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RESEARCH PAPER

Kombucha from ambarella fruit as a natural antibacterial agent against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: Kombucha tea is a traditional fermented beverage known for its probiotic benefits, produced from sweetened tea fermented with a Symbiotic Culture of Bacteria and Yeast (SCOBY). Recent innovations have focused on enhancing its functional properties using local fruit extracts. However, studies on the incorporation of *Spondias dulcis* (kedondong), a native fruit of Riau, remain limited. This study aims to formulate kombucha tea enriched with kedondong juice and evaluate its quality and antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Four formulations were prepared with kedondong juice concentrations of 0% (F1), 10% (F2), 20% (F3), and 30% (F4). Parameters observed included organoleptic properties, pH, alcohol content, microbial growth, and total acidity. Antibacterial activity was tested using the disc diffusion method, and results were statistically analyzed using One-Way ANOVA. F4 on day 7 showed the highest inhibition zones (13.30 mm for *E. coli* and 14.49 mm for *S. aureus*), with significant differences between formulations ($p < 0.05$). This study highlights the potential of kedondong-enriched kombucha tea as a functional probiotic beverage and a natural source of mild antibacterial agents, offering an innovative approach to utilizing local biodiversity in public health.

Keywords: Antibacterial, Kombucha Tea, Kedondong Fruit, *E. coli*, *S. aureus*.

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INTRODUCTION

Infectious diseases remain one of the leading causes of death worldwide, especially in developing countries, including Indonesia. Two bacteria commonly associated with gastrointestinal infections are *Escherichia coli* and *Staphylococcus aureus*. Both are part of the normal flora of the human body, but can become pathogenic under certain conditions, causing various infectious diseases [1]. The treatment of bacterial infections generally involves the use of antibiotics; however, the increasing resistance to antibiotics presents a significant challenge in healthcare. In Indonesia, irrational use of antibiotics has contributed to the acceleration of resistance. The AMRIN (Antimicrobial Resistance in Indonesia) study in 2005 revealed that out of 2,494 samples, 43% of *E. coli* isolates were resistant to antibiotics, including ampicillin (24%), cotrimoxazole (29%), and chloramphenicol (25%) [2].

This situation has encouraged the search for alternative antimicrobial therapies derived from natural sources, including the use of probiotics. One popular probiotic source is kombucha tea, a traditional fermented beverage containing lactic acid bacteria and yeasts, produced through the fermentation of a tea and sugar solution using a microbial starter known as the Symbiotic Culture of Bacteria and Yeast (SCOBY). Kombucha is known to produce bioactive compounds, including organic acids (acetic, gluconic, and lactic acid), vitamins (B complex and C), and polyphenols, which exhibit antimicrobial, antioxidant, and detoxifying activities [3].

Amid the trend of fermented health drinks, kombucha has become increasingly popular not only for its unique taste but also for its potential to improve digestive health through its probiotic content. Kombucha has anticarcinogenic, anti-inflammatory, and immunomodulatory effects, and also improves digestive system function [4]. Kombucha tea exhibits antibacterial activity against *E. coli* and *S. aureus*, with minimum inhibitory concentrations (MIC) of 25% and minimum bactericidal concentrations (MBC) of 40% for *E. coli* and 35% for *S. aureus*, respectively. Kombucha has also been shown to inhibit *Staphylococcus epidermidis* due to its antibacterial compounds [5]. Tests indicated that the MIC was achieved at a 15% concentration of 14-day fermented kombucha, with an inhibition zone of 1.37 mm [6]. Kombucha tea made with torch ginger flower and 40% sugar concentration was the most effective in inhibiting the growth of *S. aureus*, *E. coli*, and *Candida albicans*, with inhibition zones of 14 mm, 10.3 mm, and 12 mm, respectively [7].

Indonesia, being a tropical country, boasts a rich biodiversity, including local plants with potential for developing functional products. One such plant is ambarella (*Spondias dulcis*), which is widely found in Riau Province, including the Indragiri Hulu Regency. Ambarella fruit is known to contain sucrose as an energy source, vitamin C as an antioxidant, and phytochemical compounds such as flavonoids, phenolics, terpenoids, and saponins with pharmacological potential [8],[9]. A study showed that both the flesh and stem bark extracts of ambarella could inhibit the growth of *S. aureus*, with inhibition zone diameters ranging from 10.48 mm to 14.54 mm, depending on the concentration [10].

