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Full Paper

Impact of pH on succession of sourdough lactic acid bacteria communities and their fermentation properties

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Sourdough, a traditional fermented dough, is made via natural fermentation by lactic acid bacteria (LAB). Its pH changes from near neutral to acid during the subculture process. However, the product quality of subcultured sourdough depends on the unpredictable succession of LAB communities, the influential factors of which are still unclear. To elucidate one end of the LAB community succession mechanism, we evaluated the effect of pH by designing four subculture experiments using a model medium adjusted to pH 6.7, 5.5, and 4.5, as well as a natural sourdough subculture. All experiments began by inoculating a sourdough LAB mixture, and both bacterial successions and fermentative properties were monitored until ten subculture steps. In media subcultures, lactic acid production was higher in higher pH media. Three LAB genera, *Weissella*, *Pediococcus*, and *Lactobacillus*, each represented by one operational taxonomic unit (OTU), were successively detected in all subcultures. In later steps with lower pH media, an OTU closely related to *Lactobacillus brevis* dominated, replacing an OTU closely related to the *Weissella cibaria-confusa* group that was more dominant than the *L. brevis* OTU in the near-neutral pH medium. In the sourdough subculture, the three genera were also detected, while *Lactobacillus* was dominant in earlier steps due to the emergence of an OTU closely related to *Lactobacillus sanfranciscensis*. These results suggest that a lower pH is favorable for the sequence of sourdough bacterial community evolution finalizing with *Lactobacillus* domination. Further research is needed to elucidate additional factors other than pH that influence the pattern of LAB community shift.

Key words: 16S rRNA gene, *Weissella*, *Pediococcus*, *Lactobacillus*, amplicon sequencing, qPCR

INTRODUCTION

Sourdough, which consists of flour and water, is used to make baked foods such as sourdough bread and contains metabolically active microorganisms, including lactic acid bacteria (LAB) [1]. To maintain sourdough in bakeries, a successive subculture of sourdough is traditionally carried out by adding flour and water to an aliquot of previously fermented sourdough [1].

Sourdough LAB give rise to the characteristic qualities of several kinds of foods (flavor, texture, taste, and shelf life) by producing metabolites such as lactic acid, acetic acid, and ethanol [2, 3]. However, it is not easy to control sourdough LAB fermentation, due to the spontaneous occurrence of complex phase successions of LAB communities during subculture. Previous studies have characterized three phases, each dominated by the genera *Weissella*, *Pediococcus*, and *Lactobacillus*, whose succession determines the balance of metabolites in sourdough [4]. However, the mechanism of action of these three phases has not yet been elucidated, and elucidation of it would help stabilize daily sourdough fermentation.

Study of the mechanism of sourdough LAB community succession is hampered by some difficulties. This is because the LAB that emerge in subcultured sourdough differ from batch to batch due to spontaneous open-batch fermentation [4]. In addition, the LAB community succession in sourdough is hard to quantify by conventional culture-dependent analysis, as only a few species are retrieved from an agar plate, although more than 70 LAB species have been potentially present in the sourdough ecosystem [5, 6]. In contrast, the *in vitro* batch model using a laboratory-prepared medium is useful for investigating the sole effect of individual parameters on the microbial community and has been applied for the study of various fermented foods [7–10]. Wheat sourdough simulation medium (WSSM) was previously developed and used for pure culture experiments to verify physiological characteristics of sourdough LAB, such as the effect of pH on growth [11].

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One of the major characteristics of sourdough fermentation is a drop in pH proportional to the maturation of the LAB community that produces lactic and acetic acid, eventually reaching a pH of approximately 4.0 [12]. Since pH changes must induce stress on the LAB community, assessment of pH conditions is necessary to understand and control the evolution of the LAB community in sourdough. In addition, pH may influence the extent of lactic acid fermentation in sourdough. To our knowledge, however, there is only one report verifying the effect of pH on the succession of LAB communities in sourdough, and this report focused on the effect of an acidic pH of 4.0 [13]. Therefore, little is known regarding the effect of pH at values higher than 4.0.

The aim of this study was to clarify the effect of various pH ranges on the succession of sourdough LAB communities. Therefore, we analyzed both LAB community succession and metabolomics in a model sourdough, which was initiated by the inoculation of a sourdough starter LAB mixture and then subcultured for ten steps with pH 6.7-, 5.5- and 4.5-adjusted WSSMs as well as natural sourdough.

