ARTICLE

A metagenomic analysis of the microbial community structures in the winemaking stages of *'Baya'*: An indigenous heritage rice wine of Batad, Ifugao, Philippines

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ABSTRACT

Baya, an indigenous rice wine from Batad, Ifugao, Philippines, holds a significant place in community festivities, social gatherings, and rituals. Using *binukbok* as the starter culture, the fermentation process transforms the initial juice, *tonoh*, into *Baya* wine. Despite its cultural importance, traditional *Baya* wine is losing favor to commercially produced variants as well as the decreasing interest from younger generations of winemakers. To date, the identification of microbial communities in the stages of making *Baya* wine remains scientifically unstudied. This study therefore aimed to identify and analyze the microbial communities in *binukbok*, *tonoh*, and *Baya* wine, examining their relative abundance, Operational Taxonomic Units (OTUs), and microbial succession. Through this, the fermentation process of *Baya* is better understood while setting the stage for further studies relating to traditional native winemaking. By integrating modern scientific techniques with traditional practices, it helps in preserving the authenticity of the native wine. This not only improves the economic state of the region, but this also allows for the appreciation of our own culture by showcasing our heritage products and heirlooms to the present generation and the next. Samples of *binukbok*, *tonoh*, and the resulting *Baya* wine were obtained from Batad, Ifugao, and were sent for DNA extraction, amplicon sequencing, and analyzed using QIIME2. The 16S rRNA gene sequence analysis revealed common genera across all samples: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, and *Weissella*. For 18S rRNA gene sequence analysis, it was found that unassigned sequences dominated both the starter culture and wine samples, while *Lactobacillus* still predominated in the juice sample. Putative identification through manual NCBI BLAST search revealed *Saccharomyces* as the dominant OTU at the genus level. Alpha diver sity revealed increasing species richness, from juice to starter culture to wine, while beta diversity highlighted distinct microbial communities among samples. This study is a pioneering scientific report on the microbial community structures present in the winemaking stages of the Ifugao indigenous rice wine *Baya*.

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INTRODUCTION

Fermentation is a traditional preservation technique widely employed across various cultures and generations that creates distinct flavors and aromas in food and beverages, preserves food, and removes unwanted compounds (Carboni et al. 2023, Chua et al. 2024). Across Asia, popular rice wines like *Sake* in Japan, C*heongju* in Korea, and S*haoxing* in China are deeply rooted in cultural traditions and made it to the mainstream market. The Philippines, with its rich heritage and artistic traditions, produces various fermented foods concocted from a vast array of indigenous raw materials and microbial resources, reflecting traditional food preferences (Elegado et al. 2016). However, despite the longstanding tradition and cultural importance of fermented products, there have been minimal scientific investigations dedicated to these Philippine traditional products.

Nestled in the remote mountains of the Cordillera Administrative Region (CAR), in the Northern Philippines, the province of Ifugao is renowned for its awe-inspiring ancient rice terraces (Dizon et al. 2012). Within this breathtaking landscape, the tradition of *Baya* wine fermentation thrives, offering a glimpse into the cultural tapestry of Ifugao (Acabado and Martin 2020). The local tradition of wine fermentation, known locally as "*munkiwa,*" is proudly upheld by the Ifugao people, and has endured through centuries, serving as a testament to their indomitable spirit (Mahiwo 2013).

Baya wine, an indigenous rice wine, holds a significant place in Ifugao festivities, social gatherings, rituals, and ceremonies like weddings, harvests, baptisms, and funerals (Cagat 2015, Chua et al. 2024). Its production involves a detailed process that starts with selecting rice varities, typically including *ipuggo*, *botnol, ayuhip,* and *dayakot* glutinous rice varieties (Moore 2014). The rice is manually pounded, soaked in water, and partially cooked (Padang 1984). It is then mixed with *binukbok*, a native starter culture created by combining dough with the roots of a grass herb known as "*on-wad*." (Tuguinay 2009). The mixture undergoes fermentation in winnower or earthenware jars, positioned above a heated stove to expedite the process (Padang 1984, Sicat and Codamon-Dugyon 2016).

Once the desired taste is achieved—seven to ten days for a sweeter taste, and eleven days to thirty days for a stronger, bittersweet alcoholic taste—the fermented rice is carefully moved to a pot container called *gosi* or *angag*, where it is promptly covered with banana leaves to retain its warmth and initiate further fermentation (Tuguinay 2009). Over the course of approximately two to three days, the first juice, known as *tonoh or tanaw*, is collected. This mixture undergoes additional fermentation for around two weeks to reach maturity, resulting in the final product of *Baya* wine (Padang 1984, Sicat and Codamon-Dugyon 2016) (Figure 1). In some cases, the wine may undergo an extended fermentation period of up to a month to enhance its flavor profile and complexity (Sicat and Codamon-Dugyon 2016).