Although kombucha tea and ambarella fruit have been studied separately for their antibacterial properties, limited research has combined the two in a single fermented product formulation. This combination has the potential to produce a functional probiotic drink that is not only beneficial for health but also serves as a mild antimicrobial agent, utilizing local natural ingredients. The addition of ambarella fruit juice to kombucha tea is expected to enhance its antioxidant and antibacterial contents while supporting the conservation and utilization of local biological resources.

Based on this background, it is necessary to conduct research to design a kombucha tea formulation incorporating ambarella fruit juice and to evaluate its physical, chemical, microbiological properties, and antibacterial activity against *E. coli* and *S. aureus*. This study is expected to open opportunities for developing Indonesian natural-based functional probiotic beverages and provide a natural alternative in controlling antibiotic-resistant bacterial infections.

MATERIALS AND METHODS

Tools used include: analytical balance (Shimadzu® ATX224), UV-Vis spectrophotometer (Spectrum® SP-UV 300SRB), autoclave (Gea® YXQG-01), incubator (Memmert®), colony counter (Suntex Colony Counter 570®), hot plate (WiseStir®), vortex mixer (Asone®), micropipette (Nesco®), pH meter (pHep®), alcoholmeter (n. Tralles TP), blender, and glassware such as test tubes, beakers, measuring cylinders, funnels, stirring rods, Petri dishes, Erlenmeyer flasks, spatulas, volumetric pipettes, ball pipettes, dropper pipettes, microtubes, inoculation loops, calipers, rulers, and other standard microbiological laboratory equipment.

Materials include: ambarella fruit (*S. dulcis*) obtained from Marpoyan Damai Subdistrict, Pekanbaru City; *E. coli* and *S. aureus* bacteria; kombucha starter culture (SCOBY) from Indokombucha®; mineral water; black tea (Goalpara®); granulated sugar; distilled water; 70% alcohol; chloroform; chloroform ammonia; sulfuric acid (H_2SO_4); Dragendorff's reagent; magnesium powder; Liebermann-Burchard reagent; concentrated hydrochloric acid (HCl); 1% ferric chloride solution; Mayer's reagent; sterile 0.9% NaCl solution; 1% phenolphthalein (PP) indicator; buffer solutions of pH 4 and pH 7; 0.1 N NaOH; potassium hydrogen phthalate; spiritus; MRS agar (Merck®); Nutrient Agar (Merck®); Potato Dextrose Agar (Merck®); and ciprofloxacin antibiotic discs (Oxoid®).

Procedures

1. Sampling and Identification

Ambarella fruits (*S. dulcis* Parkinson) were collected from Marpoyan Damai Subdistrict, Pekanbaru City, Indonesia.

2. Sterilization of Equipment

All equipment was thoroughly cleaned and dried. Glassware with open ends was covered with cotton wrapped in gauze, wrapped in parchment paper, and sterilized at 160 °C for 2 hours in a dry heat oven.

3. Identification of Lactic Acid Bacteria (LAB) and Yeast in Kombucha Starter

LAB Identification: 1 mL of kombucha starter was pipetted and inoculated onto MRS agar in a Petri dish, then incubated for 48 hours. Colonies were picked and smeared onto a clean glass slide, fixed over a Bunsen flame until dry, and subjected to Gram staining as follows: crystal violet (1 minute), rinse with distilled water, Lugol's iodine (1 minute), rinse, alcohol decolorization (10–20 seconds), rinse, and safranin counterstain (1 minute), rinse again, and observed under a microscope [11].

Yeast Identification: A yeast colony was taken with a loop and smeared onto a clean glass slide, fixed over a Bunsen flame, then stained with three drops of lactophenol cotton blue reagent. The slide was covered with a cover glass, left for 10 minutes, and observed under a microscope to determine yeast cell morphology [12].

4. Preparation of Ambarella Fruit Juice

The ambarella fruits were peeled, and 1 kg of the flesh was washed thoroughly and chopped into small pieces. The fruit was blended with water at a 1:2 ratio (fruit: water) until it became a pulp. The pulp was filtered, and the resulting filtrate was used as ambarella fruit juice [13].

5. Preparation of Kombucha Tea

A total of 500 mL of kombucha tea was prepared using four different formulations, as shown in the formulation table. Water was boiled in a stainless-steel pan, then black tea, sugar, and ambarella fruit juice were added according to the proportions. The solution was filtered and poured into a glass jar. After cooling to room temperature, a 10% (v/v) kombucha starter was added, stirred to homogenize, and topped up with water to achieve a total volume of 500 mL. The jar was covered with a clean cloth or tissue and secured with a string or rubber band to prevent exposure to the elements.

