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Indigenous bacteria and fungi drive traditional *kimoto* sake fermentations

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ABSTRACT

Sake (Japanese rice wine) production is a complex, multistage process in which fermentation is performed by a succession of mixed fungi and bacteria. This study employed high-throughput marker-gene sequencing, quantitative PCR, and terminal restriction fragment length polymorphism to characterize the bacterial and fungal communities of spontaneous sake production from koji to product as well as brewery equipment surfaces. Results demonstrate a dynamic microbial succession, with koji and early moto fermentations dominated by *Bacillus*, *Staphylococcus*, and *Aspergillus flavus* subsp. *oryzae*, succeeded by *Lactobacillus* spp. and *Saccharomyces cerevisiae* later in the fermentations. The microbiota driving these fermentations were also prevalent in the production environment, illustrating the reservoirs and routes for microbial contact in this traditional food fermentation. Interrogating the microbial consortia of production environments in parallel with food products is a valuable approach for understanding the complete ecology of food-production systems and can be applied to any food system, leading to enlightened perspectives for process control and food safety.

Humans have employed food fermentation since time immemorial to improve the safety, stability, flavor, nutrition, and value of their agricultural products. Traditionally, these processes have been driven by indigenous fungi and bacteria originating in raw materials, in autochthonous starter cultures, or in the processing environment itself (1), organisms that are responsible for these beneficial transformative processes as well as for product spoilage (2-3). While most modern fermented foods are inoculated with defined starter cultures, traditional, uninoculated products remain celebrated for their historical and cultural significances (4), and indigenous microbial activity is often considered to increase the flavor complexity of these foods (5). The advent of high-throughput sequencing technologies has enhanced our ability to investigate the role of microbial communities in food systems with greater scale and sensitivity than ever possible (4), connecting the transmission of microbial communities in food production and food-processing environments to their impact on food products.

Sake is the traditional, national alcoholic beverage of Japan. Sake is produced from rice through the saccharification of starch by *Aspergillus flavus* subsp. *oryzae* and subsequent alcoholic fermentation by *Saccharomyces cerevisiae*. Sake brewing involves a serial propagation process, beginning with *koji*, a solid culture consisting of rice and *A. oryzae* (6) (Figure 1). Polished, steamed rice is mixed with the dried spores of *A. oryzae* and incubated for approximately 2 days. *Koji* is then pitched with more steamed rice, water, and yeast into the *moto* (*seed mash*) tank, an open mashing vessel, wherein fermentation occurs for 10-25 days. Next, the *moto* is moved to a larger vessel and mixed with increasing amounts of water, rice, and *koji* in three additions to form *moromi*, the main fermentation. *Moromi* fermentation occurs for 20-30 days, after which it is pressed, filtered, and typically pasteurized to become finished sake. Originally, sake brewing was performed entirely by autochthonous microorganisms. However, as sake fermentations are conducted in open fermenters such methods are prone to microbial contamination. Thus, most modern sake production is inoculated with pure yeast and acidified with lactic acid in the moto to inhibit the growth of undesirable organisms. In contrast, in traditional moto fermentations the growth of undesirable bacteria and wild yeast is inhibited by several factors (low pH, high concentration of sugar and nitrite, and low-temperature). In particular, lactic acid and nitrite produced by specific bacteria play an important role for inhibition of undesirable bacteria (7). After decrease of undesirable microorganisms, the indigenous yeast that is suitable for sake fermentation grows spontaneously or pure culture yeast is added. This traditional method of moto process is called “*kimoto*”.

Many studies have been conducted to reveal the microbial transitions that occur during kimoto-style sake production using culture-based techniques (7-9) but few have employed culture-independent techniques (10). In the early stages, bacteria (*Micrococcus*, *Escherichia*, *Psuedomonas*, *Enterobacter*, *Aerobacter*, and *Achromobacter*) and non-*Saccharomyces* yeasts (*Pichia* spp., *Candida* spp., *Zygosaccharomyces* spp.) have been detected (7-9). Among these, gram-negative bacteria including *Escherichia* and *Pseudomonas* initially increase. Then, lactic acid bacteria such as *Leuconostoc mesenteroides* var. *sake* and *Lactobacillus sakei* grow and produce lactic acid, leading to decreased pH (6-7, 11-12). In parallel with these microbial community changes, rice starches are saccharified by *A. oryzae* amylase activity in the *moto,* and wild yeasts and bacteria are inhibited by the low pH, high sugar concentration, and high concentration of nitrite (7). Subsequently, sake yeast (*Saccharomyces cerevisiae*) increases in the *moto* and conducts the main alcoholic fermentation (7).

To improve our understanding of *kimoto* fermentations, we analyzed the bacterial and fungal communities of *koji* and *kimoto* production in parallel with the processing environment of a North American sake brewery, using high-throughput marker-gene sequencing, quantitative PCR (QPCR), and terminal restriction fragment-length polymorphism (TRFLP). Results suggest that microbial transfer from the processing environment is responsible for driving microbial successions throughout sake fermentations.

