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Research article

Accumulation of 5-methyltetrahydrofolate and other bioactive compounds, in the course of fermentation of green tea (*Camellia sinensis*) kombucha

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ABSTRACT

Kombucha is a potential probiotic tea-based drink with increasing worldwide consumption. Studies on this probiotic beverage are growing rapidly, especially about micronutrients and microbial population. As such, the present study performed the molecular identification of the microorganism and evaluated 5-methyltetrahydrofolate content by HPLC-DAD, phenolic compounds, flavonoids, carotenoids, antioxidant activity by spectrophotometric methods, and physicochemical composition of green tea kombucha on fermentation days 1, 3, 7, 14, and 21. DNA sequencing identified the *Microbacterium* genus as predominant. However, was unable to safely determine the species level because of the rRNA 16S gene sequence similarity between four species *M. ureisolvens, M. yannicii, M. chocolatum e M. atlanticum*. The concentration of 5-methyltetrahydrofolate found on the third day was 39.12 ± 1.32 μg/mL (liquid) and 45.78 ± 8.42 μg/mL (polymeric biofilm); On the twenty-first day it was 50.87 ± 3.56 μg/mL (liquid) and 54.88 ± 3.89 μg/mL (polymeric biofilm). Total phenolic compounds increased with fermentation; however, flavonoids and carotenoids were degraded by the process. The information on 5-methyltetrahydrofolate is unprecedented and highly relevant for food guidelines, since related deficiencies can lead to fetal malformation in the first three months of pregnancy. Lastly, the best fermentation time to obtain 5-methyltetrahydrofolate and others bioactive compounds is between days 7–14. Further analyses are also encouraged to understand the bioavailability of the vitamin.

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1. Introduction

Kombucha is a traditional Asian fermented beverage made from green or black tea (*Camellia sinensis*), whose fermentation is carried out by mixed cultures of yeasts and bacteria dispersed in the liquid medium. The microorganisms in the liquid media ferment the added sugars to sustain growth and for the production of a polymeric biofilm known as SCOBY (Symbiotic Culture of Bacteria and Yeast) [1]. This biofilm contains mainly acetic bacteria from the genera *Gluconobacter* and *Komagataeibacter* and many osmophilic yeast species, especially from the genera *Brettanomyces*, *Candida*, *Saccharomyces*, *Schizosaccharomyces*, *Pichia* and *Zygosaccharomyces* [2–6].

From the last decades of the twentieth century, kombucha consumption popularized because of the potentially beneficial effects of this fermented drink. Part of these benefits would come from components in tea (*Camellia sinensis*), but the fermentation process used in kombucha production adds functional properties [1,7,8]. Metabolic products from the fermentation may be of benefit to human health. In addition to the benefits from some of the primary products, such as short-chain fatty acids, secondary metabolites benefit humans, such as phenolic compounds, flavonoids, and nutrients [9,10]. Some vitamins, such as folate, might be produced during fermentation, as has been shown in fermented milk and plant-based products [11–13]. Folate accumulation during fermentation depends on several factors, for example the bacteria and yeast strains used in the process [14,15]. However, folate accumulation during kombucha fermentation is largely unresearched. Due to it being a tea-based drink, some of the nutrients that can be analysed in kombucha are hydrosoluble vitamins. Folates, in particular, have been found not only in plants, but they have also been reported to be synthesized by folate-producing microorganisms in fermented dairy drinks [9,10]. This vitamin is commonly found in tiny amounts in nature and it is sensitive to light, oxidation, leaching, heat and other physicochemical factors [16–19]. Folates play important roles in the human body in oxidation, reduction and transfer reactions, amino acid metabolism and constitution of proteins and synthesis of nucleic acids, being important for a healthy nervous system [20–22].

The products obtained by fermentation are largely determined by environmental factors, such as temperature, pH, oxygen availability, light exposure, CO_2 concentration, and so on, along with the substrates available in terms of composition and concentrations [23,24]. Therefore, as with other fermented foods and beverages, kombucha may be enriched with desired bioactive compounds and nutrients due to the different conditions of the fermentation process, and this is of current technological interest [23,24].

The nutritional and bioactive components in kombucha are largely determined by the fermentation process. The mixed culture of microorganisms used to produce kombucha may be largely selected by environmental factors where the fermentation is conducted, and microorganisms from the local environment may be incorporated to the SCOBY. Therefore, the chemical profile of the kombucha beverage might be influenced by the location where it is produced, by the potential impacts of the local environment of production on the composition of the SCOBY colony and on the fermentation temperature, if non-controlled [15]. Therefore, it is of interest to determine the composition of kombucha in parallel to the determination of the SCOBY colony, especially when the experiments take place in non-traditional world regions of kombucha production [25–27]. In the present study, we investigated kombucha produced in the Northeast region of Brazil, in a region with tropical climate, in a coastal area.

Kombucha produced in Latin America is largely unresearched, and it is not known if microorganisms from the local environment could be incorporated in the SCOBY, and if so, how it would impact the chemical profile of the kombucha beverage. The present work aimed to analyse the content of 5-methytetrahydrofolate, total phenolics, total flavonoids, total carotenoids, antioxidant potential, and physicochemical properties of green tea kombucha produced in the Northeast of Brazil, during a 21-day fermentation period.

