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Characterization of Advanced Fermentation of Kombucha Rose Extract

Brigitta Charlotte Avila Jovier[1], Rafaelle Jovanka[1], Karina Bianca Lewerissa[1*], Binardo Adi Seno Mawarno[2]

[1]Department of Food Technology, Faculty of Health Sciences and Medicine,
Satya Wacana Christian University, Salatiga, Central Java, Indonesia
[2]Department of Food Engineering Technology, Faculty of Science and Technology, Duta Bangsa University
Surakarta, Central Java, Indonesia
Email corresponding author: karina.lewerissa@uksw.edu

ABSTRACT

With the addition of rose flower extract, kombucha offers functional health benefits that can help strengthen the immune system, particularly during periods of climate change, when the risk of illness increases. Meanwhile, rose is rich in bioactive compounds, including flavonoids, polyphenols, and vitamin C, which are known for their antioxidant and anti-inflammatory properties. The fermentation process in kombucha produces probiotics that support digestive health. Three different rose extract concentrations were added to kombucha tea for the second fermentation process, namely 20%, 30%, and 50% for 3 days. The results showed that the pH of all samples decreased during the 3-day fermentation period, with the final pH ranging from 2.5 to 2.8, and the lowest value was observed in the control sample. The highest pH was observed in the sample with 50% rose extract addition. The addition of rose extract decreased the total solid content of the products, due to the dilution of kombucha tea, which produces tiny pellicles during the fermentation process. However, it increased the antioxidant activity in the product. The higher the rose extract, the higher the antioxidant activity. Microscopic observation revealed that kombucha tea contained yeast and Gram-positive bacteria, and all samples contained more than 10^8 CFU/ml of lactic acid bacteria. The most preferable kombucha could be found in a 50% rose extract sample.

Keywords : *fermentation; functional benefits; kombucha tea; rose extract*

INTRODUCTION

Society's awareness of a healthy lifestyle has led to a trend in consuming products that are free from chemical additives and safe for consumption (Plessas, 2021). This awareness increases due to the increase in degenerative diseases, such as heart disease, diabetes, and obesity. One method that seems to be a solution for consumers' demands and preferences is fermentation. It also has several advantages, including sustainability and low cost (Yuan et al., 2024). In some cases, it also extends the shelf-life of ingredients (Paramithiotis et al., 2022; Urbonaviciene et al., 2015). Fermentation techniques also create new active compounds, retain nutritional values, increase nutritional, flavour, and product variety (Kiczorowski et al., 2022; Mojikon et al., 2022; Saud et al., 2024).

The trend in consuming fermented drinks over non-fermented ones is increasing (Kusumasari et al., 2024). Kombucha is a fermented green or black tea product with a sugar content of 5-8% resulting from a process of inoculation with a consortium of lactic acid bacteria and yeast, commonly known as SCOBY (symbiotic culture of bacteria and yeast) (Bishop et al., 2022; Soares et al., 2021). As its name suggests, SCOBY consists of a wide variety of microorganisms—including bacteria such as the *Komagataeibacter, Acetobacter, Lactobacillus*, and *Azotobacter* groups—as well as a variety of yeasts that includes *Saccharomyces, Brettanomyces,* and *Zygosaccharomyces* (Santiago-Santiago et al., 2025).The fermentation process of kombucha occurs for 8-10 days (De Filippis et al., 2018), which this fermented beverage is known to have many health benefits through various studies, such as lowering blood pressure and cholesterol, aiding weight loss, relieving arthritis, boosting

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immune response, improving constipation, inhibiting cancer proliferation and acting as an anti-hyperglycemic and antioxidant (Kitwetcharoen et al., 2023; Nissa, 2023). The health-promoting functional characteristics of kombucha arise from vital nutrients that support well-being, including chlorogenic acid, phenolic compounds, organic acids, water-soluble vitamins, and a range of micronutrients generated during fermentation. (Saito et al. (2024).

