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Enhancing antioxidant activity and flavor profile of buni fruit (*Antidesma bunius*) kombucha through optimized fermentation conditions

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Abstract

Kombucha, a fermented beverage, is a rich source of antioxidants and offers various health benefits. This study explored the potential of Buni fruit (*Antidesma bunius*) in kombucha production, evaluating its chemical, biological, and antioxidant properties and sensory perceptions of Buni Fruit Kombucha under different fermentation conditions, including sugar concentration (5% and 10%), incubation temperature (4°C and 30°C), and fermentation duration (7 and 14 days). The chemical analysis of Buni fruit kombucha revealed a consistent decrease in pH, a gradual decline in sugar concentration, and an increase in total titratable acidity, highlighting significant changes during fermentation. During the 14-day incubation period, the microbial population dynamics of kombucha showed a significant decrease in total plate count and yeast count, but an increase in beneficial lactic acid bacteria. A significant difference in antioxidant activity was observed, with the highest activity recorded at an IC_{50} value of 61.32 ± 1.24 µL/mL. Sensory evaluation revealed no significant difference in the colour of kombucha among treatments, but there are significant differences in taste and aroma. The optimal conditions for enhancing antioxidant activity and sensory properties were found to be a 5% sugar concentration and fermentation at 30°C for 7 days. GC-MS analysis revealed the presence of three major compound groups (carboxylic acids, alcohols, and esters). These findings suggest that Buni has a high potential for use in creating functional foods through fermentation, which can contribute to developing functional kombucha beverages using fruit substrates to enhance their benefits and flavour profile.

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1. Introduction

Unhealthy habits, stress, and environmental pollutants can lead to the overproduction of reactive oxygen species, disrupting the body's balance and causing oxidative stress (1,2). These free radicals can contribute to the onset of various diseases like atherosclerosis, Parkinson's, Alzheimer's, and obesity by damaging cells and tissues, triggering inflammation

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and oxidative stress. To counteract oxidative stress, it is important to consume antioxidant-rich foods and beverages containing compounds such as vitamins E, A, C, and polyphenols (3,4). Kombucha, a fermented beverage, is one example of a functional drink rich in antioxidants.

Kombucha is a fermented beverage made from black tea and a symbiotic culture of bacteria and yeast known as SCOBY. The culture includes yeast and acetic acid bacteria (5). It has gained global popularity and is believed to offer various health advantages (6). Research suggests that kombucha possesses antibacterial, anticancer, antidiabetic, and antioxidant properties, along with cardiovascular benefits, hepatoprotective effects, improved digestion, immune system stimulation, and cholesterol reduction (7,8). While tea (*Camellia sinensis*) is the most commonly used substrate in kombucha production, other alternative substrates such as herbal infusions (9), fruit juices (10), milk (11), soy (12), and others have been explored in various studies. Among these alternatives, fruit-based substrates are particularly attractive because fruits naturally contain sugars, organic acids, vitamins, and phenolic compounds that can support microbial metabolism during fermentation and contribute to the nutritional and sensory characteristics of the final beverage. This exploration aims to achieve a wider range of flavours and enhance the health benefits of kombucha (6).

One of the indigenous berry fruits found in Indonesia is the Buni fruit (*Antidesma bunius*), which has a delightful sweet-sour flavour and reddish-purple colour (13). Traditionally, communities have used this plant to treat hypertension, heart disease and anemia, as well as a natural colouring source (14,15). Buni fruit contains anthocyanins, flavonoids, and phenolic acids (16). Previous studies have identified major polyphenolic components in buni fruit (*Antidesma bunius*), including gallic acid, catechin, epicatechin, and cyanidin-3-O-glucoside (17). The total phenolic content of fully ripe buni flesh can reach 40.73 mg GAE/g, with total flavonoid content of 31.54 mg QE/g, while the anthocyanin content of the extract was reported to be 20.93 mg cyanidin-3-glucoside equivalent/L, indicating strong antioxidant potential (18). However, despite its potential health benefits, Buni fruit is not as widely used, partly due to its sour taste when consumed directly and a lack of knowledge about its nutritional content and benefits. The utilisation of Buni fruit is still limited, and its optimisation is needed to enhance its attractiveness and economic value, such as using it in functional beverages like kombucha.

During kombucha fermentation, microbial metabolism can transform phenolic compounds into more bioavailable forms, which may further increase antioxidant potential (6). Compared to commonly used kombucha substrates such as Camellia sinensis, which typically exhibit total phenolic content in the range of 4.5–13 mg GAE/g dry weight depending on tea type and processing (19). Buni fruit provides a richer phenolic profile, particularly in anthocyanins, which are largely absent in tea substrates. This suggests that Buni fruit has potential as an alternative substrate for producing kombucha with enhanced functional properties. However, despite increasing interest in fruit-based kombucha, the optimal fermentation conditions for Buni fruit kombucha remain poorly understood

Typically, the fermentation process is influenced by various factors, including type of substrate, sugar concentration, and fermentation duration (20,21). In particular, temperature plays a crucial role in regulating microbial activity, where lower temperatures can slow metabolism and alter metabolite profiles, while higher temperatures accelerate fermentation processes (22). These parameters significantly influence fermentation kinetics, physicochemical characteristics, and sensory properties of kombucha (23). To date, no study has systematically evaluated the combined effects of sugar concentration, temperature, and fermentation duration on the antioxidant and sensory profile of Buni fruit kombucha. Therefore, this study aims to determine the ideal fermentation conditions for maximising antioxidant activity and sensory profile of Buni fruit (*Antidesma bunius*) kombucha.