2. Material and methods

2.1. Materials

The kombucha culture used in the present study was obtained from home producers/consumers that crafted the kombucha beverage by home cultivating the SCOBY in *Camellia sinensis* tea added with white sugar. For analysis, the kombucha was produced in parallel experiments in the laboratory under controlled conditions, growing the SCOBY in tea added with sucrose at 25 ± 3 °C [1,6,10]. Dry green tea leaves (*Camellia sinensis*) were purchased in a local supermarket, in June 2020. The tea was prepared by infusing 75 g of dried tea leaves in 7.5 L of bi-distilled water at 90 °C, for 30–40 min. The tea was filtered by filter paper (Whatman Nº 1), and 600 g (8 %) of refined sugar cane was added. 1500 mL (20 %) of the viable colony kombucha starter (kombucha liquid with polymeric disc cut into 2 cm^3 cubic fragments) was added. The fermented kombucha was sampled (20 mL aliquots) in the days 1, 3, 7, 14, and 21, and stored at −20 °C for analysis in triplicate.

The following reagents and samples were used: Methanol (Merck®, Brazil), monobasic potassium phosphate, and phosphoric acid (Fluka®, Switzerland), with a purity level of 99 % or greater or HPLC grade. Ammonium acetate and acetone (Vetec®, Brazil), Trolox, gallic acid, ABTS, Folin-Ciocalteu (Sigma-Aldrich®, Germany), sodium hydroxide, sodium nitrite, and potassium persulfate (Neon®, Brazil) all in analytical grade. A commercial standard of 5-methyltetrahydrofolate (Sigma-Aldrich®, US), with purity greater than 99 %, was used to identify and quantify chromatographic peaks in HPLC-DAD analysis of folate in kombucha. Ultrapure water was obtained from an ultra-purification system giving water with a 18 MΩ cm conductivity and total organic carbon of 10 ppb or less at 25 °C and was used for all analyses.

2.2. Methods

2.2.1. Bacteria isolation and cultivation media

Kombucha beverage samples were collected after 11 days of fermentation and were serially diluted by a factor of 10 at each step (10^{-1} to 10^{-6}) in sterile saline solution 0.9 g/100 mL NaCl. For the polymeric disc (SCOBY), a section was aseptically collected and mechanically fragmented. The fragmented SCOBY was weighted and transferred to tubes containing sterile saline at a proportion of 1 mL of saline for each gram of sample. The tubes were vortex-mixed for 1 min and incubated for 15 min at 30 °C to allow the SCOBY-trapped microorganism cells to be transferred to the saline. To ensure the growth of even the most fastidious organisms was formulated a media based on the formulation of the well-established Man-Rogosa-Sharpe (MRS) for lactic acid bacteria (MRS). The carbohydrate of the original formulation of MRS was replaced by sucrose, since this is the most abundant carbohydrate in plant based substrate, like the green tea in the kombucha, and was add calcium carbonate for buffering and protection of acidogenic microorganisms either acetic acid or lactic acid bacteria. For cell plating, 100 μL of diluted kombucha or SCOBY suspension were plated on isolation medium with the aid of a sterile Drigalski spatula. Isolation medium was a modified SYC broth (10 g L^{-1} sucrose, 2 g L^{-1} yeast extract and 6 g L^{-1} calcium carbonate), with peptone (10 g L^{-1}), polysorbate (80 g L^{-1}), and ammonium citrate (2 g L^{-1}). The pH of this modified SYC isolation medium (miSYC) was adjusted to 7.0 ± 0.2 with proper HCl or NaOH solutions if necessary. For the solid medium, bacto-agar was added at 16 g L^{-1} prior to autoclaving. The plates were incubated aerobically at 30 °C for 96 h. Bacterial colonies were randomly collected, transferred to fresh miSYC plates, and incubated at 30 °C for 48 h for isolation and checking for colony purity. Cells isolated from bacterial colonies were used to inoculate miSYC broth and cultivated at 30 °C for 48 h. Frozen stocks were prepared by collecting cells from the liquid culture by centrifugation and suspension in $CaCO_3$-free miSYC broth containing 15 mL/100 mL glycerol. After vortex-mixing, cells were stored at −80 °C.

2.2.2. Genotyping culture isolates by 16S rRNA gene sequencing

Each isolate was reactivated from the ultra-freezer stock, cultivated at 30 °C for 48 h on miSYC plates and the colonies were collected for total DNA extraction according to Lucena et al. [28] and Mendonça et al. [29], with modifications to avoid contamination with the kombucha exopolysaccharide. Briefly, the cells were collected from the plates and suspended in lysis buffer (50 mM Tris HCl, 10 mM EDTA, adjusted to pH 8.0 with NaOH) containing 0.1 mg mL^{-1} lysozyme and 10 g L^{-1} sucrose. The tube was incubated at 37 °C for 45 min. Afterwards, 0.5 g/mL sodium dodecyl-sulfate was added to cell suspensions, mixed by inversion, and kept on ice for 15 min. To prevent interferences from exopolysaccharides, CTAB (Cetyltrimethylammonium Bromide) was added to a final concentration of 2 g/100 mL and the sample was incubated at 70 °C for 15 min. Then, the lysate was mixed 1:1 (v/v) with a phenol solution (25 g/100 mL phenol, pH 8.0, 24 mL/100 mL chloroform and 1 mL/100 mL isoamyl alcohol), mixed by inversion for 2 min and centrifuged at 12, 000×*g*, 4 °C for 15 min. The upper aqueous phase was collected, transferred to a new tube and the treatment with CTAB/phenol solution was repeated. Then, the clean aqueous phase was collected to a new tube and mixed with an equal volume of cold isopropanol. The mixture was mixed by inversion for 2 min and incubated on ice for 10 min. After incubation, the tubes were centrifuged at 12, 000×*g*, 4 °C for 15 min. The supernatant was discarded, and the DNA-containing pellet was washed with cold ethanol 70 % (v/v) solution and dried at room temperature for 2 h. The DNA pellet was suspended in sterile deionized water and the DNA concentration and level of purity were checked in a Nanodrop™ device manufactured by Thermo Scientific model 2000c. The purity parameter applied was the ration above or equal to 2.0 of the wavelength 260nm/230 nm, for phenolic contamination, and 260nm/280 nm, for cellular impurities from the DNA isolation measured through protein contamination levels. Regarding the integrity was assessed by electrophoresis in 1 % (w/v) agarose gel in 0.5 × in TAE buffer (20 mM Tris HCl,10 mM EDTA NaOH, 0.5 mM acetic acid, pH 8.0). The DNA was dyed with 0.5 μg/mL of ethidium bromide and visualized with UV-light in a photo-documenting equipment. The clean intact material was stored at −20 °C for analysis.

