Supplementary information

Title

Comprehensive analysis of the change in bacterial and chemical components and growth prediction of *Lactobacillus sakei* during *kimoto*-style fermentation starter preparation in *sake* making.

Authors

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Fig. S1

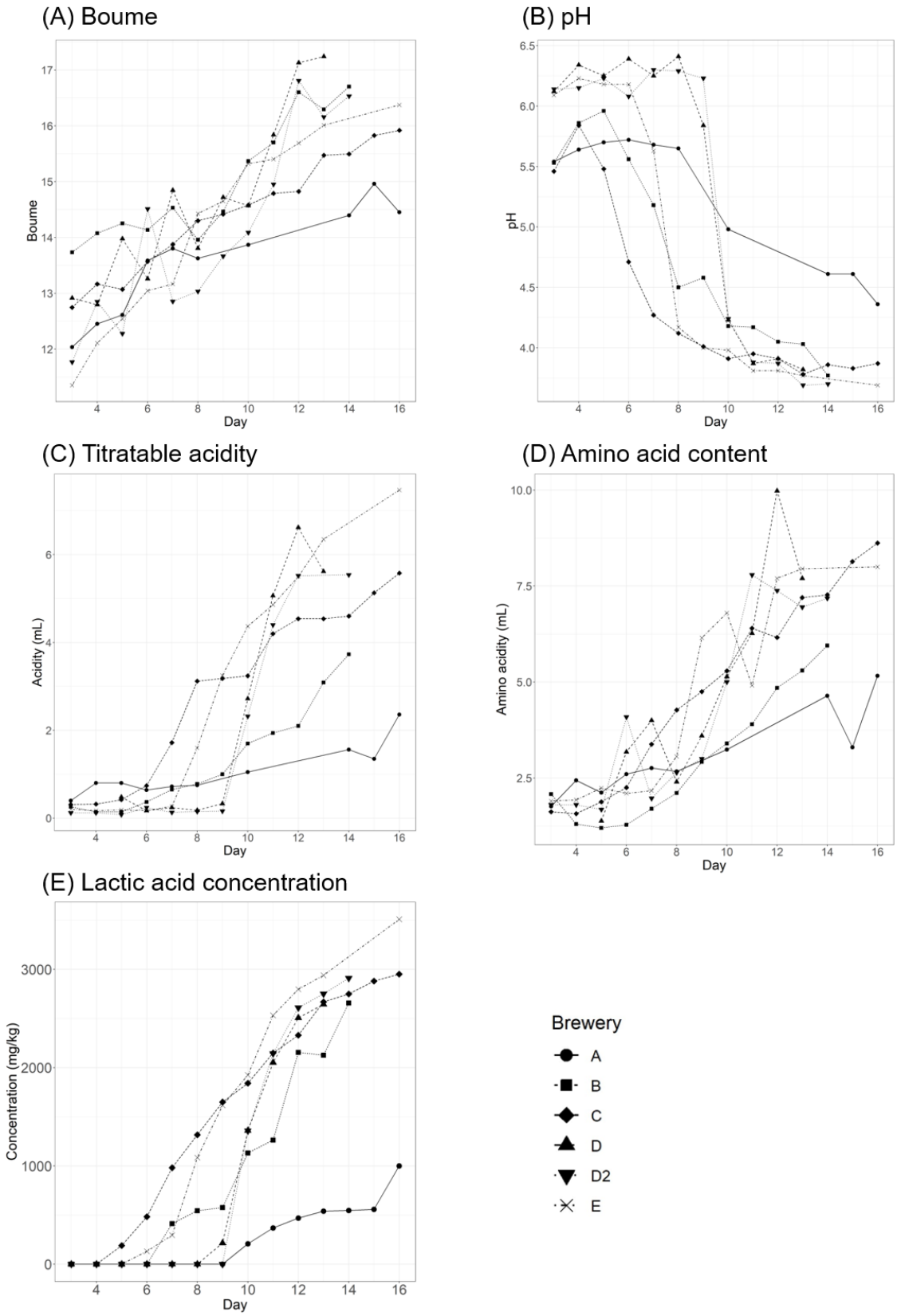
(A) Boume
Boume
Day
(B) pH
pH
Day
(C) Titratable acidity
Acidity (mL)
Day
(D) Amino acid content
Amino acidity (mL)
Day
(E) Lactic acid concentration
Concentration (mg/kg)
Day
Brewery
A
B
C
D
D2
E

Fig S2.

