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Diversity of fungi and dynamics of ethanol concentration during fermentation of porang (*Amorphophallus oncophyllus*)

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Abstract. *Helmi H, Kusmiadi R, Mahardika RG, Karsiningsih E. 2024. Diversity of fungi and dynamics of ethanol concentration during fermentation of porang (*Amorphophallus oncophyllus*). Biodiversitas 25: 542-552.* Fermentation can be an alternative to process porang (*Amorphophallus oncophyllus*). As one of the fermentation products, ethanol can degrade starch and consequently increase the purity of glucomannan in porang flour. However, ethanol can also inhibit the growth of microorganisms that play a role in fermentation. This study aimed to determine the ethanol concentration and investigate the fungal diversity during the fermentation of porang tubers. Ethanol analysis was analyzed by Headspace Gas Chromatography coupled with flame ionization detection, and Starch content was analyzed by colorimetric method. Fungal metagenome was amplified at the Internal transcribed spacer (ITS) region of nuclear DNA (rDNA) and followed by sequencing using Oxford Nanopore Technology (ONT). The result showed that the ethanol concentration increased during 48 hours of fermentation, followed by decreased starch content. The highest fungal diversity index occurred at 48 hours of fermentation. Ethanol and fungal enzymatic activity can degrade the starch. During fermentation, the *Candida parapsilosis* population increased until 24 hours after fermentation. *Cyberlindnera subsufficiens* population increased at 48 hours fermentation until 72 hours fermentation. Fungal diversity and ethanol concentration had decreased at 72 hours of fermentation, so porang fermentation should be stopped at 48 hours. *Candida parapsilosis* could be the candidate of starter for fermentation porang due to the capability of this species to produce ethanol to degrade starch.

Keywords: Fermentation, fungi, metagenome, porang, yeast

INTRODUCTION

Porang is a kind of plants that grow well in Indonesia and is still one genus of konjac plant found in hilly areas in sub-tropical regions in Southeast Asia (Impaprasert et al. 2014; Manab et al. 2016; Nurlela et al. 2019). Porang tuber is one of Indonesia's export products due to its glucomannan content (Harmayani et al. 2014; Wardani and Arifiyana 2020; Sari et al. 2022). Physical and chemical methods are the former and frequently applied to process porang. One of the rare methods to process porang tuber is fermentation. Fermentation involves microbial enzyme activity (Jung et al. 2013; Feng et al. 2018; Sharma et al. 2020). Fermented foods have many advantages, such as containing probiotics, being more digestible, rich in vitamins, amino acids, antioxidants, peptides, and antimicrobial activity (Chaudhary et al. 2018; Sharma et al. 2020; Flibert et al. 2021). Fermentation can improve the nutrition of porang fermented flour, while glucomannan content in porang has been noted as prebiotic and known as Generally Regarded as Safe (GRAS) (Wardhani et al. 2017).

Starch is the main compound of carbohydrates in porang flour, approximately 70-85% (Nurlela et al. 2019). Starch is an impurity compound in the extraction of konjac to yield glucomannan. Microorganisms can degrade starch to compounds such as ethanol and organic acids (lactic acid, acetic acid, fumaric acid). Previous studies also showed that ethanol can reduce the starch in the extraction of glucomannan from konjac and porang; ethanol can dissolve the starch but not the glucomannan (Nurlela et al. 2021).

Fungi, especially yeasts, can produce amylase and ethanol (Riesute et al. 2021). The yeast population determines the metabolite, characters, and organoleptic of food. Not all yeasts have fermentation benefits, but they are also responsible for food spoilage (Flibert et al. 2021; Riesute et al. 2021). During fermentation, there are dynamics of microorganisms depend on the condition and substrate available (Pereira et al. 2015a). The dynamic population of yeasts during fermentation was noticed in cassava sour fermentation. *Pichia kudriavzevii* and *Issatchenkia orientalis* were found only at the beginning of fermentation, while *Geotrichum candidum, Clavispora lusitaniae,* and *Rhodotorula mucilaginosa* were detected during cassava fermentation. *Candida rugosa, C. pararugosa, C. akabenensis, Cryptococcus albidus, Neurospora crassa* and *N. intermedia* were found in the final product (Reboucas et al. 2016).

However, only some microorganisms can be cultured, so the data on microbial diversity in fermented foods is often biased and does not reflect actual circumstances (Yao et al. 2021a). Hence, advanced approaches are needed to

study microbial diversity, including metagenomics (Kergourlay et al. 2015; Kimura 2018; Nam et al. 2023). Metagenome approach to investigate fungal diversity has been applied to some fermented food such as beer (Shinohara et al. 2021), cassava sour flour (Reboucas et al. 2016), and green table olives Negrinha de Freixo cv (Pereira et al. 2015a). The internal transcribed spacer (ITS) region of nuclear DNA (rDNA) has become the most sequenced region to identify fungal taxonomy at the species level (Schoch et al. 2012; Nam et al. 2023). Oxford Nanopore Technology (ONT) provides long-read sequencing that covers the full-length sequence of ITS region through a fast, cheap, and high throughput process (Shinohara et al. 2021). Since all the informative sites of the ITS region are considered, the full-length ITS sequences offer a higher level of taxonomic and phylogenetic resolution for fungal identification (Schoch et al. 2012; Siddique et al. 2022).

The fungal diversity during porang fermentation had not been reported. Fungi, especially yeasts, produce ethanol during fermentation. In porang fermentation, ethanol can dissolve starch and consequently increase the purity of glucomannan. However, ethanol is also a limiting factor for the growth of yeasts. This study aimed to determine the ethanol concentration and investigate the fungal diversity during the porang fermentation through an unculturable method followed by *Oxford Nanopore Technology* sequencing. This study was fundamental research to evoke the fungal diversity involved in porang fermentation and could be the foundation for developing an autochthonous starter for the fermentation of porang. This research was expected to contribute to the food industry for functional and nutritional fermented porang flour.

MATERIALS AND METHODS

Chemicals

DNA miniprep kits (Zymo research Catalog 4300), agarose (Sigma-Aldrich), DNA ladder (Invitrogen), Ethanol ACS reagent grade >99.8% (Sigma-Aldrich), 1-Propanol ACS reagent grade >99.5% (Sigma-Aldrich), Water ACS reagent (Sigma-Aldrich).