Materials & Methods

Sample Collection and DNA Extraction

All samples were collected from a single sake brewery located in North America. This facility produces sake using the *kimoto* method, using no starter cultures except for *A. oryzae* in their koji preparations. Koji samples were collected before inoculation with *A. oryzae*, at mixing times, and at harvest across the 48-hr preparation time of two separate batches. *Moto* and *moromi* samples from two separate production batches were collected in duplicate every other day for the first two weeks, then weekly thereafter. Biological replicates were collected from the *moromi*, which was fermented in two separate fermentation tanks. Equipment and environmental surfaces were sampled as previously described (1). Sterile cotton-tipped swabs (Puritan Medical, Guilford, ME) were moistened with sterile phosphate-buffered saline and streaked across a 100 cm^2 area of the target surface in two perpendicular series of firm, overlapping S-strokes, rotating the swab to ensure full contact of all parts of the swab tip and the surface. Samples were placed on ice and frozen immediately in a –20˚C freezer for storage. Fermentation samples were centrifuged at 4,000g prior to DNA extraction. DNA was extracted using the standard protocol for the ZR Fecal DNA MiniPrep Kit (Zymo Research, Irvine, CA), with bead beating in a FastPrep-24 bead beater (MP Bio, Solon, OH), and stored at –20˚C until further processing.

Sequencing Library Construction

Amplification and sequencing was performed as described previously for bacterial (13) and fungal communities (14). Briefly, the V4 domain of bacterial 16S rDNA genes was amplified using primers F515 (5'–*NNNNNNNN***GT**GTGCCAGCMGCCGCGGTAA–3') and R806 (5'–GGACTACHVGGGTWTCTAAT–3') (15), with the forward primer modified to contain a unique 8 nt barcode (italicized poly-N section of primer above) and 2 nt linker sequence (bold, underlined portion) at the 5' terminus. PCR reactions contained 5-100 ng DNA template, 1X GoTaq Green Master Mix (Promega), 1 mM MgCl2, and 2 pmol of each primer. Reaction conditions consisted of an initial 94°C for 3 min followed by 40 cycles of 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec, and a final extension of 72°C for 10 min. Fungal internal transcribed spacer (ITS) 1 loci were amplified with primers BITS (5'–*NNNNNNNN***CT**ACCTGCGGARGGATCA–3') and B58S3 (5'–GAGATCCRTTGYTRAAAGTT–3') (14), with a unique 8 nt barcode and linker sequence incorporated in each forward primer. PCR reactions contained 5-100 ng DNA template, 1X GoTaq Green Master Mix (Promega, Madison, WI), 1 mM MgCl2, and 2 pmol of each primer. Reaction conditions

consisted of an initial 95°C for 2 min followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, and a final extension of 72°C for 5 min. Amplicons were combined into two separate pooled samples (keeping bacterial and fungal amplicons separate) at roughly equal amplification intensity ratios, purified using the Qiaquick spin kit (Qiagen), and submitted to the UC Davis Genome Center DNA Technologies Core for Illumina paired-end library preparation, cluster generation, and 250 bp paired-end sequencing on an Illumina MiSeq instrument in two separate runs.

Data Analysis

Raw fastq files were demultiplexed, quality-filtered, and analyzed using QIIME v.1.7.0 (16). The 250-bp reads were truncated at any site of more than three sequential bases receiving a quality score < Q20, and any read containing ambiguous base calls or barcode/primer errors were discarded, as were reads with < 75% (of total read length) consecutive high-quality base calls (17). Reverse primer sequences were trimmed from the ends of ITS sequences following demultiplexing. Operational taxonomic units (OTUs) were clustered at 97% identity using the QIIME subsampled reference OTU-picking pipeline using UCLUST-ref (18) against either the Greengenes 16S rRNA database (May 2013 release) (19) or the UNITE fungal ITS database (20), modified as described previously (14). OTUs were classified taxonomically against these same databases using the QIIME-based wrappers of RDP classifier (21) (16S sequences) or a UCLUST-based classifier (Bokulich et al., submitted). Any OTU comprising less than 0.001% of total sequences for each run were removed prior to further analysis (17). Environmental surveillance heatmaps based on taxonomic abundances were visualized using SitePainter 1.1 (22).

The absolute abundance of individual bacterial and fungal taxa detected by marker-gene sequencing was estimated as the product of their relative abundances (number of sequences identified as that taxon divided by total number of sequences observed) x the observed copy number of the corresponding gene detected by QPCR (16S rRNA gene copies for bacteria, ITS copies for fungi).

Terminal restriction fragment length polymorphism (TRFLP). Lactic acid bacteria-specific TRFLP (LAB-TRFLP) was performed as described previously using the primers NLAB2F (5'-(HEX)-GGCGGCGTGCCTAATACATGCAAGT-3') and WLAB1R (5'-TCGCTTTACGCCCAATAAATCCGGA-3') (23). PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of

denaturation at 95°C for 45 sec, annealing at 66°C for 30 sec, and extension at 72°C for 45 sec, and with a final extension at 72°C for 5 min. Samples were purified using QIAquick PCR Purification Kit (Qiagen), digested using enzymes MseI and Hpy188I according to manufacturer instructions, and submitted to the UC Davis College of Biological Sciences Sequencing Facility for capillary electrophoresis fragment separation. Electropherogram traces were visualized using the program Peak Scanner v1.0 (Applied Biosystems, Carlsbad, CA) using a baseline detection value of 10 fluorescence units. Peak filtration and clustering were performed with R software using the TRFLP-STATS (24). OTUs were identified based on an empirical TRFLP database (23) and an *in silico* digest database generated with MiCA (25) of good-quality 16S rRNA gene sequences from RDP (26), allowing up to 3 nucleotide mismatches within 15 bp of the 5' terminus of the forward primer. OTUs detected by TRFLP are reported as relative abundance, or the peak area of the corresponding terminal restriction fragment(s) divided by the total peak area observed for each sample.

