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# The Common and Unique Microbiota in Sabah's Traditional Rice Wine Starter Cultures (Sasad)

(Mikrobiota Biasa dan Unik dalam Kultur Pemula Wain Beras Tradisional Sabah (Sasad))

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ABSTRACT

Rice wine is an alcoholic beverage produced through the fermentation of cereal grains, mainly rice, with microbial starters consisting of fungi and bacteria. However, the microbes in the rice wine starter cultures have never been documented comprehensively except for a few studies that looked into culturable microbes. Hence, their exact core microbiota contents, especially those unculturable microbes remained unknown. Therefore, this research aimed to identify the fungal and bacterial communities in sasad through ribosomal amplicon-based next-generation sequencing and analysis that captured both the culturable and unculturable microbiota. The results showed that two fungal phyla (Mucoromycota and Ascomycota) with five genera (*Mucor, Rhizopus, Saccharomycopsis, Wickerhamomyces*, and *Kodamaea*) and two bacterial phyla (Proteobacteria and Firmicutes) with 10 genera (*Kosakonia, Weissella, Enterobacter, Lactococcus, Pseudomonas, Bacillus, Chromobacterium, Paludibacterium, Enterococcus,* and *Gluconobacter*) were identified as the core microbiota (relative abundance > 1.00%) in the sasad samples. Some of these microbes have been reported in other starter cultures, but some are unique to the sasad (*Chromobacterium* and *Paludibacterium*). Hence, this research provides the first comprehensive report on the microbes in sasad and provides important insights into the potential roles of core microbiota. These data may be used to facilitate the development of starter cultures with defined microbial compositions for the consistent production of safe and high-quality rice wines in the future.
Keywords: Microbiota; rice wine; starter culture

ABSTRAK

Wain beras ialah minuman beralkohol yang dihasilkan melalui penapaian bijirin, terutamanya beras, dengan mikrob pemula yang terdiri daripada kulat dan bakteria. Walau bagaimanapun, mikrob dalam ramuan pemula wain beras belum pernah didokumenkan secara menyeluruh kecuali beberapa kajian yang mengkaji mikrob yang boleh dikultur. Oleh itu, kandungan mikrobiota teras yang tepat terutamanya mikrob yang tidak boleh dikultur tidak diketahui. Maka, penyelidikan ini bertujuan untuk mengenal pasti komuniti kulat dan bakteria dalam sasad melalui penjujukan dan analisis generasi akan datang berasaskan amplikon ribosom yang akan merangkumi kedua-dua mikrobiota yang boleh dikultur dan tidak boleh dikultur. Hasilnya menunjukkan bahawa dua filum kulat (Mucoromycota dan Ascomycota) dengan lima genus (*Mucor, Rhizopus, Saccharomycopsis, Wickerhamomyces* dan *Kodamaea*) dan dua filum bakteria (Proteobacteria dan Firmicutes) dengan 10 genus (*Kosakonia, Weissella, Enterobacter, Lactococcus, Pseudomonas, Bacillus, Chromobacterium, Paludibacterium, Enterococcus,* dan *Gluconobacter*) telah dikenal pasti sebagai mikrobiota teras (kelimpahan relatif > 1.00%) dalam sampel sasad. Sesetengah mikrob ini telah dilaporkan dalam kultura pemula lain, tetapi terdapat beberapa yang unik untuk sasad (*Chromobacterium* dan *Paludibacterium*). Oleh itu, penyelidikan ini merupakan laporan komprehensif pertama tentang mikrob dalam sasad dan memberikan gambaran penting tentang potensi peranan mikrobiota teras. Data ini boleh digunakan untuk memudahkan pembangunan kultur pemula dengan komposisi mikrob yang ditentukan untuk pengeluaran wain beras yang selamat dan berkualiti tinggi secara konsisten pada masa hadapan.

Kata kunci: Kultur pemula; mikrobiota; wain beras

## INTRODUCTION

Rice wine is an alcoholic beverage produced by fermenting cereal grains, principally rice, with starter cultures (Rhee, Lee & Lee 2011). Traditionally, rice wines are produced using starter cultures sold in the market or by the back-slopping technique, in which a small amount of the leftover rice wine from the previous batch is used as the starter culture. However, the starter cultures sold in the market often vary in microbial compositions, and their exact microbial contents are usually unknown. The lack of standardisation of microbial compositions in traditional stater cultures resulted in the production of different microbial metabolites, which in turn caused the variation in nutritional, chemical, and organoleptic properties among rice wines of different batches (Chim et al. 2015; Ly et al. 2018; Palaniveloo & Vairappan 2013).The inconsistency in rice wine quality would be an issue for marketing rice wine in national and international markets.

In addition, traditional starter cultures that lacked quality control may contain undesirable or pathogenic microorganisms (Chim et al. 2015). For example, several pathogenic species such as *Aspergillus nomius*, *Clostridium* sp., *Enterobacter* sp., *Escherichia coli*, *Fusarium culmorum*, *Penicillium georgiense*, and *Pseudomonas* sp. were found in the traditional starter cultures used to produce hong qu glutinous rice wine, giving rise to food safety issues (Huang et al. 2019). Starter cultures produced under barely controlled conditions might also be contaminated by methanol-producing microorganisms, leading to the production of rice wine with high methanol concentration (Abidin et al. 2019; Ohimain 2016). Besides that, unethical producers frequently add colourless methanol into alcoholic beverages to increase alcohol content and gain greater profits (Rostrup et al. 2016). However, methanol is toxic to humans, and methanol poisoning outbreaks and deaths resulting from the ingestion of fermented alcoholic beverages contaminated with methanol have been reported on a global scale (WHO 2014).

