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Culture-based analysis of fungi in leaves after the primary and secondary fermentation processes during Ishizuchi-kurocha production and lactate assimilation of *P. kudriavzevii*

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ABSTRACT

Ishizuchi-kurocha is a Japanese traditional fermented tea that is produced by primary aerobic and secondary fermentation steps. The secondary fermentation step of Ishizuchi-kurocha is mainly mediated through lactic acid bacteria. Here, we performed quantitative analyses of the culturable fungal communities at each step and identified several morphologically representative fungal isolates. While filamentous fungi (median, 3.2×10^7 CFU/g sample) and yeasts (median, 3.7×10^7 CFU/g) were both detected after the primary fermentation step, only yeasts (median, 1.6×10^7 CFU/g) were detected in the end of the secondary fermentation step, suggesting that the fungal community in tea leaves are dramatically changed between the two steps. *Pichia kudriavzevii* and *Pichia manshurica*, the prevalent fungal species at the end of the secondary fermentation step, grew well in exudate from the secondary fermentation step. *P. kudriavzevii* also grew well in media containing D- or L-lactate as the sole carbon source. The growth of the disruptant of *cyb2A* encoding a cytochrome b_2 lactate dehydrogenase in *P. kudriavzevii* was severely impaired on medium supplemented with L-lactate, but not D-lactate, suggesting that Cyb2Ap plays a crucial role in the use of L-lactate, and *P. kudriavzevii* efficiently uses both L- and D-lactate as carbon sources. Thus, lactate assimilation seems to be a key phenotype to become a prevalent species in the secondary fermentation step, and Cyb2Ap has a pivotal role in L-lactate metabolism in *P. kudriavzevii*. Further understanding and engineering of *P. kudriavzevii* and *P. manshurica* will contribute to the control of lactic acid bacteria fermentation during the fermented tea production and also to other industrial uses.

1. Introduction

Tea, which is among the most common beverages worldwide, is produced from the leaves of the tea tree, especially two prominent varieties, *Camellia sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. While the processing of green tea does not involve fermentation, the production of four types of traditional teas (Ishizuchi-kurocha, Goishi-cha, Awa-bancha, and Batabata-cha) in Japan, as well as Pu-erh tea (Yunnan, China), Lahpetso (Myanmar), and Miang (northern Thailand) include post-fermentation processes (Horie et al., 2017).

The manufacturing processes among Japanese post-fermented teas have important differences (Horie et al., 2017). For example, the production of Batabata-cha, similar to Pu-erh tea, includes an aerobic fermentation process by fungi. On the other hand, Awa-bancha production includes a fermentation process by lactic acid bacteria (LAB). Different from these post-fermented teas, Ishizuchi-kurocha and Goishi-cha include two fermentation steps. The primary fermentation step is aerobic, in which filamentous fungi play a pivotal role, while the secondary fermentation step is driven by Lactobacilli as the major contributors (Horie et al., 2017).

Abbreviations: CFU, colony forming unit; ITS, internal transcribed spacer; LAB, lactic acid bacteria; NBRP, National Bio-Resource Project; PCR, Polymerase Chain Reaction; PDA, potato dextrose agar; YNB, yeast nitrogen base

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The constituents of the fungal community of Pu-erh tea have been intensely investigated. Zhao et al. (2010) showed that the dominant filamentous fungal genus was *Aspergillus*, especially *Aspergillus niger*. More recently, Zhang et al. (2016a) reported that the dominant species in tea aged through a high-temperature pile-fermentation process were *A. niger*, *A. fumigatus*, *Rhizomucor pusillus*, and *Rasamsonia emersonii*. Similarly, culture-independent analysis conducted by Zhang et al. (2016b) found that the most abundant fungal species in raw or ripened Pu-erh tea were *Aspergillus* sp., including *A. niger*, and *Blastobotrys adeninivorans*. Although Tamura et al. (1994) characterized the microbial communities in the fermentation steps during the production of Ishizuchi-kurocha, Goshi-cha, and Awa-bancha, the fungi were only identified at the genus level, which included *Aspergillus*, *Penicillium*, and *Mucor*. Following that publication, Okada et al. (1996) isolated the filamentous fungi *A. fumigatus*, *A. niger*, *Penicillium* sp., and *Scopulariopsis brevicaulis*, and the yeast *Debaryomyces hansenii* from the primary and secondary fermentation steps, respectively, and identified the isolated molds by morphological observation, but not genetic identification.

LAB are major players in the secondary fermentation step (Horie et al., 2019), and changes in culture or media conditions such as metabolite production including lactate and pH changes from the primary fermentation step suggest that the prevalent fungal species in the secondary fermentation step exhibit distinct advantages over the prevalent species in the primary fermentation step. Ueno showed that the use of lactate by *Candida glabrata* was important for its adaptation in a mouse intestine (Ueno et al., 2011), suggesting this utility to be an important factor for prevailing. Hence, we focused on lactate utility of a prevalent fungal species in the secondary fermentation step. Cyb2p is an L-lactate cytochrome *c* oxidoreductase (cytochrome b_2) that catalyzes the conversion from L-lactate to pyruvate and the reverse reaction. Cyb2p is essential for the utilization of L-lactate by *Saccharomyces cerevisiae* (Guiard, 1985).

In this study, the culturable fungal communities in the primary and secondary fermentation steps of Ishizuchi-kurocha processing were quantitatively analyzed. In parallel, the fungal species involved in both fermentation steps were identified. The results showed that *Pichia kudriavzevii* (*Issatchenkia orientalis*, NCBI: txid4909) and *P. manshurica* (NCBI: txid121235) were the prevalent fungal species from tea leaves during the secondary fermentation step. Also, disruptants of *cyb2A* and homologues of *P. kudriavzevii* were prepared to assess fungal growth in media with L- or D-lactate as the sole carbon source.

