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Enhancing the Chemical Composition of Kombucha Fermentation by Adding Indian Gooseberry as a Substrate

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Abstract: Kombucha is a fermented tea beverage obtained by the symbiosis of yeast, acetic acid bacteria and some lactic acid bacteria, and it has many health benefits. The aim of this study was to investigate the potential of adding Indian gooseberry as a substrate to enhance the chemical properties of kombucha. In this study, traditional kombucha made from green tea was compared to kombucha made from green tea blended with various forms of Indian gooseberry, including whole fruit, dried fruit and juice. The fermentation was performed for 21 days and samples were collected every 3 days to enumerate the total number of yeast and bacteria. Physical and chemical properties, including total soluble solids, alcohol content, pH, acetic acid content, total phenolic and flavonoid content, antioxidant activity and organic acids, were analyzed. The results revealed that the dried Indian gooseberry kombucha (DIGK) demonstrated significantly high total phenolic content and total flavonoid content. In addition, DIGK had the highest D-Saccharic acid-1,4 lactone (DSL) on the 9th day of fermentation. This discovery suggests that dried Indian gooseberry can be used as an alternative substrate for kombucha fermentation to create a new type of kombucha beverage with enhanced chemical properties.

Keywords: kombucha; Indian gooseberry; antioxidant activity; organic acids

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1. Introduction

Kombucha is a fermented tea beverage with a slightly sparkling, sweet and sour taste that originated in China 2000 years ago. The traditional kombucha is produced using black tea or green tea as a substrate and fermenting by the symbiosis of yeast, acetic acid bacteria and some lactic acid bacteria [1,2]. The chemical composition of kombucha influenced by the amount of metabolites produced by microorganisms, the type of substrate used and fermentation parameters (time, temperature and amount of oxygen). Previous studies demonstrated that kombucha contains chemical components, including ethanol, organic acids (acetic, gluconic, glucuronic, citric, L-lactic, malic, tartaric, malonic, oxalic, succinic, pyruvic, usnic, and D-saccharic acid-1,4-lactone (DSL)), tea polyphenols (catechins, theaflavins and flavonoids), water-soluble vitamins (B_1, B_2, B_3, B_6, B_{12}, C, E and K), minerals (Cu, Fe, Mn, Ni and Zn), amino acids and proteins [1–6]. It has been suggested that consuming kombucha can provide multiple health benefits from its anti-inflammatory, antioxidant, antimicrobial, antidiabetic, anti-hypercholesterolemic and anti-proliferative properties. Moreover, activities as hepatoprotective, neuroprotective and treatment of digestive diseases are observed [1,7–9].

Although black tea, green tea or oolong tea are used as the main substrate for producing kombucha, there are many studies using other raw materials as an alternative substrate [8,10]. In recent studies, fruits, vegetables and herbs have been used [7], such as blueberry [11], orange [12], snake fruits [13], pomegranate, red grape, sour cherry, apple [14], African mustard leaves [15], wheatgrass [16], wax mallow flower [5], chrysanthemum, honeysuckle, mint [17], yarrow flower [18] and lemon balm [19,20]. Those results indicated that the benefits, chemical and biological properties of kombucha produced from the alternative substrates were higher than those produced from the traditional kombucha.

Indian gooseberry (*Phyllanthus emblica* Linn.) is a native fruit of India that is also found in Sri Lanka, Uzbekistan, China and Thailand as well as other countries in Southeast Asia. The fruit is a rich source of vitamin C and contains a variety of bioactive phytochemicals, including polyphenols, flavones, tannins, glycosides, terpenoids and other bioactive substances [21,22]. This fruit has been used in various traditional folk systems of medicine in Asian countries and also in the traditional Indian system of Ayurvedic medicine [23–25]. Fresh or dried fruit is used for the treatment of jaundice, diabetes, diarrhea, fever and cough, etc. [21,25–27]. Scientific studies have shown that Indian gooseberry has various biological activity such as antioxidant, antimicrobial, anti-diabetic, anti-inflammatory, anticancer, anti-hypercholesterolemia and hepatoprotective [25–27].

In this study, the potential of incorporating Indian gooseberry into kombucha production to create a new type of kombucha beverage and improve its chemical properties was explored. The study compared traditional kombucha made from green tea with kombucha from green tea mixed with different forms of Indian gooseberry, such as whole fruit, dried fruit, and juice.

2. Materials and Methods

2.1. Preparation of Kombucha Starter

Green tea leaves and starter culture were collected from Tea Gallery Group (Thailand) Co., Ltd., Chiang Mai, Thailand. The green tea broth was prepared by adding 1% (*w/v*) of green tea leaves to boiling water. After 10 min, the tea leaves were sieved and 10% (*w/v*) of sucrose was added to prepare sugared green tea. Then, 400 mL of sugared green tea was poured into a 500 mL Duran bottle, a 10% (*v/v*) starter culture was added. The Duran bottle was covered with a cloth sheet and secured with rubber bands, then it was incubated for 10 days under aerobic conditions at 30 ± 2 °C.

2.2. Preparation of Indian Gooseberry

Indian gooseberry (*Phyllanthus emblica* Linn.) was purchased from Mae Tha Market, Lamphun province, Thailand. The fruit was washed and stored at −20 °C until used, while the dried fruit was prepared by drying in a hot air oven (BINDER) at 60 °C for 5 days.

2.3. Evaluation of Indian Gooseberry as a Supplement in Kombucha Fermentation: A Comparative Study of Five Treatments

Three forms of Indian gooseberry were used as a supplement substrate for kombucha fermentation, including whole fruit, dried fruit, and juice. The experiments were divided into five treatments with the traditional kombucha (TK) being used as a control (Figure 1). The Indian gooseberry fruit and sugared green tea were mixed in 1:9 ratio (*w/v*) to prepare Indian gooseberry juice kombucha (IGJK) and Indian gooseberry kombucha (IGK). Dried Indian gooseberry and sugared green tea were mixed in the ratio of 275 g/9 l (1 kg of fresh fruit = 275 g of dried fruit) to prepare dried Indian gooseberry kombucha (DIGK). The fermented Indian gooseberry juice (IGJ) was also prepared by using Indian gooseberry in the same ratio as other treatments but without green tea (Figure 1). The fermentation process was performed by adding a 10% (*v/v*) starter culture to 400 mL of each treatment in a 500 mL Duran bottle, which was then covered with a cloth sheet and fastened with rubber bands. The bottles were incubated for 21 days under aerobic conditions at

30 ± 2°C. Samples were collected by taking three Duran bottles per treatment every 3 days for composition analysis. After that, the Duran bottles of samples were discarded.