(A) **(B)** **(C)**

Figure 1: Materials used and stages of the winemaking process of the Ifugao rice wine, *Baya*

Traditional wine production is also linked to the local economy of the Philippines as it promotes agricultural practices and provides income for many Filipino families (Gregorio 2023). However, the cultural and artistic significance of traditional wine production is gradually decreasing, overshadowed by modern commercially produced rice wines. Additionally, the diminishing interest among younger generations of winemakers further threatens the preservation of this cultural heritage. To keep its traditions and culture alive, and to deepen our understanding of this heirloom wine, scientific studies on the microbial communities involved in traditional winemaking is imperative. Such research will significantly contribute to preserving the cultural integrity of indigenous rice wines such as the *Baya* wine by laying the foundation for future research about its microbial dynamics. This approach not only fosters appreciation and cultural tourism among people, but it also strengthens the calls for protecting indigenous rights and may boost consumer interest and sales of traditional native wines. This study aimed to identify and characterize the microbial succession and diversity present in traditional *Baya* wine and its starter culture found in Batad, Ifugao, Philippines using 16S and 18S metagenomic sequencing markers.

MATERIALS AND METHODS

Sample Collection, DNA Extraction, and Amplicon Sequencing

The *Baya* wine is traditionally made using a native starter culture called *binukbok*, and the initial juice fermented for seven days, known as *tonoh*, is further fermented for twenty-four more days to produce the final wine product, *Baya*. A block of fresh starter culture (*binukbok*) and fresh samples of *tonoh* and *Baya* were purchased directly from a known traditional winemaker in Batad, Ifugao, Philippines. The collected samples were stored in their original container and were sent to MACROGEN Korea Sequencing Facility for DNA extraction and amplicon sequencing services.

DNA was extracted from the samples by MACROGEN Nucleic Acid Extraction. The quality and concentration of the extracted DNA obtained were quantified using fluorescence-based quantification. A sequencing library was constructed for 16S V3-V4 and 18S V9F-V9R using Macrogen Oligonucleotide Purification Cartridge (MOPC)-purified primers. For bacterial community, the forward primer Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and the reverse primer Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al. 2011) were used to amplify the V3-V4 region of the 16S rRNA gene. For the fungal community, the forward primer 1380F (5'-CCCTGCCHTTTGTACACAC-3') and the reverse primer 1510R (5'-CCTTCYGCAGGTTCACCTAC-3') (Amaral-Zettler et al. 2009) were used to amplify the V9F-V9R region of the 18S rRNA gene. The 300bp purified samples were then sequenced using the MiSeq platform under a paired-end setting.

Bioinformatics Processing

Raw sequenced data were processed using Quantitative Insights Into Microbial Ecology (QIIME) 2 version 2024.2 (Bolyen et al. 2019) through Jupyter notebook (Kluyver et al. 2016). Raw reverse and forward reads were assembled together using the 'join-pairs' function with a minimum overlap of 12 base pairs. Low-quality sequences were filtered out based on a PHRED score of 30, and Operational Taxonomic Units (OTUs) were clustered using Silva 138 99% OTUs full-length sequences at 97% similarity (Michael et al. 2020). Chimeric sequences and singletons were also filtered out using VSEARCH UCHIME plugins.

Taxonomic classification of 16S OTU sequences was done using a pre-trained Naive Bayes classifier with Silva 138 99% OTUs full-length sequences using the sklearn plugin (Bokulich et al. 2018, Michael et al. 2020). For 18S samples, putative identities were determined using nucleotide Basic Local Alignment Search Tool (BLASTn) due to classifier limitations (Altschul et al. 1990).

Metazoan sequences were also excluded from the analysis. For more comparable diversity indices, the dataset was rarefied at a sampling depth of 5,000 using single rarefaction based on the sample with the lowest reads or counts. The final dataset was then used for subsequent diversity analyses. Alpha diversity was assessed using Shannon diversity index, Faith's phylogenetic diversity, observed features, Pielou's evenness, Chao1, and abundance-based coverage estimator (ACE). Beta diversity was evaluated using Bray-Curtis, Jaccard, unweighted UniFrac, and weighted UniFrac distances.

RESULT AND DISCUSSION

Bacterial community structures identified based on 16S rRNA gene sequence analysis

The metagenomic analysis of the starter culture, juice, and wine samples revealed the presence of many bacterial species as shown in Figure 2. One hundred sixty-one thousand three hundred ninety-two (161,392) reads were obtained and analyzed from all the 16S samples and a total of 90 different OTUs resolved at the genus level were identified. *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, and *Weisella* are 6 genera that were found in all samples and were also identified in the study of Geng et al. (2013) to persist throughout the fermentation process. Four (4) other genera were shared by the juice and wine while the starter culture and wine sample shared 12 genera. The starter culture and juice samples do not exclusively share any OTUs. Additionally, 24 genera are unique to the starter culture sample, 2 in the juice sample, and 39 distinct genera in the wine sample.