2. Materials and methods

2.1. Tea leaf samples

Tea leaf samples from the end points of the primary fermentation step (26 samples obtained at four to six days after the fermentation) and secondary fermentation step (11 samples obtained at 12 to 25 days after the fermentation) produced between June to October in 2015, 2016, and 2017 were kindly provided from three manufacturers of Ishizuchi-kurocha in Saijo City, Ehime Prefecture, Japan.

2.2. Colony counting and isolation of fungal strains

To determine the colony forming units (CFU) of fungi, 10 g of tea leaves from each sample were weighed into filtrate Bag II (Eiken Chemical Co., Ltd., Tokyo, Japan), suspended in a 9-fold weight of sterilized water, and homogenized at 230 rpm for 1 min with a Stomacher 400 Circulator paddle blender (Seward Ltd., Worthing, UK). Then, 500 μL of each stomached sample were serially diluted by tenfold (10^{-1} to 10^{-6}) with sterilized water. Each 100-μL sample was loaded on plates containing potato dextrose agar (PDA) supplemented with 100 μg/mL of chloramphenicol, and incubated at room temperature for 2 days. Plates in which 15–200 colonies appeared were used for counting.

Several representative colonies determined morphologically from each sample were transferred to fresh PDA slants for preparation of genomic DNA. *A. niger* YM1 and *Meyerozyma caribbica* YM9 from tea leaves of the primary fermentation, and *P. kudriavzevii* (*P. kudriavzevii*) YM48 and *P. manshurica* YM63 from tea leaves of the secondary fermentation that were isolated in 2015 were used for growth analyses, as described below. *Saccharomyces cerevisiae* S288C, provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology of Japan was also used in this study.

2.3. Genomic DNA preparation and Sanger sequencing

Genomic DNA preparation, PCR amplification, and Sanger sequencing were performed as described previously (Onishi et al., 2017; Toyotome et al., 2019).

2.4. Preparation of yeast nitrogen base (YNB) liquid media supplemented with various carbon sources and filtered exudate from a secondary fermentation sample

YNB with ammonium sulfate (MP Biomedicals, LLC., Santa Ana, CA, USA) liquid media containing 2% (wt/vol) glucose (YNBG), 2% (vol/vol) L-lactate (YNBLL), or D-lactate (YNBDL) were prepared. YNBLL and YNBDL media were adjusted to pH 7.0 or pH 4.0 with 4 M NaOH before autoclaving.

Air was removed from leaves with weight stone during the secondary fermentation step. Exudate (acidity around 10%) was recovered from a bucket after the secondary fermentation and filtered with a 0.45-μm filter. The filtered exudate was stored at −25 °C until use.

2.5. Growth analysis in liquid media and exudate

The spores of *A. niger* were collected in 1 mL of 0.9% NaCl solution. *P. kudriavzevii*, *P. manshurica*, and *M. caribbica* were cultured in YNBG at 37 °C overnight, then the cells were collected and re-suspended in 0.9% NaCl solution. The spore or yeast concentration in each suspension was determined by cell-counting with an improved Neubauer hemocytometer.

The concentration of each suspension was adjusted to 4×10^5 cells/mL in each liquid medium. A 100-μL aliquot of each cell suspension was added to the wells of Round- and Flat-Bottomed 96-Well Test Plates with lid (AS ONE Co., Osaka, Japan) for endpoint analysis and for growth curve analysis, respectively. In YNBG, YNBLL, or YNBDL, the fungal cells were cultured at 37 °C. For endpoint analysis, the AnaeroPack-MicroAero cultivation system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) was used, and after culture for 48 h, the optical density (OD) in each well was determined with a GENios Pro Multifunction Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). For the growth curve analysis, plates covered with a clear plate seal was incubated at 37 °C in the microplate reader, and OD at 620 nm was determined every hour until 72 h after inoculation. Wells without fungal cells were used as blank samples.

2.6. Gene disruption in *P. kudriavzevii*

Gene disruption in *P. kudriavzevii* was performed as described below using the primers shown in Table A.1. Plasmids pHM874-8 (NBRP ID: BYP8929) and pHM785-1 (NBRP ID: BYP8919) provided by NBRP were used as templates of the hygromycin B and G418/kanamycin resistance genes, respectively. The upstream and downstream flanking genomic regions at 1 kb from the target gene *cyb2A* (JL09_g341 in *P. kudriavzevii* SD108) and the homologue *cyb2B* (JL09_g1432 in *P. kudriavzevii* SD108) and *cyb2C* (JL_09_g3241) were fused by the double-joint PCR method (Yu et al., 2004) after amplification of the resistance genes with each specific primer pair.

The fused constructs were transformed into *P. kudriavzevii* YM48 using an electroporation method (Zemanova et al., 2004) with slight modifications. Yeast cells were pretreated with 10 mM dithiothreitol before electroporation and electric pulses were applied using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA) and a 2-mm cuvette (Nepa Gene Co., Ltd., Chiba, Japan) at 2.5 kV, 200 Ω, and 25 μF. After electroporation, transformants were selected with 400 μg/mL hygromycin B (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) or 400 μg/mL hygromycin B and 400 μg/mL G418 (FUJIFILM Wako Pure Chemical Co.). The colonies were picked-up and the remaining gene copies were determined using a relative quantification by real-time PCR. The primers listed in Table A.1, THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan), and LightCycler 480 Instrument II (Roche Diagnostics, Rotkreuz, Switzerland) were used for real-time PCR. Briefly, the *P. kudriavzevii* YM48 genome was serially diluted and standard curves for a housekeeping gene *act1* and each *cyb2* gene were determined from cycle threshold (Ct) values. Ct values of disruptants based on the determined standard curves were used for relative quantification. As shown in Fig. A.1, quantification of gene copies indicated that each *cyb2* gene in single disruptants was half of that of *act1* gene copies, indicating that a single copy of each gene was disrupted. Since no gene copy was present in each double disruptant, each *cyb2* gene was not detected (Fig. A.1).