Quantitative PCR

In order to quantify net microbial biomass in sake samples and on equipment surfaces, quantitative PCR (QPCR) was used to enumerate total fungi and bacteria. QPCR was performed in 20-µL reactions containing 2 µL of DNA template, 8 pmol of each respective primer, and 10 µL of Takara SYBR 2X Perfect Real Time Master Mix (Takara Bio Inc). Total fungi were quantified using the primers BITS (5'–ACCTGCGGARGGATCA–3') and B58S3 (5'–GAGATCCRTTGYTRAAAGTT–3') (14). Reaction conditions involved an initial step at 95˚C for 30 sec, followed by 40 cycles of 5 sec at 95 ˚C, 1 min at 55 ˚C, and 1 min at 72 ˚C. For amplification of total bacteria, the primers Uni334F (5'-ACTCCTACGGGAGGCAGCAGT-3') and Uni514R (5'-ATTACCGCGGCTGCTGGC-3') (27) were used. Reaction conditions consisted of an initial hold at 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 15 sec at 95 ˚C and 60 sec at 60 ˚C. All reactions were performed in triplicate in optical-grade 96-well plates on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems). The instrument automatically calculated cycle threshold (C_T), efficiency (E), confidence intervals, and *Saccharomyces cerevisiae* cell equivalents (fungi) or 16S rRNA gene copy number (bacteria) by comparing sample threshold values (C_T) to a standard curve of serially diluted genomic DNA extracted from a known concentration of *S. cerevisiae* or *Escherichia coli* cells.

Data Accessibility

Raw marker-gene sequencing data are deposited in QIIME-DB (www.microbio.me/qiime) under the accession numbers 2278 (16S rRNA gene sequences) and 2279 (fungal ITS sequences).

RESULTS

Koji and sake preparation involve multi-stage microbial succession.

To elucidate the microbial processes involved in traditional *kimoto* sake fermentations, a combined culture-independent approach of marker-gene sequencing, QPCR, and LAB-TRFLP (23) was used to profile two separate fermentations and koji preparations in a single North American sake brewery. Results demonstrated large changes in bacterial community composition and abundance over time (Figure 2). Koji preparations were characterized by several-log bacterial growth over 48 hr, reaching a maximum of close to 10^9 16S rRNA gene copies/mL (Figure 2A-B). For the first 24 hr following inoculation, the bacterial communities appear unpredictable and differed between batches but primarily consisted of *Acinetobacter*, *Bacillus*, and *Staphylococcus*. After 24 hr, both koji batches were dominated by *Bacillus* with secondary populations of *Staphylococcus* and *Planococcaceae*. After mixing with steamed rice and water to initiate *moto* production, the bacterial communities quickly change from a koji-like profile to become dominated by *Lactobacillaceae*, *Klebsiella*, and *Lactococcus* within 4 days, accompanied by another 1-log increase in bacterial abundance to between 10^9 to 10^{10} 16S rRNA gene copies/mL (Figure 2A). As *moto* fermentation proceeded, *Lactococcus* and *Klebsiella* gradually declined, replaced by increasing populations of *Lactobacillaceae*. The onset of *moromi*, during which more steamed rice is added as the fermentation is mixed and transferred to a larger vessel, is characterized by another drastic change in bacterial community composition. *Bacillus* and *Leuconostoc* suddenly emerged in both batches before gradually decreasing during the course of fermentation. *Lactococcus, Staphylococcus*, and *Klebsiella* continued to decrease over the course of *moromi* fermentation, yielding to increasing *Lactobacillaceae* populations. However, no appreciable change in bacterial abundance occurred during the *moto*-to-*moromi* transition, nor during the remainder of the fermentation, through which it hovered around 10^8 to 10^9 16S rRNA copies/mL (Figure 2A-B). Although some of the taxonomic groups detected in these fermentations include potentially pathogenic organisms (e.g., *Klebsiella*), both batches tested finished at 10% alcohol and pH 3.7, effectively preventing growth or survival of pathogenic organisms and explaining the decreased abundance of these groups as *moromi* fermentation progressed.

Due to the limited heterogeneity of some bacterial taxonomic groups within the 16S rRNA gene V4 domain with the short read lengths achievable by marker-gene sequencing methods, the dominant bacterial taxon in sake fermentations could only be confidently identified to family level: *Lactobacillaceae*. Therefore, LAB-TRFLP (23) was used to identify which *Lactobacillaceae* were

present during the course of sake fermentations. Results identified *Lactobacillus plantarum* as the most abundant species during the course of fermentation, with large populations of *Lactobacillus parabrevis, Lactobacillus fermentum*, and a group identified as either *Lactobacillus acidophilus, Lactobacillus helveticus,* or *Lactobacillus amylolyticus* (Figure 2C). *L. plantarum, L. acidophilus,* and *L. fermentum* have all been described in sake previously (12). Consistent with the marker-gene sequencing results, *Lactococcus lactis* and other *Streptococcaceae* (most likely other lactococci) were also detected during the fermentation course.