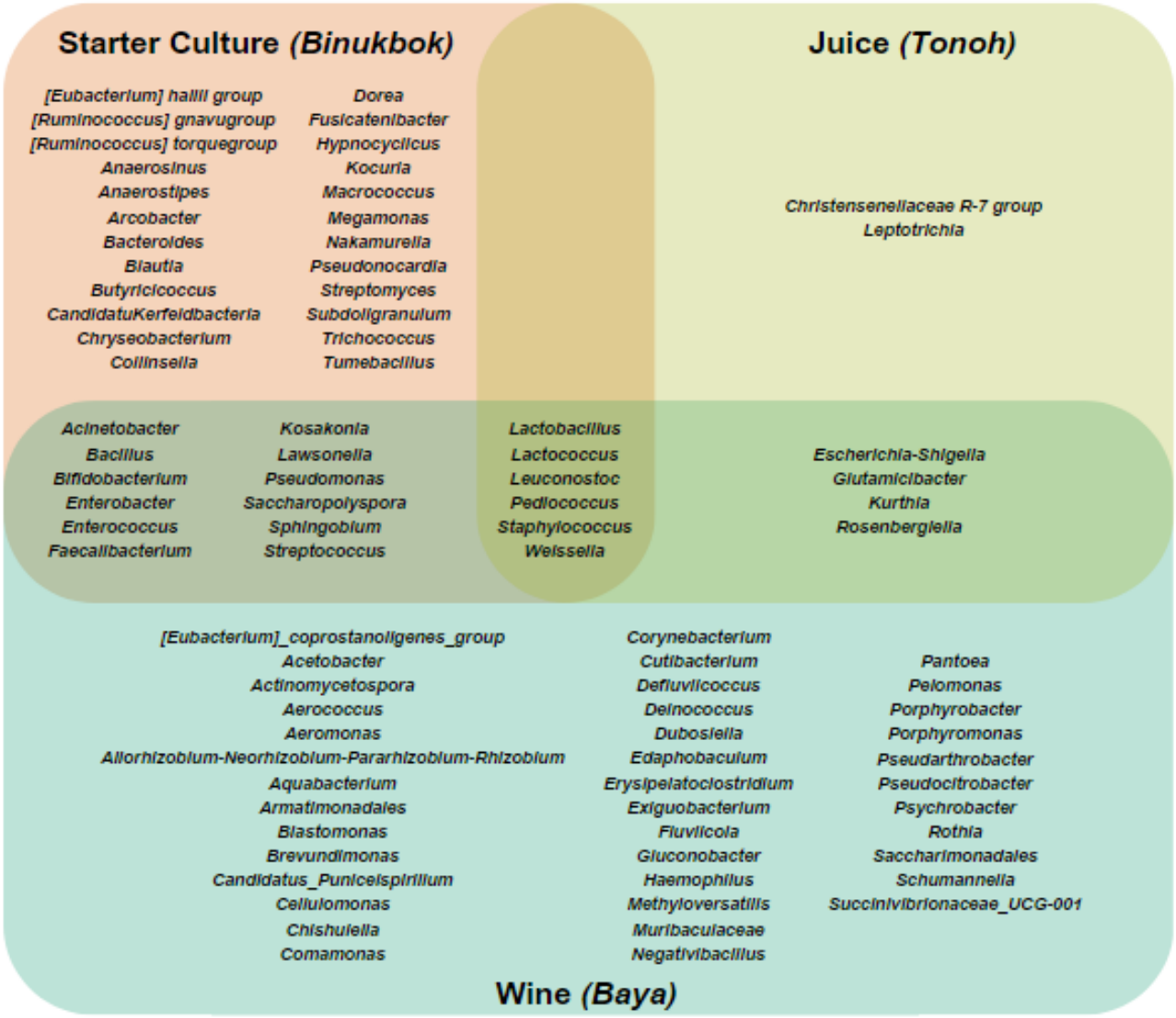

Figure 2: A three-way Venn diagram showing bacterial succession by 16S gene metagenome sequencing during *Baya* wine fermentation: starter culture (*binukbok)*, juice (*tonoh)*, and *Baya* wine.

Lactobacillus is a gram-positive, facultative anaerobe that ferments carbohydrates into lactic acid (Du et al. 2022). In this study, *Lactobacillus* was found to predominate the bacterial composition of the juice (*tonoh*), specifically *Lactobacillus acetotolerans*. Isolates of these bacteria have been reported as hard-to-culture beer spoilage bacteria found in South China breweries. Data from the study of Yin et al. (2018) indicated that *L. acetotolerans* could persist in the brewery environment, particularly in fermentation and maturation tanks, as well as in pitching starter cultures by the formation of the viable but putative non-culturable (VPNC) state.

Lactococcus species, also gram-positive, ferments hexose and glucose sugars, producing lactic acid fermented and are commonly used in dairy product production as an industrial starter culture (Onyeaka and Nwabor 2022, Mills et al. 2011, Jung et al. 2020).

The genus *Pediococcus* is a gram-positive, non-motile, and homofermentative lactic acid bacteria, that ferments glucose to produce lactic acid and is considered a common spoilage bacteria in wine (Onyeaka and Nwabor 2022, Gil-Sánchez et al. 2019). They are lactic acid bacteria that occur along with *Lactobacillus* and *Leuconostoc* which are often used in the production of traditional fermented foods as starters in plant fermentation processes (Holland et al. 2011, Todorov et al. 2022). Some of the strains of the genus caught some interest because of their capacity to synthesize exopolysaccharides, potentially contributing to wine texture, and acetic acid concentration (Gil-Sánchez et al. 2019). According to Osborne (2010), *Pediococcus* spp. have been isolated from wines worldwide. However, they are not well-studied compared with other lactic acid bacteria which is why their prevalence and impact on wine quality have still not been clearly defined.

Members of the genus *Leuconostoc* are epiphytic bacteria found on roots and are widespread in the natural environment. These gram-positive, heterofermentative bacteria are of importance in the industry for these can act as a novel starter culture for an array of fermented foods. In particular, these play a crucial role in the flavor profile and quality of fermented vegetables, dairy products, meats, and alcoholic drinks (Rosca et al. 2023, Zubaidah et al. 2020).