16S rRNA gene was amplified with the use of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers. PCR reactions were prepared by mixing 0.5 μg of DNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase in 1 × reaction buffer in 50 μL of final volume. Amplifications were performed with the first cycle of denaturation for 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 2 min 15 s at 46 °C and 1 min 15 s at 72 °C, with final extension for 7 min at 72 °C. The amplified gene products were purified via Wizard SV Gel and PCR Clean-Up System kit (Promega Co., USA), quantified and 100 ng were mixed with the ABI Prism Big Dye Terminator™ Ready Reaction Mix version 3.1 (Applied Biosystems, USA) containing one of the primers above at 1.2 μM, following manufacturer's instructions. DNA sequencing was performed in ABI Prism Sequencer device (Applied Biosystems, USA) in the platform of DNA sequencing and Gene Expression of the Biosciences Centre of the Federal University of Pernambuco. The resulting sequences were analysed with the aid of BioEdit v7.0 software and submitted to BLAST analysis at the Gene Bank/NCBI. Bacterial identification was confirmed when the query sequence showed more than 97 % similarity with a homologous sequence of a strain type in the database.

2.2.3. Determination of 5-methyltetrahydrofolate in kombucha by HPLC

The concentrations of 5-methyltetrahydrofolate (5-MTHF) in kombucha beverage and in the polymeric disc were determined by reversed-phase HPLC with diode array detection. The commercial standard used in this study were kept at −18 °C, and the stock solution was prepared at 1000 mg L^{-1}, by dissolving the standard in 1 mL NaOH 2 % (w/v) and completing the volume in a 100 mL volumetric flask with 8 mmol L^{-1} ammonium acetate at pH 6.7. The diluted solutions used for the calibration curve were prepared from the stock solution, and they were kept at 4 °C throughout the analyses [30].

The liquid chromatograph (Shimadzu®, Japan) used in this study was equipped with the following modules: controller system model CBM-20A, diode array detector (DAD) model SPD-20AV, quaternary pump model LC-20 AT, column oven model CTO-20AC,

automatic sampler model SIL-20AC. All commands were performed using LC Solution Software (version 1.25, Shimadzu®, Japan). The analyses were performed at the Multi-User Laboratory for Chemical Characterisation of Food, Drugs, and Nutritional Supplements at the Nutrition Department, Federal University of Pernambuco. HPLC analysis was performed on samples from the third and twenty-first days of fermentation.

Approximately 4 g of the sample (kombucha beverage or polymeric disc) were weighed on an analytical balance (Tecnal®, Brazil) to the nearest 0.1 mg, crushed (polymeric disc), and added to an Erlenmeyer flask and homogenized with 20 mL of 50 mmol L^{-1} ammonium acetate extraction solution. The samples were then vortexed (Fisatom®) for 1 min at medium speed and incubated at 40 °C for 10 min in a thermostated water bath (Quimis®; Brazil). Afterwards, the samples were centrifuged (5000×*g*, 4 °C, 15 min; Eppendorf 5810®) and subsequently filtered through cellulose acetate membranes (0.45 μm porosity). One mL of each extract was filtered again through 0.22 μm porosity polyvinylidene fluoride membranes, into 2.0 mL screw-capped glass vials, and taken to the chromatograph [30]. The selected method was chosen due to its high efficiency in extracting folates from vegetables for quantitative analysis.

A reversed-phase chromatographic column was used (C_{18}, 150 × 4.6 mm, 5 μm particle size; Allcrom Phenomenex). The solutions used as mobile phase were: 0.1 mol L^{-1} pH 2.0 monobasic potassium phosphate buffer (pHmeter Mettler toledo®) and methanol, both filtered through 0.22 μm porosity membrane filters (Millipore). During analysis, the isocratic mobile phase was composed of potassium phosphate buffer:methanol 85:15 (v/v), at 0.5 mL min^{-1} flow rate, and a total run time of 25 min. For each analytical run, 20 μL of either sample extract or standard solution were injected. The eluate was monitored by the DAD (200–400 nm) and a fixed wavelength of 290 nm was used for quantification of 5-THF [30].

2.2.4. 5-Methyltetrahydrofolate identification and quantification

Peak identity was determined by comparing the peak retention times in samples' chromatographic runs with those of standards, and by UV-spectral similarity, which is an equipment feature that allows comparisons between peaks' UV-spectra. Quantification was performed by external standardization using an analytical calibration curve with ten concentrations of 5-methyltetrahydrofolate ranging from 0.05 μg mL^{-1} to 100 μg mL^{-1}. The working standard solutions used for the calibration curve were individually prepared by diluting the stock solution with ammonium acetate (8 mmol L^{-1}) in volumetric flasks.