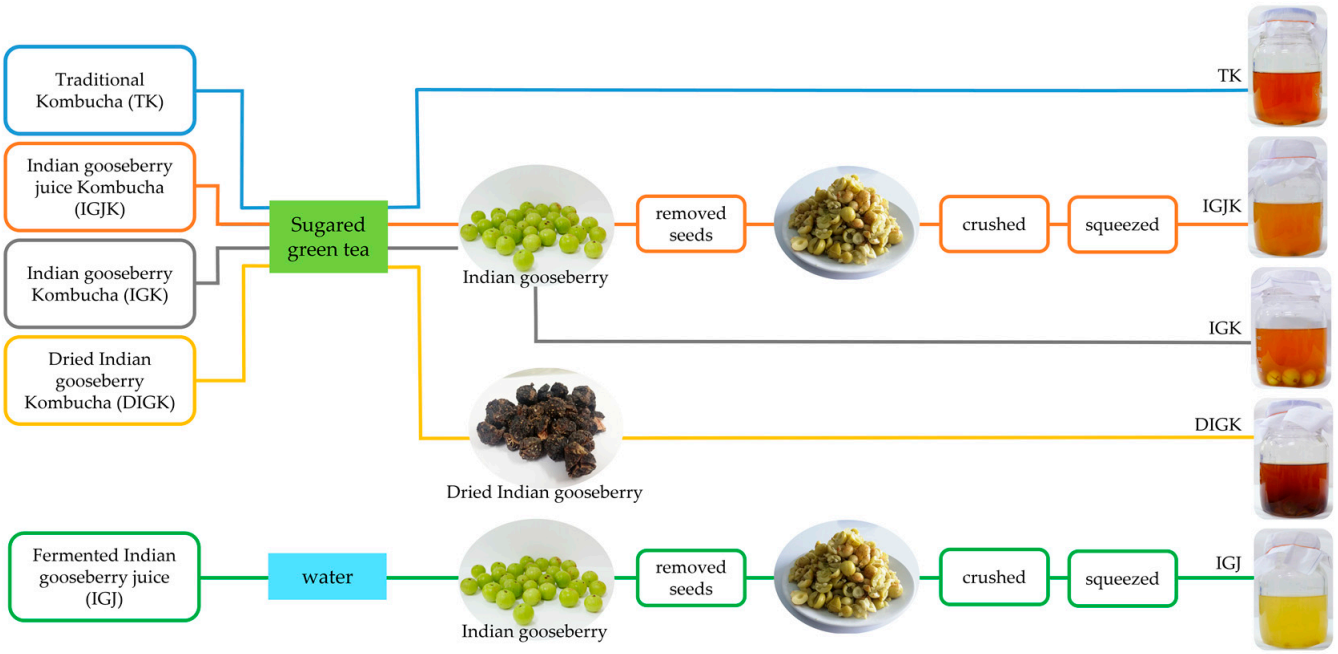

Figure 1. The preparation of kombucha fermentation: A comparative study of five treatments.

2.4. Microbiological Analysis

A serial dilution drop-plate [28] was used to count the total number of bacteria and yeast. This experiment used two types of agar media: Hestrin & Schramm (HS) agar with 100 ppm cycloheximide was used for acetic acid bacteria, and Yeast Malt (YM) agar was used for yeast. The sample was diluted with 9 mL of sterile 0.1% peptone water for 10-fold dilution. One milliliter of the sample was mixed with 9 mL of sterile 0.1% peptone water and this mixture was diluted with the peptone water for serial dilution. Twenty microliters of each dilution was dropped on each type of agar medium. The plates were then incubated at 30 °C for 2 days. The amounts of bacteria and yeast were counted and calculated in the form of log CFU/mL.

2.5. Measurement of Total Soluble Solids (TSS), Alcohol Content, pH and Acetic Acid Content

The total soluble solids (TSS, °Brix) in the samples were measured using a refractometer (TI-RBX0032A, Trans Instruments (S) Pte Ltd., Singapore), the alcohol content was measured using an ebulliometer (Dujardin-salleron, Paris, France), the pH value was measured using an electronic pH-meter (Metrohm, 713 pH meter, Herisau, Switzerland), and the total acidity of samples was determined by titrating with 0.1 N NaOH using phenolphthalein as an indicator [18]. The results were calculated and expressed as grams of acetic acid per liter of the sample.

2.6. Determination of Total Phenolic Content

The Folin–Ciocalteu colorimetric method was used to determine the total phenolic content (TPC) [15]. Twenty microliters of diluted sample was mixed with 100 µL of 10% (*v/v*) Folin–Ciocalteu reagent in a 96-well plate and allowed to stand in the dark at room temperature for 8 min. After that, 80 µL of 7.5% (*w/v*) sodium carbonate was added to the mixture and was allowed to stand in the dark at room temperature for 30 min. After incubation, the absorbance was measured at 765 nm by spectrophotometer (Genesys 20, Thermo Scientific, Dreieich, Germany). The analyses were run in triplicate and the results

were expressed as the gallic equivalent (GAE) per milliliter of sample (mg GAE/mL), using gallic acid as the calibration standard.

2.7. Determination of Total Flavonoid Content

The crystalline aluminum chloride assay was employed to quantify the total flavonoid content (TFC) in the samples [18]. Five hundred microliters of diluted sample was mixed with 150 µL of 5% (*w/v*) $NaNO_2$ solution in a test tube. After 5 min, 150 µL of 10% (*w/v*) $AlCl_3$ solution was added and allowed to stand for 6 min. Then 500 µL of 1N NaOH was added to the mixture, followed by 5 mL of distilled water. The blank solution was prepared by replacing the sample with distilled water. After mixing, the absorbance was measured immediately at 510 nm by spectrophotometer (Genesys 20, Thermo Scientific, Dreieich, Germany). Quercetin was used as a calibration reference. The analyses were run in triplicate, and the results were expressed as milligram quercetin equivalent (QE) per milliliter of sample (mg QE/mL).

2.8. Antioxidant Activity Analysis

2.8.1. DPPH Assay

Free-radical scavenging activity of the samples was determined by using 2,2-diphenyl-1picrylhydrazyl (DPPH) radical assay [29], with some modification for this work. The sample was diluted into five different concentrations. First, 100 microliters of sample was combined with 50 microliters of a 0.5 mg/mL DPPH solution in a 96-well plate and allowed to stand in the dark at room temperature for 30 min. After incubation, absorbance was measured at 517 nm by spectrophotometer (Genesys 20, Thermo Scientific, Dreieich, Germany). The control solution was prepared by replacing the sample with methanol. The radical scavenging activity of the sample was calculated using the following equation:

$$\% \text{ Radical scavenging} = \left(\frac{Acontrol - Asample}{Acontrol} \right) \times 100$$

The measurements were carried out in triplicate to ensure accuracy, and the result was presented as the amount of sample used to inhibit 50% of the free radicals, known as IC_{50}.

2.8.2. ABTS Assay

This study used a modified version of the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay [30] to determine the free radical scavenging activity of the sample. The $ABTS^{\bullet+}$ solution was prepared by mixing 7.4 mM of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution at a ratio of 1:1 (*w/w*) and kept in the dark at room temperature for 12–16 h. The $ABTS^{\bullet+}$ solution was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm. Five microliters of diluted sample was mixed with 195 µL of $ABTS^{\bullet+}$ solution in a 96-well plate and allowed to stand in the dark at room temperature for 10 min. After the incubation, the absorbance of the mixture was measured at 734 nm by spectrophotometer (Genesys 20, Thermo Scientific, Dreieich, Germany). The results were compared to a reference substance (Trolox) and presents as Trolox Equivalent Antioxidant Capacity (TEAC). To ensure accuracy, the determination was performed in triplicate.

