BIODIVERSITAS
Volume 25, Number 9, September 2024
Pages: 3201-3207

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250942

Investigation of the bacterial diversity in fermented mangrove apple (*Sonneratia caseolaris*) fruit juice kombucha using DNA metabarcoding

JELENA ANGEL VISAKHADEVI[1], SAPTO ANDRIYONO[2,♥], WORO HASTUTI SATYANTINI[3], NUR IZZATI MOHD NOH[4]

[1]Biotechnology of Fisheries and Marine Postgraduate Program, Faculty of Fisheries and Marine, Universitas Airlangga. Jl. Dharmahusada Permai Mulyorejo, Surabaya 60115, East Java, Indonesia
[2]Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga. Jl. Dharmahusada Permai Mulyorejo, Surabaya 60115, East Java, Indonesia. Tel.: +62-31-5911451, Fax.: +62-31-5965741, ♥email: sapto.andriyono@fpk.unair.ac.id
[3]Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga. Jl. Dharmahusada Permai Mulyorejo, Surabaya 60115, East Java, Indonesia
[4]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia. Skudai Hwy, 81310 Skudai, Johor Bahru, Malaysia

Manuscript received: 24 July 2024. Revision accepted: 21 September 2024.

Abstract. *Visakhadevi JA, Andriyono S, Satyantini WH, Noh NIM. 2024. Investigation of the bacterial diversity in fermented mangrove apple (*Sonneratia caseolaris*) fruit juice kombucha using DNA metabarcoding. Biodiversitas 25: 3201-3207.* Kombucha, a functional beverage produced from the symbiosis between bacteria and yeast, varies in microbial composition and biological activity depending on the raw materials used for fermentation. This study investigated the potential of mangrove apple (*Sonneratia caseolaris* (L.) Engl.) fruit juice as a novel substrate for kombucha fermentation because of its high nutritional value and underutilization. We analyzed the bacterial composition of kombucha using a molecular metabarcoding approach with the universal 16S rRNA primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T). The optimal bacterial density was observed on the 8th day of fermentation, with a value of 1.402. The pH progressively decreased during the fermentation period. This study successfully explored the diversity of bacteria in kombucha grown in apple fruit juice. The dominant bacterial taxon was acetic acid bacteria, with 75,009 reads for Acetobacteraceae. At the genus level, *Komagataeibacter* was predominant, with 74,660 reads, and at the species level, *Komagataeibacter saccharivorans* was the most abundant, with 67,502 reads. These results suggest mangrove apple fruit juice as the viable substrate for kombucha fermentation that supports a rich microbial community. This study highlights the potential of alternative substrates, such as mangrove apple fruit juice, to enhance kombucha's microbial diversity and health benefits.

Keywords: Bacteria, diversity, kombucha, mangrove apple, metabarcoding

INTRODUCTION

Kombucha is traditional fermented beverage characterized by a slightly sour taste. It is produced using a sugar solution and a microbial starter known as Symbiotic Culture of Bacteria and Yeast (SCOBY). Kombucha stands out among other brewed beverage products because it produces vitamins, organic acids, antibiotic compounds, and antimicrobial compounds (Adzadogo and Gbewoyo 2018), which are brought about by the symbiotic relationship between yeast and bacteria during fermentation (Kaewkod et al. 2019). Kombucha fermentation involves the symbiotic association between yeast and bacteria within the cellulose matrix. This matrix, formed by bacteria and yeast, is essential for the development of SCOBY (de Miranda et al. 2022). During fermentation, the yeast catalyzes the hydrolysis of sucrose to fructose and glucose via invertase, subsequently producing ethanol through the glycolytic pathway. Acetic acid bacteria oxidize ethanol to acetic acid via alcohol dehydrogenase and aldehyde dehydrogenase (Gomes et al. 2018). In commercial kombucha, various species of acetic acid bacteria, including *Gluconobacter* sp. and *Acetobacter* sp., are typically present alongside lactic acid bacteria, such as *Lactobacillus* sp. (Yang et al. 2022).

The composition of kombucha significantly influences its quality and functional properties. Different bacterial communities produce organic compounds that confer distinct health benefits (Jayabalan et al. 2014). Factors such as bacterial and yeast species, substrates, fermentation time, temperature, and pH affect microbial diversity. Kombucha is commonly categorized based on the substrate used for fermentation, with black tea, green tea, and fruit juice being the most frequently used (Kim and Adhikari 2020). Mangrove apples (*Sonneratia caseolaris* (L.) Engl.) are fruits commonly found in brackish waters. It is non-toxic, edible, and has a sour taste and distinctive aroma (Setiawan et al. 2016). Mangrove apple fruit is nutritionally rich (Ferdous et al. 2021), containing vitamins A (221.97 IU), B (5.04 mg), B2 (7.65 mg), and C (56.74 mg), along with high water content (84.76%), ash (8.4%), fat (4.82%), protein (9.21%), and carbohydrates (77.57%) per 100 g (Dari et al. 2022). Additionally, mangrove apple fruit juice contains phytochemicals, such as steroids, triterpenoids, and flavonoids, which act as antioxidants to neutralize free radicals, thereby preventing cancer, heart disease, and premature aging (Susanto et al. 2020). However, public knowledge of the nutritional content of this fruit is limited, leading to a lack of information regarding its processing

and potential uses (Salsabila et al. 2023). The versatility of kombucha allows for variations in both taste and ingredients. Studies have shown that kombucha can be produced from various substrates, including fruit juice. Different substrates can significantly affect the microbial composition and biological activity of kombucha (Jayabalan et al. 2014). The utilization of mangrove apple fruit juice as a substrate for kombucha fermentation is an innovative approach given its high nutritional value and potential health benefits.