The limit of quantification (LOQ) was defined as the lowest concentration of the analyte that reached a signal ten times above the baseline noise [31], using Equation *(1)*.

$$LOQ = (SD \times 10)/SC \quad (1)$$

Where, SD is the standard deviation of the intercept with the y-axis of at least three calibration curves, and SC is the slope of the calibration curve.

The limit of detection (LOD) was defined as the lowest concentration of the analyte that reached a signal three times above the baseline noise [31], using Equation *(2)*.

$$LOD = (SD \times 3)/SC \quad (2)$$

2.2.5. Spectrophotometric analysis of bioactive compounds

Total phenolic compounds, total flavonoids, and antioxidant activity by the ABTS radical assay were determined in the polymeric disc and in the kombucha beverage. The polymeric disc was weighed 1g at Falcon tube, and 10 mL of methanol:water 80:20 (v/v) were added, followed by vortex-mixing for 30 s and left to rest, protected from light for 24 h. Afterwards, the mixture was filtered, and the filtrate was transferred to a new Falcon tube covered with aluminum foil and stored at −20 °C until analysis. The polymeric disc was extracted in triplicate.

2.2.5.1. Determination of total phenolic compounds. Total phenolics were determined as previously described [32], with slight modifications. Two-hundred and fifty μL of kombucha beverage or the polymeric disc extract were mixed with 1.25 mL of the 10 % Folin-ciocalteu reagent in test tubes. The solutions were quickly vortex-mixed and then stored at room temperature and in the dark for 6 min. Afterwards, 1.0 mL of a 7.5 % Na_2CO_3 solution was added, and samples were incubated in a water bath (Novatecnica®, Model NT232; Brazil) at 50 °C for 5 min. After incubation the samples' absorbance was determined at 765 nm in a spectrophotometer (BEL Photonics, Brazil). A reagents' blank with deionized water was used to calibrate the spectrophotometer zero absorbance. The total phenolic content was determined using a standard curve with gallic acid, and the results were expressed as mg of gallic acid equivalent (GAE) in the polymeric disc (mg GAE/100 g) or in the kombucha beverage (mg GAE/L).

2.2.5.2. Determination of total flavonoids. Content of total flavonoids was determined by the aluminum chloride assay as described by Zhishen, Mengcheng, and Jianming [33]. Briefly, to 0.5 mL of sample (kombucha beverage or the polymeric disc extract) 2.0 mL of distilled water was added in a test tube, followed by the addition of 150 μL of 5 g/100 mL $NaNO_2$. After 5 min, 150 μL of 10 g/100 mL aluminum chloride was added to the sample tubes, and after 6 min, 1.0 mL of 1.0 mol/L NaOH and 1.2 mL of distilled water were added. The samples' absorbance was read at $\lambda = 510$ nm, against a blank solution, without the extracts. The concentration of total flavonoids in the extracts was determined based on a standard curve of catechin, and the results were expressed in mg catechin equivalents (CE) per hundred grams of the polymeric disc (mg CE/100 g) or per litre of the kombucha beverage (mg CE/L).

2.2.5.3. Determination of total carotenoids. Total carotenoids content was determined spectrophotometrically, essentially as previously described [34]. Ten mL of acetone were added over 1.0 mL of kombucha in glass tubes wrapped in aluminum foil, the mixture was vortex-mixed vigorously for 30 s and filtered through qualitative filter paper (40 Whatman®, 125 mm). The samples' absorbance was read on a spectrophotometer (BEL Photonics) at $\lambda = 470$, 645, and 662 nm, against an acetone blank. The total carotenoid contents were calculated based on its molecular absorptivity, using Equations *(3)*–*(5)* below, and were expressed in μg/100 mL.

$$Ca_{(\mu g/\ mL)} = 11.24_{A662}\text{-}2.04_{A645} \quad (3)$$

$$Cb_{(\mu g/\ mL)} = 20.13_{A645}\text{-}4.19_{A662} \quad (4)$$

$$C_{(\mu g/\ mL)} = (1000_{A470}\text{-}1.90Ca\text{-}63.14Cb)214 \quad (5)$$

2.2.6. Proximate composition, and physical and chemical parameters

The contents of ash and moisture in the polymeric disc and in the kombucha beverage were determined as described by the Association of Official Analytical Chemists [35]. Total lipid content was determined by the method of Folch, Less, and Sloane-Stanley [36]. The analyses for pH, water activity, total soluble solids by refractometry (° Brix), titratable acidity, and total sugars were performed according to official methods of the Brazilian food authority [34] using pHmeter (GEHAKA, model PG1800, São Paulo - SP, Brazil), Aqualab water activity measuring device (DECAGON, model AQUALAB 4 TE, USA), and a portable refractometer (LORBEN, São Paulo – SP, Brazil).

2.3. Statistical analysis

The tests of variance homogeneity (Bartlet's) and of normality (Shapiro-Wilk) were used to assess data distribution features. Mean values were compared by ANOVA with Tukey's post-hoc test for data passing the normality tests, and by the Kruskal-Walli's with Dunn's post-hoc test for those showing non-normal distribution. Statistical tests were run in R (R Core Team, 2021), and p-values <0.05 were considered as statistically significant.

3. Results and discussion

3.1. Kombucha culture identification by rRNA 16S genotyping

The isolation of microorganism from the tea and the Scooby of the samples suggests the microorganisms load is very low. Only from the lowest dilution (10^{-1}) showed microbial growth, 3 colonies from the tea and 4 colony for the Scooby. Molecular identification of the isolates from kombucha tea and polymeric disc materials was performed through partial amplification of rRNA 16S gene (Fig. 1) and is shown in Table 1. The microorganisms obtained were identified as belonging to the genus *Microbacterium*. However, the genotyping was unable to safely determine the species level because of the rRNA 16S gene sequence similarity between four species *M. ureisolvens*, *M. yannicii*, *M. chocolatum* and *M. atlanticum*.

