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Changes in Bioactive Compounds, Antioxidant Activities and Chemical Properties of Pickled Tea By-Product Fermentation: Promising Waste Management and Value-Added Product

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Abstract: Pickled tea is an ethnic fermented product produced using Assam tea (*Camellia sinensis* var. *assamica*) leaves. It is produced in large quantities every year and the liquid waste from its production is estimated to be up to 2500 mL per every kilogram of pickled tea production. To reduce the waste, pickled tea juice remaining from the process was developed into (1) pineapple kombucha and (2) formulated functional drinks as "value added" products. The juice used for making kombucha was collected at 15 days of pickled tea fermentation due to its high value in antioxidant activity (previous study, 2250 μmol TE per g DW). After fermenting the juice with starter culture, the properties of pineapple kombucha were assessed at 0, 1, 3, 5, 7, 9, 11 days. Results showed that the total phenolic of pineapple kombucha was reduced, while antioxidant assay (FRAP and ORAC) slightly increased. The most suitable fermentation period of pineapple kombucha was at day 3. The formulated drink was made from mixing pineapple kombucha with ginger and lemon juice at various ratios including 100:0:0, 80:10:10 and 80:15:5. The ratio 80:10:10 gave the highest TP and antioxidant activity for the functional drink. In addition, for sensory analysis, liking attribute of 80:15:5 fermented juice kombucha pineapple favor was significantly higher compared to other formulations. The study demonstrates the promising second fermentation process of by-product juice from pickled tea production for the conversion to value-added functional drink with reasonable antioxidant properties.

Keywords: assamica tea; kombucha; fermentation; theaflavin; bioactive compounds; sustainability; value added product

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1. Introduction

Assam tea (*Camellia sinensis* var. *assamica*) originates from India, and its leaf is larger than Chinese tea and is specially processed to give a strong and robust flavor, however, it is less popular to consume as dried tea compared with Chinese tea. In Southeast Asian countries such as Thailand, Myanmar and Laos PDR, Assam tea is used mainly to produce an ethnic pickled tea using the submerged fermentation technique. Pickled tea is frequently consumed as a side dish with a meal. Pickled tea may also be used as an appetizer, being savory and aromatic, or as a snack. Moreover, it is commonly served by the Northern Thai people and Burmese at ceremonial occasions such as wedding ceremonies, house gatherings or housewarming events [1].

Bioactive compounds in teas vary depending on tea variety, processing methods and post-harvest treatments. In general, tea leaves contain several bioactive compounds including flavan-3-ols, flavonols, organic acids, theaflavins, purine alkaloids and amino acid. The high level of bioactive compounds in fresh tea leaves include catechins, monomeric

flavonols, and (-)-epigallocatechin gallate (EGC) [2], while the high level of bioactive compounds in green tea leaves include flavanols, flavandiols and phenolic acids such as gallic acid, coumaric acid, and caffeic acid. Additionally, pickled tea contains theaflavins and thearubigins that occur through the polymerization and antioxidants of some phenolic compounds present in the tea leaves [2].

Fermented juice is a liquid by-product from pickled tea production and is usually discarded. The estimation of the liquid waste discarded was 2500 mL per 1 kg of pickled tea produced, leading to environmental pollution. For example, air pollution and water pollution is associated with disposal in household areas and water sources. Previous research [3] showed that pickled tea leaves contain several bioactive compounds consisting of phenolic compounds including flavonoid, catechin, caffeine, fluoride, gallate derivatives including epicatechin (EC), epicatechingallate (ECG), epigallocatechin (EGC) and epigallocatechingallate (EGCG). For pickled tea, theaflavins and thearubigins are formed by polymerization of bioactive compounds and oxidation reaction of tea polyphenols. These chemical constituents exert functional bioactivities such as antioxidant and antimicrobial activities. Moreover, Bouphun et al. [4] found eight kinds of organic acids in young and mature fresh leaves: oxalic acid, tartaric acid, formic acid, ascorbic acid, acetic acid, citric acid, succinic acid, and fumaric acid. Acetic acid content was particularly high. During pickled tea processing, leaching of bioactive compounds from tea leaves into fermented juice was associated with enzymatic reactions, and bacterial digestion occurs. Thus, the juice potentially contains bioactive compounds, and it could be processed into functional products such as kombucha and other functional beverages.

