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Research article

Characteristics and Antioxidant Activities of Kombucha from Black Tea and Roselle by a Mixed Starter Culture

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kombucha;
roselle

Abstract

Kombucha is a functional beverage fermented by the symbiosis of bacteria and yeasts and is comprised mainly of bioactive compounds and acids that have beneficial health effects. Our research aimed to determine the optimal ratio of co-culture of the bacterium *Acetobacter pasteurianus* AJ605 and the yeast *Zygosaccharomyces bailii* YN403 for kombucha fermentation in order to improve the consistency of kombucha quality so that it can be mass produced. Microbial growth, chemical characteristics, and antioxidant activities of kombucha during fermentation were analyzed. The results showed that a co-culture ratio of 8:2 (v/v) of *A. pasteurianus* AJ605 and *Z. bailii* YN403 gave the highest antioxidant activity after 10 days of fermentation, with a DPPH IC_{50} value of 25.76 µl/mL and an ABTS IC_{50} value of 8.84 µl/mL. The co-culture ratio of 8:2 showed antioxidant activity that was not significantly different ($p>0.05$) from that of SCOBY (symbiotic culture of bacteria and yeasts) alone. Additionally, during fermentation, the pH of kombucha decreased to 3.16, with increases in titratable acidity and alcohol content of 7.00 g/L and 7.96 g/L, respectively. To enhance the antioxidant activity and taste quality of kombucha, black tea and roselle were mixed at various ratios and inoculated with 10% (v/v) optimal co-culture. The results showed that a high antioxidant activity was obtained using an 8:2 (w/w) ratio of black tea and roselle, at day 10 of fermentation, with a DPPH IC_{50} value of 23.88 µl/mL and ABTS IC_{50} value of 6.11 µl/mL. Moreover, the results indicate that kombucha from black tea and roselle can be a functional beverage that has high antioxidant activity and sensory acceptability.

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1. Introduction

Kombucha has a sweet-sour flavor and is produced mainly from tea, herbs, or fruits that are infused and fermented by a symbiotic culture of bacteria and yeasts (SCOBY) [1]. Kombucha is composed of a floating cellulosic pellicle and sour liquid broth [2]. Various organic acids and bioactive compounds are produced from the microbial interactions and tea. The bioactivities of kombucha including antioxidant activities, oxidative stress reducing, and antimicrobial activities against pathogens were reported. Several types of bacteria and yeasts were found to be involved in kombucha fermentation, which depends on different sources of the kombucha starter [2, 3], resulting in inconsistencies in the production of organic compounds and unstable kombucha products. Acetic acid bacteria (AAB) (e.g., *Acetobacter*, *Gluconacetobacter*, and *Komagataibacter*) and some lactic acid bacteria are frequently found in kombucha production. In addition, various genera of yeasts including *Saccharomyces*, *Zygosaccharomyces*, *Dekkera*, and *Pichia* are commonly found in kombucha [4]. The inconsistency in the microbial starters used in kombucha production is problematic because it results in unstable flavors and undesirable properties [5]. Therefore, the main objective of this study were to determine the optimal co-culture ratio of *Acetobacter pasteurianus* AJ605 and *Zygosaccharomyces bailii* YN403, and the optimal co-culturing time for kombucha fermentation. Both microbes were isolated from kombucha fermentation and demonstrated high acid production and tolerance, respectively [6]. In addition, *A. pasteurianus* and *Z. bailii* are predominant and mostly found in tea fungi [2, 4, 7]. In previous studies, a combination of bacteria and yeast for kombucha fermentation was reported. Wang *et al*. [8] used mixed cultures of *Acetobacter pasteurianus*, *Gluconacetobacter xylinus*, and *Zygosaccharomyces bailii*, which showed high antioxidant activity and sensory properties similar to those of kombucha from SCOBY. Nguyen *et al*. [9] reported that the best combination ratio of *Dekkera bruxellensis* KN89 and *Gluconacetobacter intermedius* KN89 was 6:4; this ratio produced the highest amount of glucuronic acid in kombucha. The beneficial effects of kombucha, in particular those effectss due to its bioactive compounds and antioxidants, are enhanced by supplementing kombucha with other substrates such as fruits or herbs.

Roselle *(Hibiscus sabdariffa* L.) belongs to the Malvaceae family, and is found in tropical and subtropical countries such as India, Malaysia, Saudi Arabia, and Thailand [10]. Roselle calyxes are dried and used in food and beverages [11]. The calyx of roselle is a major source of bioactive compounds, including organic acids, anthocyanins, flavonoids, and polysaccharides [12]. The bioactive compounds in roselle have been widely examined for their antioxidant capabilities in many previous studies [11-13].

Tea (*Camellia sinensis* L.) is a native East Asian plant found in Thailand. Various types of tea depend on the production processes. Black tea is fully fermented before the dehydration process. Several bioactive compounds (e.g., phenolics and flavonoids) are transformed during the tea fermentation process, which increases thier antioxidant properties [14]. Black tea is traditionally used as a recipe for sweet black tea prepared in kombucha [2, 14].

In this study, we investigated the optimal ratio of co-culture. In addition, kombucha from optimal co-culture with mixture of black tea and roselle at various ratios was evaluated for its antioxidant activity and sensory properties.

2. Materials and Methods

2.1 Sources of microbes and commercial starter

The bacterium *Acetobacter pasteurianus* AJ605 and the yeast *Zygosaccharomyces bailii* YN403 were isolated from commercial kombucha starters obtained from Thailand [6]. These strains were stored in the collection of the Laboratory of Biology Department, King Mongkut's Institute of Technology Ladkrabang, Thailand. The commercial starter of kombucha was purchased from the Varunee Store (Bangkok, Thailand).