The kombucha microbial community and population dynamics under fermentation are not completely known, and several species still been described [37]. The diversity of bacteria and fungi found in kombucha imposes challenges for the safe identification of microorganisms, to ensure if they were present in the inoculum or if they came from environmental contamination, or even whether the species are advantageous or deleterious to the process [38,39]. The most common organisms include acetic acid bacteria, lactic acid bacteria and yeasts, either *Saccharomyces* spp. or non-*Saccharomyces* spp., although Bacteroides and even Enterobacteriaceae have also been reported [40].

The beneficial effects of the kombucha were suggested in several studies, but it is unclear the role of the microorganisms in these

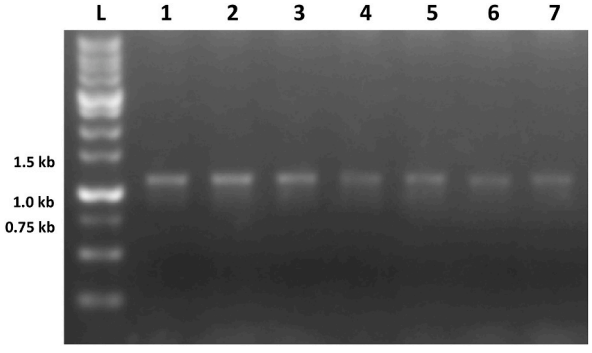

Fig. 1. PCR partial amplification of rRNA 16S gene. Lane L - Generuler of 1 kb, lane 1 to 7 are the amplification of the bacterial isolate's DNA, CA-T-1K to CA-S-7K.

Table 1
Molecular identification of the isolates from kombucha tea and polymeric disc materials.

Source	Isolate Identifier	GenBank deposit	Specie	Strain	Query cover	Identity	Mismatch
Tea	CA-T-1K	MW412501.1	*Microbacterium ureisolvens*	CFH_S00084	100	100	0
Tea	CA-T-2K	MW412501.1	*Microbacterium ureisolvens*	CFH_S00084	100	100	0
Tea	CA-T-3K	MG997081.1	*Microbacterium yannicii*	BMCPN6_2	100	100	0
Polimeric disc	CA-S-4K	MT386172.1	*Microbacterium chocolatum*	Atecer7F	100	100	0
Polimeric disc	CA-S-5K	MW412501.1	*Microbacterium ureisolvens*	CFH_S00084	100	100	0
Polimeric disc	CA-S-6K	MW412501.1	*Microbacterium ureisolvens*	CFH_S00084	100	100	0
Polimeric disc	CA-S-7K	MW412501.1	*Microbacterium ureisolvens*	CFH_S00084	100	100	0

health benefits [37,40–42]. In the present work, in both the tea and the SCOBY, only bacteria were identified, belonging to the genus Microbacterium, which encompasses organisms from a wide range of environmental sources, including species seen in plants [43]. Previous studies have also reported isolates of this genus in kombucha beverage or polymeric disc, strengthening the association of these species with this beverage [44–46]. Some species of Microbacterium genus showed probiotic features in animal models [47], including mammals [48], and might produce antioxidants [49] and antimicrobial compounds [50]. More studies are necessary to ascertain which of these properties can be attributed to the species found in kombucha.

