



Enhancing kombucha composition: Effects of sugarcane juice and cocoa honey on antioxidant properties, physicochemical, and chemical profile

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Abstract

This study aimed to evaluate the effect of different fermentation substrates on the physicochemical, chemical, and antioxidant characteristics of kombucha (T1: sugarcane juice at 15 °Brix and cocoa honey; T2: sugarcane juice at 5 °Brix and cocoa honey; T3: sucrose and cocoa honey; T4: sucrose control). The analyses included pH, soluble solids, titratable acidity, color, phenolic content, antioxidant activity, organic acids, ethanol, and volatile compounds, assessed during fermentation (0, 168, and 240 h). Reductions in soluble solids and pH were observed during fermentation. T1 showed higher production of acetic acid (7.99 g/L) and lactic acid (4.99 g/L) in 240 h. T1 and T2 showed higher alcohol content (1.99% and 1.08% ABV, respectively), classifying them as alcoholic kombuchas, whereas T3 and T4 remained non-alcoholic (<0.5% ABV). T3 and T4 showed greater antioxidant stability, with T4 showing a higher concentration of total phenolics (913.26 mg GAE/100 mL) and T3 showing a greater ABTS⁺ radical scavenging (94.91%) in 240 h, while T1 exhibited a decline in free radical scavenging. 91 volatile compounds were identified. Acetic acid 2-phenylethyl ester was abundant in T2 and T4, while ethyl decanoate was abundant in T1 and T3 in 240 h. Sugarcane juice promoted efficient fermentation, thereby increasing organic acid production, while sucrose-based kombuchas showed greater antioxidant stability. T1 increased the levels of organic acids and volatiles, whereas T3 enhanced antioxidant activity with low alcohol. T3 and T4 are non-alcoholic beverages with high antioxidant potential, whereas T1 and T2 are alcoholic kombuchas that reach different market niches.

Keywords Kombucha fermentation · Cocoa honey · Sugarcane juice · Bioactive compounds

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Introduction

Kombucha is an ancient beverage of Chinese origin, produced by fermenting sweetened green or black tea (*Camellia sinensis*) with a symbiotic culture of bacteria and yeast (SCOBY). The microbiota is mainly composed of acetic acid bacteria (AAB) such as *Komagataeibacter* (previously *Glucanoacetobacter*), *Acetobacter*, and *Gluconobacter*, along with yeasts from the genera *Zygosaccharomyces*, *Saccharomyces*, *Brettanomyces*, *Pichia*, *Candida*, and *Lachancea*, and, to a lesser extent or occasionally, lactic acid bacteria (LAB) including *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Oenococcus* [1–3]. *Komagataeibacter* is usually the predominant bacterium in SCOBYs, accounting for 50–90% of the population, and *Zygosaccharomyces* is the yeast, with a relative abundance of up to 84.1% [4–5]. During fermentation, yeasts convert carbohydrates into alcohol, while acetic acid bacteria (AAB) oxidize the alcohol to acetic acid, lowering the beverage's pH [5]. Other metabolites formed include organic acids (gluconic, succinic, lactic, malic, and citric), phenolic compounds (catechins and theaflavins), vitamins (B1, B2, B6, B12, E, and C), and amino acids [6–7].

The first fermentation of kombucha usually lasts 7 to 14 days [8]. In artisanal or small-scale production, a second fermentation is common, aiming to enhance carbonation and intensify the beverage's sensory complexity, especially when ingredients such as fruit juices or natural sweeteners are added [9]. Kombucha, once a traditional and artisanal product, has been gaining prominence in the global market, with an expected annual growth rate of 23.2% until 2027, reaching USD 10.45 billion. The flavored version is one of the main consumption trends [10].

This beverage is gaining popularity due to its claims of health benefits, which may be attributed to bioactive compounds. Among these, phenolic compounds and flavonoids stand out due to their potent antioxidant activity, which helps scavenge free radicals and reduce oxidative stress. Additionally, caffeine, alkaloids, and specific organic acids also contribute to its functional properties, offering antibacterial, anti-inflammatory, and neuroprotective effects [11–12]. Studies suggest that kombucha consumption may support physiological processes related to oxidative balance, inflammation control, and metabolic regulation [13–14].

The search for new flavors and functionalities has driven the use of alternative substrates in kombucha production, including fruit juices, banana peels, soy milk, yams, herbal infusions, molasses, almond milk, yerba mate, and purple pitanga [2, 8, 15–20]. Among these substrates, sugarcane juice and cocoa honey stand out as promising options for use in this context. Sugarcane juice is an energy-rich beverage with high sugar content (15–25%, mainly sucrose),

organic acids (0.8–2%), starch, minerals (potassium, calcium, magnesium, phosphorus, and iron), vitamins (A, C, B1, B2, B3, B5, and B6), nitrogenous compounds such as amino acids and proteins, phenolic acids (chlorogenic, cinnamic, caffeic, and sinapic acids), polyphenols and flavonoids [21–22]. Cocoa honey has a composition rich in sugars (fructose, glucose, and sucrose), fibers, vitamins (C, B3, B5, and B7), organic acids (mainly citric acid), minerals (potassium, magnesium, calcium, sodium, and phosphorus), and phenolic compounds [23].

Cocoa honey, a byproduct of cocoa processing, poses environmental risks, including soil contamination and unpleasant odors if improperly disposed of. To promote its sustainable use, various value-added products have been developed, including kefir, vinegar, spirits, beer, and kombucha [24–26]. In kombucha, cocoa honey not only enhances nutritional and sensory properties but also reduces waste and minimizes the environmental impact associated with cocoa industry residues [26].

Despite the growing number of studies on kombucha fermentation, few address the effects of combining multiple alternative substrates, particularly sugarcane juice and cocoa honey [26–28]. Understanding how these combinations affect physicochemical parameters, antioxidant potential, and volatile profile of kombucha is essential for expanding the possibilities for developing functional beverages while promoting sustainable practices. Thus, this study aimed to evaluate the impact of sugarcane juice, cocoa honey, and their combination on kombucha fermentation, compared with the traditional sucrose-based formulation. Through physicochemical analyses, including organic acid profiling, ethanol quantification, volatile compound profiling, total phenolic quantification, and antioxidant activity assessment, this research aims to deepen understanding of how alternative substrates enhance kombucha production.