Table 1. Formulation of Ambarella Kombucha Tea

Component	Formula 1 (%)	Formula 2 (%)	Formula 3 (%)	Formula 4 (%)
Black Tea	1.6 (w/v)	1.6	1.6	1.6
Sugar	10 (w/v)	10	10	10
Kombucha Starter	10 (v/v)	10	10	10
Ambarella Juice	0 (v/v)	10	20	30
Water	up to 100%	up to 100%	up to 100%	up to 100%

6. Formula Evaluation [14]

Organoleptic Test:

Visual observation of color, aroma, taste, and texture of SCOBY in each formula.

pH Measurement: pH was measured at room temperature using a calibrated pH meter with buffer solutions of pH 4 and pH 7. The electrode was immersed in the sample (~25 mL) until a stable reading was obtained. Measurements were taken three times, and the average was recorded.

Alcohol Content Test: Alcohol content was measured using an alcoholmeter. Each formula sample was placed in a 100 mL measuring cylinder, allowed to reach room temperature, and the alcoholmeter was immersed and read. Testing was conducted on fermentation day 0, 7, and 14.

(LAB): Samples were diluted with sterile distilled water at a 1:9 ratio (dilutions 10^{-1} to 10^{-7}). MRS agar was prepared by dissolving 34.1 g in 500 mL distilled water, heated, and sterilized using an autoclave (121 °C, 15 minutes). The diluted sample was added to Petri dishes containing media, homogenized using figure-eight motion, and incubated inverted at 37 °C for 48 hours. Colonies were counted using a colony counter. Determination of Total Yeast: Samples were diluted with sterile physiological saline (1 mL sample into 9 mL solution, dilutions 10^{-1} to 10^{-5}). From the last two dilutions, 1 mL each was poured into sterile Petri dishes, followed by 12 mL of sterile PDA media. Plates were incubated at 37 °C for 48 hours, and colonies were counted using a colony counter.

Determination of Total Titratable Acidity (TTA): 10 mL of kedondong kombucha was diluted to 250 mL in a volumetric flask. From this, 50 mL was transferred into a 250 mL Erlenmeyer flask and 2 drops of 1% phenolphthalein indicator were added. It was titrated with 0.1 N NaOH until a pink color persisted for 15 seconds. The volume of titrant used was recorded.

Antibacterial Activity Test: Bacterial suspensions of *E. coli* and *S. aureus* (0.3 mL) were mixed into 12 mL of Nutrient Agar (NA) in Petri dishes, homogenized, and allowed to solidify. Paper discs were dropped with 10 µL of negative control (tea without kombucha

starter), samples F1, F2, F3, F4, and positive control (ciprofloxacin 5 µg/disc). After the media solidified, the discs were placed and incubated for 24 hours at 37 °C. Microbial growth was observed, and the inhibition zones were measured using a calliper. The test was conducted in triplicate [16].

RESULTS AND DISCUSSION

Ambarella (*S. dulcis*), also known as kedondong, is a tropical fruit native to Southeast Asia, Africa, and the Caribbean. It is known for its distinctive flavor profile, which combines sweet and tangy-spicy notes depending on its ripeness. When unripe, the fruit is green, firm, and sharply sour; as it ripens, it turns yellow, with soft, juicy flesh that tastes similar to a blend of pineapple and mango.

Morphologically, the fruit is oval-shaped with smooth skin that changes color from green to yellow as it matures. Ambarella is widely consumed in various forms such as fruit salad, pickles, preserves, or juice, both in its unripe and ripe stages. Beyond its culinary uses, ambarella also plays a role in traditional medicine, particularly for treating digestive issues and haemorrhoids. Nutritionally, it is rich in vitamins A and C, which serve as important antioxidants.

This plant is extensively cultivated in tropical regions, including Indonesia, India, Sri Lanka, Malaysia, Thailand, and several Caribbean countries. In Indonesia, ambarella is commonly eaten fresh in fruit salads or processed into sweetened preserved fruits.

Figure 1. Kedondong Plant and Fruit (*S. dulcis* Parkinson)

This study formulated kombucha tea by adding varying concentrations of kedondong (*S. dulcis* Park) juice and evaluated its antibacterial activity. The formula evaluation included organoleptic test, pH, alcohol content, total LAB, total yeast, and total titratable acidity (TTA). Kombucha tea is known as a probiotic drink beneficial for health, particularly in improving digestive disorders. Kedondong fruits were used at 70–80% ripeness because, at this level, the fruit is not overly sour and contains adequate sugar and fibre.

