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Saccharomyces arboricola and Its Hybrids' Propensity for Sake Production: Interspecific Hybrids Reveal Increased Fermentation Abilities and a Mosaic Metabolic Profile

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Abstract: The use of interspecific hybrids during the industrial fermentation process has been well established, positioning the frontier of advancement in brewing to capitalize on the potential of *Saccharomyces* hybridization. Interspecific yeast hybrids used in modern monoculture inoculations benefit from a wide range of volatile metabolites that broaden the organoleptic complexity. This is the first report of sake brewing by *Saccharomyces arboricola* and its hybrids. *S. arboricola* x *S. cerevisiae* direct-mating generated cryotolerant interspecific hybrids which increased yields of ethanol and ethyl hexanoate compared to parental strains, important flavor attributes of fine Japanese ginjo sake rice wine. Hierarchical clustering heatmapping with principal component analysis for metabolic profiling was used in finding low levels of endogenous amino/organic acids clustered *S. arboricola* apart from the *S. cerevisiae* industrial strains. In sake fermentations, hybrid strains showed a mosaic profile of parental strains, while metabolic analysis suggested *S. arboricola* had a lower amino acid net uptake than *S. cerevisiae.* Additionally, this research found an increase in ethanolic fermentation from pyruvate and increased sulfur metabolism. Together, these results suggest *S. arboricola* is poised for in-depth metabolomic exploration in sake fermentation.

Keywords: sake; fermentation; hybrid; ginjo; ethanol; ethyl hexanoate; isoamyl acetate; yeast; *Saccharomyces*; metabolism

1. Introduction

As currently defined, the budding yeast clade known as *Saccharomyces* has eight members: *S. cerevisiae*, *S. paradoxus* (syn. *S. cerevisiae* var. *tetraspora*, *S. cerevisiae* var. *terrestris*), *S. uvarum* (syn. *S. bayanus* var. *uvarum*), *S. mikatae*, *S. kudriavzevii*, *S. arboricola* (syn. *S. arboricolus*), *S. eubayanus*, and *S. jurei* [1–9]. Additionally, *S. pastorianus* (syn. *S. carlsbergensis*, *S. monacensis*) and *S. bayanus* are two natural hybrids found in this group [10–12]. *S. arboricola* was first isolated from the tree bark of the *Fagaceea* family in the west and southwest of China [2] in 2008 and later also isolated in western Taiwan [5] and northern New Zealand [13]. The New Zealand isolate was reported to be highly divergent from the Chinese isolates used in this study, supporting the far East Asian origin theory for *Saccharomyces* yeast. The Chinese *S. arboricola* has a high quality annotated genomic sequence that when compared to *S. cerevisiae* shows a reciprocal translocation on chromosome (chr.) IV and XIII, inversion

on chr.VI, high presence of non-syntenic genes near telomeric regions, lack of introgression, and four novel genes [14]. Between *S. cerevisiae* and other Saccharomyces members there is a 15–30% divergence, but the divergence between S. cerevisiae strains is 0.1–0.6% [15,16]. Additionally, subpopulations of *S. paradoxus* show a 1.5–4.5% divergence, while the divergence between *S. eubayanus* and *S. uvarum* is 6% [7,17]. The nucleotide divergence of yeast within *Saccharomyces* is near the man to bird comparison, but with the advantage of a physiological and structural similarity of man to chimp [18]. Favorable properties of different *Saccharomyces* yeast can be brought together by hybridization that often results in transgressive phenotypes. Typical environmentally isolated *Saccharomyces* strains are diploid while laboratory strains are often used in the haploid form to reduce variability and industrial yeast are frequently found to be polyploidy, potentially a result of favored fermentation environment. Although interspecies hybrids of the *Saccharomyces* genus are viable, the they do not produce viable gametes or ascospores in yeast resulting in reproductive isolation as a sterility barrier [9]. Hybridization of phenotypically diverse yeast has been exploited for the improvement of industrial strains for specific purposes.

Fermenting yeasts, specifically *S. cerevisiae*, has been unwittingly domesticated throughout humanity's existence via the production of various foods and beverages, including bread, beer, wine, and sake. One factor in *S. cerevisiae* selection is its unique metabolic capabilities as a Crabtree positive yeast; they amass ethanol in the presence of oxygen. The commonly preferred eukaryotic metabolic pathway in the presence of oxygen is oxidative respiration for its high ATP yield. But in the high concentrations of six-carbon carbohydrates, such as glucose, this pathway is repressed by the Crabtree effect. Under these conditions, the energy for growth comes from the glycolysis pathway until exhaustion of six-carbon molecules causes a diauxic shift to oxidation of the two-carbon ethanol molecules into CO_2 [19,20]. Crabtree positive microbes are beneficial in the production of alcoholic beverages which have been produced for at least 9000 years, and the earliest known recording of Japanese sake is mentioned ca. 285 AD in an ancient Chinese document called *Gishiwajinden* [21–23]. Sake is the alcoholic drink generated by fermenting rice and beer is that of barley, making their development specific to regions dominated by these grains. Rice and barley diverged 50 million years ago and rice was introduced to Japan from the Chinese Yangzi region around 400 BC, many differences exist between these two grains [24,25]. During the 15th century in Bavaria, lager-brewing arose [26] and is the most popular alcoholic beverage in the world today. Since the lager beer yeast, *S. pastorianus* was discovered to be a hybrid of *S. eubayanus* x *S. cerevisiae,* interest has sparked in the use of interspecific hybrids in fermentation [27]. Originally the *S. eubayanus* parent was identified in Patagonia and the question of lager yeast's origin has generated controversy [28–31]. Yeasts originated multiple times from various fungal lineages of which one gave rise to the budding yeasts [32]. The genomic diversity with geographical boundaries and many other lines of evidence support a Far East Asia origin for *Saccharomyces* [33–35].

Beer, wine, and sake are complex chemical matrixes resulting from many metabolic and chemical reactions in which raw ingredients determine initial sugar, assimilable nitrogen, and fatty acid profiles [25,36]. The volatile metabolic profile obtained during fermentation of different carbohydrates is reflective of the raw ingredients composition [37,38]. Wine is perhaps one of the oldest biotechnologies as yeast are often found on damaged grapes and crushing of the fruit facilitates the release of the fermentable sugars [39,40]. Obtaining fermentable sugars for beer and sake is quite different. Production of beer involves a saccharification of malted barley by the grain's own enzymes to convert their starches to maltose, glucose, and maltotriose found in descending concentrations. Boiling of the wort results in less than 3% of the malt lipids available to yeast during fermentation [41]. Fermentation of green beer is accomplished in 3 days to 10 days [42] and results in an average alcohol content of 5% (vol/vol) with some brews tipping the scales around 10% (vol/vol) or more.