Kombucha has become increasingly popular due to its distinctive taste, which combines sweet, sour, and carbonated flavors that consumers highly seek after. Kombucha fermentation can take place in several stages. The first stage involves the primary fermentation of tea by SCOBY, which produces organic acids, ethanol, and a microbial biofilm on the surface of the solution (Laureys et al., 2020). The second stage, commonly referred to as secondary fermentation, is a continuation of the fermentation process, where the kombucha is separated from the SCOBY.

Secondary fermentation is generally carried out to stimulate the formation of secondary metabolites produced from the growth of microorganisms under anaerobic conditions. These secondary metabolites will increase flavor and aroma variation, create more attractive colors, and also add functional value to kombucha tea. For example, the secondary fermentation of kombucha, as studied by Wahyudi et al. (2023), which involved adding cinnamon and secang wood extracts, was found to affect pH characteristics, color, and density. Research by Tanamas et al., 2025 also states that the addition of mango juice in fermentation for 2 days affects the vitamin C content, total acid, pH, total dissolved solids, total sugar, and sensory color, aroma, taste, sparkling sensation, and overall acceptance of kombucha.

The diversity of aromatic compounds in kombucha based on their origin can be divided into three categories, namely substrate matrix (tea), sugar source, and microbial secondary metabolites. For the latter category, a number of volatile organic compounds can vary greatly depending on the duration and stage of fermentation. There are six groups of VOCs, namely carboxylic acids, alcohols, aldehydes, ketones, esters, and benzenoids. Organic acids and esters are two components produced through the metabolic pathways of bacteria found in SCOBY (Suffys et al., 2023)

The final result of the two-stage kombucha fermentation is significantly influenced by the raw materials, fermentation time, and environmental factors during the fermentation process. Differences in the ratio of tea, sugar, and initial starter concentration can lead to variations in taste, aroma, and chemical composition in kombucha products. In addition to tea, several plant parts have been used in kombucha production, either as the primary substrate or in secondary fermentation, including coffee, soursop, guava, bay leaves, betel leaves, seaweed, sappan wood, rosella, and wedang uwuh (traditional beverage from Yogyakarta, made from a mixture of various dried herbs) mainly to increase the antioxidant content (Afiani et al., 2024).

Rose extract is recognized as a healthy beverage because it contains several active ingredients derived from rose flowers, including tannins, geraniol, nerol, citronellol, flavonoids, eugenol, vitamins C, B, and E, as well as K (Wulandari & Sutardi, 2021). The high content of bioactive compounds in rose flowers makes them a potential raw material for pharmaceuticals and functional foods. The use of rose flowers as a substrate for kombucha in secondary fermentation remains limited and has not been widely studied. The purpose of this study was to produce kombucha through secondary fermentation using rose extract and to characterize the kombucha product in terms of pH, total solids, antioxidants, and sensory characteristics.

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METHODS

Material

The basic ingredients used are: kombucha SCOBY starter obtained through the marketplace, tea leaves (TongTji brand), dried rose flower petal obtained from Sruni Village, Musuk District, Boyolali, and sugar (Gulaku brand). The materials needed for analysis are: 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Merck, Germany), distilled water, Man Rogosa Sharpe Agar (MRSA) medium (Merck, Germany), and methanol solvent (Merck, Germany)

Tool

The equipment used includes a pot for boiling, scales, a sieve, a pH meter, a refractometer, and a spectrophotometer.

Research Design

The research design was a completely randomized design with three replicates. Data were analyzed using Analysis of Variance (Anova) at $\alpha = 0.05$ to determine whether there was an influence on the test parameter. The differences from Anova were further tested by Duncan's Multiple Range Test at $\alpha = 0.05\%$.

The research stages consisted of making the first fermentation kombucha, the subsequent fermentation kombucha with rose extract, and analyzing the chemical and microbiological characteristics.