2.9. Determination of Organic Acids

Five organic acids, including glucuronic acid, gluconic acid, D-saccharic acid 1,4-lactone (DSL), ascorbic acid and acetic acid, were measured by using reversed phase chromatography with Agilent 1200 series HPLC (Agilent technologies 1200 series, Santa Clara, CA, USA). The samples were filtrated through a 0.45 µm nylon membrane filter and the analysis was performed in isocratic mode with 3% methanol in 20 mM KH_2PO_4, pH 2.4. A 20 µL of filtrate sample was injected into the column (Inertsil ODS-3 C18 4.6 × 150 mm, 5 µm, GL Sciences, Tokyo, Japan) at a flow rate of 0.8 mL/min, with the

column temperature maintained at 28 °C [31,32]. The analyses were monitored at 210 nm wavelength by comparing the retention time of the standard compounds.

2.10. Statistical Analysis

The results were expressed as mean ± standard deviation. The results were evaluated using one-way analysis of variance and Duncan's multiple range test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Physical Characterization

The study examined the fermentation process of kombucha in five treatments over a period of 21 days. The treatment included traditional kombucha (TK), Indian gooseberry juice kombucha (IGJK), Indian gooseberry kombucha (IGK), dried Indian gooseberry kombucha (DIGK) and fermented Indian gooseberry juice (IGJ). The TK, IGJK and IGK had a brown color; the DIGK had a dark-brown color; and IGJ had a yellow color. The study observed the formation of floating bacterial cellulose on the surface of products and the sour smell increased as the fermentation process progressed (Figure 2).

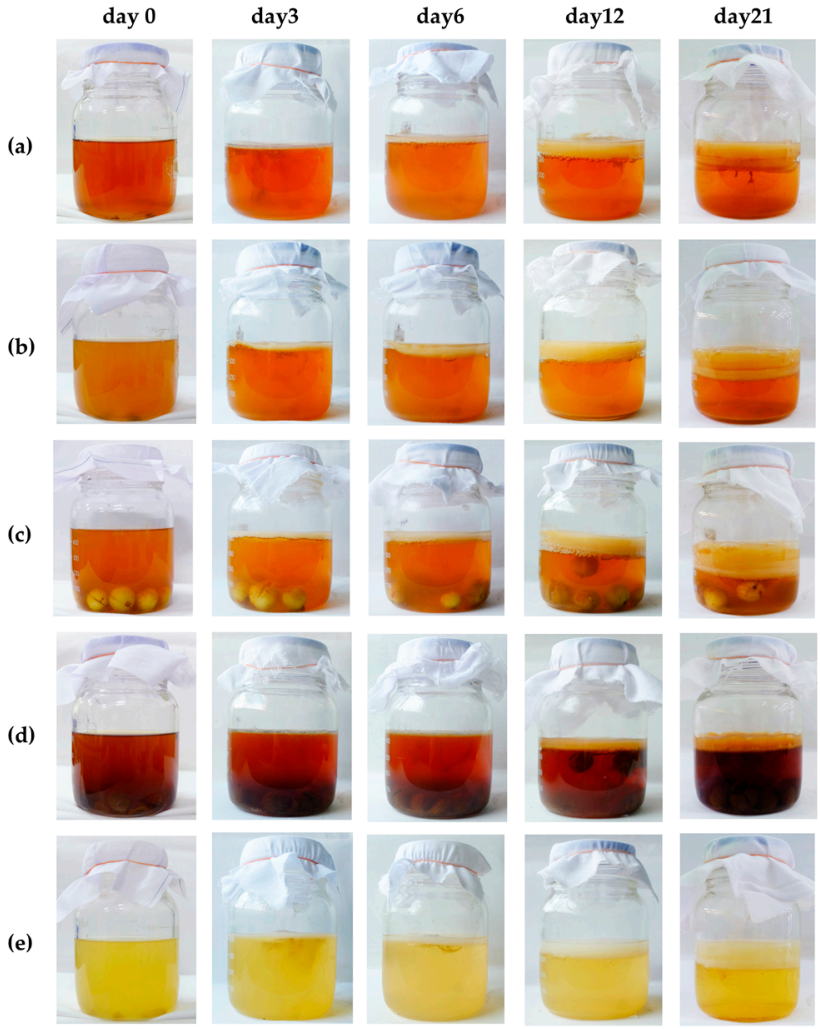

Figure 2. Differentiation of kombucha during 21 days of fermentation prepared from (**a**) traditional kombucha; (**b**) Indian gooseberry juice kombucha; (**c**) Indian gooseberry kombucha; (**d**) dried Indian gooseberry kombucha; and (**e**) fermented Indian gooseberry juice.

3.2. Microbiological Analysis

The total numbers of bacteria and yeasts during 21 days of fermentation are shown in Figure 3a,b. The total bacteria count of all treatments increased in the first three days, from 4.30–5.09 log CFU/mL to 6.02–7.06 log CFU/mL. By the end of the fermentation, the total bacteria count remained steady in the range of 5.37–6.56 log CFU/mL. Similarly, the total yeast count for all treatment increased during the first three days, from 4.30–5.09 log CFU/mL to 6.02–7.06 log CFU/mL, and slightly decreased after 6 days. The total yeast count remained stable in the range of 4.82–6.56 log CFU/mL at the end of fermentation.

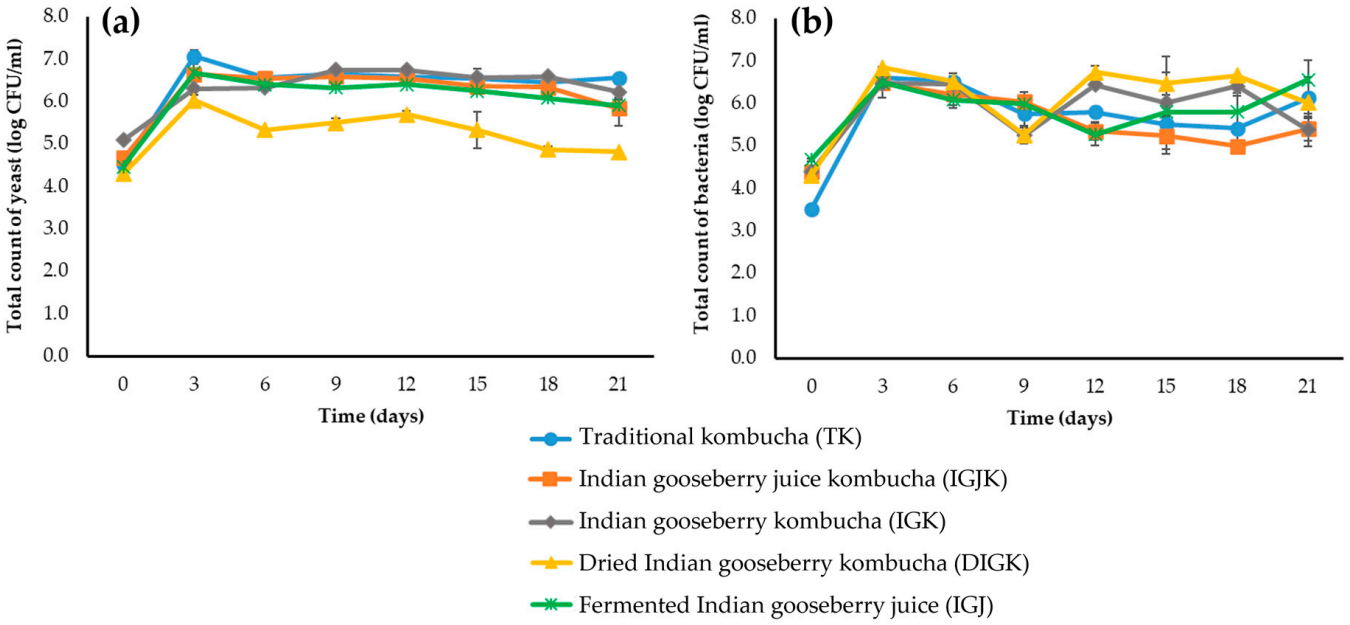

Figure 3. The total count of (**a**) bacteria and (**b**) yeast. n = 3. The error bars show ± SD.