MATERIALS AND METHODS

LAB starter preparation by blending sourdoughs

The present study made use of 152 sourdoughs previously sampled from a variety of spontaneous sourdoughs at different time points. These sourdough samples were immediately frozen and stored at −30°C until LAB starter preparation. After thawing the samples, equal amounts of each sourdough sample were pooled together in a sterile bowl. The pooled sourdoughs were manually kneaded with a sterile spoon until the sourdough mixture reached a uniform consistency. This sourdough mixture was used as the LAB starter. Separately from the LAB starter preparation, the presumed LAB survivors in each of the 152 thawed sourdoughs were visually confirmed based on growth on maltose MRS agar medium [4].

Subculture experiments with media at three different pH levels

WSSM was used as the medium to simulate wheat sourdough fermentation [11]. This medium contained 0.5 g/L of glucose, 0.5 g/L of fructose, 10 g/L of maltose, 2 g/L of sucrose, and 12 g/L of wheat peptone. Prior to autoclave sterilization, the pH of the WSSM was adjusted to 6.7, 5.5, and 4.5, respectively, by adding HCl. Three hundred milliliters of subculture was placed in a 500 mL Erlenmeyer flask for standing culture. The experiment was initiated by inoculating 20% (v/v) of the prepared sourdough LAB starter into the WSSM and continued until 10 steps of daily subculture by transferring 20% (v/v) of the cultivated medium to a fresh medium. In each subculture step, fermentation was carried out at 30°C for 8 hr, and then the mixture was stored at 4°C for 16 hr. Sampling was performed at the end of each subculture step. Measurement of pH was performed at both the start and end of each subculture step. Samples for chemical analysis, 16S rRNA gene amplicon sequencing, and quantitative real-time PCR (qPCR) were stored at −30°C until use, whereas pH measurement and LAB counts were performed immediately after sampling. To confirm the reproducibility, each subculture experiment was carried out in two batches.

Sourdough subculture preparation

Wheat flour was used to prepare wheat sourdough. The ash content of the wheat flour was 0.51% of the dry matter, and the protein content of that was 12.2% of the dry matter. The sourdough subculture was carried out in a 1.5-L plastic container covered with a lid. First, 120 g of flour and 120 g of tap water were manually kneaded with a spatula. Following that, 60 g of the prepared sourdough LAB starter was inoculated into the mixture. The conditions of both fermentation and storage were the same as the WSSM subcultures. After fermentation and storage, 60 g of the sourdough was used to inoculate a mixture of 120 g each of flour and tap water for the next subculture. The experiment was continued until 10 steps of daily subculture following the same steps as the previous method for WSSM subculture. The sampling protocols were the same as the subculture experiments with WSSM. To confirm the reproducibility, the sourdough subculture was carried out in two batches.

Measurement of pH and determination of total titratable acidity

The pH value was measured with a pH meter (F-73, Horiba, Kyoto, Japan). To assess the acidity of each subculture step, total titratable acidity (TTA) was determined using a previously described method with some modifications [14]. At the end of each subculture step, the sample was diluted 10 times with deionized water and titrated with 0.1 M NaOH to get to the initial pH of each subculture step. Both pH and TTA were measured twice per sample, and the averaged values were obtained for each batch.

Determination of sugars, organic acids, and ethanol

The determinations of maltose, glucose, lactic acid, acetic acid, and ethanol concentrations were carried out by using a biosensor (BF-7, Oji Scientific Instrument, Osaka, Japan) according to the manufacturer's instructions [4]. These determinations were performed twice per sample, and the averaged values were obtained for each batch.

Enumeration and isolation of LAB

LAB were counted as described previously [4]. Enumeration was performed twice per sample, and the averaged counts were obtained for each batch. Two to five colonies per sample of presumptive LAB were randomly selected. For isolation, selected colonies were purified by streaking twice onto the same agar medium.

Identification of LAB isolates

The genomic DNA of LAB was extracted using an alkaline boiling method previously described [4]. For all LAB isolates, the 16S rRNA gene was amplified using the primer pair 10F/800R [15]. A nucleotide BLAST [16] seach of the partial 16S rRNA gene sequences of all isolated LAB showed ≥ 99% identity to type strains in the sequence database. To confirm the results of identification at the species level, species-specific PCR and housekeeping gene sequencing were performed as described previously [4]. All primers used in this study are summarized in Supplementary Table 1 [17–21].