Advancements in bacterial identification methods, such as metabarcoding, have significantly improved the accuracy and speed of microbial analyses (Pédron et al. 2020; Francioli et al. 2021). Metabarcoding uses DNA sequencing technology to quickly and efficiently analyze and identify various types of bacteria in samples, making it a valuable tool for modern microbiological research (Andretta et al. 2019; Bukin et al. 2019; Santos et al. 2020). This technique offers more detailed results than conventional methods, enabling a comprehensive understanding of microbial communities in fermented products (Reva et al. 2015; Rué et al. 2023). Given the nutritional potential of the mangrove apple fruit and the advantages of modern microbial identification techniques, this study aims to investigate the microbial composition of kombucha fermented with mangrove apple fruit juice using DNA metabarcoding. We hypothesize that different substrates would yield distinct microbial communities, thereby enhancing the functional properties of kombucha. This research contributes to the understanding of the role of specific bacterial species in kombucha fermentation and highlights the potential of underutilized substrates, such as mangrove apple fruit, in producing high-quality, and health-promoting fermented beverages.

MATERIALS AND METHODS

This study was conducted at the Chemistry, Analytical, Food, and Microbiology Laboratories of the Faculty of Fisheries and Marine Sciences, Universitas Airlangga, Surabaya. DNA was extracted from kombucha bacteria in the laboratory of the Professor Nidhom Foundation, Surabaya, East Java, Indonesia. A metabarcoding analysis was conducted using Genetika Science Laboratories Services (Jakarta, Indonesia), after the DNA extraction process from previous experiments. We employed an exploratory laboratory research method involving direct sampling and a molecular approach. Mangrove apple fruits were collected from Wonorejo, Surabaya. Fruit juice was prepared and inoculated with a starter culture and SCOBY cellulose to create kombucha samples according to a previous study (Emiljanowicz and Malinowska-Pańczyk 2020). The samples were fermented for 12 days at room temperature (25°C), with daily pH measurements. Samples were collected at the point of optimal bacterial density during the 12-day fermentation period (Barbosa et al. 2021). The composition and dominance of fermenting bacteria were analyzed using DNA metabarcoding.

Preparation of mangrove apple fruit juice and kombucha

One kilogram of ripe mangrove apple fruit was prepared by separating the pulp from the seeds, skin, and petals. The pulp was mixed with water in a 1:1 ratio (fruit: water by volume) and mashed to form a slurry. The slurry was filtered to obtain fruit juice, and the remaining seed debris was discarded. The seed debris underwent a second filtration process with an additional liter of water to remove any remaining impurities and obtain clean fruit juice. The juice from both filtration stages was then strained through a golden mella cloth to obtain pure mangrove apple fruit juice (Putra et al. 2022). The process of making mangrove apple fruit juice kombucha was based on the method described by Emiljanowicz and Malinowska-Pańczyk (Emiljanowicz and Malinowska-Pańczyk 2020) with modifications. Pure mangrove apple fruit juice was supplemented with 10% sucrose and pasteurized at 60°C for 30 min. After pasteurization, the juice was transferred to sterilized jars and combined with a 10% starter culture and 25 g of SCOBY cellulose. The jars were covered with tissue paper, secured with rubber bands, and fermented at room temperature (25°C) for 12 days (Barbosa et al. 2021). The jars were covered with aluminum foil to prevent direct exposure to sunlight during fermentation.

Measurement of bacterial density and pH

The total bacterial density was measured daily for 12 days to monitor bacterial activity during fermentation. Bacterial density was determined using the Optical Density method with a UV-Vis spectrophotometer at a wavelength of 610 nm, as described by (Wood et al. (2019). The pH of mangrove apple fruit juice kombucha was measured daily for 12 days using a calibrated pH meter. To ensure accuracy, the pH meter electrode was rinsed with distilled water and dried before each measurement. The pH meter was then immersed in kombucha and readings were taken once the value stabilized. This procedure aimed to track changes in acidity and alkalinity throughout the fermentation process.

DNA extraction of bacteria

DNA extraction aims to obtain pure DNA for PCR amplification of bacterial DNA sequences. Before extraction, sample preparation involved transferring 300 µL of an 8-day fermented kombucha sample into a 1.5 mL microcentrifuge tube (RNAse-free). The GENEzol reagent was added to the sample at a 3:1 ratio. The samples were homogenized using a vortex mixer and incubated for 5 minutes at room temperature. For the separation phase, 200 µL of chloroform was added per 1 mL of GENEzol reagent in the homogenization sample. The sample in the microcentrifuge tube was shaken vigorously for 10 seconds and centrifuged at 15,000 rpm for 15 minutes at 4°C (Tesena et al. 2017). DNA was extracted according to the standard protocol provided with a GENEzol Reagent Kit (Taiwan).