First fermentation of kombucha is as follows: steep 5 grams of tea leaves in 100 mL of water for 5 minutes, then strain and add 10 grams of sugar (Kitwetcharoen et al., 2023). The tea solution is poured into sterilized jars, and once the solution has reached room temperature, 10% SCOBY is added to it. The jars are covered with cloth and left to ferment aerobically for 8 days. After 8 days, the first fermentation of kombucha is harvested.

The preparation of advanced kombucha fermentation with rose extract is as follows: rose extract was prepared by weighing 5 grams of rose flower petals, adding them to 500 mL of water, boiling, straining, and adding 3 percent sugar. The rose extract solution was allowed to reach room temperature before continuing with fermentation. The first harvest of kombucha solution was mixed with 20%, 30%, and 50% rose extract. Advanced fermentation was carried out in closed glass jars to support anaerobic fermentation. Advanced fermentation lasts for 3 days.

Test Parameters

pH measurement

The pH measurement was performed using a pH meter (Mutlu et al., 2018). Before using the pH meter, the indicator cathode tip was washed with distilled water. The pH meter was calibrated by dipping the cathode tip into pH 4 and 7 buffer solutions. The cathode tip was then dipped into the kombucha solution. The pH meter probe was cleaned with distilled water each time the pH of the following sample was measured. The pH measurements were taken on days 1 through 3 of the secondary fermentation.

Total dissolved solids measurement

Kombucha samples were dripped onto a refractometer prism, then covered, and the value

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of the boundary line on the refractometer eyepiece lens was read. The value obtained was the concentration of dissolved solids expressed in Brix units, as Brix indicates sugar content and is often used as an estimate for Total Dissolved Solids (TDS), since sugar is the primary component of dissolved solids in many agricultural and food products (Elisanti et al., 2023).

Antioxidant activity measurement

Antioxidant activity testing against free radicals was performed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method (Almiranti et al., 2024). The method measures the antioxidant activity based on the number of electrons donated to a free radical (Rosida et al., 2023). One gram of the sample was dissolved in 2 mL of 250 μM DPPH in 100% methanol, then homogenized using a vortex and stored in a dark place at room temperature for 30 minutes. A blank was also prepared as a control. Absorbance measurements were performed using a UV-Vis spectrophotometer at a wavelength of 517 nm. The absorbance measurement results were analyzed using the following equation:

$$Inhibition\ (\%) = \frac{blank\ absorbance - sample\ absorbance}{blank\ absorbance}\ 100\%$$

Sensory evaluation

Organoleptic testing is a testing method that uses human senses to assess the sensory attributes of a product, such as taste, aroma, color, and texture. This test is also known as sensory testing, and is very important for measuring consumer acceptance. In this research, the organoleptic test was conducted using a hedonic test with 20 untrained panelists. During the test, the panelists were asked to taste samples that had been randomly coded. After tasting one sample, the panelists were asked to rinse their mouths with plain water provided as a flavor neutralizer. Then, the panelists were asked to rate the samples based on their preference. The assessment was based on color, taste, and aroma using a 5-point hedonic scale (1: strongly dislike; 2: dislike; 3: neutral; 4: like; 5: strongly like) (Gumansalangi et al., 2019).

Measurement of total lactic acid bacteria (LAB)

Enumeration of lactic acid bacteria (LAB) was performed using the dilution and pour plate methods on special media MRSA (Man Rogosa Sharpe Agar) followed by incubation at a specific temperature and the colonies that grow are counted on plates that have a specific number of colonies. MRSA media was selected because it is designed to optimize LAB growth, does not provide sufficient nutrients for the yeast and acetic acid bacteria (AAB) present in SCOBY. In addition, SCOBY consists of a consortium of microbes, mainly a complex symbiosis of AAB and various types of yeast. A 1 milliliter sample was added to 9 milliliters of sterilized water, and the mixture was mixed using a vortex (dilution of 10^{-1}). Furthermore, serial dilutions were made up to 10^{-8} dilutions. One milliliter of the sample from 10^{-8} was placed in a sterile petri dish (Duplo), and then MRSA (cooled to 45°C) was added. The cultures were stored in an incubator for 48 hours at 37°C (Rahayu & Setiadi, 2023). After the incubation period, the actual number of colonies on plates that contain between 30 and 300 colonies were recorded. The results of this calculation are used to calculate the number of lactic acid bacteria in units of colonies per milliliter (log CFU/mL).

