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

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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 in 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 ribosomal RNA 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 between October and November 2021. The ingredients of the fermentation starter were water, polished rice, and *koji*. We did not use nitric acid or other additives for this batch. The polishing ratio of the rice was 85%. It is assumed that the microbiome is not homogeneous because the fermentation starter is produced in a large tank. Therefore, all samples were collected in duplicate to avoid sampling bias. 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 750 µl of lysis buffer supplied with the GenFind V2 DNA extraction kit (Beckman Coulter, Indianapolis, U.S.). The suspension was vortexed for 10 min, heat-treated at 100 °C for 10 min, and centrifuged for 5 min at 15,000 rpm. The supernatant was mixed with E.Z. beads (AMR, Tokyo, Japan). DNA fragmentation was performed on an MM-400 (Retsch, Haan, Germany) at a maximum speed of 3 min. The rest of the DNA purification process was performed using

115 a GenFind v2 DNA extraction kit (Beckman Coulter, Indianapolis, U.S.) following the
116 manufacturer's protocol. Finally, DNA was eluted with 80 µl of sterile water. 341F (5'-
117 TCGTCGGCAGCGTCAGATGTGTATAAGAGAGACACCTACGGGNGGCWGCAG-3')
118 and 806R (5'-
119 GTCTCGTGGGCTCGGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC
120 C-3') primers were utilized to amplify the V3-V4 region of the 16S rRNA gene by PCR (11,12).
121 Thermal cycling conditions were 95 °C for 3 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s,
122 and 72 °C for 30 s; and a final extension at 72 °C for 5 min. A second PCR was performed to
123 add sequencing adapters and dual-index barcodes to distinguish amplicons from each sample
124 using the same reaction conditions with eight cycles. Preparation of libraries and sequencing
125 were performed by paired-end sequencing of 300 bp on the Illumina MiSeq platform (Illumina,
126 Inc., San Diego USA) at GenomeRead Inc. in Kagawa, Japan.
127
128 **Microbiome analysis** The QIIME2 (version 2021.02) (13) platform was used for
129 microbiome analysis. FASTQ files were imported into the QIIME2 platform. Sequences were
130 processed using qiime dada2 denoise-paired command for quality control and classification into
131 amplicon sequencing variants (ASVs). Taxonomic analysis was run on SILVA database SSU
132 138 by qiime feature-classifier classify-sklearn (14–16). Sequence reads that were not classified
133 as any species after phylogenetic classification (Unassigned) and reads classified as chloroplast
134 and mitochondria were excluded from further analyses. Since the genus *Latilactobacillus* is a
135 taxon which relatively recently derived from the genus *Lactobacillus* (17), the SILVA database
136 SSU 138 used in this study does not reflect the reclassification of the genus *Latilactobacillus*,
137 so it was assigned as *Lactobacillus* in this study.
138
139 **Processing for comparative microbiome analysis** Sequence data from BioProject

PRJDB12939 was used for comparative analysis (8). Since 341F (5'-CCTACGGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGGTATCTAATCC-3') were used in the study, removal of one base from the 5' end of the reverse read was performed to match the read lengths of the samples obtained in our study. The fastp v.0.20.1. (18) was deployed for this processing. The method described above was repeated to perform taxonomic analysis. The depth of sequence reads (Features) differed among samples, and to normalize them, we subsampled from each sample to 5,000 reads each. Samples with fewer than 5,000 reads (S22, 23, 24, 25, 26, 28) were excluded from the diversity analysis (Table S1). Sub-sampling is an approach for inferring microbiome differences between samples and has been reported to be a suitable analytical method when analyzing new data sets (19). To evaluate the effect of sequence read counts on microbiome diversity assessment, we examined changes in the value of the Shannon index over a range of reading counts from 0-5,000 by rarefaction curves. The Rarefaction curve of the Shannon index leveled off when the number of leads was just under 500 (Table S2). Therefore, this investigation suggests that no significant changes in microbial diversity are due to the subsampling of 5,000 reads from each sample.

Statistical Analysis Kruskal-Wallis tests were used to compare the alpha diversity (Shannon diversity index) among samples. To compare differences in Beta diversity (Weighted UniFrac distance) between samples, for all PERMANOVA analyses, 5,000 trials were performed to assess the statistical significance. Q-values < 0.05 after multiple testing corrections were considered statistically significant. All multiple testing corrections were performed by computing FDRs using the Benjamini–Hochberg method, and Q-values (adjusted P-values) < 0.05 were considered statistically significant. Data visualization was performed by R version 4.2.1, the ggplot2 package version 3.4.0 and ggprism version 1.0.4.

165 # RESULTS

166 **Chemicals and Temperature Change in fermentation starters** The chemical data
167 and the average temperature of the *sake* starter are shown in Fig. 1. The amount of nitrite started
168 to rise on day 7 and reached its peak on day 11. On day 14 it was undetectable; Titratable acidity
169 (TA), a value representing total acidity, increased on day 15, followed by an increase in ethanol
170 concentration on day 27. In addition, a rapid decrease in glucose was observed along with an
171 increase in ethanol concentration on day 27. These changes in chemical data and the average
172 temperature are generally observed in *Kimoto*-style fermentation, so we suggest that the
173 fermentation proceeded well in the *Kimoto*-style fermentation starter of the *Tsuchida Sake*
174 Brewery.

