# A unique case in which *Kimoto*-style fermentation was completed with *Leuconostoc* as the dominant genus without transitioning to *Lactobacillus*

**Authors**

Kohei Ito,[1*] Ryo Niwa,[1,2] Yuta Yamagishi,[1,3] Ken Kobayashi,[1] Yuji Tsuchida,[4] Genki Hoshino,[4] Tomoyuki Nakagawa,[5] Takashi Watanabe[6]

**Affiliation**

1. BIOTA inc., Tokyo, 101-0022, Japan
2. Graduate School of Medicine, Kyoto University, Kyoto, 606-8501, Japan
3. Department of Life Science, College of Science, Rikkyo University, Tokyo, 171-8501, Japan
4. Tsuchida Sake Brewery, Gunma, 378-0102, Japan
5. Faculty of Applied Biological Sciences, Gifu University, Gifu, 501-1193, Japan
6. Gunma Industrial Technology Center, Gunma 379-2147, Japan

* Corresponding author (e-mail: kohei@biota.ne.jp)

Microbial transition in the *Kimoto*-style starter

**Abstract**

The *Kimoto*-style fermentation starter is a traditional preparation method of *sake* brewing. In this process, specific microbial transition patterns have been observed within nitrate-reducing bacteria and lactic acid bacteria during the production process of the fermentation starter. We have characterized phylogenetic compositions and diversity of the bacterial community in a *sake* brewery performing the *Kimoto*-style fermentation. Comparing the time-series changes with other *sake* breweries previously reported, we found a novel type of *Kimoto*-style fermentation which the microbial transition differed significantly from other breweries during the fermentation step. Specifically, the lactic acid bacteria, *Leuconostoc* spp. was a predominant species in the late stage in the preparation process of fermentation starter, on the other hand, *Lactobacillus* spp., which plays a pivotal role in other breweries, was not detected in this analysis. The discovery of this new variation of microbiome transition in *Kimoto*-style fermentation has further deepened our understanding of the diversity of *sake* brewing.

[**Keywords:** Microbiome, Japanese-*sake*, *Kimoto*-style, Lactic acid bacteria, Fermentation]

## INTRODUCTION

*Sake* is a traditional alcoholic beverage made from rice in Japan. In a preliminary stage of a main brewing process, a fermentation starter with purely cultured yeast is produced to prevent microbial contamination and poor fermentation and to promote smooth alcoholic fermentation. The production process is as follows; First, *Aspergillus oryzae*, which secretes amylases, is propagated on steamed rice to make *koji*. Then, *koji*, steamed rice, and water are mixed in an open-top tank. Fermentation of this mixture produces a fermentation starter. The fermentation starter is further mixed with *koji*, steamed rice, and water, and after a 3-5 week fermentation process, the fermentation mash or starter is produced. The fermentation starter is separated into *sake* and spent rice by a filter press to complete the *sake*.

The fermentation starter is divided into three styles, *Sokujo*, *Kimoto* and *Yamahai*. *Sokujo*-style fermentation starter is a modern method to make the starter culture with the addition of food-grade lactic acid. *Kimoto*-style fermentation starter is the traditional preparation method of the starter culture and is manufactured under highly acidic conditions by inducing the growth of nitrate-reducing bacteria and lactic acid bacteria properly. *Yamahai*-style is similar to *Kimoto*-style but made without grinding rice. Lactic acid inhibits contaminations of unintended yeasts and bacteria from external environments into the fermentation starter.

The microbiome compositions during *Kimoto*-style fermentation starter production show standard transitions as follows. Nitrate-reducing bacteria, which were reported to come from water (1), initially grows and produces nitrite, thereby inhibiting the growth of microorganisms that are less tolerant to nitrite. At the same time, lactic acid bacteria, especially *Leuconostoc* spp., which grow at low temperatures and have fewer nutrient requirements, increase, and then lactic acid bacteria such as *Latilactobacillus sakei*, which require strict nutrients, occupy the microbiome compositions as a predominant species. These steps are

known as a common microbial transition in *sake* brewing (2-4).

However, it was reported that some fermentation starters brewed by *Kimoto*-style in several *sake* breweries show distinctive microbial transitions and chemical changes and that this is one of the reasons for producing unique *sake* flavors among breweries, even if they use the same production process (5-8). A possible reason to explain this fact is differences in *Kuratsuki* microorganisms (microorganisms living in *sake* breweries) and the introduction of diverse microorganisms from outside of tanks during the *sake* brewing process (9,10).

The *Tsuchida Sake* Brewery (Gunma, Japan) is one of a few breweries that produce the *Kimoto*-style fermentation starter without adding yeasts and fully relies on *Kuratsuki* microorganisms to produce the fermentation starter. In addition, compared to the *Kimoto*-style fermentation in other *sake* breweries, this *sake* brewery is characterized by not using any food additives such as brewers alcohol, enzyme reagents, or activated charcoal. Furthermore, this brewery does not use sake-brewing rice but table rice for *sake* brewing. For the above reasons, the microbial community and its transition in the fermentation starter of the brewery were considered to be divergent from previous studies.

