A dark matter in *sake* brewing: Origin of microbes producing a *Kimoto*-style fermentation starter

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Abstract

In *Kimoto*-style fermentation, a fermentation starter is produced before the primary brewing process to stabilize fermentation. Nitrate-reducing bacteria, mainly derived from brewing water, produce nitrite, and the molecule proliferates lactic acid bacteria such as *Leuconostoc* spp. due to its characteristics of tolerance against low temperatures and fewer nutritional requirements. Later, *Lactobacillus* spp. is switched to the dominant genus, creating a weakly acidic condition to control wild yeasts and unexpected bacterial contamination. However, the sources of these microorganisms that play a pivotal role in *Sake* brewing have not yet been revealed. Thus, comprehensive elucidation of the microbiome is necessary. In this study, we performed 16S rRNA amplicon sequencing analysis after sampling from architectural surfaces, tools, and raw materials for making fermentation starters, including *koji*, and water in *Tsuchida Sake* brewery, Gunma, Japan. Amplicon sequence variants (ASVs) between the external environments and the fermentation starter were compared, and it was verified that the microorganisms in the external environments, such as built environments, tools, and raw materials in the sake brewery, were introduced into the fermentation starter. Furthermore, various adventitious microbes confirmed in the fermentation starter of early days and from the external environments were detected in a nonnegligible proportion in the starter, which may impact the sake' taste and flavor. These findings illuminate the uncharacterized microbial dark matter of sake brewing, the sources of microbes in *Kimoto*-style fermentation.

1. Introduction

Description of microbial transition of a fermentation starter of *Kimoto*-style *Sake*

Fermentation is an ancient biotechnology that has been used to improve the safety, storage capacity, flavor, and nutrition of food. The most modern fermented foods are manufactured by adding fermentation starters for their stability and reproducibility. In contrast, the fermentation process has traditionally been carried out by utilizing microorganisms from raw materials, starter cultures, and the environment around the fermentation batches. Among fermented foods, *Sake* is the traditional alcoholic beverage of Japan. *Sake* is made from rice through starch saccharification by *Aspergillus oryzae* and alcoholic fermentation by *Saccharomyces cerevisiae*. The beverage is traditionally brewed using a fermentation starter called *Moto*.

In the preliminary stage of the primary brewing process, *Moto* with purely cultured yeast is manufactured to prevent microbial contamination and promote smooth alcoholic fermentation. Based on the manufacturing methods of *Moto*, *Sake* is divided into three types, *Sokujo*, *Kimoto*, and *Yamahai*. *Sokujo*-style *Moto* is a modern method to add food-grade quality lactic acid and decrease pH before alcoholic fermentation by yeast. Lactic acid inhibits contaminations of unintended yeasts and bacteria from external environments into the fermentation starter. Since *Sokujo* relies on lactic acid to control the pH of *Moto*, a desirable condition can be created relatively quickly, leading to stable production of the starter. Meanwhile, the *Kimoto*-style *Moto* is a traditional preparation method for the starter culture and is manufactured by inducing the growth of lactic acid bacteria properly. Lactic acid bacteria produce lactic acid, which contributes to creating an environment conducive to the growth of yeast. The *Yamahai* style is similar to the *Kimoto* style but made without grinding rice. *Koji*, mold (*Aspergillus oryzae*) on rice, degrades solid rice and initiates alcoholic fermentation by yeast. Repeatedly, in both methods, lactic acid bacteria, which are indispensable in the production of *Sake* and *Moto*, grow without being added to the culture. In other words, these lactic acid bacteria are taken in from the external environment.

Impacts of *Kuratsuki* bacteria and adventitious microbes from external environments

The microorganisms that inhibit surrounding the fermentation reactor, or more precisely, in the breweries, are described as *Kuratsuki* in Japanese. Massive efforts have been made to investigate *Kuratsuki* microbes. One of the significant findings is the study isolating the genus *Kocuria* from sake in multiple batches from the same *Sake* manufacturer in Japan (1). The study also found that *Kocuria* was a unique genus among the 33 breweries and concluded that the isolated *Kocuria* were *Kuratsuki* microbes. In addition, several *Kuratsuki* microbes have also been isolated, including *Staphylococcus* sp. and *Bacillus* sp., as well as lactic acid bacteria considered necessary for *Sake* production (1–3). As an alternative method to identify *Kuratsuki* microbes, a study using amplicon sequencing was reported in 2014 (4). A comparison between *Kimoto*-style *Moto* and microbes on the surfaces of the brewery was conducted by 16S rRNA and ITS amplicon sequencing. They elucidated that microbes on the surfaces of fermentation equipment were commonly detected in *Kimoto*-style fermentation.