Total genomic DNA extraction

Total genomic DNA from the subcultured WSSM was extracted by the bead beating method using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) [4, 22]. Total genomic

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DNA from sourdough was extracted by a similar protocol to the extraction from the subcultured WSSM, except for using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) [4].

Bacterial community analysis by 16S rRNA gene amplicon sequencing

Bacterial community analysis was carried out as previously described [4]. The universal primers Tru27F and Tru354R were used for the first PCR (Supplementary Table 1) [23]. The amplicons were pooled in equal amounts (20 ng of each amplicon), and then the DNA concentration of the library was measured. The amplicon library was mixed with a PhiX control to a final concentration of 10 pM 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 (600 cycles).

The obtained sequences were processed using the UPARSE pipeline in USEARCH [24]. Pairs of sequence reads were merged using the fastq-mergepairs command. After quality filtering, length trimming, dereplication, and discarding of singletons, the merged sequences were clustered into 234 operational taxonomic units (OTUs) using the UPARSE-OTU algorithm. A total of 2,847,981 reads were obtained from 87 samples (median, 31,927 reads per sample; range, 11,282–83,339 reads/sample). The taxonomy of each OTU was assigned up to the genus level using the RDP 16S rRNA gene database [25]. Furthermore, the taxonomies of four representative OTUs (OTU1, 2, 3, and 5) were assigned at a closely related species level according to the top-hit species (with ≥99% similarity, except for OTU2, which showed ≥97% similarity), namely to the type strain, via BLAST using the OTU as the query.

A beta diversity analysis using unweighted UniFrac metrics was performed based on the OTU table rarified to 10,000 sequences per sample by using the beta_diversity_through_plot.py script in QIIME v1.9 [26].

Quantification of total bacterial amount by qPCR

Total bacteria were quantified by qPCR targeting the 16S rRNA gene using the primers Uni334F and Uni514R (Supplementary Table 1) as described previously [27]. Melting curve analysis was performed at the end of the qPCR cycles to check the amplification specificity. All reactions were performed in duplicate using the Rotor-Gene 6000 cycler equipped with a Rotor-Disc 72 (Qiagen, Hilden, Germany). For all assays, PCR efficiency was ≥67%, with a correlation coefficient of ≥0.99. The copy numbers of the 16S rRNA gene in the sample were quantified by comparing the cycle threshold value of the sample to a standard curve of serially diluted genomic DNA (10^3–10^7 copies/μL) extracted from *Lactobacillus plantarum* subsp. *argentoratensis* JCM 16169^T.

Regarding the four representative OTUs (OTU1, 2, 3, and 5), the estimated 16S rRNA gene copy numbers of each OTU was calculated by multiplying the corresponding relative abundance obtained from the 16S rRNA gene amplicon sequencing by the 16S rRNA gene copy numbers obtained by qPCR [28].

Nucleotide sequence accession number

The raw data of 16S rRNA gene amplicon sequences were deposited in the DNA Data Bank of Japan (DRA008897) under accession number BioProject PRJDB8699.

RESULTS

Effect of pH on lactic acid fermentation

To investigate the effect of pH on lactic acid fermentation, three subcultures with WSSM adjusted to pH 6.7, 5.5, and 4.5 were started by inoculating a starter mixture of 152 sourdough samples. Figure 1A shows the succession of pH in each subculture. Beginning with the 4th step, the final pH of each step constantly dropped to 4.0, despite the initial pH of the WSSM. Moreover, the TTA was higher when a medium with a higher pH was used (Fig. 1B). After the 4th step, the subculture with the WSSM adjusted to pH 6.7 showed approximately four-fold and two-fold higher TTA values compared with the WSSM adjusted to pH 4.5 and pH 5.5, respectively. The LAB counts exceeded 9 log CFU/mL in all subcultures after the 4th step and thereafter stabilized at a maximum level of approximately 9.5 log CFU/