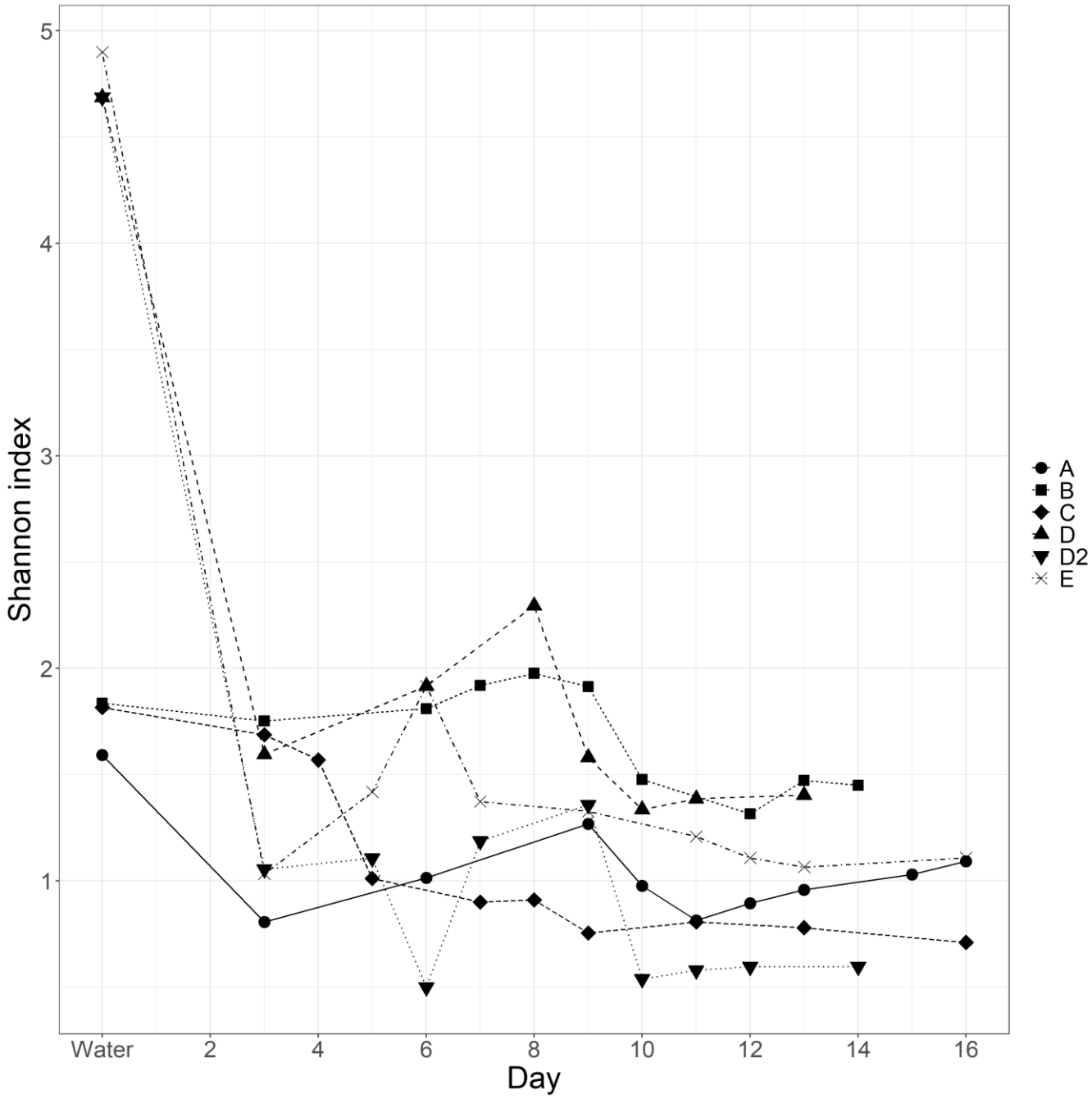

Fig S3.