In the recent past, modern biotechnology advancements have contributed to the revolution of traditional approaches and demonstrated the feasibility and importance of understanding the concepts behind the fermentation process. To date, several researchers have identified the fungal and bacterial communities in various rice wines and their starter cultures using molecular approaches (Anupma & Tamang 2020; Cai et al. 2018; Huang et al. 2018; Jiang et al. 2020; Zhao et al. 2020). However, such information on sasad, starter cultures used for the fermentation of rice wine known as tapai in Sabah, Malaysia, has not been published.

Therefore, this research aims to identify the microbial composition of sasad using amplicon-based next-generation sequencing (NGS) and provide insights into their potential contribution to rice wine fermentation. The findings in this research can benefit the food and biotechnology sectors, particularly the rice wine industry.

## MATERIALS AND METHODS

### RAW MATERIALS

Three types of sasad (SA, SB, and SC) were obtained from the local market located in Penampang, Sabah, and stored at 4 °C immediately after collection before analysis. The application to access the biological resources of Sabah for this research was approved by Sabah Biodiversity Centre (SaBC), with the access licence reference numbers JKM/MBS.1000-2/2 JLD.12 (72), JKM/MBS.1000-2/2 JLD.12 (75), JKM/MBS.1000-2/2 JLD.12 (76), and JKM/MBS.1000-2/2 JLD.12 (77).

### GENOMIC DNA (gDNA) EXTRACTION

The gDNA of fungi and bacteria were extracted from three types of sasad, each with biological triplicates using DNAeasy PowerLyzer PowerSoil Kit (Qiagen) according to the manufacturer's protocol. The starter cultures (sasad) were ground into powder before DNA extraction. The eluted gDNA was pooled and stored at -20 °C for downstream analysis.

### AMPLICON-BASED NEXT-GENERATION SEQUENCING (NGS)

The extracted gDNA samples were subjected to amplicon library preparation using two-step PCR according to Illumina's 16S metagenomic library preparation guideline. The targeted regions were amplified using locus-specific sequence primers with overhang adapters, where the fungal internal transcribed spacer 2 (ITS2) region was amplified using forward primer ITS3 [5'- overhang (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG) - GCA TCG ATG AAG AAC GCA GC -3'] and reverse primer ITS4 [5'- overhang (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G) - TCC TCC GCT TAT TGA TAT GC -3'], whereas the bacterial 16S ribosomal RNA (rRNA) V3-V4 region was amplified using forward primer 341F [5'- overhang (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG) - CCT ACG GGN GGC WGC AG -3'] and reverse primer 805R [5'- overhang (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G) - GAC TAC HVG GGT ATC TAA TCC -3'].

Dual indices were attached to the amplicons using Illumina Nextera XT Index Kit v2 according to the manufacturer's protocols. The quality of the libraries was measured using the Agilent Bioanalyzer 2100 System by Agilent DNA 1000 Kit and fluorometric quantification by Helixyte Green™ dsDNA Quantifying Reagent. The libraries were normalised and pooled according to the

protocol recommended by Illumina and subjected to NGS via the Illumina MiSeq platform (300 bp paired-end).

### AMPLICON SEQUENCE ANALYSIS

The raw amplicon paired-end reads were demultiplexed by grouping based on their barcode sequences. Quality assessment of raw reads was carried out using FastQC after the primers and adaptors were removed using Cutadapt 3.5. Paired-end reads were processed and merged using DADA2 V1.18. Chimera screening and taxonomy assignment were done using the SILVA non-redundant database V138.1. For phylogenetic analysis (UniFrac), sequence alignment was done using MUSCLE 3.8.-

## RESULTS AND DISCUSSIONS

### FUNGAL COMMUNITIES IN SASAD

The fungal communities in sasad, SA, SB, and SC are shown in Table 1. The species richness (Chao1) between the sasad samples did not differ significantly ($p > 0.05$). However, Shannon and Simpson diversity indices indicated that the fungal community in SA had the greatest abundance and evenness, followed by SB and SC ($p < 0.05$). According to the dendrogram (UPGMA) constructed based on weighted UniFrac distance matrices of fungal communities in sasad (Figure 1), the fungal communities in SB and SC have higher similarities and were more closely clustered compared to SA.

The relative abundances of different fungal taxonomic levels detected in SA, SB, and SC were illustrated using Krona charts, as shown in Figure 2. Three fungal phyla detected in the sasad samples were Mucoromycota, Ascomycota, and Basidiomycota, in which SB and SC were predominated by Mucoromycota while SA was predominated by both Mucoromycota and Ascomycota. However, Basidiomycota was found in SA and SC only. This result was consistent to Anupma and Tamang's (2020) finding which showed the presence of Mucoromycota and Ascomycota in 40 traditionally prepared dry starters collected from eight states in North East India, while Basidiomycota was present in only four of the starter cultures (chowan, dawdim, marcha, and thiat). Besides that, Zhao et al. (2020) reported Mucoromycota and Ascomycota as the predominant fungi in the fermentation of black glutinous rice wine. These results further support the hypothesis that Mucoromycota and Ascomycota were the common fungal phyla in rice wine starter cultures.

Generally, *Mucor*, *Rhizopus*, *Saccharomycopsis*, *Wickerhamomyces*, and *Kodamaea* were the five core fungal genera (relative abundance > 1.00%) identified in the sasad samples (traditional starter cultures used for the fermentation of tapai) obtained from the local market located in Penampang, Sabah, Malaysia. Similarly, Nout and Aidoo (2002) reported *Mucor* and *Rhizopus* as the fungi used to produce tapai. However, the results were contrary to the previous study by Chiang, Chye and Mohd Ismail (2006), which identified *Saccharomyces*, *Candida*, *Rhodotorula*, and *Cryptococcus* from tapai fermentation. The difference in fungal communities might be due to the variation in raw materials, preparation techniques, environmental conditions, and geographical locations (Zhao et al. 2020). As a result, sasad from different locations of Sabah are predicted to have varying microbial compositions, resulting in a variety of rice wines with unique tastes and aromas.