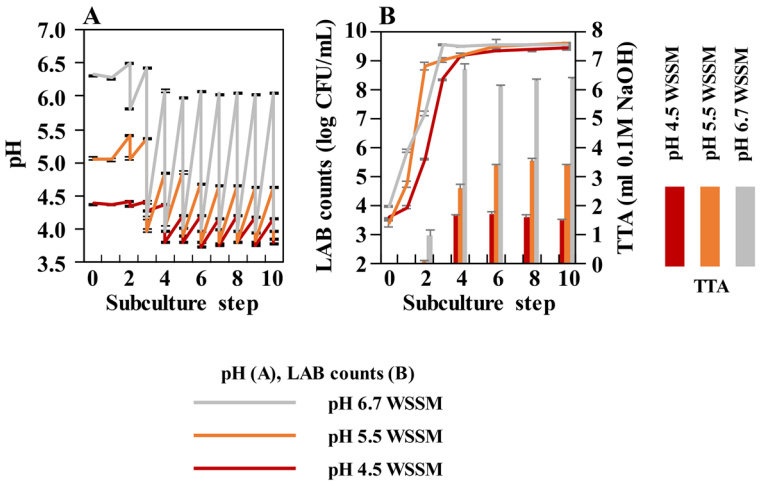

Fig. 1. Changes in pH (A), LAB counts, and TTA (B) in the three pH-adjusted WSSM subculture experiments. Data are expressed as the mean ± standard deviation of two independent batch experiments.

mL (Fig. 1B). These results indicated that the production of acids increased when using a higher pH-adjusted WSSM, while LAB growth remained constant, regardless of the pH of the WSSM.

To investigate the effect of pH in detail, products and substrates of lactic acid fermentation, i.e., lactic acid, acetic acid, ethanol, maltose, and glucose, were measured in the subcultures at the three different pH levels. At the beginning (step 0), maltose was the most abundant, with a constant concentration of approximately 48 mM (Supplementary Fig. 1). Subsequently, maltose levels decreased to 20 mM in the 4th step when using the pH 6.7 WSSM, while the concentration remained higher than 32 mM until the last step when using the pH 4.5 WSSM.

Lactic acid production differed among the three experiments. The concentration of lactic acid increased to 99 mM in the 4th step when using the pH 6.7 WSSM (Supplementary Fig. 1). When using the pH 5.5 and pH 4.5 WSSMs, the lactic acid concentration gradually increased to 72 mM and 55 mM at the last step, respectively. Moreover, ethanol concentrations also increased to a maximum of 29 mM at the 3rd step when using the pH 5.5 WSSM, and thereafter they decreased to below 10 mM, while the ethanol production was much lower in the case of the WSSMs at the other pH levels.

Bacterial community succession in WSSM subcultures

To investigate the effect of pH on the bacterial community succession, a combined culture-independent approach of 16S rRNA gene amplicon sequencing and qPCR was used to profile the microbiota at each subculture step and compare them among the experiments with the WSSMs at the different pH levels. Figure 2 shows the changes in both bacterial community composition and abundance throughout the subculture steps. At the beginning (step 0), the 16S rRNA gene counts (9.1–9.4 log copies/mL) and the bacterial community composition showed a similar profile across all experiments, a reflection of the inoculation of the starter sourdough LAB mixture. Then, *Weissella*, *Pediococcus*, and *Lactobacillus* commonly emerged as the subculture steps increased, with total 16S rRNA gene counts over 10 log copies/mL in all experiments. On the other hand, *Bacillus* appeared until the 4th step with the pH 6.7 WSSM.

In order to depict a more accurate picture of the real abundance changes at a species level, LAB species succession was assessed by qPCR and plate culture. Three representative OTUs accounting for the OTUs with the top-three highest read counts in WSSM subcultures, namely OTU1, OTU2, and OTU5, showed more than 97% identity in the 16S rRNA gene sequence to *Pediococcus pentosaceus*, *Weissella cibaria-confusa* group, and *Lactobacillus brevis*, respectively. Regardless of the difference in the pH of the WSSM, *P. pentosaceus* (OTU1) accounted for 10 to 10.5 log 16S rRNA gene copies/mL in all subcultures (Fig. 3). The colonization of *P. pentosaceus* was confirmed by detecting the colonies on the agar plates at high frequency (total of 59, 35, and 39 isolates out of 75, 76, and 76 tested isolates in the pH 6.7, 5.5, 4.5 WSSMs, respectively; Supplementary Table 2). The *W. cibaria-confusa* group (OTU2) emerged at the same level as *P. pentosaceus* in the early steps of all subcultures but decreased in later steps. Notably, the decrease in the *W. cibaria-confusa* group was more pronounced in lower pH WSSMs, and *L. brevis* (OTU5) emerged instead. *W. cibaria* was frequently identified between the 1st and 6th subculture steps with the pH 5.5 WSSM (total of 25 isolates out of 76 tested isolates) and pH 4.5 WSSM (total of 12 isolates