3. Results

3.1. Fungal community shifts from molds and yeasts in the primary fermentation step to LAB and yeasts in the secondary fermentation step

Culturable fungal cells were quantified at the end of each fermentation step (Fig. 1A). The median of fungal cell number was 8.8×10^7 (range, 2.2×10^6 to 7.9×10^8) CFU/g sample in tea leaf samples from the primary fermentation. In the primary fermentation, yeasts and molds were observed as culturable cells (Fig. 1B). Molds were detected in every sample. On the other hand, yeasts were not detected in 3 (11.5%) of 26 samples, suggesting that molds are essential to the aerobic fermentation step, while yeasts are auxiliary. In contrast to the primary fermentation samples, only yeasts were detected in all secondary fermentation samples (median number, 1.6×10^7 CFU/g sample; the range was from 8.5×10^4 to 4.8×10^8 CFU/g sample), suggesting that yeasts support the process with LAB in the secondary fermentation.

The culturable fungal species in the primary fermentation step were identified morphologically to elucidate the abundance (Fig. 1C). *Aspergillus* Section *Nigri* was the major mold in 23 (88.5%) of 26 primary fermentation samples. Internal transcribed spacer (ITS) sequencing of the picked-up colonies confirmed the presence of *A. niger sensu lato*, but not *A. tubingensis*. In 2 (7.7%) of 26 samples, *A. fumigatus* was the major mold species. Zygomycetes (*e.g.*, *Mucor* and *Rhizopus*) were found in some tea leaf samples. Overall, 43 yeast strains were obtained from fermented leaf samples after the primary fermentation including *Candida intermedia* (2 isolates), *Clavispora lusitaniae* (2), *Cutaneotrichosporon dermatis* (12), *Cyberlindnera saturnus* (1), *Debaryomyces hansenii* (2), *Dipodascus geotrichum* (2), *Meyerozyma caribbica* (4), *Rhodosporidiobolus ruineniae* (5), *Pichia kudriavzevii* (2), and *Pichia manshurica* (11). On the other hand, yeasts (43 isolates) isolated from the secondary fermentation samples except for one isolate (*Cutaneotrichosporon dermatis*) were *P. kudriavzevii* (16) and *P. manshurica* (26). These data suggest that *A. niger s.l.* and the yeasts *P. kudriavzevii* and *P. manshurica* play important roles in the primary and the secondary fermentation steps during Ishizuchi-kurocha production, respectively.

3.2. *P. kudriavzevii* have advantages in lactate-containing media

To clarify the reason why mycological abundance was shifted from the primary fermentation to secondary fermentation, we examined the growth of five fungal species in filtered exudate of the secondary fermentation. *A. niger* YM1 and *Meyerozyma caribbica* YM9 isolated from the primary fermentation samples, *P. kudriavzevii* YM48 and *P. manshurica* YM63 from the secondary fermentation samples, and *Saccharomyces cerevisiae* S288C were used in this experiment. As shown in Figs. 2 and A.2, *P. kudriavzevii* and *P. manshurica* grew better in the exudate from leaves in the secondary fermentation than the three other species analyzed here, indicating that *P. kudriavzevii* and *P. manshurica* were the prevalent species and have advantages in the environment of the secondary fermentation.

Because the exudate and fermented leaves contained large amounts of lactate produced by LAB, we assumed that efficient lactate assimilation by *P. kudriavzevii* and *P. manshurica* may be an advantage in the secondary fermentation. LAB produce large quantities of L-lactate and D-lactate, and lower the pH in the secondary fermentation. To examine the utilization of lactate, *P. kudriavzevii* and *P. manshurica* were cultured in YNB supplemented with L- or D-lactate (pH 7.0 or 4.0) and YNB supplemented with glucose as a control. As shown in Fig. 3 and Fig. A.3, *P. kudriavzevii* rapidly propagated in liquid media supplemented with lactate. Although *P. manshurica* propagated in L-lactate containing media (Fig. A.3), its growth was inefficient as compared with *P. kudriavzevii*. Further, the propagation of *M. caribbica*, *A. niger*, or *S. cerevisiae* in lactate containing media was slow (Fig. A.3).

3.3. Cyb2Ap of *P. kudriavzevii* has a pivotal role in L-lactate assimilation

We focused on the ability of the *cyb2* genes to assimilate lactate in *P. kudriavzevii*. CYB2 of *S. cerevisiae* (Guiard, 1985) and Cyb2p of *C. glabrata* (Ueno et al., 2011) are known as L-lactate dehydrogenases that convert L-lactate to pyruvate. A Protein BLAST search identified three homologues of *S. cerevisiae* CYB2 in *P. kudriavzevii* strain SD108, namely JL09_g341 (Cyb2Ap), JL09_g1432 (Cyb2Bp), and JL09_g3241 (Cyb2Cp), with Cyb2Ap having the highest homology. Next, disruptants of *P. kudriavzevii* YM48 were prepared. *P. kudriavzevii* was a diploid and harbored two copies of each *cyb2* gene. Elimination of both copies were confirmed by real-time PCR (Fig. A.1). As shown in Fig. 4, *P. kudriavzevii ΔΔcyb2A*, but not *ΔΔcyb2B* or *ΔΔcyb2C*, strongly impaired cell growth in YNBLL medium, suggesting that Cyb2Ap has a pivotal role in L-lactate assimilation. In contrast, all disruptants grew in YNBDL medium, suggesting that the three Cyb2 homologues are dispensable for D-lactate assimilation.