2. Materials and Methods

The materials used in this study included Buni fruit sourced from Yogyakarta and analytical-grade solvents and reagents. The reagents encompass Folin-Ciocalteu for phenolic content analysis and 1,1-diphenyl-2-picrylhydrazyl (DPPH) for antioxidant activity analysis. Comparative standards such as quercetin, gallic acid, and ascorbic acid, all of analytical grade, were utilised. Kombucha starter culture was obtained from the Laboratory of Microbiology, Faculty of Applied Science and Technology. The instruments employed in the research comprised standard laboratory tools, a Gas Chromatography-Mass Spectrometry (GC-MS), and a UV-Vis spectrophotometer.

2.1. Research Procedures

2.1.1. Kombucha Production

Buni fruit samples were washed with tap water, peeled, blended with water at a 1:1 (w/v) ratio, and filtered to obtain the Buni fruit juice. The process of producing Buni fruit kombucha follows the methodology described by (8). The Buni fruit juice was divided into two portions, and sugar was added to obtain final concentrations of 5% and 10%. Pasteurisation was carried out over medium heat for 30 minutes. After that, the juice was cooled to room temperature. A total of 200 mL of juice was added to each jar, followed by a 10% kombucha starter. The jars were covered with a sterile cloth. Finally, each jar was labeled according to the treatment (Table 1) with different sugar concentrations (5% and 10%) and incubation temperatures (4°C and 30°C). The incubation temperatures were set at 30°C and 4°C to represent active fermentation and low-temperature conditions, respectively. Samples were analyzed after 7 and 14 days of fermentation. The experiment was designed to evaluate the effects of sugar concentration and incubation temperature on the fermentation characteristics of Buni fruit kombucha.

Table 1. Fermentation Conditions.

Treatment	Sugar concentration (%)	Temperature (°C)
S1T1	5	4
S1T2	5	30
S2T1	10	4
S2T2	10	30

2.1.2. Chemical Analysis of Kombucha

The chemical analysis procedure comprises the measurement of pH, total titratable acidity (TTA), and reducing sugar content. The pH measurement was conducted using a pH meter. Reducing sugar content was determined by utilising the Nelson–Somogyi method (24,25) and expressed as % (w/v) glucose equivalent based on the calibration curve. TTA assessment follows (26), wherein carbon dioxide was eliminated from the fermentation broth by subjecting it to heating at 100°C in a water bath for 10 minutes. Subsequently, a 20 mL sample was extracted and titrated with 0.1 M NaOH, employing phenolphthalein as an indicator. The total titratable acidity (TTA) was then deduced in grams of acetic acid per litre of the sample.

2.1.3. Biological Analysis of Kombucha

The determination of growth density (OD) of the fermented culture was carried out at 600 nm utilising a UV-Vis spectrophotometer. Kombucha mushroom dry weight measurement followed the method outlined by (27). The separation of mushrooms from the culture involved placing them on filter paper, washing them with distilled water thrice, and subsequently subjecting them to drying at 80°C until achieving a constant weight. The quantification of Lactic Acid Bacteria (LAB) in samples of Buni fruit kombucha was conducted utilising the standard plate count method, as described by (28). Initially, 1 ml of the sample was mixed with 9 ml of sterile 0.85% NaCl to establish a 10^{-1} dilution, followed by serial dilutions ranging from 10^{-2} to 10^{-6}. Subsequently, 100 µl of each dilution was spread onto Petri dishes containing De Man, Rogosa, and Sharpe (MRS) agar and uniformly spread using a sterile plate spreader. The Petri dishes were then inverted and incubated at 37°C for 48 hours. After incubation, the colonies were enumerated and reported as colony-forming units per millilitre (CFU/mL).

2.1.4. Antioxidant Activity by the DPPH Methods

In vitro assessment of the antioxidant activity of Buni fruit kombucha was conducted using the DPPH radical scavenging method, as outlined by (29) with modification. The kombucha samples were filtered using Whatman No. 1 filter paper and used as liquid samples. Different volumes of the kombucha sample (20, 40, 60, and 80 µL) were added into the reaction mixture and adjusted with methanol to obtain a constant final volume of 2 mL, resulting in final concentrations of 10, 20, 30, and 40 µL/mL (final concentration in the reaction mixture). Each reaction mixture consisted of 1 mL of freshly prepared 0.1 mM DPPH solution and the appropriate volume of sample, followed by methanol to reach the final volume. The mixtures were incubated in the dark for 30 minutes at room temperature. Ascorbic acid was used as the reference standard under the same conditions. A control (1 mL DPPH + methanol) and a corresponding sample blank (sample + methanol without DPPH) were prepared for each concentration to correct for background absorbance due to the inherent color of the kombucha. Methanol (95%) was used as the reagent blank. After incubation, the absorbance was measured at 517 nm. The antioxidant activity was expressed as percentage of DPPH inhibition. All measurements were performed in triplicate. The IC_{50} value, defined as the concentration required to inhibit 50% of DPPH radicals, was determined by plotting percentage inhibition against sample concentration (µL/mL) using linear regression analysis. The results were expressed in µL/mL due to the liquid nature of the sample without prior extraction or dry weight determination. The percentage of DPPH inhibition was calculated using the following equation:

$$\%\ inhibition = \frac{A_{control} - \left(A_{sample} - A_{blank\ sample}\right)}{A_{control}} \times 100 \qquad (1)$$

where: A control = absorbance of DPPH solution without sample, A sample = absorbance of DPPH solution with sample, and A blank sample = absorbance of sample without DPPH (background correction).

