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EFFECTS OF DEFINED MIXED-FUNGI STARTER CULTURES ON THE NUTRITIONAL PROFILE AND SENSORY ATTRIBUTES OF FERMENTED RICE WINE LEES

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ABSTRACT

Rice wine (RW) and its fermentation residue, rice wine lees (RWL), are prepared using a traditional starter culture (TSC). The TSC contains a combination of different fungal and bacterial species that are variable in composition. This study evaluated the effects on the nutritional profile and the sensory qualities of RWL using various combinations of these fungal species as starter cultures. We isolated the three most abundant fungal species in TSC and found them to be *Rhizopus oryzae* (RO), *Mucor indicus* (MI), and *Saccharomyces cerevisiae* (SC). Although sensory evaluation revealed that the RWL produced using the RO+SC was the most preferred (n = 30) in color, aroma, sweetness, bitterness, and clarity, no significant differences were observed compared to when RO+MI+SC was used. Moreover, the RO+MI+SC-produced rice wine lees demonstrated the highest nutritional composition in terms of the following: crude protein content (11.12±0.08%), total energy (412.96±0.07 kcal/100 g), essential amino acids, and vitamin content. Hence, the combination of these three fungal species, RO, MI, and SC, as a starter culture in rice wine fermentation gives rise to rice wine lees with higher nutritional value than using the traditional rice wine starter culture.

Keywords: Traditional starter culture, *Rhizopus oryzae*, *Mucor indicus*, *Saccharomyces cerevisiae*, nutritional profile, sensory qualities.

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INTRODUCTION

Rice wine (from glutinous rice) is a commonly fermented alcoholic drink consumed in various Asian countries such as Japan, China, Korea, and the Philippines (Chao, 2002). Rice wine is mainly characterized by its alcoholic but sweet-acidic taste produced from the fermentation of polished rice, such as glutinous white rice and black pigmented rice (Sanchez, 2008). The simultaneous saccharification and fermentation of polished rice produce this rice wine (Dela Rosa & Medina, 2022; Sanchez, 2008). The rice wine is prepared using a traditional rice wine starter culture (TSC), which consists of dried discs of ground glutinous rice and added herbs (Sanchez et al., 1988; Tanimura et al., 1977). The distinct taste of this local rice wine is attributed to the TSC, which consistently produces quality rice wine. In the traditional rice wine-making process, the conventional starter culture dramatically influences the flavor, clarity, aroma, saccharifying power, alcohol content, shelf life, chemical and physical properties, and improved yield in rice wine production (Coronel et al., 1981).

The TSC is made from rice flour, ginger extract, wild grassroots (*Bidens pilosa*), and old TSC, the mother starter culture (Chay et al., 2020; Dizon et al., 2009). It contains microorganisms that convert the starch in rice to sugar and then sugar to alcohol simultaneously. These chemical processes are called saccharification and fermentation (Chao, 2002). However, the TSC is naturally made and prepared in an unsanitary process, introducing different microbial systems (Dizon et al., 2013). A previous study demonstrated that TSC contains beneficial and harmful microorganisms due to unreliable methods, unhygienic conditions, and lack of local producers' quality control and technical know-how. Moreover, species and quantities of microbial load differ from producer to producer and from one location to another, resulting in inconsistent quality products and a low degree of industrialization. (Dela Rosa & Medina, 2022; Dizon et al., 2009; Cai et al., 2019).

In the production of rice wine, a large amount of residue called the rice wine lees, is generated after filtration. RWL is discarded since its potential and use have not been thoroughly elucidated (Manaois & Morales, 2014). People consumed RWL as a snack, wherein sugar was added to it to add sweetness and flavor (Manaois & Morales, 2018). In some research, rice wine (*tapuy*) lees was used to produce flour, further utilized as a functional ingredient in *polvoron*, a Philippine snack food (Manaois & Morales, 2018). Moreover, the *polvoron* made from RWL has increased crude protein (45.03%) and dietary fiber (13.10%) levels without affecting the product's overall sensory quality and taste (Manaois & Morales, 2014). A separate study used RWL from different rice varieties in flour production. RWL flour was substituted for wheat flour in butterscotch. Results illustrated that incorporating RWL flour in butterscotch contained high levels of crude protein and dietary fiber, which may be used in developing high-fiber functional foods (Manaois & Morales, 2018). Despite these, like the rice wine, the sensory attributes and nutritional profile of RWL depend on the microbial system in the rice wine starter culture employed in the process (Dung et al., 2007). Developing a control-formulated starter culture mixture that can enhance the sensory attributes and nutritional composition of the RWL may open a great opportunity to promote the potential economic use of RWL in obtaining high-added-value natural food and upgrading the small-scale rice wine industry. Given the abovementioned gap, eight well-defined starter culture mixtures were prepared according to the microbial species and absolute proportions and quantities of the significant microorganisms isolated from the rice wine starter culture. Moreover, the performance of the traditional starter culture and the well-defined mixed-fungi starter cultures was compared in rice wine fermentation by analyzing the sensory attributes and nutritional value of the resulting RWL.

MATERIALS AND METHODS

Sample collection

The following materials were bought from a local public market in Baguio City, Philippines: 30 dried traditional rice wine starter culture (TSC) discs (approx. 10 grams per disc), 10 kilograms of white glutinous rice, and 10 kilograms of black glutinous rice.

Isolation and identification of fungi

Briefly, 10 grams of powdered TSC were homogenized in 90.0 mL of 0.85% w/v sterile saline solution. One milliliter of each diluted sample (10^{-4},10^{-5}, 10^{-6}, and 10^{-7} dilutions) was poured onto potato dextrose agar (PDA) and yeast peptone dextrose agar (YPD) supplemented with 100 ng/µL ampicillin to inhibit the growth of bacteria. The petri plates were incubated at 30 ºC for one to three days (Chay et al., 2020). The most dominant and abundant colonies were isolated and identified using the traditional method of morphological characteristics. The selected colonies were sub-cultured using new PDA and YPD plates at 30 ^{o}C for one to three days, depending on the fungi's growth, development, and type (molds or yeast). Moreover, the fungi were selected based on their ability to liquefy cooked rice and produce high sugar and ethanol content (Dung et al., 2007). The fungal cultures were isolated, purified, and stored in 20% glycerol stock. Then, the samples were sent to Macrogen in South Korea for fungal identification via ITS sequencing (ITS 4 and ITS 5). Finally, fungal isolates were used to develop the starter culture suspension in singles and combinations.