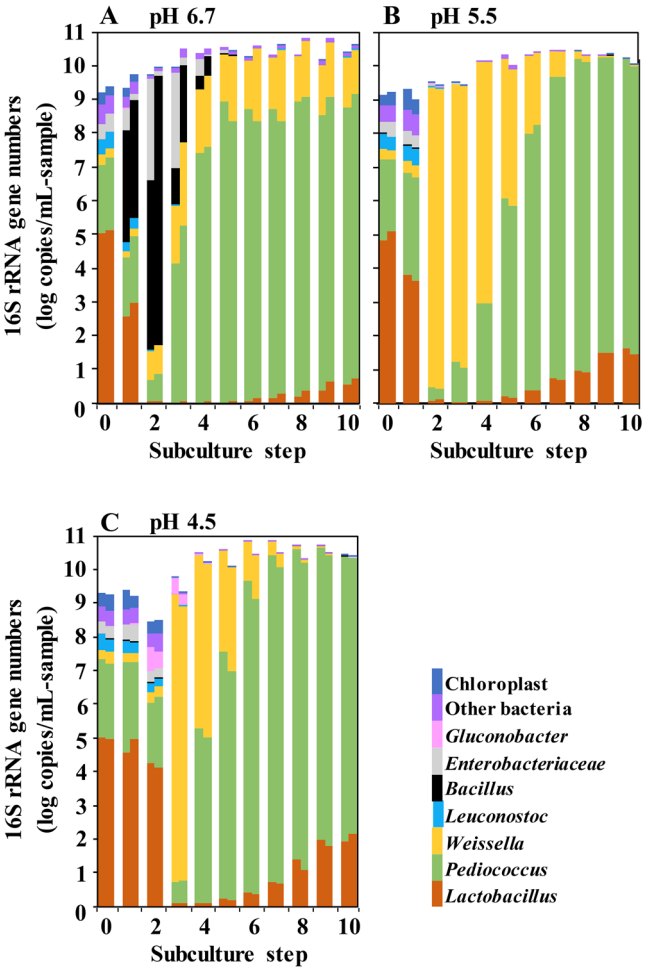

Fig. 2. Bacterial community succession in three pH-adjusted WSSM subculture experiments. The relative abundance of the bacterial community in WSSMs with pH 6.7 (A), pH 5.5 (B), and pH 4.5 (C). Each column represents two batches (left bar, batch 1; right bar, batch 2). Column height indicates the number of 16S rRNA gene copies/mL of a sample as determined by qPCR. The relative abundance (read counts/total read counts) of each bacterial taxonomy is overlaid on each bar and does not correspond to the y axis. Relative abundances higher than 5.0% for any sample are shown.

out of 76 tested isolates). In contrast, *L. brevis* was detected in the later steps of the pH 5.5 WSSM (total of 9 isolates out of 76 tested isolates) and pH 4.5 WSSM (total of 12 isolates out of 76 tested isolates).

Bacterial community succession in a natural sourdough subculture

Instead of the WSSM, natural sourdough was used for subculture and bacterial community succession. The sourdough was prepared by inoculating the same starter LAB mixture into wheat flour and water, and it was thereafter subcultured for ten steps. The succession profiles based on pH, LAB counts, and TTA were analogous to those observed in the subculture with the pH 5.5 WSSM (Supplementary Fig. 2). As observed in the WSSM subcultures, three LAB genera, *Weissella*, *Pediococcus*, and *Lactobacillus*, emerged as the predominant genera during the ten steps of subculture, whereas the community was mostly