Microscopic identification of LAB showed rod-shaped (bacilli) and round cells with purplish-blue color, arranged in chains or clusters. Meanwhile, yeast cells exhibited oval or elliptical shapes. Common yeast species found in SCOBY cultures include *Saccharomyces*, *Zygosaccharomyces*, and *Brettanomyces*. *Saccharomyces* appears round, oval, or elongated, while *Brettanomyces* is ogival-shaped and can produce a high amount of acid.

Fermentation is influenced by factors such as microbial load, pH, substrate, temperature, oxygen, and fermentation duration. The kombucha fermentation process accelerates with increasing temperature, ideally between 23–37 °C. Fermentation should be carried out in a dark, non-humid room, avoiding direct sunlight exposure as it may damage the culture [17].

Organoleptic testing showed that on day 0, before fermentation, there were no significant differences in taste, color, or aroma among all formulas. However, by days 7 and 14, the taste became more acidic due to increased kedondong juice concentration and longer fermentation. Yeast and LAB in the kombucha culture convert sugars into CO_2 gas and various organic acids, leading to increased acidity [18].

pH measurements showed that on days 0 and 7, pH values were within a safe consumption range (2.5–4.5). On day 14, formulas 3 and 4 recorded pH <2.5, requiring dilution before consumption. This pH drop is related to the production of acetic acid by kombucha during fermentation.

Alcohol in kombucha can be produced when yeast ferments glucose into pyruvate through glycolysis. Pyruvate is then decarboxylated by pyruvate decarboxylase into acetaldehyde and CO_2, which is subsequently converted into ethanol by dehydrogenase [19]. However, no alcohol content was detected in this study, likely due to the limited sensitivity of the manual alcoholmeter used. Gas Chromatography (GC) is the most recommended method for accurate and reliable alcohol detection in kombucha.

According to Fatwa of MUI (Indonesian Ulema Council) No. 10 of 2018, fermented foods and beverages are considered halal if the alcohol (ethanol) content is below 0.5% and do not contain haram or harmful substances.

A study showed that 12-day kombucha fermentation resulted in an average alcohol content below 0.5%, meeting LPPOM MUI halal standards [20]. However, halal certification of kombucha depends not only on alcohol content. The fermentation process involving yeasts like *Saccharomyces cerevisiae* may produce alcohol, and raw materials such as sugar or flavourings must also be halal. Hence, to ensure a halal status, producers must maintain an alcohol content below 0.5%, use halal-certified ingredients, and comply with the LPPOM MUI guidelines.

Total LAB was counted at 10^{-6} and 10^{-7} dilutions to obtain colony numbers within the standard count range (30–300 colonies). Results showed that LAB counts met the requirement for probiotic drinks (>10^7 CFU/mL). LAB counts increased on day 7, followed by a decrease on day 14, possibly due to reduced nutrient availability and pH decline affecting LAB viability [9].

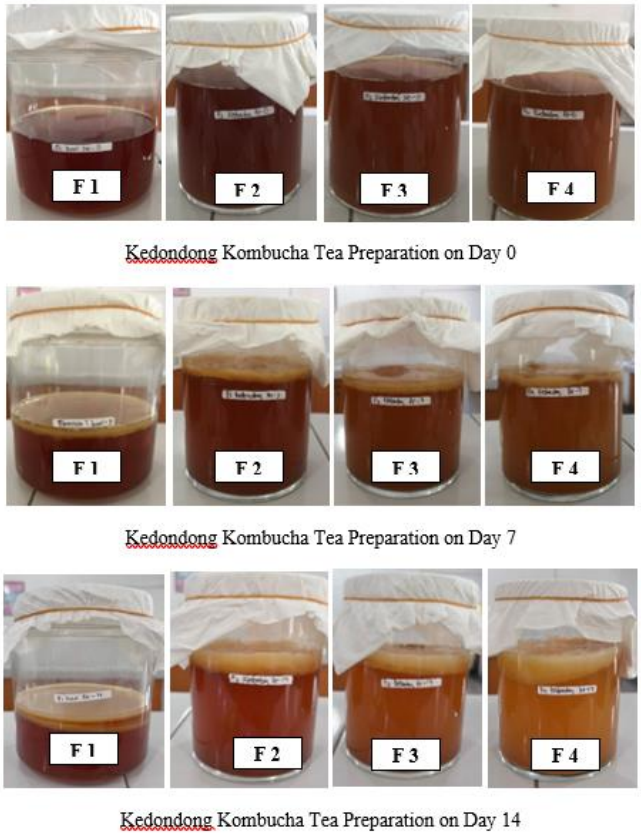


Figure 2. Kedondong Kombucha Tea Preparation on Day 0, Day 7 and Day 14 of Fermentation

