



Research article

Effect of tropical fruit pulp infusion during second fermentation of cannabis leaf-based kombucha on quality characteristics and its storage changes

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Abstract

Importance of the work: With the rising demand for functional beverages, kombucha is of interest due to its health benefits; however, the taste is harsh, making it difficult to swallow, which was resolved by using a second fermentation.

Objectives: To investigate: 1) the effects of fruit infusion during the second fermentation of kombucha on its quality characteristics; and 2) the storage changes in cannabis leaf-based kombucha.

Materials and Methods: The first fermentation was prepared using 30% cannabis leaf infusion and fermentation at 25°C for 7 d to produce the cannabis-leaf-based kombucha. The second fermentation involved infusion-blended pulps of cantaloupe, mahachanok mango or tangerine (each 10% or 15% volume per volume, v/v) into the cannabis leaf-based kombucha prior to fermentation at 25°C for 24 hr. The quality of all treatments was analyzed and microbial changes were monitored. The criteria used for the sample selection were based on the quality and lactic acid bacteria (LAB) counts. Then, changes were investigated due to storage at 4 ± 2°C for up to 30 d.

Results: The cannabis leaf-based kombucha infused with fruit pulp had higher LAB counts (6.57–6.71 log CFU/mL), polyphenol contents (0.80–0.96 mg GAE/mL) and antioxidant properties (4.70–5.11 mg Trolox/mL) than the control treatment. The phytochemicals and sufficient nutrients obtained from the fruit enhanced the LAB growth which resulted in lowering the level of acetic acid obtained from the first kombucha fermentation. Desirable physical and chemical quality properties were enhanced. The color quality changes were influenced by pH and total acidity alterations. The selected kombucha using 10% tangerine pulp maintained its quality properties, without the detection of any pathogenic microorganisms after 30 d of refrigerated storage, suggesting it remained acceptable and safe for consumption.

Main finding: The addition of fruits during the second fermentation of kombucha could effectively improve the kombucha quality with beneficial functions such as enhanced flavor, overall qualities and LAB viability.

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Introduction

The popularity of "natural fermented drinks" has gained much attention after the COVID-19 pandemic since consumer trends are shifting to healthier options (MINTEL, 2021). Kombucha is a fermented drink sold in tangy flavors offering health benefits, including antimicrobial, antioxidant and anti-inflammatory properties (Anantachoke et al., 2023). Typically, the traditional kombucha is produced using various types of tea leaves as substrate combined with a symbiotic culture of microorganisms, such as *Acetobacter* sp., *Gluconobacter* sp., *Saccharomyces* sp., *Zygosaccharomyces* sp. and *Candida* sp., that are fermented for 7–14 d. However, traditional kombucha obtained from the first fermentation contained a low number of number of lactic acid bacteria (LAB) according to Neffe-Skocińska et al. (2017); furthermore, it still had a harsh vinegar taste, a high ethanol content and a strong fermented odor (Zou et al., 2021). Numerous studies have investigated innovative kombucha products using alternative substrates, such as fruits, herb leaves, spices, agro-industrial wastes and wild plants, or by adding functional ingredients such as powdered probiotics, vitamin premixes and natural extracts. These innovations have improved kombucha's properties in terms of taste, overall quality, nutritional content, flavor characteristics, biological properties and consumer acceptability (Rahmani et al., 2019; Frolova et al., 2023; Majid et al., 2023; Miglioranza et al., 2024). Recently, the leaves of cannabis (*Cannabis sativa* L.) have gained attention as an ingredient due to the growing interest in its use in functional beverages and their promoted health benefits (Suphim et al., 2024). The extract from cannabis leaves can promote the growth of *Lactobacillus* strains (Zandani et al., 2022).

Although substrates can enhance kombucha quality, several undesirable qualities remain, including unpleasant sweetness, low natural carbonation, faded color, harsh acetic acid and degraded nutritional quality (Morales et al., 2023). In addition, the bioactivity of kombucha was reported to decrease by day 7 of the first fermentation (Frolova et al., 2023; Morales et al., 2023). A promising approach to improving these qualities is the application of a second fermentation using functional ingredients, particularly fruit pulp, to improve the sensory profile with fruity aromas and enhance antioxidant activity, where the addition of fruits or herbs has been reported prior to fermentation at 25°C for 24–48 hr under aerobic conditions (Silva Júnior et al., 2021; Frolova et al., 2023).

Tropical fruits are rich in vitamins A and C, β -carotene, flavonoids and other bioactive compounds that contribute to health benefits and are used as flavoring substances to improve the quality of functional drinks (Lebaka et al., 2021; Maciel et al., 2023). Many studies have reported on the addition of tropical fruits into the fermentation process, for example mahachanok mango, tangerines and Thai orange cantaloupes (Fonteles et al., 2012; Laophongphit et al., 2023; Maciel et al., 2023). Bioactive compounds of fruits support fermentation, as well as improving the antioxidant activity and nutritional quality of beverages (Fonteles et al., 2012). These fruits might be suitable as substrates for kombucha fermentation to enhance overall quality properties.