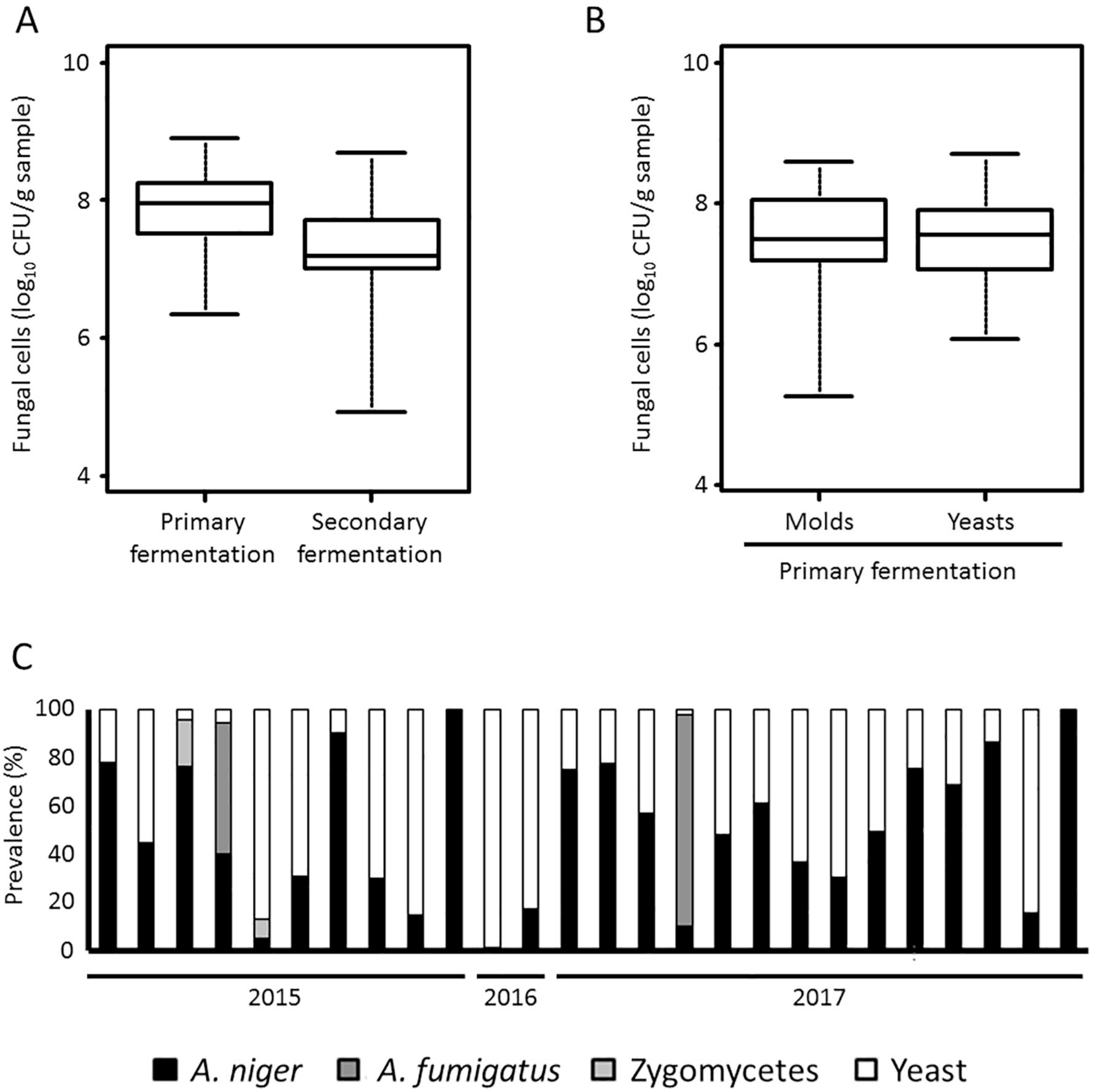

Fig. 1. The amount of culturable fungi in fermented tea leaf samples. CFUs per gram of total fungi (A) and molds and yeasts (B) in fermented leaf samples are shown in the box-plot graphs (C). The percentages of *A. niger*, *A. fumigatus*, zygomycetes, and yeasts in leaf samples from each primary fermentation step. The numbers shown below the graph indicate the manufacturing years of each sample.

4. Discussion

In this study, we quantified and qualified the fungal communities in the primary and secondary fermentation steps of Ishizuchi-kurocha production. Culturable filamentous fungi (median, 3.2×10^7 CFU/g sample) and yeasts (median, 3.7×10^7 CFU/g) were observed in the primary fermentation step, indicating that the numbers of filamentous fungi and yeasts were comparable (Fig. 1). In contrast, only yeasts were isolated from leaf samples of the secondary fermentation step. However, only aerobic culture was performed in this study, which presented the possibility that analysis of anaerobic fungi was lacking. As limitations of culture-based analysis, only culturable species can be identified, and molds may underestimate because of the tubular structure of hyphae. Nevertheless, more than 100 strains were obtained using the culture-based analysis from the primary and secondary fermentation samples and the genome data of *P. manshurica* YM63 have recently been released. Although it seems that other weaknesses may be a bias of the primer pair and remaining DNA derived from the primary fermentation step in the secondary fermentation step. However, the mycobiome analyzes by amplicon sequencing with next-generation sequencers are useful to obtain an overview of fungal flora that should be conducted in near future. The information on prevalent fungal species both fermentation steps serve as a good pilot guide of further mycobiome analyzes. *A. niger s.l.* was prevalent in most samples of the primary fermentation step. In an earlier study, *Rhizomucor variabilis* was isolated from Ishizuchi-kurocha (Yanai et al., 2008). In the present study, *R. variabilis* was not detected, although some zygomycetes species, including *Rhizopus* sp. and *Mucor* sp., were isolated from the primary fermentation samples. *A. niger* was reported as the major fungal species during the fermentation process of other teas, such as Pu-erh tea (Abe et al., 2008; Xu et al., 2005) and Goishi-cha (Okada et al., 1996), suggesting that the fungal community has changed over the period of several years from *Rhizomucor*-type to *A. niger*-type, similar to that of Pu-erh tea and Goishi-cha. *A. fumigatus* was dominant in two primary fermentation samples. Since fungal communities in the primary fermentation are unstable, as shown in Fig. 1C, the surveillance of fungal communities or the use of *A. niger* strains as a starter might prove useful for producing more stable products. Additionally, the opportunistic pathogen *A.*

