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Full Paper

Lot-to-lot variation in the microbiota during the brewing process of *kimoto*-type Japanese rice wine

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***Kimoto*-type Japanese rice wine (*sake*) has a wide variety of flavors, as the predominant microbes, including lactic acid bacteria (LAB) and nitrate-reducing bacteria, that spontaneously proliferate in the fermentation starter vary depending on the brewery. In this study, we traced the microbiota in four lots of starters manufactured in a newly established brewery and evaluated the lot-to-lot variation and characteristics of the microbiota in the brewery. The results of a 16S ribosomal RNA amplicon analysis showed that the starters brewed in the second brewing year had a more diverse microbiota than those in the first brewing year. Among the LAB predominated at the middle production stage, lactococci, including *Leuconostoc* spp., were detected in all the lots, while lactobacilli predominated for the first time in the second year. These results suggest that repeated brewing increased microbial diversity and altered the microbial transition pattern in the *kimoto*-style fermentation starters. Phylogenetic analyses for the LAB isolates from each starter identified *Leuconostoc suionicum, Leuconostoc citreum*, and *Leuconostoc mesenteroides* as predominant lactococci as well as a unique lactobacillus in place of *Latilactobacillus sakei*. We also found that a rice *koji*-derived *Staphylococcus gallinarum* with nitrate-reducing activity was generally predominant during the early production stage, suggesting that there was a case in which staphylococci played a role in nitrite production in the starters. These findings are expected to contribute to the understanding of the diversity of microbiota in *kimoto*-type *sake* brewing and enable control of the microbiota for consistent *sake* quality.**

Key words: Japanese rice wine, microbiota, lactic acid bacteria, nitrate-reducing bacteria

INTRODUCTION

Japanese rice wine (*sake*) is a traditional Japanese alcoholic beverage that is manufactured by fermenting rice using rice, rice *koji*, and water as the raw materials. *Sake* brewing is characterized as parallel fermentation consisting of the saccharification of rice starch by the amylase produced by *koji* mold (*Aspergillus oryzae*) and simultaneous production of alcohol from sugar by *sake* yeast (*Saccharomyces cerevisiae*) [1]. In the first step of *sake* brewing, *koji* mold spores are sprinkled on steamed rice and incubated at 30–40°C for two days to prepare rice *koji* (malted rice). The obtained rice *koji* is mixed with steamed rice, water, and the *sake* yeast, and the mixture is kept at a controlled temperature for 2 to 4 weeks to allow the yeast to propagate sufficiently to make a fermentation starter (*moto*). After that, the main *sake* mash (*moromi*) is prepared by adding the rice *koji*, steamed rice, and water to the fermentation starter three times over the course of 4 days and then fermented for about a month. Finally, the fermented *moromi* is separated into *sake* and *sake* lees using a filter press.

In *sake* brewing, production of the fermentation starter is an important process that determines the quality of the *sake*. Fermentation starters can be divided into two types depending on the manufacturing method. A *sokujo*-style fermentation starter is produced by adding lactic acid directly to the starter. The acid helps prevent contamination by wild microorganisms and creates an acidic environment desirable for ethanol production by yeast. Production of the other style, a *kimoto*-style fermentation starter, relies on the lactic acid bacteria (LAB) that naturally

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occur in the starter and produce a sufficient amount of lactic acid. The simplicity of the *sokujo* style distinguishes it as a modern manufacturing method, but the production method using the traditional *kimoto* style has been on the rise in recent years, as the *sake* produced using this method has diverse tastes and flavors due to the diversity of the LAB that grow naturally during the *sake*-brewing process. It has been reported that the composition of organic acids and the accumulation of ornithine in *sake* vary depending on the species of LAB [2, 3].

The *kimoto*-style fermentation method starts at a neutral pH. Thus, there is a risk of spoilage due to the growth of undesirable microorganisms. However, it is known that systematic transitions of the microbiota during production prevent spoilage [4]. In the early stage of fermentation, the starter contains wild yeasts and nitrate-reducing bacteria derived from the starting materials. Nitrate-reducing bacteria, such as *Pseudomonas* spp. and *Enterobacter* spp., convert the nitrate dissolved in the water into nitrite, which suppresses the growth of contaminating yeasts. During the middle stage of fermentation, lactococci proliferate first, followed by lactobacilli, to produce lactic acid, lower the pH, and eliminate the nitrate-reducing bacteria and wild yeast. In the late stage of fermentation, the *sake* yeast grows and produces alcohol, which eliminates the LAB grown in the middle stage, resulting in a pure *sake* yeast broth. *Leuconostoc mesenteroides* and *Latilactobacillus* (formerly *Lactobacillus*) *sakei* are well-known lactococci and lactobacilli, respectively, found during starter production [5]. However, recent metagenomic studies using next-generation sequencing technology have revealed that each *sake* brewery has a unique microbiome, including the dominant LAB species [3, 6–9]. In addition, even in the same brewery, there have been cases where the dominant LAB species have varied depending on the production year [3, 7].

Several reports have indicated that the microorganisms found in a *kimoto*-style fermentation starter originated from the rice *koji* and water or the surfaces of the brewing equipment [6, 10–13]. In particular, the same lineage of *L. mesenteroides* was found as a dominant species in a starter over the course of years and was shown to inhabit the surface of the equipment used for *sake* brewing [14]. These results imply that the LAB dominant in *kimoto*-style fermentation starters may be unique to the *sake* brewery. However, it is still unknown whether repeated *sake* brewing affects the microbial diversity, including the predominant LAB, and transition patterns in a *kimoto*-style fermentation starter.

The objectives of this study were to evaluate the lot-to-lot variation in the microbiota of *kimoto*-style fermentation starters and elucidate the characteristics of transitions in the microbiota during the manufacturing process in a newly established *sake* brewery in Hokkaido, Japan. The microbiota was analyzed on the basis of an RNA-based 16S ribosomal RNA (rRNA) amplicon analysis for four lots of starters produced in the first and second brewing years after establishment of the brewery and evaluated for the differences in the microbiota in each lot through bacterial composition and diversity analyses. In addition, we investigated the phylogenetic and physiological characteristics of the predominant LAB and staphylococci in each lot.

MATERIALS AND METHODS

Sample collection

Four lots of *kimoto*-style fermentation starters (Lots A to D) were collected from a brewery, Hekiungura, established in 2020 by Kamikawa Taisetsu Sake Brewery Co., Ltd. (Obihiro, Hokkaido, Japan). The starters were fermented for 32 days (March 10–April 10, 2021 in the first brewing year) for Lot A, 31 days (April 6–May 6, 2021 in the first brewing year) for Lot B, 30 days (October 24–November 22, 2021 in the second brewing year) for Lot C, and 30 days (February 15–March 16, 2022 in the second brewing year) for Lot D. *Sake* rice produced in Hokkaido prefecture with a polishing degree of 80% was used as a raw material. The varieties of *sake* rice used were as follows: *Suisei* for Lot A, *Kitashizuku* for Lots B and D, and *Ginpu* for Lot C. Rice *koji* (20 kg), steamed rice (40 kg), and water (55 L) were mixed in a tank and fermented for about 2 weeks. *Sake* yeasts were added when the total acidity reached approximately 3 and 5 mL, and fermentation was continued for another 2 weeks or so. The fermentation was carried out while controlling the temperature between 5 and 25°C as shown in Supplementary Fig. 1.