Kombucha is a fermented beverage produced from a mixture of steeped tea and sugar, combined with a culture of yeast strains and bacteria. Some kombucha products also have fruit juice or other flavors added during production. The combination of sugar and yeast triggers fermentation, which results in kombucha containing 0.5% or more alcohol by volume. Research has shown that kombucha possesses health-promoting effects, such as prevention of cancer, high blood pressure, and improved digestive function [5]. Kombucha has a slightly acidic, carbonated, and sweet taste. The fermentation process is associated with the symbiosis of bacteria and yeasts as kombucha starter. The common substrate of kombucha is black tea or green tea. The bacterial species of greatest abundance in kombucha culture are *Komagataeibacter* spp., *Acetobacter* spp. and *Lactobacillus* spp., while *Zygosaccharomyces* spp. and *Brettanomyces* spp. are the most abundant yeasts [6,7]. During fermentation acetic acid bacteria produce the cellulose pellicle layer and biofilm called "scoby". Based on our previously published data, pickled tea contained high levels of antioxidants and several bioactive compounds that are formed during fermentation [3]. The authors expanded the work on the by-product juice completing the sustainable production of the pickled tea process. Therefore, the aim of this study is determining the proper fermentation period of the pickled tea juice that results in highest antioxidant properties and determine the functional property of fermented juice as well as develop kombucha from pickled tea juice as a functional value-added beverage that simultaneously reduces waste.

2. Materials and Methods

2.1. Tea Leaf Harvest

Tea leaves (3rd–5th leaves from the top of the tree) were handpicked from Assam trees organically grown at Ban Si Na Pan located in Nan province in October 2019. The tea leaves were green and dark green with the length of 10–15 cm. Tea leaves were tie to wood stalks forming bunches of tea leaves (10–15 leaves). Symbiotic starter culture of bacteria and yeast (Scoby) was obtained by natural fermentation of black tea with sugar. Identification of bacteria was conducted.

2.2. Fermentation Process

2.2.1. Tea Leaves Fermentation to Obtain By-Product Fermented Juice

The tea leaves sample (200–250 g) was cleaned with running water and then steamed at 100 °C for 1 h and cooled at room temperature. Then, the steamed leaves were packed in containers and covered with cheese cloth and held under aerobic conditions for 2–3 days depending on the fungal growth rate (visible white filamentous growth on the leaves). Next, the steamed tea leaves covered with mature fungal growth were washed with water. A 1000 g portion of washed tea leaves, 8 g of salt, 8 mL of starter fermentation juice from Ban Si Na Pan and 2500 mL of DI water were combined and fermented under anaerobic condition. The by-products, fermented juice samples, were collected at the 0, 7, 15, 30, 60 days. Samples were stored at −20 °C for analysis and subsequently made into kombucha and functional drink.

2.2.2. Kombucha Fermentation with Pineapple

Fermented juice, which is a by-product of tea leaves fermentation, was used as a substrate for kombucha production. Fermented juice (from day 15, based on highest antioxidant activity) was diluted with water at a ratio 1:1.5 and addition of 10% of sucrose and 10% of fresh pineapple juice v/v were also added. The mixture was boiled until the sucrose was completely dissolved. The mixture was then cooled to room temperature. The solution was poured into a glass jar, pasteurized in a water bath(Hanyang Scientific, Seoul, Korea), and inoculated with the addition of 2.5% (w/v) scoby and 3% (v/v) of the solution from the scoby. The jar was covered with a clean white cloth that was tightly fastened. Fermentation was carried out in the dark in an incubator (Memmert Model IN110, Schwabach, Germany) at 25 °C for 11 d or until a cellulose film on the kombucha scoby was visible.

2.2.3. Formulation of Pineapple Kombucha as Functional Drink

The kombucha obtained as the second fermentation was formulated into 2 ginger and lemon flavored drinks by varying the ratio of ginger and lemon. Ratio of kombucha: ginger: lemon were varied as 160:20:20 mL and 160:30:10. All 3 ingredients were mixed and then stirred to ensure thorough mixing.

2.3. Chemical Properties Determination

2.3.1. pH Measurement and Total Soluble Solid

Changes in pH of kombucha were measured using a pH meter (F20, Mettler Toledo, Bangkok, Thailand). Total soluble solids was measured using a hand refractometer (brix N1, Atago, Tokyo, Japan) at 25 °C.

2.3.2. FRAP Determination

The antioxidant activity (AOA) of fermented juice samples was determined using the ferric reducing ability of plasma (FRAP) assay from Somsong et al. [3]. The working FRAP reagent was prepared by mixing 300 mM acetate buffer, pH 3.6, with 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM of hydrochloric acid and 20 mM of $FeCl_3 \cdot 6H_2O$ at a ratio of 10:1:1. The acetate buffer (pH 3.6) was prepared using 3.1 g sodium acetate trihydrate and 16 mL acetic acid in 1 L distilled water. 20 µL of a diluted sample was added into a well of a 96-well plate and 200 µL of FRAP working solution added. After that, samples absorbance was read at 593 nm after 30 min in a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). A standard curve was prepared using different concentrations (100–600 µM L1) of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and the results were expressed as µmol Trolox equivalents per 100 mL (µmol TE/100 mL) or µmol Trolox equivalents per gram (µmol TE/g).