Immature sake is generally produced over 41 days and involves several steamed rice additions at temperatures between 12–20 °C applying high osmotic pressure on the yeast from the rice mash ratios [43]. The highest alcohol yield in the world, 17–20% (vol/vol) without distillation, is obtained

through this process [25]. *Aspergillus oryzae*, colloquially known as koji, is a keystone organism in the production of sake from polished rice [44]. By traditional Japanese sake mash methods, koji performs a continuous saccharification of highly milled rice starches to the fermentable sugars and the budding yeast, *S. cerevisiae*, ferments these sugars into ethanol [45]. Free sugars such as glucose and oligomers are produced by the koji enzymes glucoamylase and α-amylase during the sake mash [23]. Recent work has shown that koji secretes mannitol 2-dehydrogenase which converts fructose to mannitol in the sake mash. Interestingly, *S. arboricola* efficiently catabolizes mannitol, an abundant natural energy storage molecule that *Aspergillus* sp. produce in high concentrations [46,47]. This phenotype is superior to *S. cerevisiae* metabolism of mannitol [14]. Considering the temperature tolerance and growth capabilities on mannitol, it was hypothesized that *S. arboricola* and interspecific hybrids are uniquely fit for sake fermentation with *A. oryzae* because of their metabolic access to the underutilized sugar-alcohol and temperature preference which affects the flavor active metabolites.

The aim of this study was to characterize the metabolic capabilities of *S. arboricola* and its hybrids with *S. cerevisiae* in Japanese rice wine. For the first time, sake fermentation profiling of *S. arboricola* and newly generated *S. arboricola* x *S. cerevisiae* interspecific hybrids is reported. The wine and beer brewing industries have often employed interspecific hybrids for their fermentations. *S. kudriavzevii* x *S. cerevisiae* are known to ferment wines, *S. mikatae* x *S. cerevisiae* hybrids have been actively produced for wine, and the *S. cerevisiae* x *S. eubayanus* hybrid produces the most popular beer in the world [33,48,49]. Limited research has been conducted on the metabolic capabilities of *S. arboricola* and these results suggest *S. arboricola* and the hybrids' propensity for fermentation may be suited for sake production.

2. Materials and Methods

2.1. Yeast Strains

Yeast strains of *Saccharomyces cerevisiae* included Italian wine isolate M22 [50], West African wine isolate DBVPG6044 [51], sake isolate Kyokai No. 7 known as haploid 868K7 [52], sake isolate TCR7 [53], American brewing isolate WLP001, and American brewing isolate HT01. Yeast strains of *Saccharomyces arboricola* included SA350 (syn. AS 2.3317), SA351 (syn. AS 2.3318), SA352 (syn. AS 2.3319 [14]. Hybrids SAM8c and SAM9a were between *S. arboricola* 350 x *S. cerevisiae* M22 (Table 1). A minimum of three biological replicates were used in each analysis.

Table 1. Yeast Strains Used During this Study.

Strain	Species	Isolation Source	Reference
SA350 (syn. AS 2.3317)	*S. arboricola*	Tree bark-China	Liti, G., et al. (2013)
SA351 (syn. AS 2.3318)	*S. arboricola*	Tree bark-China	Liti, G., et al. (2013)
SA352 (syn. AS 2.3319)	*S. arboricola*	Tree bark-China	Liti, G., et al. (2013)
M22	*S. cerevisiae*	Wine-Italy	Capece, A., et al. (2012)
DBVPG6044	*S. cerevisiae*	Billi wine-West Africa	Song, G., et al. (2015)
Kyokai No. 7 (syn. 868K7/ K7)	*S. cerevisiae*	Sake-Japan	Katou, T., et. al. (2008)
TCR7	*S. cerevisiae*	Sake-Japan	Horie, K., et al. (2010)
WLP001	*S. cerevisiae*	Beer, American ale	Commercially available from White Labs, USA
HT01	*S. cerevisiae*	Beer, American ale	Current study
SAM8c	Hybrid (*S. arboricola x S. cerevisiae*)	Lab, spore to spore mating	Current study
SAM9a	Hybrid (*S. arboricola x S. cerevisiae*)	Lab, spore to spore mating	Current study

2.2. Yeast Isolation

Saccharomyces cerevisiae HT01 was the only strain isolated for this study and was isolated as described previously [54] with slight modification. Briefly, beer was harvested from a can sanitized on the outside with 70% ethanol into an enrichment media (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g sucrose, 76 mL EtOH, 1 mg chloramphenicol, and 1 mL of 1-M HCl per liter). Samples were transferred to sterile tubes and incubated until signs of yeast appeared. Cultures were then streaked onto YPD media and single colonies were grown and stocked. From these stocks, the yeast culture's genomic DNA was extracted, the ITS region was amplified via polymerase chain reaction (PCR) [55], Sanger sequenced at the West Virginia University Genomic Core, and the isolated yeast was confirmed to be *S. cerevisiae* via utilizing NCBI Blast.

2.3. Serial Spot Dilutions

Serial spot dilution growth assays were conducted as described previously [56] with some modification. Briefly, OD_{600nm} growth readings were taken from fresh, overnight cultures of each yeast grown at 23 °C. For each yeast assayed, a total of 3.2×10^5 yeast cells were transferred to a well in the first column of a 96 well plate. Each yeast assayed was serially diluted 4 times in a 1:10 ratio, using sterile distilled and MilliQ (MQ) filtered water, across the rows which resulted in 5 wells each. A handheld 48 pin plate was used to place the serial dilution spots onto solid YPD agar media. Plates were then incubated at 4 °C for ten days, 10 °C for seven days, and 15 °C for four days. Growth was assessed and photographed.

2.4. Sporulation and Spore Staining

Fresh liquid yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) overnight at 23 °C in a shaking water bath. Isolates were streaked out onto YEP(EG) buffered agar plates (1% succinic acid, 1% yeast extract, 2% peptone, 2% glycerol, 2% agar, potassium hydroxide to pH 5.5, 2.5% ethanol) for 2–3 days. Single colonies were thinly patched onto Snack-Spo Zn agar plates (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, 0.005% zinc acetate, 2% agar) and incubated for 6 days. To measure spore formation, a modified Schaeffer-Fulton endospore staining technique was used. Briefly, cultures from the sporulation plates were suspended in 1ml distilled water and recovered by pipette. The suspended culture was fixed to a glass microscope slide via flame. Malachite green (10%, 0.1 g/mL) was then used to stain the spore walls for 15 min (minute/s) before decoloring and rinsing with water. Safranin (0.25%, 2.5 mg/mL) was used as a counterstain for non-sporulated cells for 1 min before rinsing with water and mounting in glycerol. Malachite green cells were counted as spores and safarin red cells were counted as non-sporulated yeast cells [57]. Images were taken under a light microscope and sporulation efficiency for each strain was calculated, reported in the results section, to select the best possibility of a successful hybridization.

2.5. Interspecific Hybridization

The ascus of the spore was digested over 1 hr. with Zymolase (25 mg/mL) at 37 °C. The digested spores were placed dropwise on YPD agar at an angle to facilitate a central streak of liquid across the plate. Micromanipulation of spores from *S. arboricola* strain 350 and *S. cerevisiae* M22 spores was performed by fiber optic needle under a light microscope using the SporePlay (Singer Instruments, Roadwater, England) dissection microscope by Singer Instruments and yielded 40 spore to spore mattings. Visual observations were conducted on the shmooing process during incubation at 25 °C. Single colonies were selected, streaked out for purification, and stocked at −80 °C with cryogenic media (1% yeast extract, 2% peptone, 15% glycerol).