Therefore, the current investigated the effect of second fermentation with various fruit pulps (cantaloupe, mahachanok mango or tangerine at 10% or 15% volume per volume, v/v). The cannabis leaf-based kombucha from the first fermentation was continually fermented with fruit pulp at 25°C for 24 hr. Then, the quality characteristic changes and LAB counts were studied that had been produced during the period of second fermentation and refrigerated storage at $4 \pm 2^\circ\text{C}$ for 30 d. It was expected that there would be improved LAB growth along with overall quality enhancement.

Materials and Methods

Raw materials and chemical reagents

Various substrates were used in the first fermentation of kombucha. Assam green tea leaves (*Camellia sinensis* (L.) Kuntze var. *assamica*) were purchased from Tea Company (Chiang Rai, Thailand) and cannabis leaves (*Cannabis sativa* sp. Hang Kra Rog Phu Phan ST1) were supplied by Kasetsart University (Chalermphrakiat Sakon Nakhon campus, Thailand). A liquid symbiotic culture of bacteria and yeast (SCOBY) for kombucha fermentation was purchased from Swan Ketogenic Company (Bangkok, Thailand). The fruits used as the substrates during the second fermentation of kombucha were mahachanok mango (*Mangifera indica* L. 'Mahachanok'), Thai orange cantaloupe (*Cucumis melo* L. var. *cantaloupensis*) and tangerine (*Citrus reticulata* Blanco, 1837) at 10 or 15% v/v. All fruits were purchased from a local market (Bangkok, Thailand). The chemical reagents were: gallic acid, Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox) and bromocresol purple and were purchased

from Sigma-Aldrich (Burlington, MA, USA). The ethanol (AR grade, 95%) was purchased from Qrec (Auckland, New Zealand). The De Man, Rogosa and Sharpe agar (MRS) was purchased from Difco (Detroit, MI, USA) and used as the medium for microbial analysis.

Preparation of cannabis leaf-based kombucha

The first fermentation of kombucha was performed according to the method of Silva Júnior et al. (2021), with a slight modification. This procedure started by preparing a sugar solution with hot water in 1 L glass jars, infusing the mixture of tea and cannabis leaves (70:30) for 15 min to produce a brewed solution that was sterilized for 15 min at 121°C. Next liquid symbiotic culture of bacteria and yeast (SCOBY) culture was inoculated into the cooled brewed tea mixed with cannabis leaves and then incubated at $25 \pm 1^\circ\text{C}$ for 7 d using a standing incubator (Sanyo Incubator MIR 523; Thanes Development Co., Ltd.; Bangkok, Thailand). Based on preliminary study, appropriate kombucha formulas were investigated with varying ratios of Assam green tea and cannabis leaves (A=100:0, B=85:15, C=70:30, D=50:50). Formula C was selected, based on its low amount of acetic acid (4.42 g/L) and high LAB count (5.53 log CFU/mL), which were desirable in the second fermentation with the various fruit pulps.

Production of cannabis leaf-based kombucha with fruit pulps in second fermentation

Preparation of fruit pulps preparation and their characteristics

The preparation was started by blending each pulp (mango flesh, cantaloupe flesh or tangerine pulp after squeezing out the juice) homogeneously using an ultra-high-speed blender (ULTRABLEND-BL935; Tefal; Rumilly, France) and treating all samples with ultrasonication using an ultrasonic bath for 10 min at room temperature (GT SONIC-D27 500W; Hongkong China) to reduce the microbial load and to extract the soluble nutrients (Fonteles et al., 2012). The obtained fruit pulps were analyzed for pH, total acidity, total soluble solids, total polyphenols content and %DPPH scavenging. Then the different fruit pulps were added separately to liquid samples of the cannabis leaf-based kombucha and the second fermentation was carried out.

Analysis of pH and total acidity of fruit pulps

The pH and total acidity were measured in triplicate to characterize the fruit pulp as the substrate during the second

fermentation of the cannabis leaf-based kombucha, following the method described by Silva et al. (2021). The pH value was determined using a digital pH meter (Model 9625; Horiba, Ltd.; Sapporo, Japan). Total acidity was analyzed according to a titrimetric method using 0.01 N NaOH solutions and phenolphthalein as the indicator (Silva et al. 2021). The concentration of total acidity was reported in grams per liter for each sample.

Analysis of total soluble solids of fruit pulps

Total soluble solids (TSS) were determined in triplicate for each fruit pulp according to the method by Silva et al. (2021). The TSS content was determined using a hand refractometer (MASTER-53M; Atago Co. Ltd.; Tokyo, Japan) and expressed as soluble solids content (°Brix).

Analysis of total polyphenol contents of fruit pulps

The total polyphenol content (TPC) in each fruit pulp was determined using the Folin-Ciocalteu spectrophotometric method, with modifications as described by Rahmani et al. (2019). A reaction mixture was prepared by combining 100 μL of the sample with 500 μL of Folin-Ciocalteu reagent (0.2 N) and allowing it to react at room temperature for 5 min. Following this, 400 μL of sodium carbonate solution (75 g/L) was added and the mixture solution was incubated for 25 min at room temperature. The absorbance value was measured at 765 nm using a spectrophotometer (Biomate 3S UV-Vis; Thermo Fisher Scientific Co. Ltd.; Waltham, MA, USA). A standard curve was established with gallic acid in the range 5–50 μM , with a coefficient of determination (R^2) of 0.9987. The results were reported as milligrams of gallic acid equivalent (GAE) per milliliter.