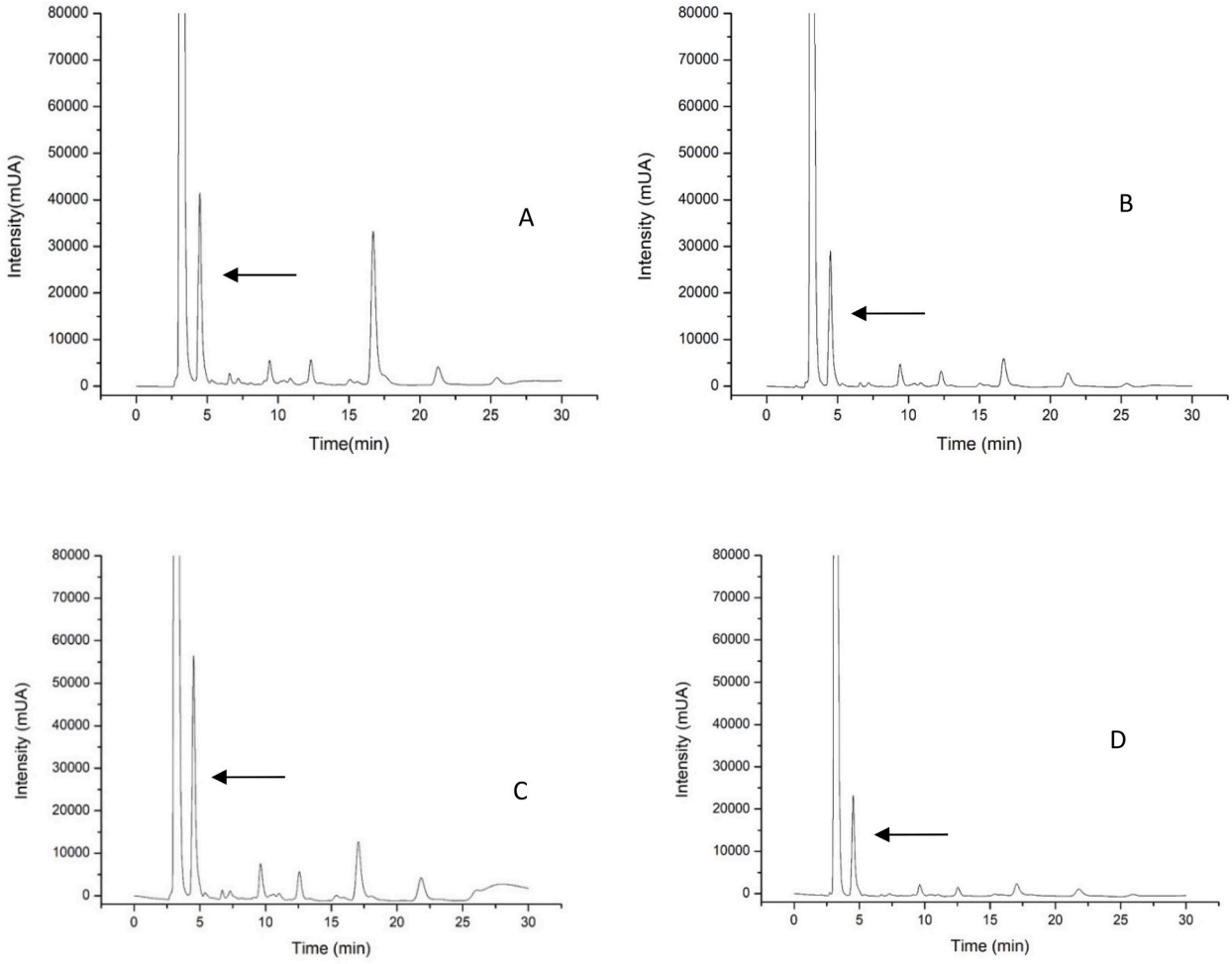

Fig. 2. Typical HPLC-DAD chromatograms obtained from the analysis of 5-methyl-tetrahydrofolate (5-*m*-THF) green tea kombucha liquid (A and C) or polymeric disc (B and D) fermented for 3 days (A and B) or for 21 days (C and D). Chromatographic conditions: chromatographic column C_{18} (150 × 4.6 mm, 5 μm particle size; Allcrom Phenomenex), isocratic elution using a 0.1 mol L^{-1} monobasic potassium buffer (pH 2.0) and methanol: water (85:15) as the mobile phase (v/v) with 20 μL injections for each analysis, with a flow rate of 0.5 mL/min^{-1} and detection by UV with a fixed wavelength of 290 nm.

3.2. 5-Methyltetrahydrofolate content in kombucha

The HPLC chromatograms show the profile of the substances detected in kombucha after three and twenty-one days of fermentation (Fig. 2). The chromatographic peak eluting at 4.5 min was annotated as 5-methyltetrahydrofolate, based on retention time, standard coelution, and spectra comparison (99 % spectra-similarity). Other chromatographic peaks seen had spectra similarities above 80 % with three forms of folates (folic acid, formyl folate, tetrahydrofolate), compared to the library available. However, as these compounds' standards were not used in the present work, it was not possible to confirm the identities of these peaks.

Table 2 shows the contents of 5-methyltetrahydrofolate in kombucha beverage and in the polymeric disc after fermentation for 3 and 21 days, and the analytical quality parameters. There was a significant increase in the 5-methyltetrahydrofolate content between three and 21 days of fermentation of 30 % in the kombucha beverage and 20 % in the polymeric disc.

Folates are essential nutrients to human cells as it exerts metabolic functions via C-transfer reactions in nucleic acids synthesis, DNA-methylation, methionine regeneration, prevention of cardiovascular diseases and of neural tube defects in fetuses, among other functions. Dietary folates exist primarily conjugated to polyglutamates, but these forms cannot be absorbed in the enterocyte membrane. The polyglutamate tail is hydrolysed in the small intestine by glutamate carboxypeptidase II, and folate is then absorbed and converted into its active forms (folyl-polyglutamates, tetrahydrofolates, H_2-folates) [51]. In this perspective, folate is in a highly bioavailable and bioactive form in kombucha, in the same structural form as it is usually found in human body, hence kombucha consumption would be beneficial to folate status.

The United States Department of Agriculture [52] recommends the daily consumption of 400 μg of folic acid for both males and female adults, and 600 μg for pregnant women. These levels prevent cardiovascular disease and anemia in adults, and neural tube defects in the developing fetus, respectively. To the best of our knowledge, until this work, scientific evidence of folates in kombucha was lacking, especially because folate accumulation occurred as the fermentation process continued [53]. Vitamin-producing microorganisms have been previously found in other fermented food products such as dairy drinks, soy-based drinks and in a potato, amaranth and chia vegan spread [11,12,14]. However, the present work seems to be the first one showing folate metabolites in a fermented tea-based drink. Future studies of vitamin metabolites in fermented beverages would be welcome.

A 200 mL portion of the kombucha produced and analysed in this study contained on average 7.82 and 10.17 mg of 5-methyltetrahydrofolate fermented for either 3 or 21 days, respectively. The amount of vitamin found in the samples analysed are much greater than the recommended dietary allowance (RDA) for folate and represent approximately 13-fold and 19-fold, respectively, of the RDA for pregnant women. This indicates that this food can be an excellent source of this vitamin in human nutrition [52].

It is important to note that green tea comes from *Camellia sinensis* leaves that have been industrially processed. Naturally occurring 5-methyltetrahydrofolate in tea leaves, which is the most common form of folate in foods, is sensitive to several physical factors such as heat, light, extreme values of pH, and leaching during processing. Therefore, technological operations like washing, bleaching, drying and freezing would lead to folate loss. Additionally, plants from the *Theaceae* family are not known for being a source of folates [53–55]. Therefore, it was expected that folates would be absent from the tea used as substrate for SCOBY growth and kombucha preparation.