PCR and sequencing of 16S rRNA gene

Amplification of 16S rRNA gene-targeted DNA sequences specific to the 16S rRNA gene. The primers 16S

27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACTT) were used (Walker et al. 2015). DNA amplification was performed using a MyCycler instrument (Bio-Rad, Boston USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 PCR cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. The final extension step was performed at 72°C for 5 minutes. PCR products were confirmed by electrophoresis. The agarose gel was prepared by dissolving agarose powder (0.5 g) in TBE buffer (50 mL of TBE buffer) and heating until a clear solution was formed. After cooling to a warm temperature, the solution was poured into an electrophoresis mold, and 0.5 µL of ethidium bromide was added. A comb was placed at one end of the mold and left until the agarose solidified. The comb was then removed and the gel was transferred to an electrophoresis tank filled with TBE buffer until the gel was submerged. Each DNA sample (2 µL) was mixed with 1 µL of loading dye and carefully loaded into the wells. Electrophoresis was performed at 100 V and 400 mA for 30 minutes. The results were visualized under ultraviolet light and photographed using a GelDoc system (Sepriana et al. 2020).

This study utilized next-generation sequencing (NGS) with the Oxford Nanopore Technologies (ONT) long-read platform. This platform involves passing a single DNA strand through a nanoporous membrane and applying a voltage difference across the membrane. Nucleotides within a pore affect its electrical resistance, and measuring the current over time can reveal the DNA base sequence passing through the pore. The electrical current signal served as the raw data collected by the ONT sequencer, followed by base calling. Base calling on the ONT device translates this raw signal into DNA sequences with the electrical signal originating from a single molecule, resulting in diverse and stochastic data (Wick et al. 2019).

Bioinformatics and data analysis

The data generated from the sequencing process included 16S rRNA sequences spanning the V1-V9 regions, provided in Fastq format with reads in both the forward and reverse directions, to achieve longer sequence lengths. The bioinformatics workflow encompasses several key steps for analyzing and interpreting sequencing data. Nanopore sequencing was conducted using MinKNOW software version 23.04.5 (https://nanoporetech.com/news/news-introducing-new-minknow-app). Raw electrical signals were translated into DNA sequences using Guppy version 6.5.7 (https://pypi.org/project/ont-pyguppy-client-lib/6.5.7/) with a high-accuracy model. Quality control (QC) of the FASTQ files was performed using NanoPlot (https://nanoplot.bioinf.be/), a tool for assessing sequencing data quality. Quality filtering was performed using NanoFilt (https://anaconda.org/bioconda/nanofilt) to ensure high-quality reads for downstream analysis.

Nanopore sequencing data were obtained using MinKNOW software version 23.04.5, an application for microbial ecological analysis that can be accessed through Linux-based computer devices. Figures were used to determine the relative abundance of each species. The relative abundance of each species was determined using OriginPro 2022 (https://www.originlab.com/2022).

RESULTS AND DISCUSSION

Density of bacteria in kombucha during fermentation

Optical density was used to measure bacterial density using spectrophotometric absorption. The optical density was calculated by measuring the absorbance of mangrove apple fruit juice kombucha samples taken daily. The results of the optical density measurements using a spectrophotometer (λ 610 nm) show different absorbance values for each observed sample (Figure 1).

The results showed that the absorbance value of mangrove apple fruit juice kombucha increased daily, with peak bacterial growth occurring on the 8th day, reaching a density of 1.47×10^5 cells/mL. From the 9th to the 12th day, there was a decrease in the absorbance value of the mangrove apple fruit juice kombucha samples, with the absorbance value on the 9th day being 1.37×10^5 cells/mL and on the 12th day being 1.08×10^5 cells/mL. This indicates that the number of bacteria increased daily until the 8th day and gradually decreased thereafter (Table 1).

Kombucha pH value during fermentation

The results showed that the pH value on day 0 was 3.20, and on day 8, the pH value was 2.77, reaching its peak on day 12 with a pH value of 2.69. The highest pH was obtained on day 1, with a pH of 3.21, while the lowest pH value was obtained on day 12, with a pH of 2.69. The pH values from days 0 to 12 tended to decrease during fermentation (Table 1).

Composition and domination of bacteria in kombucha

Metabarcoding results indicated the presence of five families, 38 genera, and 107 bacterial species in the kombucha samples made from mangrove apple fruit. However, most of these taxa exhibited very low abundance, with percentages below 0.1%.

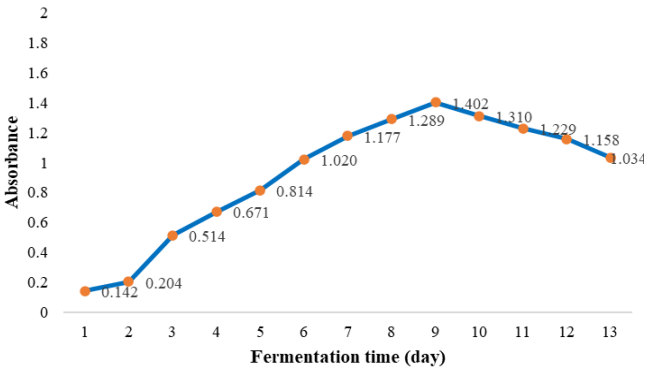

Figure 1. Optical density absorbance value of mangrove apple fruit juice kombucha during 12 days of fermentation