Antioxidant properties

The antioxidant properties were determined based on %DPPH radical scavenging activity. The DPPH assay followed the protocol by Rahmani et al. (2019). A 200 μL sample was mixed with 800 μL of a 100 μM DPPH solution in ethanol. After thorough vortexing to produce a homogeneous product, the mixture was incubated for 25 min at room temperature in a dark room. Then, each sample was measured at 517 nm using the Biomate 3S UV-VIS spectrophotometer. The results were presented as %DPPH radical scavenging activity.

Second fermentation of cannabis leaf-based kombucha infused with fruit pulps

The cannabis leaf-based kombucha samples were prepared for the second fermentation by removing the cellulose biofilm

on top of the liquid using a sterile spatula. Each type of fruit pulp (mahachanok mango, tangerine or Thai orange cantaloupe) was added to separate samples at concentrations of 10% v/v or 15% v/v to 1 L of kombucha base in glass jars with a 1 L capacity. The concentrations of 10% v/v and 15% v/v were determined based on preliminary study, which suggested as the optimal range concentrations for the second fermentation of kombucha (Silva Junior et al., 2021; Frolova et al., 2023). The six formulations were incubated under aerobic conditions at $25 \pm 1^\circ\text{C}$ for 24 hr in a standing incubator (Sanyo Incubator MIR 523; Thanos Development Co., Ltd.; Bangkok, Thailand). In this study, kombucha samples were abbreviated as 'K', with treatments names based on the added type of fruit (K-control, K-cantaloupe 10%, K-cantaloupe 15%, K-mango 10%, K-mango 15%, K-tangerine 10%, and K-tangerine 15%).

Effect of second fermentation on quality and microbial property changes in cannabis leaf-based kombucha infused with various fruit pulps

Analysis of microbial properties of fermented kombucha with fruit pulps

The LAB counts in the samples were analyzed using the pour plate method, based on the method in the Bacteriological Analytical Manual (U.S. Food and Drug Administration, 2017). Briefly, peptone water and MRS medium agar supplemented with 0.005% bromocresol purple were prepared and sterilized using autoclaving at 121°C for 15 min. The samples were prepared by serial dilution (1:9 v/v) with peptone water. A diluted sample (1 mL) was poured into a Petri dish and then the MRS agar was added. The samples were incubated at 35°C for 3 d. The number of LAB was reported in log colony-forming units (CFU) per milliliter.

Analysis of physical and chemical quality of fermented kombucha with fruit pulps

The obtained samples consisting of (the control treatment and six formulas from the second kombucha fermentation) were analyzed for pH, total acidity and TSS content, following the reported methods above. The turbidity value for all samples was measured according to a method described by Wang et al. (2022) to obtain the turbidity values. All measurements were conducted in triplicate with duplicate experiments.

Analysis of color quality of fermented kombucha with fruit pulps

The color parameters for all samples were evaluated using a colorimeter (UltraScan VIS Spectrophotometer; Hunter Lab;

Bangkok, Thailand) following the method by Zou et al. (2021). Each sample was measured 10 times and the color parameters were presented in terms of the Commission Internationale de l'Éclairage L^* , a^* and b^* values, where L^* represents lightness, a^* represents redness (+) to greenness (–) and b^* represents yellowness (+) to blueness (–).

Analysis of antioxidant properties of fermented kombucha with fruit pulps

The TPC content and DPPH antioxidant capacity of the obtained kombucha samples were determined following the procedure described by Rahmani et al. (2019). Briefly, the TPC content each sample was calculated according to the coefficient correlation of the gallic acid standard curve, as discussed above, and expressed as milligrams GAE per milliliter of kombucha. The DPPH antioxidant capacity of samples was determined according to correlation with the Trolox standard curve (range 10–140 μM ; $R^2 = 0.9987$). The results were expressed in milligrams of Trolox per milliliter of kombucha.

Effect of refrigerated storage on overall quality and microbial property changes in selected cannabis leaf-based kombucha infused with 10% tangerine pulp

The fermented kombucha infused with 10% tangerine pulp was selected based on the LAB, TPC, DPPH and turbidity values and investigated for overall quality characteristics during refrigerated storage ($4^\circ\text{C} \pm 2$) for 30 d. During the observation, the samples were stored in separate sterilized glass bottles in a refrigerator and subsamples were collected at 0, 7, 14, 21 and 30 d for analysis of pH, total acidity, TSS, turbidity, color parameters, antioxidant properties and LAB counts, according to the methods discussed above. Microbial detection was conducted for total coliform and viable counts of *Staphylococcus aureus* and *Escherichia coli* following the method described by U.S. Food and Drug Administration (2017) and the analyses was performed on day 0 and day 30.