The fungal communities of *kimoto* fermentations exhibited comparatively less complexity, consisting primarily of *S. cerevisiae* and *A. oryzae* throughout the entire course of fermentation (Figure 3). Rapid fungal growth was observed over the 48-hr course of koji preparation, from around 10^6 to 10^{10} *S. cerevisiae* cell equivalents/mL, consisting almost entirely of *A. oryzae* with minor populations of *S. cerevisiae*. Populations continue to increase and stabilize around 10^{10} cell equivalents/mL through *moto* and *moromi* stages, during which time *A. oryzae* dramatically decreases in favor of *S. cerevisiae*. Minor populations of *Wickerhamomyces anomalus* were observed sporadically in both batches in these later stages. In the initial days of batch 1 *moromi*, several other fungi were observed, including *Phoma, Aspergillus*, and an unknown *Nectriaceae*, disappearing within the first week ("Other" in Figure 3).

Processing environment is source of adventitious microbiota in sake fermentations.

Marker-gene sequencing and QPCR were also both applied to characterize the bacterial and fungal communities on equipment and surfaces within the sake brewery environment in order to observe sites of microbial transfer between the processing environment and these autochthonous sake fermentations. The adventitious microbiota detected during these sake fermentations were observed frequently throughout the brewery environment (Figure 4). The greatest abundance of bacteria and fungi were detected within the main fermentation cellar, particularly in and around the *moto*, fermentation, and aging tanks. *S. cerevisiae* (99.9% maximum relative abundance) and the *Lactobacillaceae* OTUs (70.7%) detected in the fermentations were highly abundant at these sites, as were *Bacillus* (40.8%), *Klebsiella* (10.7%), *Lactococcus* (10.3%), *Leuconostoc* (1.8%), *Staphylococcus* (3.1%), and *W. anomalus* (38.5%), all organisms detected in the *moto* and *moromi* fermentations. The koji room and rice steaming room both displayed lower bacterial and fungal abundance compared to the main cellar. Microbes detected in the fermentations were less prevalent here, but *Bacillus* and *Staphylococcus* were detected at higher abundances on equipment surfaces within these rooms, corresponding to their detection in the koji preparations. While *A.*

237 *oryzae* was the dominant fungus in koji preparations, it was detected less frequently in the
238 environment (73.5% maximum relative abundance inside koji room, 6.5% maximum elsewhere).

239

240 **Discussion**

241 *Kimoto* sake fermentations are a unique and increasingly rare fermentation tradition, employing
242 indigenous microbiota to perform a multi-stage food fermentation. These several stages of
243 production apparently involve a parallel succession of bacteria and fungi responsible for the
244 fermentation. The initial stage, koji preparation, is a semi-aerobic, stirred, solid fermentation,
245 dominated by *A. oryzae* (the only organism inoculated in the koji), *Bacillus*, and *Staphylococcus*,
246 accompanied by a complex, variable consortium of adventitious bacteria (Figure 2-3). Some of
247 these groups, e.g., *Pseudomonas*, have been detected in early sake fermentations previously but do
248 not persist (6-7), consistent with our observations. *A. oryzae* was the dominant fungus detected in
249 the koji and the koji room environment, reflecting its use as an inoculum here. The marker-gene
250 sequencing method could not distinguish subspecies of this fungus, but this OTU presumably
251 represents the subspecies *A. oryzae*, the pure commercial inoculum used in this facility (and
252 traditionally in sake and other food fermentations), not other phytopathogenic, aflatoxin-producing
253 subspecies of *A. flavus* (28).

254 The *moto*, or seed mash, is the next propagation stage involved in sake production, during which
255 prepared koji is mixed with steamed rice and water, precipitating a dramatic shift in the microbial
256 communities and initiating alcoholic fermentation (Figure 2-3). *A. oryzae*, *Bacillus*, and other koji
257 organisms rapidly declined, most likely because of decreased aerobiosis following hydration, and
258 were replaced by *S. cerevisiae*, *Lactobacillus* spp., *Lactococcus*, and *Klebsiella*. This consortium bears
259 considerable similarity to another autochthonous beverage fermentation, lambic-style coolship
260 beers (29), providing a similar niche as a grain-based sugar substrate with relatively high-pH and
261 low-alcohol prior to fermentation. While the roles of *Saccharomyces* and lactic acid bacteria in sake
262 fermentations are well characterized—alcohol production and acidification, respectively—those of
263 several other microbiota that appear in the *moto* are unclear. *Klebsiella* may play a similar role as in
264 lambic-style beers, in which enterobacteria produce short-chain fatty acids and organic acids that
265 contribute to product complexity (30). Consequently, they may be responsible for some of the more
266 pungent aromas of *kimoto* compared to modern sake production.

267 The *moromi*, or main mash, involves mixing the *moto* with increasing quantities of water and
268 steamed rice to start the main fermentation. Interestingly, *Bacillus* and *Leuconostoc* emerged at this
269 stage in both batches analyzed, as well as *Staphylococcus* in batch B, reminiscent of the bacterial
270 composition of the koji (Figure 2). Though these taxa were detected throughout the main cellar,
271 their sudden emergence in the *moromi* may suggest that the several-fold dilution of the *moto* with