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RESULTS AND DISCUSSIONS

Control Sample Measurement

Table 1 presents the pH, TDS, and antioxidant activity of kombucha tea during primary fermentation and rose extract as the secondary fermentation substrate. Kombucha tea has a low pH due to the fermentation process that has taken place for 8 days. The pH value in this research is lower than that of the pH of kombucha investigated by Nurikasari et al. (2017). Different pH values may be due to variations in the starters, tea leaves, and temperature used in this experiment. According to Amarasinghe et al. (2018), a rapid decrease in pH occurs during the first two weeks of fermentation, followed by a gradual reduction to a specific limit until the 8th week. Rose extract has a neutral pH since there is no fermentation activity.

The TDS value of kombucha tea is higher than that of rose extract, as it contains a 10% sugar addition. During fermentation, some sugars remain unconverted to organic acids and alcohol. On the other hand, adding only 3% sucrose to rose extract resulted in a low TDS value. The difference in sugar content is the cause of this difference in value. Sugar increases the TDS value because sucrose, in its crystal form, is a soluble solid. When sugar dissolves in water, it breaks down into small soluble particles, thereby increasing the total amount of solids.

Table 1 shows that both kombucha and rose extract exhibit antioxidant activity, at levels of 43.10% and 48.18%, respectively. Rose extract has functional properties, especially as an antioxidant. Total phenolic compounds in rose water extract from red rose petals were 16.40-115.04 mg GAE/g, and the vitamin C content reached 1,342.67-2,201 mg/100 g, indicating its possibility as an antioxidant (Wulandari & Sutardi, 2021). Kombucha is known to have antioxidant activity. Some authors have stated that the antioxidant activity is caused by polyphenolic compounds, including catechin-theaflavin, tearubigin, flavonoids, and other compounds present in tea (Jakubczyk et al., 2020).

Table 1. Test parameters for kombucha tea and rose extract as controls

Parameters	kombucha tea (primary fermentation	Rose extract as a secondary fermentation substrate
pH	2.77 $\pm$ 0.02	7.04 $\pm$ 0.07
TDS	6.1 $\pm$ 0.1	3.6 $\pm$ 0.1
Antioxidant activity (%)	43.10 $\pm$ 0.03	48.18 $\pm$ 0.03

pH Measurement

The pH measurement of kombucha fermented with rose extract was observed for three consecutive days. The pH value decreased with increasing time. Reduced pH occurred because sugar, a carbon source for microbial growth, was converted into organic acids in the kombucha product (Bishop et al., 2022). The fermentation process begins with the hydrolysis of sucrose into fructose and glucose by enzymes produced within the yeast cells. The yeast uses glucose and fructose to produce ethanol and carbon dioxide (Kitwetcharoen et al., 2023). The decrease in pH over time indicates that the bacteria and yeast are in an active state. The pH values of the kombucha samples ranged from 2.51 to 2.84. The lowest pH value was found in kombucha that did not contain rose extract. Since kombucha without rose extract (control) did not undergo dilution due to the addition of rose flower, it is understandable

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that it has the lowest pH value. The pH value of the control also indicated an 8-9% decrease in pH during the first fermentation to the secondary fermentation. Table 2 shows that the higher the dilution, the higher the pH value obtained. Statistical analysis revealed significant differences in pH among various concentrations of rose extract. The results show that the addition of rose extract affects the pH value of the product (Table 2). Rose extract, a secondary substrate fermentation (see Table 1), has a neutral pH, and mixing it with kombucha results in a higher pH value. The rate of pH change during fermentation is non-linear. The result aligns with research by Frolova et al. (2023), which states that the decrease in pH does not occur linearly over time.