Statistical analysis

All experiments were performed in duplicate and data were expressed as mean $\pm$ SD values. Data were analyzed using one-way analysis of variance using the SPSS Statistics software (version 22; Thaisoftup Co. Ltd.; Bangkok, Thailand). *Post hoc* analysis was conducted using Duncan's multiple range test when significant ($p < 0.05$) differences were observed.

Results and Discussion

Quality characteristics of cannabis leaf-based kombucha obtained from first fermentation

The qualities of kombucha obtained from the first fermentation were analyzed. The cannabis leaf-based kombucha had a low pH (3.53) and was a light yellow color ($L^*=58.89$, $a^*=10.57$, $b^*=52.85$). The TPC of the kombucha was 0.76 mg GAE/mL and DPPH antioxidant capacity was 4.01 mg TE/mL. The LAB count was 5.53 log CFU/mL. This sample had a harsh, vinegar-like, acetic acid taste and a strong fermented odor with less carbonation compared to the normal kombucha. Enhancement of flavor and taste have been reported due to the high LAB growth in the kombucha mixed with other alternative leaves (Wang et al., 2024). The second fermentation was performed to promote the growth of LAB.

Effect of second fermentation with various fruit pulps on changes in microbial and quality properties of cannabis leaf-based kombucha

The study investigated the appropriate types of fruit pulps (mahachanok mango, tangerine and Thai orange cantaloupe) and suitable concentrations. The second fermentation resulted in improvements to the overall qualities and has been reported to improve consumer acceptability (Frolova et al., 2023).

Characteristics of fruit pulps

The results in Table 1 indicated that the mahachanok mango pulp had the highest total acidity, TPC, %DPPH radical scavenging activity and TSS content, while having the lowest pH. These results might have been due to the various types of organic acids presented in mango, including citric acid, malic acid, oxalic acid, succinic acid, ascorbic acid and tartaric acid (Lebaka et al., 2021). In addition, flavonoids, phenolic acids, mangiferin, lutein, quercetin and tannins may have contributed

to the highest TPC content and %DPPH radical scavenging activity in mango (Alaiya and Odeniyi, 2023). The highest TSS content might have been due to the presence of pectin and sucrose in the mahachanok mango and served as the fermentable substrates (Laophongphit et al., 2023). Of the three types of fruit pulp studied, the tangerine pulp had the best odor and good color.

Changes in microbial properties of kombucha infused with fruit pulps

The fruit pulp infusion in cannabis leaf-based kombucha during the second fermentation resulted in increasing the viable counts of LAB, which indicated that the fruit pulp provided sufficient nutrients (mainly sugar and pectin) that could promote the growth of LAB (Laophongphit et al., 2023). The viable counts of LAB in all the fruit-infused kombucha samples were higher (6.57–6.71 log CFU/mL) than in the control treatment (Fig. 1), which was consistent with Laophongphit et al. (2023), who reported that fruit pulp could promote the growth of LAB because of greater amounts of soluble total sugar content and pectin that served as carbon sources for LAB.

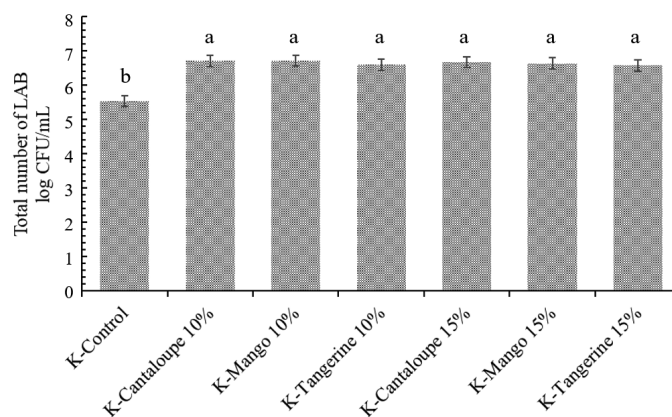


Fig. 1 Numbers of lactic acid bacteria (LAB) in cannabis leaf-based kombucha infused with various fruits pulp during second fermentation, where error bars indicate mean $\pm$ SD ($n = 7$) and different lowercase letters above columns indicate significant ($p < 0.05$) differences among treatments

Table 1 Characteristic of fruit pulps as substrate for second fermentation

Parameter	Thai orange cantaloupe	Mahachanok mango	Tangerine
pH	6.40 $\pm$ 0.03 ^a	4.53 $\pm$ 0.03 ^b	4.57 $\pm$ 0.05 ^c
Total acidity (g/L)	2.47 $\pm$ 0.09 ^c	16.56 $\pm$ 0.85 ^a	13.29 $\pm$ 0.24 ^b
Total soluble solids ($^{\circ}$ Brix)	6.05 $\pm$ 0.05 ^c	17.92 $\pm$ 0.08 ^a	9.28 $\pm$ 0.04 ^b
TPC (mg GAE/mL)	0.20 $\pm$ 0.00 ^c	0.89 $\pm$ 0.03 ^a	0.55 $\pm$ 0.02 ^b
DPPH radical scavenging activity (%)	28.304 $\pm$ 1.18 ^c	77.88 $\pm$ 0.52 ^a	49.27 $\pm$ 0.13 ^b

TSS = Total soluble solids ($^{\circ}$ Brix); TPC = Total Polyphenol Content (mg GAE/mL); DPPH = 2,2 diphenyl -1-picrylhydrazyl. Mean $\pm$ SD values in same row superscripted with different lowercase letters indicate significant ($p < 0.05$) differences.