2.2 Plant materials

Dried black tea (*Camellia sinensis* L.) was purchased from a local tea plantation in Chiang Rai Province, Thailand in September 2019. Fresh calyxes of roselle (*Hibiscus sabdariffa* L.) were harvested from a 120-days-old plant in Nakhon-Prathom province, Thailand. They were rinsed with tap water and dried in a hot air oven at 45°C for 3 days. The dried roselle and black tea were kept in zip lock bags at 4°C.

2.3 Preparation of commercial kombucha

The commercial starter (SCOBY) prepared with 3% (w/v) pellicle and 10% (v/v) of its liquid from a previous starter was inoculated into tea that had been prepared as 0.4% (w/v) black tea infused in boiled water for 15 min with 10% (w/v) sucrose. The fermentation was carried out for 10 days at 30±2°C.

Commercial kombucha was prepared in sweet black tea broth as previously described [9]. Briefly, 1% (w/v) dried black tea was infused in boiling water for 15 min, mixed with 10% (w/v) sucrose, and immediately dispensed into several 500 mL glass jars (each containing 300 mL). Then, the 3% (w/v) pellicle and 10% (v/v) liquid from SCOBY were added as inoculum [2], and it was further incubated for 10 days at 30±2°C. The microbiological and chemical properties of the samples were analyzed after 0, 3, 7, and 10 days of fermentation.

2.4 An optimal ratio of a mixed inoculum

Two starter cultures of *A. pasteurianus* AJ605 and *Z. bailii* YN403 were prepared separately in sweet black tea broth as previously described [9]. A loopfull of bacterium and yeast was separately cultured in the broth and incubated at 30°C at a speed of 150 rpm in an incubator shaker (HASUC, HSYC-2112B, China) for 24 h. The inoculum density was measured to an optical density (OD) of 0.5 at 600 nm using a UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) before mixing the two cultures. The total plate count was 6.4 log CFU/mL for bacterium and 5.9 log CFU/mL for yeast using spread plate techniques.

Cultures of *A. pasteurianus* AJ605 and *Z. bailii* YN403 were added at different ratios, and the experimental center point was set at a 5:5 ratio for 10% (v/v) into the new sweet black tea (Table 1). The total volume of the sample for each treatment contained 300 mL in a 500 mL glass jar. All samples were fermented at 30±2°C for 10 days. Samples were collected on days 0, 3, 7, and 10 to assess kombucha characterization and enumerate *A. pasteurianus* AJ605 and *Z. bailii* YN403.

Table 1. The ratios of combination starter culture (%,v/v) of *A. pasteurianus* AJ605 (bacterium; B) and *Z. bailii* YN403 (yeast; Y) for co-culture in kombucha consortium

Yeast (Y)	Bacterium (B)				
	10	**8**	**5**	**2**	**0**
0	B10Y0				
2		B8Y2			
5			B5Y5		
8				B2Y8	
10					B0Y10

2.5 Improvement of black tea kombucha by blending with roselle

Black tea and roselle were blended in different ratios (Table 2). All samples were prepared as sweet tea; 1% (w/v) of black tea, roselle, and black tea mixed with roselle at various ratios were steeped in 1 L of boiled water for 15 min and then dissolved in 10% (w/v) sucrose. The muslin cloths and jars used were sterilized in autoclave at 121°C for 15 min. The sweet teas were filtered through sterilized muslin cloth and then 300 mL of each were filled into sterilized jars of capacity 500 mL. Next, each infusion was inoculated with 10% (v/v) of optimal starter culture ratio, closed with sterilized muslin cloth, and incubated at ambient temperature (30±2°C) for 10 days. Samples were collected on days 0, 3, 7 and 10 of the fermentation period.

Table 2. The ratios of black tea (C) and roselle (R) (%w/w) for kombucha fermentation

Roselle (R)	Black tea (C)					
	1	**0.8**	**0.7**	**0.6**	**0.5**	**0**
0	C10R0					
0.2		C8R2				
0.3			C7R3			
0.4				C6R4		
0.5					C5R5	
1						C0R10

2.6 Microbiology determination

The total bacterial count was determined using the spread plate technique on glucose yeast calcium carbonate agar (GYC). Enumeration of the total yeast plate was performed on dichloran Rose-Bengal chloramphenicol agar (DRBC) (Sigma-Aldrich, USA). Serial dilutions of the kombucha samples were prepared using 0.1% (w/v) peptone. The plates were incubated at 30°C for five days [3, 15].

2.7 Kombucha characterization

2.7.1 pH and acidity determination

The kombucha samples were centrifuged at 5,000 rpm for 15 min and the supernatants were collected. The pH was determined using pH meter (SevenCompact pH Meter S220, Mettler, USA.). The total acidity was measured using the titration method described by Kaewkod *et al*. [16] and expressed as grams of acetic acid per liter of fermented sample (g/L).

2.7.2 Total sugar and alcohol contents

The total sugar content was measured using the total phenol-sulfuric method [17]. Glucose was used as a standard. The alcohol content was determined after mixing 500 µL of supernatant with 500 µL of 10% (v/v) n-propanol. Alcohol content was measured by gas-chromatography (GC-2014, Shimadzu, Japan) with FID detector, DB-1 capillary column (Agilent J&W): 30 m×0.32 mm I.D., 5.00 µm of film thickness, and column temperature at 60°C. The temperature program started at 60°C holding time for 6 min, and then increased to 130°C at rate of 20°C/min, with holding for 1.50 min. The detector temperature at 180°C, helium was used as the carrier gas and sample injection volume was 0.5 µL.