2.3.3. ORAC Determination

The assay was based on the procedure described by Somsong et al. [3]. The reaction was carried out in 75 mM phosphate buffer (pH 7.0). The samples or standard (20 µL) and fluorescein (160 µL; 120 mM) solutions were pre-incubated for 15 min at 37 °C; then 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) solution (20 µL; 480 mM) was added to initiate the reaction. The fluorescence measurement (Ex. 485 nm, Em. 520 nm) was recorded every minute for 80 min. A standard curve was prepared using Trolox (5–50 µM). The results were expressed as µmol Trolox equivalents per 100 mL (µmol TE/100 mL) or µmol Trolox equivalents per gram (µmol TE/g).

2.3.4. Total Phenolic Content (TPC)

The total phenolic content of the samples was determined based on the method of Somsong et al. [3]. Samples were diluted 10 times with distilled water and a 150 µL volume was further diluted with 2.4 mL distilled water. This was followed by adding 150 µL of diluted (1:10) Folin–Ciocalteu reagent (2 N). The solutions were mixed well for 2 min before adding 7.5% (*w*/*v*) of 300 µL Na_2CO_3 to account for an ascorbic acid correction and mixed again. After incubation for 2 h at room temperature in the dark, the absorbance at 765 nm was determined. Gallic acid concentration was used to establish the standard curve ranged from 0.01–0.1 mg/mL. The results were expressed as mg gallic acid equivalents/100 mL (mg GAE/100 mL).

2.3.5. Total Flavonoid Content (TFC)

The flavonoids content was determined by aluminium trichloride method using catechin as a reference compound [8]. A volume of 125 µL of standard was added to 75 µL of a 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min, then 150 µL of aluminium trichloride (1%) was added and incubated for 5 min followed by the addition of 750 µL of NaOH (1 M). The final volume of the solution was adjusted to 2500 µL with distilled water. After 15 min of incubation, the mixture turned pink, and the absorbance was measured at 510 nm using a microplate reader. The total flavonoids content was expressed as mg catechin equivalents per 100 mL (mg CE/100 mL) or mg catechin equivalents per gram (mg CE/g).

2.3.6. Theaflavin Determination (TR)

Quantification of tea pigment was performed using an HPLC system (Agilent technologies 1260 series, Waldbronn, Germany). Fermented tea juice samples were filtered through 0.45 µM nylon syringe filters. Then, the standard solutions and extract samples were injected in HPLC VertiSep^{TM} UPS Columns18 (150 mm × 4.6 mm, 3 µm). The mobile phases were (A) water containing 20 mL/L acetic acid and (B) 7:1 acetonitrile: ethyl acetate, by volume. The linear gradient of mobile phase B was followed: 0–15 min, from 18% to 30%, followed by 18% for 5 min. The operating column temperature was maintained at 25 °C. The injection volume was 20 µL, and the flow rate was 0.9 mL/min. The results were compared with theaflavin standards [9].

2.4. Consumer Acceptability of Functional Drink

The protocol of sensory evaluation was approved by Mahidol University Central Institutional Review Board (Protocol Code: MU-CIRB 2019/315.0912). In-house sensory evaluation was conducted at the Institute of Nutrition, Mahidol University to assess the sensory quality of the functional drinks. Samples were evaluated by 33 untrained panelists. Functional drinks (10 mL) of both formulations were served cold (10–15 °C) in a clear 1-oz plastic cup. Samples were coded with 3-digit random numbers. Each panelist was asked to rate the overall quality on a 9-point hedonic scale (9 = like extremely, 5 = neither like nor dislike and 1 = dislike extremely) based on aroma, color, appearance, tea flavor, sour, sweet, bitter, sparkling and overall acceptability. Participants rinsed their mouths with drinking water before and between tasting the samples.

2.5. Statistical Analysis

Experiments were conducted using a complete block design with at least 3 replicates for the fermented tea method, analyzing functional property of fermented tea samples and formulating fermented tea drink. The sensory evaluation was conducted in Randomized Complete Block Design (panelist as a block). The data of formulated fermented tea drink were analyzed using dependent sample *t*-test or one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. *p*-value < 0.05 were considered significant. All statistical analysis was processed using computer software (IBM SPSS Statistics 19.0, IBM, Armonk, New York, NY, USA).

3. Results and Discussion

3.1. Comparison of Bioactive Compounds in Fermented Tea Leaves and Its Fermented Juice

During fermentation there was a notable change in pH. In the experiment, there are three stages of transition. First, the pH rapidly decreased from 4.74 to 4.14 with a significant difference ($p < 0.05$) from day 0 to day 60, which correlated to an increase in acetic acid content from 0.02–0.17%. (Table 1). The changes in pH value of the fermented juice were similar in pickled tea, kombucha, and Puerh tea [10,11]. These results are in agreement with several studies where the pH of fermented tea leaves decreased from 5.35 to 4.67 within 60 days [11].