Fermentation of porang tubers

Porang tubers (*Amorphophallus oncophyllus*) were peeled, sliced with thick 1.5 mm and washed with tap water. After draining, 1 kg of sliced porang tubers was soaked with 2 L of distilled water. For 0 hours of treatment, 50 mL of soaked water was taken and prepared for ethanol and metagenomic analysis. 50 mL of fermentation water was taken after 24 hours, 48 hours, and 72 hours of fermentation for ethanol and metagenomic analysis. Before further analysis, all soaked and fermented water was kept at -20°C. After fermentation, porang tubers were drained and dried using a food dehydrator at 50°C. Dried porang tubers were mashed using a blender and filtered using a 90-mesh sieve. Next, 200 grams of fermented porang flour was taken to determine the starch content.

Ethanol analysis

Ethanol analysis was carried out using headspace gas chromatography coupled with flame ionization detection (HS-GC- FID), according to Ebersole et al. (2017), with slight modification to sample preparation. Several steps must be prepared before injection to GC: headspace condition preparation, GC condition preparation, reagent preparation, standard reference materials, sample collection, and standard and sample preparation. Headspace condition preparations included an incubation temperature of 80°C, a syringe temperature of 85°C, and a heating time of 15-20 minutes. GC-FID condition preparations included column type used was J&W DB-WAXetr (0.53 mm × 30 m, 2 μm film), initial GC oven temperature was set at 40°C, oven temperature gradient was held at 40°C for 10 minutes, then was increased at 25°C/minutes until reach at 240°C and hold at 240°C for 1 minute. The run time for GC was 20 minutes. FID temperature was set at 250°C. GC injector temperature was set at 150°C, and Helium was used as carrier gas at 7 mL/minute. The reagents that need to be prepared, namely ethanol ACS reagent grade, >99.8% (Sigma-Aldrich), 1-propanol ACS reagent grade, >99.5% (Sigma-Aldrich). Standard reference materials consisted of internal standards, namely propyl alcohol (1-propanol) purity 99.98% (Sigma-Aldrich), and standard stock solutions, namely ethanol reference standard absolute 200 proof, purity 99.97% (Sigma-Aldrich). Samples were collected from porang tuber's fermentation water at 0 hours, 24 hours, 48 hours, and 72 hours of fermentation; as much as 20 mL of samples were taken using a micropipette and poured into sterile bottle samples. Samples were kept at -20°C before analysis. Standard preparations consisted of ethanol stock solution prepared by mixing 5 mL ethanol reference standard with 95 mL water. The internal standard stock solution was prepared by mixing 5 mL 1-propanol reference standard with 95 mL water. Sample preparation was prepared by adding each fermented porang flour (10mL) sample with 0.1 mL internal standard solution and transfer into a 20 mL headspace vial. For analysis, HS-GC-FID was set up with conditions that had been mentioned previously. After HS-GC-FID was set up, a single injection of each sample and standard was injected, and chromatographic peak area response was measured. The injection volume for each sample was 200 μL. Ethanol and 1-propanol were peaks in the sample solution identified by comparison with the retention time of the ethanol standard solution.

Starch analysis

The enzymatic colorimetric method determined starch content (AOAC 2007; Zhu et al. 2016). 100 mg of porang fermented flour was added to a test tube. Next, 0.2 mL of ethanol solution (80%, v/v) was added into the tube. Next, 3.0 mL of thermostable α-amylase (Megazyme, USA) was immediately added, and the tubes were boiled for 6 minutes and stirred at 2-minute intervals. Tubes were then placed in a 50°C water bath for 5 minutes. Next, 0.1 mL of amyloglucosidase (Megazyme, USA) was added to each tube. Tubes were then stirred and incubated for 30 minutes. 10 mL of distilled water was poured into each tube and

centrifuged at 1,800 × g for 10 minutes at room temperature. Next, 1.0 mL of aliquots from the supernatant was diluted to 10 mL with distilled water. Then, 0.1 mL of this diluted solution was placed into a clean test tube. 3 mL of glucose oxidase reagent (Diagnostic System International, Germany) was added to each tube and incubated at 50°C for 20 minutes. For blanks, 0.1 mL of water was used instead of 0.1 mL of diluted solution, and the other reagents were added with the same quantity. Samples were read for absorbance at 510 nm.

Isolation of DNA metagenome and amplification

DNA extraction was carried out using DNA MiniPrep (Zymogen USA) with the following methods: 1 mL of soaked or fermentation water was put into a ZR Bashing Bead, then added with 750 µL Zymobiomic Lysis Solution for 30 minutes at 60°C before being lysed on Beat Beater for 5 minutes. In the next step, it was centrifuged at 10,000 ×g for 1 minute. After centrifugation, 400 µL of the supernatant was transferred to a Zymo Spin filter IV, where a Collection Tube was placed under the Zymo Spin filter IV. The filtrate in the collection tube was mixed with 1200 µL of DNA binding buffer. 800 µL of this mixture was put into the Zymo Spin IIIC filter and centrifuged at 10,000 ×g for 1 minute. The filtrate from the Collection tube was discarded, and the previous step was repeated in the filter: 800 µL was put into the Zymo Spin IIIC filter and centrifuged at 10,000 ×g for 1 minute before the filtrate was discarded again. Zymo Spin IIIC filter was inserted into a new Collection Tube and mixed with 400 µL of DNA wash buffer I, followed by centrifugation at 10,000 ×g for 1 minute, and the filtrate was discarded. Next, 700 µL of DNA wash buffer II was added, centrifuged at 10,000 ×g for 1 minute, and then the filtrate was discarded. 200 µL of DNA wash buffer II was added again and centrifuged at 10,000 ×g for 1 minute then the filtrate was discarded. 75 µL of DNAse free water was added to the Zymo Spin IIIC, which had a new microtube under it. Then, the filtrate was transferred from the microtube to ZymoHRCPrep, which had a new microtube at the bottom, and centrifuged at 16,000 ×g for 1 minute. The DNA quantity was calculated with the PicoGreen method using the Victor 3 fluorometer. PCR amplification was carried out using MyTaq HS Red Mix,2X (Bioline, BIO-25048), and ITS primer (ITS1-ITS4) with the method according to Ohta et al. (2023). After amplification, 2 µL PCR products were assessed by electrophoresis with 1% TBE agarose and 100 bp DNA ladder (loaded 2.5 µL). DNA concentration was determined using NanoDrop spectrophotometers.