Development of the starter culture suspension

Pure isolates were utilized as inoculants in the starter culture suspension. Eight setups of single and combinations of fungi were made. The eight setups were as follows: *Rhizopus oryzae* (RO) only, *Saccharomyces cerevisiae* (SC) only, *Mucor indicus* (MI) only, RO+MI, RO+SC, MI+SC, RO+MI+SC, and TSC as positive control. Each strain of mold and yeast was cultivated beforehand in PDA and YPD media, respectively, to make the starter culture suspension. Moreover, all single setups contain 10^6 spores/mL for molds or 10^6 cells/mL for yeast. Then, a 1:1 ratio of fungal species was utilized for double combination setups with 10^6 spores or cells per mL. Lastly, a 1:1:1 ratio was used for the triple combination setup wherein the total number of spores and cells was still 10^6 spores/cells per mL (Dung et al., 2007).

Rice wine fermentation

Rice wine (RW) was prepared by mixing 500 grams of white glutinous rice and 500 grams of black glutinous rice in a 1:1 ratio, washed, and cooked. After cooling, the steamed rice was sprayed evenly with 40 mL of the starter culture suspension containing 10^6 spores/mL for molds and 10^6 cells/mL for yeast (Dung et al., 2007) while one dried TSC disc per kilogram of rice (approx. 10 grams/per disc) was used in the preparation of the control setup. The mixture was transferred aseptically into different containers and stored in a cool, dry place at a controlled temperature of about 20 ºC. Initially, the RW was fermented aerobically by allowing the exchange of gases for three days, followed by 27 days of anaerobic fermentation. After 30 days, the mixture was filtered using sterile cheesecloth, and the RW and RWL, the fermentation residue, were collected. Then, the freshly decanted, thick, semi-solid fermentation residue was collected and kept at 4 ^{o}C until further analysis. The RWL was freeze-dried to obtain the dry matter. Then, the RWL was subjected to sensory evaluation, and the top 5 most preferred RWLs were used in the succeeding assays.

Sensory evaluation

The sensory qualities of the RWL produced from the eight setups were evaluated using the standard method of Tand & Mabesa (1998) and Chay et al. (2017) with some modifications. Approximately 10 g of RWL samples with 3-digit random numbers in clean, sterile serving plates were given to the untrained participants (distilled water was used as a palate cleanser) using the 9-point Hedonic scale. Thirty (30) panelists/potential consumers were recruited.

The panelists were chosen based on three criteria: (1) ages 21–46 years old with experience in drinking RW and regular wine; (2) residing in Metro Manila, Philippines; and (3) no allergy to alcohol. This research was approved by the University of the Philippines-Manila Research Ethics Board with UPMREB Code: UPMREB 2023-0192-EX. The panelists were asked to evaluate the sensory attributes of the RWL in terms of color, aroma, sweetness, sourness, and bitterness using a score sheet on a scale of 1 to 9, where color: 1 = extremely light color, 9 = extremely dark color; aroma: 1 = extremely weak aroma, 9 = extremely strong aroma; sweetness: 1 = extremely not sweet, 9 = extremely sweet; sourness: 1 = extremely not sour, 9 = extremely sour; bitterness: 1 = extremely not bitter, 9 = extremely bitter (Chay et al., 2017). Then, the RWL was ranked according to the panelists' overall liking or preference by getting the average of the scores provided by the participants for the eight samples. The top 5 RWLs (1 = most preferred; 9 = least preferred) based on the overall preference/acceptability of the participants were used for further analyses.

Determination of the nutritional profile

Vitamin analysis

Fat-soluble vitamins A, D, and E were analyzed using analytical grade (> 98% purity) retinyl acetate, cholecalciferol, and alpha-tocopherol standards, respectively (purchased from Sigma Aldrich, Chemicals, St. Louis, MO) according to AOAC 21st edition protocol with some modifications. The standard solutions were prepared in UPLC-grade absolute ethanol (Sigma Aldrich, > 99.8% purity) and stored in a dark flask at -20 ^{o}C. Before the analysis, the parameters for the Ultrahigh Performance Liquid Chromatography (UPLC) (Waters Acquity UPLC H-class system) instrument were set as follows: 325 nm UV detector and a 4.6 mm × 25 cm column with a 5-micrometer packing L24. The flow rate and injection volume were set at 1.0 mL per minute and 20 µL, respectively. The mobile phase system is as follows: Mobile Phase A - formic acid/water and Mobile Phase B - acetonitrile. The measurements were done in a room temperature setting with protection in subdued light. The analyte was detected at 264 nm for cholecalciferol, 327 nm for retinyl acetate, and 284 nm for alpha-tocopherol. The total run time was about 20 minutes between each injection. The water-soluble vitamins that were analyzed are Vitamins B1, B2, B3, B5, B6, B7, B9, and C using the analytical grade thiamine, riboflavin, niacin, pantothenic acid, pyridoxine hydrochloride, biotin, folic acid, cyanocobalamin, and ascorbic acid standards, respectively (purchased from Sigma Aldrich, Chemicals, St. Louis, MO). For B vitamins, the chromatographic separation and quantitative analysis were conducted using a reversed phase-UPLC C18 column with dimensions of 250 × 4.6 mm i.d. and 5-micrometer packing through an isocratic delivery (50:50 concentration of acetonitrile-water) with a flow rate of 1.0 mL/min and sample injection volume set at 20 µL. Detection was achieved using a UV detector set at 270 nm at room temperature. Additionally, Vitamin C was detected using the following UPLC parameters: reversed phase-UPLC column through isocratic delivery of solvent system (A/B 33/67; A - 0.1 M potassium acetate, pH = 4.9; B - acetonitrile: water [50:50]) with a flow rate of 1.0 mL/min and sample injection volume of 20 µL. The detection was achieved at 254 nm at room temperature. For all vitamins standards, 20 µL of standard solutions were injected, and the peak areas were determined to generate calibration curves.