Table 1. Bacterial density and pH of kombucha

Day	Value	
	Density (Cell/mL)	pH
0	$1.51x10^4$	3.20±0.01
1	$2.16x10^4$	3.21±0.01
2	$5.41x10^4$	3.05±0.01
3	$7.06x10^4$	2.94±0.01
4	$8.55x10^4$	2.90±0.01
5	$1.07x10^5$	2.88±0.02
6	$1.23x10^5$	2.82±0.02
7	$1.35x10^5$	2.79±0.01
8	$1.47x10^5$	2.77±0.01
9	$1.37x10^5$	2.80±0.03
10	$1.29x10^5$	2.75±0.01
11	$1.21x10^5$	2.73±0.02
12	$1.08x10^5$	2.69±0.01

This study revealed that the most dominant family in mangrove apple fruit kombucha was Acetobacteraceae, accounting for 99.20% of the total reads classified at the family level. Additionally, the Lactobacillaceae family was present in only 0.52% of the reads. At the genus level, the bacterial composition of mangrove apple fruit kombucha was predominantly *Komagataeibacter*, which comprised 98.74% of the reads (Figure 2), followed by *Limosilactobacillus* at 0.35%. At the species level, the bacterial composition was dominated by *Komagataeibacter saccharivorans*, representing 89.27% of the reads. Other species included *Komagataeibacter rhaeticus* (4.03%), *Komagataeibacter europaeus* (2.75%), *Komagataeibacter melomenusus* (1.25%), *Komagataeibacter xylinus* (0.37%), and *Limosilactobacillus fermentum* (0.30%).

Discussion

Bacterial growth in apple kombucha juice reflects the activity of the microorganisms in the kombucha. The nutrients within kombucha influence the increase in the bacterial count used by microorganisms for reproduction (Tran et al. 2020). This is evidenced by the daily increase in bacterial density in mangrove apple fruit kombucha, with optimal bacterial growth peaking on day eight at a density of 1.47×10^5 cells/mL, followed by a gradual decrease to 1.08×10^5 cells/mL by day 12. The fluctuation in bacterial density in kombucha can be attributed to several factors related to the fermentation processes and environmental conditions (Coelho et al. 2020). This increase occurs as microorganisms, such as acetic acid and lactic acid bacteria, begin to metabolize and produce organic acids. Suitable environmental conditions facilitate the growth of fermenting bacteria as they degrade sucrose into glucose and fructose via invertase enzymes.

These sugars serve as nutrients for the growth of fermentation bacteria (Laureys et al. 2020). The decrease in microorganism numbers from day 9th to day 12th was due to fermentation, during which bacteria produce various organic compounds that can affect their growth (Audrain et al. 2015). In particular, acetic acid significantly inhibits bacterial activity and causes bacterial death, leading to a decrease in cell density. As acetic acid accumulates daily, the environment becomes increasingly acidic, further inhibiting bacterial activity and reducing cell number and density. Anaerobic conditions and nutrient deficiencies in kombucha also reduce cell numbers during fermentation, as bacteria struggle to metabolize and function properly (Nyhan et al. 2022). Based on these findings, this study continued to ferment kombucha on day 8, when the bacterial density was optimal, to examine the bacterial composition.