Many of the fungal genera reported in this work were found in other rice wine starters cultures, such as *Mucor*, *Rhizopus*, *Saccharomycopsis*, and *Kodamaea* in starter samples for the fermentation of Chinese sweet rice wine; *Mucor* and *Rhizopus* in medombae for the fermentation of Cambodian rice wine; as well as *Mucor*, *Rhizopus*, *Saccharomycopsis*, and *Wickerhamomyces* in nuruk for the fermentation of Korean rice wine (Cai et al. 2018; Carroll et al. 2017; Chay et al. 2017). In addition, *Rhizopus*, *Saccharomycopsis*, and *Wickerhamomyces* were the predominant genera identified during the fermentation of Wuyi hong qu glutinous rice wine, while *Rhizopus* and *Saccharomycopsis* were the core fungal genera identified

TABLE 1. Alpha diversity indices of fungal communities in sasad

| Diversity indices | Starter cultures (sasad) | | |
|---|---|---|---|
| | SA | SB | SC |
| Chao1 | $20 \pm 3^{a}$ | $15 \pm 3^{a}$ | $21 \pm 2^{a}$ |
| Shannon diversity index, H' | $1.45 \pm 0.01^{c}$ | $1.32 \pm 0.05^{b}$ | $1.15 \pm 0.04^{a}$ |
| Simpson diversity index, D | $0.71 \pm 0.00^{c}$ | $0.65 \pm 0.01^{b}$ | $0.58 \pm 0.01^{a}$ |

[a-c] Different letters within a row indicate significant differences ($p < 0.05$) between samples

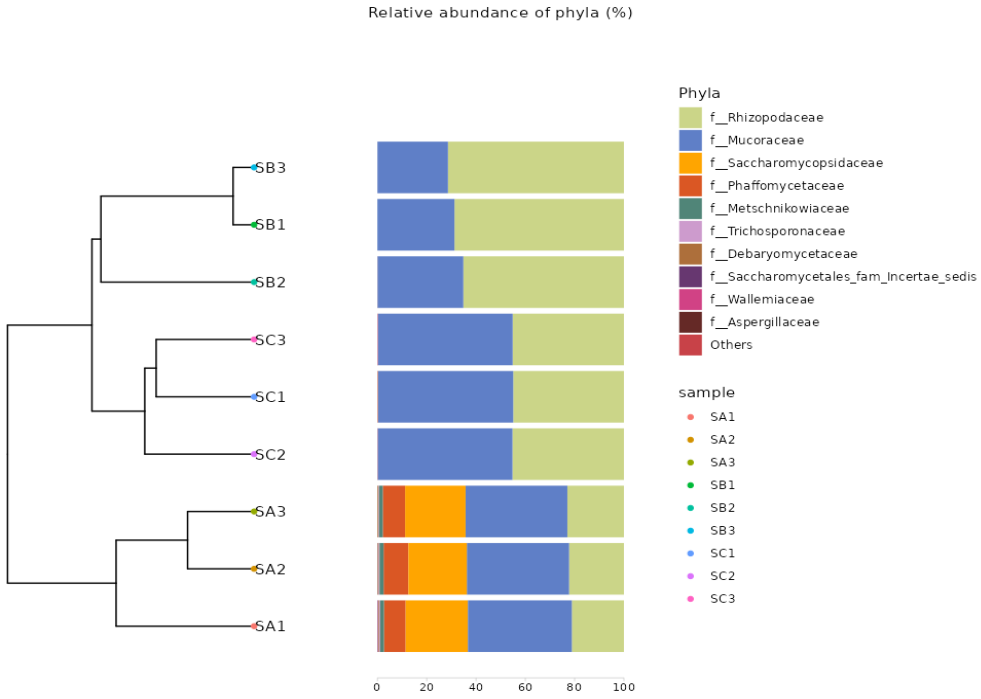


FIGURE 1. Dendrogram (UPGMA) based on weighted UniFrac distance matrices of fungal

during the fermentation of black glutinous rice wine (Huang et al. 2018; Jiang et al. 2020; Zhao et al. 2020).

The predominant fungi in sasad samples, including *Mucor* and *Rhizopus,* were fungi belonging to the phylum Mucoromycota and order Mucorales. This is in agreement with Yang et al. (2011), who reported Mucorales as the most abundant fungi in nuruk, a traditional starter culture for the fermentation of Korean rice wine. *Mucor* was found in all sasad samples, specifically 41.64 ± 0.45, 31.54 ± 3.10, and 54.69 ± 0.10% in SA, SB, and SC, respectively, where the *Mucor* sp. was most likely *M. indicus*.

*Mucor indicus*, otherwise *M. rouxianus*, *M. rouxii*, *Amylomyces rouxii* or *Chlamydomucor rouxii*, is a dimorphic fungus that can exist in both yeast and mould forms (Sharifyazd & Karimi 2017). Data from several sources have shown that both forms of *M. indicus* can efficiently produce ethanol with high yield and productivity, similar to that of *Saccharomyces cerevisiae* (Abtahi 2008; Sharifyazd & Karimi 2017; Sues et al. 2005). An advantage of *M. indicus* over *S. cerevisiae* is its ability to metabolise sugars such as hexoses (glucose, galactose, mannose, and fructose) and pentoses (arabinose and xylose) for ethanol production, where higher ethanol yields were observed under anaerobic fermentation (Sharifia, Karimi & Taherzadeh 2008; Sharifyazd & Karimi 2017; Sues et al. 2005). Therefore, *M. indicus* was classified as an ethanol-producing fungus that can be a good alternative to *S. cerevisiae* for ethanol production.