In the current study, the lower viable count of LAB in the control treatment could have been due to nutrient depletion because the carbon and nitrogen sources had already been converted into metabolites and acetic acid by the microbial communities present during the first fermentation of kombucha (Silva Júnior et al., 2021). The result agreed with Wang et al. (2024), who reported on the relationship between the number of LAB in kombucha and improvement in the flavor profile by lessening the intensity of harsh acetic and vinegar notes.

Changes in quality characteristics of kombucha infused with fruit pulps

The second fermentation affected the pH and total acidity in the cannabis leaf-based kombucha infused using various fruits (Table 2). Above all, all fruit-infused kombucha samples had significantly higher pH values (3.65–3.77) than the control treatment (3.53). These pH values were considered to support the optimal fermentation conditions for microbial growth (pH: 3.6–6.5), particularly for the LAB present in kombucha, as reported by Neffe-Skocińska et al. (2017). The, higher pH in all the fruit-infused kombucha samples could have been influenced by the buffering effects from the weak organic acids imparted from the fruits, specifically citric, ascorbic and malic acids (Lebaka et al., 2021). The total acidity levels of the fruit-infused samples were in the range 3.75–4.72 g/L, indicating an association with the pH values. These pH and total acidity values could have been influenced by the different components in the organic acids extracted from the fruits (Table 1), as well as the metabolic activity of acetic acid bacteria (AAB) and LAB for organic acids production. This agreed with Morales et al. (2023). In the current study, all samples had pH values lower than 4.2 with high percentages of acidity and high contents of LAB that were consistent with kombucha brewing standards. Specifically, the pH should be in the range 2.5–4.2 to inhibit the growth of pathogenic and spoilage microorganisms. Hence, the samples from the second

fermentation remained safe for consumption. The flavor of the fruit-infused kombucha was better than for the normal kombucha due to the generation of a fruity, sweet, floral flavor during the second fermentation. This phenomenon resulted in a product with good flavor and reduced greatly the harsh flavor of acetic acid.

The addition of fruit pulps in the second fermentation of the kombucha reduced the TSS contents to 9.71–11.00°Brix (Table 2). This reduction could have been to the effects of the second fermentation, during which yeast hydrolyzes sugars from the fruit into ethanol and organic acids as fermentation progresses. Notably, K-mango 15% had the highest TSS content of all the treatments. This might have been due to the high concentrations of water-soluble substances present in the mahachanok mango pulp (Table 1). These results were consistent with those of Frolova et al. (2023), who reported that fruit addition influenced TSS levels in kombucha, owing to the soluble substances obtained from the fruit. Furthermore, all fruit-infused kombucha had higher turbidity values than the control, with K-mango 15% being the highest (Table 2). This could have been associated with the highest TSS content present in the K-mango 15% and compounds in the mahachanok mango (Table 1). In addition, K-tangerine 10% and K-cantaloupe 10% appeared clearer. Hence, the changes in the TSS contents of the fruit-infused kombucha indicated lower sugar contents than in the control treatment, while also affecting turbidity levels and appearance.

Changes in color attributes of kombucha infused with fruit pulps

The changes in color quality of all fruit-infused kombucha treated with the second fermentation are presented in Table 3. The fruit infusion led to a decrease in the values of L*, a* and b*; consequently, the lightness, redness and yellowness were lower in all fruit-infused kombucha than in the control treatment.

Table 2 Effects of second fermentation of cannabis leaf-based kombucha (K) infused with various fruit pulps and percentages on changes in physical and chemical parameters

Formula	pH	Total acidity (g/L)	TSS (°Brix)	Turbidity value
K-Control	3.53±0.01 ^c	4.42±0.02 ^c	11.53±0.04 ^a	6.24±0.43 ^f
K-Cantaloupe 10%	3.70±0.02 ^b	3.75±0.04 ^e	9.88±0.06 ^d	15.76±0.48 ^e
K-Cantaloupe 15%	3.73±0.02 ^a	3.96±0.01 ^{de}	9.71±0.16 ^e	22.28±0.44 ^c
K-Mango 10%	3.71±0.02 ^b	3.94±0.08 ^d	10.95±0.04 ^c	32.55±0.15 ^b
K-Mango 15%	3.77±0.01 ^a	4.61±0.06 ^{ab}	11.00±0.06 ^b	39.42±0.34 ^a
K-Tangerine 10%	3.63±0.04 ^d	4.50±0.04 ^{bc}	9.93±0.05 ^d	15.86±0.90 ^e
K-Tangerine 15%	3.68±0.01 ^c	4.72±0.04 ^a	9.96±0.05 ^d	19.37±0.70 ^d

TSS = Total Soluble Solid (°Brix).
Mean ± SD values in same column superscripted with different lowercase letters indicate significant ($p < 0.05$) differences.