Table 2. pH values of kombucha with various concentrations of rose extract

Fermentation Duration	rose extract concentration			
	0%	20%	30%	50%
Day 1	2.56 ± 0.01^{d}	2.66 ± 0.01^{c}	2.74 ± 0.01^{b}	2.84 ± 0.01^{a}
Day 2	2.56 ± 0.01^{d}	2.66 ± 0.00^{c}	2.71 ± 0.01^{b}	2.83 ± 0.01^{a}
Day 3	2.57 ± 0.0^{d}	2.67 ± 0.01^{c}	2.74 ± 0.01^{b}	2.84 ± 0.01^{a}

Note: Data within the same column or same row followed by different lettersshow significantly different

Total Solids Measurement

Total dissolved solids indicate the amount of sugar and lactic acid formed during fermentation (Ningsih et al., 2019). The total dissolved solids value was in the range of 7.30-8.07. Compared to the first fermentation, the total solid of kombucha increases due to the addition of rose extract, which contains 3% sugar. However, total dissolved solids decrease as fermentation time increases. These results are consistent with those of other studies (Ningsih et al., 2019) on water kefir products made from watermelon. The decrease in total dissolved solids occurs due to the conversion of sugar into lactic acid and other organic acids. The total dissolved solids result also concides with a reduction in pH value, indicating an increase in the solution's acidity (see Table 3). The total dissolved solids in the control had the highest value compared to the other treatments because the control sample was not diluted. In addition, during kombucha fermentation, a thin biofilm formed, which could also affect the dissolved solids of the sample.

Table 3. Total dissolved solids values (Brix value) of kombucha with various concentrations of rose extract

Fermentation Duration	Rose extract concentration			
	0%	20%	30%	50%
Day 1	8.73 ± 0.06^{a}	8.50 ± 0.00^{b}	7.73 ± 0.06^{c}	7.57 ± 0.06^{d}
Day 2	8.57 ± 0.06^{a}	8.47 ± 0.56^{b}	7.67 ± 0.06^{c}	7.50 ± 0.00^{d}
Day 3	8.07 ± 0.06^{a}	7.53 ± 0.06^{b}	7.33 ± 0.06^{c}	7.30 ± 0.10^{c}

Note: Data within the same column or same row followed by different lettersshow significantly different

Antioxidant Activity

Antioxidants are molecules that fight free radicals (Martono et al., 2016), which can be harmful if their level are too high in the body. The protective action of antioxidants occurs due to their ability to be easily oxidized. The free radicals first oxidize the antioxidant, thereby protecting other molecules in cells from oxidation, which can lead to cell damage. Naturally, the body has its own antioxidant defence system to control free radicals; however, when their

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levels are too high, the body is unable to counteract them (Suarsana et al., 2013). Therefore, antioxidants from external sources, such as those that come from food or beverages, are essential to prevent oxidative damage (Rosida et al., 2023).

Figure 1 shows an increase in antioxidant activity in kombucha with the addition of rose extract. The result is consistent with numerous studies on tea drinks, which show that tea contains polyphenol compounds that act as antioxidants. According to Purwanto et al. (2022), black tea contains theaflavins, thearubigens, and their derivatives, which act as antioxidants. Some authors stated that brewing process help to break those phytochemical compounds and to dissolve in water (Widyawati & Frebriana, 2023). These compounds result from the oxidation of polyphenol enzymes formed during fermentation (Purwanto et al., 2022; Suhardini & Zubaidah, 2016). Fermentation has a beneficial effect on increasing antioxidant activity. Studies by Jacubczyk et al. (2020) indicate that kombucha exhibits higher antioxidant activity compared to unfermented tea. During fermentation, tearubigin in tea is transformed into theaflavin. In this study, the addition of rose extract enhanced the antioxidant activity of kombucha, with the highest value of antioxidant activity found in kombucha containing 50% rose extract.