Staphylococcus was observed across all samples, albeit in small quantities. They are gram-positive bacteria classified as either coagulase-positive or coagulase-negative (Namvar et al. 2014). The coagulase-negative *Staphylococcus* are utilized in starter

cultures to influence the aroma and flavor of fermented foods through its production of nitrate reductase (Khusro and Aarti 2022, Lauková 2011).

Weissella, a gram-positive, catalase-negative, and non-endospore-forming lactic acid bacteria that is commonly isolated from a diverse array of habitats including spontaneously fermenting foods and contributes to the development of flavor and aroma in fermented products as a starter culture (Fusco et al. 2018, Teixeira et al. 2021). These are known heterofermenters capable of producing lactic acid, carbon dioxide, ethanol, and acetic acid during carbohydrate fermentation. In particular, these are observed in fermented food items like fermented crop products, and cured meats where *Weissella* often serves as a starter culture (Kačániová et al. 2020, Teixeira et al. 2021).

The genus *Acinetobacter* are obligately aerobic and non-fermenting bacteria commonly found in water and soils (Howard et al. 2012). Although not fermentative, according to Malta et al. (2020), they can contribute to the taste, texture, and odor of food products. Additionally, Wei et al. (2023) mentioned that they dominate in the early stages of liquor fermentation and that they secrete various enzymes such as lipase, esterase, and pectinase. However, it is important to note that certain *Acinetobacter* species, such as *Acinetobacter baumanni,* have the potential to be pathogenic for humans (Adewoyin and Okoh 2018), showing that possible harmful microorganisms may be present in the samples.

Saccharopolyspora is the second dominant genus in the starter culture (*binukbok*) sample. This genus, along with *Acetobacter*, *Bacillus*, and *Streptomyces* was also identified in starter culture samples by Geng et al. (2013). However, as the fermentation progresses, these microorganisms disappear. The study conducted by Liu et al. (2023) stated that *Saccharopolyspora* is capable of breaking down starch and cellulose which contributed to the metabolites present in the *huangjiu* Chinese rice wine. Their results revealed the value of *Saccharopolyspora* as a key functional bacterium in fermented food products.

Relative abundance of bacterial communities

Starter Culture (Binukbok)

The starter culture or *binukbok* is composed of various raw materials such as the roots of an herb abundant in the rice fields, locally known as *on-wad*. The 16S rRNA gene sequence analysis revealed a predominant bacterial composition of *Pediococcus,* making up 82.54% relative abundance of the sample among the 45 identified genera (Figure 3). The composition also included unidentified Archaea and Bacteria, Cyanobacteria, uncultured bacteria *Actinomycetaceae* and Candidatu Kerfeld, and some unassigned taxa.

Juice (Tonoh, intermediate 2-3 days fermented product)

The 16S rRNA gene sequence analysis of the juice or *tonoh* sample was dominated by *Lactobacillaceae*, specifically *Lactobacillus* (99.87%) (Figure 3). The remaining composition includes 11 more genera: *Pediococcus, Weissella, Leuconostoc, Glutamicibacter, Staphylococcus, Rosenbergiella, Christensenellaceae R-7 group, Escherichia-Shigella, Leptotrichia, Kurthia,* and *Lactococcus*, with <1% relative abundance. Among the genus *Lactobacillus*, *Lactobacillus acetotolerans* was predominant, at 99.39% relative abundance. Species-level identification included *Lactobacillus acetotolerans, Lactobacillus fermentum, Lactobacillus brevis,* and *Staphylococcus equorum*. 18S rRNA gene sequence analysis, identified five bacterial genera: *Lactobacillus (*35.29%*), Pseudomonas (*17.65%*),* and *Pedobacter, Brevundimonas*, and *Ochrobactrum* along with an unidentified genus from the family *Rhizobiaceae*, each accounted for an equal relative abundance of 11.76%**.**

Wine product (Baya)

A total of 64 genera were identified in the 16S rRNA sequence analysis of the *Baya* wine sample. *Pediococcus* (40.69%), *Lactobacillus* (25.89%), *Weissella* (11.32%), *Leuconostoc* (9.04%), *Lactococcus* (2.67%), and *Acinetobacter* (2.27%) have >1% relative abundance as shown in Figure 3. *Lactobacillus* was the most recurrent having 6 species under it with *Lactobacillus fermentum* and *Lactobacillus acetotolerans* being the dominant taxa based on relative abundance.

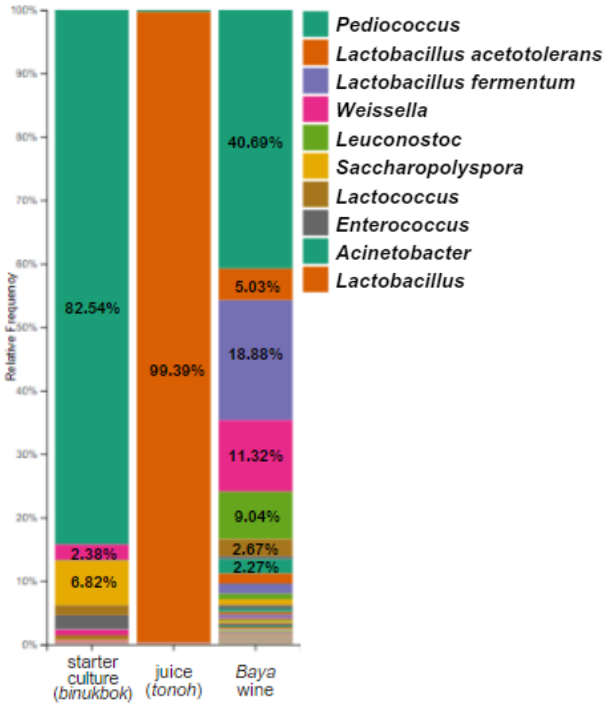

Figure 3: Relative bacterial abundance of the starter culture (*binukbok*), juice (*tonoh*), and wine (*Baya*) samples (left to right) using 16S.