Other than *Mucor indicus*, *M. circinelloides* which can produce ethanol, was commonly reported in various rice wine starter cultures (Lübbehüsen, Nielsen & McIntyre 2004). For example, both *M. indicus* and *M. circinelloides* were found in Chinese sweet rice wine starters in southern China and traditionally prepared dry starters in North East India (Anupma & Tamang 2020; Cai et al. 2018). Although Cai et al. (2018) reported *M. indicus* and *M. circinelloides* as amylolytic strains, Park et al. (2016) have confirmed that both *Mucor* spp. did not produce α-amylase; however, they did show some glucoamylase activity. In addition to ethanol, *M. indicus* can produce useful compounds, such as chitosan (for food preservation owing to its antimicrobial properties), glucosamine (for treating osteoarthritis), and polyunsaturated fatty acids, particularly gamma-linolenic acid or omega-6 fatty acid (essential for brain function, cell growth, and cardiovascular health) (Sharifia, Karimi & Taherzadeh 2008; Sharifyazd & Karimi 2017).

The other major fungus, *Rhizopus* was identified in sasad samples, SA, SB, and SC with a relative abundance of 22.10 ± 0.92, 68.42 ± 3.12, and 45.08 ± 0.16%, respectively. This confirms previous findings in the

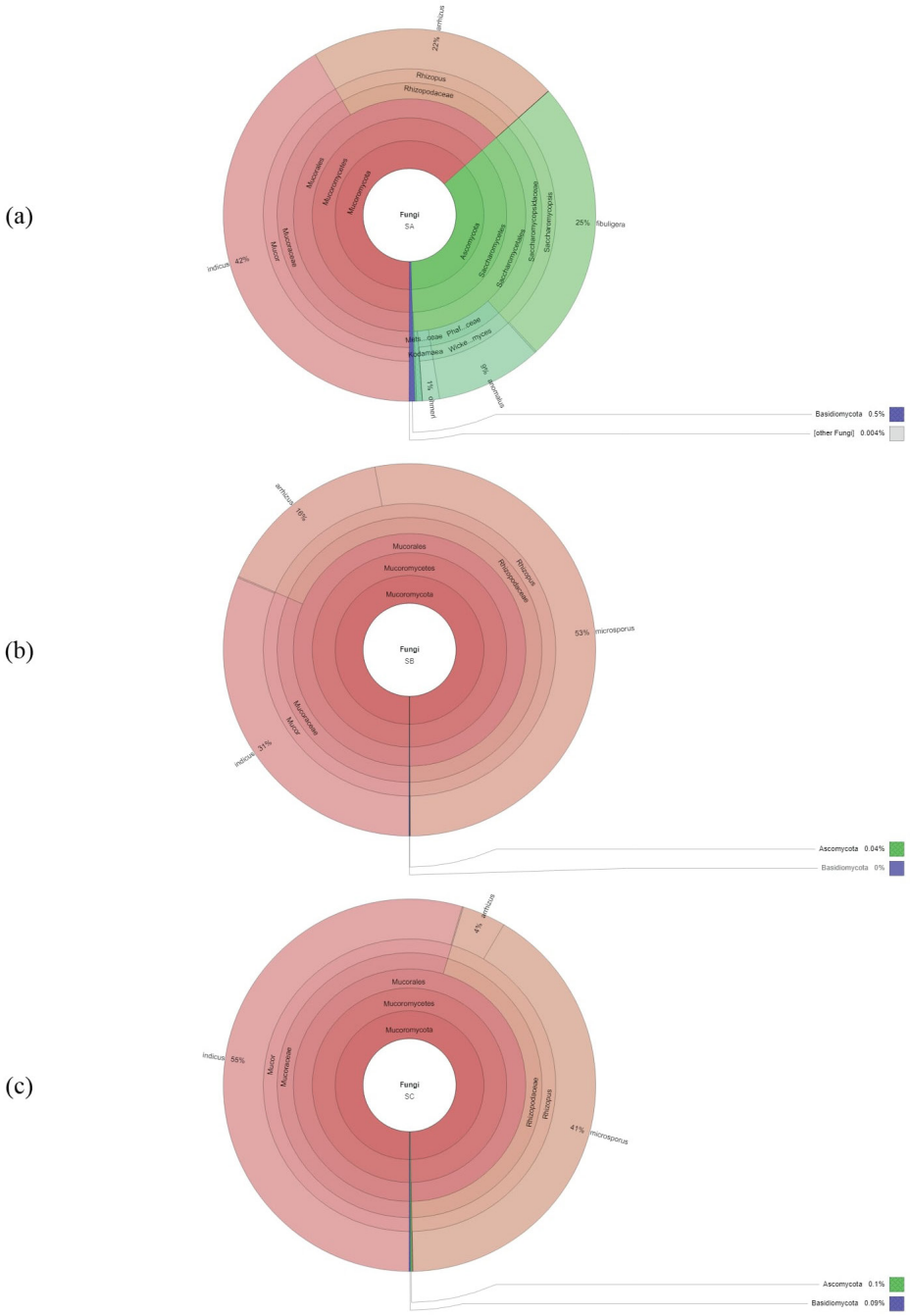


FIGURE 2. Relative abundance of fungi in sasad (a) SA, (b) SB, and (c) SC

literature that showed the predominance of *Rhizopus* in rice wine starter cultures such as Chinese sweet rice wine starters and yao qu (Cai et al. 2018; Lv et al. 2012). Besides that, *Rhizopus* was the dominant fungal genus identified throughout the entire fermentation process of black glutinous rice wine (Zhao et al. 2020). The *Rhizopus* spp. found in the sasad samples were most probably *R. microsporus* and *R. arrhizus*. These strong amylase producers were also found predominantly in bai qu, a traditional starter for hong qu glutinous rice wine (Huang et al. 2019). The ability of *Rhizopus* to produce amylase and glucoamylase shown its role in the saccharification of starch for the fermentation of rice wine (Chen et al. 2020).