Table 1. Comparison of pH, acetic acid, total phenolic, antioxidant activity (FRAP and ORAC) and total flavonoid of fermented juice at different fermentation periods.

Fermentation Period	Theaflavin (mg/100 mL)	Acetic Acid (l %)	pH	Total Phenolic (μmol GAE/100 mL)	Antioxidant Activity (mmol TE/100 mL)		Total Flavonoid (mg CE/100 mL)
					FRAP Assay	ORAC Assay	
Day 0	N/D ^{c}	0.02 ± 0.00 ^{c}	4.74 ± 0.30 ^{a}	11.93 ± 1.76 ^{b}	46.09 ± 2.25 ^{e}	215.05 ± 26.92 ^{c}	6.67 ± 3.62 ^{d}
Day 7	2.28 ± 0.69 ^{b}	0.12 ± 0.00 ^{b}	4.51 ± 0.04 ^{b}	83.02 ± 1.97 ^{a}	522.47 ± 10.79 ^{a}	1663.85 ± 163.23 ^{b}	22.08 ± 2.43 ^{c}
Day 15	3.98 ± 1.84 ^{b}	0.12 ± 0.01 ^{b}	4.42 ± 0.05 ^{c}	85.29 ± 1.43 ^{a}	395.06 ± 10.85 ^{b}	1936.16 ±125.82 ^{a}	25.19 ± 7.00 ^{bc}
Day 30	7.28 ± 0.20 ^{b}	0.13 ± 0.01 ^{b}	4.33 ± 0.20 ^{d}	81.36 ± 4.20 ^{a}	279.48 ± 4.36 ^{d}	2077.34 ± 15.86 ^{a}	29.82 ± 5.64 ^{b}
Day 60	23.21 ± 12.74 ^{a}	0.17 ± 0.02 ^{a}	4.14 ± 0.19 ^{e}	85.44± 5.56 ^{a}	294.48 ± 15.98 ^{c}	1913.78 ±107.26 ^{a}	37.61 ± 5.72 ^{a}

^{a,b,c,d,e} Means within the same column with different superscripts are significantly different ($p < 0.05$).

The production of acetic acid was due to the presence of acetic acid bacteria: cetobacterium. Previous research [4] discovered the level of acetic acid in fermented tea leaves increased during fermentation and became stable at 30 d.

The TPC of fermented juice rapidly increased significantly from day 0 to day 7, and then remained rather stable for 60 days. Conversely, TPC in fermented tea leaves during pickled tea processing slightly dropped from day 0 to day 30, and finally it decreased significantly at day 60 of fermentation (Table 2) compared with previous studies where the TPC of pickled tea was reduced approximately 5.8–26.6%. Pickled tea with a longer fermentation process has a lower phenolic content [3,12]. This could be explained through the solubility of water-soluble compounds, including phenolic compounds catechin and epigallocatechin (EGCG), which are the predominant tea polyphenols from tea leaves that leached into the fermented juice [4]. Results show that in fermented juice TPC was higher with a longer fermentation process (Table 1), which is correlated with the decrease in TPC of tea leaves.

Pickled tea can be fermented from 15 days up to a year depending on the desired degree of sourness and taste of the product. The fermentation period between 15–60 days was selected for this study since it is a commonly used range. It can be seen in Table 1 that at different fermentation periods, bioactives and antioxidants in fermented juice were different. The pH value was reduced from 4.7 to 4.1, which resulted in pickled tea moving into the acid food category. This enables the long-term storage of pickled tea.

Table 2. Comparison of total phenolic, antioxidant activity (FRAP, ORAC) and total flavonoid of different fermentation day in pickled tea leaves.

Fermentation Period	Theaflavin (mg/g)	Total Phenolic (µmol GAE/g)	Antioxidant Activity (mmol TE/g)		Total Flavonoid (mg CE/g)
			FRAP Assay	ORAC Assay	
Day 0	0.17 ± 0.01^{a}	1.64 ± 0.15^{a}	7.40 ± 1.24^{ab}	15.13 ± 0.15^{a}	9.51 ± 1.05^{a}
Day 7	0.04 ± 0.00^{b}	1.16 ± 0.18^{ab}	7.02 ± 0.32^{ab}	12.39 ± 2.47^{ab}	4.63 ± 0.14^{b}
Day 15	0.02 ± 0.00^{c}	1.40 ± 0.41^{a}	7.34 ± 0.85^{ab}	8.47 ± 0.36^{cd}	4.87 ± 0.58^{b}
Day 30	0.03 ± 0.01^{c}	1.12 ± 0.41^{ab}	7.57 ± 0.18^{a}	9.79 ± 0.05^{bc}	4.84 ± 0.08^{b}
Day60	0.01 ± 0.00^{d}	0.50 ± 0.29^{b}	5.79 ± 0.41^{c}	6.28 ± 0.45^{e}	4.12 ± 0.19^{b}

^{a,b,c,d,e} Means within the same column with different superscripts are significantly different ($p < 0.05$).