water and steamed rice introduces this microbial influx and encourages their growth until conditions re-stabilize. Spore-forming bacilli may survive rice steaming (31) and grow on the surface before alcohol increases and oxygen decreases, yielding the large populations observed in all *moromi* tanks and batches. *Bacillus* spp. are commonly reported in other rice wines and solid rice fermentations (32-38) in which the amylolytic activity of these bacteria may be an important contributor to saccharification (33). The role of bacilli in sake flavor development is unknown, but they can produce an array of ketones, acids, esters, and other compounds important to soybean fermentations (39) and may play a similar role here. *Staphylococcus* is frequently detected on human skin (40) and in food fermentations, including other Asian beverage fermentations that employ semi-solid stages similar to koji preparation (34-37). The presence of this bacterium in both the koji and the *moromi* may relate to the manual contact involved in koji production and *moromi* mixing and mashing, which is performed with a wooden paddle. The common pattern observed in rice fermentations of early dominance by *Staphylococcus* and *Bacillus* species succeeded by lactic acid bacteria has led other authors to speculate that these bacteria may produce growth factors conducive to lactic acid bacteria growth later during the fermentation (38), but this relationship has yet to be demonstrated. *Leuconostoc* species have also been frequently isolated from sake fermentations, in which it produces lactic acid (11-12). In some kimoto fermentations, *Leuconostoc mesenteroides* can directly compete with *L. sakei*, providing the opportunity for growth of wild yeasts (9).

Surprisingly, no yeasts other than *S. cerevisiae* were detected in appreciable quantities throughout any of the sake fermentations, nor within the processing environment. Non-*Saccharomyces* yeasts have been reported in other sakes previously (8), as well as in other food-processing environments (1, 41). Unlike cheese and wine production, sake production involves raw material sterilization, rice steaming, prior to any production stage. This may limit the carryover of raw material microbiota into the fermentation and into the processing areas. The low fungal biomass observed in the rice-steaming and koji-preparation rooms may be further evidence of this theory. The one non-*Saccharomyces* yeast detected in sakes and in the cellar was *W. anomalus*. This yeast is commonly detected in fermented beverages and other food products (42). It is considered typical in some food fermentations, including other rice wines (32), but causes spoilage in many foods through excessive ethyl acetate production (42). It was detected at low abundances in these *kimoto* fermentations, likely inhibited by the high alcohol concentration (42), but may be a typical member of these types of fermentations.

Most of these organisms commonly detected in these *kimoto* fermentations were also detected on equipment and other surfaces throughout the main cellar, particularly on processing equipment and fermentation tanks (Figure 4). As these fermentations rely entirely on the growth of adventitious microbiota, their presence within the cellar demonstrates the importance of surface contact for possible bi-directional transfer of these organisms between fermentations. Similarly to artisan cheese makers (1), the resident microbiota may be unique to individual breweries, potentially leading to regional differences in *kimoto* characteristics. However, the resident populations may not necessarily be stable, and likely fluctuate seasonally as previously observed in wineries (41), altering the propensity for flavor development and spoilage by indigenous microbiota on a seasonal basis in response to changing environmental conditions. This may reflect the practice of performing traditional sake fermentations only during winter months, when cooler conditions would dampen spoilage potential. Further studies across multiple sake breweries and seasons will be necessary to establish the stability and regionality of sake brewery microbiota.

This study illuminates the role of brewery-resident, adventitious microbiota in spontaneous sake fermentations. The microbial succession of these fermentations closely corresponds to the microbial consortia inhabiting the production environment, illustrating the reservoirs and routes for microbial contact in traditional food fermentations. Interrogating the microbial consortia of production environments in parallel with food products is a valuable approach for understanding the complete ecology of food-production systems. Using this model, a similar approach could—and should—be applied to any food production system, leading to enlightened perspectives for process control, spoilage prevention, and food safety.

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460 **Figure 1.** Generalized sake production schematic. Times and temperatures listed at each stage
461 represent those typically used in the sake brewery featured in this study.

462 **Figure 2.** *Kimoto* fermentations involve multi-stage bacterial succession. Bacterial community
463 abundance and structure across time for batch A (A) and batch B (B). Column height indicates QPCR
464 16S rRNA gene copy number/mL. Relative abundance of each bacterial taxon (sequence count /
465 total sequence count) derived from marker-gene sequencing, key in panel B) is superimposed on
466 each bar and does not correspond to the *y*-axis. Only taxa detected at ≥ 1% maximum relative
467 abundance are shown. (C) Relative abundance (OTU peak area / total peak area) of *Lactobacillales*
468 detected in batch A by LAB-TRFLP (23). Units along *x*-axis indicate hours (koji) or days (*moto* and
469 *moromi*) since initiation of stage. I, inoculation with *A. oryzae*; M, mixing; H, koji harvest.

470 **Figure 3.** Fungal succession of *kimoto* fermentations. Fungal community abundance and structure
471 across time for batch A (A) and batch B (B). Column height indicates QPCR *S. cerevisiae* cell
472 equivalents/mL. Relative abundance of each fungal taxon (sequence count / total sequence count)
473 derived from marker-gene sequencing, key in panel B) is superimposed on each bar and does not
474 correspond to the *y*-axis. Only taxa detected at ≥ 1% average relative abundance are shown.

475 **Figure 4**. Microbial drivers of *kimoto* fermentations are residents of the processing environment.
476 Floor plan key (top) depicts all environmental surfaces analyzed. Microbial heatmaps (below)
477 indicate estimated absolute abundance of select microbial taxa detected in high abundance in
478 *kimoto* fermentations. Total bacteria and total fungi are results of actual QPCR data, estimated
479 abundances of other taxa are the products of marker-gene sequencing relative abundance
480 (sequence count / total sequence count) x absolute abundance of the appropriate QPCR target.
481 Color gradient logarithmic scale is indicated in the key (top-right). White surfaces (fungal plots)
482 were below limit of detection.