Yeast population in kedondong kombucha tea preparations. The results showed an increase in the number of yeast colonies as fermentation time progressed. This phenomenon aligns with the Codex [21] standard, which stipulates that fermented products such as kombucha must contain a minimum yeast count of 10^4 CFU/mL to be considered microbiologically acceptable for consumption. The increase in yeast count followed the typical microbial growth curve, consisting of a lag phase (adaptation) during the first week of fermentation, followed by an exponential or logarithmic phase in the subsequent week, during which microorganisms rapidly multiply due to the availability of nutrients and a favourable environment [3].

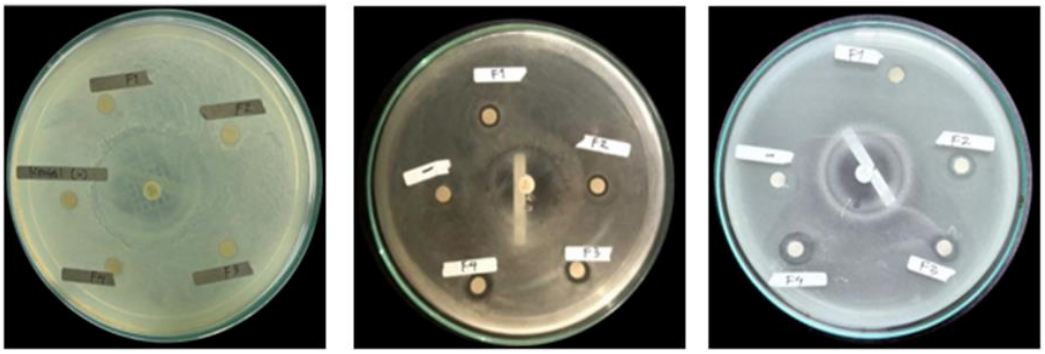
In addition, another observed parameter was titratable acidity (TTA), which reflects the total organic acid content produced during fermentation. Based on the test results, TTA levels increased progressively throughout the fermentation process. On day 0, TTA values for formulations F1, F2, F3, and F4 were 0.08%, 0.18%, 0.16%, and 0.16%, respectively. After seven days of fermentation, the TTA increased to 0.33% for F1, 0.38% for F2, 0.54% for F3, and 0.63% for F4. On day

14, TTA levels continued to rise significantly, reaching 0.50%, 0.60%, 0.68%, and 0.93% for F1, F2, F3, and F4, respectively. All these values remained within the safe consumption range according to the Indonesian National Standard (SNI, 2009), which specifies an acceptable TTA range of 0.5%–2.0%. The increase in TTA correlated directly with the decrease in pH during fermentation, which was caused by the accumulation of organic acids, particularly acetic acid, as the primary product of microbial activity in the SCOBY culture [14].

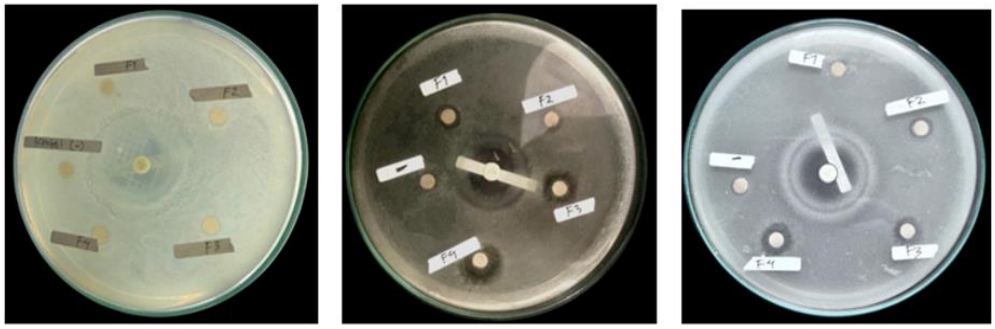
Antibacterial activity testing was also performed to evaluate the effectiveness of kedondong kombucha tea in inhibiting the growth of pathogenic bacteria, namely *E. coli* and *S. aureus*.