2.1.5. Sensory Evaluation

The sensory evaluation, following the methodology outlined by (30), aimed to gauge the preferences of panellists regarding the colour, taste, and aroma of the fermented kombucha. It was anticipated that the different fermentation stages would impact the sensory characteristics of the kombucha, leading panellists to assess their favoured Buni fruit kombucha based on colour, taste, and aroma. Utilising a hedonic scale, the assessment utilised a five-point rating system: (1) Strongly dislike, (2) Dislike, (3) Neither like nor dislike, (4) Like, and (5) Strongly like. The use of untrained panelists in this study was intended to represent general consumer preferences rather than to generate a detailed descriptive sensory profile. Therefore, the evaluation focused on sensory acceptance and perceived flavor characteristics from a consumer perspective. To minimize potential bias, the kombucha samples were presented to the panelists in a random order. The evaluation involved 30 untrained panellists with no history of taste or smell disorders, who were tasked with providing their sensory perceptions of the Buni fruit kombucha's colour, taste, and aroma.

2.1.6. Bioactive compounds screening

The analysis of bioactive compounds was conducted utilising the GC-MS method employing the Shimadzu GC-MS-QP2010 SE (Tokyo, Japan), which was equipped with a mass spectrometer detector and an autosampler. Before analysis, the kombucha sample underwent centrifugation at 12,000 rpm for 10 minutes, followed by filtration through a 0.45 µm filter membrane. Subsequently, the sample was dissolved in methanol and subjected to injection into the GC-MS with a volume of 1 µL. Separation was facilitated using an Rtx-5ms Restek column (30m x 0.25 mm ID, 0.25µm) (Bellefonte, PA, USA). The injector temperature was maintained at 230°C, while the column temperature was initially set at 70°C and gradually increased to 280°C. Helium was used as the carrier gas at a flow rate of 21.1 mL/min. Mass spectra were determined by referencing the Willey 147 & NIST14 Library. The identification of compounds was based on comparison of mass spectra with library data, and only compounds with a similarity index (SI) ≥80 was considered reliably identified.

2.2. Data Analysis

The experimental data were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was performed to determine significant differences among treatments at a significance level of $p < 0.05$. When significant differences were observed, Duncan's Multiple Range Test (DMRT) was applied as a post hoc test. Different superscript letters (a, b, c) in the tables indicate significant differences among treatments based on DMRT ($p < 0.05$).

3. Results and Discussion

3.1. Chemical Characteristics of Kombucha

Throughout the fermentation process of Buni fruit kombucha (Figure 1.), the pH levels of the various kombucha samples showed a consistent decline, ultimately reaching values between 2.63 and 2.94 after 14 days, as shown in Table 2. The lowest pH values were recorded in the S2T2 kombucha, while the highest were observed in the S2T1 kombucha. This trend aligns with previous studies, which also documented a gradual decrease in pH levels over the fermentation period, with the lowest pH values at the end of the process (31,32). The decreasing pH levels indicate increasing acidity across all kombucha samples due to the microbial breakdown of sucrose and subsequent production of various organic acids (29).

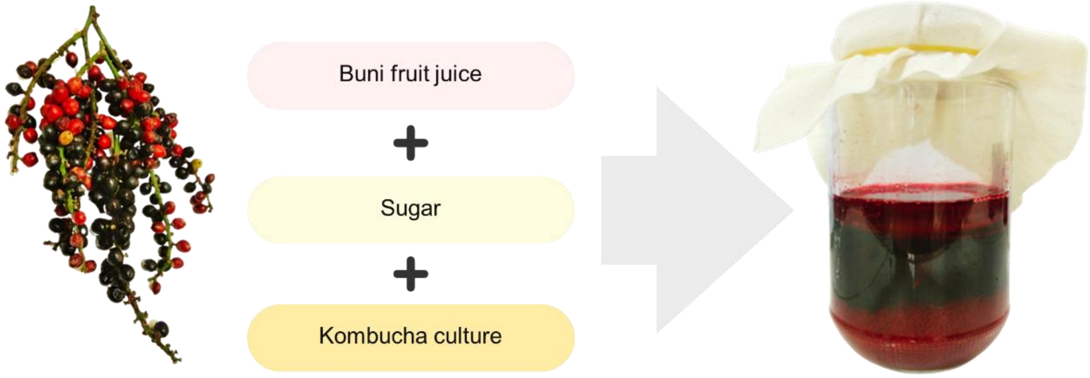

Figure 1. Buni Fruit Kombucha.

The pH values recorded in this study are consistent with those from previous research on fruit-based kombucha. For example, previous research found pH levels ranging from 3.2 to 2.7 in fruit-based kombucha after a 14-day fermentation (33). Another study reported slightly lower pH values in kombucha made with fruits like cherry, plum, apricot, strawberry, persimmon, grape, orange, and pomegranate, with pH levels between 2.03 and 2.75 after 21 days of fermentation (34). The increase in acidity is attributed to the activity of Acetic Acid Bacteria (AAB), which produce acetic acid, gluconic acid, and glucuronic acid. Additionally, the breakdown of fruit components releases fruit acids into the kombucha, further increasing acidity (35). Optimal kombucha flavour is typically achieved when the pH is between 2.5 and 3.5 (36). Producing kombucha with a pH below 2.5 is not recommended due to potential health risks from high acidity (31). However, all kombucha samples in this study exhibited safe pH levels ranging from 2.6 to 3.2.

The sugar concentration gradually decreased during fermentation as microorganisms metabolized sugar as a carbon source for growth and metabolic activity. These microorganisms metabolise the sugar throughout fermentation, converting it into organic acids and ethanol. This transformative process is initiated by yeast and bacteria, which enzymatically break down sucrose into glucose and fructose. Yeast then uses fructose via glycolysis to generate ethanol, while acetic acid bacteria metabolise glucose into gluconic acid and convert the ethanol produced by yeast into acetic acid (37). The sugar content derived from the Buni fruit can further influence the overall sugar levels within the kombucha brew. After 14 days of fermentation, samples incubated at 30°C (S1T2 and S2T2) exhibited the lowest sugar concentrations, at 3.80% and 3.90%, respectively. In contrast, higher residual sugar levels in samples incubated at 4°C (S1T1 and S2T1) can be attributed to reduced microbial metabolic activity under low-temperature conditions, which inhibits the growth and activity of mesophilic microorganisms. This observation aligns with the findings of a study that reported that higher temperatures expedite fermentation rates, as evidenced by a concurrent decrease in pH (38). This finding is also consistent with previous studies reporting that low-temperature fermentation slows enzymatic reactions and microbial growth, thereby delaying sugar utilization (22). Although microbial activity is reduced at low temperatures, it is not completely inhibited. Instead, microorganisms undergo metabolic adaptation, resulting in gradual biochemical transformations and differences in metabolite profiles compared to fermentation at higher temperatures (39,40). This explains the distinct physicochemical characteristics observed between the 4°C and 30°C treatments.