Although the occurrence of microorganisms producing folates in fermented drinks have been reported, it is important to consider the composition of the media where the microorganisms grew [11]. 5-Methyltetrahydrofolate, the most common form of folate in food, is converted to 5-methyldihydrofolate in acidic media, which still retains vitamin activity as it can be converted back into 5-methyltetrahydrofolate. The latter conversion needs a weak reducing agent, but in the absence of such a compound and if 5-methyldihydrofolate remains in the acidic medium for longer periods of time, it can suffer structural cleavage and loss of vitamin activity. Possibly, even if the folate in the acidic kombucha starter is present as 5-methyldihydrofolate, it might be converted back to 5-methyltetrahydrofolate once water, tea and sugar are added. Green tea is known to contain natural water-soluble antioxidants that could help in this activation of folates. Additionally, the water and sugar added consist of a proper medium for the microorganisms from SCOBY to grow and synthetize further folates [17,27,56].

To the best of our knowledge, this work is the first report showing 5-methyltetrahydrofolate in kombucha beverage, and this is unprecedented, as it gives this fermented drink a whole new relevance to human nutrition, as it can now be considered a source of folates. This added nutritional value could explain part of the beneficial effects attributed to kombucha, especially those related to cardiovascular disease prevention. It should be stressed that heat processing can degrade 5-methyltetrahydrofolate, therefore, if

Table 2
5-methyltetrahydrofolate content in kombucha liquid and polymeric disc on fermentation days 3 and 21, and parameters for the analytical method.

5-methyltetrahydrofolate content (mean ± S.D. μg/ml) in fermented kombucha		
Sample	3 days	21 days
Liquid	39.12 ± 1.32 μg/mL	50.87 ± 3.56 μg/mL
Polymeric disc	45.78 ± 8.42 μg/mL	54.88 ± 3.89 μg/mL
Parameters for the analytical method		
Detection limit (DL)	18.16 μg/mL	
Quantification limit (QL)	55.03 μg/mL	
Linear Coefficient (R)	0.9942	
Calibration curve	Y = 19772x+50416	

kombucha is pasteurized, for instance, folate can be easily degraded.

3.3. Total phenolics, total flavonoids and total carotenoids

Table 3 shows the concentrations of bioactive compounds (phenolic compounds, flavonoids and carotenoids) in the kombucha beverage on fermentation days 1, 3, 7, 14 and 21, and in the polymeric disc on fermentation day 21.

The content of the total phenolics compounds found in the present study was similar to Cardoso et al. [1] found in black tea, 1.09 mg GAE/mL. Black tea tends to have higher polyphenol contents than green tea, because of the processing of *Camellis sinensis* leaves to produce both tea varieties. When black tea is produced, the leaves are fermented, which stimulates the activity of polyphenol oxidases that change the color of the final product and oxidize catechins, leading to the formation of theaflavins and tearubigins, two of the main phenolic compounds in black tea [1].

However, the kombucha in the present study was formulated with a higher proportion of the SCOBY inoculum (20 % kombucha – liquid and disc) than in the study by Cardoso et al. [1] that used 13 %. This most likely provided a larger count of viable microorganisms, favoring their activity in the culture medium, especially enzyme-producing yeasts, which are responsible for transforming polyphenol polymers in their freest and most bioavailable forms. This mechanism possibly explains the increasing contents of total phenolic compounds as fermentation progressed (Table 3). The content of total phenolics in the present study increased significantly between 7 and 14 days of fermentation ($p < 0.0001$) and stabilized thereafter. Therefore, longer fermentation periods tended to favor the accumulation of total phenolic compounds in the kombucha beverage.

The contents of total flavonoids in kombucha were fairly stable during fermentation, only slightly decreasing towards the end of fermentation, at 21 days (Table 3). Total flavonoids were more concentrated in the present study than in previous reports using other media to grow the SCOBY, such as yarrow flower [27] and mushrooms [57]. However, as shown in previous studies that flavonoids may be lost as the fermentation process progresses. When compared to *C. sinensis*, kombucha had low amounts of flavonoids, suggesting that both dilution and loss in the fermentation would render kombucha a poorer source of this class of phenolic antioxidants [27].

Yeasts are known to produce carotenoids during fermentation [24] and have been previously reported in kombucha [58]. In the present study, the contents of total carotenoids tended to decrease during kombucha fermentation ($p < 0.0001$; Table 3). In days one to three of kombucha fermentation, total carotenoids were significantly higher than after 14 and 21 days ($p < 0.01$), and the carotenoid content after 7-days fermentation was not significantly different from any other. As with flavonoids, the content of total carotenoids dropped in the course of fermentation. Possibly, the acidic pH promoted by fermentation might have favored carotenoid degradation [58].

It is important to highlight that the analysis of carotenoids by spectrophotometry may suffer interference from other materials in the matrix. Chromatography studies are needed to better characterise the carotenoids in this material.

The average dietary consumption of carotenoids in Brazil is approximately 4100 μg [59], which is well below the daily recommended value of 9000 to 18,000 μg [60]. This low carotenoid intake can be improved by the habitual consumption of carotenoid-rich fruit and vegetables such as carrots, mangoes, tomatoes, and a carotenoid-rich diet could be complemented with regular kombucha doses (Table 3). Conveniently, carotenoid-rich fruit and vegetables could be mixed to kombucha to boost the drink's nutritional value.