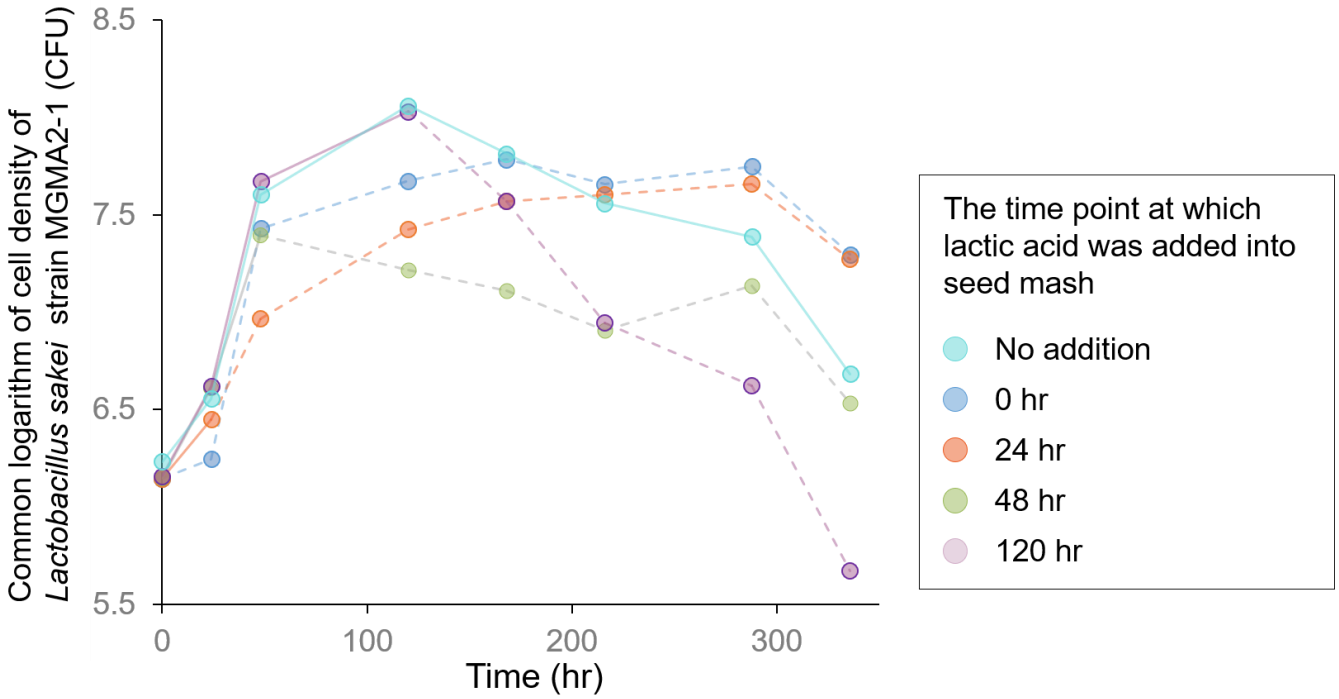
Common logarithm of cell density of Lactobacillus sakei strain MGMA2-1 (CFU)
8.5
7.5
6.5
5.5
0
100
200
300
Time (hr)
The time point at which lactic acid was added into seed mash
No addition
0 hr
24 hr
48 hr
120 hr