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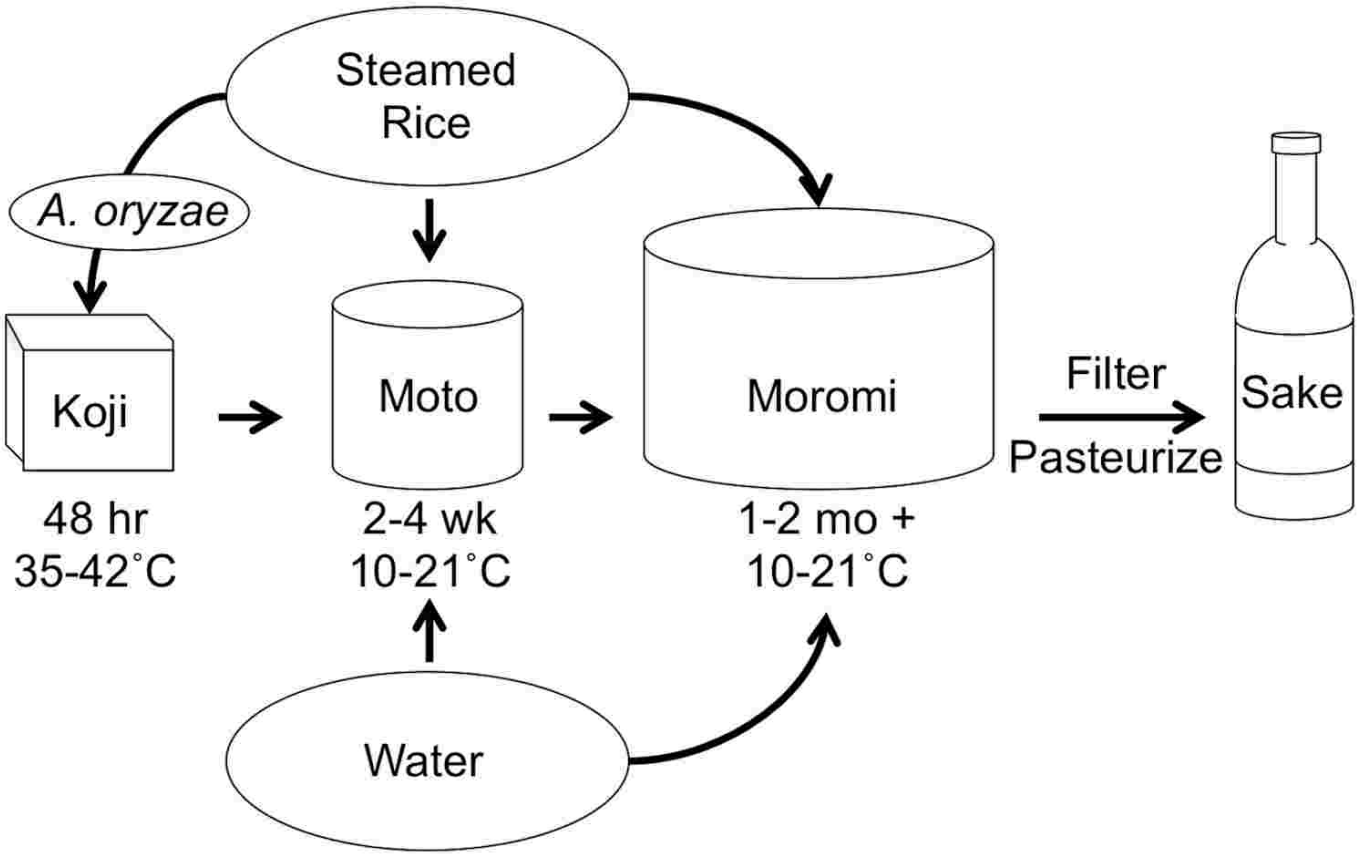
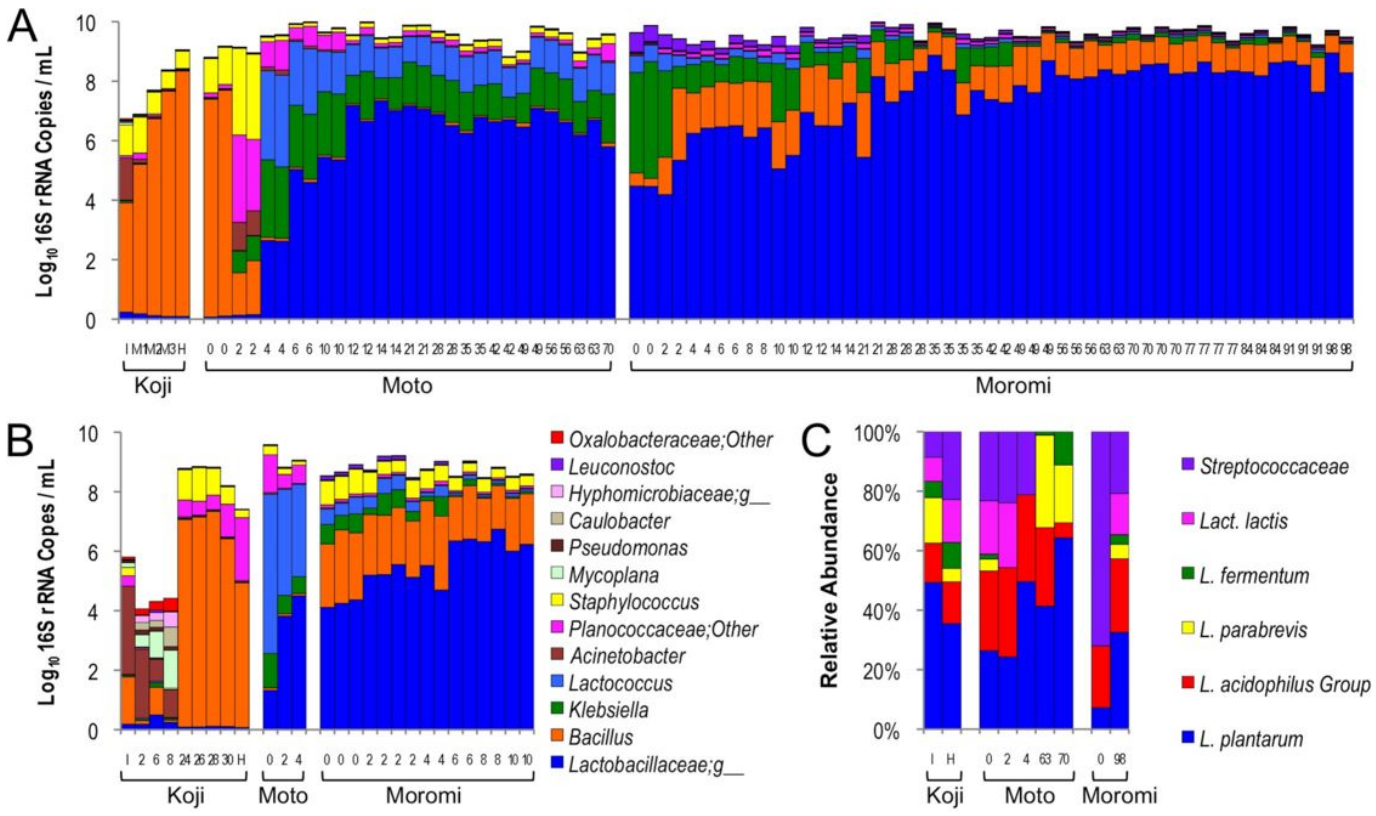