2.7.3 Bioactive compound analysis

The total phenolic content (TPC) was measured using the Folin-Ciocalteu assay modified from Singh *et al*. [18]. Kombucha samples (20 µL) were mixed with 10% (v/v) Folin-Ciocalteu reagent (100 µL), added to 80 µL of 7.5% (w/v) sodium carbonate, and further incubated for 30 min. The absorbance was measured at 765 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Germany). The TPC was calculated from the calibration curve of gallic acid using the following equation (y = 0.0044x). The TPC result was expressed as µg gallic acid equivalent per mL of kombucha sample (µg GAE/mL).

The total flavonoid content (TFC) assay was modified from that described by Hu *et al*. [19]. Briefly, 30 µL of 5% (w/v) sodium nitrite was added to 100 µL of each sample and incubated for 5 min. Then, 30 µL of 10% (w/v) aluminium chloride was mixed and incubated for 6 min followed by reaction with 200 µL of 1M sodium hydroxide. The absorbance of samples was measured at 510 nm. The TFC was calculated from the calibration curve of quercetin using the following equation (y = 0.0003x). The results were expressed as µg quercetin equivalent per milliliter of kombucha sample (µg QE/mL).

A pH differential method was used to determine the quantity of anthocyanins in the samples according to Amaya-Cruz *et al*. [20]. Samples (50 µL) were mixed with 175 µL of 0.4M sodium acetate buffer (pH 4.5) and 175 µL of 0.025M potassium chloride buffer (pH 1.0) in a 96-well plate and incubated for 30 min. The absorbance of the samples was measured at 510 nm and 700 nm. The total anthocyanin content was reported as cyanidin-3-glucoside equivalent per mL of kombucha sample (mg CGE/mL) and calculated using the following equation:

$$\text{Total anthocyanin content (mg CGE/mL)} = [((Abs_{510nm}-Abs_{700nm})pH\ 1 - (Abs_{510nm}-Abs_{700nm})pH\ 4.5) \times MW \times DF]/ (\varepsilon \times D)$$

MW: the molecular weight of cyanidine-3-glucoside (484.8 g/mol), DF: the dilution factor; ε: the molar absorptivity coefficient calculates as cyanidine-3-glucoside (26,900 $M^{-1} \bullet cm^{-1}$) and D: the height of the sample in the 96 well plate (mm).

2.7.4 Antioxidant activity determination by DPPH and ABTS assays

The DPPH and ABTS methods were modified from Miliauskas *et al*. [21]. Kombucha samples (250 µL) were mixed with 750 µL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution and incubated for 30 min in the dark. Then 150 µL of each reaction mixture was pipetted into a 96-well plate. The absorbance of the sample was measured at 517 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Germany).

The ABTS assay was performed by preparing a ABTS radical ($ABTS^{\bullet+}$) solution by mixing 7 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with 2.45 mM potassium

persulfate at a ratio of 1:1. Samples were mixed with $ABTS^{\bullet+}$ at the same ratio as in the DPPH assay and absorbance was detected at 734 nm. The absorbance of DPPH or ABTS solution (A1) and the absorbance of the sample mixed with free radical solution (A2) were measured. The percentage of scavenging activity was calculated as follows:

$$\text{Radical scavenging activity (\%)} = [(A1-A2)/ A1] \times 100$$

Antioxidant activity was reported as the concentration of kombucha that inhibited DPPH radical and $ABTS^{\bullet+}$ at 50% scavenging (IC_{50}: inhibitory concentration at 50%) [22].

2.8 Sensory evaluation

Kombucha fermented for ten days with the optimized starter culture ratios was disinfected by adding 200 ppm of potassium metabisulfite (food grade) and left for 24 h before evaluation. To determine the impact of the kind of herb plants (black tea and roselle) on consumer acceptance, the sensory evaluation was performed by 30 trained panel students (n=30) from King Mongkut's Institute of Technology Ladkrabang, who had an average age of 25±5 years. For testing, the samples were randomized. Sensory evaluation parameters including appearance (clearness, color), odor, flavor and overall acceptability were based on a 9-point hedonic scale ranging from 1 (extremely dislike) to 9 (extremely like) [23].

2.9 Statistical analysis

All experiment were performed in three independent replications (n=3), and the results were shown as mean±standard deviation (SD). Statistical analyses were performed by ANOVA and one way analysis of variance using Duncan's multiple range test in the case of significant differences ($p < 0.05$).

3. Results and Discussion

3.1 Growth characteristics of co-culture

The growth characteristics of *A. pasteurianus* AJ605 (B) and *Z. bailii* YN403 (Y) during kombucha fermentation are shown in Figure 1. The number of *A. pasteurianus* AJ605 in co-cultures of B8Y2, B5Y5, and B2Y8 ratios increased rapidly on day 3, with 6.73±0.03, 6.89±0.03, and 6.62±0.01 Log CFU/mL, respectively. The number of bacteria increased throughout the fermentation process (Figure 1). The amount of *Z. bailii* YN403 in the co-culture increased rapidly on day 3, and then gradually decreased during the fermentation period. Similar results were obtained for kombucha from SCOBY. The total bacteria cell count increased with the same number off yeast cells.

The utilization of sucrose that had been initially added to tea caused an increase in the number of both microbes [24]. A previous study indicates that during the fermentation process, yeast hydrolyzes sucrose into monosaccharides and uses it as a substrate for organic acid production by bacteria [2].

In our present study, during the last period of fermentation, yeast cell counts on co-culture and kombucha from SCOBY were found to be declining in the range between 5.43-6.42 log CFU/mL, which was lower than that of yeast monoculture (6.98 log CFU/mL). Due to the acidic conditions in kombucha caused by AAB metabolism, the increase in acetic acid production was responsible for the reduction in yeast cell counts. Another possible explanation is nutrient competition [24]. Thus, the presence of AAB can reduce the yeast population, as demonstrated in many studies [15, 24, 25].

