*, and heterofermentative LAB species, such as *L. citreum*, *W. cibaria* and *W. confusa*. After the transient domination of *P. pentosaceus* during the middle stage, the third LAB group became dominant in the sourdoughs. In sourdough A and B, the growth of *L. plantarum* and *L. brevis* was accompanied with a slight increase in lactic acid, while *L. fermentum* grew in sourdough C at the later stage, with the heterofermentation of lactic acid and ethanol. This co-domination of *L. plantarum* and *L. brevis* has also been observed in French and Italian sourdoughs (3,40,41). The competitiveness of *L. fermentum* in the sourdough ecosystem has been explained by the fact that *L. fermentum* acidifies the sourdough faster than other *Lactobacillus* species and is also capable of growing at lower pH values than other species, i.e., as low as pH 3.5 (42,43). To date, *L. fermentum* has been identified from some bakery sourdoughs in Belgium, Italy and China although this LAB species is less frequently isolated than representative sourdough LAB species such as *L. brevis* (2). These results suggest a

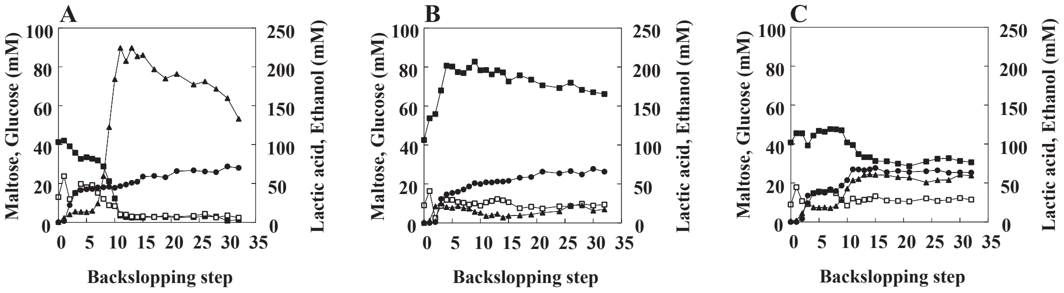

FIG. 4. Changes of fermentative chemicals during 32 backslopping steps of laboratory wheat sourdoughs. (A) Sourdough A. (B) Sourdough B. (C) Sourdough C. All determinations were independently carried out in duplicate and mean values are represented. Closed circles, lactic acid concentration; closed triangles, ethanol concentration; closed squares, maltose concentration; open squares, glucose concentration.

possibility of *L. fermentum* as a candidate starter species, as well as *L. plantarum* and *L. brevis*, which allow stable lactic acid fermentation in backslopped sourdough.

From our study, two question arose: are these *Lactobacillus* species adaptable to the other sourdough ecosystems and which *Lactobacillus* species is most applicable for this purpose. To answer these questions, further research is planned to track the bacterial community in successively backslopped sourdough from a starter containing a blend of different sourdoughs, each of which harbors different LAB species.

S. cerevisiae is the yeast that is most frequently present in fermented sourdough (44), and our results suggest that *S. cerevisiae* produced a significant amount of ethanol in sourdough A while consuming both maltose and glucose. In contrast, sourdoughs B and C mainly harbored *W. anomalus*, but only in the early to middle periods, as it began to decrease after 21 backslopping steps. This result suggests that it may be difficult for *W. anomalus* to persist in sourdough ecosystem. Although a previous study reported domination by *W. anomalus* (45), to our knowledge, this is the first study to monitor the prosperity and decline of *W. anomalus* in backslopped sourdough.

In conclusion, we investigated the dynamics of the microbial communities and chemical constituents in three backslopped sourdoughs during two months. The microbial communities in the sourdoughs commonly evolved through three phases, driven by different LAB species. In sourdoughs co-dominated by *L. plantarum* and *L. brevis*, the lactic acid concentrations gradually increased in the late stage, whereas in the other sourdough, which was solely dominated by *L. fermentum*, the lactic acid and ethanol concentrations were maintained at about the same level. The fungal communities also differed among the sourdoughs, and *S. cerevisiae* was detected in one sourdough, whereas *W. anomalus* was detected in the other two sourdoughs. High ethanol production was observed along with exhaustion of maltose and glucose in the sourdoughs dominated by *S. cerevisiae*.

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