The TPC of fermented juice rapidly increased from day 0 to day 7, and then remained relatively stable until day 60. On the contrary, the results of TFC in pickled tea leaves during pickled tea processing showed a rapid decreased from day 0 to 7, and then remained stable until 60 days of fermentation. This result for fermented tea leaves was similar with a previous report of pickled tea that was fermented using anaerobic solid-state controlled conditions [10]. The study showed that TFC of pickled tea was reduce approximately 30.8% from day 0. Hence, a longer fermentation period resulted in lower flavonoid content because the monomeric flavonoids were transformed into polymeric: TF and TR. Additionally, some compounds may have been dissolved in water such as catechin, gallic acid, TF and TR [4]. It could be seen that with a longer fermentation process, the fermented juice contained higher TFC and theaflavin (Table 1).

TF was not present in fermented juice at day 0. The TF slightly increased from 2.28 mg/100 mL at day 7 to 7.28 mg/100 mL at day 30 (Table 1). TF then rapidly increased to 23.21 mg/100 mL by day 60. Conversely, TF in pickled tea leaves had declined from 0.17 to 0.01 mg/g as shown in Table 2. For day 0, TF in pickled tea was detected because filamentous fungi that grow on the surface of tea leaves prior to the initial fermentation convert catechins into TF and TR [13–15]. The TF of pickled tea rapidly decreased from day 0 to day 7 then remained stable from day 15 to day 30, and finally, it decreased at day 60. It is possible that TF could be dissolved in water, which correlated with the increase of TF in the fermented juice (Table 1).

Antioxidant activity (AA) of pickled tea and its juice was inversely proportioned. The FRAP of fermented juice during pickled tea fermentation is shown in Table 1. The FRAP of fermented juice rapidly increased from day 0 d to day 60. On the other hand, the FRAP value of pickled tea during processing increased slightly from day 0 d to day 30, and finally it significantly decreased at 60 days of fermentation. Although FRAP usually shows strong correlation with phenolic and flavonoids, this was not the scenario with samples in the present study. This could be due to the presence of other compounds in the pickled tea that suppress the electron-donating ability of flavonoids, contributing to the FRAP values. Since the fermentation etiology is symbiotic, this may result in several complex compounds.

ORAC assay results of fermented juice during pickled tea fermentation are shown in Table 2. The ORAC of fermented juice rapidly increased from day 0 to day 7, slightly increased from day 7 to day 15 and it was relatively stable until day 60. On the other hand, the results of ORAC in pickled tea during processing showed a decrease from day 0 to day 15, increased slightly from day 15 to day 30, and then significantly decreased at 60 days of fermentation. It could be due to the presence of tannin and other proteins that inhibit hydrogen transfer [16] as well as the possible interaction of new compounds. The implementation of omics technology could be used to enhance understanding the dynamic change of this etiology.

Compared with previous studies that reported the reduction of antioxidant activity in pickled tea for FRAP and ORAC from 20.2–40.7% and 24.78–38.9%, respectively, pickled

tea produced from a longer fermentation process has lower antioxidant activity [3,17]. This could be due to the increased solubility of antioxidant compounds, including phenolic compounds: catechin, epigallocatechin (EGCG) and epicatechin (EC) [4]. It was shown in fermented juice that antioxidant activity was higher with a longer fermentation process (Table 1).

3.2. Kombucha Physiochemical Analysis

3.2.1. pH and Total Soluble Solid

Fermented juice, a by-product from pickled tea production, was used to make kombucha using scoby as the starter. The bacteria identified from the scoby film were Komagataeibacter saccharivorans, Zygosaccharomyces bailii, and Dekkera kand yeast. This shows the symbiotic ecology during fermentation. In this study, the flavor of kombucha was made into pineapple flavors. Pineapple was chosen since it would impart pleasant flavor and smell. The results of pH and total soluble solids at intervals of 0, 1, 3, 5, 7, 9, 11 days in original favor are shown in Table 3. The pH and total soluble solids during fermentation of kombucha decreased rapidly. The decrease in pH value was attributed to acid production during fermentation. There are two stages in fermentation: alcohol and acetic acid fermentations [18]. In the alcoholic fermentation stage, yeast hydrolyzes sucrose into glucose and fructose, which metabolized into ethanol may explained the decrease of total soluble solids. Concurrently, acetic acid fermentation occurred, the acetic acid bacteria used glucose to produce glucuronic acid and transform ethanol into acetic acid [19]. Therefore, total soluble solids decreased significantly at day 0, 7, 9 and 11 (Table 3).