In addition, previous studies have shown that some *Rhizopus* spp. can synthesise compounds such as lactic acid and alcohols, which in turn lead to the formation of aromatic esters that contribute to the delicate taste and aroma of rice wines (Chen et al. 2020; Huang et al. 2019). According to Chen et al. (2020), *R. arrhizus* found in jiuyao (a traditional fermentation starter for Shaoxing rice wine) was positively correlated with several flavour compounds in Shaoxing rice wine, including ethyl butanoate (ethyl butyrate) with a fruity odour, reminiscent of pineapples; ethyl dodecanoate (ethyl laurate) with a floral, fruity odour; and ethyl propanoate (ethyl propionate) with a fruity, rum-like odour (Paula Dionísio et al. 2012; Peinado, Mauricio & Moreno 2006). Furthermore, Huang et al. (2019) showed that *R. microsporus* was positively correlated with five acids (2-methyl-pentenoic acid, 3-methyl-pentenoic acid, octanoic acid, 2-octenoic acid, and benzeneacetic acid), four alcohols (isoamyl alcohol, 1-nonanol, α-methyl-benzenemethanol, and α-terpineol), and three esters (ethyl oenanthate, n-caproic acid vinyl ester, and 4-pentadecanyl butyrate) in hong qu glutinous rice wine.

Having considered the phylum Mucoromycota which predominated in all sasad samples (SA, SB, and SC), the phylum Ascomycota, which predominated in SA, needs to be taken into account. *Saccharomycopsis*, *Wickerhamomyces*, and *Kodamaea* were the core fungal genera (relative abundance > 1.00%) belonging to the phylum Ascomycota and order Saccharomycetales identified in SA, in which *Saccharomycopsis* was most abundant, followed by *Wickerhamomyces*, and *Kodamaea.*

*Saccharomycopsis* was found in SA with a relative abundance of 24.49 ± 0.78%, where the *Saccharomycopsis* sp. was most likely *S. fibuligera*. Numerous previous studies have reported *S. fibuligera* as the major amylolytic yeast in rice wine starter cultures (Cai et al. 2018; Chay et al. 2017; Limtong et al. 2002). This was supported by Carroll et al. (2017) and Tsuyoshi et al. (2005), who showed that *S. fibuligera* possessed strong α-amylase and glucoamylase activities, demonstrating its role in starch liquefaction and saccharification during rice wine fermentation. In addition, *S. fibuligera* can secrete *β*-glucosidase for hydrolysing cellulose-based oligosaccharides to produce fermentable sugars for ethanol production (Chen et al. 2020). In contrast, *S. fibuligera* was not an effective ethanol producer as it has limited alcohol fermenting ability (Limtong et al. 2002; Tsuyoshi et al. 2005).

*Wickerhamomyces*, on the other hand, was found in SA with a relative abundance of 9.30 ± 0.67%, where the *Wickerhamomyces* sp. was most likely *W. anomalus. W. anomalus*, previously known as *Candida pelliculosa*, *Hansenula anomala* or *Pichia anomala*, was one of the non-*Saccharomyces* yeasts frequently identified in various alcohol fermentation starters, including baijiu starter (strong-flavour daqu), hong qu glutinous rice wine starters (hong qu and yao qu), and Shaoxing rice wine starter (jiuyao) in China (Chen et al. 2021; Kurtzman 2011; Lv et al. 2017, 2013).

According to Atitallah et al. (2020), *W. anomalus* can efficiently ferment glucose, fructose, sucrose, and mannose to produce ethanol with high yields. This finding was consistent with that of Ruyters et al. (2015), who determined the ability of *W. anomalus* to produce ethanol in a 25% glucose fermentation medium. Their results showed that *W. anomalus* could produce up to 14% (v/v) ethanol, similar to that of *S. cerevisiae*, but with a longer fermentation period. Thus, *W. anomalus* was often reported as an efficient ethanol producer in rice wine starters such as loog-pang in Thailand and banh men in Vietnam (Limtong et al. 2002; Thanh, Mai & Tuan 2008).

Although *Kodamaea* (mainly *K. ohmeri*, formerly known as *Pichia ohmeri* and *Yamadazyma ohmeri*) accounted for 1.44 ± 0.11% of the fungal community in SA, it was reported in previous research as a possible opportunistic fungal pathogen in traditional Chinese sweet rice wine starters due to its ability to cause systemic infections in immunocompromised patients (Cai et al. 2018; Xiao et al. 2013). The occurrence of opportunistic spoilage and pathogenic microorganisms in traditional fermentation starters emphasises the importance of developing well-defined starters with quality control, especially on the microbial composition of starter cultures, to reduce the safety risks.

## BACTERIAL COMMUNITIES IN SASAD

The bacterial communities in sasad, SA, SB, and SC are shown in Table 2. The species richness (Chao1) of SB was significantly lower than SA and SC ($p < 0.05$), while Chao1 of SA and SC did not differ significantly ($p > 0.05$). However, the Shannon and Simpson diversity indices indicated that the bacterial community in SC had the greatest abundance and evenness, followed by SA and SB ($p < 0.05$). According to the dendrogram (UPGMA) constructed based on weighted UniFrac distance matrices of bacterial communities in sasad (Figure 3), the bacterial communities in SB and SC have higher similarities and were more closely clustered compared to SA.