In this study, we focused on the fermentation mechanism of the *Kimoto*-style fermentation starter from the *Tsuchida Sake* Brewery and analyzed its microbial community during the fermentation process in detail using its 16S rRNA amplicon sequencing. The *Kimoto*-style fermentation starter from the *Tsuchida Sake* Brewery possesses a distinctive microbial community compared with other *Kimoto*-style fermentation starters; specifically, the dominant genus of lactic acid bacteria was *Leuconostoc*, and the switch of the predominant lactic acid bacteria, from *Leuconostoc* to *Lactobacillus*, was not observed in the fermentation process of the *Kimoto*-style fermentation starter. Here we report a new profile of microbial transition in the *Kimoto*-style fermentation starter.

## MATERIALS AND METHODS

**Sample collection** Samples were collected for each day 1, 3, 5, 7, 9, 13, 17, 22, 28, and 33 after starting the fermentation of the fermentation starter. All samples were collected in duplicate and between October and November 2021. Fermentation starter samples used in this study were provided by *Tsuchida Sake* Brewing Company (Gunma, Japan). All samples were immediately frozen and stored until DNA extraction.

**Measurement of basic chemical components** Temperatures and chemical compositions of the fermentation starter were measured. Alcohol (ALC) and Baumé degree (Be) were measured using a DA-155 vibrating density meter for alcoholic beverages (Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan), while Acidity (TA) and Amino Acid Content (AA) were obtained using a CHA-700 multi-sample changer (Kyoto Electronics Manufacturing Co., Ltd.). The nitrite concentration was measured using a Merck Millipore MQuant nitrite test (Merck KGaA, Darmstadt, Germany).

**Total DNA extraction and high throughput sequencing** Samples were subjected to 750ul of Lysis buffer, vortexed for 10 min and heat-treated at 100°C for 10 min, centrifuged, and the supernatant was transferred to MORA beads for automated purification on a Beckman Coulter GenFindv2 after mechanical fragmentation at a maximum speed of 3 min on an MM-400. Finally, DNA was eluted with 80ul of sterile water. 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGAGACAGCCTACGGGGNGGCWGCAG-3') and 806R (5'-GTCTCGTGGGCTCGGGAGATGTGTATAAGAGACAGGACTACHVGGGGTATCTAATCC-3') primers were utilized to amplify the V3-V4 region of the 16S rRNA gene by PCR (11,12). Thermal cycling conditions were 95 °C for 3 min; 32 cycles of 95 °C for 30 s, 55 °C

111 for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. A second PCR was
112 performed to add sequencing adapters and dual-index barcodes to distinguish amplicons from
113 each sample using the same reaction conditions with eight cycles. Preparation of libraries and
114 sequencing were performed by paired-end sequencing of 300 bp on the Illumina MiSeq
115 platform (Illumina, Inc., San Diego USA) at GenomeRead Inc. in Kagawa, Japan.
116
117 **Microbiome analysis** The QIIME2 (version 2021.02) (13) platform was used for
118 microbiome analysis. FASTQ files were imported into the QIIME2 platform. Sequences were
119 processed using qiime dada2 denoise-paired command for quality control and classification into
120 amplicon sequencing variants (ASVs). Taxonomic analysis was run on SILVA database SSU
121 138 by qiime feature-classifier classify-sklearn (14-16). Sequence reads that were not classified
122 as any species after phylogenetic classification (Unassigned) and reads classified as chloroplast
123 and mitochondria were excluded from further analyses. Since the genus *Latilactobacillus* is a
124 taxon which relatively recently derived from the genus *Lactobacillus* (17), the SILVA database
125 SSU 138 used in this study does not reflect the reclassification of the genus *Latilactobacillus*,
126 so it was assigned as *Lactobacillus* in this study.
127
128 **Processing for comparative microbiome analysis** Sequence data from BioProject
129 PRJDB12939 was used for comparative analysis (8). Since 341F (5'-
130 CCTACGGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGGTATCTAATCC-3')
131 were used in the study, removal of one base from the 5' end of the reverse read was performed
132 to match the read lengths of the samples obtained in our study. fastp v.0.20.1 (18) was deployed
133 for this processing. The method described above was repeated to perform taxonomic analysis.
134 The depth of sequence reads (Features) differed among samples, and to normalize them, we
135 subsampled from each sample to 5,000 reads each. Samples with fewer than 5,000 reads (S22,