Table 3. Comparison of pH total soluble (TSS), total phenolic, antioxidant activity (FRAP, ORAC) and total flavonoid between fermentation days of pineapple kombucha.

Fermentation Period	pH	Total Soluble Solid (°Brix)	Total Phenolic (μmol GAE/100 mL)	Total Flavonoid (mg CE/100 mL)	Antioxidant Activity (mmol TE/100 mL)	
					FRAP Assay	ORAC Assay
Day 0	3.95 ± 0.01 ^{a}	16.18 ± 1.67 ^{a}	23.1 ± 0.68 ^{b}	4.83 ± 0.34 ^{a}	101.52 ± 24.22 ^{a}	431.66 ± 112.35 ^{ab}
Day 1	3.88 ± 0.00 ^{a}	16.04 ± 1.37 ^{a}	23.83 ± 5.56 ^{ab}	4.82 ± 0.38 ^{a}	101.89 ± 37.96 ^{a}	283.39 ± 39.23 ^{b}
Day 3	3.75 ± 0.01 ^{a}	15.02 ± 1.24 ^{a}	23.78 ± 2.44 ^{ab}	4.20 ± 0.90 ^{ab}	106.32 ± 22.09 ^{a}	594.71 ± 116.03 ^{a}
Day 5	3.44 ± 0.01 ^{b}	12.62 ± 0.60 ^{b}	25.05 ± 0.40 ^{ab}	3.27 ± 0.18 ^{bc}	109.16 ± 11.98 ^{a}	461.00 ± 305.12 ^{ab}
Day 7	3.30 ± 0.01 ^{b}	9.96 ± 0.87 ^{c}	26.07 ± 2.45 ^{ab}	2.66 ± 0.81 ^{c}	139.81 ± 12.63 ^{a}	506.84 ± 212.01 ^{ab}
Day 9	3.23 ± 0.01 ^{b}	8.40 ± 0.58 ^{c}	25.93 ± 1.88 ^{ab}	2.13 ± 0.50 ^{c}	129.63 ± 32.11 ^{a}	277.49 ± 76.09 ^{b}
Day 11	3.17 ± 0.01 ^{b}	7.56 ± 0.64 ^{c}	29.57 ± 1.20 ^{a}	2.12 ± 0.31 ^{c}	136.73 ± 11.68 ^{a}	281.10 ± 94.66 ^{b}

^{a,b,c} Means within the same column with different superscripts are significantly different ($p < 0.05$).

3.2.2. Total Phenolic Content and Total Flavonoid Content

The TPC values of the original kombucha fluctuated during fermentation but were not significantly different at day 3 to day 5 and day 9 to day 11 (Table 3). The shifting in TPC may be the result of complex phenolic compounds, which may degrade in the acidic environment or through enzymatic activity of bacteria or yeast in scoby. In particular, *Saccharomyces* sp., *Lactobacillus* sp., and *Acetobacter* sp., which have ability to excrete tannase, an enzyme that degrades tannins [20,21]. The TFC of the original kombucha was slightly decreased (Table 3). The TPC varied from 10.47 ± 1.18 to 10.47 ± 1.18 mg CE/100 mL. The significant tuning point of the TFC value occurred at day 5 (8.13 ± 0.68 mg CE/100 mL) (Table 3). Consequently, on the first day, the highest content of catechin occurred in fermented tea leaves [22,23] Thereafter, during kombucha fermentation, the catechins formed dimeric catechins called theaflavins, a larger compound called pro-anthocyandins, and very large oligomers and polymers called thearubigens. The chemical structures of these flavonoid oligomers in tea are very complex and have yet to be entirely characterized [24,25].

3.2.3. Antioxidant Activity

The antioxidant activity slightly increased during fermentation (Table 3). Antioxidant activity fluctuated during fermentation. The antioxidant activity decreased at day 3. After day 5 of fermentation the antioxidant activity increased slightly and was similar with TPC, with the significantly higher content at day 9 on FRAP for the highest content at day 7, then for the fermentation period of ORAC. The fluctuation of antioxidants might be due to the activity of the microorganism during the fermentation process and the stability of some antioxidant compounds. The microorganisms may compete to degrade polyphenolic compounds [14]. Zhao and Shah [26] reported that after fermentation, TPC increased in phenolic acids, derivatives, and flavan-3-ols and decreased in flavonols. In the present study, we found that TPC increased but total flavonoid deceased.

3.3. Formulation of Functional Drink

The pineapple kombucha was further formulated (Figure 1) by dilution and mixing with ginger and lemon (Table 4).