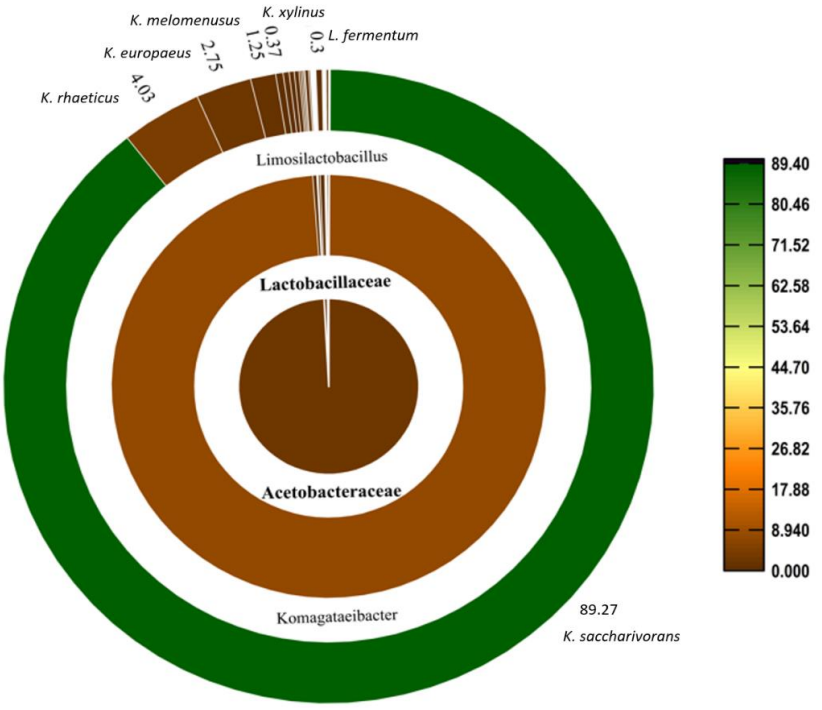

Figure 2. Bacteria proportion percentage in kombucha

The increasing popularity of kombucha has driven extensive research into new fermentation strategies using different raw materials, ultimately resulting in unique microorganisms and metabolite profiles in kombucha products. The bacterial composition in kombucha is typically dominated by lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Hooi et al. 2023). These bacteria are crucial for the production of bioactive compounds that contribute to the health benefits of kombucha. Diversifying the fermentation methods and using different raw materials can create products with varied flavor characteristics and enhance the health benefits of kombucha by increasing the content of bioactive compounds produced by microbial activity, particularly by bacteria. Several studies have identified Lactic Acid Bacteria (LAB) such as *Lactobacillus* and *Lactococcus*, which produce lactic acid, and Acetic Acid Bacteria (AAB) such as *Gluconobacter* and *Acetobacter*, which produce acetic acid, as critical bacteria involved in kombucha fermentation (Wang et al. 2022). This study reports the results of a metabarcoding analysis of the dominant bacterial composition in kombucha prepared from mangrove apple fruits. These findings indicate that kombucha made from mangrove apple fruit contains five families, 38 genera, and 107 bacterial species. Most taxa analyzed showed very low abundance, with percentages below 0.1%. Only two microbial families, Acetobacteraceae and Lactobacillaceae, were dominant. Only *Komagataeibacter* and *Limosilactobacillus* were identified at the genus level. Bacterial classification at the family level revealed the highest dominance of Acetobacteraceae, comprising 99.20% of the total reads. This family includes various genera of acetic acid bacteria, with *Komagataeibacter* (98.74%) being the dominant genus in kombucha. Several species from this genus also dominated the bacterial community in kombucha, including *Komagataeibacter saccharivorans* (90.42%), *Komagataeibacter rhaeticus* (4.07%), *Komagataeibacter europaeus* (2.78%), *Komagataeibacter melomenusus* (1.26%), and *Komagataeibacter xylinus* (0.37%). Acetic acid bacteria play a crucial role in kombucha fermentation by oxidizing ethanol into acetic acid, giving kombucha a distinctive taste (Harrison and Curtin 2021).

These bacteria also convert glucose into gluconic acid and use the ethanol produced by yeast to generate acetic acid by oxidizing ethanol through the activities of alcohol and aldehyde dehydrogenases (Laureys et al. 2020). Gluconic acid is further oxidized by AAB to 2-ketogluconic acid and 2,5-diketogluconic acid. Glucose can also be oxidized to glucuronic acid by AAB in kombucha, which is believed to exhibit strong detoxifying properties. These bacteria also produce D-saccharic acid 1,4-lactone, a compound thought to have health benefits as it acts as a competitive inhibitor of β-glucuronidase, an enzyme involved in cancer formation by hydrolyzing conjugated glucuronides into carcinogenic aglycone compounds in the intestines (Martínez Leal et al. 2018). *Komagataeibacter* is an acetic acid-producing bacterium responsible for cellulose pellicle production. This species can accumulate 10-20% acetic acid in the medium (Laureys et al. 2020).

Lactobacillaceae also contributed to the kombucha microbiota composition, representing approximately 0.5% of the reads. This family includes various genera of lactic acid bacteria, with *Limosilactobacillus* (0.35%) being the dominant genus. At the species level, *Limosilactobacillus fermentum* had a relative abundance of approximately 0.30% of the total reads. Lactic acid bacteria play significant and diverse roles in kombucha fermentation, including converting sugars into organic acids such as lactic and acetic acids, stabilizing kombucha, and contributing to its distinctive taste (Coelho et al. 2020). These bacteria produce organic compounds that enhance the flavor of beverages, complement the taste of tea, and provide the desired complexity. *Limosilactobacillus* has probiotic properties that promote digestive health by maintaining the gut microbiota balance. *Limosilactobacillus fermentum* also acts as an antioxidant and anti-inflammatory agent. Several studies have reported the health benefits of *Limosilactobacillus fermentum*, including reduced glucose levels, modulation of the intestinal immune system, and reduction in intestinal inflammation (de Luna Freire et al. 2024). Therefore, this bacterium is crucial for kombucha fermentation and for the quality and health benefits of the final beverage (Akinyemi et al. 2024). Crotti et al. (2016) reported that kombucha dominated by *Komagateibacter* genus during the fermentation process contained high concentrations of acid, as these bacteria are known for their high acid tolerance (Crotti et al. 2016). This also explains the minimal presence of LAB during kombucha fermentation, as most LAB do not grow below pH 3.5 (Semjonovs et al. 2017).

Based on the reported results, the bacterial composition of kombucha is influenced by the type of substrate and raw material used (Emiljanowicz and Malinowska-Pańczyk 2020; de Miranda et al. 2022). Different results were reported in a study on green tea-fermented beverages, which showed that the bacterial composition was dominated by *Glucanoacetobacter*, *Oenococcus*, *Gluconobacter*, and *Lactobacillus*, with the highest dominance of *G. oboediens* and *G. saccharivorans* (Coton et al. 2017). Lemon ginger kombucha has a dominant bacterial composition in *Lactobacillus* genus, with the dominant species being *L. nagelii* (Yang et al. 2022). This difference in composition is due to the compounds in the raw materials, which can affect bacterial diversity. During kombucha fermentation, symbiotic relationships develop, and many selective pressures affect bacterial diversity, particularly in kombucha. Consequently, this influences substrate consumption and metabolite production. One of the most significant selective pressures during kombucha fermentation is acid stress due to the production of organic acids by yeast, AAB, and sometimes LAB (Laureys et al. 2020).