Materials and methods

Kombucha preparation

The teas were prepared by infusing 10 g of green tea (*Camellia sinensis*) in 1 L of boiling potable water for 10 min. The infusions were cooled to room temperature (25 °C) and transferred to 3 L glass jars (14.5 × 25 cm (width × height)) having 3% of a symbiotic culture of bacteria and yeast (SCOBY, Companhia dos Fermentados, Barueri, São Paulo, Brazil) and 10% starter (fermented kombucha) [16]. SCOBY from Companhia dos Fermentados comprises a complex microbial consortium, predominantly AAB, including *Komagataeibacter* and *Acetobacter*, LAB, including *Lactobacillus* and *Pediococcus*, and yeasts,

Zygosaccharomyces, *Saccharomyces*, *Brettanomyces*, and *Pichia* [29].

The kombucha fermentations were conducted in two stages (Table 1). In both stages, fermentations were performed in triplicate at $25 \pm 3^\circ\text{C}$. The first fermentation lasted 168 h, and the second 72 h, for a total of 240 h [5, 6, 17].

The experimental design was based on a literature review [22, 24]. During the first fermentation, treatments 1 and 2 (T1 and T2) were prepared with pasteurized sugarcane juice (60°C for 30 min) at 15 °Brix (T1: 650 mL/L of sugarcane juice and 350 mL/L of green tea infusion) and 5 °Brix (T2: 250 mL/L of sugarcane juice and 750 mL/L of green tea infusion). In treatments 3 and 4 (T3 and T4), 50 g of sucrose and 950 mL of green tea infusion were used. The final beverage volume after preparations was 1 L, and all four treatments underwent the first fermentation (168 h).

At the end of the first fermentation, the beverages were filtered and transferred to sanitized 1.5 L plastic bottles (1 mL/L peracetic acid for 15 min) for the second fermentation. In this stage, 55.5 mL/L of pasteurized cocoa honey (60°C for 30 min) with a soluble solids content of 18% was added to T1, T2, and T3, while 10 g/L of sucrose was added to T4. The T4 treatment corresponds to the conventional method of kombucha production and was used as a control for the other fermentations. Sugarcane juice was purchased at a local street market in Lavras (Minas Gerais, Brazil), while the cocoa honey was obtained from São Luiz Farm, in Linhares (Espírito Santo, Brazil).

From each repetition, 30 mL aliquots were collected at the initial time (0 h), at 168 h (end of the first fermentation), and at the final time (240 h, end of the second fermentation). The samples were stored at -18°C until analysis.

Physicochemical analyses

The pH values were determined using a QUIMIS digital pH meter. The soluble solids content (°Brix) was measured with a digital refractometer (Hanna Instruments HI 96801 Refractometer), with results expressed as a percentage of soluble solids. Titratable acidity was performed using titration with NaOH (0.1 N), using phenolphthalein as the endpoint indicator. The results were expressed as a percentage of acetic acid [30].

Color analysis was performed using a CR-400 Konica Minolta spectrophotometer, employing the CIELAB/LCh color space. The L^* variable represents lightness, ranging from black ($L^* = 0$) to white ($L^* = 100$); a^* and b^* are chromaticity coordinates that ranges from green ($-a^*$) to red ($+a^*$) and from blue ($-b^*$) to yellow ($+b^*$); C^* is the Chroma and represents the color saturation; and hue angle (h°) is $\arctan(b^*/a^*)$, where the arcs from 0° to 90° , 90° to 180° , 180° to 270° and 270° to 360° correspond to the colors varying from red to yellow, yellow to green, green to blue and blue to red, respectively. The following Eq. 1 defines the total color difference:

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

The color variation, according to the ΔE^* value, can be classified as imperceptible (0–0.5), slightly perceptible (0.5–1.5), perceptible (1.5–3.0), highly visible (3.0–6.0), or excellent (> 6.0) [31].

Total phenolics and antioxidant activity

Quantification of total phenolics by the Fast Blue method

Total phenolics were determined using the method proposed by Medina [32]. For the analysis, 4 mL of the sample was added to a test tube along with 0.4 mL of a 0.01% Fast Blue solution and 0.4 mL of 5% NaOH. After 1 h of rest in the dark, readings were taken using a spectrophotometer at 420 nm. The results were expressed in mg of gallic acid equivalent (GAE) per 100 mL of sample.

Antioxidant activity by the phosphomolybdenum complex method

The phosphomolybdenum complex assay was performed according to the methodology described by Prieto et al. [33]. For the reaction, 50 μL of the sample was mixed with 450 μL of distilled water and 1.5 mL of the phosphomolybdenum complex, and then incubated at room temperature in the dark for 120 min. The absorbance was measured at 695 nm using a spectrophotometer, and the results were expressed as mg of ascorbic acid (AA) per 100 mL of sample.

Table 1 Information on the treatments used in the experiment

Treatment	First fermentation				Second fermentation	
	Sugarcane juice (°Brix)	Sugarcane juice (mL/L)	Green tea infusion (mL/L)	Sucrose (g/L)	Cocoa honey* (mL/L)	Sucrose (g/L)
T1	15	650	350	-	55.5	-
T2	5	250	750	-	55.5	-
T3	-	-	950	50	55.5	-
T4	-	-	950	50	-	10

*Soluble solids content of pure cocoa honey = 18%

Antioxidant activity by the ABTS+ method

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) radical reduction assay followed the method of Re et al. [34] with some adaptations. The absorbance was measured using the ABTS+ radical cation control in comparison to the sample at 734 nm. The results were calculated and expressed as the percentage of ABTS+ free radical scavenging (%FRS ABTS+).

Chromatographic analyses

HPLC analysis for organic acids and ethanol

The determination of organic acids (oxalic, citric, succinic, lactic, and acetic) and ethanol was performed using high-performance liquid chromatography (HPLC, Shimadzu, model LC-10Ai, Shimadzu Corp., Japan) [4]. A UV-Vis detector (SPD-10Ai) was used for organic acids, while a refractive index detector (RID-10Ai) was used for ethanol. A Shimadzu Shim-pack SCR-101 H ion-exclusion column (7.9 mm × 30 cm) was used at 50 °C. The sample pH was adjusted to 2.1 using a 200 mM perchloric acid solution. The eluent was a 16 mM perchloric acid solution with a pH of 2.1 and a flow rate of 0.6 mL/min. Calibration curves were constructed using organic acid standards (0.125–8.00 g/L) and ethanol (0.125–16.00 g/L). The obtained areas were plotted as a linear curve, and the equation of the curve was used to estimate the concentration in the sample. All standards were purchased from Sigma Aldrich.