Table 3 Effects of second fermentation in cannabis-leaf-based kombucha infused with various fruit pulps and percentages on color change

Formula	L* (lightness)	a* (redness)	b* (yellowness)
K-Control	58.89±0.06 ^a	10.57±0.02 ^a	52.85±0.07 ^a
K-Cantaloupe 10%	38.5±0.78 ^d	3.94±0.05 ^g	23.62±0.21 ^f
K-Cantaloupe 15%	32.36±1.11 ^e	4.04±0.09 ^f	22.79±0.30 ^g
K-Mango 10%	23.65±0.02 ^f	6.71±0.03 ^b	34.07±0.15 ^c
K-Mango 15%	21.87±0.02 ^g	9.18±0.06 ^c	34.99±0.15 ^b
K-Tangerine 10%	45.73±0.33 ^b	4.44±0.05 ^e	27.84±0.13 ^e
K-Tangerine 15%	41.49±0.03 ^c	5.46±0.04 ^d	29.58±0.11 ^d

Mean ± SD values in same column superscripted with different lowercase letters indicate significant ($p < 0.05$) differences.

The second fermentation improved the color quality of the cannabis leaf-based kombucha, which initially had a slightly paler yellow color after the first fermentation. For example, the kombucha samples infused with tangerine or cantaloupe pulp had a moderate yellow color; however, kombucha infused with the mahachanok mango pulp had a darker yellow color. These color changes may have been attributed to the microbial biotransformation and enzyme activity performed by AAB and LAB during organic acid production, which affected pigment stability under acidic conditions (Chen et al., 2023). Carotenoids were the main pigment found in the observed fruits and are stable at neutral pH (Damodaran and Parkin, 2017). Thus, the kombucha fermentation-induced acidity influenced the pigment stability under low pH, which further affected color changes. These results agreed with Chen et al. (2023), who reported that fermentation produced color changes through the biotransformation of the pigment compounds found in fermented drinks.

Changes in antioxidant properties of kombucha infused with fruit pulps

The effect of a second fermentation generally increased the TPC content (0.80–0.96 mg GAE/mL) and DPPH antioxidant capacity (4.70–5.11 mg Trolox/mL) obtained in all fruit-infused kombucha samples (Figs. 2A and 2B). This result was based on from the antioxidant in the tea and cannabis leaves from the first fermentation together with the fruit pulp infusion. These observations also may have been influenced by the phytochemical profiles present in each fruit type (Table 1) and were consistent with Silva Júnior et al. (2021). Notably, tangerine is rich in bioactive compounds, particularly ascorbic acid and flavanols (Maciel et al., 2023); consequently, these compounds contributed to the high antioxidant properties observed in the K-tangerine 10% sample. During the second fermentation of the kombucha, other factors, such as biotransformation processes, might have affected the TPC

content and antioxidant capacity by facilitating microbial activity and released enzymes (glucosidase and pectinase) that converted the complex polyphenols into simpler phenol compounds (Kim et al., 2023). The microbial strains presented in SCOBY kombucha included various strains of the genera *Dekkera* and *Komagataeibacter*, as well as LAB, which reacted with polyphenols through hydrolysis, oxidation, isomerization and structural transformations, leading to an increase in the antioxidant properties of the kombucha (Cele et al., 2022; Kim et al., 2023). Therefore, the addition of various fruits during the second fermentation enhanced the polyphenol content and antioxidant properties of the cannabis leaf-based kombucha. Based on the results of the quality analysis, the sample with 10% tangerine produced the highest TPC and DPPH values, as well as having low turbidity and high LAB counts. Hence, this sample was selected for further study of quality changes during refrigerated storage.