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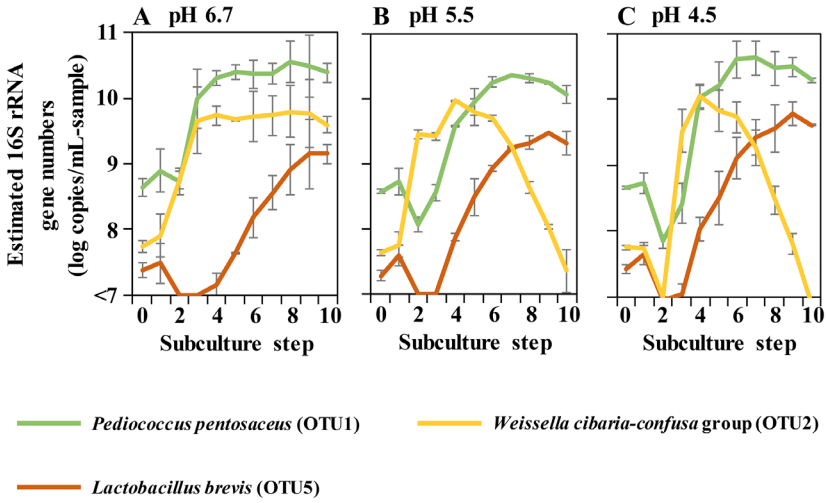

Fig. 3. Individual successions of the three representative OTUs (OTU1, 2, and 5) for pH 6.7 (A), pH 5.5 (B), and pH 4.5 WSSM (C). Closely related species of each OTU were assigned by BLAST. Estimated numbers of 16S rRNA gene copies were calculated by multiplying the number of 16S rRNA gene copies with the corresponding relative abundance of each OTU. The estimated 16S rRNA gene copies of OTU3 were closely related to those obtained for *Lactobacillus sanfranciscensis*, namely ≤ 7.8 log copies/mL. Data are expressed as the mean ± standard deviation of two independent batch experiments.

occupied in later steps by *Lactobacillus* due to the rapid domination of OTU3, which was closely related to *Lactobacillus sanfranciscensis* (Fig. 4). *L. sanfranciscensis* could be identified from the 3rd to the last steps by colony analysis (total of 15 isolates out of 68 tested isolates, Supplementary Table 2).

A principal coordinate analysis using unweighted UniFrac distance among the samples revealed the succession of the bacterial community, the pattern of which was shared among the four subcultures using the WSSM at the three different pH levels and natural sourdough (Fig. 5). The microbiota community shifted in a similar direction, namely PC1 positive to PC1 negative, along with the steps of subculture. The samples from the sourdough subcultures were localized in the PC3 positive region with the domination of *L. sanfranciscensis* (OTU3). The results indicated that the bacterial community evolution in sourdough subcultures was qualitatively realized through WSSM experiments.

DISCUSSION

In this study, we examined the effects of pH levels on the bacterial community and lactic acid fermentation in sourdough by using three pH-adjusted WSSMs. It was suggested that lowering the pH increased the undissociated form of acids, which suppressed the acid production by LAB. Lactic acid production was shown to be increased when using a higher pH WSSM. This can be explained by the expansion of the pH range available for lactic acid fermentation by resident LAB, with the lowest pH allowing lactic acid fermentation being approximately 4, as shown in Fig. 1. It should be noted that more ethanol was produced in the WSSM with a pH of 5.5. This can be explained by the domination of the genus *Weissella*, which is known as a heterofermentative LAB, from the 2nd to 5th steps. Ethanol production due to the growth of the heterofermentative LAB *Lactobacillus fermentum* has also been observed in spontaneous sourdough fermentation [4], but it was not observed in this study.

A noticeable shift of LAB from the *W. cibaria-confusa* group to *L. brevis* was observed in the subculture experiments using the pH 5.5 or pH 4.5 WSSM but not the pH 6.7 WSSM. It has been reported that *W. cibaria* can grow quickly in a medium with pH 6.8 but that growth inhibition can be observed in a pH 4.5 medium [29]. Moreover, another study reported that the growth of *Weissella* is negatively influenced by a pH 4.5 medium compared with a pH 6.5 medium [30]. In contrast, although *L. brevis* grows relatively slower than other *Lactobacillus* species and *Weissella* during sourdough fermentation [31], *L. brevis* can overcome the inhibitory effect of low pH by producing ammonia via the arginine deiminase pathway [32, 33]. It was suggested that a mildly acidic environment drives the succession of LAB communities in sourdough by the early establishment of a niche which is favorable for acid-tolerant LAB, such as *L. brevis* and *L. sanfranciscensis*. The domination of *P. pentosaceus* was observed in all subcultures, but this decreased in the sourdough as the number of subculture steps. This suggested that a controlling factor other than pH influences the decline of *P. pentosaceus* in the sourdough environment. In addition, members of the *Bacillus* genus, a genus of bacteria that includes the foodborne pathogens such as *Bacillus cereus*, grew in the earlier steps of the pH 6.7 medium but not with the lower pH media. This indicated that acidic pH driving by LAB such as *Weissella* could prevent unfavorable bacteria in the sourdough ecosystem.