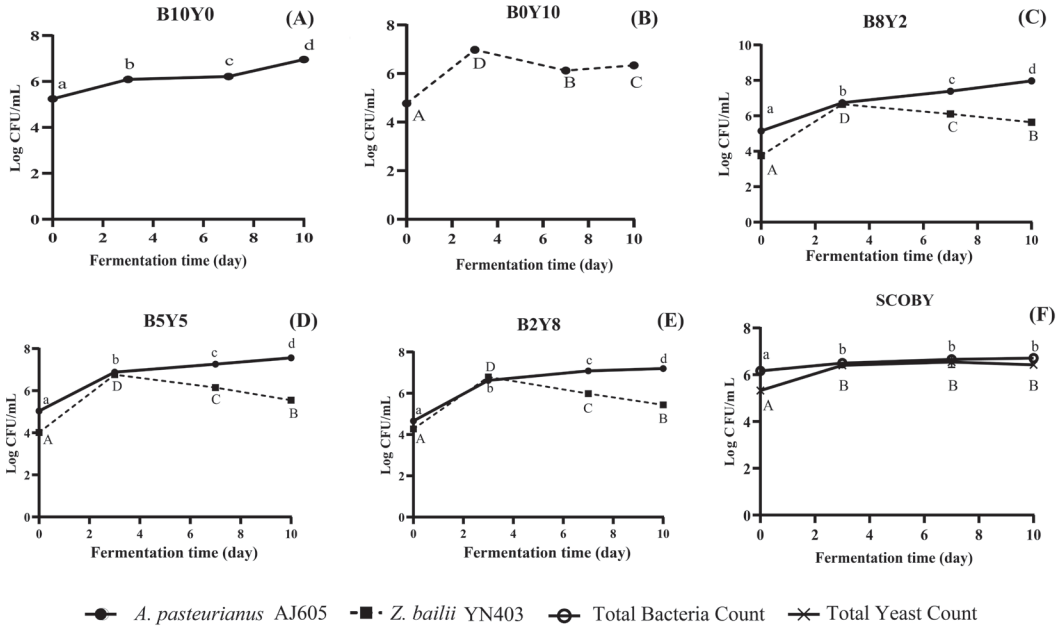

Figure 1. Microbial populations of *A. pasteurianus* AJ605 and *Z. bailii* YN403 in sweet black tea during the fermentation. B10Y0: *A. pasteurianus* AJ605 monoculture (A); B0Y10: *Z. bailii* YN403 monoculture (B); B8Y2, B5Y5, B2Y8: co-culture of *A. pasteurianus* AJ605 and *Z. bailii* YN403 (C-E); SCOBY: total bacteria count and total yeast on kombucha from commercial starter (F); Means with the various letter are significantly different ($p<0.05$) (n=3).

Previous studies have shown that microbial changes occur during fermentation. For instance, the total count of two species of AAB and *Z. bailii* CICC3267 decreased after day 2 of black tea kombucha fermentation [8]. According to Jayabalan *et al*. [25], bacteria and yeast increased until day 9 of fermentation, and then decreased. The total number of bacteria and yeast was highest on days 5 and 7 in the Pu-erh kombucha [26].

3.2 Chemical properties of black tea kombucha using co-culture

A comparison between the pH of kombucha fermented with monocultures, co-cultures and SCOBY is shown in Figure 2A. The pH of kombucha samples decreased during fermentation. On day 10, the pH of all co-culture ratios ranged from 3.02 to 3.16, which was higher than the kombucha from SCOBY. In addition, the pH value of SCOBY decreased to 2.62±0.10. These pH values are related to the amount of titratable acid found in the samples.

With increasing fermentation time, the titratable acidity of the kombucha from the co-culture increased. On day 10, the total acid contents of the samples ranged from 7.00 to 7.6 g/L. These were less than the ones in which SCOBY was used (8.69 g/L) (Figure 2B). SCOBY contains various bacterial species. Tran *et al*. [24] reported that bacterial species *Acetobacter indonesiensis, Acetobacter papaya and Komagataeibacter saccharivorans* were found in the cellulose and broth of kombucha. In addition, Gaggia *et al*. [27] identified *Komagataibacter* spp. and *Gluconobacter* spp. in kombucha.