Tracking the microbiomes from the external environment of *Moto* and its changes during the fermentation period

To characterize the source of *Kuratsuki* microbes more deeply, we conducted 16S rRNA amplicon sequencing at *Tsuchida Sake* Brewery in Gunma Prefecture, Japan. At *Tsuchida Sake* Brewery, they produce yeast-free and bacteria-free *Moto*, which means external microbiomes initiate the preparation of *Moto*. We reported their unique microbial transition in the fermentation starter in 2021 (5). Utilizing the previous data, we compared microbial communities between the built environment and the starter culture by counting shared amplicon sequencing variants (ASVs), showing identical sequences in the V3-V4 region of 16S rRNA. Our results suggest that adventitious microbes from the built environment create the initial microbial status, and *Kuratsuki* microbes giving successive fermentation, are screened during competitions in the fermentation.

2. Materials and methods

Sample collection

Samples were collected from 10 locations, including floor, raw materials, and tools in the brewery, by a swabbing method (Figure 1). All samples were collected at *Tsuchida Sake* Brewing Company (Gunma, Japan) between October and November 2021. Sterile cotton-tipped swabs (Isohelix Swab: SK-3, Cell Projects Ltd, Harrietsham, UK) were moistened with lysis buffers (BuccalFix Stabilization and Lysis Buffers: BFX, Cell Projects Ltd, Harrietsham, UK) and streaked across an area of the target surface for 3 minutes. Samples were immediately frozen and stored until DNA extraction. All samples were collected in duplicate except for water. Samples shown as *Moto* are reported previously and utilized for comparison with the environmental samples in this study (5). The tools were utilized before the fermentation period of the fermentation starter, and only the tanks were in contact with the fermentation starter during fermentation.

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 a GenFind v2 DNA extraction kit (Beckman Coulter, Indianapolis, U.S.) following the manufacturer's protocol. DNA was eluted with 80 µl of water. Primers 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACACCTACGGGNGGCWGCAG-3') and 806R (5'-GTCTCGTGGGCTCGGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') were utilized to amplify the V3-V4 region of the 16S rRNA gene, and adapter sequences were added by KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland) Thermal conditions for PCR were 95 °C for 3 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. Secondary PCR was performed with forwards and reverse primer sequences designated by Illumina. The thermal condition was set to be the same as the first PCR but for 8 cycles. After AMpure XP purification (Beckman Coulter, Indianapolis, U.S.), the libraries were sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, USA) by paired-end sequencing of 300 bp. Preparation of DNA samples and libraries and amplicon sequencing were performed at GenomeRead Inc. in Kagawa, Japan. We deposited the raw fastq files as PRJDB14823 on BioProject.

Microbiome analysis

Microbiome analysis was performed as previously reported (5). Briefly, raw FASTQ files were imported into the QIIME2 platform as qza files. Denoising and quality control of reads was performed by qiime dada2 denoise-paired, and reads were classified into amplicon sequencing variants (ASVs) (6). The SILVA database SSU 138 was utilized with qiime feature-classifier classify-sklearn for taxonomic assignment (7,8). ASVs classified as chloroplast, mitochondria, and unassigned were excluded from statistical analysis. Although the genus *Latilactobacillus* is a taxon that was recently derived from the genus *Lactobacillus* (9), the SILVA database SSU 138 did not include

this reclassification of the genus. Thus, the conventional taxonomic name *Lactobacillus* was adopted in this study. One sample from the cold room floor did not have enough sequencing depth and was excluded from the downstream analysis. The depth of sequence reads differed among samples, and we subsampled from each sample to 4,724 reads each to normalize the data. Subsampling is an approach for inferring microbiome differences between samples and has been reported to be a suitable analytical method when analyzing new datasets (10). To evaluate the effect of sequence read counts on microbiome diversity assessment, we examined changes in the value of the number of ASVs over a range of reading counts from 0-8,000 by rarefaction curves. The rarefaction curves of the number of ASVs were leveled off when the number of reads reached approximately 4,000 (Supplementary Figure 1).