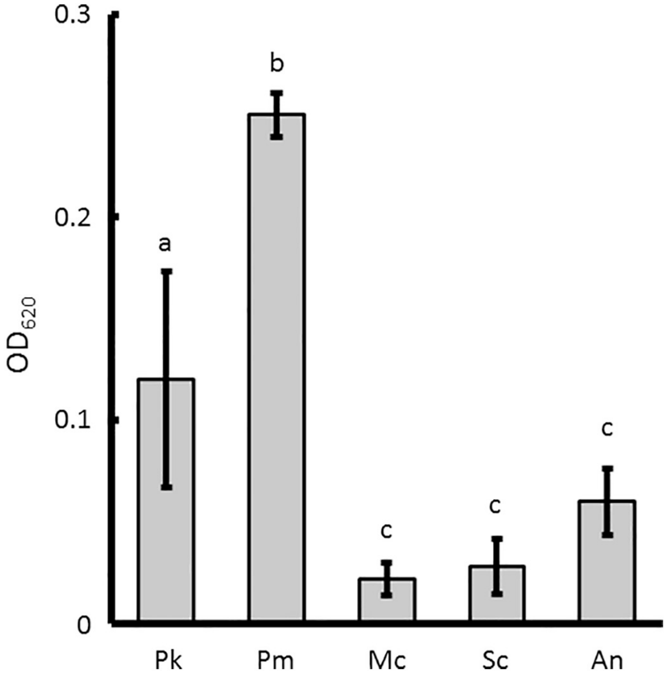

Fig. 2. Fungal growth in exudates from a secondary fermentation sample. The bars show the optical density (OD) at 620 nm after culturing for 96 h under microaerobic conditions. Pk, *Pichia kudriavzevii*; Pm, *Pichia manshurica*; Mc, *Meyerozyma caribbica*; Sc, *Saccharomyces cerevisiae*; An, *Aspergillus niger*.

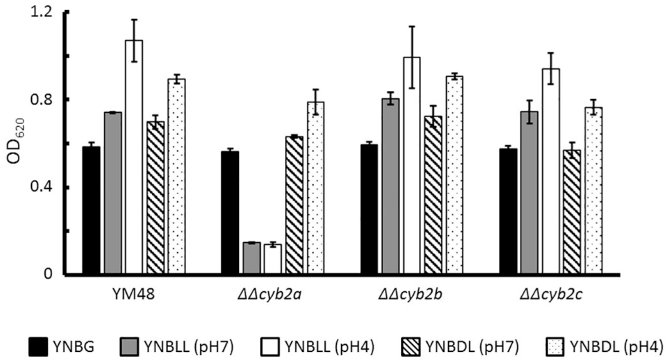

Fig. 4. Fungal growth of *cyb2A*, *cyb2B*, or *cyb2C* disruptant in media supplemented with glucose or lactate. The bars show the OD at 620 nm after culturing for 48 h under microaerobic conditions. Filled bars, YNBG; gray bars, YNBLL (pH 7); open bars, YNBLL (pH 4); hatched bars, YNBDL (pH 7); dotted bars, YNBDL (pH 4).

fumigatus was also found in few samples. A preliminary experiment using *A. oryzae* as a secure starter showed *A. oryzae* to be a prevalent species at the end of the primary fermentation step. Currently, we possess and identified 71 *A. niger* isolates and believe that the data and isolates obtained in this study will contribute to production of more stable Ishizuchi-kurocha end samples.

In contrast to the primary fermentation step, *P. kudriavzevii* and *P. manshurica* were isolated from the secondary fermentation step. Of the LAB, *Lactobacillus plantarum* was the most prevalent strain and a major player in the fermentation step (Horie et al., 2019). As reported in a review article by (Dalié et al., 2010), LAB and associated products can potentially control filamentous fungi, as some *L. plantarum* strains show antifungal activity (Russo et al., 2017), due to the antifungal potency of lactic acid. As shown in Fig. 2, the growth of *A. niger* was not detected in the cell-free exudate from the secondary fermentation, which is consistent with the findings of an earlier study (Russo et al., 2017). Propagation of the yeasts *S. cerevisiae* and *M. caribbica* was also not detected in the exudate for 48 h after inoculation. In contrast, *P. kudriavzevii* and *P. manshurica* were growable in the exudate. We hypothesized that this difference was due to the ability to assimilate lactic acid, rather than an antifungal effect. As shown in Fig. 3, examination of these yeast species and *A. niger* using synthetic media supplemented with different carbon sources indicated that *P. kudriavzevii* rapidly propagated in media supplemented with lactic acid. Notably, pH had no effect on growth. Conversely, *P. manshurica* propagated inefficiently on media supplemented with lactic acid compared with *P. kudriavzevii*. It is noteworthy that other compositions derived from tea leaves and metabolites produced by LAB might inhibit other fungi found in the primary fermentation and promote *P. manshurica* and *P. kudriavzevii*. The growth of *P. manshurica* was promoted particularly in the exudate of the secondary fermentation. In a further study, we will examine those components that promote this growth.