2.6. Genomic DNA Extraction

Alkaline lysis genomic DNA extraction was performed on fresh, overnight cultures by harvesting (0.3×10^7) cells via cold centrifugation at 7500× *g* for 3 min. Cells were washed twice with phosphate-buffered solution (PBS) before NaOH (0.02 M) addition and subsequent incubation at 100 °C for 10 min. The cellular solution underwent cold centrifugation at 4 °C for 3 min before 250 µL of supernatant was mixed with 50 µL acetic acid (2.5 M) and then 300 µL of 2-propanol. Cold centrifugation was performed again for 15 min before the supernatant was discarded. Ethanol (80% v/v) was added to the DNA pellet to lightly mix the solution before a 5 min cold centrifugation. The DNA was dried at room temperature and resuspended in TE buffer.

2.7. Polymerase Chain Reaction

DNA concentrations were measured by OD_{260nm} via a glass black cell cuvette. PCR confirmed *S. arboricola* 350 and *S. cerevisiae* M22 (SAM hybrids) interspecific hybridization. Two multiplexed species-specific primer sets were used, detailed previously [58]. The oligonucleotide sequences used for primers were as follows; for *S. arboricola* sarbF1 (GGC ACG CCC TTA CAG CAG CAA TCG) and sarbR2 (TCG TCG TAC AGA TGC TGG TAG GGC GCT); for *S. cerevisiae* scerF2 (GCG CTT TAC ATT CAG ATC CCG AG) and scerR2 (TAA GTT GGT TGT CAG CAA GAT TG ATC). A master mix for the PCR reactions was made on ice and consisted of PCR reagents from kit: KOD-Plus-Neo (product code KOD-401) ordered from TOYOBO, Japan. The PCR Touchdown [59] thermocycle protocol was used. During gel electrophoresis [60], the *S. cerevisiae* band generated at 150bp (base pairs) and *S. arboricola* generated at 349 bp.

2.8. Sake Fermentations

Freshly grown cells (2.0×10^9) were measured by OD_{600nm}, harvested from YPD via centrifugation at 3200× *g* for 3 min, and washed in MilliQ water twice. The harvested yeast, rice, koji, lactate, and water were mixed together in mason jars covered with loose lids. Incubation occurred at 15 °C for 2 weeks with an initial remixing of the mash occurring after the first 24 h. Fermentation was monitored daily by mass reduction of the sake mash, which corresponded to CO_2 production. Fermentation trials were performed in triplicate. Post-fermentation, the sake mash was harvested into conical tubes by centrifuged at 4 °C and 3200× *g*. The supernatant was transferred to storage at 4 °C for further analysis. Statistical analysis of ethanol content was validated by one-way ANOVA with a two-tailed student's *t*-test post-hoc analysis and ordered difference report for each day of fermentation.

2.9. Chromatography Analysis

2.9.1. Gas Chromatography-Flame Ionization Detection

For gas chromatography-flame ionization detection (GC-FID) of the sake supernatant, ribitol (syn. adonitol) (0.2 mg/mL) was supplied as an internal standard. Samples were freeze-dried via liquid nitrogen bath followed by vacuum chamber drying for 6 h. The freeze-dried samples were mixed with 100 µL of methoxylamine diluted with pyridine (20 mg/mL] and incubated at 30 °C for 90 min with agitation (1500 rpm). The sample was mixed with 50 µL of N-methyl-N-TMS-trifluoroacetamide (MSTFA) incubated for 30 min at 37 °C with agitation and transferred into a glass vial for chromatography metabolome analysis. A Cp-sil8CB column from Agilent, model #CP8751 (30 mm, 0.25 mm, 0.25 µm) was used to assess the sake fermentation samples. The injection was at 230 °C with a 1 mL/min helium carrier gas flow rate. The column temperature was held at 80 °C for 2 min before rising 10 °C/min until 320 °C was achieved, the temperature was held for 12 min before reaching a 320 °C detector. The Shimadzu Corporation (Shimadzu Manufacturing Company, Kyoto, Japan) model GC-2014AFSC 100 V was used in performing the GC-FID analysis.

2.9.2. Head Space-Solid Phase Microextraction

For the head space-solid phase microextraction (HS-SPME), the sake supernatant samples were prepared with NaCl and methyl hexanoate (40 mg/100 mL) was supplied as an internal standard. In a glass vial, the solution was agitated under 80 °C heat with a stir bar (160 rpm) for 15 min. A syringe was inserted into the vial to expose a SPME fiber (100 µm PDMS) for 15 min. The fiber was retracted into the syringe before removal from the vial and inserted into the injector for a 5 min exposure. A DB wax column from Agilent, model #122-7032 (30 mm, 0.25 mm, 0.25 µm) was used. The injection was at 200 °C with a 0.95 mL/min helium carrier gas flow rate. The column temperature was held at 60 °C for 1 min before rising 3 °C/min until 100 °C was achieved, the rate increased to 15 °C/min until 200 °C was reached and held for 4 min before reaching a 320 °C detector. The Shimadzu Corporation (Shimadzu Manufacturing Company, Kyoto, Japan) model GC-2014AFSC 100 V was used in performing the HS-SPME analysis.

2.9.3. Gas Chromatography–Mass Spectroscopy

For gas chromatography–mass spectroscopy (GC-MS) of endogenous metabolites, stationary phase yeast cultures were grown in YPD liquid media. Stationary phase was defined at an OD_{600nm} of 2.0. Cells (4×10^7) were harvested, washed, and freeze-dried. An extraction solution (chloroform:methanol:MQ [2:5:2]) with ribitol (0.2 mg/mL) as the internal standard was added to the freeze-dried samples and incubated for 30 min at 37 °C under agitation (1500 rpm). The samples were centrifuged, and the supernatant was harvested. MQ water was added to the supernatant and centrifuged. The supernatant was concentrated under vacuum centrifugation (−100 kPa) at 37 °C. MQ water was added, and the water-soluble metabolites were freeze-dried. Methoxyamine (20 mg/mL) was added to the samples and incubated for 90 min at 30 °C under agitation (1500 rpm). MSTFA (*N*-methyl-*N*-trimethylsilyl trifluoroacetoamide) was added to the samples and incubated for 30 min at 37 °C with agitation (1500 rpm). Samples were supplied in glass vials for GC-MS analysis using an Agilent DB-5MS+DG (30 m, 0.25 mm, 0.25 um) column, splitless injection, and helium carrier gas (1 mL/min). The column temperature rose from 80 °C at a rate of 13 °C/min until reaching a 320 °C detector. The Agilent Technologies, Inc. (Agilent Technologies, Santa Clara, California, United States of America) model 7890 B was used for the GC system, model 5977 A was used for the MS system, and the National Institute for Standards and Technology (NIST) mass spectral library (United States Department of Commerce, Gaithersburg, MD, USA) was used.