136 23, 24, 25, 26, 28) were excluded from the diversity analysis (Table S1). Sub-sampling is an
137 approach for inferring microbiome differences between samples and has been reported to be a
138 suitable analytical method when analyzing new data sets (19). To evaluate the effect of
139 sequence read counts on microbiome diversity assessment, we examined changes in the value
140 of the Shannon index over a range of reading counts from 0-5,000 by rarefaction curves. The
141 Rarefaction curve of the Shannon index leveled off when the number of leads was just under
142 500 (Table S2). Therefore, this investigation suggests that no significant changes in microbial
143 diversity are due to the subsampling of 5,000 reads from each sample.
144
145 **Statistical Analysis** Kruskal-Wallis tests were used to compare the alpha diversity
146 (Shannon diversity index) among samples. To compare differences in Beta diversity (Weighted
147 UniFrac distance) between samples, for all PERMANOVA analyses, 5,000 trials were
148 performed to assess the statistical significance. Q-values < 0.05 after multiple testing
149 corrections were considered statistically significant. All multiple testing corrections were
150 performed by computing FDRs using the Benjamini–Hochberg method, and Q-values (adjusted
151 P-values) < 0.05 were considered statistically significant.
152

153 ## RESULTS

154 **Chemicals and Temperature Change in fermentation starters** The chemical data
155 and the average temperature of the *sake* starter are shown in Fig. 1. The amount of nitrite started
156 to rise on day 7 and reached its peak on day 11. On day 14 it was undetectable; Titratable acidity
157 (TA), a value representing total acidity, increased on day 15, followed by an increase in ethanol
158 concentration on day 27. In addition, a rapid decrease in glucose was observed along with an
159 increase in ethanol concentration on day 27. These changes in chemical data and the average
160 temperature are generally observed in *Kimoto*-style fermentation, so we suggest that the

161 fermentation proceeded well in the *Kimoto*-style fermentation starter of the *Tsuchida Sake*
162 Brewery.
163
164 **Phylogenetic composition of the microbiome found in the fermentation starter** To
165 track the microbial transition in the fermentation starter during the fermentation, we performed
166 16S rRNA amplicon sequencing. Results demonstrated large changes in relative abundances of
167 the bacterial genera (Genus) during the early to late stages (Fig. 2).
168 The genera with average relative abundances higher than 5% in the early stage (day 1
169 to day 7) of the fermentation starters were *Methylobacterium-Methylorubrum* (23.0%),
170 *Anaerobacillus* (16.7%), *Bacillus* (9.4%), and *Pseudomonas* (9.3%) (Table S3). The relative
171 abundances of these bacterial genera decreased significantly within days 7-9.
172 *Methylobacterium* spp. was reported to utilize methanol as a source of carbon and energy and
173 is widely found on the leaf surface of plants, including rice (20,21). *Anaerobacterium* spp. is
174 salt-tolerant and halophilic and has been found in lakes and soils in arid regions (22,23). It is
175 reported that *Bacillus* spp. may live in *sake* breweries as *Kuratsuki* bacteria (24). *Bacillus* spp.
176 have also been detected in *koji* and are known to be associated with the production of 4-vinyl
177 guaiacol according to previous studies (9,25,26).
178 The bacterial genera with average relative abundances higher than 5% in the late stage
179 (days 17-33) were *Rahnella1* (18.1%), *Serratia* (13.9%), *Hafnia-Obesumbacterium* (8.6%), and
180 *Carnobacterium* (6.2%) (Table S3). *Leuconostoc, Rahnella, Serratia, Hafnia-*
181 *Obesumbacterium*, and *Carnobacterium* were the bacterial genera that accounted for a large
182 proportion of the bacteria found in the late stage (days 17-33). *Leuconostoc* spp. has been
183 detected in several fermented foods (27,28). *Serratia* spp. have been identified by previous
184 studies from *Yamahai-moto* (6), *Rahnella* spp. from *Yamahai* (29), and Chinese sauerkraut (30).
185 *Hafnia-Obesumbacterium* spp. has been detected in brewer's yeast along with *Rahnella* spp.,