DNA sequencing

Library preparations were conducted using PCR Barcoding kits from Oxford Nanopore Technology with the method according to Ohta et al. (2023); sequencing DNA used kits from Oxford Nanopore Technology. Nanopore sequencing was operated by MinKNOW software version 23.04.5. Basecalling was performed using Guppy version 6.5.7 with the high-accuracy model (Wick et al. 2019). FASTQ file quality was visualized using NanoPlot, and quality filtering was performed using NanoFilt (De Coster et al. 2018; Nygaard et al. 2020). Filtered reads were classified using a Centrifuge classifier (Kim et al. 2016). The Fungi index was built using the NCBI ITS RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Downstream analysis and visualizations were performed using Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and RStudio using R version 4.2.3 (https://www.R-project.org).

RESULTS AND DISCUSSION

Ethanol and starch analysis

The concentration of ethanol and starch content during fermentation is shown in Table 1. Ethanol increased until 48 hours and decreased at 72 hours of fermentation. Starch content increased at 24 hours of fermentation and decreased at 48 and 72 hours.

The quantity of DNA metagenome and quality of PCR product

The quantity of extracted DNA metagenome was shown in Table 2 that all DNA had concentration (3.1 to 51.2) with the ratio of A260/A280 ranged 1.97 to 2.07 that passed the quality for downstream analysis or further analysis (~1.8). These sample concentrations could continue to amplify (PCR process). The agarose gel electrophoresis photo of the PCR product (Amplification of gDNA with Primer ITS1-ITS4) can be seen in Figure 1. The quality of genomic DNA was visualized by agarose gel showed that the DNA size was approximately 400-700bp.

Table 1. The dynamics of ethanol concentration during fermentation. Data were acquired from HS-GC-FID analysis in three replicates using propanol as the internal standard

Samples	Ethanol concentration (ppm)	Starch content (%)
0 hours	234.63±4.31	50.52±0.31
24 hours	574.26±15.12	53.56±0.15
48 hours	813.85±0.85	46.83±0.20
72 hours	253.76±2.02	46.82±0.09

Table 2. The quantity of extracted DNA metagenome during fermentation. Data were acquired from the Nanodrop Spectrophotometer

Samples	Volume (µL)	Concentration (ng/µL) Nanodrop	A260/A280	A260/A230
0 hour	50	3.1	2.73	0.97
24 hours	50	3.6	2.68	1.19
48 hours	50	5.2	2.70	1.01
72 hours	50	51.2	1.97	2.07

Alpha diversity of fungi during fermentation

Figure 2 shows the alpha diversity. Alpha diversity describes the mean species diversity in samples. The richness, dominance, and diversity index changed during 72 hours of fermentation. Figure 2 showed that samples 48 hours had the highest richness of fungi while samples 72 hours had the highest dominance within the sample. Based on diversity indices (Shannon, Simpsons, and In Simpsons) showed that 48 hours had the highest diversity. Samples at 24 hours had the lowest index diversity (Figure 2).

Fungal abundance

The top 10 of the highest fungi at phylum, class, family, and genus levels can be seen in Figure 3. All of the samples were dominated by fungi Ascomycota, at the ordo level, dominated by Saccharomycetes. At the family level, dominated by Debaryomycetacae at 0 and 24 hours; at 48 hours, dominated by Debaryomycetacae and Phaffomycetacae; at 72 hours dominated by Phaffomycetacae. Genus Candida dominated 0 and 24-hour samples, while Cyberlindnera dominated 48 and 72 hours. There were differences in yeast species among samples. *Candida parapsilosis* was the dominant yeast at 0 and 24 hours, while *Cyberlindnera subsufficiens* was the dominant yeast at 48 and 72 hours.

Figure 4 showed that the pavian output and Krona during fermentation. Krona visualization intuitively explored the relative abundances within the complex hierarchies of metagenomic classifications. The count in Krona was based on the numReads from Centrifuge Report. *Candida parapsilosis* population increased until 24 hours of fermentation and then decreased. *Cyberlindnera subsufficiens* population increased at 48 hours fermentation until 72 hours fermentation. The species of fungi in porang at 0 hours were dominated by *Candida parapsilosis*, *Clavispora lusitaniae*, and *Metschnikowia drosophilae*. The dominant species of fungi at 24 hours were *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*. The dominant species of fungi at 48 hours fermentation were *Cyberlindnera subsufficiens*, *Candida parapsilosis*, *Scheffersomyces spartinae* while 72 hours were *Cyberlindnera subsufficiens*, *Clavispora lusitaniae*, and *Cyberlindnera saturnus.*

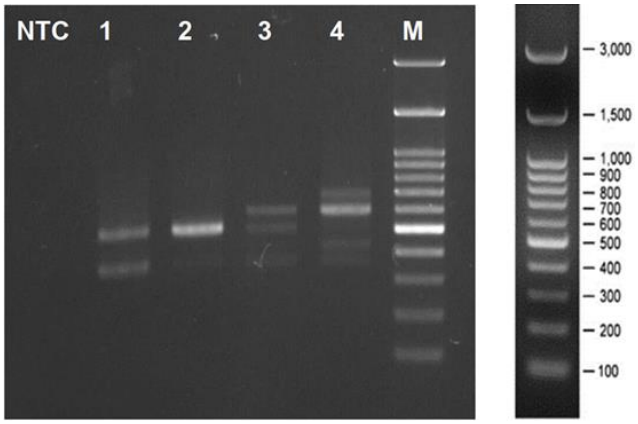

Figure 1. The quality of extracted DNA metagenome on agarose gel. Lane 1. 0 hours fermentation, lane 2. 24 hours fermentation, lane 3. 48 hours fermentation, 4. 72 hours fermentation, M: marker