The ability of lactate assimilation was examined with the use of *cyb2* gene disruptants. A homologue of *CYB2*, a *cyb2A* disruptant of *P. kudriavzevii*, severely impaired growth on YNBLL, but not YNBDL. These data indicate that *cyb2A* has a pivotal role in L-lactate assimilation. DLD1 is the major mitochondrial D-lactate dehydrogenase in *S. cerevisiae*, which oxidizes D-lactate to pyruvate. A homologue was also found in *P. kudriavzevii*, suggesting that the enzyme has a role in D-lactate assimilation. Although we attempted the disruption of the gene, but no *dld1* disruptants were obtained. Therefore, further studies are warranted to determine whether *dld1* in *P. kudriavzevii* is important for assimilating D-lactate. *P. manshurica* is also a major fungus in the secondary fermentation and common in fermentations and food spoilage. *P. kudriavzevii* and *P. manshurica* are broadly used for industrial applications, especially food production (Douglass et al., 2018). The *P. kudriavzevii* and *P. manshurica* strains isolated in this study may provide further useful resources as starters for the secondary fermentation. We recently sequenced and released the genome data of *P. manshurica*

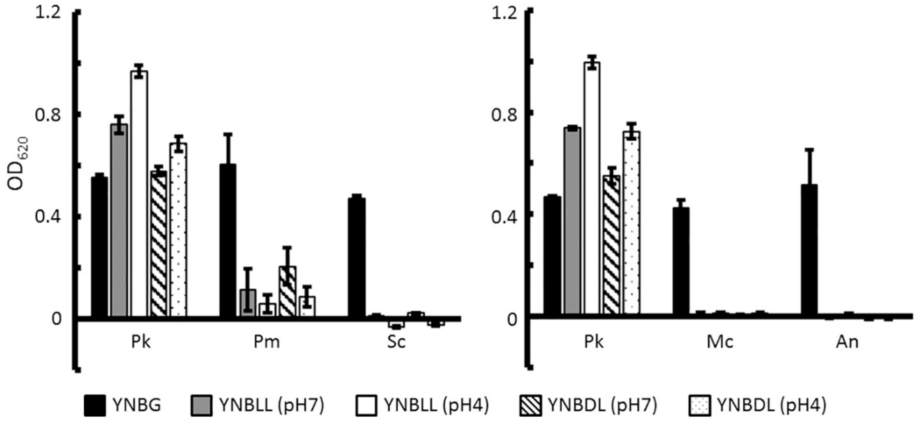

Fig. 3. Fungal growth in media supplemented with glucose or lactate. The bars show the OD at 620 nm after culturing for 48 h under microaerobic conditions. Filled bars, YNBG; gray bars, YNBLL (pH 7); open bars, YNBLL (pH 4); hatched bars, YNBDL (pH 7); dotted bars, YNBDL (pH 4). Pk, *Pichia kudriavzevii*; Pm, *Pichia manshurica*; Mc, *Meyerozyma caribbica*; Sc, *Saccharomyces cerevisiae*; An, *Aspergillus niger*.

YM63 isolated from the secondary fermentation step. Further understanding and engineering of lactate metabolism in *P. kudriavzevii* and *P. manshurica* will contribute to not only the control of LAB fermentation during the fermented tea production, but also other industrial uses.

In summary, we elucidated the culturable fungal communities and the shift between two fermentation steps during Ishizuchi-kurocha production. These results are useful reference data to produce Ishizuchi-kurocha stably and securely as well as to extend current knowledge. Our identification of the efficient propagation of *P. manshurica* and *P. kudriavzevii* in the secondary fermentation environment presents advantageous information about the fermentation steps. We also determined the gene *cyb2A* to play a pivotal role in L-lactate assimilation in *P. kudriavzevii*.

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Appendix A

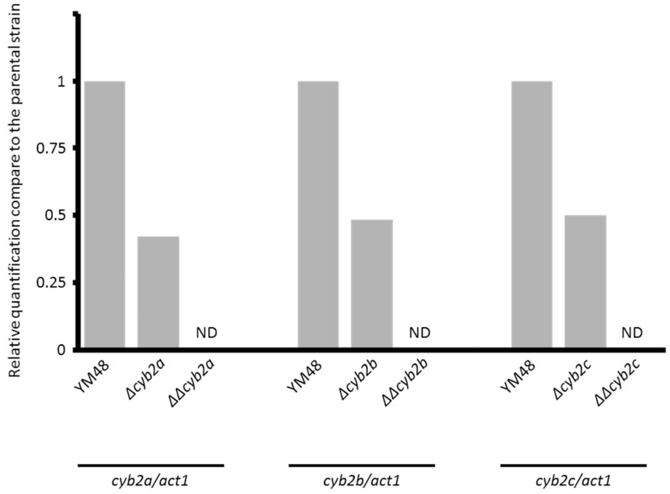

Fig. A.1. Quantification of each *cyb2* gene to confirm disruption. Each single disruptant (*Δcyb2*) possesses a half copies of each *cyb2* compared with the number of *act1* copies. Each *cyb2* gene was not detected in each double disruptant. ND, not detected.

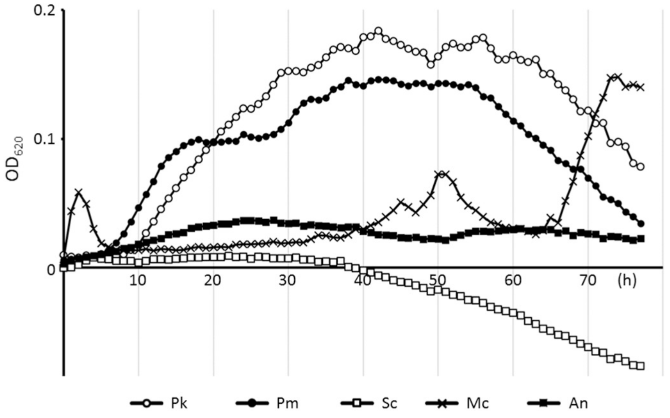

Fig. A.2. Growth curves of *Pichia kudriavzevii* (Pk), *Pichia manshurica* (Pm), *Saccharomyces cerevisiae* (Sc), *Meyerozyma caribbica* (Mc), and *Aspergillus niger* (An) in exudate from the secondary fermentation step. Those curves were drawn by averaging the data at each point of time across the two (Pk) or three (others) replicates.