Volatile compounds determination by gas chromatography coupled to mass spectrometry (GC/MS)

Volatile compounds were extracted by headspace solid-phase microextraction (SPME-HS). A 50/30 µM divinylbenzene/carboxy/polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA) and a manual holder were used for the extraction. The 5 mL samples were placed in vials with 1 g of NaCl, heated at 60 °C, equilibrated for 15 min, and extracted for 30 min [16].

The volatile compounds were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). A GC-MS-QP2010 SE system (Shimadzu) equipped with a Carbowax 20 M capillary column (30 m × 0.25 mm × 0.25 µm) was operated at 50 °C for 5 min. The temperature increased at 3 °C/min until it reached 190 °C, then was held for 10 min. The carrier gas was high-purity helium at a constant flow rate of 1.2 mL/min. Injections were performed in splitless mode for 2 min. After 3 min, mass spectra were continuously acquired from 45 to 1000 m/z. The electron-ion source temperature was 230 °C, and the quadrupole

temperature was 150 °C [8]. The volatile compounds were identified by comparing their mass spectra with those in the NIST 11 library and using the GC/MS Solutions software (version 4.11).

Data analysis

The experiment was conducted in a completely randomized design (CRD). A 4 × 3 factorial arrangement was employed, comprising four levels of substrates in the first/second fermentations (T1: 15 °Brix sugarcane juice and cocoa honey; T2: 5 °Brix sugarcane juice and cocoa honey; T3: sucrose and cocoa honey; T4: control); and three fermentation times (0, 168, and 240 h), with three replications, totaling 36 experimental units. The analyzed variables, except for volatile compounds, were subjected to analysis of variance (ANOVA), and means were compared using the Tukey test ($p \leq 0.05$) with the SISVAR[®] statistical software version 5.7. Principal Component Analysis (PCA) was performed based on the areas of the volatile compound classes, using the correlation matrix to assess the distribution of these compounds among the treatments at the beginning (0 h) and the end of fermentation (240 h).

Results and discussion

Physicochemical parameters

The variables pH, titratable acidity, soluble solids, total phenolics, antioxidant activity, organic acids, and ethanol were influenced by the interaction between fermentation time and substrates ($p \leq 0.05$) (Tables 2, 3 and 4).

As expected, at the beginning of the first fermentation, the highest soluble solids content was observed in T1, which received sugarcane juice at 15 °Brix (Table 2). The soluble solids content of the substrate used in T1 is 2.6 times higher than that of the substrates used in T2, which justifies the results. In general, a reduction in soluble solids content was observed during 168 h of fermentation (first fermentation), regardless of the substrate used ($p \leq 0.05$). It was noted that the reduction in soluble solids in beverages with sugarcane juice (T1: 14.69% to 11.03%, and T2: 5.66% to 3.63%) during the first fermentation was higher than in beverages with sucrose (T3: 5.10% to 4.36%, and T4: 4.96% to 4.23%). Sugarcane juice, primarily composed of sucrose, may have favored the growth of *Saccharomyces cerevisiae* and *Brettanomyces anomalus* during fermentation. These yeasts exhibit invertase activity, enabling them to metabolize sucrose into glucose and fructose. As sugars are converted into ethanol and CO₂, their consumption decreases the soluble solids [21, 22, 35].

Table 2 Physicochemical parameters during fermentation

Treatments	Fermentation time (h)	Soluble solids (%)	pH	Titrateable acidity (% acetic acid)
T1	0	14.96±0.06 ^{aA}	4.42±0.01 ^{aA}	0.14±0.01 ^{aB}
T2	0	5.66±0.06 ^{bA}	4.12±0.02 ^{bA}	0.12±0.01 ^{aB}
T3	0	5.10±0.01 ^{cA}	3.84±0.02 ^{cA}	0.11±0.01 ^{aC}
T4	0	4.96±0.15 ^{cA}	3.83±0.01 ^{cA}	0.13±0.00 ^{aC}
T1	168	11.03±0.06 ^{aB}	3.06±0.01 ^{aB}	1.83±0.05 ^{aA}
T2	168	3.63±0.16 ^{cB}	3.00±0.02 ^{aB}	1.00±0.03 ^{bA}
T3	168	4.36±0.11 ^{bB}	3.04±0.03 ^{aB}	0.34±0.04 ^{cB}
T4	168	4.23±0.15 ^{bB}	3.10±0.02 ^{aB}	0.38±0.02 ^{cB}
T1	240	10.26±0.35 ^{aC}	3.05±0.00 ^{bB}	1.78±0.04 ^{aA}
T2	240	3.66±0.06 ^{dB}	2.98±0.02 ^{bB}	1.01±0.01 ^{bA}
T3	240	4.36±0.15 ^{bB}	3.05±0.01 ^{bB}	0.56±0.05 ^{cA}
T4	240	4.13±0.06 ^{cB}	3.15±0.03 ^{aB}	0.48±0.03 ^{dA}

Means±standard deviations in the same column, within each fermentation time, followed by different lowercase letters, indicate that the treatments differ from each other according to Tukey's test ($p \leq 0.05$). Meanwhile, the means±standard deviation in the same column, referring to the same treatment at different fermentation times, followed by different uppercase letters, indicate differences over time, according to Tukey's test ($p \leq 0.05$). Fermentations: T1: Sugarcane juice (15 °Brix) and cocoa honey; T2: Sugarcane juice (5 °Brix) and cocoa honey; T3: Sucrose and cocoa honey; and T4: Control