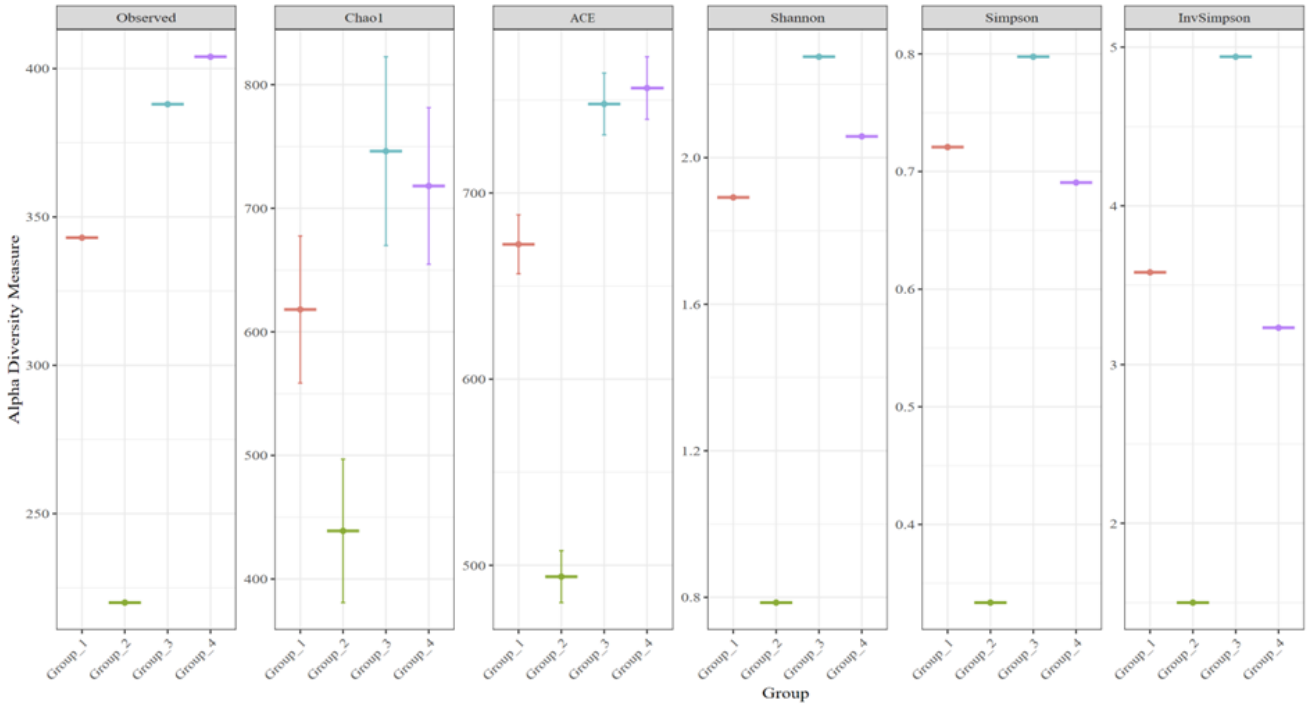

Figure 2. The richness, dominance, and diversity index changes during 72 hours of fermentation. Group 1: 0 hour fermentation, Group 2: 24 hours fermentation, Group 3: 48 hours fermentation, Group 4: 72 hours fermentation