3.3. Total Soluble Solids (TSS), Alcohol Content, pH and Acetic Acid Content

At the end of fermentation, only the total soluble solids (TSS) of the DIGK treatment increased slightly, while the TSS of the other treatments decreased steadily during fermentation, as shown in Figure 4a. The highest TSS was found in DIGK at 13.00 °Brix, following by the values of 8.00 ± 0.00, 7.33 ± 0.47, 7.00 ± 0.00 and 5.33 ± 0.62 °Brix for IGJ, IGK, TK and IGJK, respectively.

Although DIGK had the highest TSS when compared with other treatments, the alcohol content of DIGK remained unchanged (0%) as shown in Figure 4b. The alcohol content of TK and IGJ slightly increased, while IGK steadily increased after 6 days of fermentation. The alcohol content of IGJK steadily increased until 12 days of fermentation and then decreased until the end of fermentation (as shown in Figure 4b). The highest alcohol content was found in IGK, which was 2.13% ± 0.31, while the alcohol content of TK, IGJK and IGJ was 0.88% ± 0.17, 0.65% ± 0.14 and 0.40% ± 0.08, respectively.

The initial pH of all treatments was in the range of 2.65–3.96. It can also be seen that the pH of every treatment decreased until day 9 of fermentation, then remained constant until the end of fermentation, as shown in Figure 4c. The final pH values of IGJ, IGK, TK, DIGK and IGJK were 2.23 ± 0.01, 2.33 ± 0.00, 2.36 ± 0.01, 2.49 ± 0.01, and 2.55 ± 0.03, respectively. On the other hand, the acetic acid content increased as the days passed (Figure 4d). The highest acetic acid content was found in IGJK (46.68 ± 2.75 g/L, the lowest was found in DIGK (6.72 ± 0.48 g/L), while IGK, IGJ and TK had acetic acid content of 31.56 ± 4.34, 24.68 ± 1.66 and 24.36 ± 0.10 g/L, respectively.

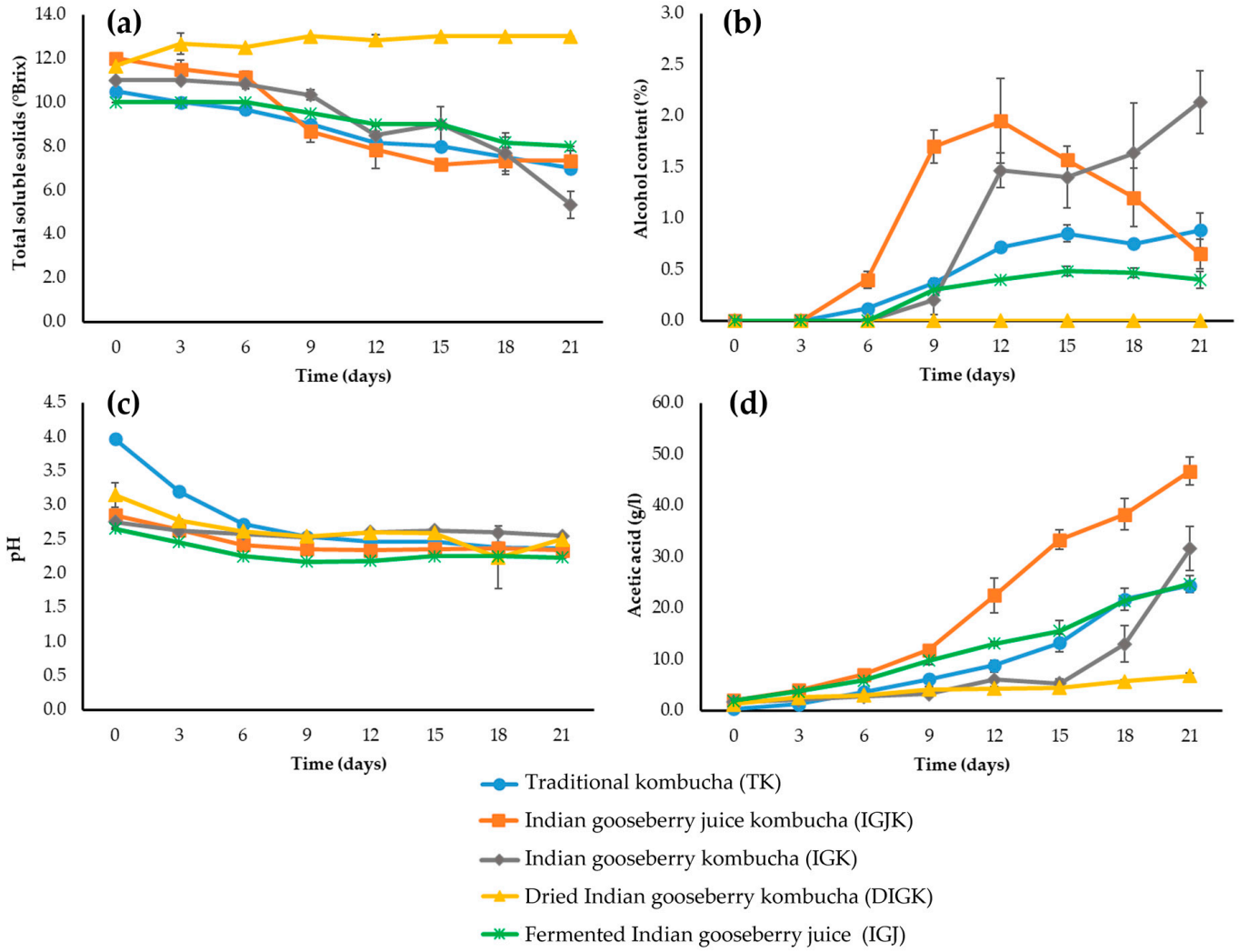

Figure 4. Effect of kombucha fermentation on (**a**) total soluble solids; (**b**) alcohol content; (**c**) pH; and (**d**) acetic acid content. n = 3. The error bars show ± SD.

3.4. Total Phenolic Content (TPC)

The total phenolic content (TPC) of every treatment increased during the first 3 days and then decreased slightly between 3 and 6 days of fermentation, as shown in Figure 5a. After that, the total phenolic content of TK and IGJ remained constant, while the TPC of IGJK, IGK and DIGK increased slightly until the end of fermentation. The results indicated that the DIGK had a significantly higher TPC ($p < 0.05$) compared to other treatments, while TK had lowest TPC (Table 1). It should also be noted that the TPC in every treatment of the kombucha that contained Indian Gooseberry was higher than in traditional kombucha. DIGK was 9.6 times higher than that of TK, followed by IGJK, IGK and IGJ.