Table 3 Organic acids and ethanol determined during fermentation

Treatments	Fermentation time (h)	g/L				
		Succinic acid	Lactic acid	Acetic acid	Ethanol	% ABV
T1	0	0.69±0.02 ^{aB}	-	-	-	-
T2	0	0.30±0.00 ^{bB}	-	-	-	-
T3	0	0.19±0.00 ^{cA}	-	-	-	-
T4	0	0.19±0.00 ^{cC}	-	-	-	-
T1	168	0.79±0.02 ^{aA}	4.86±0.04 ^{aA}	7.99±0.09 ^{aA}	11.16±0.10 ^{aB}	1.41±0.01 ^a
T2	168	0.36±0.01 ^{bA}	3.66±0.01 ^{bA}	5.35±0.15 ^{bA}	5.77±0.04 ^{bB}	0.73±0.01 ^b
T3	168	0.19±0.00 ^{dA}	-	2.58±0.01 ^{cA}	0.97±0.01 ^{dB}	0.12±0.00 ^d
T4	168	0.26±0.01 ^{cA}	-	2.44±0.02 ^{dA}	1.34±0.03 ^{cB}	0.17±0.00 ^c
T1	240	0.78±0.01 ^{aA}	4.17±0.02 ^{bB}	7.94±0.03 ^{aA}	15.74±0.21 ^{aA}	1.99±0.03 ^a
T2	240	0.37±0.00 ^{bA}	4.40±0.02 ^{aB}	5.46±0.01 ^{bA}	8.52±0.06 ^{bA}	1.08±0.01 ^b
T3	240	0.19±0.00 ^{dA}	0.76±0.01 ^{cA}	0.97±0.04 ^{dB}	2.29±0.02 ^{cA}	0.29±0.00 ^c
T4	240	0.22±0.00 ^{cB}	-	1.98±0.01 ^{cB}	1.57±0.03 ^{dA}	0.20±0.00 ^d

Means±standard deviations in the same column, within each fermentation time, followed by different lowercase letters, indicate that the treatments differ from each other according to Tukey's test ($p \leq 0.05$). Meanwhile, the means±standard deviation in the same column, referring to the same treatment at different fermentation times, followed by different uppercase letters, indicate differences over time, according to Tukey's test ($p \leq 0.05$). ABV=Alcohol by volume. Fermentations: T1: Sugarcane juice (15 °Brix) and cocoa honey; T2: Sugarcane juice (5 °Brix) and cocoa honey; T3: Sucrose and cocoa honey; and T4: Control

As predicted, the reduction in soluble solids observed during the first fermentation was accompanied by an increase in titrateable acidity, leading to a decrease in pH, regardless of the substrate used ($p \leq 0.05$) (Table 2). The highest titrateable acidity was observed in T1 at 168 h (1.83%). However, during the second fermentation, no significant differences ($p > 0.05$) in titrateable acidity or pH were observed between T1 and T2. In contrast, T3 and T4 showed substantial increases ($p \leq 0.05$) in titrateable acidity. The reduction in soluble solids and pH, and the increase in titrateable acidity, are attributed to the microbial activity of yeasts and bacteria present in the SCOBY. Yeasts utilize carbohydrates as a substrate for ethanol production, while AAB uses ethanol to produce acetic acid, thereby lowering

the pH [26]. There is also a relationship between pH values and soluble solids content, which decreases as fermentation progresses, indicating an increase in the concentration of organic acids produced throughout the fermentation process [36].

Kombucha has a characteristic acidic taste, and pH is a critical factor in the safety of kombucha beverages [16, 36]. The beverages (240 h) had pH values ranging from 2.98 to 3.15. A safe pH range for kombucha beverages is 2.5–4.2, according to Normative Instruction n° 41 [37]. These pH values inhibit the growth of pathogens such as *Bacillus cereus*, *Clostridium perfringens*, and *Clostridium botulinum*. The beverage's antimicrobial effects are mainly attributed to acetic acid [38]. Variations in pH can affect

Table 4 Evaluation of the antioxidant potential of kombuchas during the fermentation process

Treatments	Fermentation time (h)	Total phenolics (mg GAE/100 mL)	Phosphomolybdenum complex (mg AAE/100 mL)	ABTS+ (% FRS)
T1	0	720.87±6.15 ^{aB}	728.35±12.68 ^{aA}	89.39±3.20 ^{bA}
T2	0	502.39±4.61 ^{bC}	241.89±3.17 ^{bA}	95.10±1.91 ^{aA}
T3	0	531.74±6.15 ^{bC}	277.75±15.85 ^{bB}	94.25±2.36 ^{abA}
T4	0	506.74±7.69 ^{bB}	268.79±22.19 ^{bB}	94.67±2.26 ^{aA}
T1	168	821.96±13.83 ^{bA}	459.34±0.00 ^{aB}	21.08±0.19 ^{cB}
T2	168	1099.13±83.01 ^{aA}	148.85±4.76 ^{cB}	78.78±1.61 ^{bC}
T3	168	827.39±36.89 ^{bA}	293.45±15.85 ^{bB}	91.65±0.49 ^{aA}
T4	168	846.96±9.22 ^{bA}	286.72±6.34 ^{bAB}	91.09±4.04 ^{aA}
T1	240	354.57±10.76 ^{cC}	433.56±36.46 ^{aB}	15.65±2.81 ^{cC}
T2	240	727.39±3.07 ^{bB}	135.40±7.93 ^{cB}	86.14±2.48 ^{bB}
T3	240	762.17±46.12 ^{bA}	390.96±17.44 ^{aA}	94.91±1.16 ^{aA}
T4	240	913.26±47.65 ^{aA}	313.62±9.51 ^{bA}	94.67±1.40 ^{aA}

Means±standard deviations in the same column, within each fermentation time, followed by different lowercase letters, indicate that the treatments differ from each other according to Tukey's test ($p \leq 0.05$). Meanwhile, the means±standard deviation in the same column, referring to the same treatment at different fermentation times, followed by different uppercase letters, indicate differences over time, according to Tukey's test ($p \leq 0.05$). GAE: Gallic acid equivalent; AAE: Ascorbic acid equivalent; %FRS: Free radical scavenging percentage. Fermentations: T1: Sugarcane juice (15 °Brix) and cocoa honey; T2: Sugarcane juice (5 °Brix) and cocoa honey; T3: Sucrose and cocoa honey; and T4: Control

the synthesis and stability of phenolic compounds, thereby impacting the beverage's antioxidant activity [36].

Both fermentations, especially the first one, promoted an increase in the L^* value, meaning a lightening of the beverage (Table S1, Supplementary Material). At the end of fermentation, T1 and T2 stood out as the lightest. This highlights the impact of sugarcane juice on the L^* value of the beverages, making them lighter compared to those that used sucrose as a substrate in the first fermentation.