The principal coordinate analysis showed that the sequence of the bacterial community evolution in the natural sourdough subcultures was realized, to a certain extent, in the WSSM experiments. However, the passage of bacterial community shifts was more rapid in the natural sourdough subcultures due to *L. sanfranciscensis,* which is probably the species most adapted to the sourdough environment [34]. As *L. sanfranciscensis* was hardly detected in WSSM subcultures (≤ 7.8 log copies/mL of 16S rRNA gene numbers), the anaerobic condition or certain extra nutrients in sourdough may modulate the succession of the bacterial community [3, 35]. It was reported that *L. sanfranciscensis* is stimulated by the anaerobic condition in a sourdough-like

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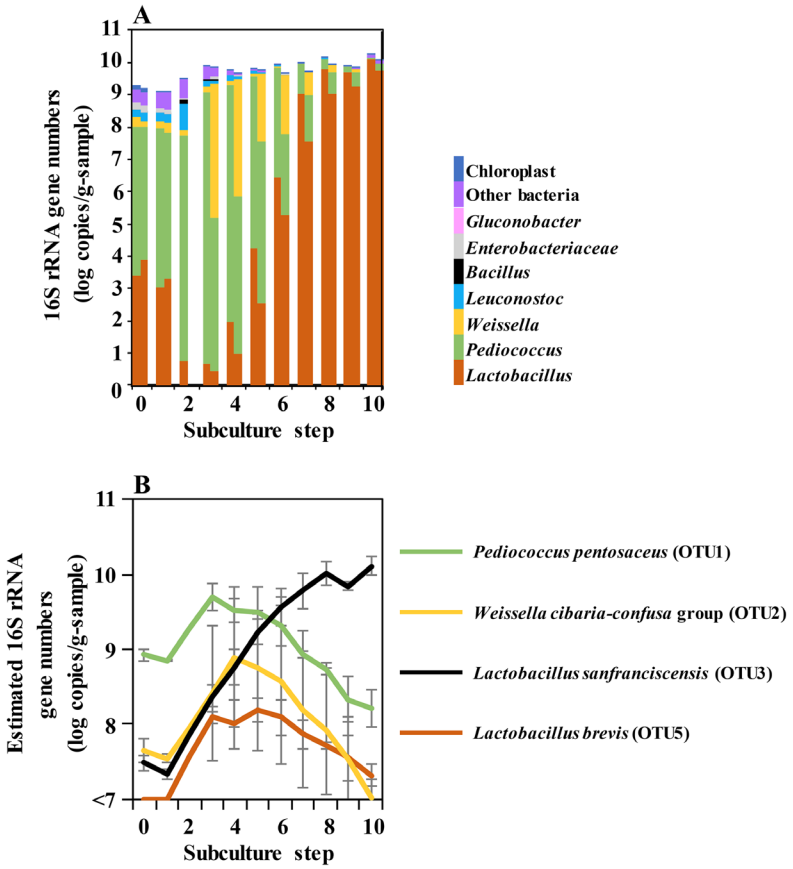

Fig. 4. Changes in bacterial community succession in the sourdough subculture experiment (A). Column height indicates the number of 16S rRNA gene copies/g of a sample as determined by qPCR. The relative abundance (read counts/total read counts) of each bacterial taxonomy is overlaid on each bar and does not correspond to the y axis. Relative abundances higher than 5.0% for any sample are shown. Each column for a bacterial community represents two batches (left bar, batch 1; right bar, batch 2) except for the 2nd step, for which only the results of batch 1 are shown. Individual successions of four representative OTUs, OTU1, 2, 3, and 5, for the sourdough subculture (B). Closely related species of each OTU were assigned by BLAST. Estimated numbers of 16S rRNA gene copies were calculated by multiplying the number of 16S rRNA gene copies with the corresponding relative abundance of each OTU. Data are expressed as the mean ± standard deviation of two independent batch experiments.

environment [35]. Further research is planned to evaluate the dissolved oxygen levels of both WSSM and sourdough subcultures in order to investigate the reason for the *L. sanfranciscensis* outgrowth. Furthermore, indigenous microorganisms residing in both sourdough ingredients and the bakery environment may interact with the sourdough bacterial community [36, 37].