2.9.4. Data Analysis

The peak area of identified compounds was divided by the peak area of the internal standard for normalization of the data. Normalized peak areas of the compounds and ethanol concentrations measured during sake fermentations were auto-scaled and used as independent variables in the loading plots for construction of the principal component analysis (PCA). Statistical analysis was performed by soft independent modeling by class analogy (SIMCA) methodology by SIMCA version 13.0.3.0 (Umetrics, Umeå, Sweden) computer software. Analysis of variance (ANOVA), post-hoc students t-test, and post hoc Tukey-Kramer honest significance test (Tukey HSD), $p < 0.05$ was used for statistical significance. Deviations from this statistical analysis procedure, if occurred, was reported in individual results or methods sections. Heatmap was generated through MetaboAnalyst 4.0 [61]. Heatmaps were based on data normalization by internal reference feature, cubed root transformation, pareto/range data scaling for endogenous/sake metabolites, clustering by Ward's method, and Euclidean distance measurements.

3. Results

Endogenous metabolome characterization of *S. arboricola* was put in context of industrially relevant *S. cerevisiae* yeast. Two yeast strains were chosen to represent either beer, wine, or sake fermentation industries. WLP001 and HT01 represented beer, M22 and DBVPG6044 represented wine, K7 and TCR7 represented sake production. SA350, SA351, and SA352 were used to represent wild *S. arboricola* (Table 1). Endogenous metabolite profiling was visualized in a hierarchical clustering heatmap that showed clustering of yeast by industry and species (Figure 1A). Ward's linkage clustering algorithm created two main clusters separated by species with the exception of West African bili wine strain DBVPG6044 that grouped within *S. arboricola*. The yeast in this cluster, located in the right half of the heatmap, all showed a low concentration of endogenous amino acids. Beer strains, sake strains, and wine strain M22 composed the other cluster on the left half. Beer strains paired together, and sake strains paired together with Italian wine strain M22. These industrial strains had similar but varying metabolic profiles displaying high endogenous levels of many amino and organic acids.

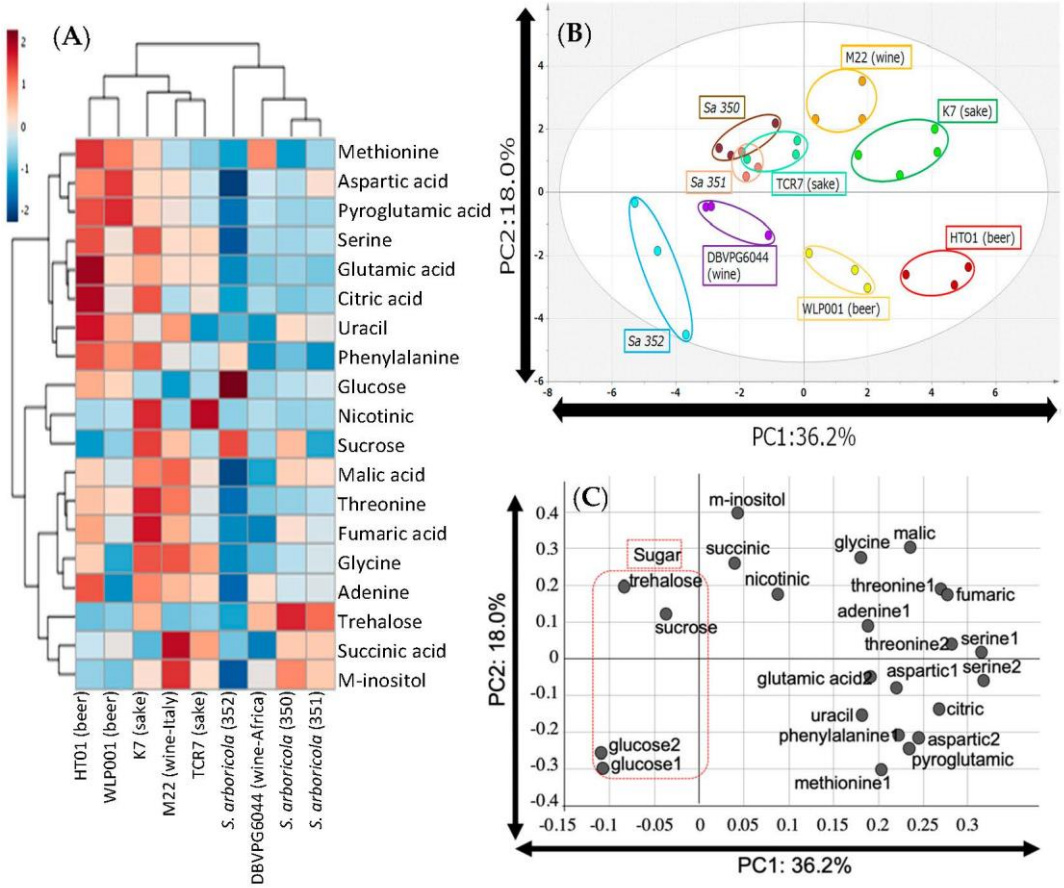

Figure 1. Yeasts endogenous metabolic profiles. (**A**) Hierarchical clustering heatmap of endogenous metabolite profiles between strains and species. Relatively high compound concentrations were shown in varying degrees of red. Relatively low compound concentrations were shown in varying degrees of blue. Shown as average concentration per sample groupings of three replicates. (**B**) Principal component analysis (PCA) score plot of *Saccharomyces* yeast explaining 17.4% of the variance on PC2 and 31.6% of the variance on PC1. (**C**) PCA loading plot. Saccharide content is circled to show importance in the PC1 variance. PCA and loading plot generated through SIMCA analysis. Three replicates were used for each strain.

A score plot generated through principal component analysis separated the strains into general industry-specific groups (Figure 1B). A total of 19 metabolites identified by GC-MS were used as variables in the construction of the loading plot (Figure 1C). Sugar utilization played a major role in separation of the strains. *S. arboricola* strains were grouped to the left while beer and sake strains grouped opposite to the right on principal component (PC1) explaining 36.2% of the sample variance and separating the groups by species. On the axis of PC2, sake yeast grouped high, wine yeasts were neutral/variable, and beer yeasts were low explaining 18.0% of sample variance and separating the yeast by their industrial niche domestication. Glucose, sucrose, and trehalose negatively contributed to PC1, which exemplified sugar uptake differences and was inferred to translate to fermentation aptitude. Beer brewing strains were found to be the most inclined yeasts in this regard and *S. arboricola* were found to be the least adept. A third component, explaining 12.7% was used to create a 3D PCA plot of the endogenous metabolites (Figure S1). Citric acid, malic acid, fumaric acid, glutamic acid, nicotinic acid, succinic acid, aspartic acid, threonine, serine, glycine, phenylalanine, pyroglutamine, methionine, uracil, inositol, and adenine positively contributed to PC1. A loading matrix was constructed for the variables (Table S1). Glycine, threonine, malic acid, fumaric acid, nicotinic acid, succinic acid, sucrose, trehalose, inositol, and adenine positively contributed to PC2. Phenylalanine, pyroglutamine, methionine, uracil, aspartic acid, and glucose contributed negatively to PC2, which distinguished the beer brewing strains from the sake strains. These two groups are represented in the heatmap's industrial cluster. Factors from the heatmap show the difference between sake and beer yeast include the endogenous inositol levels; which were high in sake yeast and affect membrane fluidity during osmotic stress such as an industrial sake mash.