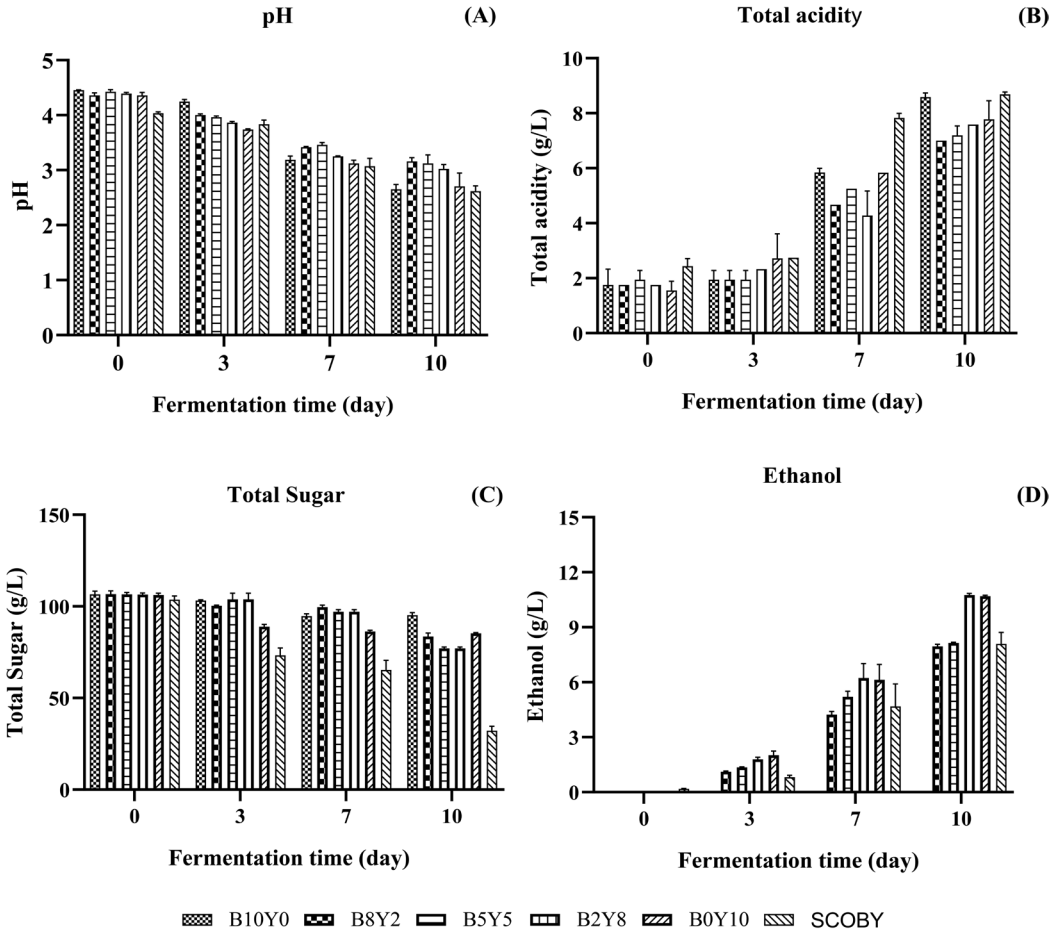

Figure 2. The changes in chemical properties during fermentation in black tea kombucha fermented with various ratios of *A. pasteurianus* AJ605 and *Z. bailii* YN403, and commercial starter (SCOBY). pH (A), Total acidity (B), Total sugar content (C) and Alcohol content (D). All results are shown as mean ± S.D. of three replicates.

In addition, numerous strains of AAB, such as *A. xylinum*, *A. pasteurianus*, *A. aceti*, and *Gluconobacter oxydans*, are predominant strains with a strong ability to produce acids such as acetic acid, D-saccharic acid-1,4-lactone (DSL), and gluconic acid [2, 8]. As shown by Leonarski *et al*. [28], many strains of AAB, such as *Komagataibacter* and *Gluconobacter*, have been found in kombucha fermentation. The final acetic acid achieved was 16.35 g/L. The increase in titratable acidity decreased the pH during kombucha fermentation. However, the acid contributes to a sour flavor in the kombucha beverage, and a high concentration of acid may have a negative effect on consumer acceptance. Additionally, consuming acidic beverages may increase the risk of gastropathy, and kombucha has high acidity during the lengthy fermenting process [9].

The total sugar content of co-culture samples significantly decreased ($p<0.05$) from 103-105 g/L to 83-77 g/L on day 10 of fermentation, and remained higher than that of SCOBY (Figure

2C). *Zygosaccharomyces bailii* YN403 probably produces an invertase enzyme that hydrolyzes sucrose to generate monosaccharides, which are then converted to alcohol and organic acids [6]. The number of viable bacterial cells gradually increased with fermentation time for the B10Y0 ratio, indicating that *A. pasteurianus* AJ605 utilized sucrose as a carbon source [8, 21]. Similarly, Biyik and Coban [29] reported that *A. pasteurianus* HBB6 could utilize sucrose as a carbon source for cellulose production.

A significant amount of ethanol was observed in this study. On the last day of fermentation, the alcohol concentrations of co-cultures B8Y2, B5Y5, and B2Y8 were 7.96±0.10, 8.15±0.30, and 10.76±0.09 g/L, respectively (Figure 2D). As *A. pasteurianus* AJ605 oxidized ethanol to produce acetic acid, the alcohol levels in these co-cultures were lower than those in kombucha from SCOBY. Among the co-culture samples, the B8Y2 presented the lowest alcohol concentration (7.96±0.10 g/L) (Figure 2D). Kombucha is a non-alcoholic beverage; therefore, it does not contain a high alcohol concentration.

Many studies have investigated the chemical composition of kombucha [8, 15, 16, 26]. Sucrose is hydrolyzed to glucose and fructose by yeast invertase [30]. This was consistent with the decrease in total sugar concentration. Bacteria and yeast utilize monosaccharides for growth and metabolism. Acidity was produced by *A. pasteurianus* AJ605, which caused a decrease in pH. During the anaerobic fermentation stage, yeast produces ethanol, which leads to an increase in ethanol. Moreover, during fermentation, bacteria can oxidize ethanol to acids.

3.3 Bioactive compounds of black tea kombucha using co-culture

The benefits of kombucha are mostly attributable to its bioactive chemicals [4]. Our study showed that microbial co-culture caused changes in bioactive compounds presented in kombucha samples. As shown in Table 3, the total phenolic content (TPC) of all samples increased during fermentation and slightly decreased afterwards. On day 7, the B8Y2 ratio had the highest TPC (615.61±7.84 µg GAE/mL), which was not significantly different from that of SCOBY (599.60±22.75 µg GAE/mL).

Black tea contains many flavonoid compounds such as catechins, thearubigin, and theaflavin [16]. Similar to TPC, the kombucha samples had the highest amount of total flavonoid (TFC) on day 7 of fermentation. The B8Y2 and kombucha from SCOBY had the highest TFC ($p<0.05$) with 1216.48±4.10 and 1220.22±4.38 µg QE/mL, respectively. All kombucha sample ratios were lower than the initial fermentation (Table 3). Consistent with our results, Jakubczyk *et al*. [31] found that the TPC and TFC of white, green, black, and red tea fermented with a commercial kombucha starter culture decreased at the end of the fermentation period.