175

176 **Phylogenetic composition of the microbiome found in the fermentation starter** To
177 track the microbial transition in the fermentation starter during the fermentation, we performed
178 16S rRNA amplicon sequencing. Results demonstrated large changes in relative abundances of
179 the bacterial genera (Genus) during the early to late stages (Fig. 2). Taxonomic compositions
180 of day 7 were markedly different between duplicates.

181 The genera with average relative abundances higher than 5% in the early stage (day 1
182 to day 7) of the fermentation starters were *Methylobacterium-Methylorubrum* (23.0%),
183 *Anaerobacillus* (16.7%), *Bacillus* (9.4%), and *Pseudomonas* (9.3%) (Table S3). The relative
184 abundances of these bacterial genera decreased significantly within days 7-9. *Anaerobacterium*
185 spp. is salt-tolerant and halophilic and has been found in lakes and soils in arid regions (20,21).
186 It is reported that *Bacillus* spp. may live in *sake* breweries as *Kuratsuki* bacteria (22).

187 The bacterial genera with average relative abundances higher than 5% in the late stage
188 (days 17-33) were *Rahnella1* (18.1%), *Serratia* (13.9%), *Hafnia-Obesumbacterium* (8.6%), and
189 *Carnobacterium* (6.2%) (Table S3). *Leuconostoc, Rahnella, Serratia, Hafnia-*

190 *Obesumbacterium*, and *Carnobacterium* were the bacterial genera that accounted for a large
191 proportion of the bacteria found in the late stage (days 17-33). *Leuconostoc* spp. has been
192 detected in several fermented foods (23,24).
193
194 **Comparison with other breweries** To characterize the microbial transition in this
195 brewery, we compared our data with a previous study that investigated the microbial transition
196 in four *sake* breweries (8). Time-series changes in the microbial diversity (Alpha diversity;
197 Shannon index) for each sample are shown (Table 1). *Tsuchida Sake* brewery was confirmed
198 to have a higher microbial diversity (Shannon index) of the fermentation starters (Q-value <
199 0.05) compared to the other breweries (Table S4).
200 A Principal Coordinate Analysis (PCoA) by Weighted UniFrac distance to visualize the
201 differences (Beta diversity) among the microbiomes of each sample showed that the *Tsuchida*
202 *Sake* Brewery formed a different cluster (q-value < 0.007) than the other breweries (Fig. 3 and
203 Table S5).
204 The relative abundances of the genus *Lactobacillus* and *Leuconostoc* in each brewery
205 during the preparation are shown in the line graphs (Fig. 4). The genus *Lactobacillus* became
206 the dominant species in all other breweries, but the relative abundance of the genus
207 *Lactobacillus* did not increase during the entire preparation in the *Tsuchida Sake* Brewery. On
208 the other hand, the genus *Leuconostoc* increased in only three breweries, and in all but the
209 *Tsuchida Sake* Brewery, their abundance eventually decreased and shifted to the genus
210 *Lactobacillus*. In contrast, in the *Tsuchida Sake* Brewery, the genus *Leuconostoc* maintained a
211 constant high relative abundance during the fermentation.
212

213 # DISCUSSION

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

224 In addition, another study reported that *Kimoto*-style fermentation is characterized by
225 the detection of multiple lactic acid bacteria compared to *Sokujo-moto* (25), so the process of
226 Kimoto-style fermentation itself may contribute to the diversity of taste in different *sake*
227 breweries. *Sokujo-moto* is a modern type of fermentation starter which includes lactic acid to
228 maintain a low pH, thus preventing microbial contamination from the brewery (5). As indicated
229 in this study, specific microbiome transitions were observed that were different from the
230 microbial transitions reported by Takahashi *et al.*, suggesting that the microbiome is unique in
231 individual breweries and that these microbial transition factors that characterize them need to
232 be investigated (8).

233 Some studies reported that only one lactic acid bacteria appears and the transition does
234 not occur (26), and no *Lactobacillus* spp. are found (27), but to the best of my knowledge this
235 is the first study proving this trend of microbiome shift in a manner of uncultured analysis
236 method.

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

238 than *L. mesenteroides*, *Lactobacillus* sp., and di-tripeptides including asparagine, which is
239 produced by *koji* mold degradation, are growth factors, and that pH and temperature affect the
240 growth of *L. sakei* (8,28,29). In this study, the peak of nitrite concentration was relatively late
241 at day 11, and the increase in acidity (TA; Titratable acidity) was delayed accordingly,
242 suggesting a longer survival period of adventitious bacteria that were initially introduced (Fig.
243 2). Therefore, there is a possibility that the growth of *L. sakei* was inhibited by specific changes
244 in nutrients and temperature.