The color evaluation of the beverages showed that the treatments, at the beginning (0 h) and at the end of fermentation (240 h), exhibited distinct colors (Fig. 1). The difference in color between the treatments at the start and end of fermentation was excellent for T1 (16.7), T2 (17.1), and T3 (11.5). At the same time, in T4, it was very noticeable (5.1). This variation may be due to a decrease in pH and microbial activity, which can alter or degrade pigments, cellulose, and pectin in the medium [39].

Organic acids and ethanol

Organic acids are the main products of kombucha fermentation, directly impacting the beverage's pH. Kombucha has a pleasant, acidic, fruity, and carbonated taste, primarily associated with the organic acids and CO_2 produced during fermentation [40]. Table 3 presents the changes in the main organic acids of kombucha during fermentation. The levels of organic acids increased significantly during fermentation compared to the initial time (0 h), as observed for titratable acidity.

Citric acid and oxalic acid were not detected during fermentation in any treatment. The highest concentration of succinic acid was in treatment T1 at 168 h and 240 of fermentation (0.79 g/L and 0.78 g/L, respectively), with

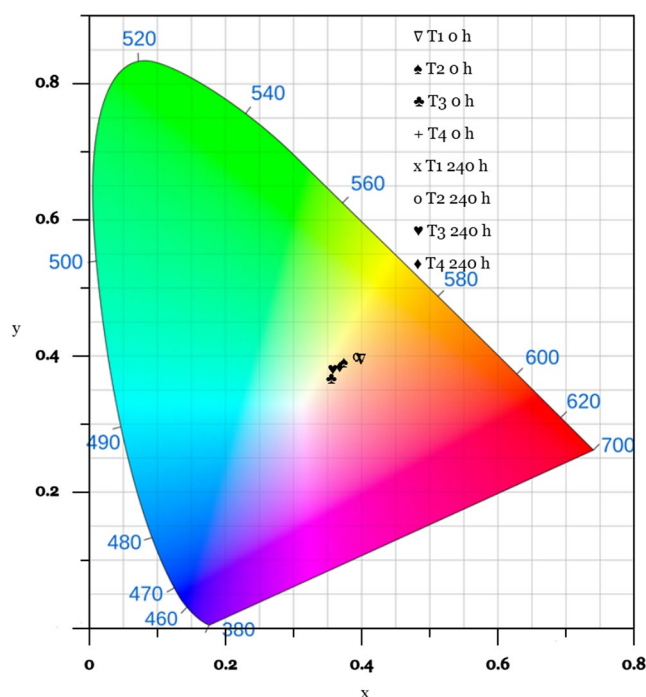


Fig. 1 CIE chromaticity diagram at the beginning of fermentation (0 h) and at the end of fermentation (240 h). Fermentations: T1: Sugarcane juice (15 °Brix) and cocoa honey; T2: Sugarcane juice (5 °Brix) and cocoa honey; T3: Sucrose and cocoa honey; and T4: Control

no difference between times ($p > 0.05$). Succinic acid can impart a fresh flavor to fermented foods [41]. The most abundant organic acid was acetic acid, and its concentration was significantly influenced by the type of substrate, with the highest concentrations in treatment T1 at 168 h (7.99 g/L) and 240 h (7.94 g/L) of fermentation, with no significant difference between these times ($p > 0.05$).

The production of acetic acid during fermentation is generally associated with AAB metabolism, particularly genera such as *Acetobacter*, *Gluconobacter*, and *Komagataeibacter* [42, 43]. Acetic acid is the predominant component in kombucha. It is produced by the oxidation of ethanol [40], imparting a pungent, acidic odor that contributes to the vinegar-like taste characteristic of kombucha [1, 44, 45]. Thus, a metabolic interaction exists, as the acetic acid produced stimulates yeast, and the ethanol produced by yeast promotes AAB to produce more acetic acid [39, 46].

Compared with acetic acid, lactic acid has milder, longer-lasting acidity [47]. The highest lactic acid concentration was also found in treatment T1 (4.86 g/L) at 168 h of fermentation. The lowest concentration of lactic and acetic acids was found in 240 h from treatment T3 (0.76 and 0.97 g/L, respectively). The detected lactic acid is likely the result of microbial metabolism by LAB, including genera such as *Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Leuconostoc*. Beyond influencing flavor, lactic acid reduces pH and enhances the microbiological safety of kombucha through its antimicrobial properties [3, 48, 49]. The lower concentrations of acetic and lactic acid in T3 may be attributed to the composition of the cocoa honey added to the sample, which resulted in insufficient glucose for their synthesis during fermentation [50].

The lower concentrations of acetic and lactic acids in sucrose treatments (T3 and T4), compared to those with sugarcane juice (T1 and T2), can be attributed to the more complete composition of sugarcane juice, which contains simple sugars, minerals, vitamins, and amino acids that promote fermentative activity. In contrast, sucrose provides fewer nutrients to microorganisms, thereby reducing microbial activity and metabolite formation [21, 22].

Kombucha is commonly marketed as a non-alcoholic beverage. Non-alcoholic beverages must contain less than 0.5% ABV [51]. Since kombucha is a fermented beverage, alcohol production is inevitable. However, kombucha manufacturing companies have developed a new category of alcoholic kombucha called “kombucha beer” or “hard kombucha”, which has an alcohol content of 0.5% ABV or higher [52].

Ethanol concentrations varied during fermentation, and it was produced in both the first and second fermentations using different substrates. After 240 h of fermentation (beverage), T3 (0.29% ABV) and T4 (0.20% ABV) exhibited low ethanol concentrations and were classified as non-alcoholic beverages according to Brazilian legislation. According to the legislation, kombuchas with up to 0.5% ABV are considered non-alcoholic [37]. The highest ethanol concentration was in T1, at 1.99% ABV, followed by T2 at 1.08% ABV. Both were classified as alcoholic kombuchas, which are considered alcoholic when their ABV ranges between

0.6% and 8.0% [37]. During fermentation, the sugar concentration in the substrate influences the ABV of kombucha. The increase in ethanol concentrations in kombucha from treatments T1 and T2 may be related to the increase in yeast population density, such as *S. cerevisiae*, present in SCOBY and starter (fermented kombucha) [53].