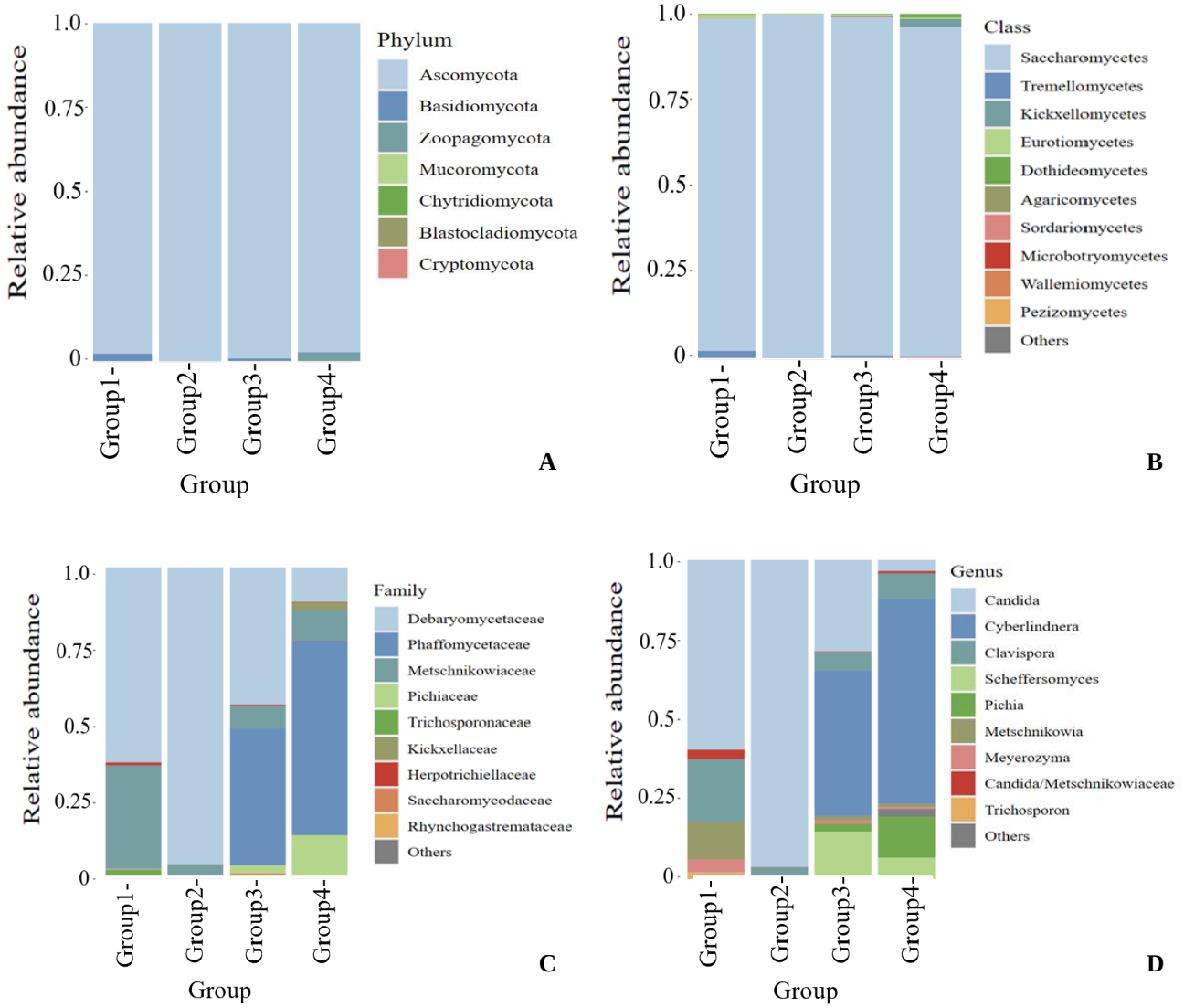

Figure 3. Relative abundance of fungi during fermentation. Group 1: 0 hours fermentation, Group 2: 24 hours fermentation, Group 3: 48 hours fermentation, Group 4: 72 hours fermentation. a. phylum, b. class, c. family, d. genus

Clustering of samples during fermentation

Based on the figure 5, we can see that there is a clustering of the samples. PCoA showed that the length of fermentation can change the diversity of bacteria. The 48 and 72-hour samples were clustered at the same cluster, while the 0 and 24 hours were in another cluster.

Discussion

This study's ethanol concentration and starch content dynamics occurred during fermentation (Table 1). The highest ethanol concentration occurred at 48 hours of fermentation. Based on Table 1, starch also decreased at 48 hours of fermentation. The optimum enzymatic activity of fungi caused this condition during fermentation to degrade starch and produce ethanol. Fungi have amylase activity to degrade starch (Bhattacharjee et al. 2019; Parapouli et al. 2020). According to Kubrat et al. (2016), amylase activity in fermentation could be induced in the presence of starch. Amylase hydrolyzes starch into smaller polymers containing glucose (Bhattacharjee et al. 2019; Parapouli et al. 2020; Cha´vez-Camarillo et al. 2022). Fermenting fungi metabolizes Glucose as ethanol (Bhattacharjee et al. 2019; Cha´vez-Camarillo et al. 2022); α-amylase and ethanol were applied to purify glucomannan in porang tubers (Kumoro et al. 2018; Wardhani et al. 2019; Nurlela et al. 2021).

Yeasts are responsible for the production of ethanol (Chaudhary et al. 2018; Cason et al. 2020). At 72 hours of fermentation, the ethanol concentration decreased. According to Joshi et al. (2018), degradation or inhibition of product formation can occur by external inhibitors or the production concentration itself. As in the case of ethanol production, once the ethanol concentration reached a threshold, it inhibited ethanol production. In line with this study, the ethanol decreased at 72 hours of fermentation might be caused by the accumulation of ethanol production at 48 hours, which consequently inhibited ethanol production.