3.5. Total Flavonoid Content (TFC)

The total flavonoid content (TFC) of all treatments followed a similar pattern to the total phenolic content (TPC), as shown in Figure 5b. The results indicated that DIGK had a significantly higher TFC ($p < 0.05$) compared to other treatments, while TK and IGJ had lower TFC compared to the other treatments ($p < 0.05$) (Table 1). It can clearly be seen that the kombucha fermented from a combination of green tea and Indian gooseberry had a higher TFC, ranging from 3.5 to 4.4 times when compared with the kombucha fermented from green tea only.

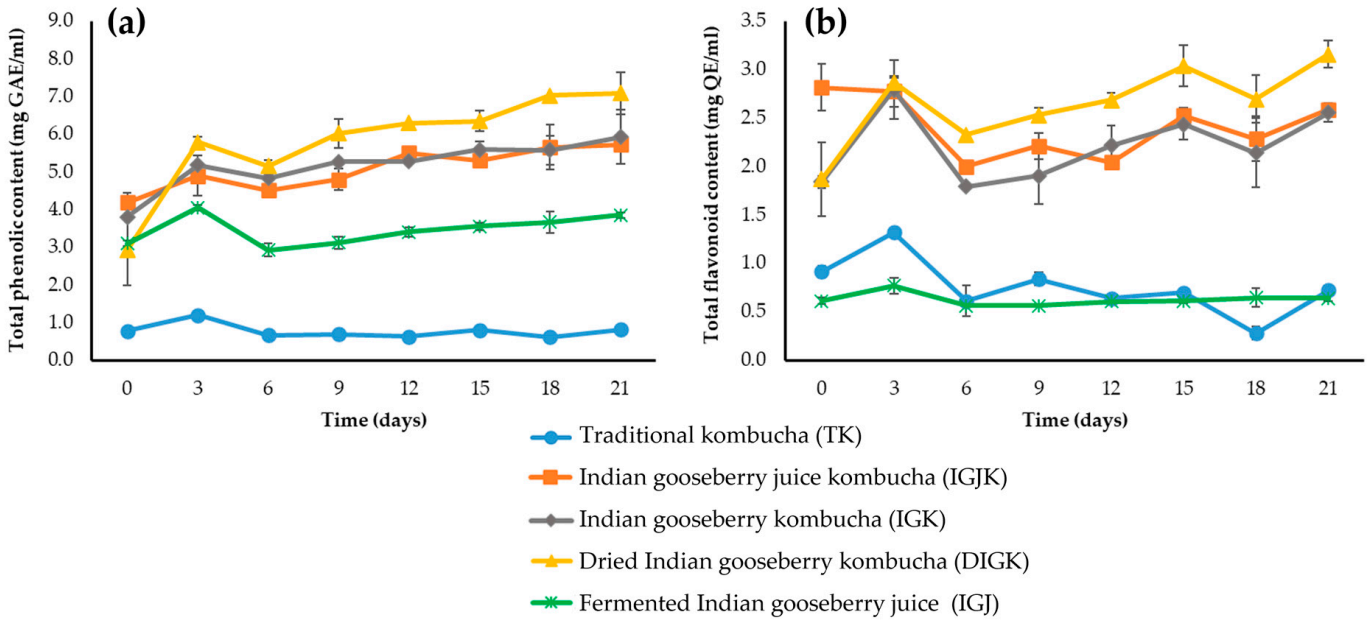

Figure 5. Effect of kombucha fermentation on (**a**) total phenolic content and (**b**) total flavonoid content. n = 3. The error bars show ± SD.

Table 1. Total phenolic, flavonoids and antioxidant activity of traditional kombucha (TK), Indian gooseberry juice kombucha (IGJK), Indian gooseberry kombucha (IGK), dried Indian gooseberry kombucha (DIGK) and fermented Indian gooseberry juice (IGJ) after 21 days of the fermentation process.

Analysis	Samples				
	TK	IGJK	IGK	DIGK	IGJ
Total phenolic content (mg GAE/mL)	0.82 ^{d} ± 0.02	5.72 ^{b} ± 0.10	5.92 ^{b} ± 0.72	7.08 ^{a} ± 0.55	3.85 ^{c} ± 0.08
Total flavonoid content (mg QE/mL)	0.72 ^{c} ± 0.05	2.59 ^{b} ± 0.05	2.55 ^{b} ± 0.09	3.16 ^{a} ± 0.14	0.64 ^{c} ± 0.03
% Inhibition at 3 µL/mL (%)	ND	66.12 ^{b} ± 1.87	70.83 ^{ab} ± 8.05	77.51 ^{a} ± 1.46	33.48 ^{c} ± 1.11
IC_{50} value by DPPH assay (µL/mL)	19.30 ^{c} ± 1.64	2.28 ^{a} ± 0.03	2.10 ^{a} ± 0.25	1.93 ^{a} ± 0.02	4.19 ^{b} ± 0.09
TEAC (mg/mL)	1.31 ^{d} ± 0.14	14.69 ^{b} ± 0.50	17.24 ^{a} ± 2.13	19.15 ^{a} ± 1.08	11.04 ^{c} ± 0.37

Values are expressed as mean ± standard deviation. Different letters in the same row indicate significantly different values ($p < 0.05$). ND: not detected.

3.6. Antioxidant Activity Analysis

3.6.1. DPPH Assay

The results of the antioxidant activity test indicated that four treatments of Indian gooseberry kombucha had higher antioxidant activity compared to the traditional kombucha (TK) treatment, as shown in Figure 6a. At a concentration of 3 µL/mL, the TK had no percentage inhibition of DPPH radicals, while the IGK and DIGK treatments had high percentages of inhibition, as shown in Table 1. DIGK, IGK and IGJK showed strong scavenging activity with IC_{50} value of 1.93 ± 0.02, 2.10 ± 0.25 and 2.28 ± 0.03 µL/mL, respectively, while TK showed scavenging activity with IC_{50} value of 19.30 µL/mL. The IC_{50} value of DIGK was 10 times greater than that of TK, as shown in Table 1.