The relative abundances of different bacterial taxonomic levels detected in SA, SB, and SC were illustrated using Krona charts, as shown in Figure 4. Two core bacterial phyla (relative abundance > 1.00%) detected in the sasad samples were Proteobacteria and Firmicutes, in which Proteobacteria was predominant in SB and SC. In contrast, the most abundant bacterial phylum in SA was Firmicutes, followed by Proteobacteria. This finding was consistent with that of Zhao et al. (2020) and Li et al. (2020), who showed that Firmicutes and Proteobacteria predominated the entire fermentation process of black glutinous rice wine and baijiu. However, Proteobacteria was more abundant in the early stages of black glutinous rice wine fermentation, whereas Firmicutes was more abundant in the early stages of baijiu fermentation. Throughout the fermentation of rice wine, the relative abundance of Proteobacteria decreased as the relative abundance of Firmicutes increased, and *vice versa*. The difference in the initial bacterial composition was greatly affected by the type of starter cultures used, while the changes in bacterial communities were dependent on the microbial community succession.

A total of 10 core bacterial genera (relative abundance > 1.00%), namely *Kosakonia*, *Enterobacter*, *Pseudomonas*, *Chromobacterium*, *Paludibacterium*, and *Gluconobacter* in the phylum Proteobacteria as well as *Weissella*, *Lactococcus*, *Bacillus*, and *Enterococcus* in the phylum Firmicutes, were identified from the sasad samples. Many of the core bacterial genera reported in this work were found in other rice wine starters, such as *Bacillus*, *Kosakonia*, and *Weissella* in traditional Chinese sweet rice wine starters (China), as well as *Bacillus*, *Enterococcus*, *Lactococcus*, and *Weissella* in banh men (Vietnam) (Cai et al. 2018; Thanh, Mai & Tuan 2008). Furthermore, *Bacillus*, *Gluconobacter*, *Kosakonia*, and *Weissella* were discovered in black glutinous rice wine fermentation, while *Bacillus*, *Lactococcus*, and *Weissella* were the core bacterial genera identified during the fermentation of Wuyi hong qu glutinous rice wine (Jiang et al. 2020; Zhao et al. 2020)

In contrast to earlier findings by Chiang, Chye and Mohd Ismail (2006), who isolated lactic acid bacteria (LAB), including *Lactobacillus*, *Lactococcus*, and *Pediococcus* from tapai fermentation, LAB found in the sasad samples used for the tapai fermentation in this study was *Weissella*, *Lactococcus*, and *Enterococcus*. The difference in bacterial compositions was most likely caused by the differences in raw materials, preparation techniques, environmental conditions, and geographical regions (Zhao et al. 2020). Additionally, different identification methods might yield different results. For example, the NGS technology used in this study allows for the analysis of complex culturable and unculturable microbial communities, providing more accurate and cost-effective results than culture-based methods that detect only culturable organisms (Huang et al. 2019).

*Weissella*, *Lactococcus*, and *Enterococcus* were the LAB classified in phylum Firmicutes and order Latobacillales that accounted for more than 70% of the bacterial community in the sasad sample SA, in which *Weissella* is a heterofermenter that produces lactic acid, alcohol, and carbon dioxide, while *Lactococcus* and *Enterococcus* are homofermenters that produce lactic acid as the sole product from sugars (Blandino et al. 2003). Similarly, these LAB were found in traditional Vietnamese alcohol fermentation starters known as banh men (Thanh, Mai & Tuan 2008). Previous studies have also shown that LAB such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Weissella* were found abundantly in Chinese rice wine starters (bai qu) (Huang et al. 2019). In addition, LAB such as *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella*, were detected throughout the fermentation of black glutinous rice wine, suggesting that LAB might play an important role in rice wine fermentation (Jiang et al. 2020).

According to Abriouel et al. (2006), LAB were widely found in various fermented foods and beverages due to their ability to survive in low-pH environments during fermentation. It is believed that LAB can produce organic acids during fermentation, providing an acidic environment that favours the growth of fungi for alcoholic fermentation and suppresses the growth of spoilage and pathogenic microorganisms (Niu et al. 2012; Thanh, Mai & Tuan 2008). For example, *Weissella*, one of the predominant LAB in Cambodian traditional dried starters (dombea), was positively correlated with malic acid; while *Lactococcus* found throughout the fermentation of Gutian hong qu glutinous rice wine, contributed to an increase in citric acid (Jiang et al. 2020; Liu et al. 2020; Ly et al. 2018).

Another core bacterial genus identified and classified in the phylum Firmicutes was *Bacillus*, which presented more abundantly in SC (3.69 ± 0.77%) compared to SB (0.87 ± 0.44%) and SA (0.21 ± 0.01%). Likewise, *Bacillus* was found in hong qu starters and dominated the fermentation of glutinous rice wines (Huang et al., 2019, 2018; Jiang et al., 2020). In addition, Cai et al. (2018) and Thanh, Mai and Tuan (2008) reported *Bacillus* as amylolytic strains or starch degraders in Chinese sweet rice wine starters and Vietnamese alcohol fermentation starters, respectively. Zhao et al. (2020) came to a similar result, suggesting that *Bacillus* can secrete hydrolytic enzymes such as amylase, acidic protease, and plasmin, promoting the formation of various flavour compounds in rice wine. This is consistent with a previous study conducted by Jiang et al. (2020), which showed that *Bacillus* was positively correlated with organic acids (butanoic acid, citric acid, lactic acid, and tartaric acid), alcohol (benzyl alcohol), esters (ethyl 9-decenoate and octanoic acid ethyl ester), and alkane (tridecane).