186 suggesting that it may produce high pH beer by inhibiting fermentation reaction (31,32).
187 *Carnobacterium* spp. are frequently detected in the natural environment and food products,
188 previous studies reported producing of bacteriocins in some species (33).
189
190 **Comparison with other breweries** To characterize the microbial transition in this
191 brewery, we compared our data with a previous study that investigated the microbial transition
192 in four *sake* breweries (8). Time-series changes in the microbial diversity (Alpha diversity;
193 Shannon index) for each sample are shown (Table 1). *Tsuchida Sake* brewery was confirmed
194 to have a higher microbial diversity (Shannon index) of the fermentation starters (Q-value <
195 0.05) compared to the other breweries (Table S4).
196 A Principal Coordinate Analysis (PCoA) by Weighted UniFrac distance to visualize the
197 differences (Beta diversity) among the microbiomes of each sample showed that the *Tsuchida*
198 *Sake* Brewery formed a different cluster (q-value < 0.007) than the other breweries (Fig. 3 and
199 Table S5).
200 The relative abundances of the genus *Lactobacillus* and *Leuconostoc* in each brewery
201 during the preparation are shown in the line graphs (Fig. 4). The genus *Lactobacillus* became
202 the dominant species in all other breweries, but the relative abundance of the genus
203 *Lactobacillus* did not increase during the entire preparation in the *Tsuchida Sake* Brewery. On
204 the other hand, the genus *Leuconostoc* increased in only three breweries, and in all but the
205 *Tsuchida Sake* Brewery, their abundance eventually decreased and shifted to the genus
206 *Lactobacillus*. In contrast, in the *Tsuchida Sake* Brewery, the genus *Leuconostoc* maintained a
207 constant high relative abundance during the fermentation.
208

209 # DISCUSSION

210 **Comparative analysis revealed unique dynamics of lactic acid bacteria in the**
211 ***Tsuchida Sake* Brewery** A previous study reported three major microbial transition profiles
212 in sake brewing from the point of switches occurring in lactic acid bacteria: (1) lactic acid
213 bacteria increase overall, but *Lactobacillus* spp. remain more abundant than *Leuconostoc* spp.,
214 (2) *Lactobacillus* spp. are more abundant in the beginning, but *Leuconostoc* spp. become
215 dominant later, and (3) *Leuconostoc* spp. remain more abundant than *Lactobacillus* spp. (8).
216 Surprisingly, in the brewery we sampled, the genus *Leuconostoc* was detected as the dominant
217 species even on day 33, the last day of brewing, and only low abundances of the genus
218 *Lactobacillus* was detected throughout the entire preparation, indicating a microbial transition
219 profile differs from previous reports.

220 In addition, another study reported that *Kimoto*-style fermentation is characterized by
221 the detection of multiple lactic acid bacteria compared to *Sokujo-moto* (29), so the process of
222 Kimoto-style fermentation itself may contribute to the diversity of taste in different sake
223 breweries. *Sokujo-moto* is a modern type of fermentation starter which includes lactic acid to
224 maintain a low pH, thus preventing microbial contamination from the brewery (5). As indicated
225 in this study, specific microbiome transitions were observed that were different from the
226 microbial transitions reported by Takahashi *et al.*, suggesting that the microbiome is unique in
227 individual breweries and that these microbial transition factors that characterize them need to
228 be investigated (8).

229 Some studies reported that only one lactic acid bacteria appears and the transition does
230 not occur (34), and no *Lactobacillus* spp. are found (35), but to the best of my knowledge this
231 is the first study proving this trend of microbiome shift in a manner of uncultured analysis
232 method.

233 A previous study revealed that *L. sakei* has a more stringent amino acid requirement

234 than *L. mesenteroides*, *Lactobacillus* sp., and di-tripeptides including asparagine, which is
235 produced by *koji* mold degradation, are growth factors, and that pH and temperature affect the
236 growth of *L. sakei* (8,36,37). In this study, the peak of nitrite concentration was relatively late
237 at day 11, and the increase in acidity (TA; Titratable acidity) was delayed accordingly,
238 suggesting a longer survival period of adventitious bacteria that were initially introduced (Fig.
239 2). Therefore, there is a possibility that the growth of *L. sakei* was inhibited by specific changes
240 in nutrients and temperature.

241 In addition, D-amino acids are attracting attention as new taste components of *sake*, D-
242 alanine is reported to be produced by amino acid racemases from *L. mesenteroides* with low
243 temperature (38). *Leuconostoc* spp. capable of high D-amino acid production have also been
244 isolated (28), therefore, the presence of a high abundance of *Leuconostoc* spp. may contribute
245 significantly to the taste of *sake*, and need to isolate and culture *Leuconostoc* spp. found in this
246 brewery for bacterial genome sequencing and detection of unique metabolism pathways.

247

248 **Nitrate-reducing bacteria are a major factor in microbial transitions** In this study,
249 *Pseudomonas* spp. was detected in the middle stage, and the peak of nitrite reaction was
250 observed on the 11th day (Fig. 1). On the other hand, previous studies on *Yamahai-moto*
251 fermentation starters, which is a sake brewing recipe similar to *Kimoto*-style and use lactic acid
252 bacteria for maintaining the high acidity in the starter, reported cases where *Pseudomonas* spp.
253 were not detected and no nitrite production was observed (5,39). It is considered that the genus
254 *Pseudomonas* is easily lysed by exposure to alcohol due to its cell surface structure (40).
255 Therefore, we believe that *Pseudomonas* spp. was detected until the middle stage, where the
256 alcohol level rose relatively late, after day 20.