Measurement of basic chemical components

The filtrate obtained from the starters through a paper filter was used for the determination of chemical components. The Baume degree was measured using a specific gravity hydrometer. The reading for the filtrate of a *kimoto*-style starter at 15°C was taken as the Baume degree. The total acidity was defined as the volume of 0.1 M NaOH aqueous solution required to neutralize 10 mL of the filtrate of a starter. To detect the point of neutralization, a few drops of a bromothymol blue solution was added to the filtrate as an indicator. The pH was measured using a pH meter (Eutech Instruments Pte. Ltd., Singapore). The concentrations of lactic acid and ethanol in the starters were evaluated using an HPLC system (LC-10AT, Shimadzu Co., Kyoto, Japan), an RSpak KC-811 column (Resonac Corporation, Tokyo, Japan) maintained at 50°C, a mobile phase of 6 mM perchloric acid, and a differential refractive index detector (RI- 201H, Resonac Corporation). After the filtrate of the *kimoto*-style starter was centrifuged (20,000 g, 2 min), the obtained supernatant was diluted 10-fold with Milli-Q water. Solutions of lactic acid and ethanol were used as standard solutions, and calibration curves showing the relationship between the concentration and peak area were constructed. The nitrite concentration was measured based on naphthylethylenediamine absorptiometry. During starter preparation, 1 mL filtrate samples were collected on days 2 to 14 and centrifuged at 20,000 g for 2 min. The supernatant was then applied to an Amicon Ultra centrifugal filter (3 K, Merck, Darmstadt, Germany) to obtain a low molecular weight fraction. Subsequently, 50 µL of a solution containing 1% (w/v) sulfanilamide dissolved in 10% (v/v) hydrochloric acid was added to an equal volume of the sample, the mixture was allowed to stand for 15 min, and 50 µL of a 0.12% (w/v) naphthylethylenediamine solution was then added. After incubation at room temperature for 20 min, the absorbance at 540 nm was measured using a microplate reader (SH-9000Lab, Corona Electric Co., Ltd., Hitchinaka, Ibaraki, Japan). Sodium nitrite was used as the standard to calculate the concentration of nitrite in the starters.

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Viable cells counting

The numbers of viable microorganisms in the *kimoto*-style starters at each fermentation stage were counted based on a dilution plating method using selective media and expressed as colony-forming units (CFU) per gram of starter. Viable LAB counts were determined by counting colonies anaerobically grown on de Man, Rogosa and Sharpe (MRS) agar plates (Biokar Diagnostics, Allone, France) containing 50 µg/mL cycloheximide and 50 µg/mL sodium azide. Total aerobic bacteria counts were determined by counting colonies grown on Reasoner's Agar No.2 (R2A) plates (Shiotani M.S. Co., Ltd., Amagasaki, Hyogo, Japan) containing 50 µg/mL cycloheximide. Viable yeast counts were determined by counting white circular colonies grown on potato dextrose agar (PDA) plates (Difco, Detroit, MI, USA) containing 100 µg/mL chloramphenicol. All cultivations were performed at 30°C.

RNA extraction and cDNA synthesis

Approximately 1 g of a *kimoto*-style starter was added to 10 mL of NAP Buffer (0.019 M EDTA, 0.018 M sodium citrate trisodium salt dihydrate, 3.8 M ammonium sulphate, pH 5.2) [15] in a 50 mL conical tube and stored at −20°C until RNA extraction. To extract RNA, samples in the NAP buffer were suspended using a vortex mixer and left at room temperature for 1 min. The upper layer was transferred to a new tube and centrifuged at low speed (200 g, 5 min, 4°C). The supernatants containing microbial cells were transferred to a new tube and centrifuged at high speed (20,000 g, 3 min, 4°C). RNA was extracted from pellets using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), followed by DNase I digestion of contaminated DNA and purification using an RNeasy Mini kit (Qiagen, Venlo, the Netherlands). A SuperScript® III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to synthesize cDNAs of each sample according to the manufacturer's instructions.

16S rRNA gene amplicon analysis

The 16S rRNA gene amplicon was obtained based on a two-step PCR. In the first PCR, approximately 550 bp fragments of the 16S rRNA V3–V4 region were amplified from a cDNA sample using TaKaRa ExTaq (Takara Bio Inc., Kusatsu, Japan) and 16S rRNA Amplicon PCR primers (Supplementary Table 1). The reaction conditions were as follows: incubation at 98°C for 1 min, followed by 25 cycles of denaturation (98°C, 10 sec), annealing (55°C, 30 sec), and extension (72°C, 30 sec), and then incubation at 72°C for 5 min. The obtained PCR product was purified using Agencourt AMPure XP (Beckman Coulter Inc., Brea, CA, USA). In the second PCR, the purified first PCR product was used as a template, and an adapter was added using the primer pair Nextera XT Index Kit v2 set A or B (Illumina, Inc., San Diego, CA, USA) to amplify an approximately 630 bp DNA fragment using PrimeSTAR Max (Takara Bio Inc., Kusatsu, Japan). The reaction conditions were as follows: incubation at 94°C for 2 min, followed by 8 cycles of denaturation (98°C, 10 sec), annealing (55°C, 5 sec), and extension (72°C, 30 sec). The obtained second PCR product was purified using Agencourt AMPure XP. All samples were prepared to equal concentrations and collected in a tube. The library was submitted to Novogene Japan (Tokyo, Japan), and sequencing analysis was performed based on the paired-end method using a NovaSeq system (Illumina, Inc.).

Data analysis for the microbiome

The data obtained from Illumina sequencing were analyzed using QIIME 2 software ver. 2023.2 (https://qiime2.org). PCR errors were corrected using DADA2, and the resulting reads were classified based on the amplicon sequence variants (ASVs). Each ASV was classified at the genus level and species level based on the SILVA ribosomal RNA gene database [16]. The α-diversity (Shannon index) was calculated using QIIME 2. The β-diversity was plotted based on a principal coordinate analysis (PCoA) of the Bray–Curtis index using MicrobiomeAnalyst 2.0 [17].