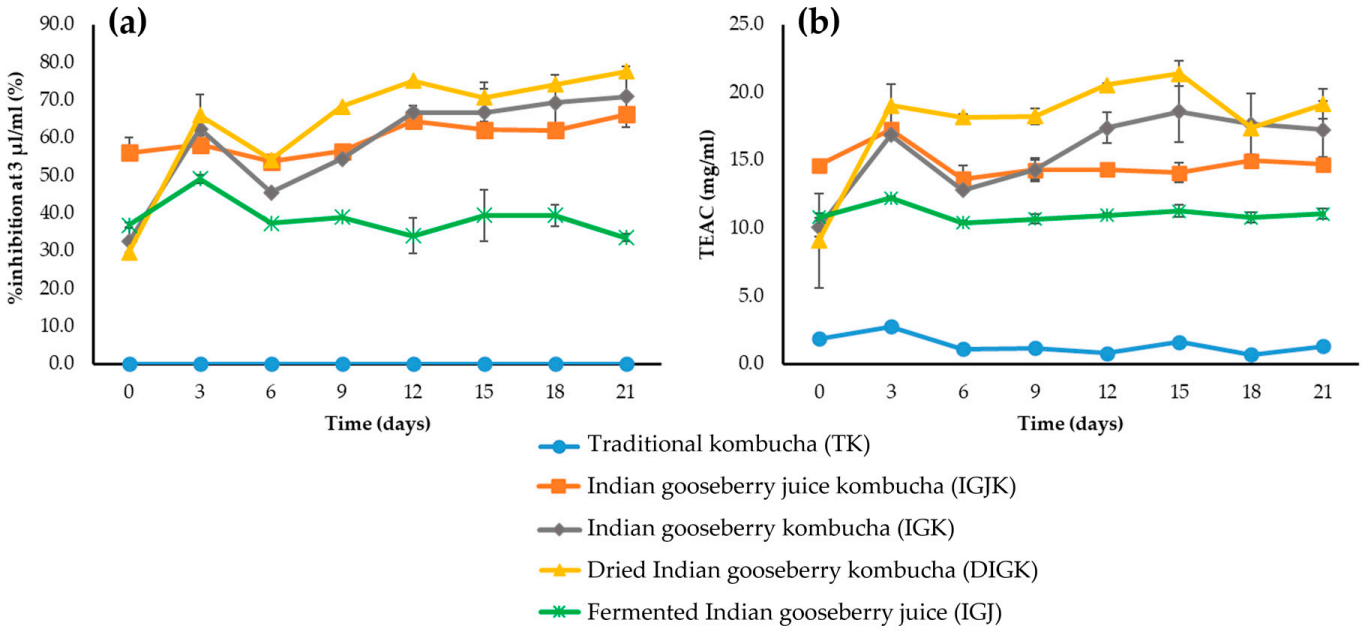

Figure 6. Effect of kombucha fermentation on (**a**) percent inhibition by DPPH assay and (**b**) Trolox Equivalent Antioxidant Capacity (TEAC) by ABTS assay, n = 3. The error bars show ± SD.

3.6.2. ABTS Assay

Following the DPPH assay results, the results of the antioxidant capacity test also indicated that four treatments of Indian gooseberry kombucha had significantly higher antioxidant capacity ($p < 0.05$) compared to the TK treatment as shown in Figure 6b. During fermentation process at 6 to 15 days, the Trolox Equivalent Antioxidant Capacity (TEAC) was highest in DIGK. Although at the end of the fermentation period, there was no statistically significant difference between the TEAC values of IGK and DIGK ($p > 0.05$), the antioxidant capacity of DIGK and IGK was 14.6 and 13.2 times higher than the antioxidant capacity of TK (as shown in Table 1).

3.7. Organic Acids

Five organic acids (glucuronic acid, gluconic acid, D-saccharic acid 1,4-lactone, ascorbic acid and acetic acid) were measured from samples by using HPLC. The chromatograms of these five standard organic acids and the organic acid profiles of the five treatments are shown in Figure 7. According to the results (Table 2), gluconic acid and acetic acid were the main organic acids in traditional kombucha, while ascorbic acid and acetic acid were the main organic acids in the other treatments. However, glucuronic acid was not detected in any samples. The change of organic acids during 21 days of fermentation are also shown in Figure 8.

The gluconic acid content gradually increased until the end of fermentation, with the highest content found in DIGK, followed by IGJ and IGJK. For the D-Saccharic acid 1,4-lactone (DSL), the highest value was at day 9 of fermentation, which was significantly ($p < 0.05$) highest in DIGK (4.04 mg/mL). The ascorbic acid content of Indian gooseberry kombucha was significantly ($p < 0.05$) higher than TK, and was highest in DIGK (6.95 mg/mL). Lastly, the acetic acid content also increased until 21 days of fermentation, and the highest acetic acid content was found in DIGK with 78.28 mg/mL.

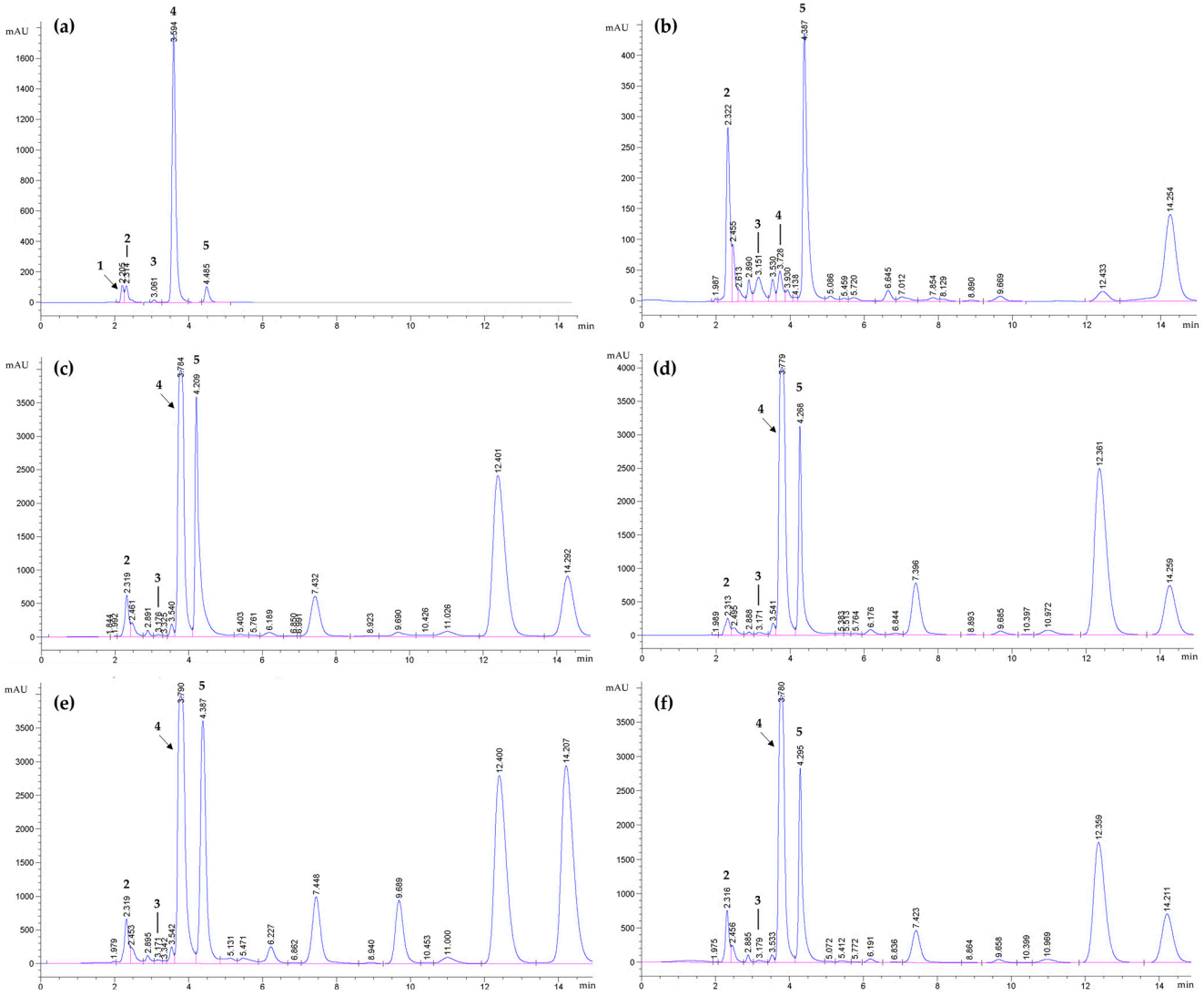

Figure 7. HPLC chromatogram of (**a**) the peaks of five standard organic acids; (**b**) traditional kombucha (TK); (**c**) Indian gooseberry juice kombucha (IGJK); (**d**) Indian gooseberry kombucha (IGK); (**e**) dried Indian gooseberry kombucha (DIGK); and (**f**) fermented Indian gooseberry juice (IGJ) are presented as organic acids. Peak 1, glucuronic acid; Peak 2, gluconic acid; Peak 3, D-Saccharic acid-1,4-lactone (DSL); Peak 4, ascorbic acid and Peak5, acetic acid.