245 In addition, D-amino acids are attracting attention as new taste components of *sake*, D-
246 alanine is reported to be produced by amino acid racemases from *L. mesenteroides* with low
247 temperature (30). *Leuconostoc* spp. capable of high D-amino acid production have also been
248 isolated (24), therefore, the presence of a high abundance of *Leuconostoc* spp. may contribute
249 significantly to the taste of *sake*, and need to isolate and culture *Leuconostoc* spp. found in this
250 brewery for bacterial genome sequencing and detection of unique metabolism pathways.

251

252 **Nitrate-reducing bacteria are a major factor in microbial transitions** In this study,
253 *Pseudomonas* spp. was detected in the middle stage, and the peak of nitrite reaction was
254 observed on the 11th day (Fig. 1). On the other hand, previous studies on *Yamahai-moto*
255 fermentation starters , which is a *sake* brewing recipe similar to *Kimoto*-style and lactic acid
256 bacteria for maintaining the high acidity in the starter play an important role, reported cases
257 where *Pseudomonas* spp. were not detected and no nitrite production was observed (5,31). It is
258 considered that the genus *Pseudomonas* is easily lysed by exposure to alcohol due to its cell
259 surface structure (32). Therefore, we believe that *Pseudomonas* spp. was detected until the
260 middle stage, where the alcohol level rose relatively late, after day 20.

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

263 other factors in the early stage (Fig. 1 and 2). This suggests that the presence or absence of
264 nitrate-reducing bacteria such as *Pseudomonas* spp. may be one of the factors that cause
265 differences in the microbial transitions in different breweries.

266 Taxonomic compositions of day 7 were markedly different between duplicates. Since
267 the fermentation starter is fermented in a large tank and mixed with a stirring stick, it is
268 considered that the microbial composition in the tank was not homogenous. In addition, 16S
269 rRNA sequencing analysis possibly show subtle differences in each experiment due to
270 experimental methods (33).

271

272 **The beneficial and harmful bacteria for *sake* brewing** *Bacillus* spp. have also been
273 detected in *koji* and are known to be associated with the production of 4-vinyl guaiacol
274 according to previous studies (9,34,35). In contract, the amylolytic activity of *Bacillus* spp. may
275 play an important role in saccharification in the fermentation starter (9,36). The previous study
276 that have examined the microbiome of *Kaburazushi* reported that *Staphylococcus* spp. and
277 *Bacillus* spp. produce the components necessary for lactic acid bacteria growth (36). *Serratia*
278 spp. have been identified by previous studies from *Yamahai-moto* (6), *Rahnella* spp. from
279 *Yamahai* (25), and Chinese sauerkraut (37). *Hafnia-Obesumbacterium* spp. has been detected
280 in brewer's yeast along with *Rahnella* spp., suggesting that it may produce high pH beer by
281 inhibiting fermentation reaction (38,39), it may also affect flavor in *sake* brewing. It is known
282 that *Carnobacterium* spp. inhibits the growth of spoilage-associated bacteria and foodborne
283 pathogens and has been estimated as useful bacteria for food preservations in various foods,
284 including cold-smoked salmon, Ricotta Fresca cheese, and cooked ham (40–42). Therefore
285 *Carnobacterium* spp. may contribute to preventing the growth of adventitious bacteria in *sake*
286 brewing. A previous study (43) reported that *Methylobacterium* spp. were cultured in botrytized
287 wine after 51 days of fermentation, suggesting that they may have also survived in the

288 fermentation starter.

289 We found significant changes in relative abundances of these bacteria such as *Rahnella1,*
290 *Serratia, Hafnia-Obesumbacterium* during the early to the late stages (Fig. 2). But These
291 bacteria were not found during the early stage. Therefore, these bacteria may have been
292 contaminated outside tanks during the middle stage (days 9-13). And these bacteria maintained
293 high relative abundance and may have been grown in the fermentation starter for some time.

294

295 **Environmental microbiome mapping in the *sake* brewery** This study allowed us
296 to detect the bacterial communities with phylogenetic composition during culturing of the
297 fermentation starter comprehensively. In the early stage, a complex and varied microbiota is
298 constructed as adventitious bacteria are contaminated by the built environments, tools, and raw
299 materials used in the brewery. Since the *sake* brewing process is conducted in an open system,
300 a variety of adventitious bacteria may contaminate the brewing's *sake* from building
301 environments, tools, and raw materials used in the brewery. Investigating the microbiome from
302 architectural surfaces and tools in *sake* breweries may reveal the origin of adventitious bacteria
303 in early *sake*. It has been suggested that these adventitious bacteria may affect the quality and
304 taste of *sake*, so they need to be clarified (25,44). There are still many unexplained aspects of
305 *Kuratuski* microorganisms, and we believe that this scientific elucidation of the traditional
306 Japanese liquor will provide significant insights into food microbiology.