Calculation of shared ASVs

We obtained sequence data from BioProject PRJDB13924 reported by the previous study (5). Shared ASVs in this study were defined as ASVs that were shared by more than 1% in both environmental and *Moto* samples. The calculation was conducted by R version 4.2.1 and Phyloseq (11) version 1.40.0.

Statistical analysis

Kruskal–Wallis tests were used to compare the relative abundances among sample types. Mann–Whitney U tests were used to compare all combinations of elements within the sample types. All multiple testing corrections were performed by computing FDRs using the Benjamini-Hochberg method, and adjusted P values < 0.05 were considered statistically significant. Differentially abundant genera between groups were identified using linear discriminant analysis (LDA) effect size (LEfSe) analysis (12). Only bacterial genera reaching the LDA score threshold of 3.0 and with a p value lower than 0.01 are shown. Data visualization was performed by R version 4.2.1, the ggplot2 package and ggprism (13).

3. Results

The phylum-level bacterial profiles in the *Sake* brewery

The relative abundances of the top 7 phyla are shown as a heatmap (Figure 2A). The bacterial profiles of architecture (i.e., floors) showed relatively high abundances of *Bacteroidota*, *Proteobacteria*, *Firmicutes,* and *Actinobacteria*. Tools (wood board, wood stand, cloth, tank) showed similar trends as architecture, but cloth and tank showed less abundance in *Bacteroidota* and more abundance in *Deinococcota*. Raw materials (*Koji* and water) are rich with *Firmicutes* and *Proteobacteria*. The relative abundances of the four phyla were significantly different among the three sample types (Figure 2B-E). *Firmicutes* had the highest median of over 30% for raw materials, and Mann–Whitney U tests also showed a significant difference in raw materials against architecture (p-value = 0.05) and tools (p-value = 0.016) (Figure 2B). Otherwise, architecture and tools showed higher relative abundances in *Bacteroidota*, *Deinococcota*, and *Actinobacteria* (Figure 2C-E).

Distributional patterns of bacterial genera associated with *Kimoto*-style fermentation

The top 15 genera are shown in a stacked bar graph (Figure 3); the rest are noted as the *remainder*. *Sphingomonas* was widely detected with a high abundance ratio within samples grouped in architecture (4.2~33.5%). 28.3% and 27.5% *Halomonas* were detected from the *Sandan-jikomi* (three-step preparation for *Moromi,* fermentation mash) room floor. *Koji* had high abundances in *Anaerobacillus* (30% and 8.7%, respectively) and *Staphylococcus* (20.1% and 4.7%, respectively). The heatmaps sorted by *Firmicutes*, *Bacteroidota*, *Deinococcota*, and *Actinobacteria* are shown in Figure 3B-E to identify the unique genera within each sample. The relative abundance of the genus *Lactobacillus*, considered to be necessary for brewing, was found in more than 1% of only one sample of the cold room floor (3.6%) and the wood board (2.8%) (Supplementary Figure 2B). Both surface types *Leuconostoc*, the dominant species of *Moto* in the brewery, was found in the tank, *Koji* making room floor, *Dekoji* room floor, and cold room floor (Supplementary Figure 2B). *Bacillus* was widely detected in water (8.7% and 1.1%, respectively), wood board (8.3% and 0%, respectively), and cloth (8.8%, and 1.7%, respectively) (Supplementary Figure 2B). LFfSe analysis identified *Anaerobacillus*, *Methylobacterium*, *Escherichia*, and *Delftia* as differentially abundant in raw materials compared with other sample types (Supplementary Figure 3).

Changes in the relative abundances of microbes from the external environment during *Moto* fermentation

Principal coordinates analysis (PCoA) based on unweighted UniFrac distances was performed (Figure 4A). The dots were divided into three major groups according to their sample types. Raw materials and tools are located between *Moto* and the group of architecture. Architectural samples differ substantially from *Moto*.

The results of the shared ASV analysis are shown as a heatmap (Figure 4B). Bacteria confirmed on the surface of all raw materials and tools showed shared ASVs with *Moto* samples throughout Day 1 to Day 9. The *Koji* making room also showed them from Day 1 to Day 5. The ratio of shared ASVs increased on Day 3 and Day 5. The tank and cloth reached 83% and 81%, respectively, on Day 9. Day 13 showed a decrease in the ratio of the tank (26%) and cloth (23%) and an increase in the cold room (6%) and

Dekoji room (6%). From Day 17 to Day 33, only the tank, *Dekoji* room floor, and cold room floor showed shared ASVs. The tank, cold room, and *Dekoji* room showed more than 40% in the later days. Further details of ASV-assigned genera shared by Moto on Day 33 are shown in Table 2. ASVs assigned to *Pseudomonas* in *Moto* were shared between the tank and cloth, and *Serratia* and *Hafnia-Obesumbacterium* were shared by cloth. Tank, cold room, and *Dekoji* room showed *Leuconostoc* sharing with *Moto*. The shared ASV of *Leuconostoc* consisted of a single ASV unit.