Fig. S4

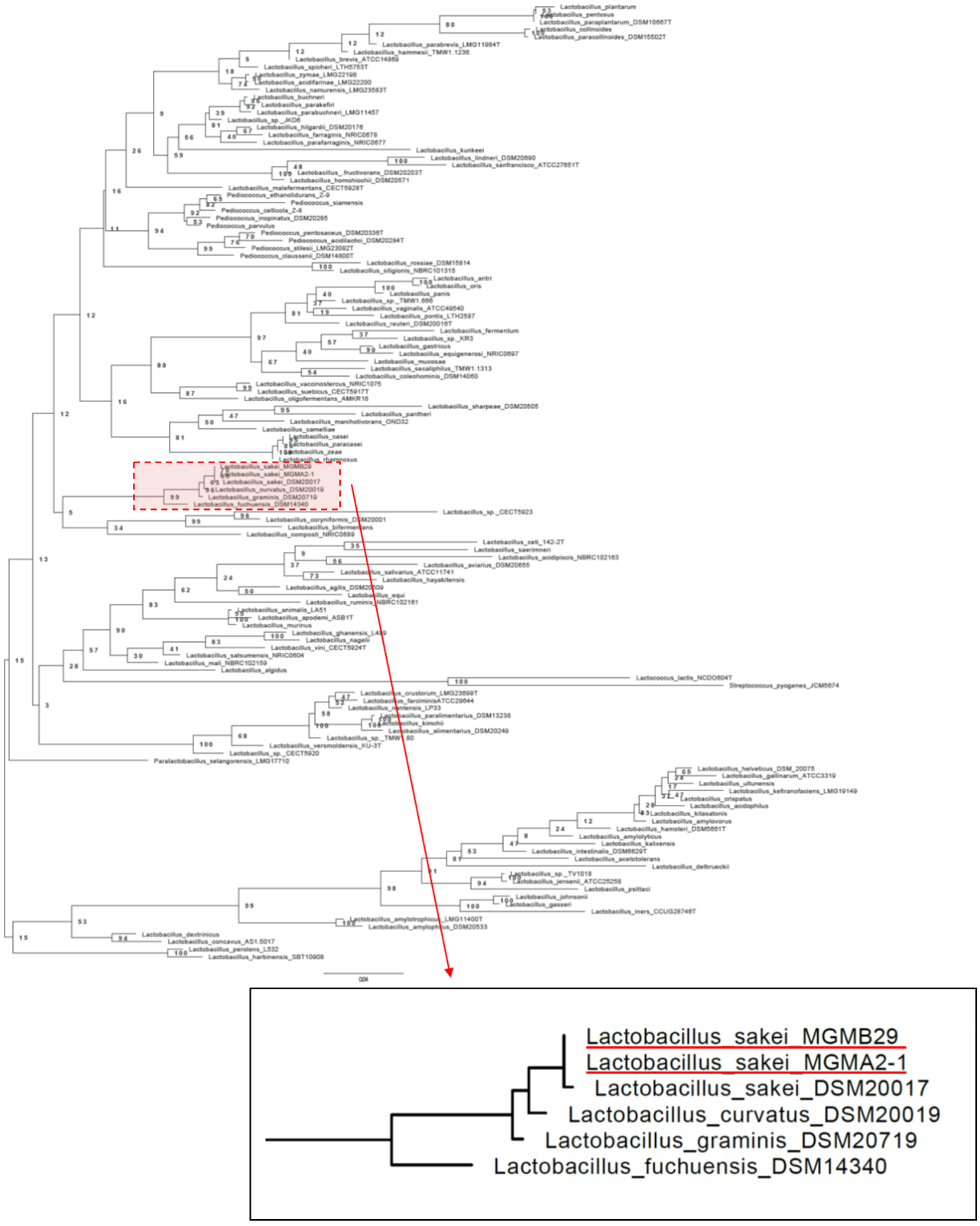
Lactobacillus_sakei_MGMB29
Lactobacillus_sakei_MGMA2-1
Lactobacillus_sakei_DSM20017
Lactobacillus_curvatus_DSM20019
Lactobacillus_graminis_DSM20719
Lactobacillus_fuchuensis_DSM14340