Isolation and phylogenetic analyses of lactic acid bacteria

Each lot of the *kimoto*-style starters was streaked on MRS agar plates, and the colonies grown on the plates were randomly selected and transferred twice to obtain pure cultures. M17 agar medium (Biokar Diagnostics) was used to isolate *Lactococcus* spp. To identify the isolates, the bacterial cells from a single colony were suspended in 50 µL of BL buffer (40 mM Tris-HCl, 1% [v/v] Tween 20, 0.5% [v/v] Nonidet P-40, 1 mM EDTA, pH 8.0), 40 µL of sterile distilled water, and 10 µL of proteinase K (1 mg/mL) and then incubated first at 60°C for 20 min and then at 95°C for 5 min. After centrifugation at 20,000 g for 1 min, the supernatant was collected and used as the PCR template. The genomic regions of the 16S rRNA, *rpoA*, and *pheS* genes in each isolate were obtained based on PCR using the oligonucleotide primers 27F and 1492R for 16S rRNA, rpoA-21-F and rpoA-23-R for *rpoA*, and pheS-21-F and pheS-23-R for *pheS* (Supplementary Table 1). All gene fragments were amplified using TaKaRa ExTaq and purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). The nucleotide sequences of those PCR products were determined by Eurofins Genomics (Tokyo, Japan) based on the Sanger method using the primers referred to earlier.

The genera of the isolates were determined based on the obtained 16S rRNA gene sequences using the RDP Classifier [18]. The isolates were grouped into clusters with completely matched 16S rRNA gene sequences. The concatenated sequences of *rpoA* and *pheS* were aligned using the ClustalW program [19] with the reference sequences. Neighbor-joining trees were constructed in the MEGA X software [20], and 1,000 bootstrap replicates were used to generate a consensus tree.

The API 50 CHL identification test (bioMérieux Japan Ltd., Tokyo, Japan) based on the carbohydrate fermentation pattern of LAB was performed according to the manufacturer's instructions. The results were recorded after 24 and 48 hr, and identification was conducted using the APIWEB database identification software (bioMérieux Japan Ltd.).

Ethanol stress tolerance test in lactic acid bacteria

Fully grown cultures of LAB strains were diluted to OD_{660}=0.5 with sterile water, and then the dilutions were inoculated to 4 mL each of MRS liquid medium containing 0, 2.5, 5, 7.5, 10, 12.5 or 15% (v/v) ethanol in a test tube. After culture at 30°C for 3 days, the turbidity (OD_{660}) was measured.

Isolation and identification of staphylococci grown on rice koji

Rice *koji* samples used for each of the lots were suspended in a sterile 0.85% (w/v) sodium chloride solution, serially

diluted, streaked onto an R2A agar medium containing 50 mg/L cycloheximide, and incubated at 30°C for 3 days. The yellow colonies grown on the R2A agar plates were randomly selected and transferred twice to obtain pure cultures. The nucleotide sequences of the 16S rRNA gene in staphylococci were obtained as described above.

Nitrate-reducing activity in staphylococci

Fully grown cultures of *Staphylococcus* strains were diluted to OD_{660}=0.3 in a 0.85% (w/v) sodium chloride solution, and 50 µL of the dilutions was impregnated onto paper discs (8 mm, ADVANTEC, Co., Ltd., Tokyo, Japan). Each disc was immediately placed onto the surface of a R2A agar plate and incubated at 30°C for 24 hr. A solution containing 1.5% (w/v) agar, 0.05% (w/v) potassium nitrate, and 1% (w/v) Griess-Romijn Reagent (1% [w/v] 1-naphthylamine, 10% [w/v] sulfanilic acid, 89.5% [w/v] tartaric acid) was overlaid to observe the development of a red color around the paper disc indicating nitrate reduction ability. The presence of the *napG* gene in the genome of staphylococci was evaluated based on PCR using TaKaRa ExTaq and the SGnarG-F1/SGnarG-R1 and SGnarG-F2/SGnarG-R2 pairs of oligonucleotide primers (Supplementary Table 1). These primers were designed with reference to the genome sequence of *Staphylococcus gallinarum* FCW1 (accession no. CP086207.1), yielding products of 450 bp from SGnarG-F1/SGnarG-R1 and 490 bp from SGnarG-F2/SgnarG-R2.

Data availability

Illumina short-read sequences were deposited in the DDBJ Sequence Read Archive under the identifier DRA017211 (DRX 489702 to DRX 489773). DNA sequences of the 16S rRNA, *rpoA*, and *pheS* genes determined based on the Sanger method were deposited in the GenBank/EMBL/DDBJ under the accession codes listed in Supplementary Tables 2 and 3.

RESULTS

Chemical contents of the kimoto-style fermentation starters during manufacturing

We analyzed four lots of the *kimoto*-style fermentation starters (Lots A to D) manufactured in the first and second brewing years after the establishment of a new brewery. The daily changes in the product temperature, Baume degree, total acidity, pH, and concentrations of ethanol, lactic acid, and nitrite are shown in Supplementary Fig. 1. Lactic acid accumulated faster in Lot C than in the other lots. Thus, an increase in the total acidity and a decrease in pH were also faster in Lot C. In addition, ethanol also accumulated faster in Lot C than in the other lots. Nitrite was detected in all the lots, with relatively high concentrations in Lot D that peaked on day 8 (0.752 ppm).

Viable cell counts of microorganisms

To investigate microbial transitions during the production of each *kimoto*-style starter, the numbers of the viable yeast, aerobic bacteria, and LAB were counted at an interval of a few days. The total numbers of viable aerobic bacteria in the early stage of fermentation (days 1–6) ranged from 10^5 to 10^7 CFU/g, which were higher than those of LAB in all the lots (Fig. 1). The results indicate that aerobically grown bacteria other than LAB dominated in the early stage. The numbers of viable LAB gradually increased from days 6 to 8 and reached 10^8–10^9 CFU/g. After the addition of the *sake* yeast, the viable yeast counts increased, and the ethanol concentration increased accordingly. The number of viable bacteria drastically decreased after the ethanol concentration in the starters reached around 6% (v/v). However, the LAB in Lot B survived even after the development of ethanol, showing about 10^3 to 10^4 CFU/g at the late stage of fermentation.

Microbiome analysis

We performed an RNA-based 16S rRNA amplicon analysis, a technique that allows the targeting of more viable bacteria [21], to evaluate the microbiota during the manufacture of Lots A–D. The number of high-quality short reads obtained by Illumina sequencing ranged from 19,502 to 165,384 (Supplementary Table 4). The rarefaction curves showed sufficient saturation even with the lowest number of reads (19,502 reads; Supplementary Fig. 2), indicating that the sample sizes were adequate for microbiome analysis.

The daily changes in α-diversity based on the Shannon index for each lot are shown in Fig. 2A. The indexes were initially low for all the lots but increased with the growth of LAB after a few days and then peaked. The maximum values of the Shannon index were higher in the starters manufactured in the second year (2.41 on day 7 for Lot C, 2.09 on day 11 for Lot D) than those in the first year (1.74 on day 6 for Lot A, 1.13 on day 6 for Lot B).