4. Discussion

Bacterial profiles differed by sample types

We found different phyla profiles between the raw materials and environmental samples (architecture and tools). Architectural samples (i.e., floors) showed microbial diversity compared with the rest of the two sample types (Figure 2A, Figure 4A). Previous studies indicate that floors in indoor environments are known to be rich in microbes (14,15). For this reason, we included floor samples rather than walls and ceilings. Built environments are also complex structures, and microbial communities differ by conditions such as surface materials and the possibility of human interaction. In fermentation starter production, the manufacturer inputs multiple ingredients and uses tools. Also, *Moto* is produced in multiple rooms based on the steps of production. These direct interactions with sources of microbes can make microbiomes in fermentation starters of early days exceedingly divergent. *Firmicutes* are detected in high abundance on dry (e.g., floors, cabinets, microwaves) and cold (e.g., inside of the refrigerators) surfaces in the kitchen (16) and refrigerator surfaces (17), but in this study, they were most abundant in raw materials (Figure 2B).

Genus-level composition (Figure 3A) shows that *Koji* samples have *Bacillus*, *Anaerobacillus*, *Staphylococcus,* and *Sphingomonas*. These genera were confirmed in *Sokujo*-style *Moto* by Sanger sequencing of their 16S rRNA sequences in the previous report (18). In this study, these genera were especially detected in the architectural samples. As other features, *Koji* is made by bare hands; therefore, it is considered that *Staphylococcus*, a well-known skin microbiota (19), may have been contaminated in the *Koji* and the floors.

We reported that *Leuconostoc* was dominant in the fermentation of *Moto*, and the switch of the predominant lactic acid bacteria from *Leuconostoc* to *Lactobacillus* was not observed in the fermentation process of this brewery (5). Several studies reported that *Lactobacillus* was detected in tanks (4,20)that can be directly in contact with raw materials and *Moto*. However, *Lactobacillus* was detected only on the cold room floor and the wood board. In contrast, *Moto* did not contact these sites. However, *Leuconostoc* was detected in the tank of *Moto*. This result suggests that appreciable concentrations of *Leuconostoc* are introduced from the tank to the fermentation reaction of *Moto*. This consideration is consistent with findings in a previous report (4) that surface contact is the most significant predictor of bacterial composition in the *Sake* brewery. We also concluded that the sources of *Kuratsuki* microbes are possibly surfaces of tools and raw materials that have high chances of direct contact with *Moto*. Therefore, the *Moto* in *Tsuchida Sake* Brewery may not have shown the lactic acid bacteria switch from *Leuconostoc* to *Lactobacillus* in the fermentation process due to the lack of the sources of *Lactobacillus*.

Shared ASV analysis revealed the interaction between environmental samples and *Moto*

In the PCoA of unweighted UniFrac distances (Figure 4A), raw materials are located near the fermentation starter because they are an input of Moto. Next are the tools that are in direct contact with *Moto*. The floor is not in direct contact with the fermentation starter and thus differs substantially from the group structure of *Moto*. In this study, the floor was employed as the architecture group. This is based on our expectation that microbes may fall on the floor during manufacture. However, experimental data indicate

that surfaces that can be in direct contact may have a more significant impact on *Moto*. The bacterial profile of brewing tools has yet to be fully revealed, and further research is needed.

In this study, we investigated how bacteria are transferred to *Moto* as a source. Bacteria on the tools and architectures were confirmed in the early stage of *Moto* (Figure 4B). Thus, the shared ASVs results indicate that *Leuconostoc*, which plays an essential role in *Moto*, was screened within adventitious microbes and eventually became the dominant species.