The changes in the TPC and TFC during fermentation were heavily influenced by the different ratios of bacteria and yeast inoculum. Several previous studies showed that some bacteria and yeasts could degrade polyphenols [25, 31]. The kinetics of bacteria and yeast increased after inoculation in kombucha fermentation, which may have increased the phenolic content [16]. The increase in TPC and TFC is caused by enzymes secreted by microbes, such as phytase, α-galactosidase, and tannase, which hydrolyze complex phenolic compounds into small molecules. However, oxidative polymerization in derivatives of phenolic content generated higher molecular weights and might have affected the lower detection of TPC and TFC [1].

3.4 Antioxidant activities of black tea kombucha using co-culture

DPPH and ABTS scavenging capacities were measured to investigate antioxidant activity, and the results are shown in Table 3. The IC_{50} value, which is a commonly used parameter to assess antioxidant activity, was defined as the concentration of kombucha required to achieve 50% radical

Table 3. The changes in TPC, TFC and antioxidant properties of black tea kombucha fermented with various ratio of *A. pasteurianus* AJ605 (B) and *Z. bailii* YN403 (Y), and commercial starter (SCOBY)

	Ratio	Fermentation time (Days)			
		0	3	7	10
TPC (µg GAE/mL)	B10Y0	513.11±3.23^{a}	544.92±8.72^{ab}	568.53±3.99^{b}	513.18±5.12^{c}
	B8Y2	516.76±3.58^{a}	558.63±5.24^{a}	615.61±7.84^{a}	588.48±5.85^{a}
	B5Y5	517.12±4.14^{a}	530.86±5.08^{bc}	544.22±5.27^{c}	497.11±3.62^{d}
	B2Y8	519.68±2.41^{a}	524.09±1.88^{c}	537.65±5.99^{c}	528.26±3.74^{b}
	B0Y10	520.40±4.53^{a}	545.71±8.72^{ab}	537.08±7.24^{c}	532.48±7.77^{b}
	SCOBY	519.27±26.82^{a}	534.35±16.14^{bc}	599.60±22.75^{a}	597.37±10.88^{a}
TFC (µg QE/mL)	B10Y0	1024.44±7.52^{b}	1030.57±10.05^{b}	1152.78±4.20^{b}	905.92±4.49^{c}
	B8Y2	1023.61±5.88^{b}	1069.45±16.02^{a}	1216.48±4.10^{a}	993.93±23.63^{b}
	B5Y5	1024.81±2.80^{b}	1026.67±6.01^{b}	1170.37±11.88^{b}	855.03±18.75^{d}
	B2Y8	1024.63±2.74^{b}	1031.85±3.94^{b}	1137.96±9.25^{b}	851.11±5.85^{d}
	B0Y10	1028.89±4.19^{b}	1034.47±3.18^{b}	1039.27±25.21^{c}	854.26±4.46^{d}
	SCOBY	1053.00±18.27^{a}	1088.89±31.80^{a}	1220.22±4.38^{a}	1175.00±24.86^{a}
IC_{50} DPPH (µL/mL)	B10Y0	45.37±0.76^{a}	36.73±5.16^{ab}	32.09±0.87^{bc}	27.33±0.65^{c}
	B8Y2	45.53±3.55^{a}	31.56±1.00^{b}	31.19±1.05^{cd}	25.76±1.95^{d}
	B5Y5	45.40±1.50^{a}	40.94±5.42^{a}	32.84±0.48^{b}	30.33±0.77^{b}
	B2Y8	42.65±0.54^{a}	38.19±1.02^{a}	32.16±0.58^{bc}	33.28±0.53^{a}
	B0Y10	42.99±0.58^{a}	40.27±2.06^{a}	34.72±0.61^{a}	34.01±0.38^{a}
	SCOBY	44.07±2.03^{a}	31.46±1.02^{b}	30.18±0.66^{d}	25.02±0.42^{d}
IC_{50} ABTS (µL/mL)	B10Y0	11.73±0.29^{b}	11.69±0.20^{b}	11.29± 0.10^{b}	10.11±0.63^{ab}
	B8Y2	11.91±0.26^{b}	11.14±0.71^{bc}	9.98±0.26^{d}	8.84±0.09^{c}
	B5Y5	11.89±0.04^{b}	11.81±0.24^{b}	11.11±0.23^{c}	9.98±1.21^{b}
	B2Y8	11.86±0.11^{b}	11.46±0.46^{b}	10.71±0.18^{b}	9.97±0.19^{b}
	B0Y10	12.84±0.23^{a}	12.44±0.04^{a}	12.13±0.30^{a}	10.58±0.11^{a}
	SCOBY	11.19±0.18^{c}	10.67±0.10^{c}	9.10±0.05^{e}	8.72±0.16^{c}

Total Phenolic content: TPC, Total Flavonoid content: TFC, gallic acid equivalent: GAE, quercetin equivalent: QE. All results showed as mean±S.D. of three replicates (n=3). Different superscript letters in the column of each fermentation time differ significantly at $p<0.05$.

scavenging activity. A lower IC_{50} value indicates high antioxidant activity. The results demonstrated that the radical scavenging ability of kombucha produced from co-culture increased with fermentation time. Kombucha prepared using the B8Y2 ratio and SCOBY had the lowest IC_{50} value on day 10 of the fermentation period for both DPPH and ABTS radical scavenging activities.

Kombucha contains various types of bioactive compounds, including antioxidant substances such as TPC, TFC, ascorbic acid, and D-saccharic acid-1,4-lactone (DSL) [2]. Changes in TPC and TFC also affect the antioxidant activity of kombucha [32]. Jakubzyck *et al*. [31] observed that the DPPH scavenging activity in kombucha increased with TPC and TFC throughout the fermentation period, while the study from Wang *et al*. [8] and Gaggia *et al*. [27] demonstrated

the antioxidant activity of kombucha increased with lower levels of polyphenol, flavonoid, and catechin detected during fermentation. This is because the complex phenolic content was degraded into derivatives, which might then have been responsible for the high antioxidant activity.