Table 2. Organic acid content of traditional kombucha (TK), Indian gooseberry juice kombucha (IGJK), Indian gooseberry kombucha (IGK), dried Indian gooseberry kombucha (DIGK) and fermented Indian gooseberry juice (IGJ) at 21 days of the fermentation process.

Organic Acid Content (mg/mL)	Samples				
	TK	**IGJK**	**IGK**	**DIGK**	**IGJ**
Glucuronic	ND	ND	ND	ND	ND
Gluconic	10.46 ^{b} ± 0.82	13.34 ^{a} ± 0.97	6.93 ^{c} ± 1.68	15.35 ^{a} ± 1.39	15.03 ^{a} ± 0.39
DSL	0.75b ^{c} ± 0.11	0.45 ^{c} ± 0.17	1.04 ^{ab} ± 0.31	1.26 ^{a} ± 0.01	0.44 ^{c} ± 0.13
Ascorbic	0.14 ^{e} ± 0.07	6.28 ^{c} ± 0.02	6.69 ^{b} ± 0.10	6.95 ^{a} ± 0.21	5.91 ^{d} ± 0.06
Acetic	16.71 ^{d} ± 1.06	36.21 ^{b} ± 0.92	36.24 ^{b} ± 1.59	78.28 ^{a} ± 0.67	31.76 ^{c} ± 0.82

Values are expressed as mean ± standard deviation. Different letters in the same row indicate significantly different values ($p < 0.05$). ND: not detected.

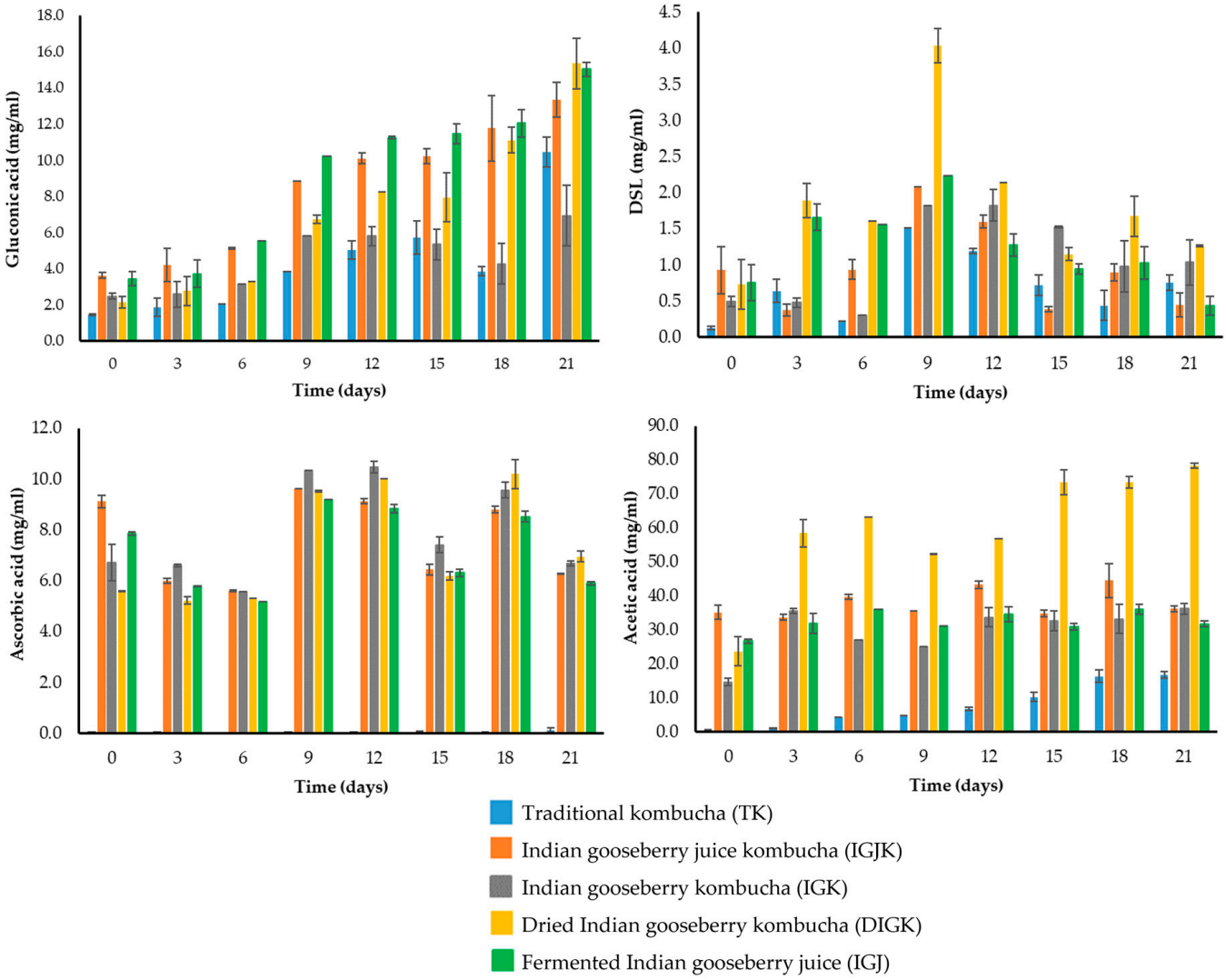

Figure 8. Organic acid content in kombucha samples, n = 3. The error bars show ± SD.

4. Discussion

The formation of bacterial cellulose on the surface of kombucha was observable after the first 3 days of the fermentation, and the cellulose continued to become thicker as the fermentation progressed. The cellulose yield increased due to the fermentation conditions and the synergistic microbial metabolism of yeast and acetic acid bacteria [33,34].

The population of yeast and bacteria in the kombucha also increased around 3 days after the fermentation, then remained stable until 21 days of fermentation. In kombucha, yeast and bacteria interact cooperatively and competitively, but overall it is a symbiotic relationship [34]. Yeast breaks down sucrose into glucose and fructose using invertase enzymes [13]; this product can be used to produce ethanol by yeast, as well as by acetic acid bacteria for cellulose biosynthesis and organic acid synthesis [15]. Moreover, the ethanol stimulates a cellulose-synthase mechanism to produce cellulose [34].