This temperature-dependent variation in microbial activity also affects organic acid production. During fermentation, the total titratable acidity (TTA), expressed as acetic acid concentration, gradually increased, with the highest value (11.04%) observed after 14 days in the S2T2 treatment. Samples incubated at 30°C (S1T2 and S2T2) exhibited significantly higher TTA compared to those incubated at 4°C, indicating more active metabolic conversion of sugars into organic acids. This outcome aligns with findings from another study that noted a significant association between titratable acidity and fermentation temperature (41). However, the TTA value observed in the S2T2 treatment (11.04%) is relatively high for a beverage and may negatively affect palatability and consumer acceptance. Kombucha is known to contain various primary organic acids, including acetic, lactic, gluconic, and glucuronic acid, which collectively contribute to the observed pH drop from 5 to 3 within 10-14 days of fermentation, indicating their increasing concentration over time (42). Acetic acid bacteria (AAB) play a crucial role in this process by utilising ethanol, glucose, and fructose to produce a spectrum of organic acids, such as acetic acid, gluconic acid, glucuronic acid, ascorbic acid, and succinic acid, alongside other beneficial compounds. This metabolic activity effectively reduces the concentration of ethanol and sugar content in kombucha, thereby shaping its final composition and characteristics (43).

Table 2. Chemical Characteristics of Buni Fruit Kombucha.

Fermentation Day	Samples	pH	Reducing Sugar (%)	Total Titratable Acidity (%)
7	S1T1	3.22 ± 0.10^{d}	4.50 ± 0.49^{cd}	3.48 ± 0.30^{a}
	S1T2	3.24 ± 0.13^{d}	4.40 ± 0.19^{bcd}	8.64 ± 0.45^{e}
	S2T1	3.24 ± 0.04^{d}	4.80 ± 0.24^{d}	3.96 ± 0.12^{b}
	S2T2	3.00 ± 0.19^{c}	4.30 ± 0.33^{abcd}	9.84 ± 0.15^{f}
14	S1T1	2.84 ± 0.08^{bc}	4.20 ± 0.29^{abc}	5.28 ± 0.14^{c}
	S1T2	2.78 ± 0.11^{ab}	3.80 ± 0.32^{a}	9.60 ± 0.24^{f}
	S2T1	2.94 ± 0.07^{bc}	4.40 ± 0.23^{bcd}	5.76 ± 0.30^{d}
	S2T2	2.63 ± 0.09^{a}	3.90 ± 0.22^{ab}	11.04 ± 0.22^{g}

Values are expressed as mean ± standard deviation for triplicate analysis. Different superscript letters indicate significant differences ($p < 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.2. Biological Characteristics of Kombucha

Based on the data presented in Table 3, bacterial growth within the kombucha culture, quantified by OD at 600nm, exhibited a steady increase from the initiation of fermentation until the process was completed. Notably, there was no discernible variance in bacterial growth, as indicated by OD600 readings across the various treatment conditions. However, the analysis of the dry weight of the kombucha mushroom revealed a significant difference ($p < 0.05$) between the different treatment groups. Specifically, the S2T2 treatment exhibited the highest dry weight of the kombucha mushroom following a 14-day incubation period. This observation aligns with the findings of Aun and Eung, which suggest that higher incubation temperatures can expedite the fermentation process, forming thicker kombucha mushrooms (38). These structures, commonly referred to as kombucha pellicles, act as microbial biofilms that serve as inoculums for subsequent kombucha fermentation and represent a distinctive feature of the beverage. These cellulosic biofilms are generated by Acetic Acid Bacteria (AAB) (44).

During the 14-day fermentation period, significant changes were observed in the microbial population of kombucha. On day 7, there was a notable difference in the total plate count between the S1T2 and S2T2 samples (6.05 ± 0.15 and 6.09 ± 0.15) compared to S1T1 and S2T1 (4.23 ± 0.09 and 4.44 ± 0.34). By day 14, the total plate counts for S1T2 (4.70 ± 0.05) and S2T1 (4.85 ± 0.16) decreased significantly compared to their day 7 counts, though they remained higher than S1T1 and S2T2. The decrease in aerobic bacterial count during prolonged fermentation was aligned with the findings of Tomar (45). The decline in aerobic bacterial count during prolonged fermentation, as reported by Tan et al., is attributed to increasingly acidic conditions and reduced nutrients (28). This pattern is commonly reported in kombucha fermentation and reflects natural microbial succession within the SCOBY, where microbial populations increase during early fermentation and subsequently decline as environmental conditions become more selective (46). As fermentation progresses, organic acid accumulation lowers the pH, creating a selective environment in which acid-tolerant microorganisms, particularly acetic acid bacteria (AAB), become dominant, while other microorganisms decline. This is also consistent with the increase in lactic acid bacteria (LAB) observed in this study, indicating that certain acid-tolerant microbial groups remain active. Therefore, the reduction in microbial counts represents a shift toward acid-adapted populations rather than a loss of symbiotic functionality.