The increase in antioxidant activity was not linear. Other compounds formed during fermentation may function as antioxidants. According to Khasbullah et al. (2024), lactic acid bacteria multiply during fermentation, producing secondary metabolites that, in addition to having anti-inflammatory health effects, also have antioxidant effects.

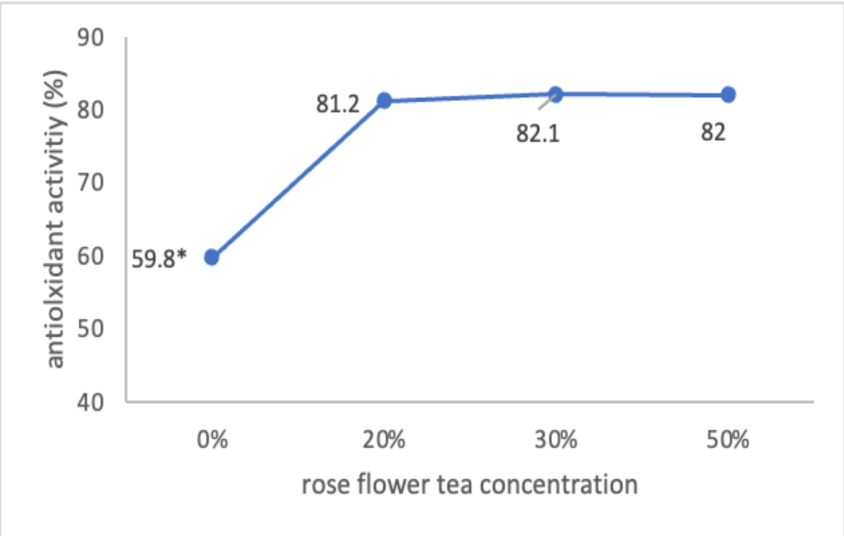

Figure 1. Antioxidant activity of kombucha rose flower on the third day of advanced fermentation (*Chu & Chen, 2006)

Lactic acid bacteria measurement

Microbiological observation revealed that the number of LAB in all samples exceeded 10^8 CFU/mL. The amount of LAB is an indicator of the efficacy of fermentation and its potential as a functional drink (Kusumasari et al., 2024). The result indicated that rose extract is compatible with LAB for growth. A study by Neffe-Skocińska et al. (2017) demonstrated that the development of LAB in kombucha is influenced by temperature. The growth of LAB is in a temperature range of 24-28 °C, which lasts up to 21 days. The temperature condition for making second-fermentation kombucha in this study was conducted at 25 °C.

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Organoleptic Characteristic

Kombucha with various concentrations of rose extract is shown in Figure 1.The results of organoleptic testing of kombucha products with various concentrations of rose extract on 20 untrained panellists are shown in Table 4. All aspects of the assessment parameters were above 3, indicating that the panellists rated the products as acceptable (more than neutral). The results show that the most preferred kombucha product is the product with the highest addition of rose extract, namely 50%. The higher the rose extract added, the better the color rating compared to the control and other treatments. The panellists least preferred the control kombucha (without adding rose extract) for the color parameter. The red colour of rose extract is suspected of influencing the product's colour, with a redder hue being preferred by consumers. The red color of rose extract, caused by its anthocyanin content, influences consumers' perceptions. The varying values in Table 4 for the color parameter are caused by the brain's perception of the actual color of the tea, so that the intensity of the red color in the drink affects the final preference score.

The results show that the most preferred kombucha product has the highest addition of rose extract, namely 50%. The higher the rose extract added, the better the color rating compared to the control and other treatments. The panellists preferred the kombucha with a 50% addition of rose extract for its color.