257 The structure of the microbiome changed significantly before and after the production
258 of nitrite, suggesting that nitrate-reducing bacteria may also affect microbial transitions and

259 other factors in the early stage (Fig. 1 and 2). This suggests that the presence or absence of
260 nitrate-reducing bacteria such as *Pseudomonas* spp. may be one of the factors that cause
261 differences in the microbial transitions in different breweries.
262
263 **Environmental microbiome mapping in the *sake* brewery** This study allowed us
264 to detect the bacterial communities with phylogenetic composition during culturing of the
265 fermentation starter comprehensively. In the early stage, a complex and varied microbiota is
266 constructed as adventitious bacteria are contaminated by the built environments, tools, and raw
267 materials used in the brewery. Since the sake brewing process is conducted in an open system,
268 a variety of adventitious bacteria may contaminate the brewing *sake* from building
269 environments, tools, and raw materials used in the brewery. Investigating the microbiome from
270 architectural surfaces and tools in *sake* breweries may reveal the origin of adventitious bacteria
271 in early sake. It has been suggested that these adventitious bacteria may affect the quality and
272 taste of sake, so they need to be clarified (29,41). There are still many unexplained aspects of
273 *Kuratuski* microorganisms, and we believe that this scientific elucidation of the traditional
274 Japanese liquor will provide significant insights into food microbiology.
275
276
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**Data availability**

The BioSample, DRA/SRA, and BioProject accession numbers for the sequence reported here are SAMD00513369-SAMD00513388, DRR393497-DRR393516, and PRJDB13924 respectively.

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## Figure legends

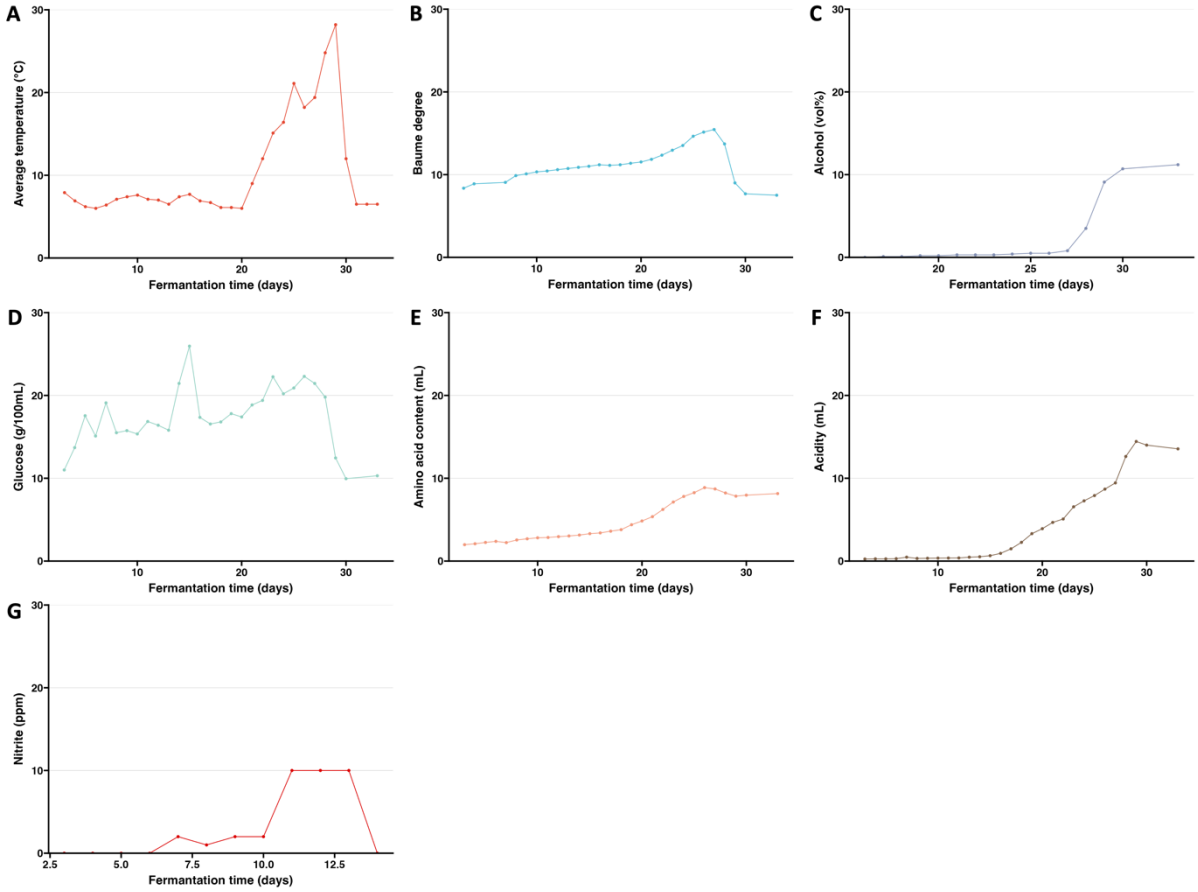


FIG. 1. Time-series data of chemical concentrations, degree, and average temperature. Average temperature (A), Baume degree (B), Alcohol (C), Glucose (D), Amino acid content (E), Acidity (F), Nitrite (G).