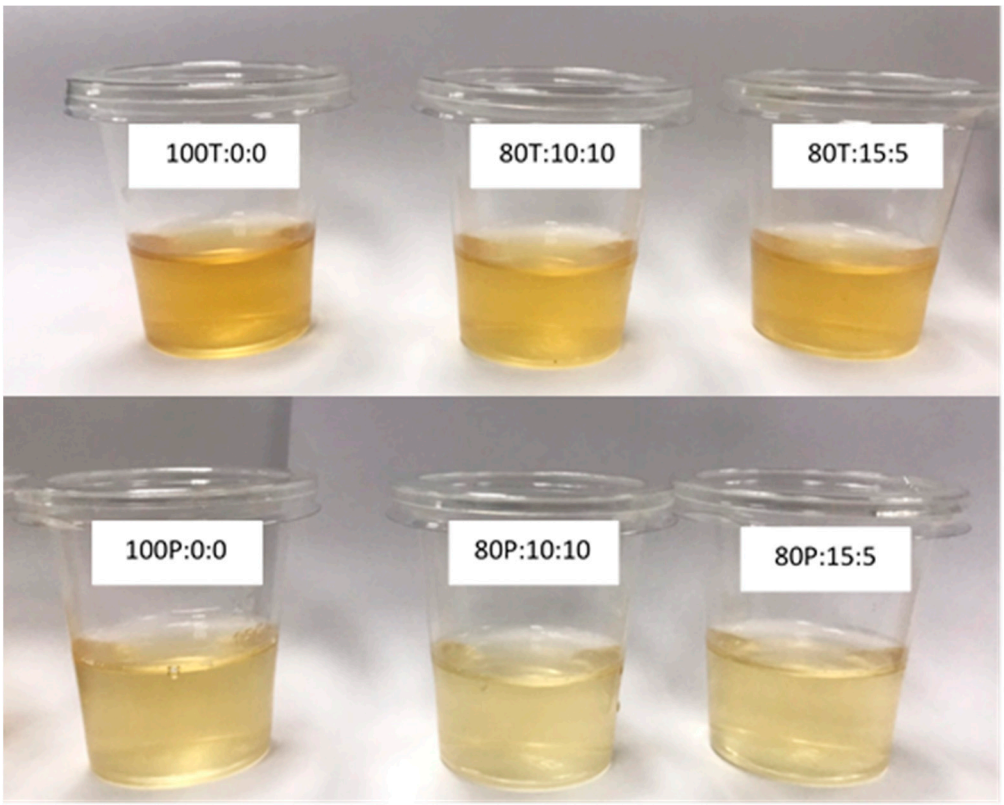

Figure 1. Appearance of fermented kombucha juice (mixing ratios for Kombucha: ginger: lemon are 100T:0:0; 80T:10:10; 80T:15:5; 100P:0:0; 80P:10:10 and 80P:15:5, where T is fermented juice kombucha original flavour with fermentation at 5 days, *p* is Fermented juice kombucha pineapple flavour with fermentation at 3 days).

Evaluation of formulated ginger and lemon kombucha was performed by 33 untrained anellists composed of 78.89% female and 21.21% male, between 23–60 years old. The sensory attributes tested included, color, odor, tea flavor, sour, sweet, bitter, sparkling, and overall acceptability. The 9-point hedonic scale was used for the quality assessment with the liking scores ranged from 1 (dislike extremely) to 9 (like extremely).

Table 4. Comparison of pH total soluble solid (TSS), total phenolic, antioxidant activity (FRAP, ORAC) and total flavonoid between formulation of fermented juice kombucha with ginger and lemon.

Sample	pH	Total Soluble Solid (°Brix)	Total Phenolic (μmol GAE/100 mL)	Total Flavonoid (mg CE/100 mL)	Antioxidant Activity (mmol TE/100 mL)	
					FRAP Assay	ORAC Assay
100P:0:0	3.23 ± 0.00 ^{b}	15.47 ± 0.12 ^{a}	14.81 ± 2.60 ^{c}	8.55 ± 0.78 ^{c}	100.35 ± 11.85 ^{b}	228.87 ± 37.47 ^{c}
80P:10:10	2.99 ± 0.10 ^{cd}	12.33 ± 2.02 ^{b}	15.28 ± 5.19 ^{c}	5.26 ± 0.36 ^{d}	135.20 ± 37.78 ^{b}	209.71 ± 39.38 ^{c}
80P:15:5	3.06 ± 0.00 ^{c}	11.73 ± 0.12 ^{b}	10.99 ± 1.95 ^{c}	6.35 ± 0.78 ^{d}	73.91 ± 12.98 ^{b}	140.42 ± 23.12 ^{d}

Mixing ratios for Kombucha: ginger: lemon are: 100T:0:0; 80T:10:10; 80T:15:5; where T is fermented juice kombucha original flavor with fermentation at 5 days. ^{a,b,c,d} Means within the same column with different superscripts are significantly different ($p < 0.05$).