307

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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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457 **Figure legends**

458

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

462

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

465

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

468

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

470

471 --------

472 **Tables**

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

474

475 TABLE S1. Total sequence reads per sample.

476

477 TABLE S2. Rarefaction curve of the Shannon index.

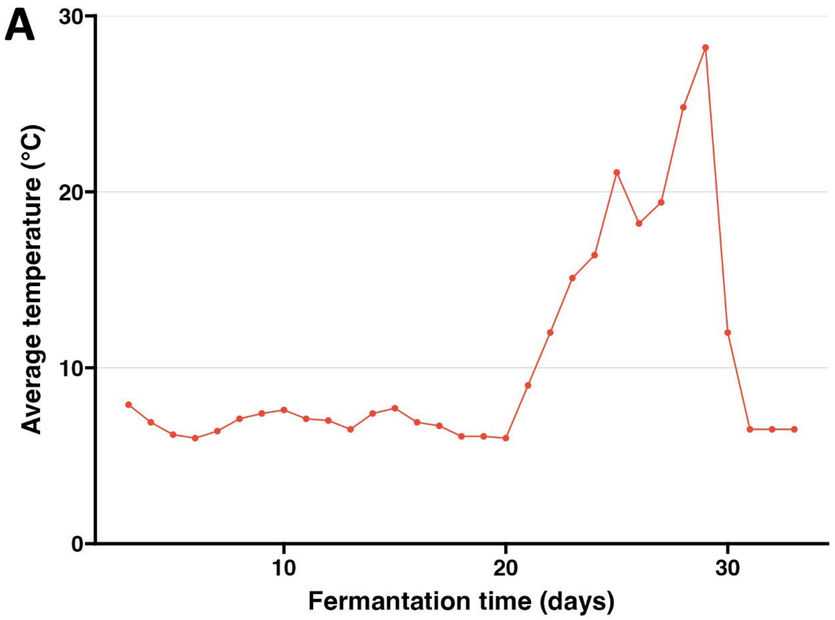
478

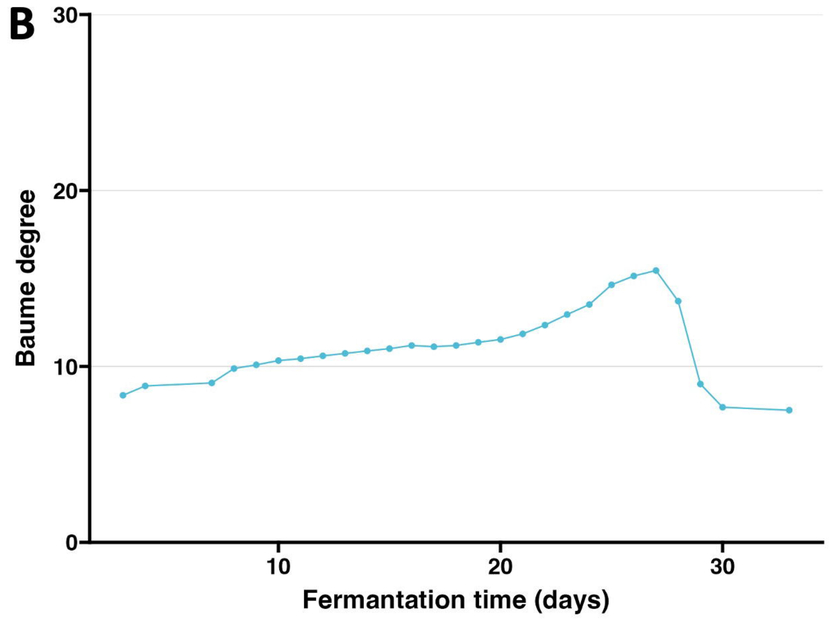
479 TABLE S3. Relative abundances of taxonomy.