The potential of *Saccharomyces* hybridization to broaden metabolic capabilities and sensory complexity from volatile metabolites was explored using *S. arboricola* and *S. cerevisiae*. Sporulation efficiency was used in selection of strains for direct-mating hybridization as industrial strains are typically poor sporulators. *S. arboricola* 350 and *S. cerevisiae* M22 yielded the highest sporulation efficiency at 69% and 91% respectively (Figure S2A). Selection of the *S. cerevisiae* industrial strain relied on sporulation results to optimize spore-spore mattings and given M22 grouped similar to the sake strain metabolism in Figure 1B, M22 was choosen. Other *S. arboricola* strains sporulated at 68% for 351 and 20% for 352. Interspecific SAM (*S. arboricola* 350 and *S. cerevisiae* M22) hybrids, SAM8c and SAM9a, generated double bands representing genomic input from both species. Two spore-to-spore mating colonies were advanced to lab-scale sake fermentation after confirmation of hybrid state by species-specific multiplexed PCR (Figure S2B). Cryotolerance of *S. arboricola* and the hybrids was shown as substantial growth at 4 and 10 °C after ten and seven days, respectively (Figure 2). At 15 °C, there was no perceivable difference between yeast growth. Thermosensitivity was not detected using the serial spotting dilution assay in temperatures up to 30 °C, slow growth of *S. arboricola* cultures during extreme temperature highs in the summer months was observed.

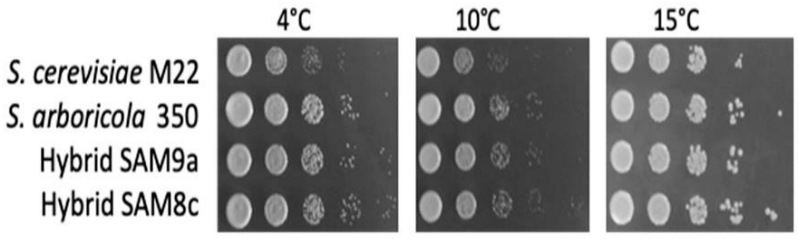

Figure 2. Cryotolerance of parental and hybrid yeasts. Serial spot dilution growth assay of parental *S. cerevisiae* M22, parental *S. arboricola* 350, and the SAM hybrids. Three different temperatures of 4 °C, 10 °C, and 15 °C was used in assessment of their cryotolerance. Yeast were incubated on solid YPD agar for ten days at 4 °C, seven days at 10 °C, and four days at 15 °C.

To determine if important sake flavor molecules changed with hybridization, ethyl hexanoate, isoamyl acetate, and ethanol fermentation was measured. In fact, it was found that *S arboricola*, *S. cerevisiae*, and the hybrids metabolite profiles after lab-scale sake mash fermentation trials were different. The hybrids had a more vigorous fermentation, yielding a higher alcohol content than *S. cerevisiae* by day seven ($p = 0.031$) of the 14 day trial fermentation (Figure 3A). A one-way ANOVA with a two-tailed student's t-test post-hoc analysis and ordered difference report for each day of fermentation was generated in determine ethanol content significance (Table S2). The gap between fermentation rates declined over time trending towards non-significance between all samples if more time was allotted. The fermentations were stopped as the fermentation rate slowed down and a fair amount of ethanol 10%+ was produced. The fermentation trials were designed with time constraints as this is often a major factor in production settings and attenuation was secondary. The final alcohol yield at day 14 was significant between *S. cerevisiae* and the hybrids ($p = 0.038$), while *S. arboricola* was not significantly different than either hybrid nor *S. cerevisiae*. Hybrid SAM9a had the most productive fermentation rate, followed by hybrid SAM8c, *S. arboricola* 350, and *S. cerevisiae* M22. The mean final alcohol content of sake produced was 12.92% for the hybrids, 12.15% for *S. arboricola* 350, and 11.46% for *S. cerevisiae* M22.

Detection of volatile aromatic compounds allows insight into flavor and sensory attributes of the final sake produced and HS-SPME technology in the gas chromatography system was utilized. Sake fermented with *S. arboricola* contained significantly less ethyl hexanoate (syn. ethyl caproate) (0.207 mg mL^{-1}, st error 0.016) than *S. cerevisiae* (0.316 mg mL^{-1}, st error 0.019, $p = 0.005$), or the hybrids (0.308 mg mL^{-1}, st error 0.014, $p = 0.003$) (Figure 3B). Sake fermented by *S. arboricola* contained a concentration of isoamyl acetate (0.368 mg mL^{-1}, st error 0.064) that was high compared to *S. cerevisiae* (0.259 mg mL^{-1}, st error 0.064, $p = 0.25$) and the hybrids (0.252 mg mL^{-1}, st error 0.045, $p = 0.17$), but fell short of a significant *p*-value (Figure 3C). Ethyl hexanoate imparts apple or aniseed and isoamyl acetate imparts a banana-like attribute to human sensory receptors. Whole metabolite heatmap by sample was created showing a separation of *S. arboricola* from the hybrids and M22 individual samples when volatile compounds were used (Figure S3A) or a separation of all three when non-volatile metabolites were used (Figure S3B).

Sake fermentation profiles were visualized in a hierarchical clustering heatmap with hybrids clustering in closer proximity to the *S. arboricola* yeast (Figure 4A). Parental strain *S. cerevisiae* M22's sake metabolite profile contained high amounts of glucose, malic acid, threonine, and valine while parental *S. arboricola*'s profile contained high amounts of citric acid, aspartic acid, glutamic acid, leucine, isoleucine, alanine, phenylalanine, inositol, and glycerol. Parental profiles almost completely opposed one another with only serine, succinic acid, and urea showing similar parental concentrations. M22 was the most distant parent to the hybrids' profile. The heatmap of the hybrid strains showed a mosaic blending of the two parental species. Metabolomic analysis using principal components clearly separated interspecific hybrids from each parent based in a PCA score plot (Figure 4B). A total of 20 metabolites, including 17 non-volatile compounds, were identified by GC-FID and 3 volatile compounds identified by HS-SPME These were used as variables in construction of the loading plot (Figure 4C). Principal component (PC1) separated samples by their species and explained 43.2% of the variance between the samples. Fermentation profiles showed a separation of the two *Saccharomyces* species on opposite ends of PC1 and the hybrid yeast grouped to the middle. PC2 separated the hybrid from the parental samples by the novel hybrid fermentation features that explained 23.1% of the sample variance. Threonine, valine, malic acid, glucose, ethyl hexanoate, and dimethyl trisulfur (DMTS) positively contributed to PC1. Phenylalanine, isoleucine, leucine, alanine, aspartic acid, citric acid, glutamic acid, succinic acid, inositol, glycerol, ethanol, and isoamyl acetate were amino acids, organic acids, and other molecules that negatively contributed to PC1. Ethanol, DMTS, phenylalanine, ethyl hexanoate, glycerol, isoleucine, glutamic acid, valine, and threonine negatively contributed to PC2. Serine, succinic acid, malic acid, glucose, urea, isoamyl acetate, aspartic acid, inositol, and alanine positively contributed to PC2. A loading matrix was constructed for the variables (Table S3).