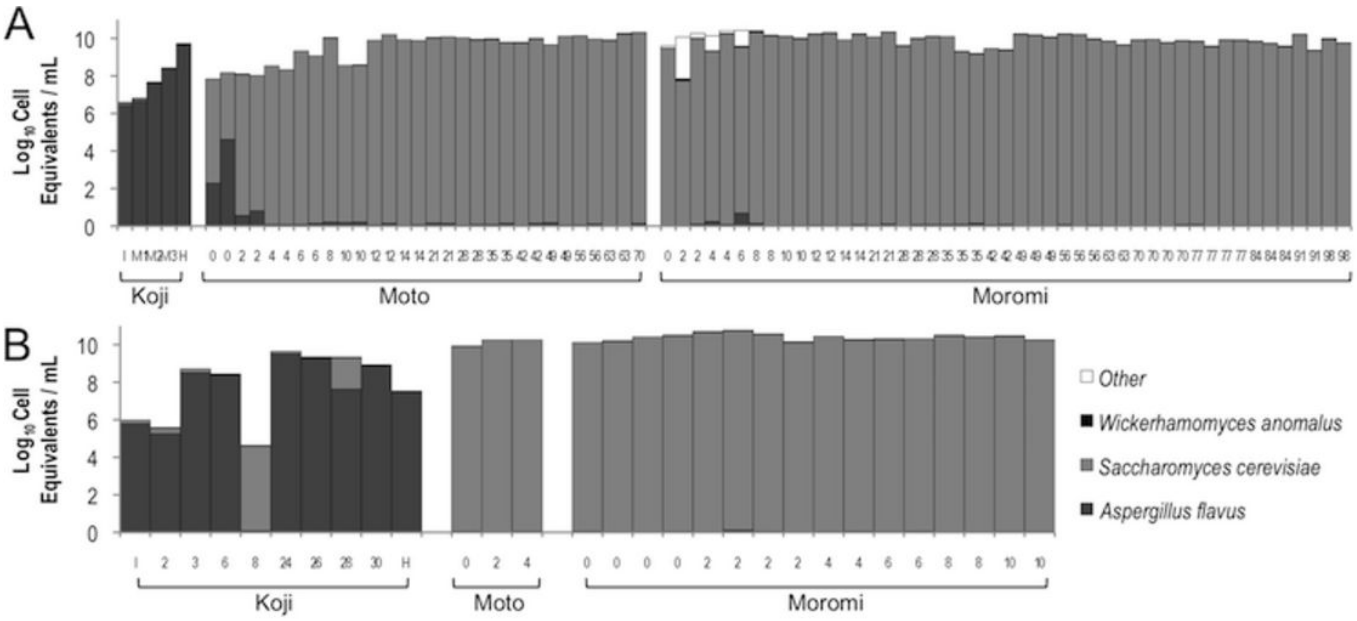
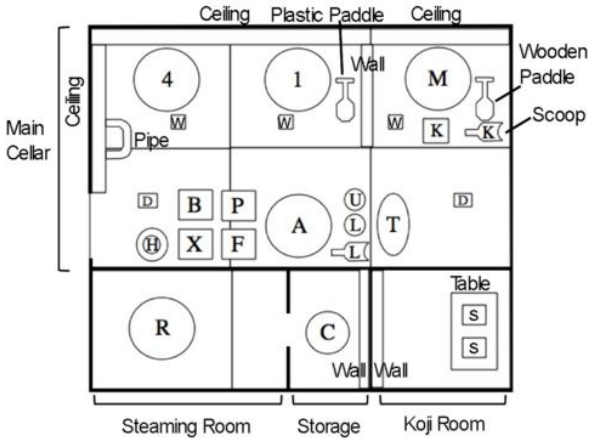
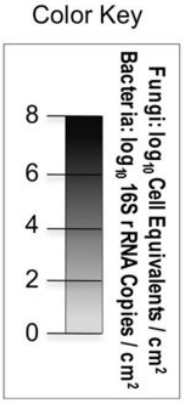