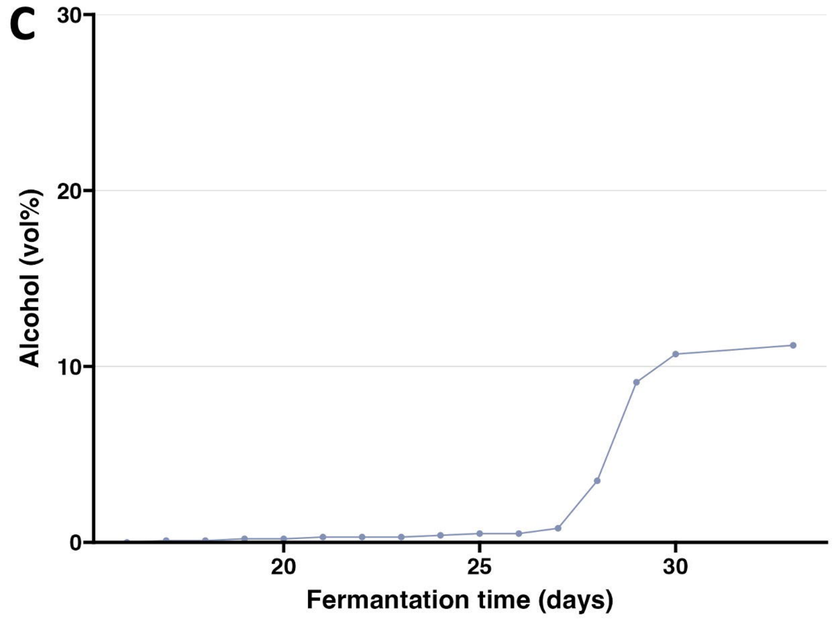
480

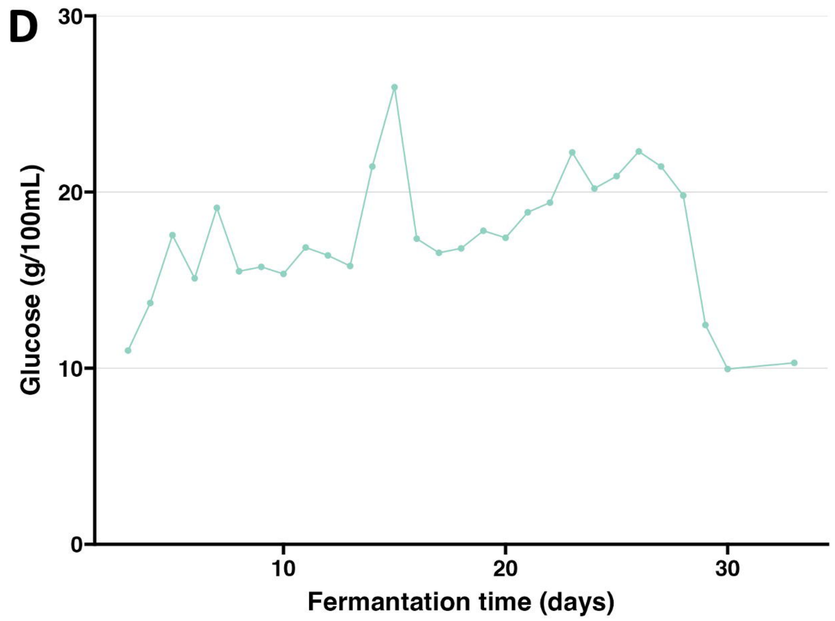
481 TABLE S4. Statistical analysis of the microbial diversity of each brewery.

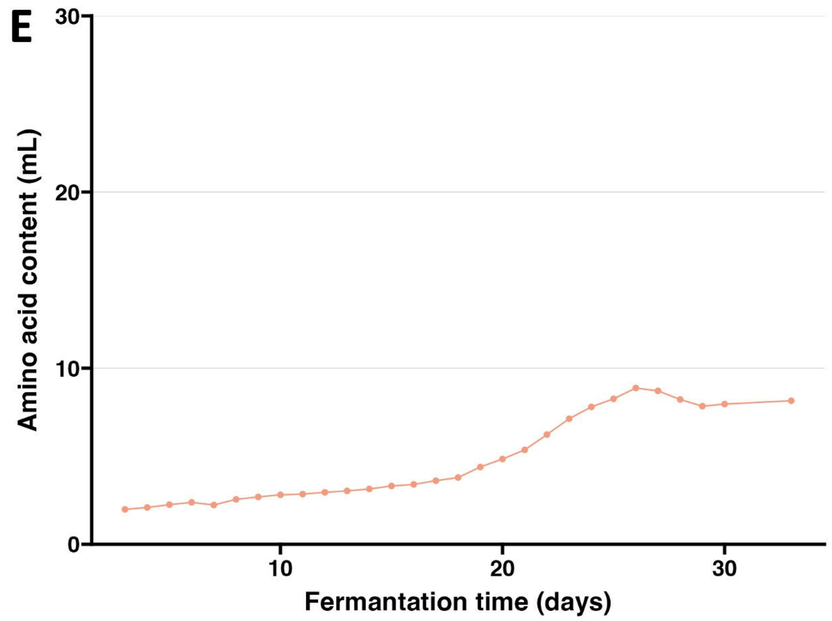
TABLE S5. Statistical analysis of the differences of each brewery.

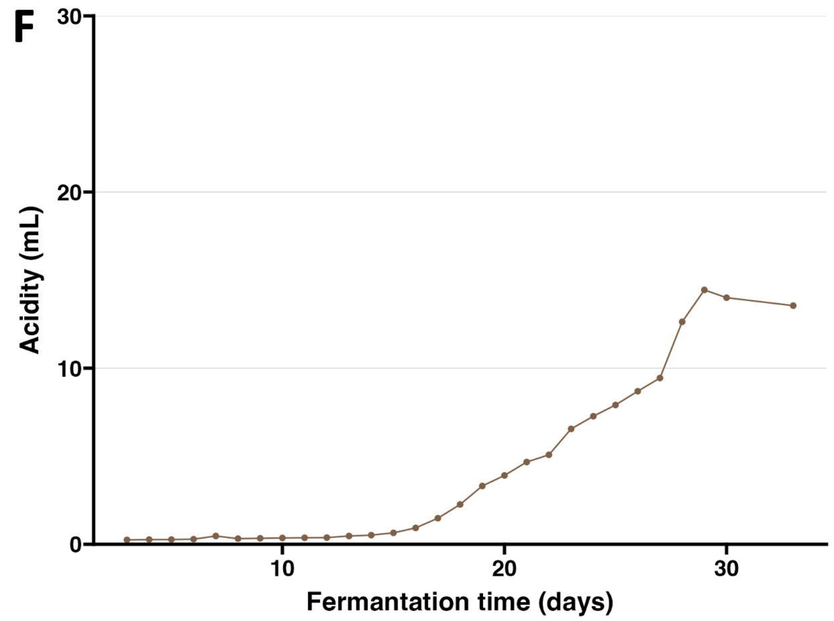

A
Average temperature (°C)
Fermantation time (days)