TABLE 2. Alpha diversity indices of bacterial communities in sasad

| Diversity indices | Starter cultures (sasad) | | |
|---|---|---|---|
| | SA | SB | SC |
| Chao1 | 120 ± 5 [b] | 77 ± 2 [a] | 134 ± 10 [b] |
| Shannon diversity index, H' | 2.14 ± 0.05 [b] | 1.45 ± 0.03 [a] | 3.07 ± 0.05 [c] |
| Simpson diversity index, D | 0.69 ± 0.01 [b] | 0.50 ± 0.01 [a] | 0.91 ± 0.01 [c] |

[a-c] Different letters within a row indicate significant differences ($p < 0.05$) between samples

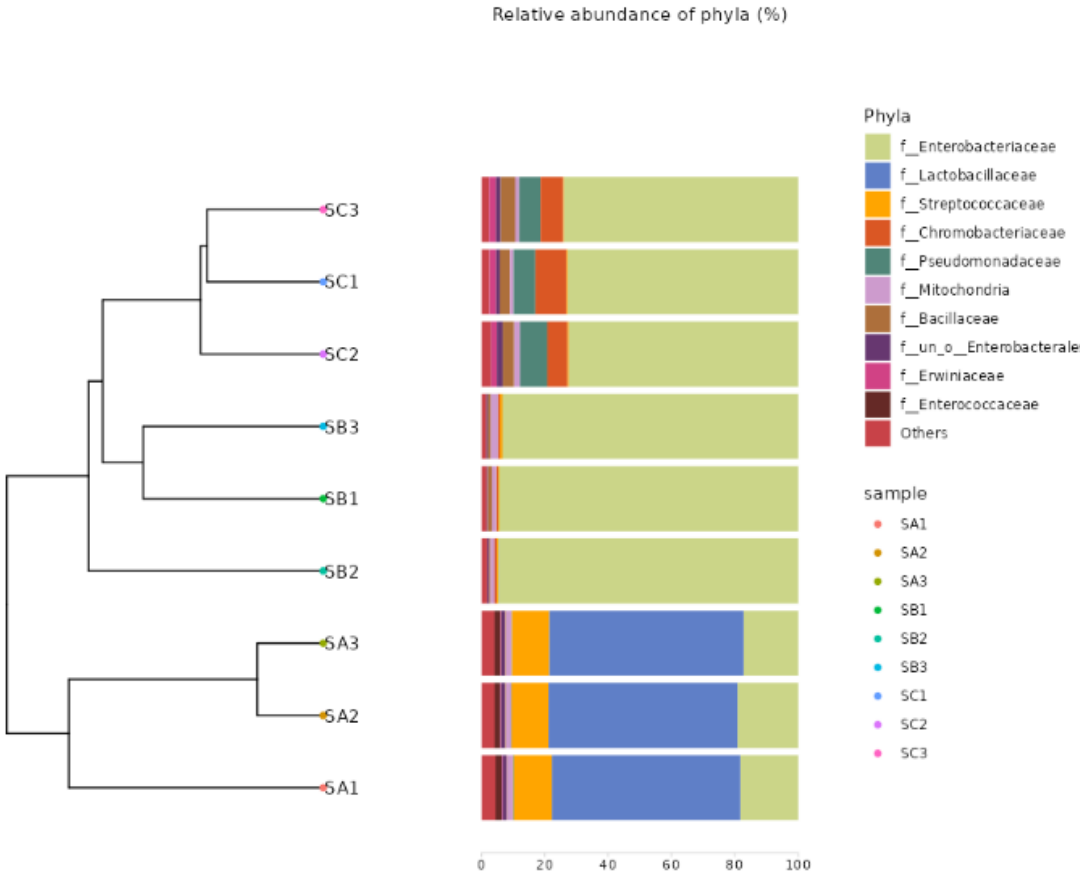


FIGURE 3. Dendrogram (UPGMA) based on weighted UniFrac distance matrices of bacterial