Several other factors can also affect the bacterial composition during fermentation, including pH, temperature, and fermentation time. Low pH enables acid-resistant bacteria to survive in such an environment, inhibiting pathogenic bacterial growth (Hafsari and Farida 2021). In this study, the pH value tended to decrease during fermentation, ranging from 3.20 on day 0 to 2.69 on day 12 of fermentation. Previous studies suggest that the optimal

pH range for consumable kombucha is between 2.5 and 3.5, with fermentation lasting 8-12 days, pH values below 2,5 pose health risks due to excessively high acetic acid content, while pH above 4.2 can affect kombucha quality and safety by allowing pathogenic bacteria to grow (Cardoso et al. 2020). Other supportive factors, such as temperature, can affect the growth of specific bacterial species, with optimal growth for acetic acid bacteria at 25-30°C and lactic acid bacteria at 25-40°C. Maintaining an optimal temperature during kombucha fermentation can also enhance bacterial growth and enzyme activity, thereby improving fermentation efficiency. Simultaneously, the optimal fermentation time also supports an optimal bacterial composition. According to the Food and Drug Administration (FDA), kombucha beverages are not recommended for human consumption after more than ten days of fermentation (Chakravorty et al. 2016). The metabarcoding results, which report on the bacterial composition in kombucha made from mangrove apples, indicate that the environment largely shapes the bacterial community in this fermented beverage. The predominance of acetic acid bacteria in kombucha made from mangrove apples is attributed to the natural characteristics of the raw material, namely, its sour taste, which results in a relatively low initial pH for kombucha compared to previous studies (Naufal et al. 2022). This condition is highly conducive to the growth of acetic acid bacteria, which tend to thrive in more acidic environments than lactic acid bacteria (Fabricio et al. 2022).

In conclusion, metabarcoding analysis of the bacterial composition in mangrove apple kombucha revealed that environmental factors significantly shape the microbial communities that develop during fermentation. While less abundant than the Acetobacteraceae family, the presence of other bacterial species suggests complex interactions within the kombucha microbial ecosystem, which may influence the final product's flavor, texture, and potential health benefits. The study confirmed the diversity of symbiotic bacteria in kombucha, with *Komagataeibacter saccharivorans* being the predominant species, accounting for 89.27% of the bacterial population in mangrove apple fruit kombucha. These findings support the hypothesis of a bacterial consortium in kombucha. After an 8-day fermentation, the kombucha reached a pH of 2.77, with a peak bacterial count of 1.47×10^5 nanoparticle cells/mL. These results provide valuable insights into the diversity of lactic acid bacteria in kombucha and contribute to a deeper understanding of its microbial ecology.

ACKNOWLEDGEMENTS

The authors acknowledge the support of the National Research Priority Research Grant for Higher Education for the fiscal year 2023, Grant No. 0536/E5/PG.02.00/2023, under contract No.1335/UN3.LPPM/PT.01.03/2023. This grant facilitated the investigation and implementation of research on metabarcoding for biodiversity studies in Indonesia.

REFERENCES

Adzadogo RS, Gbewoyo WSK. 2018. Comparison of physicochemical properties and anti-microbial activities of tea and cocoa-based kombucha. Bioteknologi 15: 33-37. DOI: 10.13057/biotek/c150104.

Akinyemi MO, Ogunremi OR, Adeleke RA, Ezekiel CN. 2024. Probiotic potentials of lactic acid bacteria and yeasts from raw goat milk in Nigeria. Probiot Antimicrob Protein 16 (1): 163-180. DOI: 10.1007/s12602-022-10022.

Andretta M, Almeida T, Ferreira L, Carvalho A, Yamatogi R, Nero L. 2019. Microbial safety status of Serro artisanal cheese produced in Brazil. J Dairy Sci 102 (12): 10790-10798. DOI: 10.3168/jds.2019-16967.

Audrain B, Farag MA, Ryu C-M, Ghigo J-M. 2015. Role of bacterial volatile compounds in bacterial biology. FEMS Microbiol Rev 39 (2): 222-233. DOI: 10.1093/femsre/fuu013.

Barbosa CD, Uetanabaro APT, Santos WCR, Caetano RG, Albano H, Kato R, Cosenza GP, Azeredo A, Góes-Neto A, Rosa CA. 2021. Microbial-physicochemical integrated analysis of kombucha fermentation. LWT 148: 111788. DOI: 10.1016/j.lwt.2021.111788.

Bukin YS, Galachyants YP, Morozov I, Bukin S, Zakharenko A, Zemskaya T. 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data 6: 190007. DOI: 10.1038/sdata.2019.7.

Cardoso RR, Neto RO, dos Santos D'Almeida CT, do Nascimento TP, Pressete CG, Azevedo L, Martino HSD, Cameron LC, Ferreira MSL, de Barros FAR. 2020. Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. Food Res Intl 128: 108782. DOI: 10.1016/j.foodres.2019.108782.

Chakravorty S, Bhattacharya S, Chatzinotas A, Chakraborty W, Bhattacharya D, Gachhui R. 2016. Kombucha tea fermentation: Microbial and biochemical dynamics. Intl J Food Microbiol 220: 63-72. DOI: 10.1016/j.ijfoodmicro.2015.12.015.

Coelho RMD, de Almeida AL, do Amaral RQG, da Mota RN, de Sousa PHM. 2020. Kombucha. Intl J Gastron Food Sci 22: 100272. DOI: 10.1016/j.ijgfs.2020.100272.