On day 7, S2T2 had the highest yeast count at 5.54 ± 0.53. However, by day 14, yeast counts generally decreased across all samples, with S1T2 and S2T1 showing significant reductions to 3.97 ± 0.14 and 4.04 ± 0.06, respectively. This reduction in yeast population is likely due to the declining sugar concentration as fermentation progresses, with yeast converting sucrose into glucose and fructose to produce ethanol, which may then be converted to acetic acid by AAB during kombucha fermentation (23). The decrease in yeast count on day 14 is consistent with findings reported by (27). The reduced sugar concentration and increasingly acidic conditions contribute to the decrease in yeast count (28). Klawpiyapamornkun et al. also observed an initial increase in yeast count on day 3, followed by a decline on day 6, with counts stabilising until the end of fermentation (47).

Lactic acid bacteria (LAB) play dual roles by enriching flavours and potentially offering health benefits. Although LAB species such as *Lactobacillus* and *Lactococcus* may be present in kombucha's microbial consortium, their consistency varies (48). The total count of LAB in treatments S1T1 and S2T1 (incubated at 4°C) significantly rose from day 7 to day 14 of fermentation, while in treatments incubated at 30°C (S1T2 and S2T2), the LAB count peaked at day 7, with no marked increase from day 7 to day 14. This disparity is likely due to the impact of incubation temperature on LAB proliferation, with elevated temperatures accelerating fermentation rates (38). Some Kombucha products have been found to contain LAB, making up to 30% of the bacterial population (49). While LAB is not essential for kombucha production, several studies have emphasised its importance in kombucha fermentation. Research has shown that introducing *Lactobacillus paracasei* enhances antibacterial properties and impacts glucuronic acid content (50). LAB also contributes to improved antioxidant and antimicrobial activities (51), as well as increased production of D-saccharic acid-1,4-lactone (DSL) and glucuronic acid (52).

During the Buni fruit kombucha fermentation process, there is a significant decrease in the total plate count and yeast count, especially in S1T2 and S2T1 samples. However, the lactic acid bacteria count increases significantly across all samples. This shift in microbial population dynamics, characterised by a decrease in yeast and overall microbial load but an increase in beneficial lactic acid bacteria.

Acetic acid bacteria (AAB) are the predominant bacteria in kombucha cultures (49). These bacteria transform ethanol, which yeast produces during sugar fermentation, into acetic acid. This transformation gives kombucha its distinctive tangy flavour and lowers the pH, creating an acidic environment. Additionally, acetic acid bacteria convert glucose into gluconic acid, adding to the complexity of kombucha's flavour profile (53). The rise in both acetic acid and lactic acid bacteria is accompanied by an increase in organic acid concentration, which is associated with the reduction in aerobic bacteria and yeast counts (45).

In addition, the high total titratable acidity (TTA), which reached 11.04% at the end of fermentation, likely contributed to the observed decline in microbial populations. Such conditions reflect the accumulation of organic acids and are known to impose stress on microbial cells, particularly affecting less acid-tolerant microorganisms such as yeast. However, this does not indicate a complete inhibition of the SCOBY consortium, as acid-tolerant microorganisms, especially acetic acid bacteria (AAB) and lactic acid bacteria (LAB), remain active and continue contributing to organic acid production and system stability under acidic conditions (54). Therefore, the reduction in total microbial counts observed after 14 days may reflect microbial adaptation and selection within the SCOBY rather than a collapse of the system. This condition may have dual implications for product stability. On one hand, the low pH and high acidity enhance microbiological safety and may improve shelf stability by inhibiting contaminant growth. On the other hand, excessive acid accumulation may negatively affect sensory quality and reduce microbial diversity, potentially limiting the functional and probiotic characteristics of the kombucha during extended storage.

Table 3. Biological Characteristic of Buni Fruit Kombucha

Fermentation Day	Kombucha Sample	Optical Density (600 nm)	Mushroom dry weight (g)	Total plate count (Log CFU/mL)	Yeast (Log CFU/mL)	Lactic Acid Bacteria (Log CFU/mL)
7	S1T1	0.88 ± 0.07^{a}	1.41 ± 0.03^{a}	4.23 ± 0.09^{a}	4.54 ± 0.36^{ab}	3.42 ± 0.18^{a}
	S1T2	1.12 ± 0.10^{b}	2.22 ± 0.16^{c}	6.05 ± 0.15^{d}	5.00 ± 0.36^{bc}	5.19 ± 0.37^{b}
	S2T1	1.11 ± 0.08^{b}	1.39 ± 0.07^{a}	4.44 ± 0.34^{ab}	5.04 ± 0.25^{bc}	3.87 ± 0.17^{a}
	S2T2	1.13 ± 0.09^{b}	2.83 ± 0.13^{d}	6.09 ± 0.15^{d}	5.54 ± 0.53^{c}	5.22 ± 0.21^{b}
14	S1T1	1.33 ± 0.17^{b}	1.55 ± 0.08^{a}	4.36 ± 0.18^{a}	4.80 ± 0.77^{b}	5.56 ± 0.63^{b}
	S1T2	1.28 ± 0.18^{b}	2.65 ± 0.11^{d}	4.70 ± 0.05^{bc}	3.97 ± 0.14^{a}	5.48 ± 0.31^{b}
	S2T1	1.20 ± 0.14^{b}	1.85 ± 0.12^{b}	4.85 ± 0.16^{c}	4.04 ± 0.06^{a}	5.69 ± 0.44^{b}
	S2T2	1.27 ± 0.05^{b}	4.33 ± 0.20^{e}	4.23 ± 0.11^{a}	4.39 ± 0.16^{ab}	5.30 ± 0.22^{b}

Values are expressed as mean ± standard deviation for triplicate analysis. Different superscript letters indicate significant differences ($p < 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.3. Antioxidant Activity by the DPPH Methods