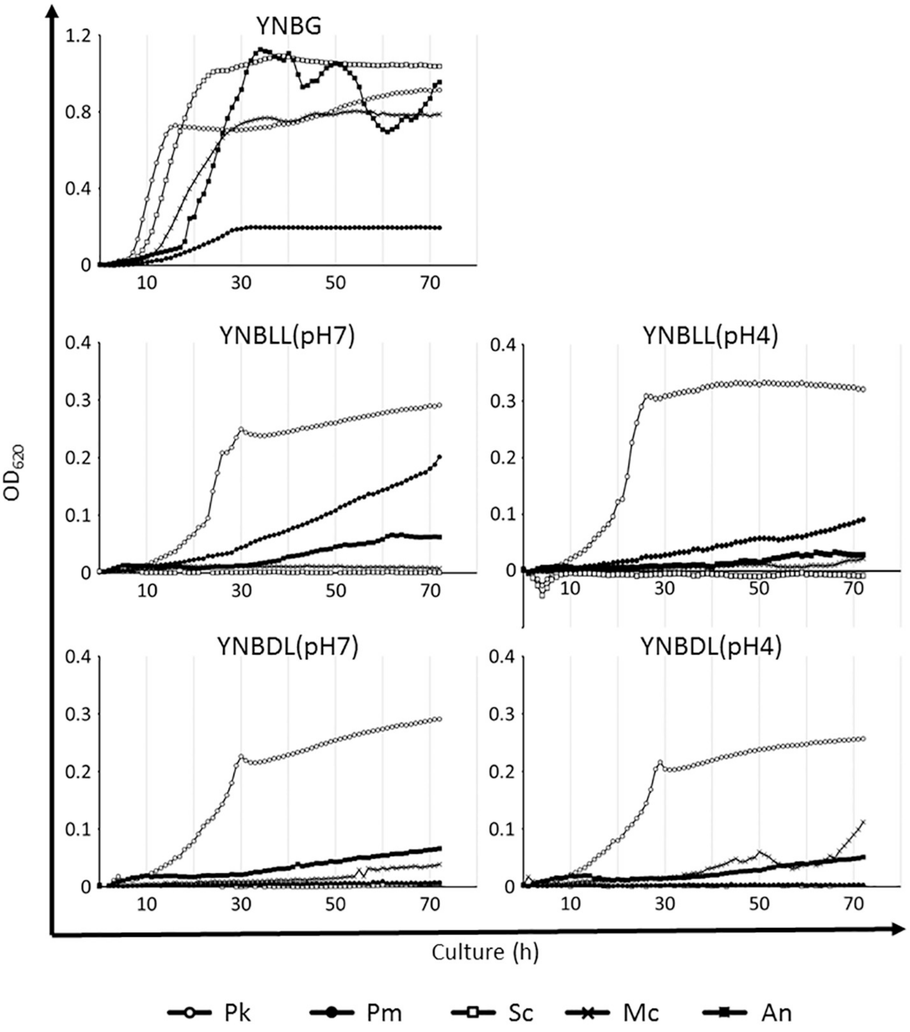

Fig. A.3. Growth curves of *Pichia kudriavzevii* (Pk), *Pichia manshurica* (Pm), *Saccharomyces cerevisiae* (Sc), *Meyerozyma caribbica* (Mc), and *Aspergillus niger* (An) cultured in YNBG, YNBLL (pH 7), YNBLL (pH 4), YNBDL (pH 7), or YNBDL (pH 4). Those curves were drawn by averaging the data at each point of time across the three replicates.

Table A.1
Primers used for this study.

Name	Sequence (5′ to 3′)	Purposes and reference
Primers for species identification		
ITS4	TCCTCCGCTTATTGATATGC	Primer to amplify and sequence ITS region. (White et al., 1990)
ITS5	GGAAGTAAAAGTCGTAACAAGG	Primer to amplify and sequence ITS region. (White et al., 1990)
NL1	GCATATCAATAAGCGGAGGAAAAG	Primer to amplify and sequence partial region of 28S rDNA. (Kurtzman and Robnett, 1997)
NL4	GGTCCGTGTTTCAAGACGG	Primer to amplify and sequence partial region of 28S rDNA. (Kurtzman and Robnett, 1997)
Primers for *cyb2A* disruption		
IoCyb2a-upF	ACTTGTTTTCCTCTTTCCCCTAGAG	Forward primer to amplify *cyb2A* upstream region in *P. kudriavzevii*.
IoCyb2a-upR	GGAGGGTATTCTGGGCCTCCATGTCTGTGTGCAACTAGGTTTATGTGGAG	Reverse primer to amplify *cyb2A* upstream region in *P. kudriavzevii* with 25-bp 5′-terminal region of hygromycin B resistance gene amplicon or G418/kanamycin resistance gene amplicon.
IoCyb2a-hygroRdownF	CTCGTCCGAGGGCAAAGGAATAGGTTTGTTGGAAATATATTATTCATAAAGGC	Forward primer to amplify *cyb2A* downstream region in *P. kudriavzevii* with 25-bp 3′-terminal region of hygromycin B resistance gene amplicon.
IoCyb2a-kanRdownF	CGTATGTGAATGCTGGTCGCTATACTGTTGTTGGAAATATATTATTCATAAAGGC	Forward primer to amplify *cyb2A* downstream region in *P. kudriavzevii* with 25-bp 3′-terminal region of G418/kanamycin resistance gene amplicon.
IoCyb2a-downR	TTAAACAACCCAACACAGACCATTC	Reverse primer to amplify *cyb2A* downstream region in *P. kudriavzevii*.
Primers for *cyb2B* disruption		
IoCyb2b-upF	CGTGCGCCTGGTCGATTTTCACCTTCTTTG	Forward primer to amplify *cyb2B* upstream region in *P. kudriavzevii*.
IoCyb2b-upR	GGAGGGTATTCTGGGCCTCCATGTCTTTGAAGGTATTATCACTGCTGTTG	Reverse primer to amplify *cyb2B* upstream region in *P. kudriavzevii* with 25-bp 5′-terminal region of hygromycin B resistance gene amplicon or G418/kanamycin resistance gene amplicon.