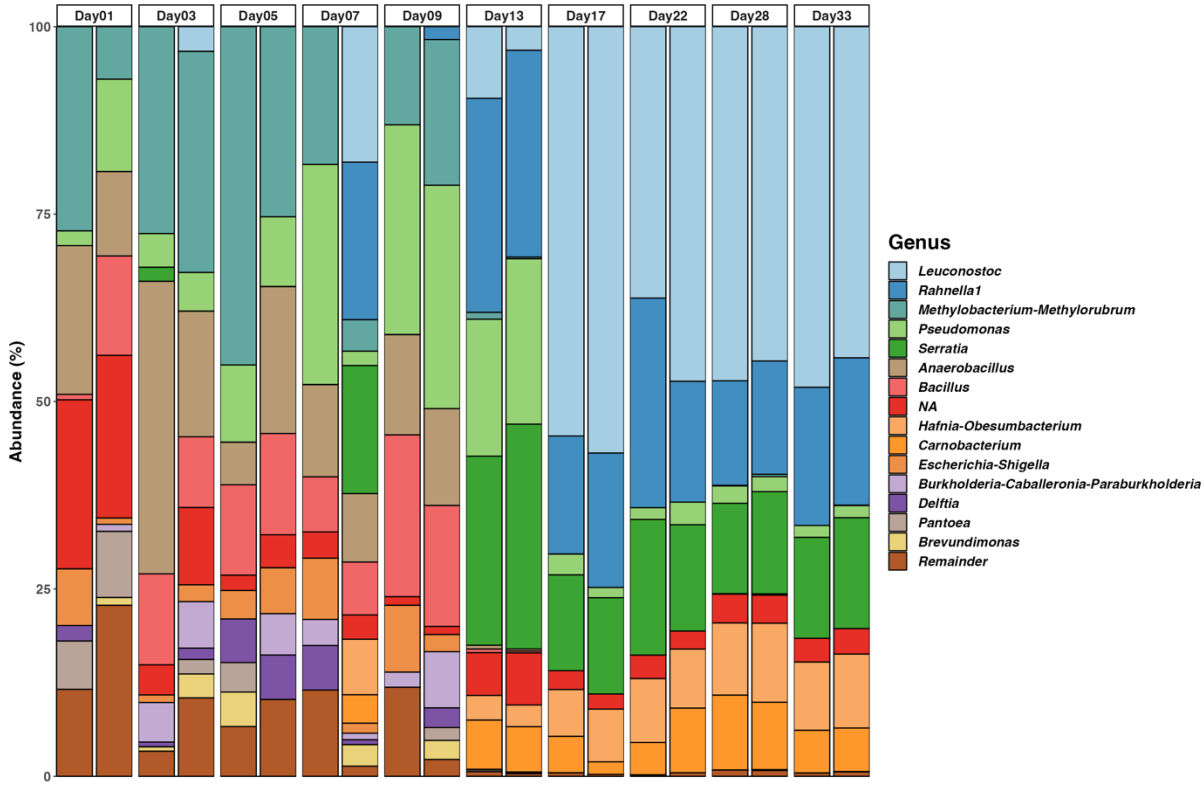


FIG. 2. Taxonomic composition of microbiomes during *Kimoto*-style fermentation starter in *sake* brewing. The top 15 genera are listed, and the rest are noted as "Remainder".