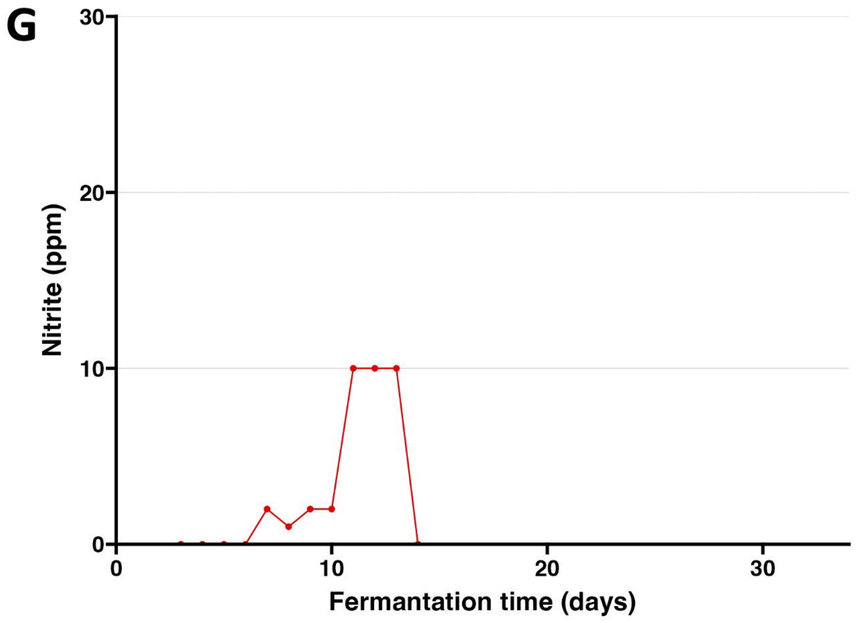

B
Baume degree
Fermantation time (days)