Technical limitations in 16 rRNA amplicon sequencing

Because of the limited classification resolution of 16S rRNA amplicon sequencing, it is challenging to rigorously predict microbial sharing between fermentation starters and the environment. Calculating shared ASVs or statistical methods is a convenient way to track microbial sources. However, high-resolution sequencing, such as metagenome sequencing, must be performed to clarify precisely which places or sources in bacteria affect fermentation. Lineages of bacteria between two groups can be compared by metagenome-assembled genomes (MAGs) and show more precision in tracking at the level of whole chromosomal sequences, not just the restricted regions of 16S rRNA.

We reported that the ethanol concentration increased as fermentation progressed (5). According to ethanol production from yeast, bacteria should decrease gradually. However, 16S rRNA amplicon sequencing is a DNA-based detection method, and it is impossible to discern between viable and nonviable bacteria. In fermented foods, viable bacteria can be considered to have a significant influence, and conventional microbiology experiments or flow cytometry with staining is required to verify the viable cells. In addition, fungi also play a pivotal role in *Sake* fermentation. Therefore, metagenome sequencing is necessary to unveil the interaction between bacteria and fungi, especially for fermentation utilizing *Koji*, *Aspergillus oryzae*, and *Saccharomyces cerevisiae*.

Also, microbes in the built environment are not as abundant as in fermented food. *Kuratsuki* microbes inhibit in breweries in a heterogeneous manner. In our samples, we confirmed the variety of relative abundances in some genera between duplicated experiments (Figure 3). This can explain by biases in library preparation, including PCR or sampling bias in each experiment. Multiple sampling points in the exact locations can give higher resolutions in rare ASVs, and shared ASVs analysis can show precision results.

Tracking *Kuratsuki* microbiota as a community structure and their impact on sake brewing

Tracing the dynamic network of microbial communities further contributes to understanding this traditional liquor. According to the previous study, microbial diversity decreases with the number of fermentation days of *Moto* (4). Another study performing microbiome analysis of *Moto* from five different *Sake* breweries reported that the bacterial communities of *Moto* were very diverse, but that diversity decreased over time through multiple microbial transition patterns (3). It was also reported that ethanol produced by yeast inhibits the growth of adventitious microbes (1). Metabolomic dynamics can also affect the velocity of dynamics in microbiomes and should be considered for the experiments' designs.

Furthermore, some adventitious microbes have been found to possess activity

interfering with the growth of bacteria that spoil *Sake*, potentially benefiting *Sake* brewing (21) or producing beneficial chemical components that contribute to the flavor, taste, and quality of *Sake* (22).

Mapping the microbiomes in *Sake* breweries may also be important in terms of quality and safety in *Sake* production (23). In the past, studies have yet to comprehensively examine changes in microbes sharing between environmental samples (architecture and tools) and *Moto* in a brewery during the fermentation process with a lens of amplicon sequencing. Previous studies have isolated *Kocuria* and *Bacillus* as viable microbes (2,24), indicating that certain microbes can live in *Moto*, such as lactic acid bacteria and yeast, and may be involved in *Sake* brewing. The possibility of horizontal gene transfer among microorganisms in *Sake* breweries (24) has also been demonstrated; instead of targeting only specific bacteria, such as lactic acid bacteria, the community-level structure should be targeted as *Kuratsuki* microbiota. Isolation of viable microbes and whole genome sequencing can give a deep insight into gene transfer.

Finally, understanding microbial diversity and dynamics are about preserving traditional food culture and food production safety. Sake requires attention to spoilage, such as microbial contamination and spoilage, which can cause spoilage and may be helpful for safety and quality control. This research can be an essential milestone in unraveling the mysteries of fermentation phenomena. Because each brewery produces different flavors of sake with different ingredients and tools, it is necessary to further research to profile microbiomes in sake breweries. Additionally, these contributions can lead to process control, spoilage prevention, food safety, and the protection of traditional cultures.

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repositories/repositories and accession numbers (s) can be found in the article.

Author Contributions

K.I. and R.N. conceived this study. K.I. and K.K. designed the experiments. K.I. and R.N. wrote the original draft. R.N. performed microbiome analysis. K.I. and R.N. performed the statistical analysis. Y.T., G.H and K.K. collected samples and provided the records required for discussion. T.N. supervised the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare no competing interests.

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Samples were collected with the help of members of the *Tsuchida Sake* Brewery. GenomeRead Inc. performed amplicon sequencing. All authors thank Morgenrot Inc. for providing the computational environment for the analysis. R.N. is a graduate student of the Medical Innovation Program at Kyoto University and supported by the JST SPRING program, Grant Number JPMJSP2110.