Amino acid analysis

The essential amino acids in the RWL were quantified using UPLC (Waters Acquity UPLC H-class system) consisting of a high-pressure binary pump, autosampler, thermostat, and a photodiode array detector from 190−700 nm wavelength according to the AOAC method 21st edition with some modifications. Briefly, one-gram crude RWL samples were dissolved in 5 mL of distilled water, followed by acid hydrolysis using 5 mL of 6 N HCl. The hydrolyzed samples were then filtered through a 0.45-micrometer filter

and diluted with the citrate buffer (pH = 2.5) for amino acid analysis (Inoue et al., 2023). The analytical grade (> 98% purity, purchased from Sigma Aldrich, Chemicals, St. Louis, MO amino acid (His, Ser, Arg, Gly, Met, Asp, Glu, Thr, Ala, Pro, Lys, Val, Ile, Leu, Tyr, Phe, Gln, Trp, Cys, and Ala) standard solutions in a concentrated form (2.5 mmol/L) were used. Then, standard solutions of amino acids (400 µL) were mixed with 250 µL of internal standard (DL-2-aminobutyric acid) and diluted to a 10 mL volumetric flask. Moreover, 400 µL of RWL sample was added with 250 µL of internal standard. The chromatographic separation of the 20 amino acids and samples was conducted using the AccQ-Tag Ultra C-18 column with the following dimensions: 2.1 mm × 100 mm × 1.7 µm. The solvent system is composed of two eluents: eluent A - ammonium formate (84%), formic acid (6%), and acetonitrile (10%); eluent B - acetonitrile (98%), formic acid (2%) by volume. The injection volume was set to 1.0 µL, the flow rate at 1.0 mL/min, the column temperature at 55 ^{o}C, and the wavelength of the PDA optical detector at 260 nm. The total analysis time was 12 minutes. The relative abundance of the essential amino acids was expressed as milligrams of amino acid per kilogram of RWL sample.

Proximate analysis

The proximate analysis determined the crude fiber, crude protein, crude fat, total energy, and total carbohydrates of the RWL samples fermented using the different starter cultures based on the AOAC method 21st ed. (2019).

The quantitative determination of crude protein was done using the Kjeldahl method according to the AOAC Official Method 2001.11. The Kjeldahl nitrogen value and % crude protein was computed using the following formulas:

$$\text{Kjeldahl nitrogen}, \% = (\text{Vs} - \text{VB}) \times \text{M} \times 14.01 \text{W} \times 10$$

$$\text{Crude protein}, \% = \% \text{Kjeldahl N} \times \text{F}$$

where: Vs is the volume of standardized acid used to titrate the sample; VB is the volume of standardized acid used to titrate the reagent blank; M is the molarity of the standard HCl; 14.01 is the atomic weight of Nitrogen; 10 is the factor to convert mg/g to percent; F is the factor to convert N to protein; and W is the weight of the sample.

The crude fiber content of the RWL samples was determined using the Wendee method. Approximately 2.0 grams of the RWL sample were placed in a Soxhlet flask with 100 mL of 1.25% sulfuric acid. The solution was refluxed for 1 hour and was washed with distilled water. Then, the Soxhlet was filled with 100 mL of 1.25% NaOH and was refluxed again for 1 hour. The resulting digested solution was filtered and will be dried in an aluminum dish at 105 ^{o}C for 2 hours. The weight of the dried sample was recorded. Lastly, the dried samples were placed in a furnace for 2 hours at 550 ^{o}C. The sample's mass loss was recorded, and the crude fiber was expressed as % crude fiber.

The crude fat of the RWL samples was determined using the Soxhlet method. Approximately 2.0 grams of samples were placed in a filter paper. Then, the filter paper was placed in a Soxhlet distilling setup. About 125 mL of hexane was added to the Soxhlet flask and was distilled for 1 hour until the hexane layer in the upper portion was clear. Next, the filter paper and solvent were recovered. The Soxhlet flask was dried in a drying oven. Finally, the dried oven was cooled to room temperature, and the mass of the flask plus the fat were recorded. The crude fat was expressed as % crude fat.

The total carbohydrates and total energy were calculated using the following formula:

$$\text{Total carbohydrates} = 100 - (\text{ash} + \text{moisture} + \text{crude fat} + \text{crude protein})\%$$

$$\text{Total energy} = 4(\%\text{curde protein}) + 9(\%\text{crude fat}) + 4(\text{total carbohydrates})$$

Statistical analyses

The effects of intervention and control (TSC) were compared via a one-way analysis of variance (ANOVA). All experiments were done in triplicate. Means were compared using Tukey's Honest Significant Difference (HSD) test. For all statistical analyses, a *p*-value of 0.05 was deemed statistically significant. If applicable, a post hoc Bonferroni statistic was employed to check the data's validity.

RESULTS

The representative fungal colonies were selected based on their appearance of growth on the PDA and YPD media. The agar block technique was utilized for mold identification through morphological characteristics. The same morphological tests were conducted for the identification of yeast colonies (Alexopoulus et al., 1996; Frazier et al., 1998).

Isolation of fungi

The first fungal colony was observed to possess hyphae, which is characteristic of a mold strain. The fungal colony was observed with a white to creamy-ish yellow cottony mycelia (young culture), which turned grayish as it aged with the development of sporangia and was fast-growing. The fungal culture was found to have a non-septate mycelium, erect simple and branched sporangiophores, transparent and long hyphae, terminal, globose to spherical multi-spored sporangia with a well-developed subtending columella, sporangium, and greyish to brownish sporangiospores. Moreover, the fungi did not possess stolon and rhizoid which distinguishes morphological characteristics of *Mucor* sp. Then, ITS 4 and ITS 5 sequencing confirmed it to be *M. indicus* IHEM 24907. The same fungal species were isolated in *Bubod* (Chay et al., 2017), *Banh men* (Dung et al., 2007), and *Hong Qu* (Xie et al., 2016). The representative photomicrographs of *M. indicus* are shown in Figure 1.