Fig. S5

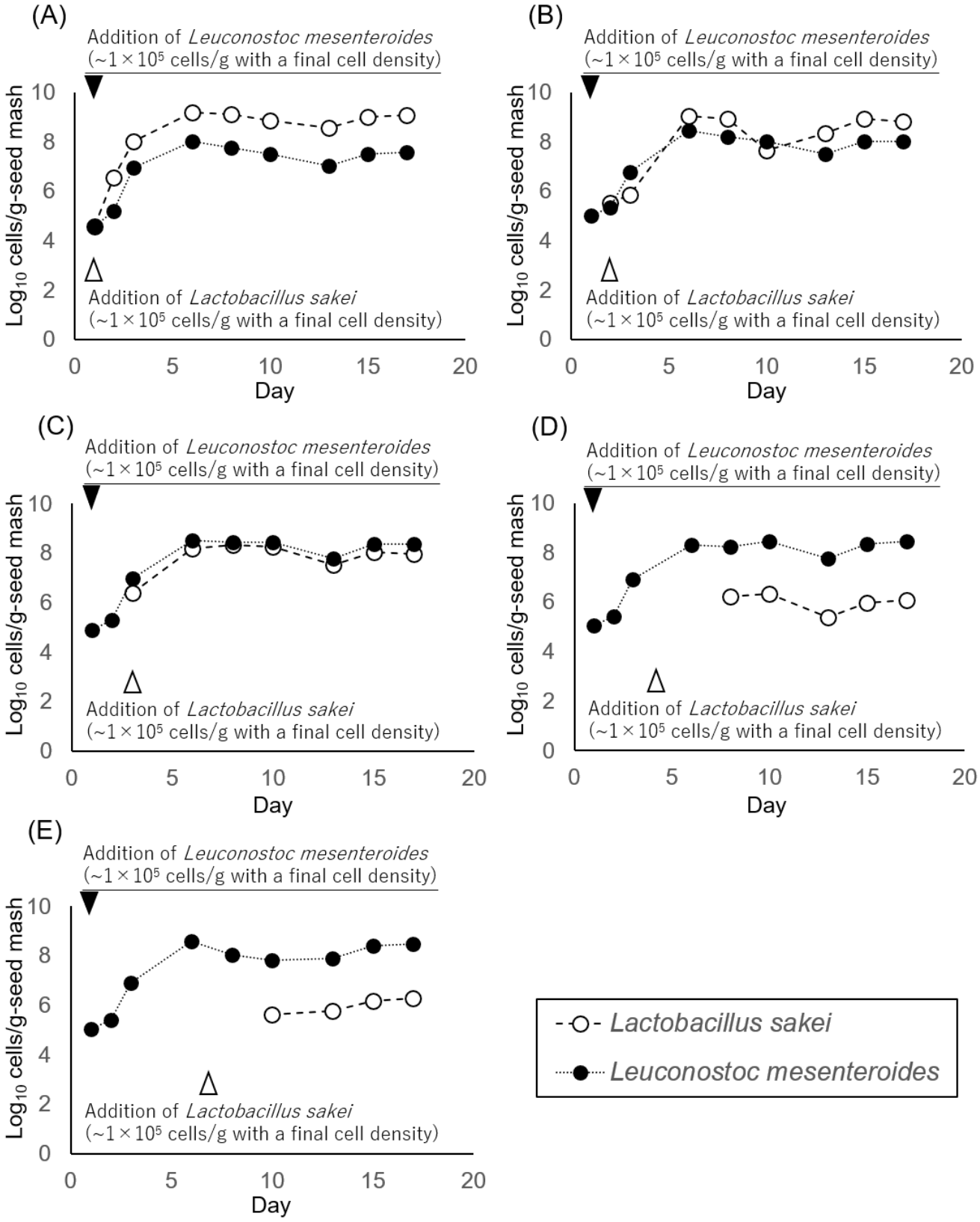
(A)
Addition of *Leuconostoc mesenteroides*
(~1 × 10⁵ cells/g with a final cell density)
Addition of *Lactobacillus sakei*
(~1 × 10⁵ cells/g with a final cell density)
Log10 cells/g-seed mash
Day
(B)
Addition of *Leuconostoc mesenteroides*
(~1 × 10⁵ cells/g with a final cell density)
Addition of *Lactobacillus sakei*
(~1 × 10⁵ cells/g with a final cell density)
Log10 cells/g-seed mash
Day
(C)
Addition of *Leuconostoc mesenteroides*
(~1 × 10⁵ cells/g with a final cell density)
Addition of *Lactobacillus sakei*
(~1 × 10⁵ cells/g with a final cell density)
Log10 cells/g-seed mash
Day
(D)
Addition of *Leuconostoc mesenteroides*
(~1 × 10⁵ cells/g with a final cell density)
Addition of *Lactobacillus sakei*
(~1 × 10⁵ cells/g with a final cell density)
Log10 cells/g-seed mash
Day
(E)
Addition of *Leuconostoc mesenteroides*
(~1 × 10⁵ cells/g with a final cell density)
Addition of *Lactobacillus sakei*
(~1 × 10⁵ cells/g with a final cell density)
Log10 cells/g-seed mash
Day
Lactobacillus sakei
Leuconostoc mesenteroides

Figure legends

Fig S1. The changes of Boume (A), pH (B), titratable acidity (C), amino acid content (D), and the concentration of lactic acid (E) during the preparation of seed mash.

Fig S2. The changes in the Shannon index during the preparation of seed mash.

Fig S3. The effect of lactic acid on the growth of *Lactobacillus sakei* strain MGMA2-1 in seed mash. Lactic acid was added to the seed mash at 0 hr, 24 hr, 48 hr, or 120 hr after inoculation (no addition as control). The results before the addition of lactic acid are indicated with solid lines and the results after the addition of lactic acid are indicated with dashed lines. The materials and methods used in this experiment are described in section 6 of the "Additional materials and methods" portion of this supporting information.

Fig S4. Phylogenetic analysis between the strains isolated in this study and the genera *Lactobacillus* and *Pediococcus*. We carried out the multiple alignment of DNA sequence with MAFFT (1), and then, estimated the relationship among species by RAxML (2), and finally, visualized the phylogenetic tree by using Figtree (3).

Fig S5. The difference in the growth of *Lactobacillus sakei* depending on the growth of *Leuconostoc mesenteroides*. (A), Simultaneous inoculation of *L. sakei* and *L. mesenteroides*; (B), Inoculation of *L. sakei* one day after *L. mesenteroides* inoculation; (C), Inoculation of *L. sakei* two days after *L. mesenteroides* inoculation; (D), Inoculation of *L. sakei* three days after *L. mesenteroides* inoculation; (E), Inoculation of *L. sakei* six days after *L. mesenteroides* inoculation. The materials and methods used in this experiment are described in section 7 of the "Additional materials and methods" portion of this supporting information.