Figure and Tables

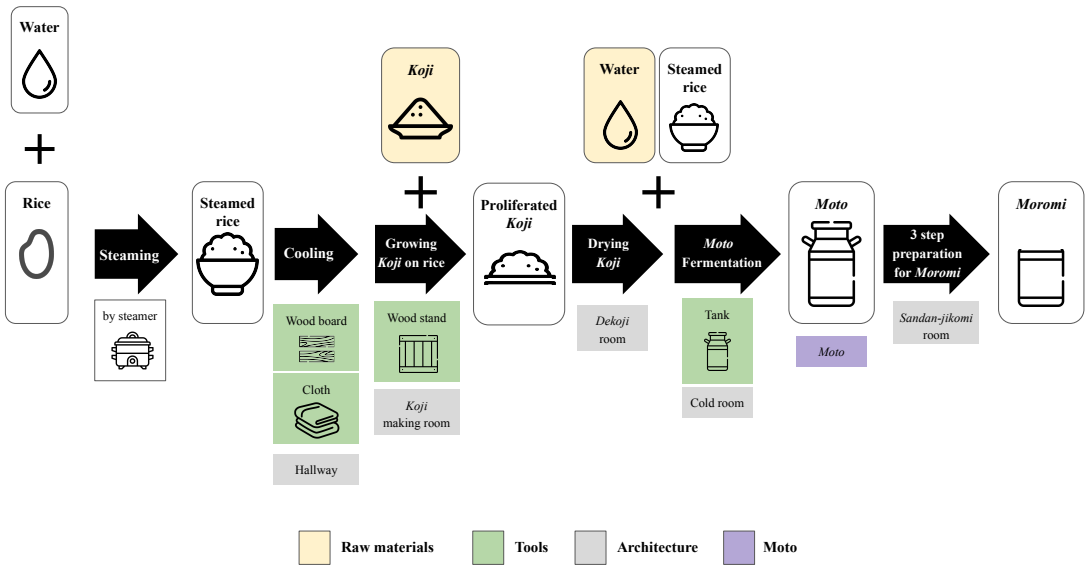

Figure 1. An overview of multiple processes for *Sake* brewing with sampling targets in the study. The group of *Architecture* includes the brewery's floor samples. The group of *Raw materials* includes water and *Koji*. The group of *Tools* includes the wood board, wood stand, cloth, and tank for *Moto* Fermentation.