M	Moto Tank
1,4	Fermentation Tanks
K	Koji Scoop/Bucket
A	Aging Tank
T	Mixing Tub
U	Moromi Bucket
L	Lees Scoop/Bucket
B	Bottler
P	Moromi Press
X	Heat Exchanger
F	Filter Press
D	Drains
H	Transfer Hoses
R	Rice Steamer
C	Rice Container
W	Wooden Beams
S	Koji Sheets





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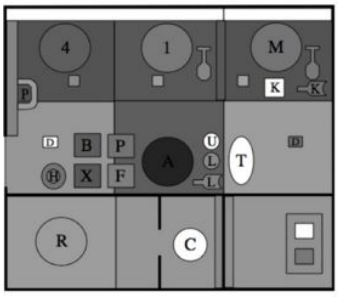
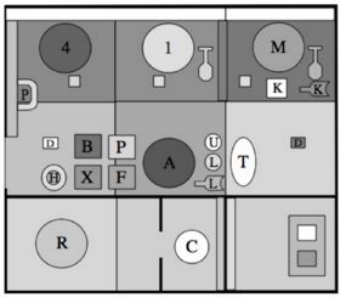


W. anomalus



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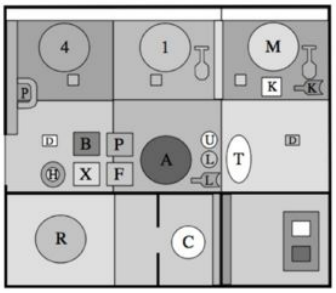
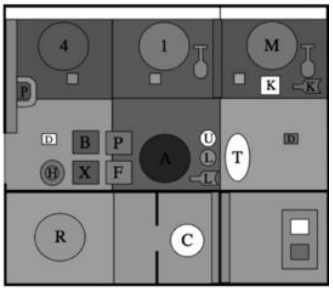
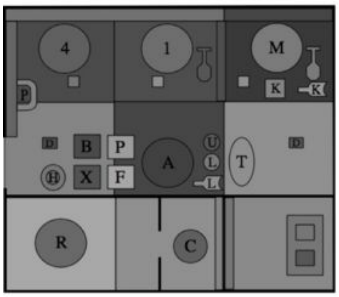


Total Fungi



***Lactobacillaceae*; g__**



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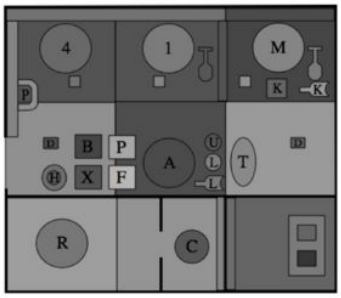

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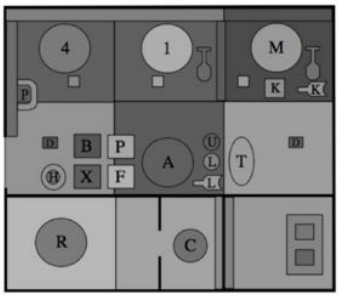

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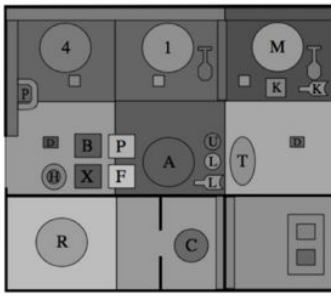

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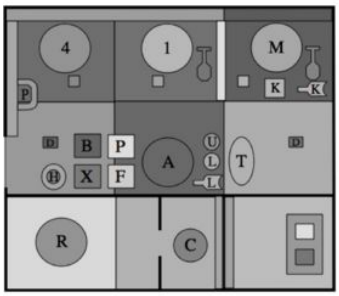

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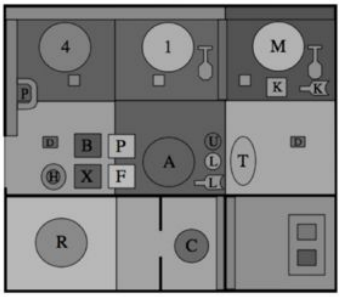
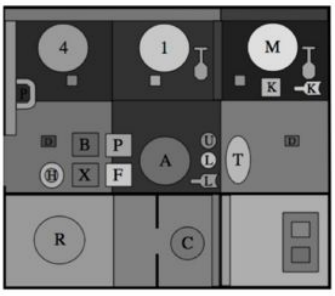


***Staphylococcaceae*; Other**



Total Bacteria