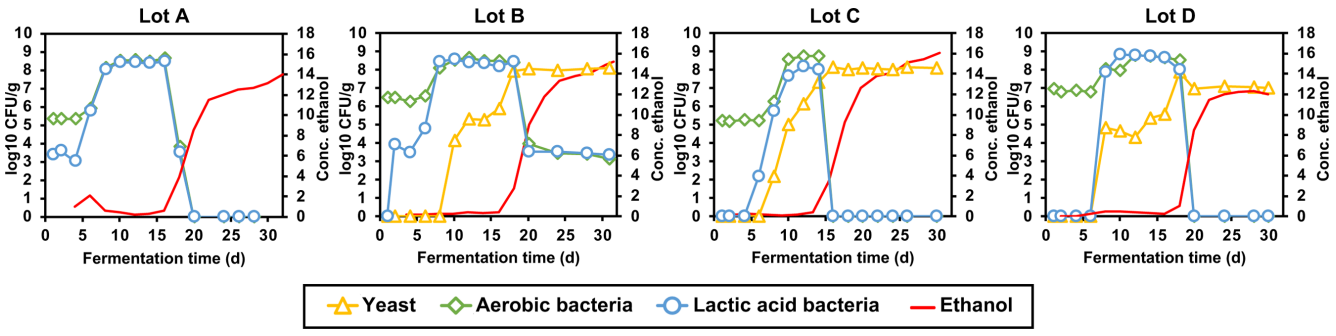

Fig. 1. Viable cell counts of microorganisms during the production of *kimoto*-style fermentation starters. The viable cell counts of yeast (yellow open squares), total aerobic bacteria (green open diamonds), and lactic acid bacteria (blue open circles) are shown. The red lines in each chart indicate the concentration of ethanol in the starter. The viable cell counts are expressed as colony-forming units per 1 g of starter (CFU/g). Measurements were performed in triplicate and mean values are shown. The viable cell counts of yeast were not analyzed for Lot A.

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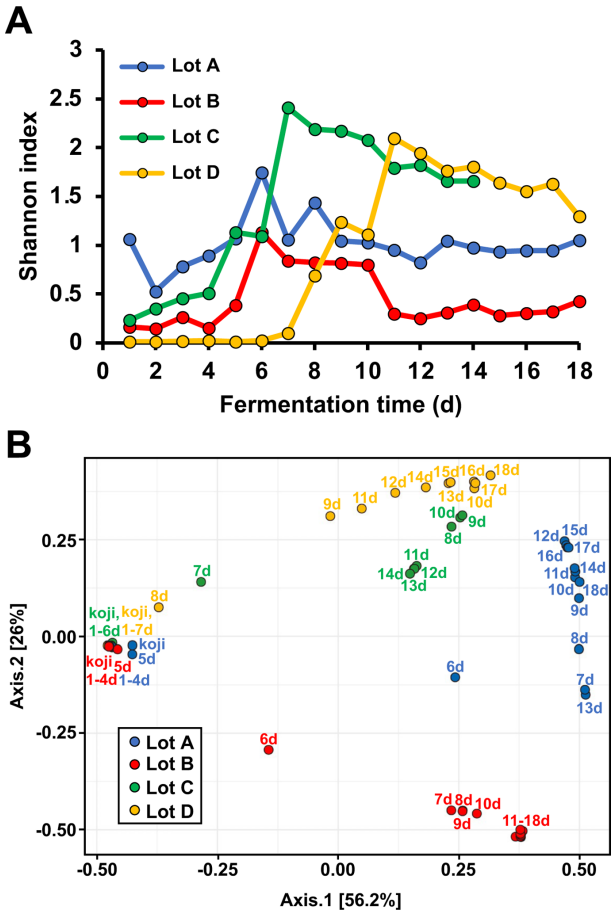

Fig. 2. Alpha and beta diversity for each lot of the *kimoto*-style fermentation starters. (A) Shannon diversity index. (B) Principal coordinate analysis (PCoA) plot based on the Bray-Curtis dissimilarity. Lot A, Lot B, Lot C, and Lot D are represented by blue, red, green, and yellow, respectively.

A β-diversity analysis based on the Bray-Curtis distance was performed, and the resulting PCoA plots are shown in Fig. 2B. The plots of the samples on days 1–5 were located close to each other for all the lots. In contrast, the plots of the samples obtained during the middle stage of fermentation revealed a certain distance between different lots. The plots for Lots C and D were relatively close together but were still clustered away from each other. These results suggest that the microbiota at the early stage of fermentation consisted of common aerobic bacteria between lots but that the LAB grown at the middle stage varied greatly from lot to lot.

The bacterial compositions of the *kimoto*-style fermentation starters during production and the rice *koji* added to each lot are shown at the genus level in Fig. 3A. During the early stages of fermentation (days 1–5), *Staphylococcus* was commonly detected in all the lots with an occupancy >90%. A high dominance of *Staphylococcus* was also detected in the rice *koji*, suggesting that the staphylococci detected in the starters were derived from the rice *koji*. Nitrate-reducing bacteria commonly found in *kimoto*-style fermentation starters, such as *Pseudomonas*, *Achromobacter, Macrococcus*, and *Enterobacter* spp., were not predominant at early stages in any of the samples examined in this study. In the middle stage of fermentation, *Leuconostoc* became predominant in Lots A, B, and D. In addition, *Staphylococcus*, *Lactobacillus*, and *Enterobacteriaceae* were detected in Lot D as minor genera. The bacterial composition in Lot C was quite different from those in the other lots, showing high occupancies of *Lactobacillus* and *Lactococcus* and low occupancies of *Leuconostoc*.

Diversity of lactic acid bacteria

To understand the detailed LAB microflora for each lot, we evaluated the relative abundance at the species level, focusing only on the *Lactobacillales* order in the rice *koji* and *kimoto*-style fermentation starters during production (Fig. 3B). The results revealed that the LAB flora greatly varied from lot to lot, but *L. mesenteroides* and *Leuconostoc citreum* were detected in all the lots. Almost all of the LAB present in Lots A and B were lactococci, including *L. mesenteroides*, *L. citreum*, and *Lactococcus lactis*. On the other hand, *Latilactobacillus* (detected as *Lactobacillus*) *curvatus* and other lactobacilli were detected in Lots C and D. Lot B had the simplest LAB flora and was predominated by *L. mesenteroides*, while Lot C had a complex flora that included *L. lactis*, *Lactococcus raffinolactis*, *L. citreum*, *L. curvatus*, and *Lactobacillus* sp. Furthermore, the species detected in the rice *koji* for Lots A, B, and C were consistent with the dominant species in the corresponding *kimoto*-style fermentation starters (Fig. 3B).