Table S1

Brewery	Day	Raw data (read counts)	Trimmed data (read counts)	Brewery	Day	Raw data (read counts)	Trimmed data (read counts)
A	Water	186684	132093	D/D2	Water	166177	119633
A	3	219916	100853	D	3	262104	111571
A	6	399475	182880	D	6	471635	211082
A	9	286715	145997	D	8	243076	125834
A	10	283768	146351	D	9	340410	187989
A	11	219608	112070	D	10	337912	193701
A	12	208253	110015	D	11	240036	137172
A	13	319282	164446	D	13	384228	211387
A	15	296010	154099	D2	3	248942	169504
A	16	300352	164201	D2	5	261700	180443
B	Water	182479	129869	D2	6	241564	185738
B	3	329809	141009	D2	7	190075	133020
B	6	212806	110757	D2	9	203901	136897
B	7	277819	138360	D2	10	2614391	2144656
B	8	335061	166051	D2	11	183196	136812
B	9	364185	187812	D2	12	222330	164419
B	10	378420	201999	D2	14	192073	143679
B	12	402294	219398	E	Water	187416	134389
B	13	455222	232825	E	3	391011	184801
B	14	503726	250195	E	5	419436	203100
C	Water	215074	58667	E	6	360901	172438
C	3	332889	87808	E	7	346717	183637
C	4	268827	97130	E	9	398518	206283
C	5	435131	198500	E	11	349137	177959
C	7	377875	192360	E	12	273156	147285
C	8	370359	182087	E	13	318846	172865
C	9	300165	154732	E	16	310996	164000
C	11	155989	81248				
C	13	258311	213827				
C	16	228955	124239				

***** The sequence after trimming the primer region and removing low-quality sequences and chimera sequences.

Table S2

We have provided the data about chemical composition in the Microsoft Excel file (Table S2).

Table S3

We have provided the data about microbial community in the Microsoft Excel file (Table S3).

Formula

[Log phase] $$\Delta C = \sum_{k=1}^{n} \int \rho_k \cdot N_0 \cdot e^{ln2 \cdot t / g_k} \, dt \quad \cdots\cdots\cdots \quad (1)$$

[Stationary phase] $$\Delta C = \sum_{k=1}^{n} \int \rho_k \cdot N_{max} \, dt \quad \cdots\cdots\cdots \quad (2)$$

ΔC : The increase in the amount of lactic acid between sampling points

ρ_k : Lactic acid production rate of bacterium ‘k’ between sampling points

g_k: Generation time of bacterium ‘k’ between sampling points

N_0: Initial amount of bacterium ‘k’

N_{max}: Maximum amount of bacterium ‘k’

Additional materials and methods.

1. Analysis of sugar.

We extracted sugar compound from the seed mash by the method used in previous studies with some modifications (4, 5). First, 2 grams of sample was weighed out into a 50 mL conical polypropylene tube (Corning Inc., Corning, NY), and 30 mL of ultrapure water was added to the conical tube. The conical tube was heated in a microwave for 40–60 sec at 600W until just before boiling, and then vortexed at 1,500 rpm for 10 min. After vortexing, the tube was centrifuged at 3,000 ×g for 15 min. Then, the supernatant was filtered through a paper filter (Advantec No.5A, Advantec Toyo, Tokyo). The sugar remaining on the pellet was extracted twice with ultrapure water, and the water was filtered through the paper filter. The sample solution obtained was diluted to 40 mL, and 0.4 mL of the sample solution was mixed with 0.1 mL of 1,000 mg/L Mannose as an internal standard and 0.5 mL of acetonitrile and placed at 4˚C overnight. The sample solution was filtered using a 96-well filter plate (AcroPrep™ 96 Filter Plates, 350 µL well, Product number 5045, Pall Corp., Port Washington, NY) by centrifugation at 1,000 ×g for 10 min. The HPLC conditions are shown below.

Mobile phase A	2mM NaOH, 2mM NaCl solution/Acetonitrile = 10/90
Mobile phase B	Ultrapure water/Acetonitrile = 90/10
Derivatization reagent	20 mM Arginine, 380mM Boric acid, 152 mM KOH (pH9.1)
Column	HILICpak VG-50 4E, 4.6×250mm, 5µm
Oven temperature	40 ˚C
Derivatization temperature	150 ˚C
Detector	Fluorescence Detector (Excitation: 320 nm, Emission: 430nm) (Waters, 474 Fluorescence Detector)
Injection volume	5 µL
Flow rate	1.0 mL/min (mobile phase), 2.0 mL/min (derivatization reagent)

Time (min)	Concentration of mobile phase B (%)
0.01	0
15.00	0
40.00	25
55.00	75
60.00	75