Identification of microbial community structure based on 18S rRNA gene sequence analysis

The sequences from the starter culture (*binukbok*) were matched with a reference database, but could not be identified, suggesting the presence of potentially novel or poorly characterized eukaryotic microorganisms. Further, the 18S rRNA gene sequence analysis revealed several unassigned eukaryotic taxa. To accurately identify these sequences, further studies are required. Additionally, updating existing databases and developing a more specific classifier tailored to these samples is necessary.

For the fermented juice sample or *tonoh*, the 18S rRNA gene sequence analysis identified five bacterial genera: *Lactobacillus (*35.29%*), Pseudomonas (*17.65%*),* and *Pedobacter, Brevundimonas*, and *Ochrobactrum* along with an unidentified genus from the family *Rhizobiaceae*, each accounted for an equal relative abundance of 11.76%**.** Consistent with the 16S rRNA, the juice sample showed dominance of *Lactobacillus*, a common lactic acid bacterium present in fermented products (Ibrahim 2016). The remaining genera, however, are not typical agents of fermentation, and their roles in food fermentation have not yet been well-studied. Moreover, the detection of only bacterial reads in the 18S rRNA bioinformatics analysis can be attributed to poor primer specificity and a bacteria-rich sample, preventing the detection of eukaryotic species (Kounosu et al. 2019). The V9 region of the 18S rRNA gene, used in this study, is deemed less suited for taxonomic resolution due to its short sequence length, unlike the V4 region, which has shown higher variability and taxonomic accuracy (Maritz et al. 2017 as cited in Zahedi et al. 2019).

For the final wine product *Baya*, the 18S rRNA gene sequence analysis showed predominantly unidentified sequences, which were manually identified by performing a BLAST search against the NCBI nucleotide database. Notably, the majority of the unassigned sequences belong to genus *Saccharomyces.* Other taxa resolved at the genus level include *Saccharomycopsis, Wickerhamiella, Kodamea, Starmerella, Hyphopichia, Nakaseomyces, Pichia, Meyerozyma,* and *Xeromyces.*

Saccharomyces, a well-known fermentative yeast, was found to be dominant in the sample, aligning with its essential role in alcohol production. *Saccharomyces cerevisiae*, capable of fermenting both glucose and fructose, is commonly employed in non-inoculated wine fermentation, although its ethanol yield is relatively low (Maicas and Mateo, 2023, Tronchoni et al. 2022). *Saccharomyces pastorianus*, the most abundantly observed taxon in the wine sample, has a homologous match of 53% to *S. cerevisiae* and produces lower levels of acetic and malic acid compared to *S. cerevisiae* (Stewart 2014, Dimopoulou et al. 2020). Similarly, *Saccharomyces paradoxus* can degrade malic acid and produces low ethanol and malic acid but has a high glycerol production (Costantini et al. 2021). *S. paradoxus* also ferments lactose and adds floral and fruity aroma to the wine (Nikulin et al. 2020).

In addition to *Saccharomyces*, nine (9) other genera were identified in the wine samples. *Saccharomycopsis* is an ascomycete known for producing amylolytic enzymes, which plays a role in breaking down starch in rice or cassava during fermentation (Farh et al. 2017). On the other hand, the non-saccharomyces yeasts *Kodamaea*, *Pichia*, *Wickerhamiella*, and *Starmerella* contribute to the wine's unique aroma by influencing the production of alcohol, ester, and aldehyde. *Kodamaea* produces key aromatic compounds such as 3-methyl-1-butanol and ionone, which impart a brandy and rose aroma (Wang et al. 2024). *Pichia,* commonly found in fermented products, can contribute to its flavor through its ability to synthesize esterase enzymes by promoting the production of flavor compounds such as ethyl ester (Xu et al. 2022). In similar manner, *Wickerhamiella versatilis* increases ethyl ester content, facilitating in the development of wine aroma, while *Starmerella etchellsii* enhances benzeneacetaldehyde content which is a carbonyl compound whose flavor notes can be incorporated into the wine's overall aroma (Wang et al. 2022, Duan et al. 2018).

Amylomyces, specifically *Amylomyces rouxii,* is commonly found in Southeast Asian starter cultures for fermented food capable of synthesizing the enzyme amyloglucosidase that enables the breakdown of starch to glucose during the fermentation process (Saito et al. 2004, Delva et al. 2022). *Nakaseomyces* along with *Saccharomyces*, is known to produce high ethanol levels and low biomass during respiro-fermentative fermentation, where yeast uses both respiration and fermentation to produce energy (Legrand et al. 2016). Additionally, this yeast was found actively producing proteins and enzymes essential in the spontaneous fermentation of a rice liquor (Lv et al. 2023). *Meyerozyma guilliermondii* functions in improving and stabilizing the physical color of wines due to the high enzymatic activity of its hydroxycinnamate decarboxylase which produces pyranoanthocyanin adducts (Benito et al. 2019). The presence of a spoilage yeast *Hyphopichia burtonii*, was also observed which can lead to the spoilage in food and beverages (Chamroensakchai et al. 2021). While it was only rarely observed, spoilage yeast may survive under stress conditions and flourish when other microorganisms are not competitive (Loureiro and Malfeito-Ferreira 2003). Hence, it is imperative to monitor yeast contamination during the process of winemaking.