Isolation of lactic acid bacteria

In the above amplicon analysis, the species-level resolution might have been low because only the V3–V4 region of 16S rRNA gene was analyzed. Therefore, in order to investigate the exact phylogeny of the predominant LAB and alteration of the flora that dominated in the *kimoto*-style fermentation starters, the LAB were isolated from the MRS agar plates prepared for each lot. In total, 54 strains were isolated from Lot A, 58 strains were isolated from Lot B, 15 strains were isolated from Lot C, and 22 strains were isolated from Lot D. The nucleotide sequences of their 16S rRNA genes were then obtained (Supplementary Table 2). The results of the genus determination using the RDP Classifier revealed that the isolates belonged to *Leuconostoc*, *Latilactobacillus*, or *Lacticaseibacillus* (Table 1). A total of 117 strains belonging to *Leuconostoc* were further classified into 6 clusters (LEU1 to LEU6) based on the homology of their 16S rRNA gene sequences (<100% identity). There was no difference in the sequences among the isolates identified as *Latilactobacillus* and *Lacticaseibacillus.* The number of isolates belonging to the cluster from each lot and its collection time are summarized in Table 1. LEU1 strains were detected in three of the lots, excluding Lot C, and were frequently detected in the early stages of fermentation (days 1–6). The strains belonging to LEU3, LEU5, and LEU6 were specifically detected in Lots A, C, and D, respectively. The strains of *Latilactobacillus* were detected after day 7 in Lots C and D, which were manufactured in the second brewing year.

Our microbiome analysis showed that *Lactococcus* spp. was particularly abundant in Lot C (Fig. 3) but that this bacterium was not isolated from the MRS medium (Table 1). Therefore, lactococcal strains were selectively isolated from Lot C sample collected on day 10 using the M17 medium. The 16S rRNA gene sequences of the isolates (LC1, LC2, LC3) were consistent with *L. lactis* strain NBRC100933^{T}, suggesting that at least Lot C contained *L. lactis.*

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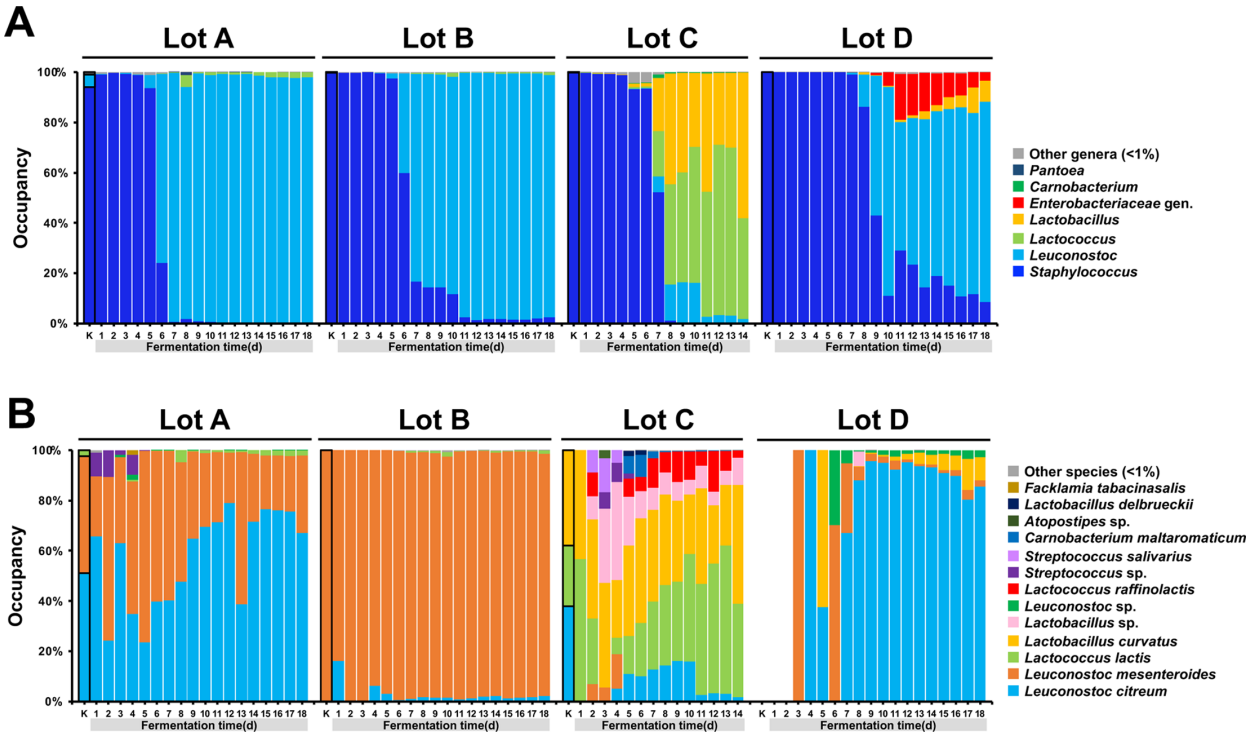

Fig. 3. Taxonomic composition in the manufacturing process of the *kimoto*-type fermentation starters. (A) Genus-level taxonomic composition in the manufacturing process of four lots of the *kimoto*-type fermentation starters. Each taxon detected at an abundance of ≥1% is shown. Genera are indicated in the right inset. The "K" on the horizontal axis indicates the rice *koji*. (B) Species-level taxonomic composition focusing on *Lactobacillales* in the manufacturing process of four lots of the *kimoto*-type fermentation starters. Each taxon detected at an abundance of ≥1% is shown. Species are indicated in the right inset. In Lot D, no reads classified into the order *Lactobacillales* were detected in the rice *koji* or the samples from days 1 and 2. The LAB genus and species names in the inset are based on the old classification, as the SILVA database at the time of analysis did not support the new classification of LAB.

Table 1. Classification of lactic acid bacteria isolated from the *kimoto*-style fermentation starters

Genus[a]	Cluster[b]	Lot A[c]			Lot B[c]				Lot C[c]		Lot D[c]		
		1–6 d	7–12 d	13–18 d	1–6 d	7–12 d	13–18 d	19 d–	1–6 d	7–12 d	1–6 d	7–12 d	13–18 d
Leuconostoc	LEU1	18	9	9	13	0	0	0	0	0	3	4	4
	LEU2	0	6	5	5	15	5	0	0	0	0	0	0
	LEU3	3	1	3	0	0	0	0	0	0	0	0	0
	LEU4	0	0	0	0	0	0	0	5	6	1	0	0
	LEU5	0	0	0	0	0	0	0	0	1	0	0	0
	LEU6	0	0	0	0	0	0	0	0	0	0	0	1
Latilactobacillus	LAT1	0	0	0	0	0	0	0	0	3	0	1	8
Lacticaseibacillus	LAC1	0	0	0	0	0	0	20	0	0	0	0	0

[a]Genus of the isolate was determined by Ribosomal Database Project (RDP) Classifier.
[b]The clusters were distributed based on the homology of their 16S rRNA gene sequences.
[c]The values indicate the isolated strain number in each fermentation period.

Phylogeny of lactic acid bacteria

To understand the detailed phylogenies of each lactic acid bacterium, we selected strains representative of the clusters of LAB from each lot and constructed a phylogenetic tree based on the concatenated sequences of their housekeeping genes, *rpoA* and *pheS*, since 16S rRNA gene sequence similarity is not always sufficient for species discrimination of *Leuconostoc* and *Lactobacillus* spp. [22, 23]. In addition, representative strains were applied to API 50 CHL identification tests based on their ability to ferment various carbohydrates.