The kombucha had a low pH around 2.8–4.0 (slightly acidic, mildly sweet and moderately sparkling). The fermented kombucha juice without mixing with ginger juice and lemon juice had lower pH and moderate TSS (Table 5), which might contribute to the decreased acidity and sweetness of the kombucha. Therefore, the panelist had preference for kombucha with subtle tastes (i.e., less sour and medium sweet). The liking scores of fermented juice kombucha were higher than 6, suggesting that the kombucha samples were acceptable to the panelists. As reported previously in the acceptability study of several types of soy whey kombucha beverages, the overall acceptability scores ranged from 2.5 (dislike very much) to 6.7 (like slightly) on a 10-point scale. Soy whey kombucha at day-8 fermentation had a 2 score and soy whey kombucha at day-6 fermentation had a 6.7 score. Soy whey kombucha at day 8 fermentation had a pungent and intense sour smell. Consequently, the panelists gave low ratings for kombucha that was too acidic. Moreover, Soy whey kombucha had 2.5 score associated with cloudy color, thus, the lower scores associated with the opaque appearance and dark color of kombucha. The rather low ratings could be explained by panelist's perceptions of kombucha [5].

Table 5. Sensory evaluation of functional drink, which is the mixture of kombucha with ginger and lemon at different ratios.

Sensory Attribute	100P:0:0	80P:10:10	80P:15:5
Color	6.09 ± 1.82 ^{a}	5.48 ± 2.11 ^{a}	6.03 ± 1.62 ^{a}
Oder	6.16 ± 1.61 ^{a}	6.13 ± 1.94 ^{a}	6.13 ± 1.77 ^{a}
Tea flavor	6.26 ± 1.67 ^{ab}	6.25 ± 1.80 ^{ab}	6.71 ± 1.65 ^{a}
Sour	6.23 ± 2.10 ^{ab}	6.31 ± 1.65 ^{ab}	6.72 ± 1.51 ^{a}
Sweet	5.77 ± 1.10 ^{ab}	5.97 ± 2.18 ^{ab}	6.61 ± 1.50 ^{a}
Bitter	6.00 ± 1.10 ^{ab}	6.03 ± 2.02 ^{ab}	6.50 ± 1.61 ^{a}
Sparking	5.75 ± 1.85 ^{ab}	6.06 ± 1.98 ^{ab}	6.23 ± 1.93 ^{a}
Overall acceptability	6.44 ± 1.81 ^{a}	6.50 ± 1.68 ^{a}	6.84 ± 1.42 ^{a}

Mixing ratios for Kombucha: ginger: lemon were 100P:0:0; 80P:10:10 and 80P:15:5, P is Fermented juice kombucha pineapple flavor with fermentation at 3 days. Rated on a 9-point hedonic scale. Means ± standard deviations of 33 panelists. ^{a,b} Means within the same row with different superscripts are significantly different ($p < 0.05$).

4. Conclusions

Kombucha beverages were successfully produced by using fermented tea juice, a waste by-product from pickled tea production. Theaflavin and antioxidant properties of pickled tea and fermented juice of pickled tea production were analyzed. Fermented juice was analysed for pH and acetic acid. TPC, FRAP, and ORAC values of fermented juice were higher at day 15 of pickled tea leaves production. The TPC, FRAP, and ORAC values of pickled tea were higher at day 15 of pickled tea production. Under conditions evaluated in the present study, a 15-day fermentation was determined optimal for pickled tea production.

The kombucha beverages developed are suitable for health promotion, having significantly higher total phenolic and antioxidants following fermentation. Based on ORAC

testing of pineapple flavor kombucha, day 3 had the highest antioxidant activity. The fermented juice kombucha, both original flavor at day 5 and pineapple flavor at day 3, were formulated with ginger drink and lemon juice. The highest TP and antioxidant activity (FRAP, ORAC) of fermented juice kombucha, both original flavor and pineapple flavor, were 80:10:10 of ratio. However, overall sensory acceptability was significantly higher in fermented juice kombucha in the pineapple flavor. Various factors may contribute to these changes, including climate, season, and varieties. In terms of selecting high antioxidant tea or pineapple, attention must be focused on plantation, season and species. Moreover, the value-added products are environmentally friendly in terms of waste reduction and provide social and economic benefits. For future studies, it would be interesting to analyze shelf-life properties of fermented juice kombucha, both original flavor and pineapple flavor. The fermented juice kombucha could be blended with other fruits or herbs, which may increase antioxidant properties.

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