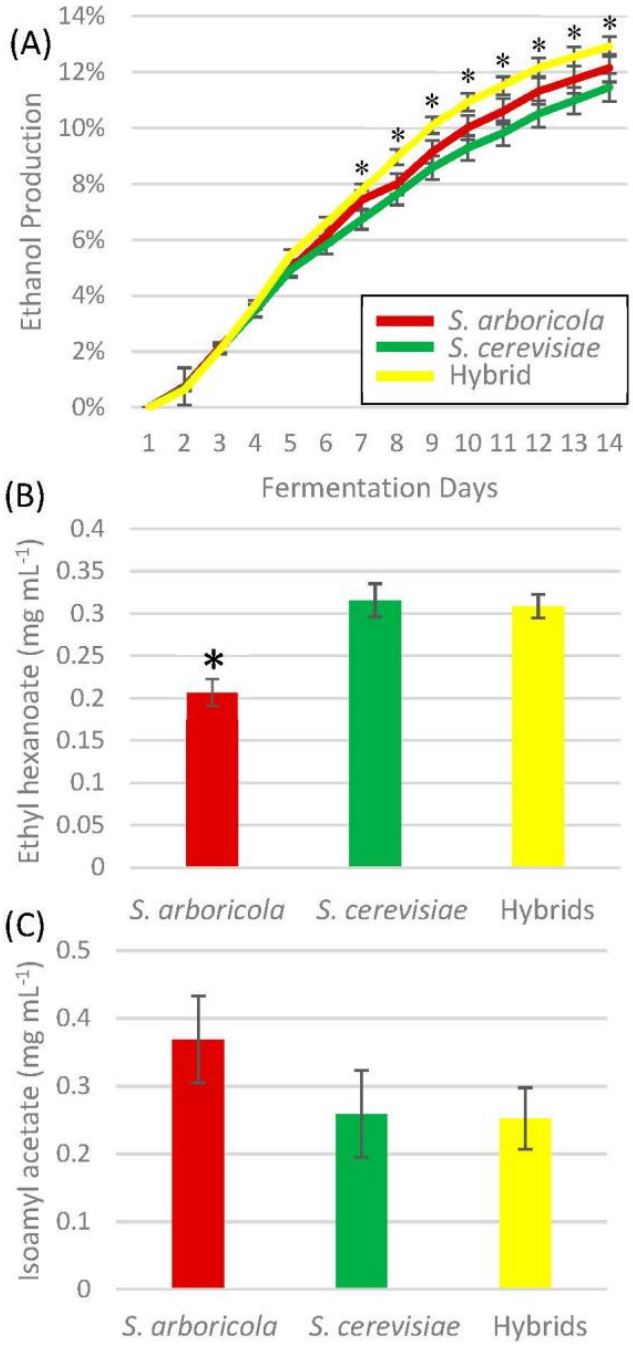

Figure 3. Phenotypic characteristics of hybrids and parental yeast. (**A**) Mean alcoholic fermentation rates of *S. arboricola* 350, *S. cerevisiae* M22, and the SAM hybrids are shown as a line graph representing the alcohol content over 14 days in time. Standard error bars shown in line graph (**B**) Bar chart depicting the production of ethyl hexanoate in mg mL^{-1}. (**C**) Bar chart depicting the production of isoamyl acetate in mg mL^{-1}. Statistical analysis performed by ANOVA, post-hoc students *t*-test, and post-hoc Tukey-HSD, $p < 0.05$.

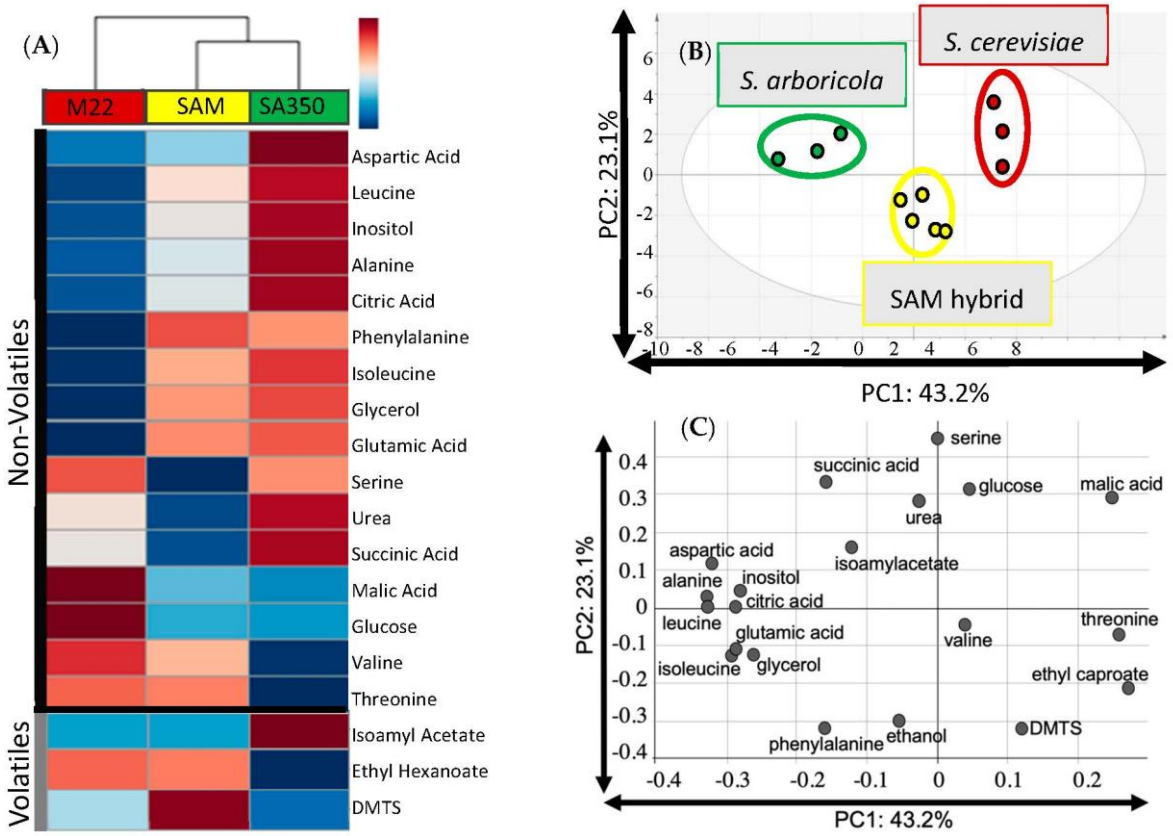

Figure 4. Sake fermentation profiles of *S. arboricola* interspecific hybrids and parental strains. (**A**) Hierarchical clustering heatmap of sake fermentation profiles between strains and species. Relatively high compound concentrations were shown in varying degrees of red. Relatively low compound concentrations were shown in varying degrees of blue. Shown as average concentration per sample groupings of three replicates each. (**B**) PCA score plot of sake fermentation profiles obtained for *S. cerevisiae*, *S. arboricola*, and the SAM hybrids. (**C**) PCA loading plot of sake fermentation profiles obtained for *S. cerevisiae*, *S. arboricola*, and the SAM hybrids. PC1 explained 43.2% and PC2 explained 23.1% of sample variance. PCA and loading plot generated through SIMCA analysis. Three replicates were used for each strain.