Table 2. Antibacterial Activity Test Results of Kedondong Kombucha Tea against *E. coli* and *S. aureus*

Parameter	Day	F1	F2	F3	F4
Taste	0	Sweet	Sweet	Sweet-sour	Sweet-sour
	7	Sweet	Sweet	Sweet-sour	Sour
	14	Sweet-sour	Sweet-sour	Sour	Sour
Aroma	0	Tea aroma	Tea aroma	Kedondong tea	Kedondong tea
	7	Kombucha tea	Kombucha tea	Kedondong kombucha	Kedondong kombucha
	14	Kombucha tea	Kombucha tea	Kedondong kombucha	Kedondong kombucha
Color	0–7	Dark brown	Dark brown	Dark brown	Dark brown
	14	Dark brown	Dark brown	Light brown	Light brown
pH	0	3.37	3.18	3.08	3.02
	7	2.92	2.78	2.63	2.60
	14	2.94	2.66	2.46	2.42
Alcohol (%)	All	0	0	0	0
Titratable Acidity (%TTA)	0	0.08	0.19	0.16	0.16
	7	0.34	0.39	0.56	0.65
	14	0.50	0.60	0.68	0.93
Total LAB (CFU/mL)	0	9.1×10^{8}	2.28×10^{9}	2.14×10^{9}	1.42×10^{9}
	7	9.1×10^{8}	1.22×10^{9}	1.05×10^{9}	8.1×10^{8}
	14	5.2×10^{8}	5.2×10^{8}	3.9×10^{8}	4.1×10^{8}
Total Yeast (CFU/mL)	0	1.37×10^{6}	1.65×10^{6}	1.39×10^{6}	1.51×10^{6}
	7	8.2×10^{5}	1.08×10^{6}	9.9×10^{5}	1.21×10^{6}
	14	1.20×10^{6}	1.39×10^{6}	9.5×10^{5}	1.56×10^{6}
Antibacterial Activity (mm)					
E. coli	0	7.38±0.33	7.67±0.19	7.77±0.49	7.95±0.36
	7	9.00±0.48	9.60±0.27	11.18±0.40	13.30±0.46
	14	9.54±0.08	9.54±0.08	11.00±0.20	11.24±0.38
S. aureus	0	7.94±0.29	7.61±0.19	7.97±0.28	8.17±0.32
	7	10.06±0.75	9.40±0.24	12.19±0.44	14.18±0.40
	14	9.17±0.33	9.18±0.31	11.21±0.44	12.92±0.22



Inhibition Zones on Days 0, 7, and 14 Against *E. coli*



Inhibition Zones on Days 0, 7, and 14 Against *S. aureus*

Figure 3. Results of the Antibacterial Activity Test of Ambarella Kombucha Tea Formulas Against *E. coli* and *S. aureus*

According to the Clinical and Laboratory Standards Institute [15], antibacterial activity is classified as weak if the inhibition zone diameter is ≤14 mm. In this study, the highest antibacterial inhibition was observed on day 7 of fermentation against *S. aureus*, with a zone diameter of 14.18 mm. The relatively high antibacterial activity on day 7 is likely associated with the rapid growth phase of lactic acid bacteria (LAB), during which sufficient nutrients were still available to support the production of secondary metabolites with antibacterial properties. However, by day 14, LAB counts decreased significantly as they entered the stationary or death phase, caused by substrate depletion and increasing acidity, leading to reduced antibacterial activity [3]. Furthermore, inhibition against *S. aureus*, a Gram-positive bacterium, was higher than against *E. coli*, a Gram-negative bacterium. It is due to the more complex and thicker cell wall structure of Gram-negative bacteria, which impedes the penetration of antimicrobial compounds into the bacterial cell.

CONCLUSION

Based on the research, kombucha tea with kedondong (*S. dulcis* Park) extract shows potential as a probiotic beverage with antibacterial activity. Formula 4 on day 7 of fermentation produced the largest inhibition zone against *S. aureus* (14.18 ± 0.40 mm), although still classified as weak inhibition. Throughout 14 days of fermentation, pH decreased, titratable acidity increased, and LAB and yeast counts met Codex standards for probiotics. No alcohol was detected in any formula. Phytochemical screening revealed the presence of phenolics, flavonoids, and saponins, which may contribute to the antibacterial effects. ANOVA showed significant differences ($p < 0.05$) among formulas.

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