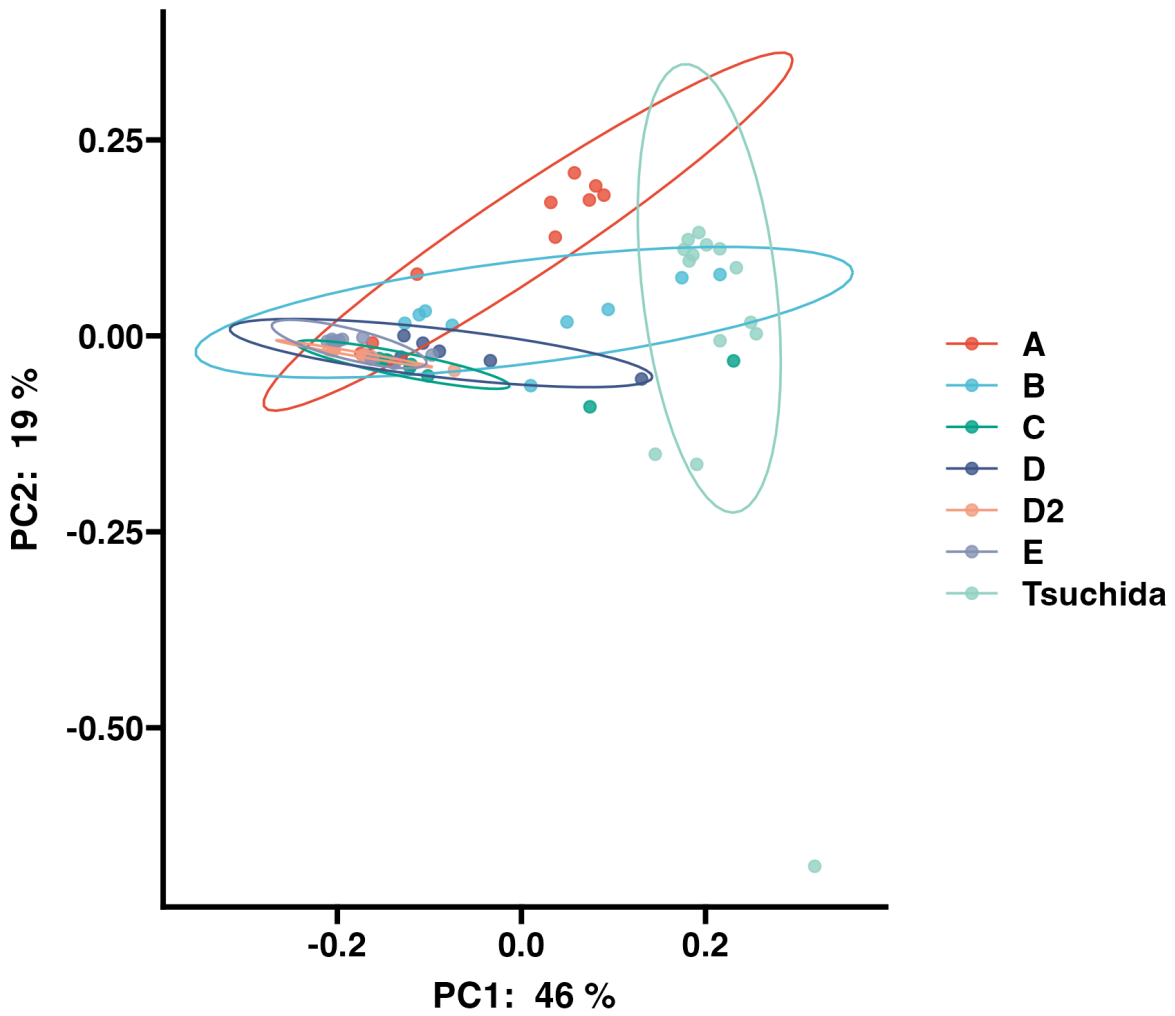


FIG. 3. Principal coordinate analysis (PCoA) of 5 *sake* breweries. Samples are compared using the weighted UniFrac distance metrics.