The lowest IC$_{50}$ concentration of Buni Fruit Kombucha necessary to inhibit the DPPH free radical by 50% was determined to be 61.32 ± 1.24 µL/mL. Antioxidant activity, expressed as IC_{50}, varies in kombucha made from different fruits; for example, snake fruit (*Salacca zalacca*) has an IC_{50} of 5.46 µg/mL (30) and blackberry (*Aronia melanocarpa*) has an IC_{50} of 791 ± 7.4 µg/mL (55). Fermentation of blackberry kombucha (*Vaccinium myrtillus*, *Ribes nigrum*, and *Aronia melanocarpa*) shows more variety of biologically active compounds that do not cause cytotoxic effects, exhibit strong antioxidant properties, and reduce oxidative stress in both human cells and yeast cells (55). The antioxidant activity of Buni Kombucha fruit is markedly influenced by the duration of fermentation, with the highest activity observed in treatment S1T2, which underwent a 7-day incubation period (Table 4). This finding aligns with a previous study, which also found peak antioxidant potential in kombucha after 7 days of fermentation (1). An increase in antioxidant activity in kombucha made from fruit juice has been found in several studies, such as Noni Fruit (*Morinda citrifolia*) (56), ginseng fruit (*Panax ginseng*) (57), apple (58), and Red goji berry (*Lycium barbarum*) (59). Similar results were observed in the antioxidant activity of kombucha made from Gac and Mango fruit, which increased until the 14th day of fermentation (60). Overall, previous studies consistently indicate that fermentation with kombucha cultures enhances the antioxidant activity of fruit-based beverages. During microbial fermentation, both indigenous and bound phenolic compounds are subjected to extensive transformation by yeasts and other microorganisms present in the SCOBY consortium. These microbes produce various hydrolytic and oxidative enzymes, including β-glucosidase, esterase, decarboxylase, and phenolic acid reductase, which facilitate the release and structural modification of phenolic compounds from plant cell wall matrices (61,62). This process increases the solubility and bioavailability of phenolics and enhances their antioxidant capacity. Previous studies on kombucha produced from maqui berry (*Aristotelia chilensis*) juice have also reported that fermentation promotes the release and accumulation of phenolic compounds and enhances interactions between phenolic compounds and anthocyanins (co-pigmentation), which are associated with improved antioxidant responses depending on fermentation conditions (63).

In addition, although the total anthocyanin content may decrease during fermentation, this reduction does not necessarily correspond to a proportional decline in antioxidant potential, as anthocyanins undergo various transformation pathways. Anthocyanins, which are commonly present as glycosylated forms such as cyanidin-3-O-glucoside, can be hydrolyzed into anthocyanidins by β-glucosidase, with the aglycone forms often exhibiting higher antioxidant activity. Furthermore, during fermentation, anthocyanins may react with microbial metabolites such as pyruvate and acetaldehyde, leading to the formation of more stable derivatives, including pyranoanthocyanins (e.g., vitisins) and polymeric pigments (64). These derived compounds exhibit enhanced chemical stability and are suggested to retain antioxidant-related properties.

All samples of Buni fruit kombucha showed decreased antioxidant activity by the 14th day, consistent with findings by Amarasinghe et al. indicating diminishing antioxidant activity with prolonged fermentation (65). This decrease is primarily attributed to continued microbial metabolism, which transforms phenolic compounds with high radical scavenging activity into less active forms through oxidation, polymerization, or conversion into simpler metabolites (66). In addition, the accumulation of organic acids and the progressively acidic environment, as reflected by decreasing pH values in this study, may further destabilize bioactive compounds and reduce their effectiveness as hydrogen or electron donors in DPPH radical scavenging. Prolonged fermentation may also lead to nutrient depletion, limiting the availability of precursors required for maintaining antioxidant-active compounds (67). This dynamic explains why the highest antioxidant activity was observed at day 7, where the balance between phenolic release, biotransformation, and compound stability is optimal. Beyond this point, degradative and conversion processes appear to dominate over biosynthetic and release mechanisms. Therefore, the antioxidant profile of kombucha is highly dependent on achieving a critical fermentation window, where bioactive compound accumulation is maximized before degradation becomes predominant, as also reflected in the IC_{50} values obtained in this study. It is crucial to recognize that the antioxidant potential of kombucha varies depending on factors such as fermentation duration, substrate composition, and microbial diversity in the culture (68).

Table 4. Antioxidant Activity of Buni Fruit Kombucha

Fermentation Day	Kombucha Sample	% Inhibition at 100 µL/mL (%)	IC_{50} value by DPPH assay (µL/mL)
7	S1T1	75.54 ± 0.19^{c}	68.50 ± 0.51^{d}
	S1T2	80.63 ± 1.62^{d}	61.32 ± 1.24^{a}
	S2T1	78.73 ± 0.61^{d}	64.95 ± 0.52^{b}
	S2T2	71.75 ± 1.21^{b}	71.83 ± 0.27^{e}
14	S1T1	71.18 ± 1.52^{b}	71.00 ± 0.72^{e}
	S1T2	75.68 ± 1.23^{c}	66.60 ± 0.39^{c}
	S2T1	76.34 ± 0.42^{c}	65.25 ± 0.84^{b}
	S2T2	67.24 ± 1.06^{a}	75.03 ± 0.91^{f}

Values are expressed as mean ± standard deviation for triplicate analysis. Different superscript letters indicate significant differences ($p < 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.4. Sensory evaluation

Sensory evaluation in this study reflects consumer-based perceptions, as it was conducted using untrained panelists to assess general acceptability and perceived flavor characteristics rather than to generate a detailed descriptive flavor profile. The results of the sensory evaluation of Buni Kombucha fruit are shown in Table 5. The panellists generally accepted all treatments. However, significant differences ($p < 0.05$) were observed in the preference scores for taste and aroma, indicating variation in consumer preference among samples, while no noticeable difference was found in colour preference. For colour, there was a slight decrease in scores from Day 7 to Day 14 for all samples, with S1T2 having the highest score on Day 7 (3.93 ± 0.13).