Coton M, Pawtowski A, Taminiau B, Burgaud G, Deniel F, Coulloumme-Labarthe L, Fall A, Daube G, Coton E. 2017. Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. FEMS Microbiol Ecol 93 (5): fix048. DOI: 10.1093/femsec/fix048.

Crotti E, Chouaia B, Alma A, Favia G, Bandi C, Bourtiz K, Daffonchio D. 2016. Ecology and physiology. In: Matsushita K, Tayoma H, Tonouchi N, Okamoto-Kainuma A (eds). Acetic Acid Bacteria. Springer, Japan. DOI: 10.1007/978-4-431-55933-7.

Dari DW, Junita D, Arsita Y, Meilina M, Meylani V. 2022. Chemical characteristics of juice of mangrove apple (*Sonneratia caseolaris*) added with sugar. Intl J Front Life Sci Res 2 (1): 18-28. DOI: 10.53294/ijflsr.2022.2.1.0023.

de Luna Freire MO, Cruz Neto JPR, de Albuquerque Lemos DE, de Albuquerque TMR, Garcia EF, de Souza EL, de Brito Alves JL. 2024. *Limosilactobacillus fermentum* strains as novel probiotic candidates to promote host health benefits and development of biotherapeutics: A comprehensive review. Probiotics Antimicrobial Proteins 16 (4): 1483-1498. DOI: 10.1007/s12602-024-10235-1.

de Miranda JF, Ruiz LF, Silva CB, Uekane TM, Silva KA, Gonzalez AGM, Fernandes FF, Lima AR. 2022. Kombucha: A review of substrates, regulations, composition, and biological properties. J Food Sci 87 (2): 503-527. DOI: 10.1111/1750-3841.16029.

Emiljanowicz KE, Malinowska-Pańczyk E. 2020. Kombucha from alternative raw materials-The review. Crit Rev Food Sci Nutr 60 (19): 3185-3194. DOI: 10.1080/10408398.2019.1679714.

Fabricio MF, Mann MB, Kothe CI, Frazzon J, Tischer B, Flôres SH, Ayub MAZ. 2022. Effect of freeze-dried kombucha culture on microbial composition and assessment of metabolic dynamics during fermentation. Food Microbiol 101: 103889. DOI: 10.1016/j.fm.2021.103889.

Ferdous M, Hasan M, Ali M, Mehedi N, Islam M. 2021. Mangrove apple (*Sonneratia caseolaris*): A promising fruit in Patuakhali coast of Bangladesh. Bangladesh J Agric 9-17. DOI: 10.3329/bjagri.v46i1-6.59969.

Francioli D, Lentendu G, Lewin S, Kolb S. 2021. DNA metabarcoding for the characterization of terrestrial microbiota-pitfalls and solutions. Microorganisms 9 (2): 361. DOI: 10.3390/microorganisms9020361.

Gomes RJ, de Fatima Borges M, de Freitas Rosa M, Castro-Gómez RJH, Spinosa WA. 2018. Acetic acid bacteria in the food industry: Systematics, characteristics and applications. Food Technol Biotechnol 56 (2): 139. DOI: 10.17113/ftb.56.02.18.5593.

Hafsari AR, Farida WN. 2021. pH characteristics of black tea kombucha culture with different sugar types in batch-culture fermentation. Gunung Djati Conference Series, Bandung. [Indonesian]

Harrison K, Curtin C. 2021. Microbial composition of SCOBY starter cultures used by commercial kombucha brewers in North America. Microorganisms 9 (5): 1060. DOI: 10.3390/microorganisms9051060.

Hooi SL, Dwiyanto J, Toh KY, Tan G, Chong CW, Lee JWJ, Lim J. 2023. The microbial composition and functional roles of different kombucha products in Singapore. CyTA-J Food 21 (1): 269-274. DOI: 10.1080/19476337.2023.2190794.

Jayabalan R, Malbaša RV, Lončar ES, Vitas JS, Sathishkumar M. 2014. A review on kombucha tea-microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. Comprehensive Rev Food Sci Food Saf 13 (4): 538-550. DOI: 10.1111/1541-4337.12073.

Kaewkod T, Bovonsombut S, Tragoolpua Y. 2019. Efficacy of kombucha obtained from green, oolong, and black teas on inhibition of pathogenic bacteria, antioxidation, and toxicity on colorectal cancer cell line. Microorganisms 7: 700. DOI: 10.3390/microorganisms7120700.

Kim J, Adhikari K. 2020. Current trends in kombucha: Marketing perspectives and the need for improved sensory research. Beverages 6 (1): 15. DOI: 10.3390/beverages6010015.

Laureys D, Britton SJ, De Clippeleer J. 2020. Kombucha tea fermentation: A review. J Am Soc Brewing Chem 78 (3): 165-174. DOI: 10.1080/03610470.2020.1734150.

Martínez Leal J, Valenzuela Suárez L, Jayabalan R, Huerta Oros J, Escalante-Aburto A. 2018. A review on health benefits of kombucha nutritional compounds and metabolites. CyTA-J Food 16 (1): 390-399. DOI: 10.1080/ 19476337.2017.1410499.

Naufal A, Harini N, Putri DN. 2022. Chemical and sensory characteristics of kombucha instant drink from red dragon fruit peel (*Hylocereus polyrhizus*) based on sugar concentration and fermentation duration. Food Technol Halal Sci J 5: 137-153. DOI: 10.22219/fths.v5i2.21556.