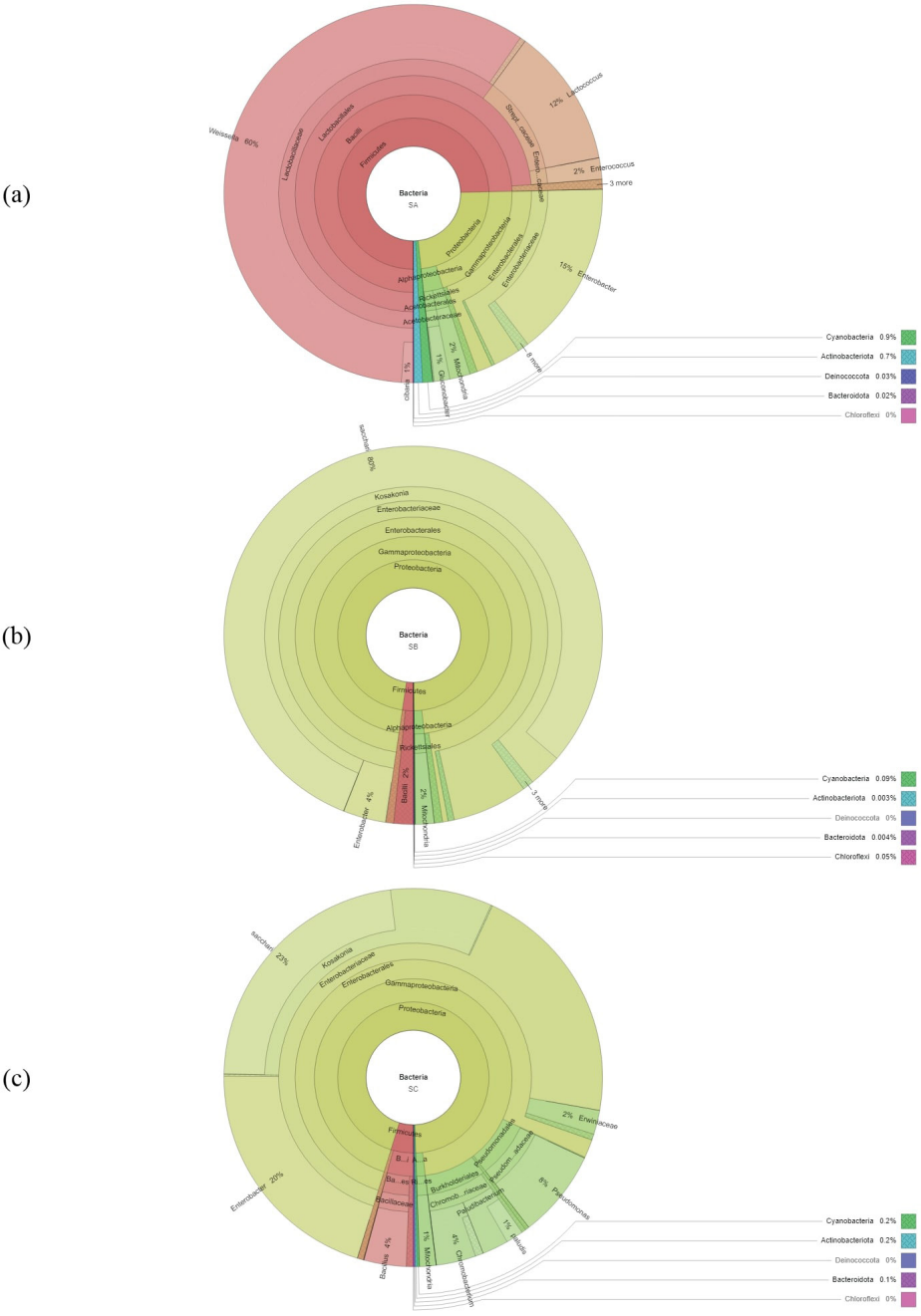


FIGURE 4. Relative abundance of bacteria in sasad (a) SA, (b) SB, and (c) SC

Meanwhile, *Kosakonia* belonging to the phylum Proteobacteria, was the predominant bacterial genus found in sasad samples SB (83.15 ± 0.63%) and SC (31.29 ± 5.18%), while the *Kosakonia* sp. identified from the sasad samples was most likely *K. sacchari*, a type of Gram-negative bacteria commonly isolated from the root of sugar cane (Gu et al. 2014). Several studies have shown that *Kosakonia* is one of the safest aroma-producing bacteria contributing to the sensory quality of rice wines. For example, Xiao et al. (2022) found that *Kosakonia* was positively correlated with 2-methylpropan-1-ol (isobutanol) and 3-methylbutan-1-ol (isoamyl alcohol), the key aroma compounds that contribute to the flavour of the Chinese rice wines (Chen & Xu 2010; Chen et al. 2020; Yang et al. 2017; Yu, Ding & Ye 2012). In addition, Chen et al. (2022) showed that *Kosakonia* was positively correlated with volatile organic compounds such as hexanal and 3-octen-2-one in hong qu rice wine. Zhao et al. (2020) came to a similar conclusion, indicating *Kosakonia* as one of the major contributors to the production of volatile flavour compounds in black glutinous rice wine.

Along these, *Enterobacter* that predominated the sasad samples may also contribute to the flavour of rice wines. Several studies have shown the presence of *Enterobacter* in traditional starter cultures such as jiuyao, xiaoqu, and rice wine koji in China; and alcoholic beverages such as Chinese rice wine and Taiwanese millet alcohol (Chao et al. 2013; Chen et al. 2020; Lü et al. 2017; Xiao et al. 2022; Zhao et al. 2022). According to Liu et al. (2020), *Enterobacter* was one the most prevalent bacteria discovered throughout the fermentation of Gutian hong qu glutinous rice wine and was abundantly found during the later phase of fermentation. However, Chen et al. (2022) and Li et al. (2020) found that the abundance of *Enterobacter* decreased as the fermentation of rice wine proceeded. It is believed that the synergistic interaction of microorganisms and biochemical reactions can lead to the production of flavour compounds, which in turn provide rice wines with their delicate aroma and taste (Jiang et al. 2020).

Other than that, *Pseudomonas* was identified in sasad sample SC with a relative abundance of 7.49 ± 1.00%. Even though *Pseudomonas* was discovered during the fermentation of various rice wines such as makgeolli, hong qu glutinous rice wine, and black glutinous rice wine, this bacterial genus was commonly reported as an opportunistic contaminant that can influence the quality of rice wine or lead to the spoilage of rice wine produced (Huang et al. 2019; Lü et al. 2017; Nile 2015; Zhao et al. 2020).

Meanwhile, *Chromobacterium* and *Paludibacterium* were not commonly identified in rice wines nor their starters, whereas *Gluconobacter* was discovered in loog-pang, a Thai rice wine starter (Chaijamrus & Mouthung 2011). Hence, those three genera are probably contaminants that did not contribute much to the rice wine-making process.

## CONCLUSIONS

Conclusively, the core fungi identified from the sasad samples in this study include amylolytic fungi (*Rhizopus* and *Sacchromycopsis*) and ethanol-producing fungi (*Mucor* and *Wickerhamomyces*). This finding broadly supports the work of other studies in this area, which discovered that both amylolytic and ethanol-producing fungi should be employed for successful rice wine fermentation (Cai et al. 2018; Tsuyoshi et al. 2005). Additionally, most of the fungi identified, including *Rhizopus* (*R. microsporus* and *R. arrhizus*), *Sacchromycopsis* (*S. fibuligera*), and *Wickerhamomyces* (*W. anomalus*) might play a significant role in the flavour formation in rice wines. Considering all the evidence presented, it is reasonable to conclude that the core bacteria identified from the sasad samples contribute mainly to the flavour development of rice wines. In addition, amylolytic *Bacillus* can hydrolyse starch, while acid-producing bacteria such as LAB (*Weissella*, *Lactococcus*, and *Enterococcus*) and acetic acid bacteria (*Gluconobacter*) may promote the growth of fungi by acidifying the fermentation mash, affecting the final composition of rice wines. Together, this study provides important insights into the microbial composition of sasad (traditional starter cultures used for the fermentation of tapai) and the potential role of fungi and bacteria in rice wine fermentation.

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