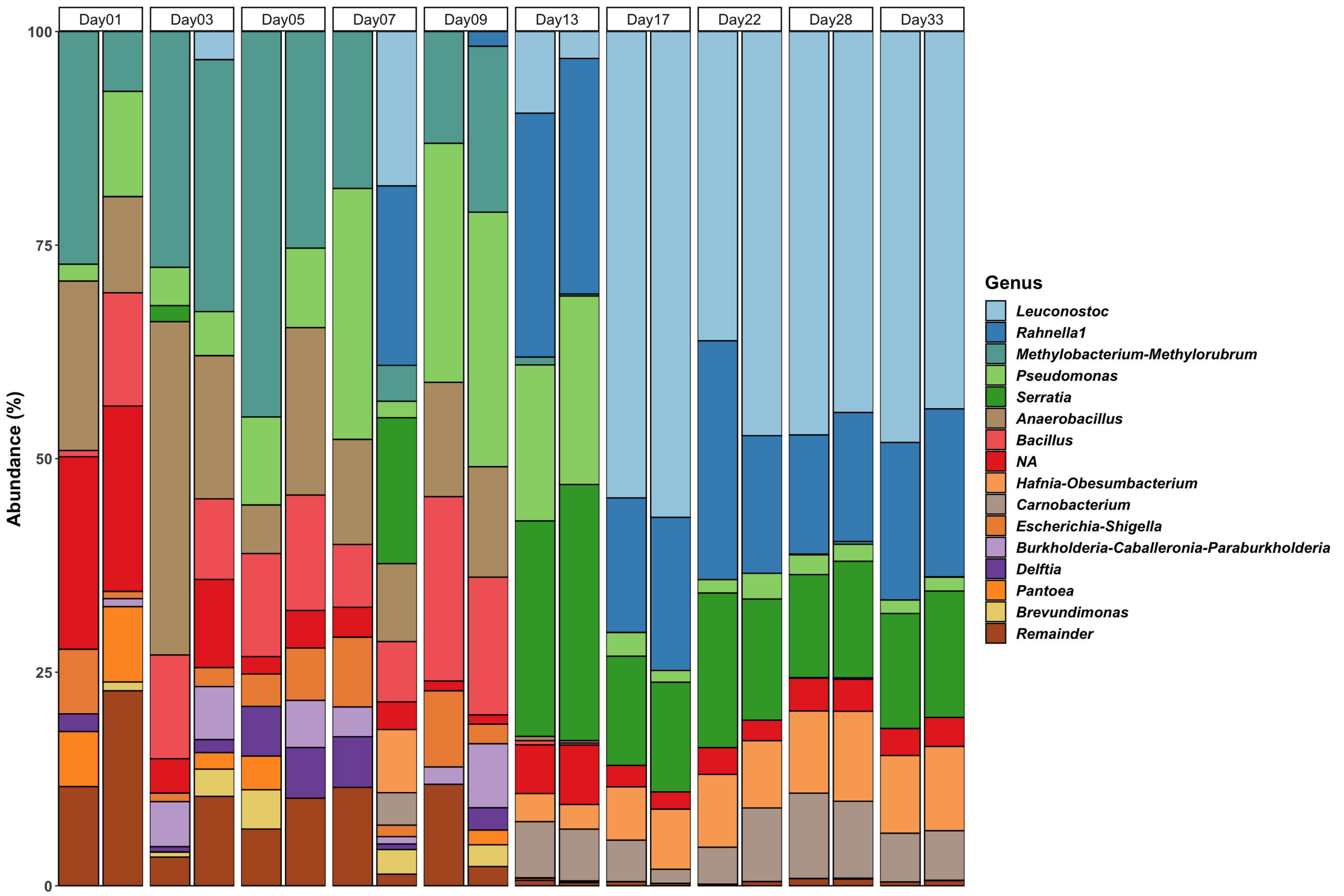

C
Alcohol (vol%)
Fermantation time (days)

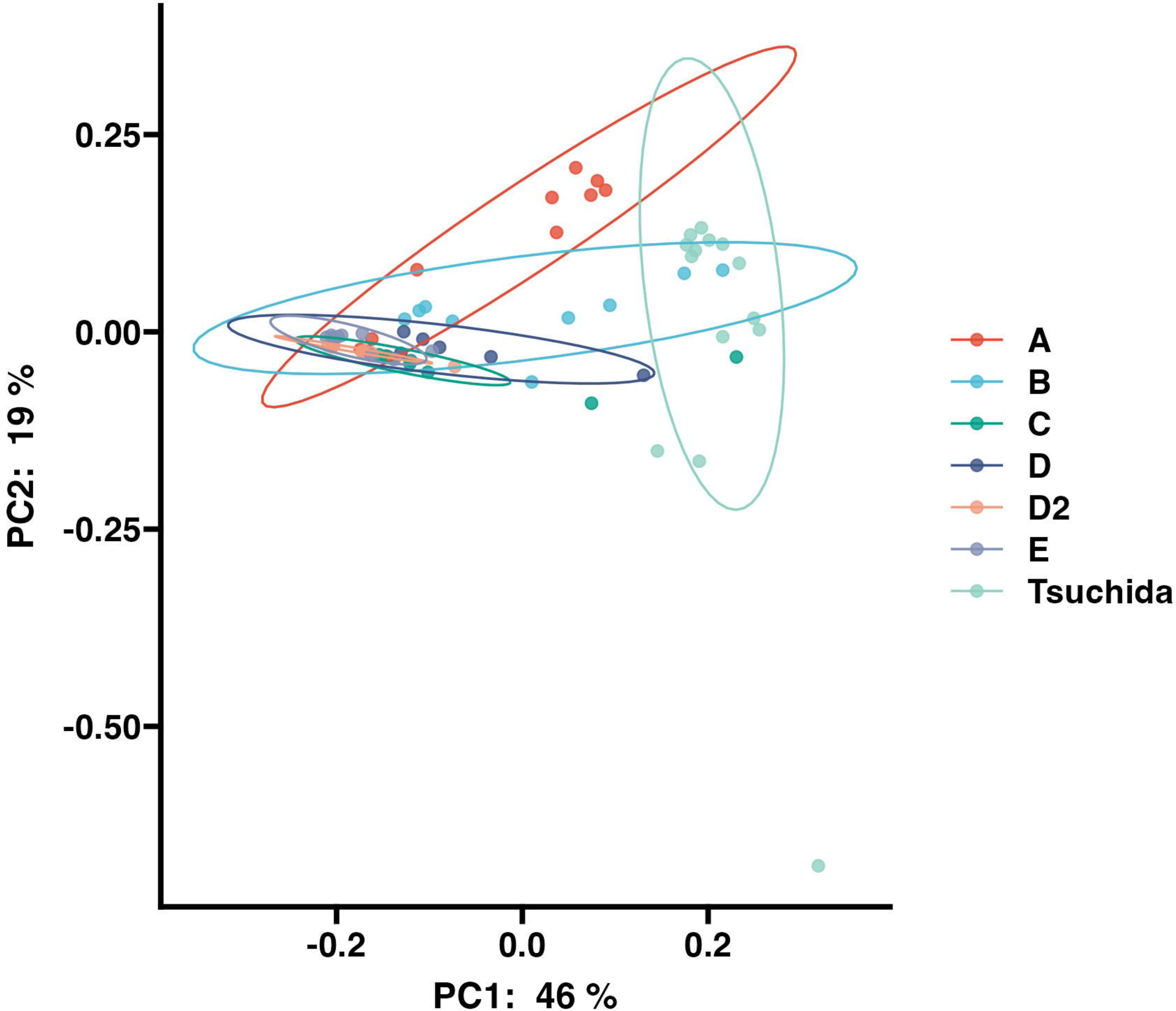

D
Glucose (g/100mL)
Fermantation time (days)