Therefore, although alcoholic kombuchas may represent a new consumer niche, their acceptance may be limited, since studies indicate that alcohol consumption, even in small amounts, may be associated with health risks [54]. Furthermore, kombucha is widely recognized as a functional and health-promoting beverage, which may create a conflict in consumer perception when the product is alcoholic.

Antioxidant potential

Phenolic compounds, widely distributed secondary metabolites in plants, exhibit high antioxidant capacity, with the potential to reduce free radicals, prevent oxidative damage, and contribute to the prevention of chronic diseases [54–57]. The total phenolic compound concentration analysis, determined using the Fast Blue method, revealed significant differences between treatments and fermentation times ($p \leq 0.05$) (Table 4).

At the beginning of fermentation (0 h), T1 showed the highest total phenolic concentration (720.87 mg GAE/100 mL). Sugarcane juice is a source of bioactive compounds, including total phenolics, such as hydroxycinnamic acids (caffeic and sinapic acids), and flavonoids, such as tricetin, apigenin, and luteolin. Therefore, the higher initial phenolic concentration is attributed to the polyphenolic contribution of sugarcane juice, which compensates for the lower amount of green tea in this treatment [22]. Throughout fermentation, phenolic concentration increased, with T2 reaching 1,099.13 mg GAE/100 mL at 168 h. The increase in total phenolics may be related to the action of enzymes released during fermentation, which promotes the de-esterification of catechins substituted with 3-galloyl [58]. However, by the end of fermentation (240 h), a significant reduction in total phenolics was observed across all treatments, with T1 showing the greatest reduction (354.57 mg GAE/100 mL). At the same time, T4 maintained the highest concentration (913.26 mg GAE/100 mL).

The phenolic composition of kombucha can be influenced by various factors, including the type of tea used and the presence of additional substrates such as cocoa honey, and sugarcane juice, which can modify antioxidant activity throughout fermentation [26, 59]. During fermentation, various transformations, including degradation, oxidation, epimerization, and hydrolysis, can alter the structure and levels of phenolics, thereby releasing bound phenolics and forming new metabolites with altered antioxidant capacity

[15, 18]. Thus, fermentation can both generate and consume phenolic compounds, resulting in variations in the beverage's antioxidant potential [36]. The cocoa honey used in T2 and T3 may have contributed to phenolic stability by providing additional bioactive compounds, including flavonoids, polyphenols, and catechins [26, 60]. These phenolics act by neutralizing free radicals through mechanisms such as hydrogen transfer, electron transfer, and metal chelation [61, 62].

The antioxidant activity, quantified using the phosphomolybdenum complex method, showed significant variation among treatments. Initially (0 h), T1 exhibited the highest antioxidant concentration (728.35 mg AAE/100 mL). During fermentation, an increase in antioxidant activity was observed in T3 and T4, while a reduction was noted in T1 and T2. Despite the decline at the end of fermentation, T1 still maintained higher values than the other treatments. The decrease in antioxidant activity in sugarcane juice-based treatments (T1 and T2) may be related to the biotransformation of phenolic compounds by microbial enzymes, including acid hydrolysis and depolymerization, and to the acidic pH of kombucha, which can alter the structure and functionality of the present antioxidant compounds [63].

The evaluation using the ABTS+ assay revealed a distinct pattern throughout the fermentation process. Initially, all treatments exhibited high free radical scavenging percentages, ranging from 89.39% (T1) to 95.10% (T2). By the end of fermentation (240 h), T1 exhibited a sharp decline in antioxidant activity (15.65%), whereas T3 and T4 maintained high values (94.67%). The higher percentages observed in T2 and T3 may be attributed to the enhanced production or preservation of bioactive metabolites, such as polyphenols and organic acids. These compounds may exhibit a greater capacity to neutralize free radicals and compete with oxygen in scavenging nitric oxide [64].

The phenolic composition and antioxidant activity of kombucha may vary depending on the substrate used. The reduction in antioxidant capacity in kombucha with higher sugarcane juice concentrations (T1) may be attributed to the conversion of bioactive compounds into metabolites with lower antioxidant activity during fermentation [36]. Compounds with high antioxidant potential may undergo structural modifications that result in a decrease in their ability to neutralize free radicals, due to the action of microbial enzymes. Structural elements, such as the number of phenolic hydroxyl or methoxyl groups, the presence of hydroxyl groups in flavones, as well as free keto and carboxylic groups, among other specific properties of phenolic compounds, can influence their antioxidant activity. Nevertheless, such structural changes can also enhance the antioxidant activity of these molecules or modify their

effectiveness against different types of free radicals, such as nitric oxide, superoxide, and hydroxyl radicals [11, 65].

Treatments containing sucrose in the first fermentation and cocoa honey in the second fermentation (T3) and sucrose in both the first and second fermentations (Control, T4) exhibited greater antioxidant stability over time. T1 experienced a significant reduction in antioxidant activity by the end of the first fermentation (168 h) and continued to decrease by the end of the second fermentation (240 h). Whereas T2 antioxidant activity was increased by 240 h. Higher sugar concentrations in T1 may have intensified the metabolism of yeasts and bacteria, resulting in increased production of organic acids, volatile compounds, and ethanol. However, this accelerated microbial activity may also have promoted the rapid degradation of phenolic compounds, resulting in lower conversion to secondary antioxidant metabolites. Part of these compounds may have been precipitated, oxidized, or transformed into forms with lower redox activity [56, 59, 61, 62]. In contrast, T2, with a lower sugar concentration, provided a more balanced fermentative metabolism, favoring the preservation of bioactive compounds and greater antioxidant stability throughout the process [18, 55].

The biotransformation of phenolic compounds during fermentation is directly related to microbial activity, the formation of organic acids, and interactions among bioactive compounds in the substrates. The reducing power of kombucha underscores its electron-donating capacity, which reflects the beverage's antioxidant potential [66]. Green tea remains one of the primary sources of antioxidant compounds, providing kombucha with a relevant bioactive profile for health [55].