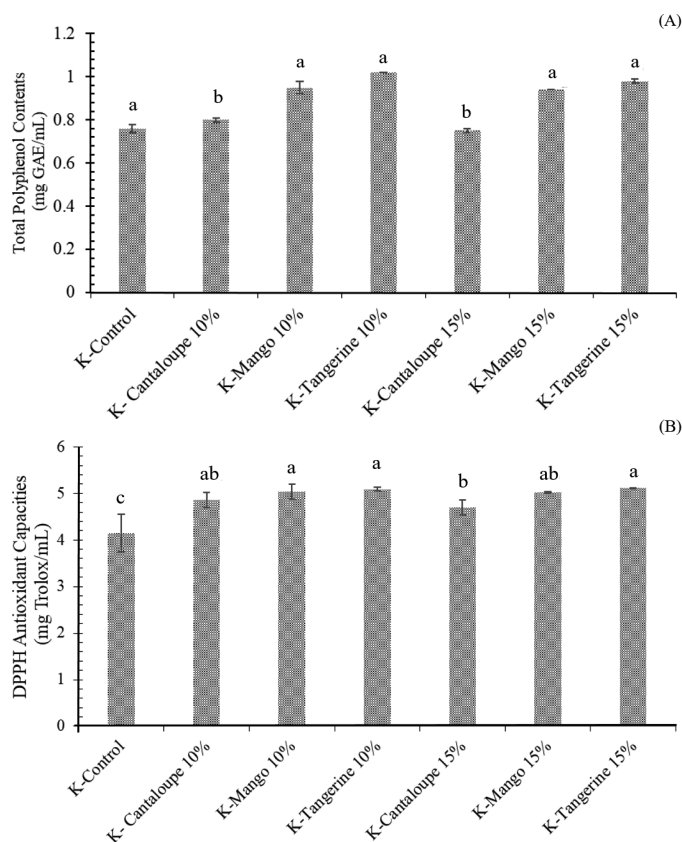


Fig. 2 Antioxidant properties of cannabis leaf-based kombucha infused with various fruits pulp during second fermentation: (A) total polyphenol contents (TPC); (B) 2,2-diphenyl-1-picrylhydrazyl (DPPH), where error bars indicate mean ± SD ($n = 7$) and different lowercase letters above columns indicate significant ($p < 0.05$) differences among the treatments

Effect of refrigerated storage on overall quality and microbial properties changes in selected cannabis leaf-based kombucha infused with 10% tangerine pulp

The cannabis leaf-based kombucha infused with 10% tangerine pulp was further investigated during 30 d of storage. The changes in the physical, chemical, antioxidant and microbial properties of the selected product during refrigerated storage are discussed below.

Physical and chemical quality characteristics of selected kombucha treatment

The effects of refrigerated storage of the selected product on the changes in pH and total acidity are presented in Table 4. The pH and total acidity are crucial in analyzing food safety and sensory appeal (Villarreal-Soto et al., 2018). Based on the current results, the pH of the product tended to decrease during storage, whereas the total acidity tended to slightly increase, reaching its peak (4.94 g/L) on day 30 of storage. This stable pH value could have been due to the buffering effects of the citric acid from the tangerine pulp, which could have prevented major pH changes (Silva et al., 2021). The increase in total acidity could be attributed to the further production of organic acids due to microbial action, specifically the AAB and LAB strains (Majid et al., 2023). Thus, these results agreed with other studies, suggesting that the pH and total acidity of this observed kombucha remained within the safe and acceptable boundaries for human consumption based on a pH range of 2.5–4.2 and a maximum total acidity of 5 g/L (Villarreal-Soto et al., 2018; Majid et al., 2023).

During the storage period, the TSS content remained stable, while the turbidity values fluctuated (7.46–16.11), reaching the significantly highest value on day 14 of storage (Table 4).

The product was kept in tightly closed bottles during storage, resulting in limited oxygen permeation. As a result, there was no yeast activity detected for further sucrose hydrolysis (Tran et al., 2024), which resulted in the stable TSS content. The fluctuation in turbidity may have been due to the dissolved substances released during the second fermentation, which was associated with bacterial metabolism under anaerobic conditions (Dartora et al., 2023; Tran et al., 2024).

The color values of L*, a* and b* tended to fluctuate during refrigerated storage (Table 4). The lightness of the sample tended to decrease and the sample had darkened after storage for 30 d. The redness and yellowness coloring were pale. On day 30 of storage, the color was characterized by a moderate yellow with less darkness. These color changes were attributed to alterations in the structure of active pigments, which could have been affected by microbial communities and their released enzymes during fermentation (Chen et al., 2023). Furthermore, the color degradation could have been influenced by a lower pH and oxidation due to light and oxygen exposure (Damodaran and Parkin, 2017). These results agreed with Majid et al. (2023), who reported similar color discoloration due to microbial action altering the active pigments under acidic conditions.

Over the storage period from day 0 to day 30, both the TPC content and DPPH antioxidant capacity of the kombucha decreased, reaching their lowest values of 0.48±0.07 mg GAE/mL and 4.65±0.01 mg Trolox/mL, respectively (Table 4). The decrease in the TPC and DPPH values could be attributed to microbial metabolism activity during post-fermentation. This phenomenon is affected by the activity of *Dekkera* and *Acetobacter*, as well as the enzymes released within the SCOBY culture, including phytases, β-galactosidase, tannase and cellulase, which can either modify

Table 4 Effects of refrigerated storage on physical and chemical characteristics of cannabis leaf-based kombucha infused with 10% tangerine pulp