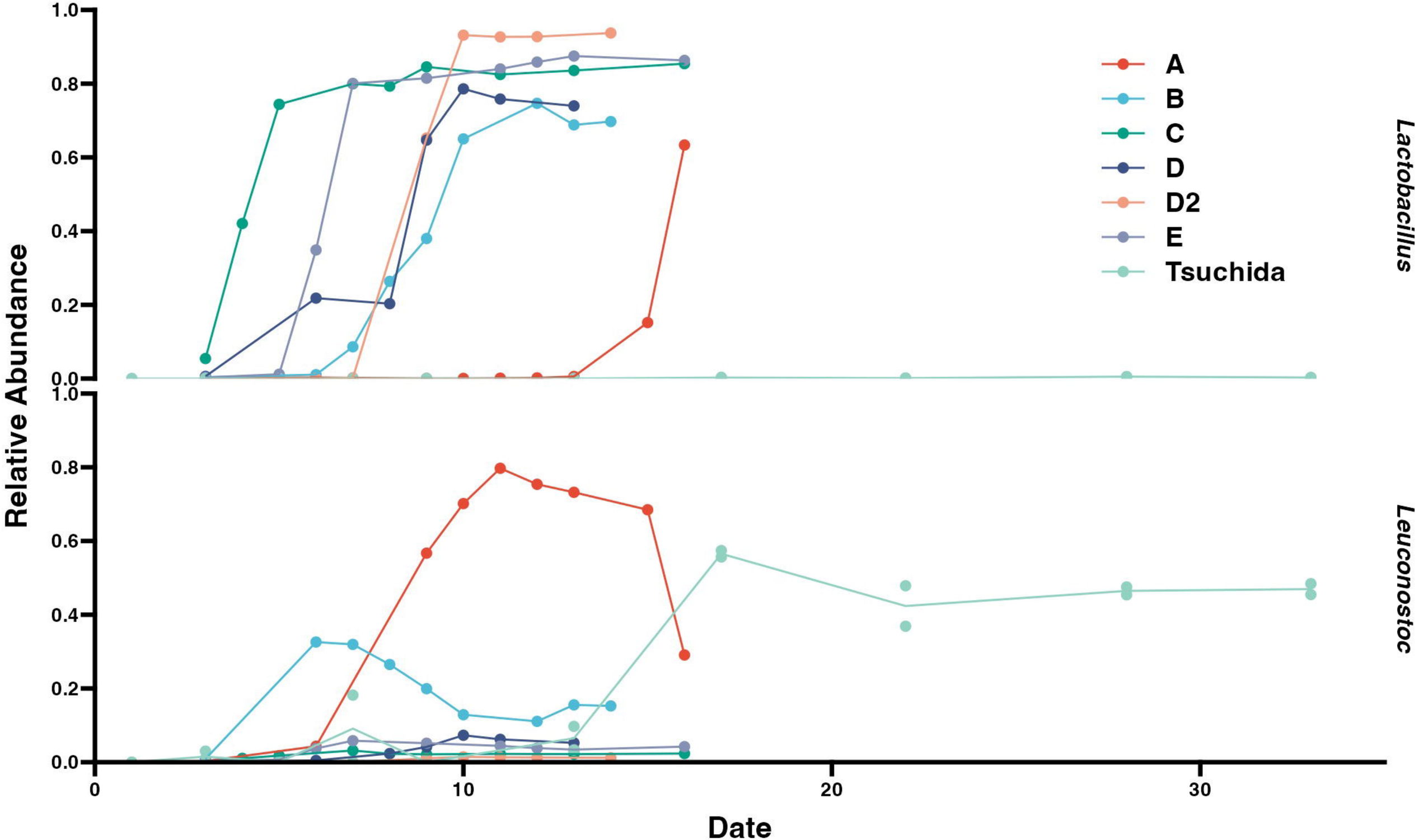

E
Amino acid content (mL)
Fermantation time (days)


F
Acidity (mL)
Fermantation time (days)


G
Nitrite (ppm)
Fermantation time (days)


Day01
Day03
Day05
Day07
Day09
Day13
Day17
Day22
Day28
Day33
Abundance (%)
100
75
50
25
0
Genus
Leuconostoc
Rahnella1
Methylobacterium-Methylorubrum
Pseudomonas
Serratia
Anaerobacillus
Bacillus
NA
Hafnia-Obesumbacterium
Carnobacterium
Escherichia-Shigella
Burkholderia-Caballeronia-Paraburkholderia
Delftia
Pantoea
Brevundimonas
Remainder


0.25
0.00
-0.25
-0.50
PC2: 19 %
-0.2
0.0
0.2
PC1: 46 %
A
B
C
D
D2
E
Tsuchida


Relative Abundance
1.0
0.8
0.6
0.4
0.2
0.0
Lactobacillus
Leuconostoc
A
B
C
D
D2
E
Tsuchida
0
10
20
30
Date

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