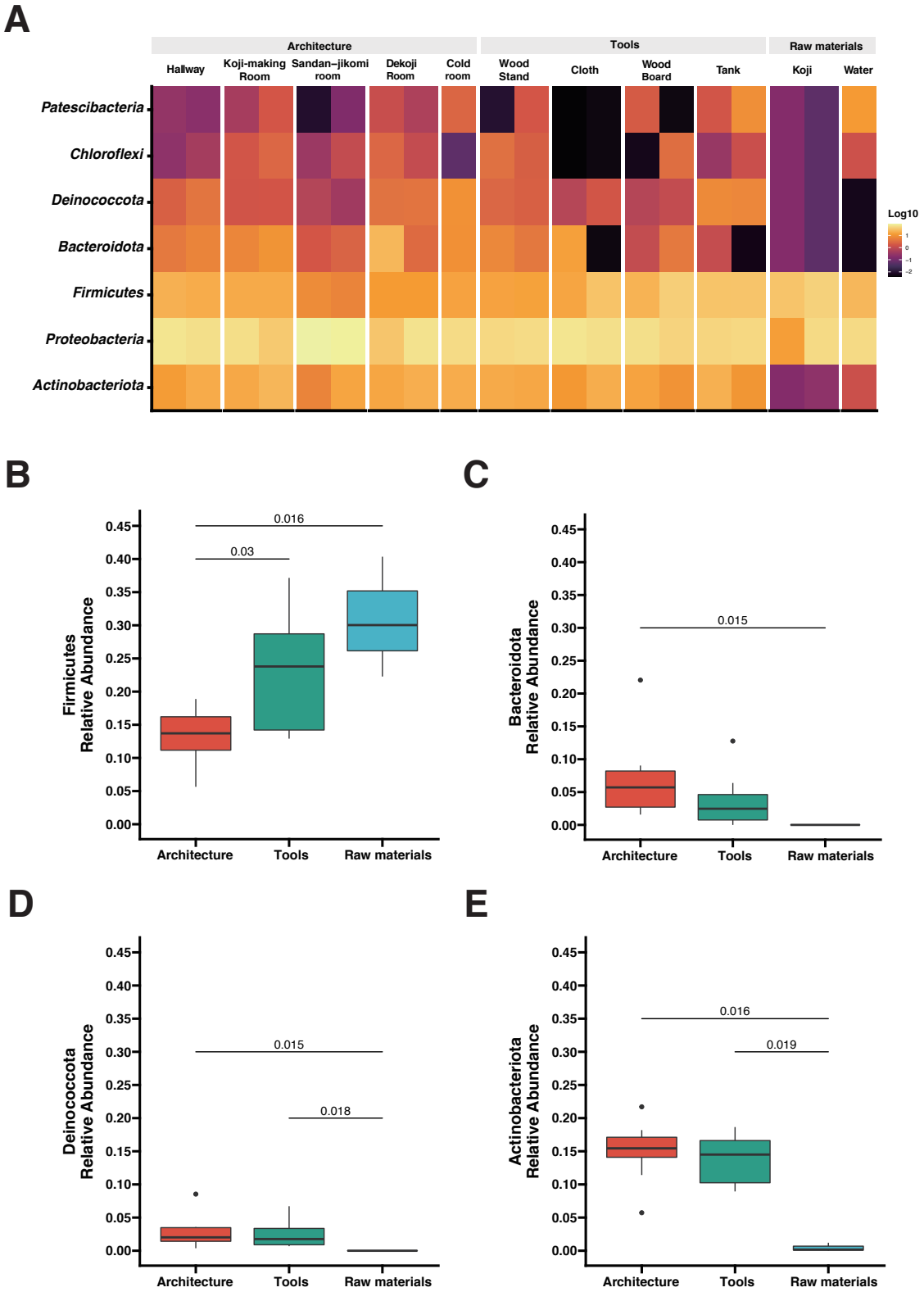

Figure 2. (A) Taxon abundance heatmap at the phylum level. Heatmap depicts the relative abundances (log10 scale) of the top 7 phyla across all surfaces. (B-E) Box plot representation for phylum level comparison among each group. Four phyla (*Firmicutes*, *Bacteroidota*, *Deinococcota*, *Actinobacteriota*) were compared across groups in the

380 brewery. Statistical analysis was computed using Mann-*U* tests.
381

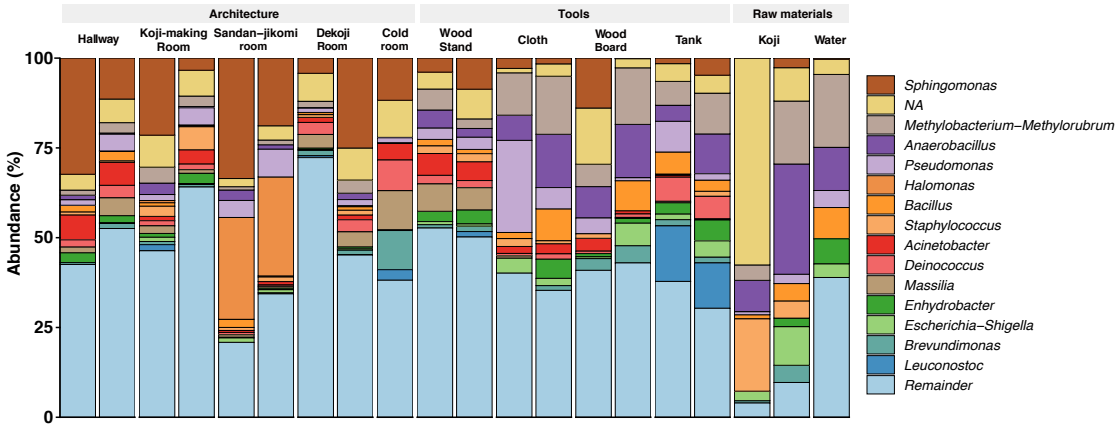

Figure 3. Genus level taxonomic composition across the environmental samples.