It has been shown previously that as the fermentation progresses, the total soluble solids (TSS) decrease, while the alcohol content increases, as a result of the yeast's glycolysis pathway, which metabolizes glucose into pyruvate, then converts pyruvate into ethanol and carbon dioxide [35]. However, in this study, the TSS of the DIGK treatment did not decrease, which is likely due to the presence of phenolic compounds in the dried ingredient that affected metabolic activity of the yeast, resulting in no detection of alcohol content. The study from Fletcher and Baetz in 2020, indicated that phenolic compounds, such as

ferulic acid, vanillin, and 4-hydroxybenzoic acid in plants used for bioethanol production, can inhibit enzymes in yeast that have the effect of limiting the amount of sugars available, product yield, and fermentation qualities [36]. At 21 days after fermentation, the alcohol content in each kombucha treatment ranged from 0.65% to 2.13%, which was higher than the legal limit of 0.5% (*v*/*v*) for classification as a non-alcoholic beverage [37].

The increase in acetic acid concentration and decrease in pH value in kombucha during fermentation is due to acetic acid bacteria using glucose and ethanol to produce organic acids, specifically acetic acid, which is the main product in kombucha [6,15].

Similar trends were seen between the total phenolic content (TPC) and the total flavonoid content (TFC), reaching a peak after 3 days of fermentation and declining 3–6 days later. After 6 days of fermentation, the TPC and the TFC of kombucha that contain all forms of Indian gooseberry increased slightly, while traditional kombucha and fermented Indian gooseberry juice remained constant until the end of fermentation. Kaewkod et al. (2019) produced kombucha from black tea, oolong tea, and green tea. They also found that TPC peaked at 3 days after fermentation started, then decreased and remained stable until 15 days of fermentation [32].

The kombucha produced from green tea blend with all forms of Indian gooseberry showed higher TPC and TFC than the traditional kombucha produced from green tea and Indian gooseberry juice alone. The greatest amount of TPC and TFC was found in kombucha made by blending dried Indian gooseberry with green tea. The increase in TPC and TFC due to Indian gooseberry is a rich source of polyphenols, and other bioactive substances [22,38], while green tea is also rich in polyphenols but low in vitamins, organic acids, and minerals [3,39]. Several studies indicate that the fermentation process can enhance TPC and TFC, and the blending of alternative substrates can also boost TPC and TFC in comparison to traditional kombucha. For instance, Silva et al. (2021) studied kombucha fermentation made from *Malvaviscus arboreus* Cav. (wax mallow flower) and found that TPC of this kombucha increased after fermentation [5]. Another report from Zubaidah et al. (2018) produced kombucha from *Salacca zalacca* (Gaerth.) Voss (snake fruit) also found that TPC and TFC increased after fermentation [13]. According to Vitas et al. (2020), the degradation of polyphenols (present in vegetables, fruits and tea leaves) during the fermentation process of kombucha [40] leads to the increase in TPC and TFC. Polyphenols are known as bioactive compounds, which have several phenol structures on each molecule [3], and the microbes in kombucha secrete an enzyme during the fermentation process that degrades polyphenols into small molecules, which contribute to an increase in TPC and TFC [13,16].

Our study also found that the antioxidant activity, as measured by DPPH assay and $ABTS^{\cdot+}$ assay had a similar trend to the total phenolic content. The increase in antioxidant activity is caused by the increase in chemical compounds, such as phenolic compounds, flavonoids, ascorbic acid and other organic acids, during fermentation [1]. From our study, we found that the blending of dried and whole Indian gooseberry had significantly higher antioxidant activity ($p < 0.05$) compared to other treatments. Various studies have demonstrated that Indian gooseberry has antioxidant activity due to ascorbic acid, polyphenolic compounds, flavonoids and tannins. Furthermore, the antioxidant properties differ due to the forms used and the extraction procedures [21]. According to a report from Umamaheswari et al. (2019), the results from DPPH assay indicated that dried powder of Indian gooseberry showed 97% inhibition of free radical scavenging activity, which was higher than the 57% inhibition from fresh juice [41].

Acetic acid, gluconic acid and glucuronic acid are the organic acids that are mostly found in kombucha [42]. However, in this study, glucuronic acid was not present in any of the treatments, probably because of the differences between the yeast and bacteria community in starter culture, time, temperature, sucrose concentration, and type of substrate [43]. For gluconic acid, it is always more prevalent than glucuronic acid in general [39,42]. This acid is produced by acetic acid bacteria from glucose and increases with fermentation time [6].

The ascorbic acid content in kombucha blended with different forms of Indian gooseberry were higher than traditional kombucha, due to the high concentration of ascorbic acid in the fruit [38], this acid is also derived from glucose which is synthesized by bacteria in kombucha [18]. Acetic acid is the most distinctive product of kombucha fermentation, which is produced from ethanol by acetic acid bacteria [18]. Acetic acid also increased with fermentation time, which was highest in DIGK. D-Saccharic acid-1,4-lactone (DSL), a derivative of D-glucaric acid, is the product from the glucuronate pathway. This acid is important in kombucha due to its health benefits, detoxifying properties, and antioxidant properties [9,31]. The DSL content showed the highest concentration at 9 days of fermentation, specifically in DIGK, but decreased with further fermentation.

Using Indian gooseberry as an alternative substrate in kombucha fermentation has been found to be highly effective in enhancing phenolic content. At the end of the fermentation process, the relative increase in phenolic content ranged from 21% to 121%, which is higher than in traditional kombucha, where the relative increase was only 4% (Table S4). Moreover, the relative increase of flavonoid content was found in the fermentation process of IGJ, IGK and DIGK ranging from 4% to 69% (Table S5). This suggests that Indian gooseberry is an excellent source of polyphenols, as previous reports have shown that it contains high levels of phenolic compounds and flavonoids [38,44].

However, the relative increase of organic acids was not influenced by the use of Indian gooseberry because the fermentation process of kombucha made from Indian gooseberry had a lower effect on increasing organic acids than traditional kombucha (Table S6). The relative increase suggested that the organic acids in kombucha from Indian gooseberry were higher than those in traditional kombucha because the Indian gooseberry has higher initial levels of organic acids.

Factors such as the microorganisms in kombucha, type of substrates, fermentation time, temperature and pH also affect the total phenolic content, total flavonoid content, antioxidant activity and organic acid content [2,6]. Further studies on the pilot scale as well as the optimal ratio of dried Indian gooseberry are therefore crucial in enhancing the chemical composition of kombucha products.

5. Conclusions

Indian gooseberry can be a promising addition to traditional kombucha fermentation. Using Indian gooseberry in different forms, such as whole fruit, dried-fruit and juice, can result in an increase in phenolic compounds, flavonoids, antioxidant activity and organic acids in the final kombucha product. Among the different forms of Indian gooseberry, the dried fruit showed the best properties and may therefore be a suitable alternative substrate for the development of new kombucha products in the future. These findings indicate that Indian gooseberry has the potential to add new dimensions to the traditional kombucha fermentation process and could be used to create new and unique kombucha products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030291/s1, Table S1: Total phenolic content changes during fermentation; Table S2: Total flavonoid content changes during fermentation; Table S3: The results of HPLC analysis of organic acids during fermentation; Table S4: Relative increase of phenolic content; Table S5: Relative increase of flavonoid content; Table S6: Relative increase of organic acids.

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