The phylogenetic tree with type strains of several *Leuconostoc* species shown in Fig. 4A indicates that the representative strains of LEU1 (A1-5, B4-2, D8-4) and LEU2 (A15-1, B16-1) were most closely related to *Leuconostoc suionicum* DSM 20241^T and *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* DRC1506^T, respectively. The results of the API 50 CHL identification tests showed that the strains in LEU1 and LEU2 were both *L. mesenteroides*, as *L. suionicum* is not included in the APIweb database (Supplementary Table 5). However, our biochemical analysis using the API 50 CHL identification tests revealed that

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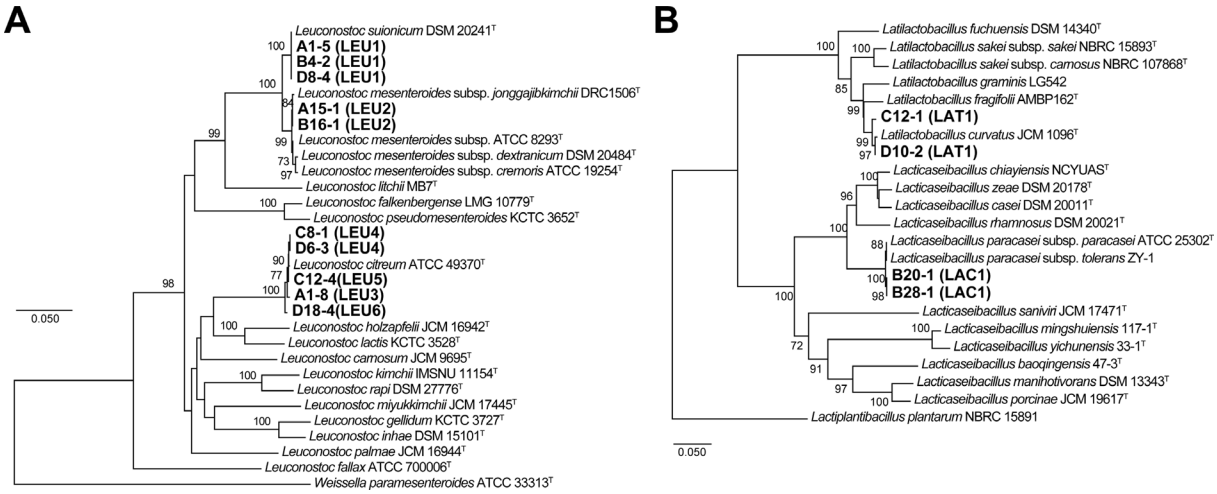

Fig. 4. Phylogenies of lactic acid bacteria isolated from the *kimoto*-style fermentation starters. (A) Phylogenetic tree based on the concatenated nucleotide sequence of the *rpoA* and *pheS* genes of *Leuconostoc* isolates. The tree was constructed using the neighbor-joining method. *Weissella paramesenteroides* ATCC 33313^T was used as the outgroup. (B) Phylogenetic tree based on the concatenated nucleotide sequence of the *rpoA* and *pheS* genes of *Latilactobacillus* and *Lacticaseibacillus* isolates. *Lactiplantibacillus plantarum* NBRC 15891^T was used as the outgroup. Numbers at the nodes are the percentages of 1000 bootstrap replications supporting that partition (values ≥70% are shown). The scale bars show the number of base substitutions per nucleotide. Representative isolates are indicated in bold. The accession numbers for the reference nucleotide sequences of *rpoA* and *pheS* are shown in Supplementary Table 8.

A1-5, B4-2, and D8-4 did not generate acid from D-raffinose, while A15-1 and B16-1 did (Supplementary Table 6). This metabolic feature of the representative strains of LEU1 (A1-5, B4-2, D8-4) was consistent with that of *L. suionicum* strain DSM20241^T [24], suggesting that the strains in the LEU1 cluster belong to *L. suionicum* and that those in the LEU2 cluster belong to *L. mesenteroides*.

The representative strains from LEU3 (A1-8), LEU4 (C8-1, D6-3), LEU5 (C12-4), and LEU6 (D18-4) were most closely related to *L. citreum* ATCC 49370^T in the phylogenetic tree, but each belonged to a different lineage (Fig. 4A). The API 50 CHL test results also showed that the representatives in LEU3, LEU4, and LEU5 were identified to be *L. citreum* (Supplementary Table 6). On the other hand, D18-4, which was grouped in LEU6, had a significantly different carbohydrate fermentation ability compared with the strains in LEU3 to LEU5 and was not identified as *L. citreum* by APIWEB (Supplementary Table 6). These results indicate that the species of the strains in the LEU3, LEU4, and LEU5 clusters was *L. citreum* and that the species of the strains in LEU6 was unable to be identified.

Latilactobacillus strains C12-1 from Lot C and D10-2 from Lot D were most closely related to *L. curvatus* JCM 1096^T, but these two strains were distinct in the phylogenetic tree (Fig. 4B). The C12-1 strain was clearly separated from the clade containing JCM 1096^T and had a unique carbohydrate fermentation ability (Supplementary Table 5). The APIWEB identification test was unable to identify the species for the C12-1 strain (Supplementary Table 6). Thus, this strain was considered to be a phylogenetically and physiologically unique lactic acid bacterium.

All isolates classified as *Lacticaseibacillus* were found only in Lot B and were isolated from the starter at the late fermentation stage, at which high concentrations of ethanol (>10% [v/v]) were present. The representatives B20-1 and B28-1 were most closely related to *Lacticaseibacillus paracasei* in the phylogenetic tree based on the sequences of housekeeping genes (Fig. 4B) and the API 50 CHL test (Supplementary Table 5).

Ethanol tolerance of lactic acid bacteria

The representative LAB were cultured in the presence of 0–15% (v/v) ethanol and compared for growth. The results shown in Supplementary Table 7 revealed that none of the *Leuconostoc* and *Latilactobacillus* representatives grew in the presence of 10% (v/v) or more ethanol, while *L. paracasei* B28-1 and B20-1 grew even in the presence of 12.5% (v/v) ethanol.

Nitrate-reducing activity in the rice koji-derived Staphylococcus *strains*

Nitrite was detected in all the lots (Supplementary Fig. 1), but commonly observed nitrate-reducing bacteria (such as *Pseudomonas*, *Achromobacter, Macrococcus*, and *Enterobacter*) were not predominant in the early fermentation samples in our microbiome analysis (Fig. 3A). On the other hand, rice *koji*-derived *Staphylococcus* spp. were predominant at this stage. Therefore, we hypothesized that rice *koji*-derived *Staphylococcus* was involved in the nitrite production in the *kimoto*-style fermentation starters manufactured in this brewery.