Interestingly, an unpredictable succession of LAB communities also occurs along with changes in the pH level in the fermentation of other natural foods [22, 38, 39]. It has been reported that the same kind of bacterial relay comprised of *Weissella*, *Enterococcus*, *Pediococcus*, and *Lactobacillus* occurs in fermented rice brans that are traditionally used for pickling vegetables in Japan [22]. It should be noted that the bacterial relay takes place in association with a shift in pH, which is consistent with our observations in this sourdough study. Moreover, in kimchi, which is a traditional fermented vegetable dish in Korea, the abundance of *Lactobacillus* expands in the bacterial community, which mainly consists of three LAB genera (*Weissella*, *Leuconostoc*, and *Lactobacillus*), while the pH drops to 4.2–4.5 during the fermentation process [39]. These similarities in the mechanism of bacterial community shifts suggest that a drop in pH driven by the LAB community is a key parameter for stable open-batch fermentation with consistent microbiota changes.

In conclusion, a sequence of bacterial community evolution mainly comprising three LAB genera was observed in the WSSM subcultures as well as in the sourdough subculture. The succession, with a shift from *Weissella* to *Lactobacillus*, was accelerated under lower pH conditions. However, the *Lactobacillus*-dominant microbiota observed in the sourdough subculture due to the outgrowth of *L. sanfranciscensis* was not realized in the WSSM subcultures. Further studies are required to elucidate additional factors other than pH that play a role in the pattern of the LAB community shifts.

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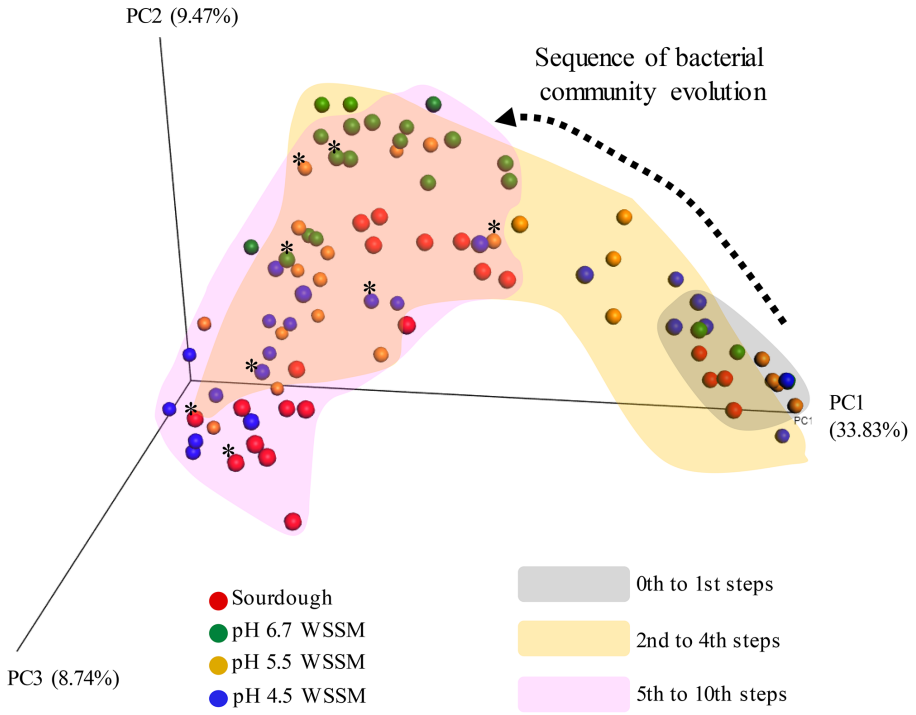

Fig. 5. Principal coordinate analysis of 16S rRNA amplicon sequencing data from WSSM subcultures adjusted to pH 6.7, 5.5, and 4.5 and the sourdough subculture. The unweighted UniFrac metrics were used for the computation. Asterisks indicate the last subculture steps.

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