The variations in antioxidant potential, as evaluated by the phosphomolybdenum complex and ABTS+ methods, arise from distinct mechanisms of action. The phosphomolybdenum assay is based on the analyte's conversion of Mo^{6+} to Mo^{5+} , followed by formation of a green phosphate complex in an acidic medium [61, 62]. In the ABTS method, the oxidation of the molecule by oxidizing agents generates its ABTS+ radical cation, which exhibits intense coloration. Antioxidant capacity is determined by the ability of tested compounds to reduce this coloration through direct interaction with the radical [61].

Volatile compounds

The volatile composition of kombuchas produced using different treatments was evaluated at the beginning of fermentation (0 h) and in the final beverage (240 h). A total of 91 volatile compounds were detected in both the initial fermentation stage and the kombucha beverages, classified into

the following groups: acids (10), alcohols (16), aldehydes (4), alkanes (3), esters (22), furans (2), ketones (6), lactones (2), phenols (7), terpenoids (7), and others (12) (Table S2, Supplementary Material).

During fermentation, microorganisms transform the chemical profiles of the tea and the substrate, producing aromatic compounds that influence the sensory characteristics of the kombucha. Natural plant compounds are converted into ketones, alcohols, and esters through fatty acid metabolism. Enzymes such as lipoxygenase promote the oxidation of these acids, generating various volatile compounds according to microbial activity, pH, and process conditions [67].

The largest classes of volatile compounds detected were esters, alcohols, and acids (Fig. 2). Among the acids found in the beverages after both fermentation stages, acetic acid was predominant in T2, T3, and T4. In contrast, octanoic acid was the primary compound in T1. Volatile acids are key aromatic compounds in fermented beverages. During kombucha fermentation, acetic acid is one of the main organic acids produced by AAB, contributing to the beverage's characteristic pungent and acidic aroma and flavor [43].

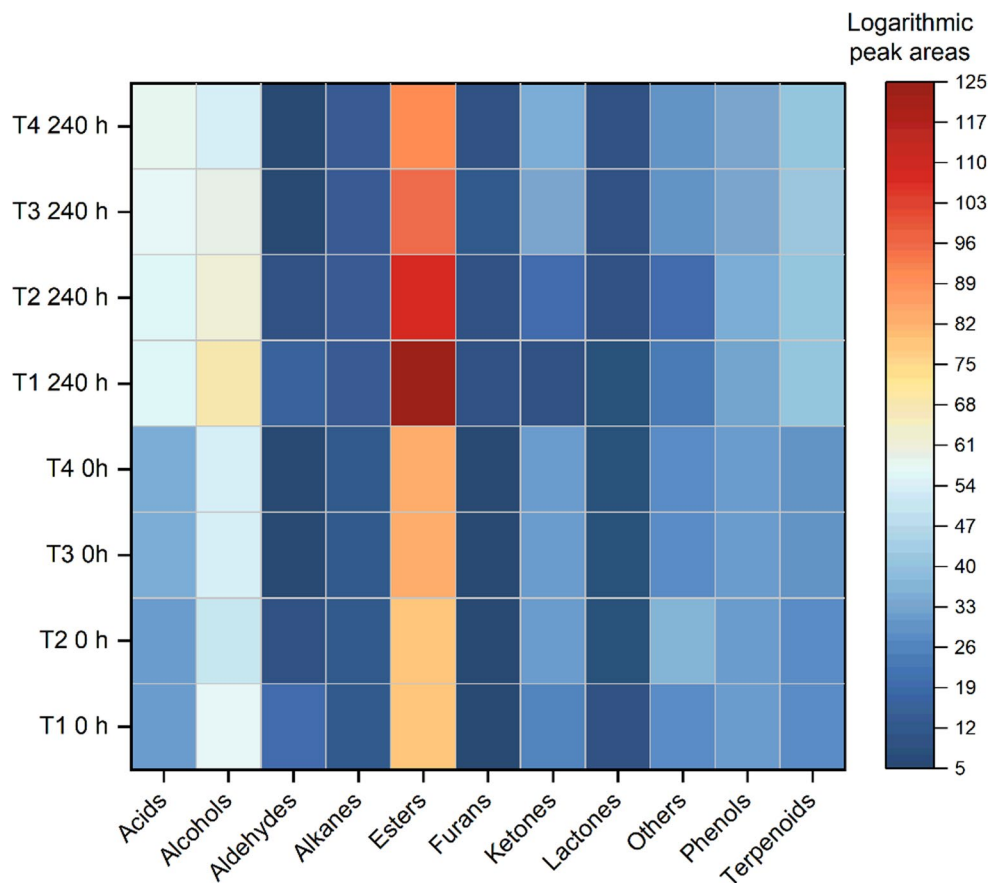
Regarding alcohol, phenylethyl alcohol was the most abundant in the final beverage. In the ester group, ethyl decanoate predominated in T1 and T3, whereas acetic acid,

2-phenylethyl ester, was the dominant ester in T2 and T4. Esters and alcohol, such as phenylethyl alcohol and acetic acid, 2-phenylethyl ester, were already present in the substrates at the beginning of fermentation. These compounds increased during fermentation and may contribute to the development of descriptors such as fruity, honey, spicy, rose, lilac, floral, fresh, and cocoa notes in the kombucha [68]. Esters and alcohols are generally produced by yeasts such as *Saccharomyces*, *Brettanomyces*, and *Pichia* [69, 70].

Esters were detected at the beginning of fermentation, but were also produced throughout the process. The highest concentrations of esters at the end of fermentation (240 h) were observed in T1 (124.3 log₁₀ peak areas), followed by T2 (108.0 log₁₀ peak areas). This chemical class is essential aromatic components, primarily responsible for defining fruity and floral notes in beverages. Their formation is closely linked to the metabolism of fatty acids and amino acids through intracellular yeast metabolism [70, 71]. The most abundant esters were decanoic acid, ethyl ester (5.62–6.67 log₁₀ peak areas), and acetic acid, 2-phenylethyl ester (5.89–6.37 log₁₀ peak areas), which contribute to sensory perceptions of apricot, banana, pineapple, strawberry, floral, honey, and tea [68].