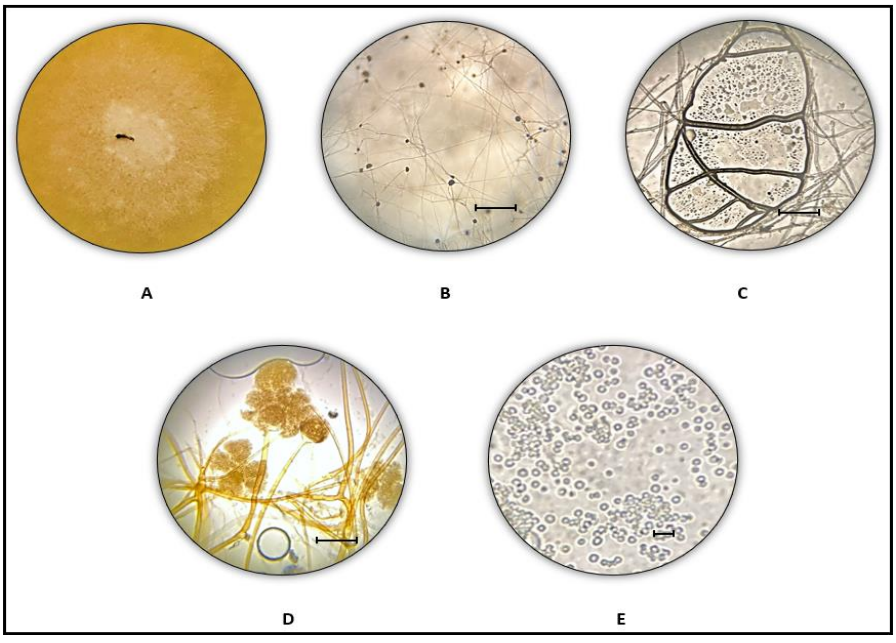

Figure 1. Photomicrographs of *Mucor indicus* IHEM 24907 (A) growing mycelium; (B) branches of sporangiophore at 4x magnification; (C) non-septate hyphae; (D) erect sporangiophore with large spherical multi-spored sporangia with columellae; (E) sporangiospores. Scale bar = 20 µm

The second mold strain isolated showed similar characteristics to the *M. indicus* with a stolon and pigmented rhizoids at the branch point of the sporangiophore, a morphological characteristic of *Rhizopus* spp. (Frazier et al., 2018). The ITS 4/ITS 5 sequencing revealed that it is *R. oryzae* strain PCNB1279. The representative photomicrographs of *R. oryzae* strain PCNB1279 are shown in Figure 2.

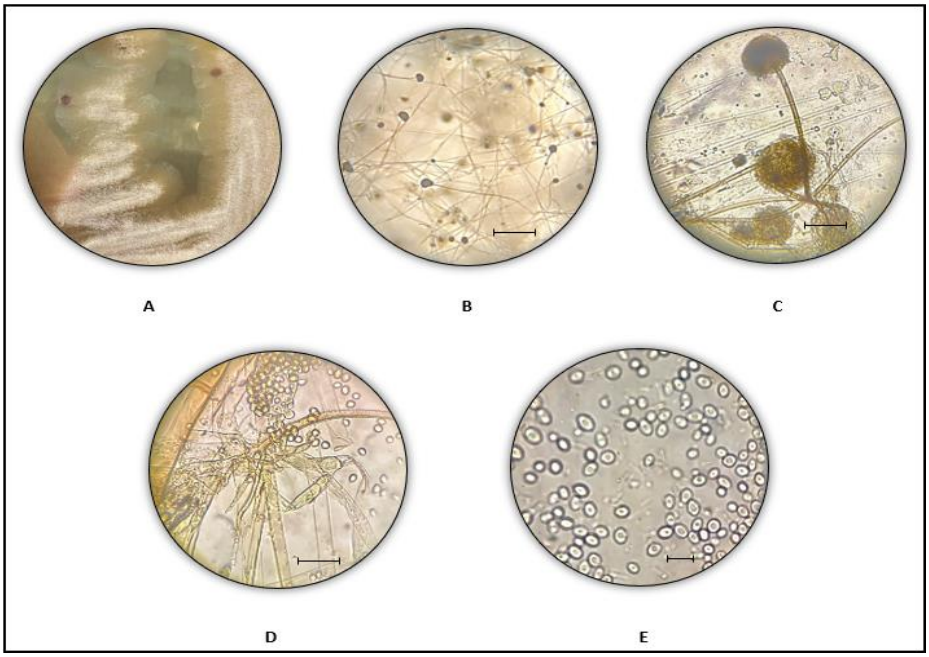

Figure 2. Photomicrographs of *Rhizopus oryzae* PCNB1279 (A) growing mycelium; (B) branches of sporangiophore; (C) sporangia with stolon; (D) septate and rhizoidal hyphae; (E) sporangiospores. Scale bar = 20 µm

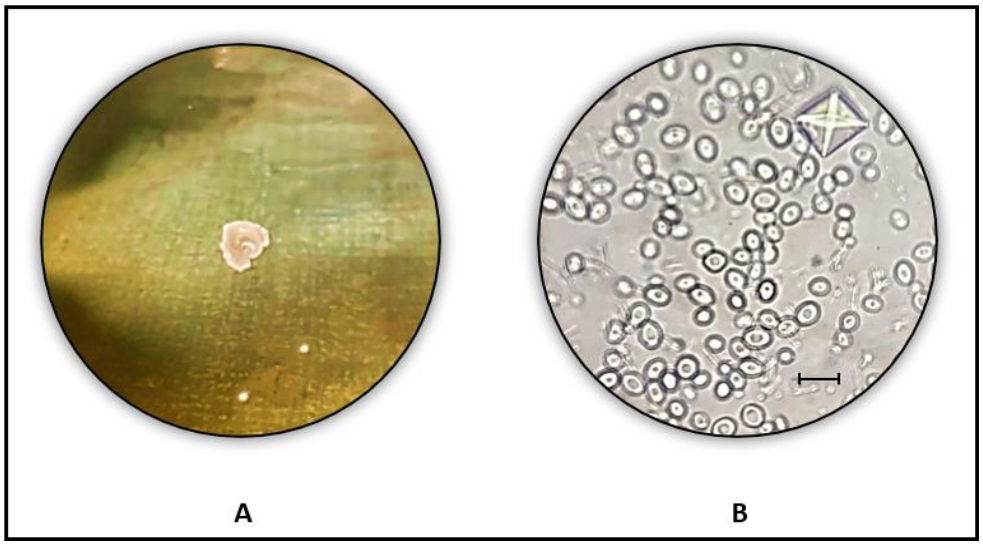

Figure 3. Photomicrograph of *Saccharomyces cerevisiae* L26A (A) growing colony (B) spherical to ovoid, unicellular yeast cells at 40x magnification with budding. Scale bar = 10 µm

The third fungus isolated manifested a characteristic of a yeast with globose to elongate yeast-like cells. The ITS 4 and 5 sequencing validated it as *S. cerevisiae* L26A. The representative photomicrographs of *S. cerevisiae* are shown in Figure 3. The results of the ITS sequencing analysis of the three fungal strains can be accessed in the supplementary material.