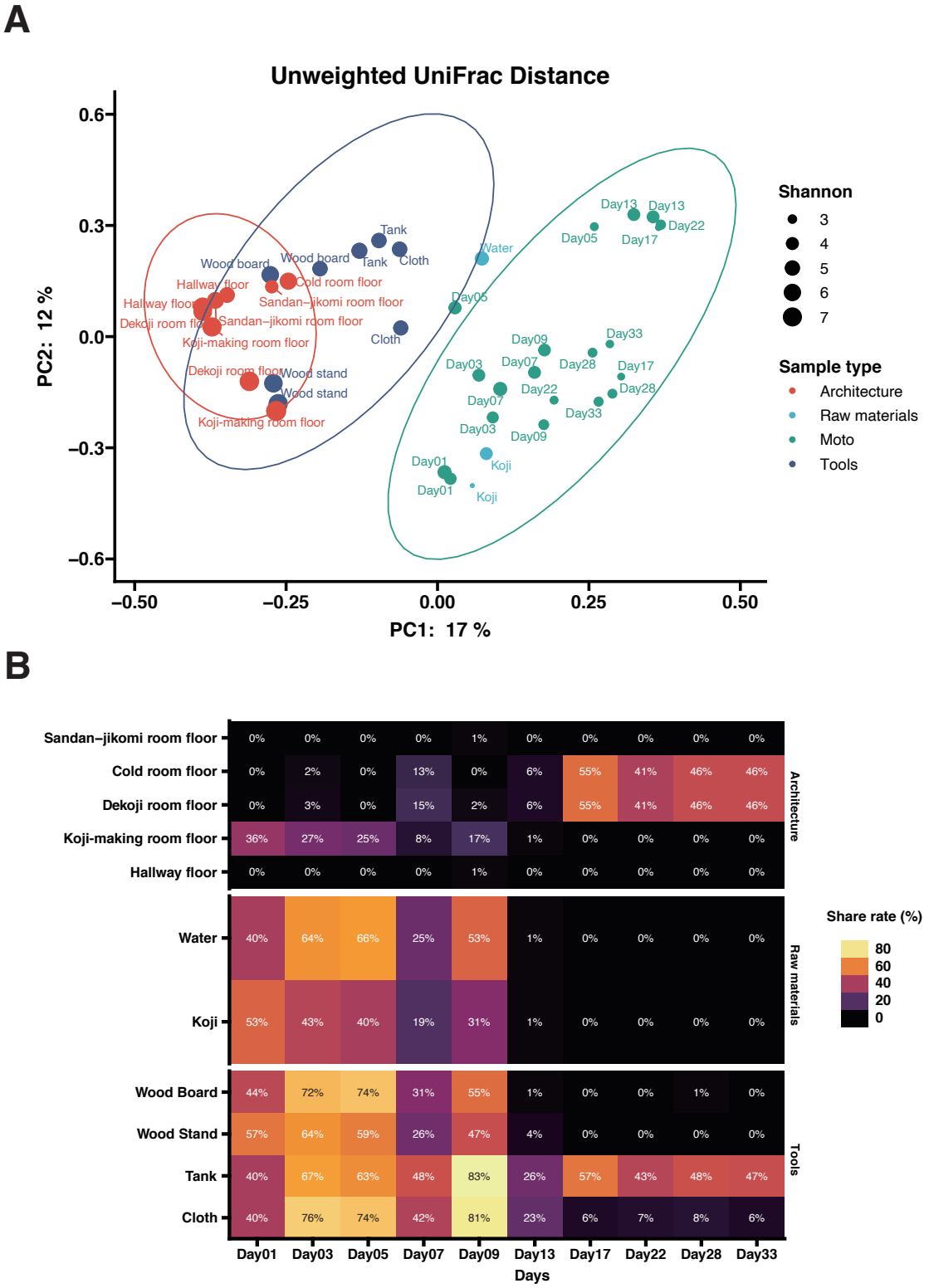

Figure 4. (A) Principal-coordinate analysis (PCoA) by Weighted UniFrac distance of environmental samples and *Moto* samples. The dot size shows the values of Shannon diversity of each sample. (B) Time-series changes in the relative abundance of shared ASVs in *Moto* during the *Kimoto*-style fermentation period. More than 40% of the

microbiomes in *Moto* on Day 33 shared the ASVs with the tank surface, cold room floor, and *Koji*-making room floor samples.

Table 1. The taxon of shared ASV (Genus-level) between *Moto* on Day33 and other materials

Taxon	Confidence	Shared surface types
Pseudomonas	0.999	Tank, Cloth
Leuconostoc	0.999	Tank, *Koji* room floor, Cold room floor
Serratia	0.904	Cloth
Hafnia-Obesumbacterium	0.858	Cloth

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