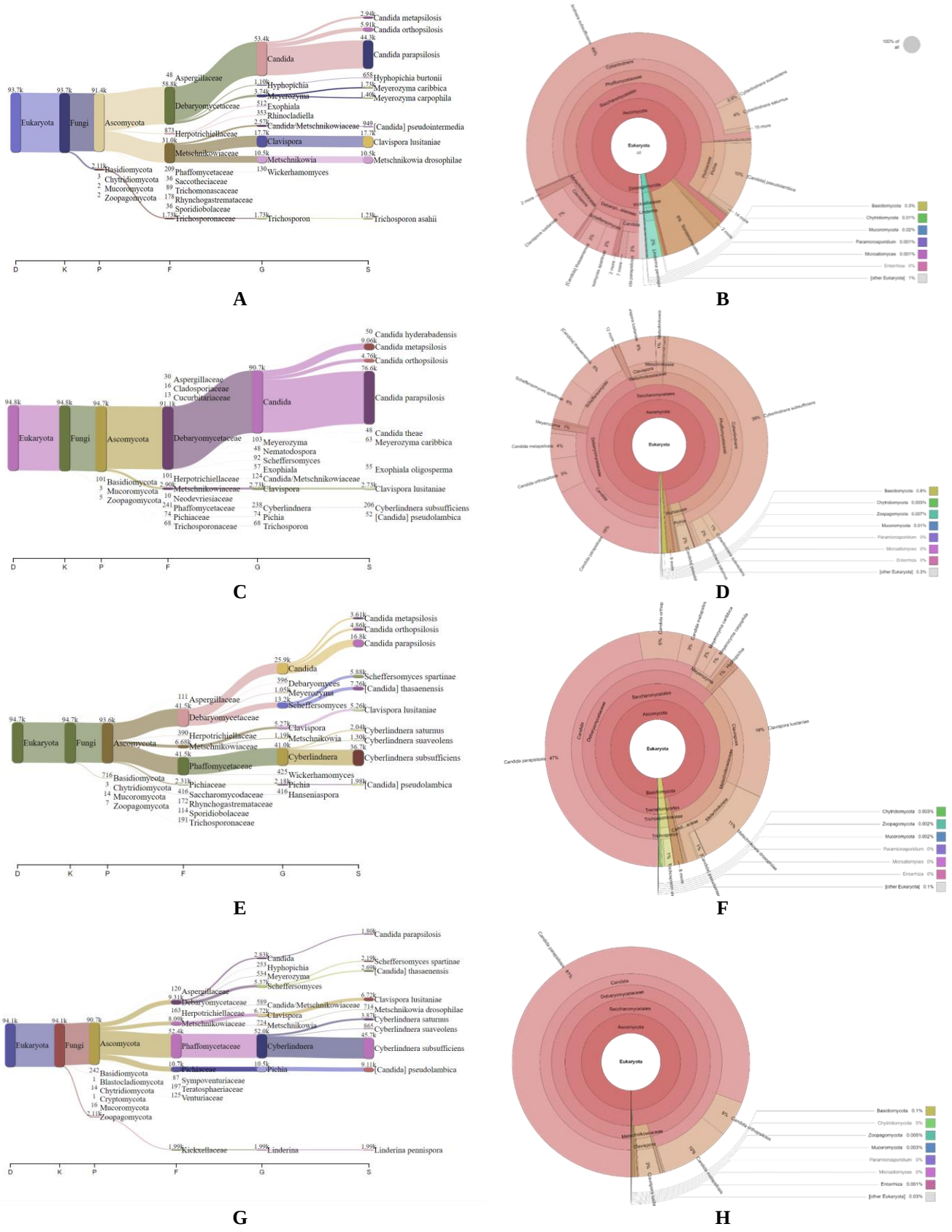

Figure 4. Pavian output for hierarchical visualization and Krona of fungal composition for each sample. a and b (pavian output and krona of 0-hour fermentation), c and d (pavian output and krona of 24 hours fermentation), e and f (pavian output and krona of 48 hours fermentation), g and h (pavian output and krona of 72 hours fermentation)

The quantity of DNA metagenome showed that DNA was good enough, with a ratio of A260/A280 ranged 1.97 to 2.73 (Table 2). The ratio of absorbance 260 and 280 showed the purity of DNA and RNA. A ratio of ~1.8 showed that DNA is pure, not contaminated with protein, phenol, or other contaminants (O'Neill et al. 2011; Lucena-Aguilar et al. 2016). The ratio of absorbance 260 and 230 is also used to measure the purity of DNA. A previous study showed that ITS1-ITS 4 had 400-700bp (Siddique et al. 2022), in line with this study (Figure 1). The alpha diversity showed the diversity and richness of the microorganism community (Yao et al. 2021b). Chao1 showed the richness within the sample; ACE showed the dominance within samples; Shannon, Simpsons, and In Simpson showed the diversity within samples (Calero Preciado et al. 2021). Samples of 48 hours of fermentation had the highest richness and diversity (Figure 2). The diversity and richness of fungi increased due to the availability of simple sugar. In the first fermentation stage, fungi still used simple sugars, but the simple sugars slowly decreased and ran out, so within 24 hours, diversity decreased because only certain species can survive using this substrate; at 48 hours of fermentation, complex substrate or polymer degraded to be simple sugar. The conditions of fermentation supported the growth of fungi. The simple sugar, such as glucose, was metabolized to ethanol. Based on Table 1, ethanol concentration reached the highest concentration (813.85 ppm) at 48 hours of fermentation. At 72 hours of fermentation, diversity decreased because the substrate had been used, so there was only a little substrate left. Moreover, some toxic compounds as the results of metabolism, such as ethanol, can inhibit the growth of fungi (Pérez-Gallardo et al. 2013; Rodrigues et al. 2015; Varize et al. 2022). The highest alcohol production at 48 hours of fermentation could inhibit the growth of fungi after 48 hours of fermentation. At 72 hours of fermentation, the highest dominance occurred (Figure 2), possibly caused by only certain fungi surviving in the toxic environment.

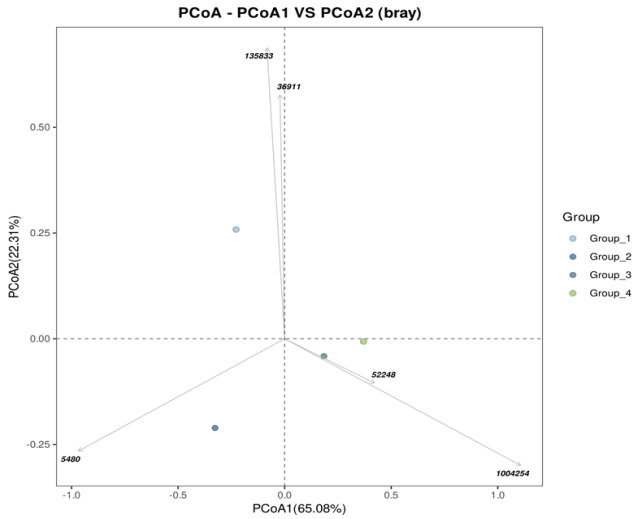

Figure 5. PCoA of clustering samples of the fermentation. Group 1: 0 hour fermentation, Group 2: 24 hours fermentation, Group 3: 48 hours fermentation, Group 4: 72 hours fermentation

Ascomycota dominates all of the samples; the phylum Ascomycota played a major role in the production of fermented foods (Cason et al. 2020). Some fermented foods, such as beer and fermented raspberry, are dominated by the phylum Ascomycota (Cason et al. 2020; Yao et al. 2021b). Saccharomycetes were the highest class for all samples. Saccharomycetes were well-known as yeast, and some species in this class involved fermentation, such as baking and brewing (Suh et al. 2006). Different from basidiomycota yeast, the group of class Saccharomycetes had cell wall polysaccharide composition dominated by β glucans, G+C content lower than basidiomycota yeast, bud formation typically holoblastic, generally more fermentative, more copiotrophic, and at the same time specialized nutritionally, more fragrant and mostly hyaline, living in tend to liquid and rich organic acid niches (Suh et al. 2006). More fermentative characters made the class of Saccharomycetes often found in fermented food.

At the family level, Debaromycetaceae was the most abundant family at 0 hours and 24 hours, while Phaffomycetaceae was the most abundant family at 48 hours and 72 hours. Debaromycetaceae is the family of order Sacharomycetes along with the families Metschnikowiaceae, Dipodascaceae, Lipomycetaceae, Pichiaceae, Phaffomycetaceae, Trichomonascaceae, Trigonopsidaceae (Christinaki et al. 2022). Some genera of Debaromycetace are *Candida* and *Meyerozyma*.