Sensory evaluation

The RWL samples shown in Figure 4 were subjected to sensory evaluation. The color of the RWL made from the various treatments produced a moderately to intensely dark color with a score ranging from 7.2 points to 8.5 points, with the RO+MI+SC setup being the darkest and the SC setup being the lightest. The dark color of this RWL may be attributed to the high tannin and anthocyanin content of the black glutinous rice variety (Bhattacharyya & Roy, 2018). Regarding the aroma, the RWL of the RO+MI setup displayed a strong alcoholic aromatic odor, followed by the MI+SC setup and the RO+SC with a score of 8.2, 7.5, and 7.3 points, respectively. Moreover, this clearly shows that the MI is responsible for the strong aromatic odor of RWL as the top 2 setup with most pungent alcoholic, aromatic odor contained MI as one of the microorganisms presents. The RO+SC setup tasted the sweetest and was the least sour among the setups. The MI+SC setup is the second sweetest but exhibits the sourest aftertaste among the eight setups. Lastly, the RO+MI+SC setup was the least bitter among the eight setups. Because of these, the ranking of the RWL in terms of the participant's overall preference includes RO+SC (rank 1), RO+MI+SC (rank 2), and MI+SC (rank 3) with mean scores of 1.8, 2.2, 2.2, respectively. This clearly shows that the setup made from RO+SC and RO+MI+SC were the two most preferred rice wine lees. Based on the sensory evaluation, the top 5 RWL setups are RO+SC, RO+MI+SC, MI+SC, TSC, and RO+MI. However, it is noteworthy that the three most preferred RWLs contain a common fungal species, SC. Thus, to check the effect of SC on the nutritional profile of the RWL, the RO+MI setup was discarded, and the SC setup was added instead. Hence, the final 5 RWL setups were RO+SC, RO+MI+SC, MI+SC, SC, and TSC. The sensory profile of the RWL is shown in Figure 5, and the data is summarized in Table 1.

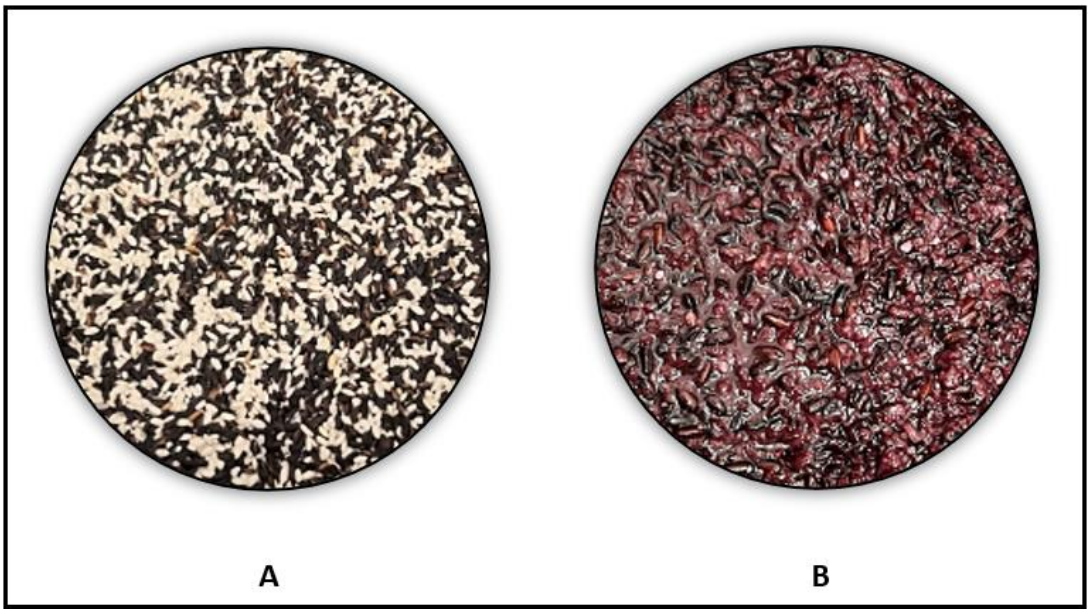

Figure 4. Picture of (A) uncooked mixture of white and black glutinous rice and (B) rice wine lees