According to the findings of our study, kombucha from the B8Y2 ratio had a high amount of bioactive compounds and antioxidant activity, which were comparable to those of kombucha from SCOBY. Therefore, this ratio was selected for further study.

3.5 Improvement of black tea kombucha by blending with roselle

The TPC, TFC, and total anthocyanin content (TAC) of kombucha prepared with different ratios of black tea and roselle were investigated. The TPC and TFC of kombucha samples increased during fermentation and reached their highest values on day 7 (Table 4). Among the black tea mixed with roselle tea kombucha samples at day 7, the sample with ratio of black tea to roselle of 8:2 (C8R2) had the highest TPC and TFC, which were 571.24±3.59 µg GAE/mL and 978.52±20.50 µg QE/mL respectively, which slightly decreased on day 10. The conversion of TPC and TFC occurs because of microbial activity during fermentation [3, 31]. Consistent with our study, Gaggia *et al*. [27] demonstrated that after initial fermentation, the TFC of black tea and rooibos kombucha significantly decreased.

Anthocyanin is a pigment that gives the roselle calyx a reddish-purple color and gives tea a red color with a sour flavor. Many studies have shown that anthocyanins are water-soluble bioactive compounds that affect antioxidants [13, 33]. The TAC of kombucha samples decreased slightly throughout the fermentation period. On day 10, the TAC ranged from 0.70±0.03 to 2.26±0.06 mg CGE/mL, as shown in Table 4. In many conditions, including low pH, light, ascorbic acid, and enzymes, anthocyanins are unstable and easily degraded [11, 13]. TAC degradation may be caused by increased acidity and microbial metabolism during kombucha fermentation. Mangani *et al*. [34] also observed that yeast metabolism influenced anthocyanin degradation in wine.

Table 4 shows the antioxidant values of black tea mixed with roselle and black tea kombucha during fermentation. The IC_{50} values of DPPH and ABTS decreased during fermentation. At the initial stage of fermentation, kombucha prepared from different ratios of black tea and roselle had lower IC_{50} values than that of roselle kombucha ($p<0.05$). On day 10 of the fermentation period, kombucha produced from black tea mixed with roselle at a ratio of 8:2 had the highest DPPH and ABTS scavenging activities, with IC_{50} for DPPH and ABTS of 22.82±0.27 µL/mL and 6.11±0.39 µL/mL, respectively. The differences in both scavenging activities were derived from the polarity, ionic condition, and stereochemistry of the antioxidant compounds, all of which influence antioxidant determination [1, 13].

Anthocyanin has been identified as a major source of antioxidant activity in roselle and has nutraceutical benefits with positive effects on human health [11, 34, 35]. The two major anthocyanin compounds found in roselle are cyanidin and delphinidin, which are degraded into three derivatives that may contribute to an increase in antioxidant activity [11, 36].

Kombucha prepared from black tea and roselle at a ratio of 8:2 was found to be the optimal ratio. This ratio produced kombucha that had a higher amount of bioactive compounds with higher antioxidant activities than other co-culture ratios of black tea mixed with roselle, and it also produced product with similar properties to black tea kombucha.

3.6 Sensory properties

To compare sensory properties including the assessment of the perception and acceptance of the product: Table 5 shows the sensory evaluation results of kombucha fermented by co-culture of bacteria and yeast at an 8:2 ratio and prepared using black tea, black tea mixed with roselle, and

Table 4. The changes in TPC, TFC, TAC and antioxidant properties during fermentation of kombucha prepared from various ratios of black tea (C) and roselle (R)