As a result of counting the yellow colonies that grew after culturing the rice *koji* on the R2A agar medium, 10^5–10^8 CFU/g were detected, and the number increased with each production (Table 2). Two strains were isolated from the agar plates on which each lot of rice *koji* was cultured to identify their species and evaluate their nitrate-reducing activity. The 16S rRNA gene sequences of all the isolates (KA1, KA2, KB1, KB2, KC1, KC2, KD1, and KD2) completely matched that of *S. gallinarum* strain VIII1^T (accession no. NR_036903.1). In a plate assay using Griess–Romijn Reagent, all the isolates developed a red color around their colonies (Table 2, Supplementay Fig. 3). However, no color development was observed for the LAB isolated in this study. Moreover, *narG* encoding a nitrate reductase alpha subunit was detected by PCR of the genomic DNA of each *Staphylococcus*

Table 2. Viable counts and nitrate-reducing activity in *Staphylococcus* spp. grown on rice *koji*

Lot	Viable cell number of staphylococci in rice koji (cfu/g)	Isolated strain	Nitrate-reducing activity in the isolates	*narG* possession in the isolates
A	4.3×10^5	KA1, KA2	positive	positive
B	n.d.	KB1, KB2	positive	positive
C	1.1×10^6	KC1, KC2	positive	positive
D	9.9×10^7	KD1, KD2	positive	positive

n.d.; not determined.

isolate (Table 2, Supplementary Fig. 4). These results suggest that rice *koji*-derived *S. gallinarum* was mainly involved in the production of nitrite in the *kimoto*-style fermentation starter manufacturing process of this *sake* brewery.

DISCUSSION

In this study, we traced the microbiota in four lots of *kimoto*-style fermentation starters manufactured in a brewery based on an RNA-based 16S rRNA amplicon analysis and further investigated the phylogenies of predominant LAB. The results of the microbiome analysis showed that the starters brewed in the second brewing year (Lots C and D) had more diverse microbiotas than those in the first brewing year (Figs. 2A and 3). It was also shown that the microbial diversity was less pronounced and that rice *koji*-derived *Staphylococcus* was highly predominant at the initial fermentation stage in all the lots. In contrast, the LAB dominant at the middle stage varied between lots, even within the same brewing year (Figs. 2B and 3). Among the LAB predominant at the middle stage, lactococci, including *Leuconostoc* and *Lactococcus*, were detected in all the lots, while lactobacilli dominated for the first time in the second brewing year. Taken together, these results suggest that two years of repeated brewing in the brewery resulted in an increase in the microbial diversity, the emergence of lactobacilli, and alteration of the predominant LAB in the *kimoto*-style fermentation starters.

Regarding the microbial source in the case of a *kimoto*-style fermentation starter, recent studies on the environmental microbiota suggested that the rice *koji* and water, as well as the fermentation tank that comes into direct contact with the starter, are important determining factors for the bacterial composition of the starter [6, 9]. In previous studies, it was observed that LAB inhabited rice *koji* at levels of 10^2–10^3 cells/g and became the source of the dominant LAB in starters [12–14]. In the present study, we showed that the predominant LAB species in the *kimoto*-style fermentation starters varied from lot to lot, while the predominant LAB species detected in the rice *koji* of Lots A, B, and C were consistent with those in the corresponding starter (Fig. 3B). These results are in line with those of the earlier studies suggesting that the rice *koji* is the source of the predominant LAB during the production of *kimoto*-style fermentation starters in a brewery [12–14].

Our results also showed that the same lineage of *L. suionicum* was detected in both the first and second years of brewing (Table 1, Fig. 4), suggesting the possibility that *L. suionicum* had already established a habitat in the brewery during the first year. Previous studies by Masuda *et al.* [14] showed that identical strains of *L. mesenteroides*, which is closely related to *L. suionicum,* were present over several years, suggesting that the strains form a habitat within the surfaces of wooden fermentation tanks. Since our *kimoto*-style fermentation starters were manufactured using stainless-steel tanks, it is unlikely that LAB survived on tank surfaces beyond a brewing season. However, based on the fact that the LAB in the starters were derived from the rice *koji*, it can be speculated that *L. suionicum* formed a habitat on wooden equipment (such as boxes and stands) used to make the rice *koji*. On the other hand, the same lineages of *L. citreum* and *Latilactobacillus* spp. were not isolated in the different brewing years (Table 1, Fig. 4). These results suggest that *L. citreum* and *Latilactobacillus* spp. may be less capable of forming a habitat on brewing equipment than *L. suionicum* and may have been introduced from other sources.

As a general model of transitions in the LAB flora at the middle stage of the production of *kimoto*-style fermentation starters, it is widely regarded that lactococci, specifically *L. mesenteroides*, become predominant first, followed by lactobacilli, specifically *L. sakei* [1, 4, 11]. In this study, the LAB flora in the starters were diverse among the lots and quite different from the general model mentioned above in the following three points. First, the LAB flora in Lots A and B was dominated by lactococci but did not show the emergence of lactobacilli. Similar transitions in LAB flora have been reported previously [9, 11, 25]. Second, *L. sakei* was not detected in all the lots examined, while the lactobacilli taxonomically related to *L. curvatus* dominated in Lots C and D. The phylogenetic tree created based on the housekeeping gene sequence and carbohydrate fermentation profile revealed that the C12-1 strain isolated from Lot C in particular is a unique lactic acid bacterium that is different from the type strain of *L. curvatus* in terms of phylogenetic and physiological aspects (Fig. 4; Supplementary Tables 5 and 6). Recent studies have suggested that *L. sakei* is the most important bacterium for the accumulation of lactic acid in a *kimoto*-style fermentation starter and has the ability to metabolize arginine to an ornithine related to the umami taste [8]. Therefore, it is expected that the outcomes of the development of lactic acid and flavor components in the *kimoto* starters investigated in the present study, in which unique lactobacilli were predominant, would differ from those in a starter in which *L. sakei* is dominant. Finally, *L. citreum* and *L. suionicum* were often the dominant lactococci in the present study, and in Lot C, *Lactococcus* spp. showed a high predominance rate. This is the first report in which *L. suionicum* was identified as a predominant LAB in a *kimoto*-style fermentation starter. It is possible, however, that strains recognized as *L. mesenteroides* in previous studies also contained *L. suionicum*, as some lineages of *L. mesenteroides* have recently been reclassified as *L. suionicum* [24]. Although *L. suionicum* and *L. mesenteroides* coexisted in the starters in Lots A and B, the former was isolated more frequently from the early-stage fermentation samples than the

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latter (Table 1). Particularly in Lot B, *L. suionicum* was highly predominant on days 1–6. From days 7–18, however, this species was not detected, and *L. mesenteroides* became predominant instead. These results suggest the occurrence of a microbial transition among *Leuconostoc* species in the *kimoto*-style fermentation starter.