60.01	0
70.00	0

2. Analysis of organic acid.

We extracted organic acid compound from the seed mash by the following method. First, 2 grams of sample was weighed out into a 50 mL conical polypropylene tube, and 8 mL of ultrapure water was added to the conical tube. The conical tube was vortexed at 1,500 rpm for 15 min, and then the tube was centrifuged at 5,000 ×g for 5 min. Then, the supernatant was filtered through a paper filter (Advantec No.5A). The organic acid remaining on the pellet was extracted twice with ultrapure water, and the water was filtered through the paper filter. The sample solution obtained was diluted to 10 mL, and 0.9 mL of the sample solution was mixed with 0.1 mL of 5,000 mg/L isovaleric acid as an internal standard. The sample solution was filtered using a 96-well filter plate (AcroPrep™ 96 Filter Plates, 350 µL well, Product number 5045) by centrifugation at 1,000 ×g for 10 min. The HPLC conditions are shown below.

Mobile phase	5mM p-Toluenesulfonic Acid (Isocratic elution)
Buffer solution	5mM p-Toluenesulfonic Acid, 100µM Ethylenediaminetetraacetic acid, 20mM Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane
Column	SCR-102H × 1, Shim-Pack SCR-102H 4.6×250mm, 5µm × 2
Oven temperature	40 ˚C
Detector	Conductivity Detector (Shimadzu Corp, Kyoto, Japan, CDD-10AVP)
Injection volume	10 µL
Flow rate	0.8 mL/min (mobile phase), 0.8 mL/min (buffer solution)

3. Analysis of nitric acid and nitrous acid.

We extracted nitric acid and nitrous acid from the seed mash by the following method. First, 2.5 grams of sample was weighed out into a 50 mL conical polypropylene tube, and 20 mL of water heated at approx. 80 ˚C was added to the conical tube. The conical tube was vortexed at 1,500 rpm for 30 sec, and then 5.0 mL of 0.5M NaOH and 5.0 mL of 9% Zinc acetate were added to the tube. The tube was shaken at 80 ˚C for 15 min, and then the sample was placed on water for cooling. The sample was centrifuged at 3,000 ×g for 15 min, and

the supernatant was filtered through a paper filter (Advantec No.5A). The nitric acid and nitrous acid remaining on the pellet were extracted twice with ultrapure water, and the water was filtered through the paper filter. The sample solution obtained was diluted to 50 mL with ultrapure water. The sample solution was filtered using a 96-well filter plate (AcroPrep™ 96 Filter Plates, 350 µL well, Product number 5045) by centrifugation at 1,000 ×g for 10 min and analyzed by HPLC under the following conditions.

Mobile phase	5mM Sodium Dihydrogenphosphate, 5mM Disodium Hydrogenphosphate 100mM Sodium Perchlorate (Isocratic elution)
Column	Asahipak NH2P-50 4E, 4.6×250mm, 5µm
Oven temperature	30 ˚C
Detector	UV-VIS (Shimadzu Corp., SPD-10AVP)
Injection volume	20 µL
Flow rate	0.8 mL/min

4. Analysis of amino acid.

We extracted amino acid from the seed mash by the following method. First, 2 grams of sample was weighed out into a 50 mL conical polypropylene tube, and 18 mL of 10% 5-Sulfosalicylic Acid solution was added to the conical tube. The conical tube was vortexed at 1,500 rpm for 5 min, and then 11.1 mL of 1M NaOH was added to the tube. The tube was centrifuged at 3,000 ×g for 15 min, and the supernatant was filtered through a paper filter (Advantec No.5A). The amino acid remaining on the pellet was extracted twice by 100 mM citric acid solution (pH 2.2), and the citric acid buffer solution was filtered through the paper filter. The sample solution obtained was diluted to 40 mL with citric acid solution. The sample solution was filtered using a 96-well filter plate (AcroPrep™ 96 Filter Plates, 350 µL well, Product number 5045) by centrifugation at 1,000 ×g for 10 min and derivatized using the AccQ-Tag Ultra Derivatization Kit (Waters Corp., Milford, MA) according to the manufacturer's instructions after the addition of norvaline as an internal standard (final concentration 50 µM). The UHPLC conditions are shown below.