	Ratio	Fermentation time (Days)			
		0	3	7	10
TPC (µg GAE/mL)	C10R0	513.11±3.23^{a}	584.29±5.77^{a}	626.32±5.60^{a}	550.63±4.73^{a}
	C8R2	497.32± 2.01^{b}	507.32±9.61^{b}	571.24±3.59^{b}	525.63±6.14^{b}
	C7R3	473.84±3.98^{c}	475.40±5.13^{c}	558.33±6.40^{c}	486.21±5.94^{c}
	C6R4	471.97±5.20^{c}	473.96±9.78^{c}	525.34±6.16^{d}	480.85±5.28^{c}
	C5R5	404.76±7.13^{d}	433.95±8.70^{d}	460.54±6.27^{e}	413.12±4.07^{d}
	C0R10	236.57±7.93^{e}	249.05±4.04^{e}	251.01±2.54^{f}	220.53±6.50^{e}
TFC (µg QE/mL)	C10R0	1034.38±7.40^{a}	1090.09±7.12^{a}	1228.26±0.71^{a}	1000.59±2.02^{a}
	C8R2	736.67±8.82^{b}	854.72±4.44^{b}	978.52±20.50^{b}	779.72±5.46^{b}
	C7R3	702.41±6.44^{c}	746.11±4.75^{c}	811.02±8.04^{c}	630.93±1.16^{c}
	C6R4	680.19±7.23^{d}	740.00±12.62^{c}	770.65±9.71^{d}	595.00±4.81^{d}
	C5R5	506.11±8.39^{e}	576.48±3.78^{d}	582.41±14.91^{e}	443.33±3.33^{e}
	C0R10	142.41±5.48^{f}	245.93±27.90^{e}	217.78±6.67^{f}	148.89±3.33^{f}
TAC (mg CGE/mL)	C10R0	0.00±0.00^{f}	0.02±0.05^{e}	0.01±0.01^{f}	0.00±0.00^{f}
	C8R2	1.11±0.06^{e}	1.11±0.02^{d}	0.89±0.06^{e}	0.70±0.03^{e}
	C7R3	1.64±0.07^{d}	1.29±0.05^{d}	1.13±0.03^{d}	1.04±0.04^{d}
	C6R4	1.96±0.07^{c}	1.93±0.08^{c}	1.64±0.11^{c}	1.24±0.09^{c}
	C5R5	3.56±0.03^{b}	3.06±0.16^{b}	2.57±0.08^{b}	2.26±0.06^{b}
	C0R10	8.33±0.15^{a}	7.91±0.21^{a}	6.41±0.11^{a}	5.44±0.09^{a}
IC_{50} DPPH (µL/mL)	C10R0	46.40±0.95^{b}	29.71±1.13^{e}	24.58±0.61^{d}	21.98±0.60^{d}
	C8R2	39.02±1.57^{c}	31.83±3.06^{de}	24.88±0.05^{d}	23.88±0.55^{cd}
	C7R3	38.80±0.96^{c}	33.63±2.74^{cd}	27.75±0.28^{c}	25.36±0.48^{c}
	C6R4	38.26±1.41^{c}	36.18±1.66^{bc}	34.35±0.28^{b}	27.34±0.96^{b}
	C5R5	46.95±1.80^{b}	39.39±2.29^{b}	33.70±0.77^{b}	27.52±1.08^{b}
	C0R10	110.92±1.35^{a}	95.36±0.14^{a}	64.32±3.16^{a}	47.6±2.11^{a}
IC_{50} ABTS (µL/mL)	C10R0	11.51±0.30^{d}	10.21±0.16^{c}	9.90±0.55^{e}	6.01±0.19^{d}
	C8R2	11.28±0.30^{d}	10.58±0.31^{c}	10.13±0.05^{de}	6.11±0.39^{d}
	C7R3	11.79±0.81^{cd}	11.53±0.40^{c}	10.99±0.20^{cd}	10.13±0.21^{c}
	C6R4	12.66±0.36^{c}	12.49±0.34^{bc}	11.16±0.89^{c}	10.27±0.18^{c}
	C5R5	14.99±0.09^{b}	13.95±0.31^{b}	13.78±0.28^{b}	11.02±0.34^{b}
	C0R10	41.00±0.84^{a}	36.35±2.46^{a}	25.41±0.56^{a}	22.53±0.14^{a}

Total Phenolic content: TPC, Total Flavonoid content: TFC, Total Anthocyanin content: TAC, gallic acid equivalent: GAE, quercetin equivalent: QE, cyanidin-3-glucoside equivalent: CGE. All results shown as mean±S.D. of three replicates (n=3). Different superscript letters in the column of each fermentation time differ significantly at $p<0.05$.

roselle as substrates. The preference scores varied according to the individual characteristics of the substrate ratio. The kombucha samples with a high concentration of roselle had a higher score for color and clearness. The preference scores for odor, taste and overall C8R2 ratio were 7.00±1.72, 6.13±1.81, 6.93±1.34, respectively, with a significantly higher difference ($p<0.05$) than the other ratios. The increase of roselle level created more sour taste, which made overall likeness and taste score tend to decrease. Roselle addition creates a clearer beverage because it does not contain sediment from tea. Therefore, the likeness scores for color and clearness increased.

As a results, kombucha from the C8R2 ratio demonstrated a high total phenolic content and lower IC_{50} of DPPH and ABTS. In addition, product at the C8R2 ratio scored well on sensory determination.

Table 5. Effects of kombucha prepared from different ratios of black tea and roselle in each ratio on the sensorial evaluation of flavored kombucha samples

Ratio	Color	Clearness	Odor	Taste	Overall
C10R0	5.27±1.80^{b}	6.60±1.48^{b}	5.33±2.31^{b}	5.10±1.52^{b}	4.93±1.26^{c}
C8R2	5.33±1.21^{b}	6.83±1.32^{b}	7.00±1.72^{a}	6.13±1.81^{a}	6.93±1.34^{a}
C7R3	6.43±1.25^{a}	6.67±1.60^{b}	4.23±1.98^{c}	5.23±0.82^{b}	6.03±1.35^{b}
C6R4	6.57±1.43^{a}	6.80±1.45^{b}	3.80±1.79^{c}	4.30±2.18^{b}	5.27±1.80^{bc}
C5R5	7.03±1.33^{a}	7.13±1.34^{ab}	3.70±1.39^{c}	4.60±1.16^{b}	5.33±1.79^{bc}
C0R10	7.03±1.35^{a}	7.63±1.00^{a}	4.67±2.15^{bc}	4.83±2.21^{b}	4.80±1.92^{c}

All results showed as mean ± S.D and different superscript letters in the column differ significantly at $p<0.05$.

4. Conclusions

To sum up, in kombucha fermentation, the co-culture system using bacteria and yeast plays an important role in the chemical properties and antioxidant activities of the kombucha. Our kombucha recipe has contributed to the development of traditional kombucha beverages. The maximum antioxidant activity was observed in kombucha made from a consortium at B8Y2 of the bacterium *A. pasteurianus* AJ605 and the yeast *Z. bailii* YN403 after 10 days of fermentation. Furthermore, mixing black tea with roselle resulted in different antioxidant activities. The C8R2 of black tea: roselle gave a higher antioxidant activity than other ratios of black tea with roselle, and the result was similar to that of black tea kombucha. The phenolic content, flavonoids, and anthocyanins from roselle also contribute to its antioxidant activity. Kombucha prepared from black tea mixed with roselle had a better sensory score than black tea kombucha. Our results clearly demonstrated that this new formulation produced different flavors and beverages with high antioxidant activity, and the selected mixed starter culture improved the consistency and quality of the kombucha product.

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