(*continued on next page*)

Table A.1 (*continued*)

Name	Sequence (5′ to 3′)	Purposes and reference
IoCyb2b-hygroRdownF	CACTCGTCCGAGGGCAAAGGAATAGGTGTCTGATATTTGCTAAATTGAAATGAAC	Forward primer to amplify *cyb2B* downstream region in *P. kudriavzevii* with 25-bp 3′-terminal region of hygromycin B resistance gene amplicon.
IoCyb2b-kanRdownF	CGTATGTGAATGCTGGTCGCTATACTGGTCTGATATTTGCTAAATTGAAATGAAC	Forward primer to amplify *cyb2A* downstream region in *P. kudriavzevii* with 25-bp 3′-terminal region of G418/kanamycin resistance gene amplicon.
IoCyb2b-downR	TCCTGATCTCACAGCACATTTCTAC	Reverse primer to amplify *cyb2A* downstream region in *P. kudriavzevii.*
Primers to amplify selection marker gene cassette		
TEFp-F	GACATGGAGGCCCAGAATACC	Forward primer to amplify hygromycin B resistance gene in pHM874-8 or G418/kanamycin resistance gene in pHM785-1.
Hygro-R	ACCTATTCCTTTGCCCTCGGACGA	Reverse primer to amplify hygromycin B resistance gene in pHM874-8.
KanR-R	CAGTATAGCGACCAGCATTCACATACG	Reverse primer to amplify G418/kanamycin resistance gene in pHM785-1.
Primers for *cyb2C* disruption		
IoCyb2c-upF	TCTTCAAGTCTCCCGATTCC	Forward primer to amplify the upstream region of *cyb2C* in *P. kudriavzevii.*
IoCyb2c-upR	TGGTCTCGTCAAAGTATTTGCAAGGAC	Reverse primer to amplify the upstream region of *cyb2C* in *P. kudriavzevii.*
IoCyb2c-downF	TTTACGAATCCATGTCTGATTCAAGTTGGG	Forward primer to amplify the downstream region of *cyb2C* in *P. kudriavzevii.*
IoCyb2c-downR	TCTCAAATGTTTTTCTTTTGATTTACTTAAATACTAA	Reverse primer to amplify the downstream region of *cyb2C* in *P. kudriavzevii.*
DIoCyb2c-F	CAATAGTTATTTCTGAGTTTCTTATGGCACATTGTCCTTGCAAATACTTTGACGAGACCAGACATGGAGGCCCAG	Forward primer to amplify hygromycin B resistance gene in pHM874-8 or G418/kanamycin resistance gene in pHM785-1 with 60-bp upstream region adjacent to *cyb2C* gene in *P. kudriavzevii.*
DIoCyb2c-R	AAGGGAGAAATGTGGAATACATTTTCACATCCCAACTTGAATCAGACATGGATTCGTAAACTTCGAGCGTCCCAA	Reverse primer to amplify hygromycin B resistance gene in pHM874-8 with 60-bp downstream region adjacent to *cyb2C* gene in *P. kudriavzevii.*
DIoCyb2cKanR-R	CCCAACTTGAATCAGACATGGATTCGTAAACAGTATAGCGACCAGCATTCACATACG	Reverse primer to amplify G418/kanamycin resistance gene in pHM785-1 with 25-bp downstream region adjacent to *cyb2C* gene in *P. kudriavzevii.*
Primers for real-time PCR		
IoCyb2a-F	TCGATTTGGGTGATTCCGCA	Forward primer to check the disruption of *cyb2A* in *P. kudriavzevii* by real-time PCR.
IoCyb2a-R	TTAGTCCACGACTTGACCGC	Reverse primer to check the disruption of *cyb2A* in *P. kudriavzevii* by real-time PCR.
IoCyb2b-F	TCTTTGTCACTGTGGACGCT	Forward primer to check the disruption of *cyb2B* in *P. kudriavzevii* by real-time PCR.
IoCyb2b-R	TGGCAATGCACCAGAGGTAG	Reverse primer to check the disruption of *cyb2B* in *P. kudriavzevii* by real-time PCR.
IoCyb2c-F	AACATTTTGAGGGCATGCGG	Forward primer to check the disruption of *cyb2C* in *P. kudriavzevii* by real-time PCR.
IoCyb2c-R	GGCGAGGCACTCTCCATAAA	Reverse primer to check the disruption of *cyb2C* in *P. kudriavzevii* by real-time PCR.
IoACT1-F	CCGGTTTTGCCGGTGATGAT	Forward primer to amplify partial *act1* in *P. kudriavzevii* by real-time PCR.
IoACT1-F	CCTTTTGGCCCATACCAACCA	Reverse primer to amplify partial *act1* in *P. kudriavzevii* by real-time PCR.

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