Buni fruit contains anthocyanins, which give it a reddish-purple colour (16). Studies on maqui berry kombucha have shown that anthocyanins remain stable during 7 days of fermentation, providing the best color intensity response (63). The total anthocyanin content in blackberry (*Vaccinum myrtillus*, *Ribes nigrum*, and *Aronia melanocarpa*) increases with kombucha culture fermentation (55). However, in this study, sensory evaluation results indicated that all panellists gave the same preference score for colour since all treatments were a deep purple, resulting in no significant difference ($p < 0.05$) between samples.

The panelists particularly favored the taste and aroma of S1T2, which was incubated for 7 days. This preference could be due to its moderate pH level (3.24), resulting in a less sour taste and medium sugar content (4.40%), offering an appealing flavor profile for the panelists. The sweetness of kombucha may be attributed to residual sugars that have not yet been metabolized by the SCOBY, which can influence the final flavor profile of the beverage (69).

In addition, the balance between decreasing sugar content and increasing acidity during fermentation plays a key role in shaping the aftertaste and mouthfeel of kombucha. Samples fermented for 7 days, particularly S1T2, exhibited a smoother mouthfeel and a mild aftertaste due to residual sugars and moderate acidity. A higher initial sugar concentration at the same fermentation time leads to a sweeter kombucha. Higher temperatures and lower initial sugar content in kombucha were linked to fermented, yeasty aromas, fizzy mouthfeels, and sour and bitter tastes (70). In contrast, samples with higher acidity, particularly S2T2, showed lower preference scores for taste and aroma, especially at Day 14. This suggests that excessive acid accumulation during fermentation may lead to overly sour and less acceptable flavor profiles. Prolonged fermentation (14 days) led to a sharper, more astringent aftertaste and a thinner mouthfeel, associated with lower sugar levels and higher total titratable acidity. However, the use of untrained panelists may limit detailed sensory interpretation. Therefore, future studies should employ trained panelists and descriptive sensory analysis.

Table 5. Sensory evaluation of Buni Fruit Kombucha

Samples	Color		Taste		Aroma	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
S1T1	3.90 ± 0.17^{a}	3.77 ± 0.21^{a}	3.17 ± 0.16^{c}	3.17 ± 0.14^{c}	3.33 ± 0.24^{de}	3.10 ± 0.12^{cd}
S1T2	3.93 ± 0.13^{a}	3.70 ± 0.22^{a}	4.23 ± 0.26^{d}	1.97 ± 0.06^{a}	3.60 ± 0.12^{e}	2.10 ± 0.17^{a}
S2T1	3.67 ± 0.18^{a}	3.63 ± 0.12^{a}	2.77 ± 0.23^{b}	4.07 ± 0.13^{d}	2.83 ± 0.15^{bc}	3.56 ± 0.19^{e}
S2T2	3.73 ± 0.36^{a}	3.37 ± 0.37^{a}	2.67 ± 0.23^{b}	2.13 ± 0.22^{a}	2.70 ± 0.32^{b}	2.20 ± 0.16^{a}

Values are expressed as mean ± standard deviation for triplicate analysis. Different superscript letters indicate significant differences ($p < 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.5. Bioactive compounds analysis

Volatile organic compounds (VOCs) play a significant role in determining the aroma profile of kombucha. Gas chromatography-mass spectrometry (GC-MS) analysis reveals the presence of three major compound groups—carboxylic acids, alcohols, and esters—in Buni fruit kombucha (Figure 2 and Table 6). These compounds, including decanoic, hexadecanoic, hexanoic, and octanoic acids, are synthesized during kombucha fermentation (71).

In addition to their chemical presence, the detected volatile compounds can be associated with specific aroma characteristics in kombucha. Hexadecanoic acid (9.49%) and octadecanoic acid (17.22%) are commonly associated with fatty, waxy, and oily aroma attributes. Similar aroma descriptors for long-chain fatty acids have been reported in previous studies (71). However, the relatively high abundance of these compounds may influence the sensory perception of the product, potentially leading to less preferred aroma characteristics in certain treatments.