Microbial Succession

At the start of the fermentation process of the *Baya* wine, *Pediococcus* and *Weisella* were dominant in the starter culture (*binukbok*). In the juice sample (*tonoh*), *Pediococcus* dropped to 0.28% while *Lactobacillus acetotolerans* predominated at 99.39%. In the wine sample (*Baya*), *Pediococcus* rebounded to 40.69% dominance. During ethanol fermentation, *Pediococcus* is known to produce high concentrations of acetic acid, lowering the pH, resulting in an acidic environment favorable for *Lactobacillus acetotolerans,* which thrives at pH levels as low as 2.9 and 6% acetic acid (Gil-Sánchez et al. 2019, Goto et al. 2018). In contrast, *Pediococcus* growth is only favored at pH levels greater than 3.50, causing its decline in intermediate stages (Osborne, 2010). *Weisella* showed a similar trend with *Pediococcus* where it decreased in the juice sample and increased in the wine sample due to its tolerance for pH levels above 4.0 (Fusco et al. 2015). As the pH decreases, microorganisms that cannot tolerate acidic conditions, such as *Weissella* and the other bacteria in the starter culture sample that have ceased to grow, are inhibited or outcompeted, allowing the *Lactobacillus acetotolerans* to dominate and cause the shift in microbial dominance.

In the wine sample, *Lactobacillus acetotolerans* decreased while *Lactobacillus fermentum* emerged. As the fermentation progresses, sugar is reduced and the alcohol content increases due to ethanol production, which alters the environment and makes it more habitable for other organisms like *Lactobacillus fermentum* that have higher ethanol tolerance (Otegbayo et al. 2020). *Lactobacillus fermentum* thrives in later stages of fermentation when alcohol levels are higher, nutrients are depleted, and metabolic byproducts accumulate, demonstrating its ability to utilize a wide range of nutrients and tolerate metabolic byproducts (Ibrahim 2016, Zhao et al. 2019). This versatility enables it to adapt more effectively to the available nutrient sources and thrive in diverse fermentation environments that might inhibit other microorganisms.

Microbial Diversity

Samples were classified into three categories, starter culture (*binukbok*), juice (*tonoh*), and wine (*Baya*). To look for consistent patterns of microbial diversity in each sample, multiple alpha diversity indices were assessed. The values showed consistent increase in the species richness from juice (lowest number of taxa), progressing through starter culture, and reaching wine (highest number of taxa) (Table 1). This pattern was observed across all the tested alpha diversity metrics, despite the differences in index measures. The consistency of the pattern implies that there is stable and predictable microbial succession within the samples (Ortiz-Álvarez et al. 2018).

Table 1: Summary of Alpha Diversity Indices per sample.

Alpha Diversity Index	Starter Culture (*binukbok*)	Juice (*Tonoh*)	Wine (*Baya*)
Shannon	1.864193507	0.091041605	4.031180615
Faith's PD	4.350702822	1.474525514	7.990649982
Observed Features	124	16	257
Pielou's Evenness	0.268067426	0.022760401	0.503543551

The observed greater microbial diversity in the wine could potentially be attributed to the recruitment of various microbial taxa during the fermentation process. Keystone microorganisms in fermentation processes such as *Lactobacillus* and *Saccharomyces* influence microbial diversity by modifying the environment to a more favorable habitat, enhancing niche complementarity (Boynton and Greig 2016). Further, dominant species may also exert antagonistic interactions to wine spoilage microorganisms, consequently increasing diversity (Boynton and Greig 2016, Mas et al. 2016).

Greater microbial diversity in wine potentially translates to a wider range of microbial functions. The activity of different microbial species and strains significantly impacts the organoleptic profiles of wine, enhancing its complexity and sensory richness (Mas et al. 2016). This can be attributed to the end products of microbial activity—acids, alcohols, and aromatic compounds— directly impacting wine aroma and flavor. These compounds can also interact with juice components that can either enhance or mask varietal characters. In line with this, the characteristic microbial diversity observed in *Baya* wine is crucial as it imparts the indigenous wine's authenticity and typicity. The microbial population unique to *Baya* wine, its microbial fingerprint, provides the wine with a distinctive character.

Beta diversity compares the diversity among different samples, focusing on the relatedness and differences in the composition of microbial communities. Calculated beta diversity indices particularly: Bray-Curtis, Jaccard, unweighted UniFrac distance, and weighted UniFrac distance were also subjected to principal coordinate analysis (PCoA) to investigate the variation in microbial communities across all samples and observe how the samples would cluster in an ordination space. Bray-Curtis dissimilarity emphasizes both composition and relative abundance as it measures not only compositional differences between samples but also the differences in the overall counts, or their relative abundance (Greenacre 2017). Jaccard distance is based on the presence or absence of data but does not include abundance information. For UniFrac distances, the occurrence table and phylogeny diversity or the sequence distance are taken into account. In weighted UniFrac distance, phylogenetic differences between microbial communities are measured, taking into account both the presence or absence of lineages and their relative abundance. In contrast, unweighted UniFrac only considers the presence or absence of lineages.