4. Discussion

This is the first report of sake brewing by *Saccharomyces arboricola* and its hybrids. The exploratory metabolic analysis of *S. arboricola*, a neglected *Saccharomyces* yeast lacking metabolic evaluation, adds to the novelty of this study. Through direct mating of *S. arboricola* to *S. cerevisiae*, interspecific hybrids gained a higher final ethanol content over *S. cerevisiae* and a higher ethyl hexanoate over *S. arboricola*. It was found that the low levels of endogenous amino/organic acids clustered *S. arboricola* apart from the industrial strains by principal component analysis and hierarchical clustering heatmapping. In sake fermentations, hybrid strains showed a mosaic profile of parental strains except where parental profiles were similar. This metabolic analysis suggested *S. arboricola* had higher transportation activity of pyruvate to the mitochondria and a lower amino acid net uptake than *S. cerevisiae*. Additionally, hybrid metabolic analysis suggested an increase in ethanolic fermentation from pyruvate and increased sulfur metabolism. Together, these results suggest *S. arboricola* is poised for in-depth and focused metabolomic exploration.

Endogenous metabolite comparison of nine industrially relevant and diverse yeast by GC-MS provided a separation of metabolomic profiles where industry and species appeared to be drivers of distinction in both heat map clustering and PCA. The internal composition is characteristic of their metabolic tendencies and influences the environment in which many endogenous metabolic

reactions occur. Of the industrial yeast, sake yeast separated from beer yeast by PC2 of the PCA (Figure 1B) which is characterized by the glucose to fructose ratio, inositol level, as well as the organic and amino acid profile. The endogenous inositol levels were high in sake yeast and affect membrane fluidity during osmotic stress such as an industrial sake mash. Sugar utilization played a major role in separation of the strains as evident in the loading plot which highlighted the importance of sugar metabolism. Metabolic distinctions between the glucose to fructose ratio of beer to sake or wine yeasts can be inferred to resemble the kinetic differences of hexokinase-mediated sugar phosphorylation. The glucose to fructose ratio between wine strains was correlated with the kinetics of hexokinase-mediated sugar phosphorylation and the inherent properties of each unique strain [62,63]. High amounts of endogenous inositol and trehalose were characteristic of *S. arboricola*. Furthermore, serine and glycine were found to be low in the *S. arboricola* cluster and are important glycolytic metabolites biosynthesized from 3-phosphoglycerate or alternatively through the gluconeogenic pathway utilizing glyoxylate [64]. Organic and amino acid metabolism shift between alternative biosynthesis pathways and their byproducts likely play a role in the difference seen between strains. Additional genetic and environmental factors are also suspected to play a role in strain metabolomic profiles as seen by increased copy number of *MAL* (maltose) genes in sake and beer yeast, yet wine strains tend to lose this attribute because their typical industrial fermentation sugar availability lacks maltose [65]. Copy number variations (CNV) are concentrated in the sub telomeric regions, with chr. I, VII, VIII, IX, X, XII, XV, and XVI being more susceptible to variation [65]. Gene ontology related to carbon and nitrogen metabolism, ion transport, and flocculation are prone to CNV [66,67] which often results in deletions rather than amplifications of genetic material [65]. The CNVs are thought to underline niche adaptation and domestication [65,66].

Flavor-active elements formed during yeast fermentation can be divided into five key groups: sulfur-containing molecules, organic acids, higher alcohols, carbonyl compounds, and volatile esters [68–71]. Of these classifications, volatile esters are the largest and are responsible for the highly desired fruity character in several alcoholic beverages [72]. Production of ethyl hexanoate and isoamyl alcohol is maximized during fermentations at 15 °C [73,74]. Ethyl hexanoate imparts apple or aniseed and isoamyl acetate imparts a banana-like attribute to human sensory receptors. Ethyl hexanoate, a MCFA (medium chain fatty acids) ester with a 0.17–0.21 mg mL^{-1} flavor threshold, is an important flavor molecule for ginjo sake [68,75]. The fruity ester, formed from hexanoic acid or hexanoyl CoA with ethanol, imparts a light, sweet, apple complexity to the delicate and fragrant beverage. Ginjo sake is produced from rice with the outer 40% of the grain polished away to remove the hull, bran, and part of the starchy endosperm, leaving 60% of the rice to create the sake mash. During the sake fermentation trials, *S. arboricola* produced significantly less ethyl hexanoate than either the hybrid or *S. cerevisiae* wine strain M22. Increased inositol concentrations have been shown to decrease production of ethyl hexanoate during fermentation [76]. This relationship was seen in PC1 of the hybrids and suggests *S. arboricola*'s inositol biosynthesis pathway is more active, in turn producing low ethyl hexanoate levels seen in this study. Additionally, *S. arboricola*'s production of isoamyl acetate showed a trend towards a significant increase over the hybrid or *S. cerevisiae* M22 (Figure 3C). Isoamyl acetate, an acetate ester with a 0.6–1.2 mg mL^{-1} flavor threshold, imparts a distinct banana character typical of hefeweizen yeast [68]. Hybrid strains produced a pleasant aromatic profile containing both ethyl hexanoate and isoamyl acetate. Ethanol hexanoyl transferase, Eht1, is believed to catalyze the formation of ethyl hexanoate [68]. Alcohol acetyltransferase genes Atf1 and Atf2 are responsible for the total biosynthesis of isoamyl alcohol and divergence in the Atf proteins has been suggested to influence the whole ester aroma profile of yeast strains [72]. Protein identity between *S. arboricola* and *S. cerevisiae* for Atf1 was 81%, Atf2 was 36%, and Eht1 was 84%. Together the aromatic ester profile of *S. arboricola* was unique in the combination of low ethyl hexanoate and isoamyl alcohol concentrations are speculated to be owing from inositol lipid activity and variation in factors affecting Atf activity. This area of research is merits further investigation.