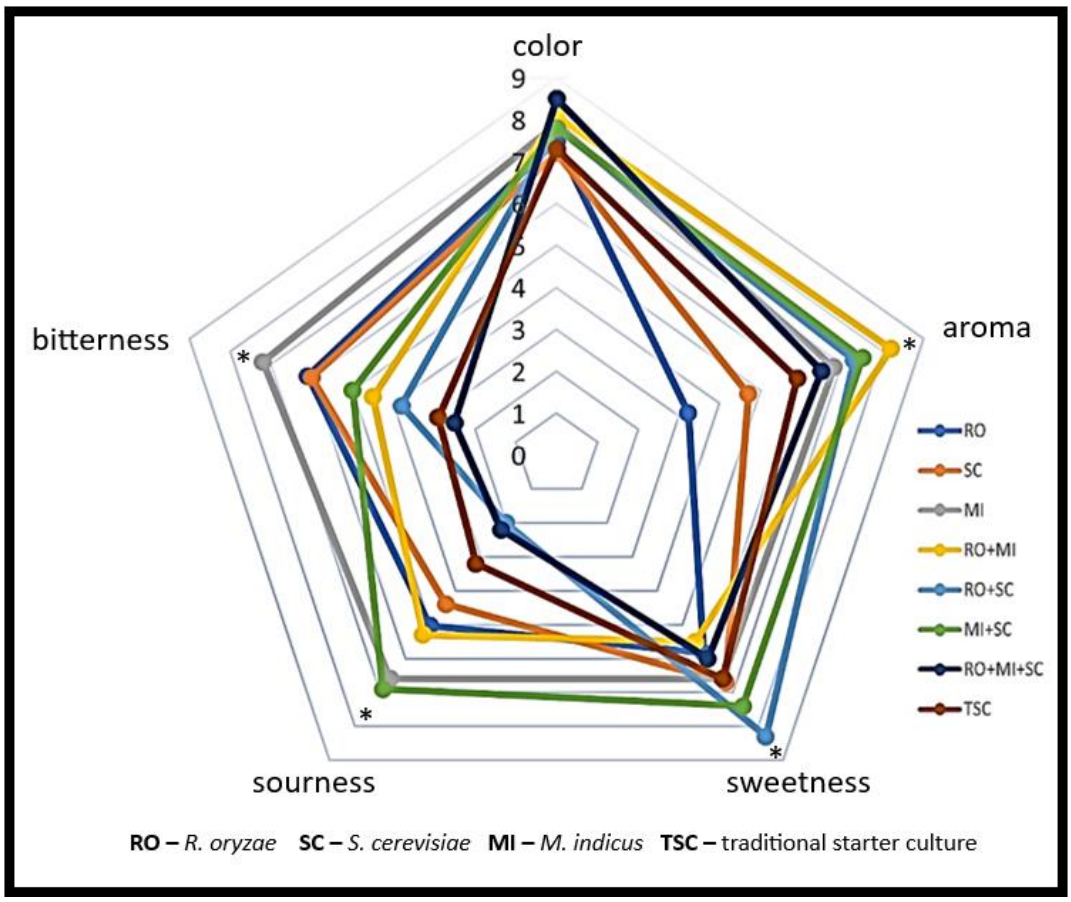

Figure 5. Sensory evaluation of rice wine lees (RWL) produced using various starter culture mixtures. Data points represent the means of each sensory parameter. All sensory attributes showed significant differences (*p < 0.05) across the different starter culture mixtures against traditional starter culture (TSC) except for color. Means were compared against the control (TSC) using ANOVA and post hoc Tukey HSD statistics with *p < 0.05

Table 1. Mean values ± standard deviation of the sensory attributes of the rice wine lees (RWL) prepared using different starter culture mixtures

Treatments	Color	Aroma	Sweetness	Sourness	Bitterness	Overall Acceptability
TSC	7.3 ± 1.5	5.9 ± 0.9	6.6 ± 1.3	3.2 ± 0.8	2.9 ± 0.8	4.0 ± 1.0
RO+MI+SC	8.5 ± 0.5*	6.5 ± 1.1*	6.0 ± 0.8	2.2 ± 1.3*	2.5 ± 1.0	2.2 ± 0.8*
MI+SC	7.8 ± 0.9	7.5 ± 1.0*	7.4 ± 1.1*	6.9 ± 0.8*	5.0 ± 0.9*	2.2 ± 1.0*
RO+SC	7.8 ± 0.7	7.3 ± 0.7*	8.3 ± 0.8*	2.0 ± 0.7	3.8 ± 0.7*	1.8 ± 0.8*
RO+MI	8.1 ± 0.7*	8.2 ± 0.7*	5.5 ± 1.1	5.3 ± 0.7*	4.5 ± 0.5*	6.2 ± 1.4*
MI	7.9 ± 1.2*	6.8 ± 1.2*	6.6 ± 0.8	6.6 ± 1.1*	7.2 ± 0.8*	6.7 ± 1.2*
SC	7.2 ± 0.7	4.7 ± 1.1	6.7 ± 1.2	4.4 ± 1.3*	6.0 ± 0.8*	6.6 ± 0.9*
RO	7.5 ± 1.2	3.2 ± 1.2	5.8 ± 1.3	5.0 ± 0.8*	6.1 ± 0.9*	6.3 ± 1.2*

Notes: For the overall acceptability, the higher the score, the less preferred rice wine lees. Abbreviations: RO - *Rhizopus oryzae*; MI - *Mucor indicus*; SC - *Saccharomyces cerevisiae*; TSC - traditional starter culture. *Significant values (P < 0.05) according to Tukey HSD and post hoc Bonferroni statistics.

Nutritional profile of RWL

The freeze-dried RWL was subjected to proximate analysis to determine the relative composition of crude fiber, fat, protein, carbohydrates, and total energy. It has been observed that the RO+MI+SC and RO+SC contained the highest crude protein among the five setups, with values of 11.12 ± 0.08% and 10.06 ± 0.01%, respectively. These results were consistent with the crude protein content of raw milled rice (Sanchez, 1988). Additionally, these two setups were also the highest crude fat-containing RWL, with an average value of 6.76 ± 0.00% and 6.75 ± 0.00%, respectively, similar to the crude fat content of Philippine rice wine (*tapuy)* lees (Manaois & Morales, 2014; Manaois & Morales, 2018). The setup SC showed the highest crude fiber content (7.33 ± 0.03%), followed by the MI+SC with 7.19 ± 0.01%. Regarding total carbohydrates, the TSC displayed the highest amount with a mean value of 80.87 ± 0.10%, followed by SC with 80.26 ± 1.03% and MI+SC with 79.90 ± 0.03%. However, the RO+MI+SC showed the highest total energy content of about 412.96 ± 0.07 kcal/100 g of sample. Table 2 summarizes the proximate composition of the five RWLs.

Table 2. Proximate composition of the dried rice wine lees (RWL) prepared using different starter culture mixtures

Proximate Composition	TSC	RO+MI+SC	MI+SC	RO+SC	SC
Crude protein (%)	7.25 ± 0.01	11.12 ± 0.08*	7.89 ± 0.00*	10.06 ± 0.01*	8.53 ± 0.00*
Crude fat (%)	5.00 ± 0.10	6.76 ± 0.00*	5.02 ± 0.03	6.75 ± 0.00*	3.88 ± 1.01
Crude fiber (%)	6.89 ± 0.00	6.21 ± 0.02*	7.19 ± 0.01*	5.26 ± 0.06*	7.33 ± 0.03*
Total carbohydrates (%)	80.87 ± 0.10	75.97 ± 0.10*	79.90 ± 0.03	76.87 ± 0.07*	80.26 ± 1.03
Total energy (kcal/100 g)	397.42 ± 0.50	412.96 ± 0.07*	396.33 ± 0.19	397.71 ± 0.24*	390.06 ± 4.97*

Notes: RO - *Rhizopus oryzae*; MI - *Mucor indicus*; SC - *Saccharomyces cerevisiae*; TSC - traditional starter culture. *Significant values ($P < 0.05$) according to Tukey HSD and post hoc Bonferroni statistics.