PCoA was performed to measure the dissimilarity between the starter culture, juice, and wine samples as shown in Figure 5 and Figure 6. The PCoA plot generated 3 axes illustrate 100% of the total variance among the 3 samples wherein the starter culture (*binukbok*), juice (*tonoh*), and wine (*Baya*) are represented by a sphere, diamond, and square, respectively. All four-distance metrics show the same trend wherein the three samples are ordinated far from each other, which indicates that the species present and their relative abundance per sample are distinct, suggesting that the microbial community structure across all samples are unique from each other in terms of composition and abundance of species.

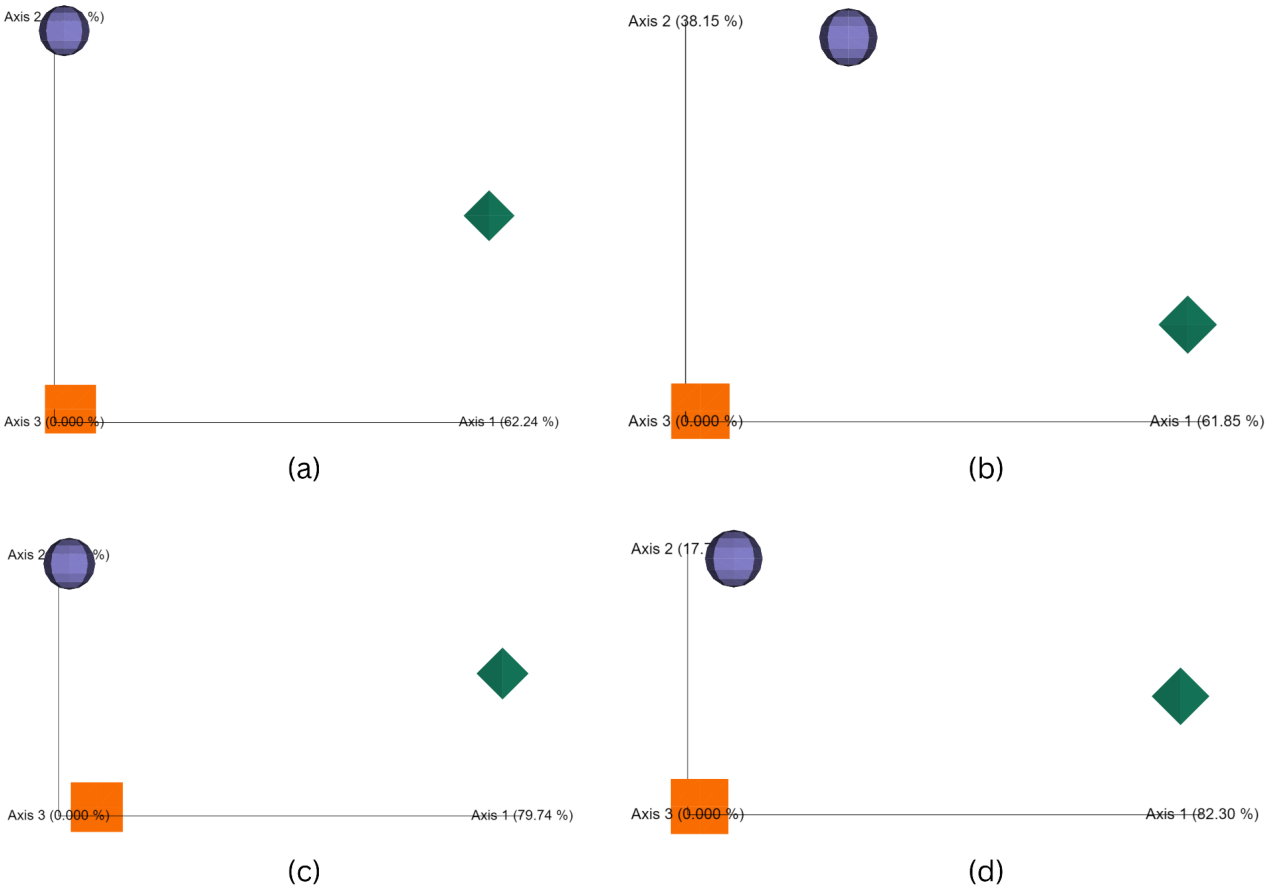

Figure 4: Principal coordinate analysis (PCoA) plots showing the beta diversity between the starter culture or *binukbok* (sphere), juice or *tonoh* (diamond), and wine or *Baya* (square) samples using the following four distance measures: (a) Jaccard distance, (b) Unweighted Unifrac, (c) Bray Curtis Dissimilarity, and (d) Weighted Unifrac.