For taste parameters, there is an irregular pattern, where the highest addition of rose extract (50%) has the most preferred taste, followed by no addition (0%), 30% addition, and the least preferred is 20% addition. This is because some panellists are not yet accustomed to tasting flavours of tea beyond the original tea flavour, including kombucha tea, which is characterized by a combination of sour, slightly sweet, and fizzy flavours. The addition of a slightly sweet floral flavour, and some that taste slightly bitter, may have influenced the panellists' preferences.

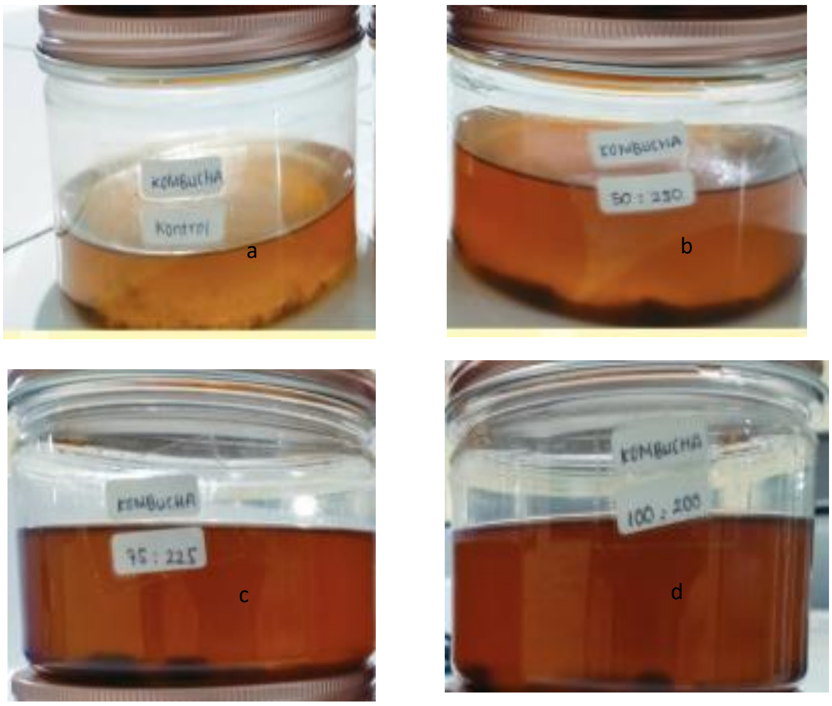

Figure 1. Kombucha product with different concentration of rose extract
(a) 0% (b) 20%) (c) 30% and (d) 50%

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The aroma rating ranged from 3.2 to 3.4 for the treatments. The results show that the products were well-received by panelists. Statistical analysis indicates that there is no significant difference among samples for all parameters tested. However, the formulation with 50% rose extract is the best choice to obtain the most important benefit from the product.

Table 4. Organoleptic test results of rose extract kombucha on the third day of advanced fermentation

Rose extract concentration	Sensory parameter			
	Color	Taste	Aroma	Score
0%	3.4 ± 0.7	3.7 ± 0.7	3.2 ± 0.6	3
20%	3.3 ± 0.2	3.4 ± 0.6	3.4 ± 0.7	4
30%	3.5 ± 0.3	3.6 ± 1.2	3.3 ± 0.5	2
50%	3.7 ± 0.5	4.1 ± 0.3	3.3 ± 0.2	1

CONCLUSION

The advanced fermentation of kombucha with the addition of rose extract resulted in increased antioxidant activity, lower total dissolved solids, and higher pH values compared to the control. The addition of rose extract at the highest concentration resulted in the most consumer-preferred kombucha product, as indicated by the highest score for sensory parameters tested in this study. The addition of 50% rose extract concentration resulted in significantly higher levels of bioactive compounds, which function as antioxidants, compared to other treatments. Further research is needed to determine the optimal concentration of the extract and external factors such as fermentation time in order to produce kombucha with higher acceptance levels and greater functional benefits.

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