Terpenoids are a significant chemical group in kombucha beverages, primarily derived from tea and exhibiting fruity,

Fig. 2 Heatmap of volatile compound classes detected in kombucha at the beginning of fermentation (0 h) and at the end of fermentation (240 h). Fermentations: T1: Sugarcane juice (15 °Brix) and cocoa honey; T2: Sugarcane juice (5 °Brix) and cocoa honey; T3: Sucrose and cocoa honey; and T4: Control



sweet, citrusy, herbal, and floral aromas [69]. Geraniol was the commonly detected terpenoid across all treatments, contributing floral notes reminiscent of geranium and rose [49]. The terpenoids 1,5,7-octatrien-3-ol, 3,7-dimethyl-, and 5,9-undecadien-2-ol, 6,10-dimethyl- were not detected at the beginning of fermentation in any treatment but appeared in all treatments in the final beverage as fermentation products.

Principal Component Analysis (PCA) was used to correlate the chemical classes of volatile compounds with different treatments (Fig. 3). Principal components 1 and 2 explained 85.93% of the total variance for the treatments at the beginning of fermentation (0 h) and in the final beverage (240 h).

At the beginning of fermentation, T2, T3, and T4 were associated with ketones and other compounds. By the end of fermentation (240 h), T1 and T2 were correlated with esters, alcohols, and aldehydes, whereas T3 and T4 were associated with lactones, phenols, acids, furans, terpenoids, and alkanes. The concentrations of different chemical classes vary among treatments, potentially influencing the product's sensory profile. Alcohols and esters are associated with fruity and floral aromas, aldehydes contribute green and buttery notes, and terpenoids impart citrus and herbal characteristics, all of which are considered desirable in kombucha [49, 68–71]. In contrast, phenolic compounds are associated with smoky, spicy, or medicinal descriptors, which can adversely affect sensory quality [44].

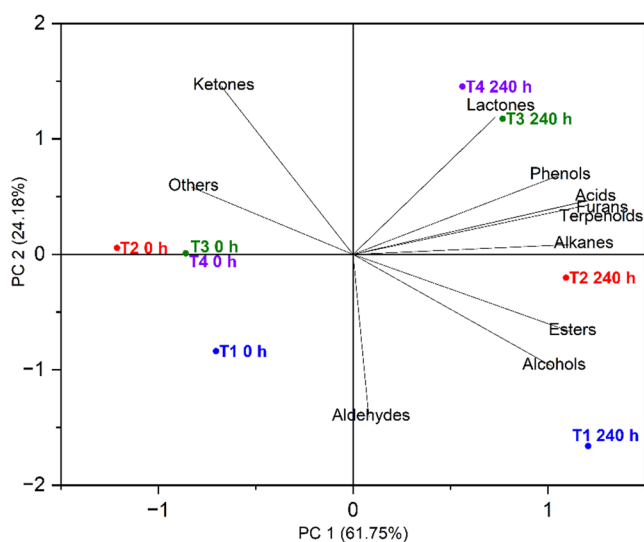


Fig. 3 Principal Component Analysis (PCA) of classes detected in kombucha at the beginning of fermentation (0 h) and at the end of fermentation (240 h). Fermentations: T1: Sugarcane juice (15 °Brix) and cocoa honey; T2: Sugarcane juice (5 °Brix) and cocoa honey; T3: Sucrose and cocoa honey; and T4: Control

These metabolites may originate from substrates or microbial activity during fermentation. Throughout this process, microbial metabolism intensifies the production of alcohols, esters, volatile acids, aldehydes, and other compounds. Yeasts mainly synthesize esters and alcohols through the Ehrlich pathway and fatty acid metabolism, while volatile acids derive from the breakdown of carbohydrates, amino acids, and lipids [71].

The differences observed between treatments suggest that substrate composition plays an essential role in modulating microbial activity and, consequently, in the biosynthesis of volatile compounds. Notably, esters and higher alcohols often act synergistically to reinforce floral and fruity notes. On the other hand, the accumulation of phenolic compounds in T3 and T4 may contribute spicy or medicinal nuances that can mask or contrast with the delicate notes derived from esters and terpenoids. These interactions suggest that the sensory quality of kombucha is not solely determined by the presence of specific volatile compounds, but also by the balance between compounds that contribute positively to flavor and aroma.

This study presents new perspectives for the formulation of kombucha using alternative substrates widely available in tropical regions, such as sugarcane juice and cocoa honey, which are often underutilized. Sugarcane juice favored microbial growth and fermentative efficiency, intensifying the production of organic acids and volatile compounds. In contrast, cocoa honey contributed to bioactive compounds such as phenolic acids, polyphenols, flavonoids, vitamins, and amino acids that increased antioxidant activity, while also promoting environmental benefits by valuing agro-industrial byproducts. These findings demonstrate the versatility of regional raw materials in modulating the chemical and functional properties of kombucha. The availability of alcoholic (T1 and T2) and non-alcoholic (T3 and T4) beverages broadens potential applications across market segments, including health-conscious consumers, the functional beverage sector, and even the alcoholic beverage market. The results of this study reinforce the technological feasibility and industrial potential of using tropical substrates, suggesting scalability and alignment with consumer trends prioritizing sustainability, health, and innovation in the functional beverage market. Although each formulation has demonstrated specific advantages, future studies may focus on analyzing microbial load, especially pathogenic microorganisms, and cytotoxicity, in addition to sensory analysis correlating consumer acceptance with bioactive and physico-chemical characteristics.

Conclusion

This study shows that the use of different substrates influences the physicochemical, chemical, and antioxidant properties of kombucha. The use of sugarcane juice, sucrose, and cocoa honey resulted in significant differences in °Brix, acidity, organic acid profile, antioxidant activity, and volatile compounds, demonstrating the direct impact of the substrates on the final characteristics. The treatment with sugarcane juice at 15 °Brix (T1) stood out for its high alcohol content, organic acids (particularly acetic and lactic), and higher concentrations of volatile compounds, including esters and alcohols. Meanwhile, the sucrose treatments (T3 and T4) exhibited greater antioxidant activity, particularly when combined with cocoa honey (T3).

Accordingly, sucrose and cocoa honey treatment (T3) stood out for its high antioxidant activity and greater production of volatile compounds. The alcohol content of T3 and T4 remained below 0.5%, making them viable alternatives for consumers seeking non-alcoholic beverages. In contrast, the treatments with sugarcane juice (T1 and T2) exhibited higher alcohol content, positioning them as attractive options for consumers exploring new niches of alcoholic kombuchas.

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Declarations

Competing interest The authors do not have any conflicts of interest.

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