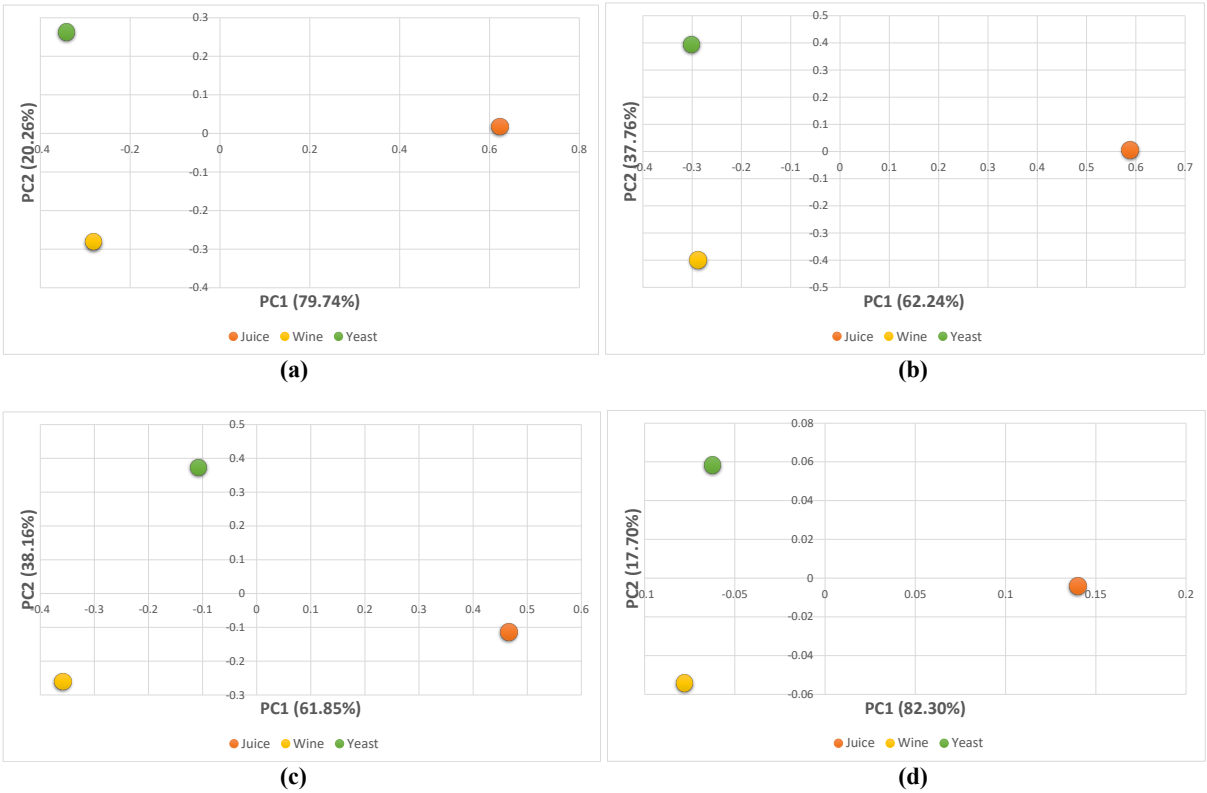

Figure 5: Principal coordinate analysis (PCoA) plots showing the beta diversity of the following four distance measures: (a) Jaccard distance, (b) Unweighted Unifrac, (c) Bray Curtis Dissimilarity, and (d) Weighted Unifrac.

These results imply that there are temporal changes occurring as the winemaking process progresses from the starter culture to extracting the first juice and finally to completing the fermentation process to yield the final beverage product, the *Baya* rice wine. While the samples share similarities in terms of the genera present, their overall structure and size of the microbial community differs in each stage. These changes may be associated with the fermentation process which affects the bacterial composition present in each stage (Piao et al. 2015).

At present, the study mainly focuses on the identification of microbial communities and the succession across different samples used and produced in the production of Baya wine. Parameters such as pH levels, sugar content, and other fermentation parameters were not included, and exclusion of such parameters may affect in understanding the role of each factor in the emergence and succession of the microorganisms. This is the limitation of the study. However, the identified microorganisms' respective niches can be inferred from existing literature. Furthermore, the identification of the samples of the study was limited to the genus level as not all microorganisms found using the classifier had species identification. Improvement of the current existing database and classifier used is warranted to achieve a better grasp of the identities of the microbial communities.

CONCLUSION

The 16S rRNA gene sequence analysis revealed common genera found in the solid starter culture (*binukbok*), in the intermediate fermented juice (*tonoh*) and in the final wine product (*Baya*) samples namely: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, and *Weissella*. For 18S rRNA gene sequence analysis, unassigned sequences dominated both the starter culture and wine samples, while *Lactobacillus* still predominated in the juice sample. Putative manual identification through manual NCBI BLAST search revealed *Saccharomyces* as the dominant OTU at the genus level. Alpha diversity revealed increasing species richness from juice to starter yeast to wine, while beta diversity highlighted distinct microbial communities among samples. Further studies should analyze more samples for comprehensive results, use ITS rRNA sequencing analysis for more fungal community identification, and explore environmental factors affecting fermentation. Additionally, updating existing reference databases or curating specific databases can improve OTUs identification specific to the samples. Comparisons with commercially produced rice wines and incorporating proteomics for functional insights are also recommended.

Despite its cultural importance, traditional *Baya* rice wine is a rarity and is losing favor to commercially produced variants as well as the decreasing interest from younger generations of winemakers. To date, this report is a pioneering study to provide scientific information on the identification and succession of microbial community structures involved in the stages of traditional winemaking of the Ifugao indigenous rice wine *'Baya'* which is one of the rich, varied and treasured indigenous products of the Ifugao people and the rest of the Cordillera region of the Philippines.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest related to this publication.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

All authors contributed to the study's conceptualization, data collection, analysis, manuscript preparation, and have approved the final version.

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