3.4. Physicochemical analyses

Values found for pH decreased as the fermentation progressed (days 1, 3, 7, 14 and 21) pH 3.34 ± 0.00 (1), 3.07 ± 0.01 (3), 3.07 ± 0.00 (7) and 2.97 ± 0.08 (14), 2.06 ± 0.10 (21). A similar value was found by Villarreal-Soto and collaborators (2019), with a stable pH value of 2.7 on the fifteenth day of fermentation. Like the current study, Xia and collaborators [61] and Villarreal-Soto and collaborators [62] analysed green tea kombucha and found a reduction in pH and an increase in titratable acidity of the kombucha paralleled to the progression of the fermentation time.

The longer fermentation directly impacts on the increased activity of the lactic and acetic microorganisms, the consumption of substrate to produce energy, and the subsequent production of secondary metabolites. Part of these metabolic compounds is composed of organic acids such as lactic acid, glucuronic acid and acetic acid as the main organic acid. The production of these acids in the medium lowers the pH and keeps the drink safer from a lot of external microbiological contamination [1,10].

Other parameters for the kombucha characterisation remained stable throughout the fermentation process. The water activity (a w)

Table 3
Total phenolics compounds, total flavonoids and total carotenoids in fermented kombucha liquid and polymeric disc.

Fermentation time (days)						
Analysis	1	3	7	14	21	Polymeric Disc
Total Phenolics mg GAE/mL	1.05 ± 0^{c}	1.08 ± 0^{c}	1.24 ± 0^{c}	1.86 ± 0.02 ^{ab}	1.94 ± 0.02 ^{ab}	0.09 ± 0
Total Flavonoids mg CE/mL	0.07 ± 0.00^{a}	0.07 ± 0.00^{ab}	0.07 ± 0 ^{ab}	0.07 ± 0 ^{ab}	0.06 ± 0.00^{b}	0.00 ± 0
Total Carotenoids μg/mL	0,538 ± 0.36^{a}	0,466 ± 3.12^{a}	0,409 ± 0.89 ^{ac}	0,266 ± 0.69 ^{bc}	0,246 ± 0.69 ^{bc}	ND

GAE = Gallic acid equivalent. CE = Catechin equivalent. ND not detected. Data expressed as mean ± standard deviation. Anova One-way was used as the statistical method, with a level of significance ($p < 0.05$), Letters indicate significant differences after Dunn test.

was found to be 0.99 ± 0.00 and 0.99 ± 0.01 for the liquid and the polymeric biofilm respectively, and humidity with an average of the kombucha liquid and polymeric biofilm of 93.06 ± 0.57 and 92.33 ± 0.59 respectively, during the whole fermentation process [27].

The dry matter increased significantly from day one to day three, then decreased significantly again until days 14 and 21, which were not different from each other. The ash content remained below 0.1 % during all fermentation times, with an average of 0.03 % ± 0.02 and 0.02 % ± 0.01 for the kombucha liquid and polymeric biofilm, respectively. The ash content only showed a significantly higher content on day 21 when compared to days 1, 7, and 14 [27].

The titratable acidity increased throughout of the fermentation process. In the present study, the titratable acidity went from 0.27 ± 0.00 to 0.35 ± 0.01; 0.46 ± 0.02; 0.82 ± 0.12 and 1.17 ± 0.17 on days 1, 3, 7, 14 and 21 respectively and 1.38 ± 0.01 for the polymeric biofilm. These values are within limits and accepted as normal as there is an increase in the organic acids synthesized by the microorganisms [1,10,27].

Overall, the lipids did not vary a lot over the fermentation time. Only fermentation day 7 had a significantly lower lipid concentration than day 21 ($p < 0.001$). The analysis of lipids showed an overall average percentage of 0.3 % ± 0.12 of lipids in the kombucha liquid and 0.6 % ± 0.19 in the polymeric biofilm [1,6,61].

4. Conclusion

It is necessary to expand studies of the genus *Microbacterium* in order to clarify the species or species in dominance, however the accumulation of 5-methyltetrahydrofolate in kombucha is cited for the first time in the literature. The fermented green tea kombucha proved to be a promising source of 5-methyltetrahydrofolate. Prolonging the fermentation leads to degradation of some bioactive compounds, as well as compromises its acceptance by the consumers. The values found on the present study suggest that the ideal fermentation time to obtain the nutritional benefits is between 7 and 14 days of fermentation. As folates are susceptible to cleavage in the presence of oxidants or reducers, it is advised that the drink is be consumed on its own, without the addition of other ingredients that might compromise its benefits. Taking this information into account, consumers can still benefit from the 5-methyltetrahydrofolate and bioactive compounds intake, while maintaining the sensory quality and safety of consumption in relation to the acidity of the beverage.

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CRediT authorship contribution statement

Samuel de Santana Khan: Writing – original draft, Visualization, Resources, Funding acquisition, Formal analysis. **Vanessa Bordin Vieira:** Validation, Supervision, Methodology, Formal analysis. **Ana Carolina dos Santos Costa:** Writing – review & editing, Resources. **Arthur Victor da Silva:** Validation, Methodology, Formal analysis. **Allyson Andrade Mendonça:** Writing – original draft, Supervision, Formal analysis, Data curation. **Marcos Antonio de Morais Junior:** Writing – review & editing, Supervision. **Dayane da Silva Santos:** Validation, Formal analysis. **Alexandre Guedes Torres:** Writing – review & editing. **Maria Inês Sucupira Maciel:** Writing – review & editing. **Emmanuela Prado de Paiva Azevedo:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

None.

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