Parameter	Day 0	Day 7	Day 14	Day 21	Day 30
Physical characteristics					
pH	3.63±0.01 ^d	3.72±0.01 ^a	3.69±0.01 ^b	3.68±0.01 ^b	3.65±0.01 ^d
TSS (°Brix)	9.93±0.06 ^b	10.20±0.06 ^a	10.00±0.00 ^b	10.00±0.00 ^b	10.00±0.00 ^b
Turbidity	15.76±0.54 ^a	7.46±0.44 ^c	16.11±1.06 ^a	12.09±1.33 ^b	9.94±0.95 ^c
L* value	45.73±0.45 ^d	78.15±0.34 ^a	66.52±0.51 ^b	51.45±0.47 ^c	45.32±0.34 ^c
a* value	4.44±0.07 ^a	0.48±0.07 ^c	1.72±0.06 ^d	3.42±0.05 ^b	3.23±0.03 ^c
b* value	27.84±0.36 ^d	34.24±0.17 ^b	33.78±0.13 ^c	41.36±0.32 ^a	27.21±0.10 ^c
Chemical characteristics					
Total acidity (g/L)	4.50±0.24 ^b	4.26±0.09 ^b	4.75±0.09 ^{ab}	4.86±0.28 ^a	4.94±0.14 ^a
TPC (mg GAE/mL)	0.84±0.14 ^a	0.48±0.04 ^c	0.67±0.02 ^b	0.70±0.03 ^b	0.72±0.01 ^b
DPPH (mg Trolox/mL)	5.09±0.03 ^a	4.91±0.04 ^b	4.93±0.02 ^b	4.74±0.11 ^c	4.65±0.01 ^c

TSS = Total soluble solids (°Brix); TPC = Total Polyphenol Content (mg GAE/mL); GAE = Gallic acid equivalent; DPPH = 2,2 diphenyl-1-picrylhydrazyl. Mean ± SD values in same row superscripted with different lowercase letters indicate significant ($p < 0.05$) differences.

or degrade the structure of polyphenol compounds (Emiljanowicz and Malinowska-Pańczyk, 2020; Kim et al., 2023). Polyphenol degradation can reduce radical scavenging abilities, leading to a decrease in antioxidant properties (Kim et al., 2023). The current result was in agreement with another study investigating functional drinks that reported a decrease in the TPC and antioxidant properties during storage (Anuyahong et al., 2020).

Microbial characteristics of selected kombucha treatment

Throughout the storage period, the viable count of LAB in the fermented kombucha fluctuated from day 0 to day 30 (Fig. 3). This may have been due to the detected ethanol content and continued production of organic acids that could have hindered LAB growth under stressful conditions (Majid et al., 2023). In addition, specific LAB strains can be expected to grow under anaerobic conditions with limited nutrients (Fusco et al., 2015). Consequently, these factors affected the fluctuation in the viable count of LAB in the fermented kombucha during refrigerated storage. Similar results were reported by Majid et al. (2023). Furthermore, the microbial assessment conducted on days 0 and 30 of refrigerated storage indicated the absence of spoilage or pathogenic microorganisms in the cannabis leaf-based kombucha infused with 10% tangerine pulp (Table 5). Thus, this product could be considered as safe for human consumption, in agreement with the Kombucha Brewers International Code of Practice (2021).

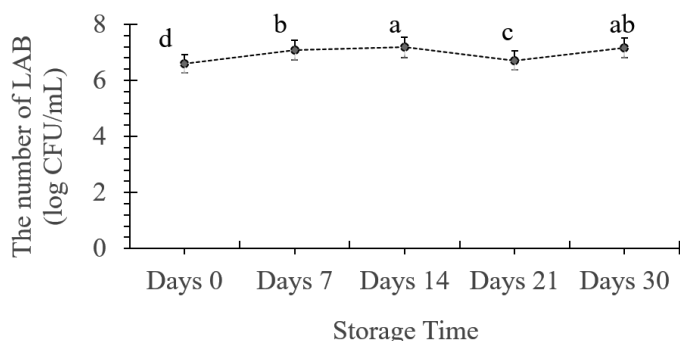


Fig. 3 The numbers of lactic acid bacteria (LAB) in cannabis leaf-based kombucha infused with 10% tangerine pulp during refrigerated storage, where error bars indicate mean $\pm$ SD ($n = 5$) and different lowercase letters indicate significant ($p < 0.05$) differences among the treatments.

Table 5 Microbial characteristics in cannabis leaf-based kombucha infused with 10% tangerine pulp for food safety appraisal

Parameter	Day 0	Day 30
Total coliform (MPN/mL)	< 3.0	< 3.0
<i>Escherichia coli</i> (MPN/mL)	< 3.0	< 3.0
<i>Staphylococcus aureus</i> (colonies/mL)	< 10 est	< 10 est

MPN = Most probable number est = estimated.

Conclusions

The second fermentation of cannabis leaf-based kombucha with infused pulps of tangerine, mahachanok mango or Thai orange cantaloupe affected the overall quality properties and LAB count. The abundance of LAB in the fruit-infused kombucha was a key to improving the taste, flavor, quality and functionality of the resulting kombucha. All fruit-infused kombucha samples had higher levels of polyphenols and antioxidant capacity than the control treatment. The second kombucha fermentation with fruit pulp contributed to an increase in total acidity, as well as lowering the pH and soluble solids. In addition, the color attributes changed, depending on the pigment compounds detected in each infused fruit pulp. Furthermore, the turbidity decreased and this influenced the appearance of the product. After 30 d of refrigerated storage, the pH of the fruit-infused kombucha was lower than 4.5 with a high number of LAB and no pathogenic microorganisms, thereby indicating that the product was safe for consumption.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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