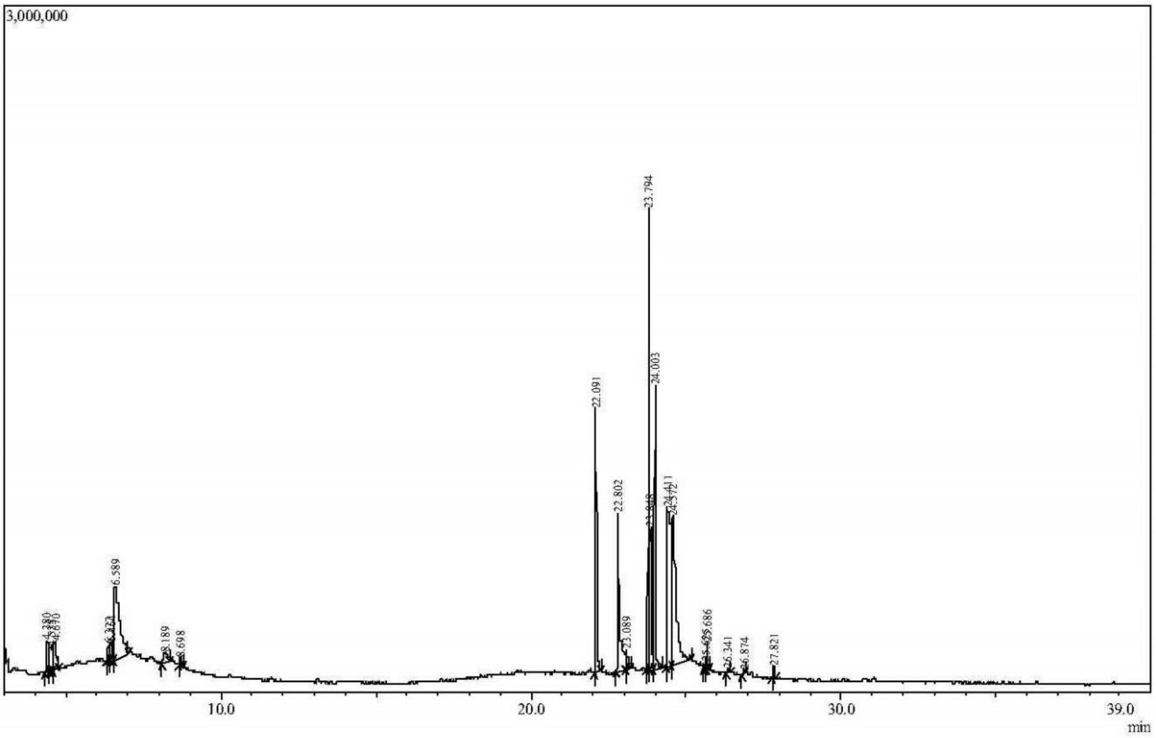

Figure 2. Chromatogram of GC-MS analysis of Buni Fruit Kombucha.

Table 6. Bioactive compound of Buni Fruit Kombucha.

Class	Compound	Formula	Retention Time	% Area	Similarity Index
Carboxylic acid	Hexadecanoic acid	$C_{16}H_{32}O_2$	22.802	9.49	95
	Octadecanoic acid	$C_{18}H_{36}O_2$	24.572	17.22	92
Alcohol	2-Furanmethanol	$C_5H_6O_2$	6.589	11.56	85
Ester	Hexadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	22.091	7.49	96
	Octadecanoic acid, methyl ester	$C_{20}H_{40}O_2$	24.003	7.21	97
	Eicosanoic acid, methyl ester	$C_{22}H_{44}O_2$	25.686	0.69	94
	Docosanoic acid, methyl ester	$C_{24}H_{48}O_2$	27.821	0.56	92

Furfural alcohol, alternatively known as 2-furan methanol, is a noteworthy compound in kombucha, contributing significantly to its flavour profile. Its presence is not limited to kombucha alone but extends to various sources like coffee aroma, tea, wheat bread, crispy bread, soybean, cocoa, rice, and potato chips (72). The alcohol compound 2-furanmethanol (11.56%) detected in this study is characterized by sweet, caramel-like, and roasted aroma notes, which are generally perceived as pleasant and contribute to aroma complexity (73). Alcohols, which are commonly detected in kombucha, are primarily produced through yeast metabolism during fermentation (74). Through mixed fermentation, non-Saccharomyces yeast strains like *Zygosaccharomyces* sp. and *Dekkera* sp. enrich VOC profiles, thereby enhancing aroma and facilitating the formation of organic acids via oxidation (75). These alcohols, along with aldehydes, serve as crucial substrates for synthesising organic acids and esters through the metabolic pathways of bacteria within the kombucha culture (71). As the fermentation process unfolds, microbial activity alters the aroma profile of kombucha, giving rise to a diverse array of molecules (72).

Esters were identified as one of the key volatile groups contributing to the aroma profile of Buni fruit kombucha. In this study, esters such as hexadecanoic acid methyl ester (7.49%) and octadecanoic acid methyl ester (7.21%) were detected at relatively notable levels, indicating their potential role in aroma formation. The presence of alcohols and carboxylic acids, which act as precursors in esterification reactions during fermentation, may support the formation of these ester compounds (76). Esters are widely associated with fruity, sweet, and pleasant aroma characteristics (77), which may help balance the sour and acidic characteristics of kombucha, leading to better sensory acceptance.The chemical profile of Buni fruit kombucha consisted of fatty acids, alcohols, and esters, which may collectively influence the sensory profile depending on their volatility and odor activity. These identified volatile compounds provide insight into the types of aroma-active compounds formed during fermentation. Therefore, variations in aroma preference among treatments (Table 5) may be partially related to differences in fermentation conditions that influence the formation and balance of these compounds. During fermentation, microbial activity modifies the aroma profile of kombucha by generating a diverse range of metabolites (37).

4. Conclusions

The study demonstrates that Buni fruit kombucha has significant potential as a functional beverage with antioxidant properties. Optimal fermentation conditions involving a 5% sugar concentration and an incubation temperature of 30°C for 7 days resulted in the highest antioxidant activity and favorable sensory profile. These results highlight the potential of Buni fruit for the development of functional fermented beverages, supporting its utilization as a value-added product with potential health benefits. Further studies should focus on standardizing the initial substrate, as well as employing trained panelists and advanced descriptive sensory methods to achieve a more detailed characterization of flavor changes. Additionally, future work should investigate the bioactive potential and underlying mechanisms of Buni fruit kombucha.

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Author Contributions

O.R.A. and T.W. conceptualization; O.R.A and N.I. methodology; P.N. and C.J. performed the experiments; N.I. and P.N. analyzed the data; O.R.A. writing original draft preparation; O.R.A and P.N. writing-review and editing; O.R.A. supervision; O.R.A and T.W. funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The use of humans in the sensory analysis was conducted in accordance with the Declaration of Helsinki and has been approved by the Ethics Committee on Research Involving Human Beings of Universitas Ahmad Dahlan (EC No.012305078 on June 16, 2023)

Data Availability Statement

The data supporting the findings is available within the article.

Conflicts of Interest

All authors declare no conflict of interest, financial or otherwise.

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