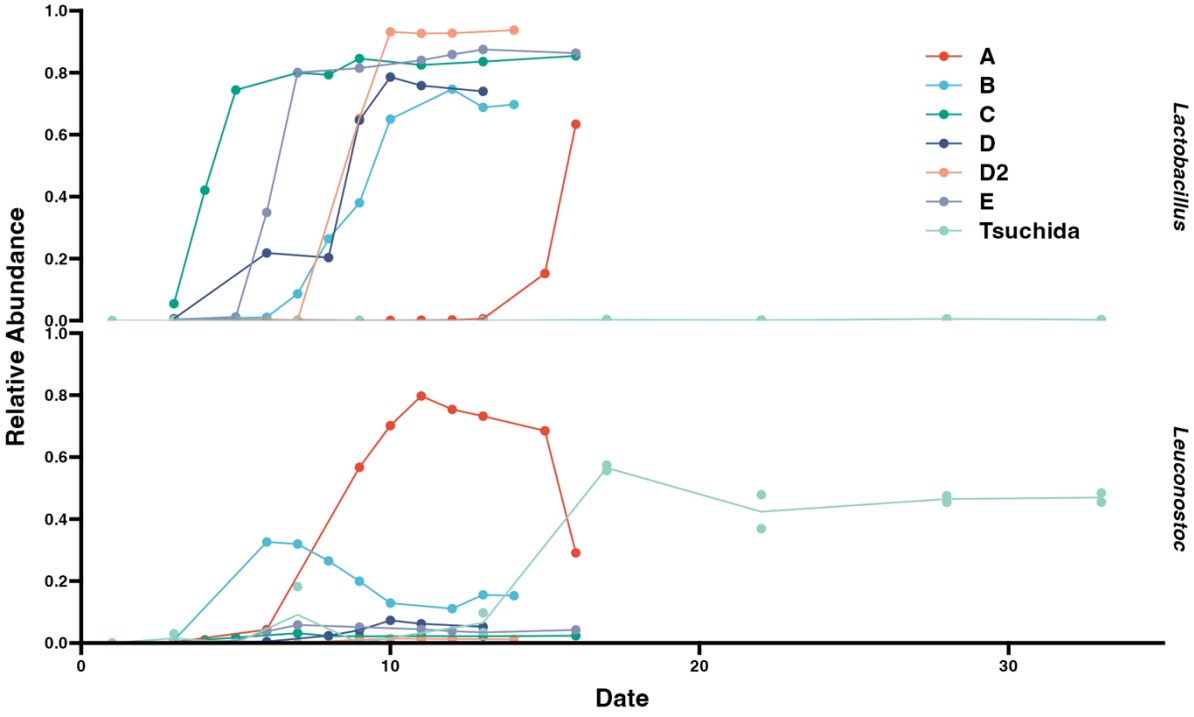


FIG. 4. Line plots of relative abundances of *Lactobacillus* and *Leuconostoc*.

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# Tables

TABLE 1. Time-series changes in Shannon index for each sample.

Microbial transition in the *Kimoto*-style starter

| days | sample_type | brewery | Shannon index |
|---|---|---|---|
| 5 | shubo | Tsuchida | 4.083626084 |
| 7 | shubo | Tsuchida | 4.483739465 |
| 9 | shubo | Tsuchida | 4.00914674 |
| 9 | shubo | Tsuchida | 3.260447879 |
| 13 | shubo | Tsuchida | 3.955204079 |
| 13 | shubo | Tsuchida | 3.953384193 |
| 17 | shubo | Tsuchida | 2.683731987 |
| 17 | shubo | Tsuchida | 2.54526448 |
| 22 | shubo | Tsuchida | 3.246816955 |
| 22 | shubo | Tsuchida | 2.970864866 |
| 28 | shubo | Tsuchida | 2.966174624 |
| 28 | shubo | Tsuchida | 3.072856664 |
| 33 | shubo | Tsuchida | 2.925714709 |
| 33 | shubo | Tsuchida | 3.056336844 |
| 3 | shubo | A | 0.075588383 |
| 6 | shubo | A | 0.47124225 |
| 9 | shubo | A | 1.748384977 |
| 10 | shubo | A | 1.517717158 |
| 11 | shubo | A | 1.252412288 |
| 12 | shubo | A | 1.496213641 |
| 13 | shubo | A | 1.682703746 |
| 15 | shubo | A | 1.732575907 |
| 16 | shubo | A | 1.486121819 |
| 3 | shubo | B | 2.38193983 |
| 6 | shubo | B | 2.933967722 |
| 7 | shubo | B | 3.090082101 |
| 8 | shubo | B | 3.294985987 |
| 9 | shubo | B | 3.142318413 |
| 10 | shubo | B | 2.2285432 |
| 12 | shubo | B | 1.835421019 |
| 13 | shubo | B | 2.113163833 |
| 14 | shubo | B | 2.012528982 |
| 3 | shubo | C | 1.837678435 |
| 4 | shubo | C | 2.042470055 |
| 5 | shubo | C | 1.28555966 |
| 7 | shubo | C | 1.261663511 |
| 8 | shubo | C | 1.221165987 |
| 9 | shubo | C | 1.037990141 |
| 11 | shubo | C | 1.10982519 |
| 13 | shubo | C | 1.085215814 |
| 16 | shubo | C | 1.016089562 |
| 3 | shubo | D | 1.812960613 |
| 6 | shubo | D | 2.714883385 |
| 8 | shubo | D | 3.393236556 |
| 9 | shubo | D | 2.492923589 |
| 10 | shubo | D | 2.097860823 |
| 11 | shubo | D | 2.214411678 |
| 13 | shubo | D | 2.233278552 |
| 3 | shubo | D2 | 0.541406715 |
| 5 | shubo | D2 | 0.55204851 |
| 6 | shubo | D2 | 0.460579254 |
| 7 | shubo | D2 | 0.725185898 |
| 9 | shubo | D2 | 2.65814023 |
| 10 | shubo | D2 | 1.727365975 |
| 11 | shubo | D2 | 1.722444728 |
| 12 | shubo | D2 | 1.675929835 |
| 14 | shubo | D2 | 1.64604447 |
| 3 | shubo | E | 0.806481485 |
| 5 | shubo | E | 1.332881408 |
| 6 | shubo | E | 2.553896711 |
| 7 | shubo | E | 1.644847183 |
| 9 | shubo | E | 1.56711282 |
| 11 | shubo | E | 1.427864233 |
| 12 | shubo | E | 1.328343618 |
| 13 | shubo | E | 1.247446865 |
| 16 | shubo | E | 1.325637366 |