Genus Candida was dominated at 0 and 24 hours of fermentation at the genus level. Genus *Candida* has been reported to be present in fermented food and used as a starter for some fermented foods (Ramos et al. 2011; Riesute et al. 2021; Mariyam et al. 2023). Candida species, such as *Candida tropicalis* and *Candida glabrata*, were involved in fermentation of some of the traditional starter cultures fermented food of Assam's community (Parasar et al. 2017). Isolates of *Candida* assimilated citrate, malate, and propionate poorly but showed exceedingly appreciable metabolism in pyruvate (Parasar et al. 2017). At the first stage of fermentation, simple sugar was assumed abundant. At the first step of glucose metabolism, one glucose is catabolized to be 2 moles of pyruvate. The high pyruvate in fermentation water might make the suitable condition for the growth of *Candida*. Genus *Cyberlindnera* was dominated at 48 and 72 hours of fermentation. Genus *Cyberlindnera* was known for its higher ester production (Bellut et al. 2019). Ester can be produced from the reaction of alcohol and carboxylic acid, such as acetic or lactic acid (Saerens et al. 2010; Ravotti et al. 2018) (Saerens et al. 2010; Ravotti et al. 2018). In line with ethanol concentration (Table 1), the highest alcohol concentration occurred at 48 hours of fermentation. The dominance of *Cyberlindnera* at the end of fermentation is due to the alcohol and carboxylic acid resulting from glucose metabolism. Ester is a volatile compound that can influence the aroma of fermented products (Saerens et al. 2010) (Saerens et al. 2010).

During the fermentation of porang tuber, there was a dynamic fungi population. *Candida parapsilosis* population increased until 24 hours of fermentation and then decreased. *Cyberlindnera subsufficiens* population

increased at 48 hours fermentation until 72 hours fermentation. The dynamics of fungi also noticed in Cabernet Sauvignon grape must fermentation depended on the glucose and fructose consumption, oxygen level, and alcohol content (Bagheri et al. 2015). The oxidative yeasts, including *A. pullulans*, *Rhodotorula* spp., *R. diobovatum*, and *Cryptococcus* spp., were the initial yeasts population in the must grape. After 5 days of fermentation, *Candida parasilopsis* and *S. cerevisiae* dominated the must grape from the biodynamic vineyard. *S. cerevisiae* had the highest abundance of fungi at the end of fermentation after consuming 50% and 75% glucose (Bagheri et al. 2015). In line with this study, alcohol content was one of the factors that determined the dynamics population of fungi. Moreover, the simple and complex sugars were assumed to determine the fungi dynamics population during the porang fermentation.

The species of fungi in porang at 0 hours were dominated by *Candida parapsilosis*, *Clavispora lusitaniae*, and *Metschnikowia drosophilae*. The must from the biodynamic vineyard displayed a high incidence of *C. parapsilosis*, and this yeast remained dominant throughout the fermentation of wine (Bagheri et al. 2015). *Candida parapsilosis* was fungi that dominated in naturally fermented cotton seed and rice beverages produced by Brazilian Amerindians (Ramos et al. 2011), cassava fermentation attiéké (Flibert et al. 2021), Black gram (vadai) (Adebo et al. 2022), fermented dairy product (curd cheese) (Riesute et al. 2021). *Candida parapsilosis* was also used as a starter for xylitol production (Furlan and De Castro 2001) and cacao bean fermentation (Mariyam et al. 2023). Previous studies showed that *Candida parapsilosis* could use various carbon sources. In line with the statement from Mu et al. (2005) and Ramos et al. (2011), who stated that *C. parapsilosis* could use different kinds of carbon sources, namely glucose, starch soluble, potato starch, maltose, sucrose, and trehalose, *Candida parapsilosis* can use glucose to produce ethanol (Furlan and De Castro 2001). *Candida parapsilosis* can proliferate in high concentrations of glucose (Furlan and De Castro 2001). It could be inferred that at 0 hours fermentation contained high glucose.

Clavispora lusitaniae was fungi found in cassava fermentation (Reboucas et al. 2016), naturally fermented cotton seed and rice beverage produced by Brazilian Amerindians (Ramos et al. 2011), and sauerkraut (Satora et al. 2020). *Clavispora lusitaniae* can used maltose, sucrose, glucose, cellobiose and trehalose (Ramos et al. 2011). *Clavispora lusitaniae* can produce ethanol from glucose and cellobiose. According to Suh et al. (2006), *Clavispora* and *Metschnikowia* showed uniform nutritional profiles, which were often found in association with herbivorous invertebrates. Both of these yeasts favored some carbon sources, such as plant sugar, namely sucrose, maltose, and other α-glucosides and β-glucosides, as well as sorbose, mannitol, glucitol, and N acetyl-D-glucosamine. In this study, *Clavispora* and *Metschnikowia* dominated at the same fermentation time, indicating that both genera had the same nutritional profile.

The genus *Metschnikowia* is also (Saerens et al. 2010) found in the grape (Parapouli et al. 2020). This genus was also isolated from insects such as stingless bees, bettles, and drosophilids (Bowles et al. 2001; Mills and Philosophy 2018). *Metschnikowia drosophilae* was found in morning glory (*Ipomoea* sp.) flowers and associated with *Drosophila bromeliae* on Grand Cayman Island. This species can ferment glucose and trehalose and can assimilate galactose, trehalose, cellobiose, salicin, L-sorbose, D-xylose, ethanol (slow), glycerol, ribitol (slow), xylitol, mannitol, glucitol, lactic acid (slow), succinic acid, citric acid (slow), malic acid (slow), D-gluconic acid (weak), glucono-Δ-lactone (weak), N- acetyl-glucosamine, and hexadecane (slow). This species can not grow on inulin, sucrose, raffinose, melibiose, lactose, maltose, melezitose, methyl-α-D-glucoside, starch, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methanol, 1-propanol, 2-propanol, 1-butanol, erythritol, galactitol, meso-inositol, 2-keto-gluconic acid, D-glucosamine, acetone, or ethyl acetate (Bowles et al. 2001). This species' slow capability to assimilate ethanol and some acids is presumed to be why this species only had a high abundance at 0 hours of fermentation. Bowles et al. (2001) supported these reasons that *M. drosophilae* showed slow capability in the assimilation of ethanol, lactic acid, citric acid, malic acid, and D-gluconic acid. Some studies about species from the genus of *Metschnikowia* also supported these reasons (Lachance and Bowles 2004; Vicente et al. 2020). *Candida parapsilosis*, the highest abundance species at 0 and 24 hours of fermentation, can produce ethanol. The alcohol production made the unsuitable environment for *Metschnikowia drosophilae*.