Mobile phase A	AccQ Tag Ultra Eluent A
Mobile phase B	AccQ Tag Ultra Eluent B
Column	AccQ-Tag Ultra, 2.1×100 mm, 1.7 µm

Oven temperature	60 ˚C
Detector	Mass spectrometer (Waters, Quattro Premier XE)
Injection volume	5 μL
Flow rate	0.7 mL/min

Time (min)	Concentration of mobile phase B (%)
Initial	0.1
0.54	0.1
5.74	9.1
7.74	21.2
8.04	59.6
8.05	90.0
8.64	90.0
8.73	0.1
10.0	0.1

5. Analysis of amine.

We extracted amine from the seed mash by the following method. First, 2.5 grams of sample was weighed out into a 50 mL conical polypropylene tube, and 5 mL of 20% trichloroacetic acid solution and 20 mL of ultrapure water were added to the conical tube. The conical tube was vortexed at 1,500 rpm for 5 min, and then the tube was centrifuged at 3,000 ×g for 15 min. The supernatant was filtered through a paper filter (Advantec No.5A). The amine remaining on the pellet was extracted twice by 4% trichloroacetic acid solution, and the solution was filtered through the paper filter. The sample solution obtained was diluted to 50 mL with 4% trichloroacetic acid solution. The sample solution was filtered using a 96-well filter plate (AcroPrep™ 96 Filter Plates, 350 μL well, Product number 5045) by centrifugation at 1,000 ×g for 10 min, and 9 μL of the sample was derivatized using the AccQ-Tag Ultra Derivatization Kit (Waters Corp.) according to the manufacturer's instructions after the addition of heptylamine as an internal standard (final concentration 40 μM). The UHPLC conditions are shown below.

Mobile phase A	AccQ Tag Ultra Eluent A
Mobile phase B	AccQ Tag Ultra Eluent B

Column	UPLC BEH C18, 2.1×100 mm, 1.7 μm
Oven temperature	55 ˚C
Detector	Mass spectrometer (Waters, Quattro Premier XE)
Injection volume	2 μL
Flow rate	0.5 mL/min

Time (min)	Concentration of mobile phase B (%)
Initial	1.0
0.54	1.0
2.54	5.0
3.54	15.0
4.54	30.0
7.05	90.0
7.65	90.0
7.75	1.0
8.70	1.0

6. Effect of lactic acid on the growth of *Lactobacillus sakei* during seed mash preparation.

We prepared seed mash by using 10 g of sterilized pregelatinized rice and 16.6 mL of autoclaved pH 6.4-adjusted *koji* medium in order to remove the influence of degradation of the rice by *koji* enzymes on the nutrition of the seed mash. The ingredients were mixed in a 50 mL conical polypropylene tube. We inoculated *L. sakei* strain MGMA2-1 into the seed mash. A volume of 266 μL of 10% lactic acid solution was added to the seed mash at 0 hr, 24 hr, 48 hr, or 120 hr after inoculation (no addition as control). We determined the colony-forming units (cfu) of *L. sakei* by the spiral plating method.

7. Co-culture condition of *L. sakei* and *Leuconostoc mesenteroides* during seed mash preparation.

We prepared seed mash by using 10 g of sterilized pregelatinized rice and 16.6 mL of autoclaved pH 6.4-adjusted *koji* medium in order to remove the influence of degradation of the rice by *koji* enzymes on the nutrition of the seed mash. The ingredients were mixed in a 50 mL conical polypropylene tube. *L.*

mesenteroides NBRC100496 was purchased from National Institute of Technology and Evaluation, Japan. *L. mesenteroides* NBRC100496 was inoculated at the start time of the culture, and *L. sakei* MGMA2-1 was inoculated into the seed mash simultaneously with the *L. mesenteroides* inoculation, or one, two, three, or six days after the *L. mesenteroides* inoculation. The seed mash was placed at 10°C in an incubator for 17 days and sampled on day 1, day 2, day 3, day 6, day 8, day 10, day 13, day 15, and day 17 (day 1 refers to the day the culture was started). Genomic DNA of *L. sakei* or *L. mesenteroides* in the sample collected was extracted from the samples in the same way used in next-generation sequencing analysis and determined by qPCR analysis using a primer set as follows: For the quantification of *L. sakei*, CTCCAAGTCATTAAAGTTTACTCAC (forward primer), CATCCGTTGAACCTAAACCTTCTTG (reverse primer); for the quantification of *L. mesenteroides*, GAAGTACGTGATACATCATTACGTG (forward primer), CACGAACCATCATGTTGACACCTGG (reverse primer). The PCR was carried out in a total volume of 10 µL using a KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Wilmington, MA) containing 0.2 µM of each primer. The thermal cycler program of the PCR was 95C° for 3 min, 45 cycles at 95°C for 10 sec, 61°C (*L. sakei*) or 65°C (*L. mesenteroides*) for 30 sec, and 72°C for 30 sec using the LightCycler® 96 System (Roche Diagnostics, Switzerland).

References

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