The amino acid content of the freeze-dried RWL was determined using the UPLC method. The result showed that the various treatments significantly differed regarding amino acid content. The RO+MI+SC and TSC contained significant amounts of histidine compared to the other treatments. Regarding serine content, the setup RO+MI+SC and RO+SC had the highest serine-containing RWL. Overall, the setup RO+MI+SC displayed the highest amounts of histidine, threonine, leucine, isoleucine, phenylalanine, tryptophan, and methionine, considered essential amino acids. Thus, using the starter culture containing a 1:1:1 ratio of RO+MI+SC resulted in the highest amount of essential amino acids in RWL. The summary of the amino acid content results can be viewed in Table 3.

The water-soluble and fat-soluble vitamin content of the five RWL was determined using the UPLC method. Based on the data, the RO+SC demonstrated the highest vitamin B complex content, as shown in Table 4. However, the RO+MI+SC displayed a significant amount of vitamins A, C, D, and E with an average value of about 359.87 ± 0.11, 52.43 ± 4.32, 66.65 ± 0.45, and 20.32 ± 0.32, respectively, which are the highest values out of all the five treatments. Moreover, the RO+MI+SC and RO+SC setups showed higher amounts of vitamins than the control group (TSC). The same result was reported, wherein the vitamin C content was significantly improved in fermented rice compared to uncooked rice (Hor et al., 2022). Thus, it can be concluded that the combination of fungal cultures generated a

higher vitamin content than the TSC. This may be attributed to the synergistic effect of the pure isolates rather than the effects of the various fungal species present in the TSC, which comprises both beneficial and unnecessary fungi.

Table 3. Summary of the amino acid content (in mg/kg) of the rice wine lees (RWL) prepared using different starter culture mixtures

Amino Acid	TSC	RO+MI+SC	MI+SC	MI+SC	SC
His	41.30 ± 6.25	42.07 ± 7.55	37.89 ± 4.06	28.27 ± 2.69*	31.61 ± 4.43
Thr	0.96 ± 0.09	1.78 ± 0.11	1.17 ± 0.03	1.75 ± 0.19*	0.74 ± 0.04
Leu	0.48 ± 0.02	1.61 ± 0.05*	0.92 ± 0.02*	1.10 ± 0.01*	0.66 ± 0.04*
Ile	1.06 ± 0.03	4.88 ± 0.07*	3.71 ± 0.11*	3.79 ± 0.03*	2.63 ± 0.17*
Phe	1.92 ± 0.05	4.19 ± 0.06*	4.06 ± 0.10*	3.76 ± 0.04*	2.86 ± 0.15*
Trp	0.03 ± 0.01	0.18 ± 0.01*	0.14 ± 0.01*	0.16 ± 0.01*	0.10 ± 0.01*
Met	0.53 ± 0.03	3.15 ± 0.20*	1.34 ± 0.12*	1.67 ± 0.01*	1.19 ± 0.11*
Ala	2.23 ± 0.08	3.06 ± 0.07*	3.58 ± 0.11*	3.76 ± 0.02*	3.04 ± 0.17*
Pro	0.35 ± 0.02	1.19 ± 0.07*	1.36 ± 0.06*	1.21 ± 0.02*	0.76 ± 0.05*
Lys	1.60 ± 0.06	0.97 ± 0.05*	1.31 ± 0.05*	1.19 ± 0.03*	1.17 ± 0.07*
Tyr	1.79 ± 0.05	2.69 ± 0.09*	3.45 ± 0.10*	3.80 ± 0.06*	2.66 ± 0.13*
Val	1.83 ± 0.06	2.10 ± 0.19	3.13 ± 0.28*	3.35 ± 0.05*	2.43 ± 0.07*
Gly	3.55 ± 0.82	2.60 ± 0.05	2.86 ± 0.03	2.75 ± 0.03	2.73 ± 0.10
Asp	2.20 ± 0.07	3.76 ± 0.11*	5.28 ± 0.14*	4.52 ± 0.13*	4.32 ± 0.23*
Glu	1.77 ± 0.03	1.57 ± 0.06*	2.13 ± 0.04*	1.79 ± 0.08	1.91 ± 0.05
Arg	2.45 ± 0.09	3.21 ± 0.08	2.03 ± 0.08*	2.66 ± 0.12*	1.54 ± 0.07*
Ser	219.30 ± 3.99	333.55 ± 16.23*	290.40 ± 3.79*	329.63 ± 9.96*	237.80 ± 10.55*

Notes: RWL produced using RO+MI+SC starter cultures showed higher contents of essential amino acids (bold letters) compared to the other setups and control (TSC group). RO - *Rhizopus oryzae*; MI - *Mucor indicus*; SC - *Saccharomyces cerevisiae*; TSC - traditional starter culture; RWL - rice wine lees. *Significant values ($P < 0.05$) according to Tukey HSD and post hoc Bonferroni statistics.

Table 4. Water- and fat-soluble vitamin content (in µg/100 g dry matter) of the rice wine lees (RWL) using different starter culture mixtures

Vitamins	TSC	RO+MI+SC	MI+SC	RO+SC	SC
A	305.25 ± 0.44	359.87 ± 0.11*	300.48 ± 2.33	354.23 ± 2.57*	312.94± 1.21*
B_1	20.87 ± 4.50	25.64 ± 0.45	20.62 ± 1.45	26.38 ± 1.25	25.09± 5.04
B_2	30.11 ± 2.90	34.78 ± 7.89	24.59 ± 2.43*	54.78 ± 1.93*	54.93± 3.22*
B_3	31.33 ± 3.94	29.29 ± 1.89*	31.21 ± 2.33	32.33 ± 1.37	31.45 ± 2.09
B_6	38.36 ± 3.66	32.37 ± 1.46*	23.18 ± 4.54	42.19 ± 2.47*	21.74 ± 0.62
C	32.67 ± 2.55	52.43 ± 4.32	40.29 ± 1.48*	44.23 ± 0.33*	43.84 ± 1.99
D	55.92 ± 0.72	66.65 ± 0.45	52.72 ± 0.89	65.88 ± 1.43	60.23 ± 0.34 *
E	10.58 ± 0.25	20.32 ± 0.32*	7.23 ± 0.23*	17.34 ± 1.02*	11.11 ± 0.02

Notes: RWL produced using RO+MI+SC starter culture showed higher contents of fat-soluble vitamins compared to the other setups and control (TSC group). RO - *Rhizopus oryzae*; MI - *Mucor indicus*; SC - *Saccharomyces cerevisiae*; TSC - traditional starter culture; RWL - rice wine lees. *Significant vales ($P < 0.05$) according to Tukey HSD and post hoc Bonferroni statistics.