The microbial community of Lot C was particularly different compared with the other lots (Fig. 3). The exact reason why this difference occurred is unclear, but a seasonal effect might have been involved, as Lot C was the only one brewed early in the brewing year. In the brewery, *sake* is manufactured from around September to June every year, and the extensive cleaning of the room for making rice *koji,* known as the *kojimuro*, during the off-season might have influenced the microbiota of the rice *koji*. Additionally, because Lot C was produced using a different variety of *sake* rice from the other lots, the difference in rice variety might also be related to the difference in microbiota in the rice *koji* and starters. The final product brewed with Lot C had differences in total acidity and amino acid degrees compared with those of the other lots (data not shown). These differences might have involved the microbiota in the starter, but further evaluation of the chemical components related to taste and flavor using chromatographic technologies is needed to deeply understand the impact of the microbiota on *sake* quality.

Most of the LAB predominant during the middle stage do not survive the late stage of fermentation due to the high concentrations of ethanol produced by the *sake* yeast [1, 11]. In the present study, the ethanol-tolerant *L. paracasei* in Lot B was found to persist in the late stage of fermentation. We cannot rule out the possibility that *L. paracasei* accidentally contaminated the lot, but it is also possible that the differences in the microbiota during fermentation enabled the survival of the ethanol-tolerant bacteria. Lot B had the lowest microbiota diversity compared with the other lots, consisting almost exclusively of *L. mesenteroides* and *L. suionicum* at the mid-fermentation stage (Table 1, Fig. 3B), which presumably led to reduced competition for essential nutrients. Numerous reports have shown that many strains belonging to *L. citreum*, *L. curvatus*, and *L. lactis* produce bacteriocins, which are proteins or peptides that inhibit the growth of similar or closely related bacterial strains [26, 27]. In particular, the culture supernatants of *L. citreum* obtained from rice *koji* reportedly inhibited the growth of several LAB strains [28]. Therefore, it is possible that the difference in the bacterial flora is a contributing factor for the survival of the ethanol-tolerant LAB in the late stage of fermentation.

L. paracasei has been frequently detected in *moromi* and *sake* lees in the brewery investigated in the present study, although no adverse effects on *sake* quality have been observed to date (data not shown). Interestingly, an attempt to diversify *sake* quality by utilizing the malolactic fermentation ability of a *L. paracasei* strain has been reported [29]. It has also been reported that inoculation with the dead cells of *L. paracasei* isolated from *sake* lees has the effects of alleviating atopic dermatitis and improving insulin resistance [30, 31]. Therefore, it is possible that the presence of *L. paracasei* in *moto* and *moromi* has a positive effect on the quality and functionality of *sake*.

Staphylococcus was commonly detected during the early stages of fermentation in all the lots investigated in the present study (Fig. 3). A high dominance of *Staphylococcus* was also detected in the rice *koji*, suggesting that the staphylococci grown in the starters were derived from the rice *koji*. *Staphylococcus* spp. are widespread in nature and also known to be present on the human skin [32]. Since the rice *koji* is made with bare hands, there is a possibility that staphylococci may have contaminated the rice *koji* via the people involved in its production. The results of viable cell counting and bacterial identification showed that the number of viable *Staphylococcus* spp. in the rice *koji* increased in a later lot and that *S. gallinarum* with the same 16S rRNA gene sequence was isolated even after repeated brewing (Table 2), suggesting that this bacterium might have already established habitats in the *kojimuro*. Similarly, *Staphylococcus* spp. were previously detected in *kimoto*-style fermentation starters [3, 7], in rice *koji* [6, 10, 33], and on the floor and in equipment for *sake* brewing [6, 9].

A strain of *S. gallinarum* isolated from fermented coconut water was reported to have the genes encoding nitrate reductase within its genome and a nitrate-reducing activity [34]. Our results also indicate that the *S. gallinarum* strains isolated from the rice *koji* prepared in the brewery have nitrate reduction activity and the responsible genes (Table 2). Several reports have suggested that *Pseudomonas* spp., *Macrococcus* spp. *Achromobacter* spp., and *Enterobacter* spp. have the ability to play a role as nitrate-reducing bacteria in the early stage of *kimoto*-style starter fermentation [1, 11, 12]. However, such commonly found nitrate-reducing bacteria were not predominant in the present study (Fig. 3A). The present results indicate that this brewery is unique in that *S. gallinarum* is responsible for the production of nitrite during the initial production process of the *kimoto*-style starters. The higher nitrite concentration in Lot D than in the other lots may be due to the larger number of viable *Staphylococcus* that grew in the rice *koji* (Table 2) or the presence of *Enterobacteriaceae* on and after the nineth day of production (Fig. 3A).

Previous reports have suggested that the transition from lactococci to lactobacilli at the middle stage of fermentation depends on the type of nitrate-reducing bacteria that predominate in the early stage [35, 36]. It has been suggested that this is because rapid consumption of nitrite occurs when *Enterobacter* spp. are predominant, whereas this is less likely to occur when *Pseudomonas* spp. are predominant, resulting in higher amounts of nitrite remaining in the starter. The tolerance of *L. mesenteroides* to nitrite is lower than that of *L. sakei*, suggesting that the transition to lactobacilli occurs when *Pseudomonas* predominates but not when *Enterobacter* does [35, 36]. According to our results, the same species of staphylococci was predominant at the initial stage of fermentation in all the lots, but the predominance of lactobacilli at the middle stage was only detected in Lots C and D. In addition, no correlation was observed between the amount of nitrite accumulation and the predominant LAB species (Fig. 3 and Supplementary Fig. 1). These circumstances suggest that the predominance of *Staphylococcus* spp. does not affect the transition to lactobacillus in the *kimoto*-type starter.

Based on our microbiota analyses of the *kimoto*-style fermentation starters manufactured in the newly established brewery for two brewing years, we concluded the following. (1) The diversity of microbial community increased and the transition pattern of LAB at the middle stage of manufacturing changed in the second year compared with that in the first year. (2) The rice *koji* was the major microbial source determining the microbiota during starter production. (3) There was a case in which rice *koji*-derived *S. gallinarum* played a role in nitrite production in

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the early stage of fermentation. In addition, we confirmed certain distinctive features of the microbiota in the brewery, including that *L. suionicum*, *L. citreum*, and *L. mesenteroides* were detected as predominant lactococci and also that a unique lactobacillus was detected in place of *L. sakei*. These findings are expected to contribute to not only enhancing our understanding of the diversity of microbiota in *kimoto*-style *sake* brewing but also to enabling control of the microbiota in the rice *koji* and *moto* for consistent *sake* quality by adjustment of brewing conditions (such as the temperature and pH) and addition of appropriate LAB. In future research, we will focus on elucidating the factors responsible for the formation of the LAB flora in rice *koji* and how a specific lactic acid bacteria and *Staphylococcus* affect the quality of *sake*. In addition, we will continue to investigate whether the microbial diversity at the brewery will increase further or remain stable.

CONFLICT OF INTEREST

The authors declare no competing interest.

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