Nyhan LM, Lynch KM, Sahin AW, Arendt EK. 2022. Advances in kombucha tea fermentation: A review. Appl Microbiol 2 (1): 73-103. DOI: 10.3390/applmicrobiol2010005.

Pédron J, Guyon L, Lecomte A, Blottière L, Chandeysson C, Rochelle-Newall E, Raynaud X, Berge O, Barny M-A. 2020. Comparison of environmental and culture-derived bacterial communities through 16S metabarcoding: A powerful tool to assess media selectivity and detect rare taxa. Microorganisms 8: 1129. DOI: 10.3390/microorganisms8081129.

Putra MAP, Nirmala D, Andriyono S. 2022. Study on the addition of carrageenan stabilizer in the preparation of mangrove rosella syrup. Jurnal Perikanan Unram 12: 480-492. DOI: 10.29303/jp.v12i3.349. [Indonesian]

Reva ON, Zaets IE, Ovcharenko LP, Kukharenko OE, Shpylova SP, Podolich OV, de Vera J-P, Kozyrovska NO. 2015. Metabarcoding of the kombucha microbial community grown in different microenvironments. AMB Express 5: 124. DOI: 10.1186/s13568-015-0124-5.

Rué O, Coton M, Dugat-Bony E, Howell K, Irlinger F, Legras J-L, Loux V, Michel E, Mounier J, Neuvéglise C. 2023. Comparison of metabarcoding taxonomic markers to describe fungal communities in fermented foods. Peer Commun J 3: e97. DOI: 10.24072/pcjournal.321.

Salsabila DI, Machfidho A, Salsabila RA, Anggraini AV, Prasetyo AD, Rahmatullah AA, Ramadhani NH, Maharani DR, Husna AI. 2023. Pedada mangrove (*Sonneratta caseolaris*) fruit processing as syrup in the Sukorejo Area, Gresik. Sewagati 7 (1): 106-112. DOI: 10.12962/j26139960.v7i1.445.

Santos A, van Aerle R, Barrientos L, Martinez-Urtaza J. 2020. Computational methods for 16S metabarcoding studies using Nanopore sequencing data. Comput Structur Biotechnol J 18: 296-305. DOI: 10.1016/j.csbj.2020.01.005.

Semjonovs P, Ruklisha M, Paegle L, Saka M, Treimane R, Skute M, Rozenberga L, Vikele L, Sabovics M, Cleenwerck I. 2017. Cellulose synthesis by *Komagataeibacter rhaeticus* strain P 1463 isolated from Kombucha. Appl Microbiol Biotechnol 101: 1003-1012. DOI 10.1007/s00253-016-7761-8.

Sepriana C, Sumiati E, Jekti DSD, Zulkifli L. 2020. Identification and inhibition test of clove plant flower endophytic bacterial isolates (*Syzygium aromaticum* L.) against pathogenic bacteria. Jurnal Penelitian Pendidikan IPA 6 (1): 101-106. DOI: 10.29303/jppipa.v6i1.340. [Indonesian]

Setiawan E, Efendi R, Herawati N. 2016. Pedada Fruit Utilization (*Sonneratia caseolaris*) in Jam Making. [Doctoral Dissertation]. University Riau, Riau. [Indonesia]

Susanto A, Rifkowaty EE, Rosmalinda, Kurniawan T, Assorudin Sentap-Dalong, JR, Pawan KD. 2020. Engineering of nanoencapsulant preparation of pedada fruit extract (*Sonneratia caseolaris*) as a natural antioxidant and the resulting physicochemical properties. Jurnal Saintika Unpam 2 (2): 97-108. [Indonesian]

Tesena P, Korchunjit W, Taylor J, Wongtawan T. 2017. Comparison of commercial RNA extraction kits and qPCR master mixes for studying gene expression in small biopsy tissue samples from the equine gastric epithelium. J Equine Sci 28 (4): 135-141. DOI: 10.1294/jes.28.135.

Tran T, Grandvalet C, Verdier F, Martin A, Alexandre H, Tourdot-Maréchal R. 2020. Microbiological and technological parameters impacting the chemical composition and sensory quality of kombucha. Compre Rev Food Sci Food Saf 19 (4): 2050-2070. DOI: 10.1111/1541-4337.12574.

Walker AW, Martin JC, Scott P, Parkhill J, Flint HJ, Scott KP. 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome 3: 26. DOI: 10.1186/s40168-015-0087-4.

Wang B, Rutherfurd-Markwick K, Zhang X-X, Mutukumira AN. 2022. Kombucha: Production and microbiological research. Foods 11 (21): 3456. DOI: 10.3390/foods11213456.

Wick RR, Judd LM, Holt KE. 2019. Performance of neural network basecalling tools for Oxford Nanopore sequencing. Genome Biol 20: 129. DOI: 10.1186/s13059-019-1727-y.

Wood J, Osman A, Wade S. 2019. An efficient, cost-effective method for determining the growth rate of sulfate-reducing bacteria using spectrophotometry. MethodsX 6: 2248-2257. DOI: 10.1016/j.mex.2019.09.036.

Yang J, Lagishetty V, Kurnia P, Henning SM, Ahdoot AI, Jacobs JP. 2022. Microbial and chemical profiles of commercial kombucha products. Nutrients 14 (3): 670. DOI: 10.3390/nu14030670.