At 24 hours of fermentation, the fungi species were dominated by *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*. Previously, *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis* were clustered into one group. *Candida metapsilosis* was categorized as *C. parapsilosis* group II, while *Candida orthopsilosis* was categorized as *C. parapsilosis* group II. Cluster analysis of sequence polymorphisms, gene ITS, and real-time PCR showed that the three groups consistently separated and further proposed as *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*, respectively (Giri and Kindo 2015; Tavanti et al. 2020). *Candida parapsilosis* and *Candida metapsilosis* were detected at the fermentation of sourdough at 0 days fermentation and 3 days fermentation of buckwheat honey fermentation of uninoculated wort (Bednarek et al. 2019) and cigar tobacco leaves fermentation (Jia et al. 2023). *Candida parapsilosis* and *orthopsilosis* were also detected in naturally fermented cotton seed and rice beverages produced by Brazilian Amerindians (Ramos et al. 2011). The high glucose content in water fermentation at 0 and 24 hours was presumed to be why *Candida parapsilosis* had the highest abundance at 0 and 24 hours of fermentation. The increase in ethanol was assumed to be caused by this species due to this species could produce ethanol.

The dominant species of fungi at 48 hours fermentation were *Cyberlindnera subsufficiens*, *Candida parapsilosis*,

Scheffersomyces spartinae. *Cyberlindnera subsufficiens* was a starter to produce a fruity and non-alcoholic beer (Bellut et al. 2019). *Scheffersomyces spartinae* produced the volatile organic compound, which is categorized as a derivative of alcohols, namely 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-phenylethanol (Zou et al. 2023). Those compounds had antifungal effects (Zou et al. 2023) that can inhibit other fungi. *Candida parapsilosis* still survived and dominated at porang fermentation due to the capability of this yeast to adapt to stress conditions (Mariyam et al. 2023). *Candida parapsilosis* can produce extracellular matrices or biofilm containing large amounts of carbohydrates and β-1,3 glucan with increasing glucose concentrations (Silva et al. 2011; Pereira et al. 2015b). Nguyen and Nosanchuk (2011) Nguyen and Nosanchuk (2011) studied the behavior of *C. parapsilosis* strains in stress conditions, such as high glucose levels. They concluded that yeast cells could convert the excessive glucose levels into lipid droplets (Pereira et al. 2015a). Yeast cells protect themselves from the toxic effects of free fatty acids generated during de novo fatty acid biosynthesis from glucose (Pereira et al. 2015a). At 48 hours of fermentation, *C. parapsilosis* was still dominated, which caused the ethanol production still increase until 48 hours of fermentation.

At 72 hours of fermentation, the fungi species were dominated by *Cyberlindnera subsufficiens, Clavispora lusitaniae*, and *Cyberlindnera saturnus.* Some *Cyberlindnera,* such as *C. subsufficiens* and *Cyberlindnera saturnus,* can produce low ethanol as the metabolite of the product of carbohydrate metabolism (Liu and Quek 2016). Low ethanol and high ester production are two major reasons the genus *Cyberlindnera* is used as a starter in low alcohol and fruity aroma of alcohol fermentation. *Cyberlindnera saturnus,* which formerly was known as *Williopsis saturnus* also used as a starter for beer fermentation, beet fermentation, and apple cider fermentation (Aung et al. 2015; Liu and Quek 2016; Bellut et al. 2019). The high population of *C. lusitaniae* assumed that fungi can use a mixture of carbon sources. In a mixture of carbon sources, *C. lusitaniae* used glucose first and other carbon sources after glucose exhaustion (Ochoa-Chacón et al. 2022). Although *C. subsufficiens* and *Cyberlindnera saturnus* can produce low ethanol and *C. lusitaniae* can produce ethanol, ethanol concentration has decreased. It can be assumed that some ethanol production had been metabolized to be ester by *C. subsufficiens* and *Cyberlindnera saturnus.*

Based on the relative abundance of bacterial species, the difference in length of fermentation caused the differences in dominant fungal diversity. Principle Coordinate Analysis (PCoA) results showed that the sample groupings were formed based on the fungal composition. Group 1 (0 hours fermentation) and Group 2 (24 hours fermentation) showed fungal composition similarities, while Group 3 (48 hours fermentation) and Group 4 (72 hours fermentation) showed fungal composition similarities (Figure 5). The length of fermentation affects the fungal composition in some fermented foods, such as shrimp sauce (Lee et al. 2014). At 0 and 24 hours of fermentation, the fungal community used more simple carbohydrates; at 48 and 72 hours of fermentation, it assumed that the fungal community used the higher carboxylic acid and ethanol. At the early stage of fermentation (0 and 24 hours fermentation), the fungal community metabolized glucose to ethanol and some carboxylic acids. At the end of fermentation (48 and 72 hours), ethanol and carboxylic acid changed to ester volatile compounds.

Fermentation of porang tubers can be stopped at 48 hours fermentation. The diversity of fungi and yeasts enzymatic activities was high at this time. Starch, as one of the impurity materials of glucomannan extraction, was lower due to the degradation effect of ethanol and amylase. Moreover, at 48 hours of fermentation, *Cyberlindnera subsufficiens* was dominated by fungi. *Cyberlindnera subsufficiens* could produce an ester and fruity aroma, improving the quality of fermented porang tubers. *Candida parapsilosis* can be the candidate of starter for fermentation porang due to the ability of this species to produce ethanol. Ethanol which is produced during fermentation, can inhibit the growth of undesired or spoilage microorganisms in the fermentation process. Ethanol can be a carbon source to support the growth of *Cyberlindnera subsufficiens*. *Candida parapsilosis* can produce killer toxins to inhibit the growth of undesired or pathogenic microorganism. Moreover, this species resistance to stressfull condition such as acidity and high ethanol concentration. This species can produce aroma-active compounds (de Melo Pereira et al. 2022). Although this species have been used as starter for cocoa and coffe fermentation, butit is consideration to determine the genomic of this species which is confirmed by patogenity test and human clinical trials for the safety starter. Adding a starter can shorten the fermentation time and maintain the quality of the fermented product.

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