DISCUSSION

Rice wine is an alcoholic drink commonly produced in various Asian countries and is characterized by its sweet-alcoholic taste and an alcohol content that ranges from 13–19% (Sanchez, 2008). In the present study, defined starter culture mixtures were prepared using a single and a combination of fungal species isolated and purified from the traditional rice wine starter culture (TSC) used in rice winemaking. The starter culture used in the production of rice wine significantly affects the physicochemical properties, sensory attributes, and bioactivities such as lifespan extending property (Chua et al., 2024) of the resulting rice wine and its fermentation residue, rice wine lees (Coronel et al., 1981; Chay et al., 2020; Dizon et al., 2009; Dizon et al., 2013). The TSC contains diverse fungal and microbial species, both beneficial and harmful microbes, and the relative amount of these microorganisms is different from one TSC to another (Sanchez, 2008; Dela Rosa & Medina, 2022; Dizon et al., 2009; Cai et al., 2019). This introduces issues regarding the reproducibility and inconsistency of the quality of the rice wine products with a very low degree of industrialization. Hence, it is less competitive than other wine brands in the local and international markets. Thus, the need to standardize rice winemaking in terms of a well-defined starter culture with improved quality and taste arises.

Using a well-defined starter culture mixture, the combination of RO, MI, and SC significantly improved the quality and taste of the RWL compared to traditionally prepared rice wine lees. Moreover, the sensory evaluation revealed that RWL produced using the RO+MI+SC had a sweet and aromatic sensory attribute, which the 30 participants preferred. This result showed that local consumers preferred an RWL product with sweet-tasting characteristics and little alcoholic taste. Furthermore, this result may be attributed to Filipinos' preference for sweet products (Cornell et al., 2020; Villanueva, 2021).

The nutritional value of the RWL was significantly enhanced by employing the RO+MI+SC starter culture mixture. Using RO+MI+SC starter culture mixture significantly increased crude protein, fiber, fat, carbohydrates, and energy contents of the RWL. The increase in crude protein content of RWL may be attributed to the metabolic processes mediated by the microorganisms present in the fermentation setup, resulting in an influx of proteins that adhered to the surface of the lees. The same observation was reported during the post-fermentation period of Manzoni Bianco wine (Vincenzi et al., 2011). Additionally, research has shown that the protein concentrations of wine decreased with time, which may be attributed to protein agglutination and precipitation, increasing the crude protein content of the wine residue (Lira et al., 2013).

Moreover, the protein generated by microorganisms may be further metabolized, producing the essential amino acids in varying amounts and classes. It is well established that the release of free amino acids is attributed to the proteolytic activity of the enzymes produced by the microorganisms in the mixture. Subsequently, these amino acids play an essential role in enhancing the flavor and taste development of the RWL. For instance, glutamic and aspartic acids are the two primary amino acids responsible for improving the taste. At the same time, serine, glycine, and alanine are accountable for the sweetness of food (Mau et al., 1998), which is validated by the high concentration of serine, as shown in Table 3. The same result was obtained when the amino acid content of Chinese rice wine was determined (Xie et al., 2016). However, the large amounts of arginine, lysine, and valine may cause the bitter taste of the RWL (Mau et al., 1998). Apart from the flavor enhancement properties of these free amino acids, these amino acids are also essential in human metabolic processes.

Thus, the consumption of the RWL produced by the RO+MI+SC may benefit humans. Previous publications revealed that rice contains water-soluble and fat-soluble vitamins (Ito et al., 2019; Wasan et al., 2022). This paper presented that using the

RO+MI+SC starter culture mixture produced a wide array of beneficial vitamins. The increased vitamin C content of the RWL may act as a potent antioxidant when consumed (Hor et al., 2022). Furthermore, the RWL produced by the RO+MI+SC enriched the innate vitamin A content of the rice as well as the vitamin B complex. These vitamins may be produced because of the degradation process of the biomolecules via the endogenous microbiological enzymes secreted by the microorganisms present in the mixture (Sharma et al., 2020). In a separate study, the lees from Philippine rice wine were reported to contain a significant amount of phenolics, which increased the lifespan of the *Caenorhabditis elegans* model (Chua et al., 2024). By combining all these, it can be inferred that the underutilized RWL is nutritious and supported by high amounts of crude protein, total carbohydrates, total energy, amino acids, and vitamins. Thus, the RWL can be used to develop functional, value-added food products available to consumers and, at the same time, resolve the problem of food waste management in the rice wine industry (Chua et al., 2024). Additionally, this research may open a great opportunity to promote the potential economic use of RWL, upgrading the small-scale rice wine industry.

CONCLUSION

This study demonstrated the effects of various fungal species as starter culture mixtures in preparing rice wine lees (RWL). It has been shown that using pure and combinations of fungi isolated in the traditional rice wine starter culture significantly improved and yielded better sensory qualities. Moreover, the nutritional profile in terms of vitamins, amino acids, proximate, and total energy content of the RWL produced using the prepared starter culture was far better and superior to the lees produced using the traditional starter culture. The results suggested that the RO+MI+SC starter culture mixture has excellent potential as an enhanced starter culture compared to the conventional starter culture in producing rice wine lees with improved quality, taste, essential amino acids, and vitamin content, indicating high nutritional value. Lastly, this work presented an effective use of a defined starter culture mixture for producing RWL at a laboratory scale. Further research is needed to develop this defined starter culture mixture to be deemed suitable for rice wine and rice wine lees production on a commercial scale.

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Supplementary material: Detailed information regarding the ITS sequencing analysis of the three fungal strains can be accessed and downloaded through this link: https://rb.gy/nanoxd.

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