Interspecific hybrids are commonly employed in agricultural and horticultural settings where hybrid vigor provides an increased or transgressive phenotype [77]. In oenology *Saccharomyces* hybrids, such as *S. cerevisiae* x *S. kudrazevii*, are often employed [78,79]. Many times *Saccharomyces* hybrids are able to yield more ethanol during fermentation under lab controlled environments [80], and these results support previous findings. The use of *S. arboricola* in sake production is novel and no other study had *S. arboricola's* metabolism as the primary focus of the manuscript. To date several labs have investigated *Saccharomyces* hybrids, one study included *S. arboricola* in a beer brewing context while one other included *S. arboricola* among their hybrids in a biofuel context [80,81]. This research showed that in a sake mash, SAM interspecific hybrids produced a more vigorous fermentation in a short amount of time which may be beneficial if used or further developed in a production context. Of the range of temperatures for *Saccharomyces* growth, *S. arboricola* is cryotolerant and thermosensitive [2,80,82]. Cryotolerance is a common attribute of yeast hybrids as seen in the lager yeast *Saccharomyces pastorianus*, which is a hybrid between *S. cerevisiae* x *S. eubayanus* [7,83–85]. This phenotype has proven beneficial for the cold fermentations typical of lagering beer or brewing sake. The ability to ferment at colder temperatures allows for more volatile compounds to be retained and a decrease in bitter or acidic taste [86]. The mechanism by which thermal phenotypes are determined has not been fully elucidated, but mitotype, ploidy, and lipid composition are factors [80,86–91]. Lipid profiles with a higher number of MCFA attributes to cryotolerance of *S. cerevisiae* x *S. kudriavzevii* hybrids [92]. At colder temperatures, increasing the MCFA composition and reducing saturated and unsaturated fatty acid composition allows increased fluidity of the membranes.

Previous reports show that wine yeast produce lower amino acidity and less succinic acid, but more malic acid than sake yeast [93]. Yeast strain has a major impact on metabolic profiles as seen in this study's two wine strains. During fermentation, organic acid production is attributed to the metabolism of pyruvate in the mitochondria and the reverse tricarboxylic acid cycle (TCA) cycle in the cytoplasm [94,95]. Most succinic acid and malic acid are produced through mitochondrial TCA cycle, however reductive TCA can supplement their production during sake fermentation [95]. These amino and organic acidity profiles are metabolically unique to individual strains and these may contribute a favorable fresh acidity such as malate while succinate contributes an unfavorable acidity to sake. During sake metabolic analysis, PC1 was inferred to represent the amino acid net uptake and mitochondrial transportation of pyruvate which contributes to the amino and organic acidity profiles (Figure 4C). Of the sake metabolites detected, 75% of the compounds were produced in high abundance in the sake by *S. arboricola* as compared to *S. cerevisiae* which produced 44% of the compounds in high amounts. *S. arboricola* was considered to have a lower amino acid net uptake than *S. cerevisiae* strains in this study. The abundance of residual amino acids detected in the sake medium and the low abundance of endogenous metabolites were considered in these findings. Moreover, membrane fluidity and amino acid and organic acid transport of *S. arboricola* has not been investigated and merits further research.

The generated interspecific hybrids were considered to have vigorous glycolysis and fermentation of pyruvate to ethanol compared to parental strains. The reduced malic acid, succinic acid, and glucose (Figure 4A) compared to the increased ethanol yield (Figure 3A) supported these claims. The hybrid condition was distinctly exemplified in principal component 2 of the sake metabolite score plot. In this study, where parental metabolic profiles were similar, the hybrids revealed an inversion of these compounds in the heatmap as seen in DMTS, serine, urea, and succinic acid. DMTS is associated with the stale aroma of Japanese sake called hineka. Recycling of sulfur via the methionine salvage pathway produces DMTS-P1, a precursor of this aromatic compound [96]. Mercaptans (thiols) typically have a low sensory threshold and the hybrids were considered to have an active sulfur metabolism. Additionally, urea is a yeast waste product from arginase activity and can be catabolized to form succinate in the cell [97]. Mechanistic elucidation of these metabolic variances is beyond the scope of this initial report of sake fermentation by *S. arboricola* and the hybrids, further research is required.

Research for *S. arboricola* yeast is very limited and one other report [80], to the author's knowledge, has explored their use and their interspecific hybrids' use in beverage fermentation. During their wort fermentation trials, *S. arboricola* produced high levels of ethyl hexanoate at 0.35 mg mL^{-1}, while this study revealed low production at 0.207 mg mL^{-1} by substituting sake mash for wort. Yeast strain and raw ingredients certainly play a role in the final fermentation product qualities. The chemistry of sake and beer have some similarities, but there are also many differences including raw materials such as rice or barley and the presence of *A. oryzae* in the sake mash. These results indicate koji was an integral part of the sake mash converting rice carbohydrate into fermentable sugars. *Aspergillus* spp. produce mannitol at high levels [46,47] and *S. arboricola* has great growth utilizing mannitol [14], but the bioavailability of this sugar for fermentation by *S. arboricola* remains unexplored. This metabolic capability likely facilitated the increase in fermentation performance seen in this study. *S. arboricola* and their hybrids may be uniquely fit for use in sake fermentations.

In two recent large phylogenetic analysis of 157 and 1011 *Saccharomyces cerevisiae* strains, an Asian cluster was found basal to the root of the trees [34,65] suggesting little divergence from their wild ancestors in comparison to the highly diverged beer1 clade. Although 1011 genome study was a great step in unmasking the lost ecology and domestication of natural *Saccharomyces* yeast, it had an overrepresentation of beer brewing strains. In order to dive deeper into the Out of Asia Origin Hypothesis, many more Asian and diverse strains will need to be sequenced and compared phylogenetically. The investigation into *S. arboricola*'s biotechnological value yielded promising results, but the dynamics between *A. oryzae*'s mannitol production and *S. arboricola*'s mannitol utilization merits further research. Exploration of the biotechnical potential of the *Saccharomyces* clade is only now beginning and understanding the diversity of yeast facilitates humanity's exploitation of *Saccharomyces*.

5. Conclusions

This study explored the novel use of *S. arboricola* in Japanese rice wine known as sake, traditionally produced from polished rice with *A. oryzae* during winter months. The metabolites in the sake produced from *S. arboricola*, *S. cerevisiae*, and their interspecific hybrids were distinct by PCA analysis. *S. arboricola* had a lower amino acid net uptake than *S. cerevisiae* and the hybrids showed a mosaic profile of parental strains. Hybrid strains had a more vigorous ethanolic fermentation in the sake mash than *S. cerevisiae* but had a higher ethyl hexanoate yield than *S. arboricola*. In the ginjo style of Japanese sake, ethanol and ethyl hexanoate are important sensory metabolic compounds. *S. arboricola* differed from *S. cerevisiae* industrial strains because of lower levels of endogenous amino/organic acids, suggesting a lower amino acid net uptake. The endogenous metabolite study highlighted the impact of strain selection which industry niche has unwittingly had an influence in each strains metabolism. The diversity of the *Saccharomyces* clade of budding yeast is expansive and future studies focused metabolic phenotypes of new strains and novel applications will most likely surface in the following years to come.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2311-5637/6/1/14/s1.

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