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Impact of fermentation conditions on the production of bioactive compounds with anticancer, anti-inflammatory and antioxidant properties in kombucha tea extracts

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ABSTRACT

The production of natural bioactive compounds through the fermentation of plants has increased in recent years. The biological activities of the extracts obtained from the fermentation of black tea with the kombucha consortium were evaluated. To improve the productivity of these compounds two different vessel geometries were used and successive extractions with solvents of increasing polarity were performed. Forty-seven compounds were identified by GC-MS, including several organic acids and phenolic compounds. Total phenolic content, pH value, and antioxidant, antiproliferation and anti-inflammatory activities were measured after 21 days of fermentation. A higher surface/height (s/h) ratio seemed to enhance the anti-inflammatory activity of kombucha tea, resulting in IC₅₀ value of $9.0 \pm 0.1 \mu\text{g/mL}$ compared to $24.3 \pm 0.2 \mu\text{g/mL}$ with the lowest ratio. Regarding the anticancer activity, the highest inhibition percentage of 55.3% at 50 $\mu\text{g/mL}$ against the HCT-116 human colon carcinoma cell line was obtained with the ethyl acetate extract after 21 days of fermentation compared to the value of 8% obtained with the same extraction solvent using the non-fermented black tea. These results showed that fermentation may improve the biological activities of the tea and that the production of bioactive compounds can vary depending on the fermentation conditions.

1. Introduction

Kombucha is a beverage of Manchurian origin obtained from sweet tea infusions which are fermented by a mixed microbial consortium composed of bacteria (e.g., *Komagataeibacter xylinum*, *Acetobacter xylinoides*, *Gluconobacter oxydans*, *Gluconacetobacter hansenii*, *Oenococcus oeni*, *Komagataeibacter europaeus*, *Lactobacillus* sp...) and yeasts (e.g., *Saccharomyces* sp., *Schizosaccharomyces pombe*, *Zygosaccharomyces kombuchaensis*, *Torulaspota delbrueckii*, *Brettanomyces* sp...) [1–4]. During kombucha fermentation, black tea compounds such as flavonoids, amino acids and phenolic acids [5] together with sucrose

undergo a transformation by the action of yeasts and bacteria. Many metabolites, such as organic acids (glucuronic, acetic), vitamins (C, B₁, B₂, B₁₂) and ethanol, are produced by this complex microbial consortium [4,6,7]. Among them, some are able to inhibit the growth of potentially contaminating bacteria. Kombucha tea is also known for being a functional food with several benefits [8], such as neurodegenerative disease prevention, blood pressure reduction, antioxidant activity, hypoglycemic effect, detoxification activity and anticancer properties, which are attributed to the produced metabolites during fermentation. This fermentation also leads to the formation of a solid polymeric structure ("bacterial cellulose") that floats on the surface of

Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; BHB, 3-tert-butyl-4-hydroxybenzoic acid; CAFF, caffeine; CAA, caffeic acid; C, catechin; CGA, chlorogenic acid; CA, P-coumaric acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC, (–)-epicatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; FA, ferulic acid; GA, gallic acid; HCT-116, human colon cancer cell line; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; LOX, lipoxygenase; MCF-7, breast cancer cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; NSAID, nonsteroidal anti-inflammatory drugs; R2, coefficient of correlation; RI, refractive index; RUT, rutin hydrate; s/h, surface/height; s/v, surface/volume, specific interfacial area; TAX, taxifolin; TB, theobromine; TCA, trans-cinnamic acid; TMCS, chlorotrimethylsilane; TPC, total phenolic content; UPLC, ultra performance liquid chromatography

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the culture medium because of the activity of certain strains such as *Komagataeibacter xylinus* or *Gluconacetobacter* spp. Moreover, other species that are not part of the Kombucha consortium are also capable to produce this cellulosic biofilm, such as *Aerobacter*, *Agrobacterium*, *Azotobacter*, and *Rhizobium*, *Salmonella* [9]. As the fermentation advances, this cellulosic biofilm keeps widening and forming new layers that accumulate one over the other, and after 7–14 days, the cellulosic biofilm may reach a thickness between 8 and 12 mm. Its formation as well as fermentation kinetics can be influenced by several parameters such as, temperature, pH, dissolved oxygen, type of sugars, fermentation time, and vessel geometry [10–12]. The last parameter is especially important in kombucha fermentation because it is performed in static conditions. Therefore, the activity of acetic acid bacteria, which are strictly aerobic, will highly depend on the transfer of oxygen from the air into the fermentation broth [10]. Several studies have shown the impact of the interfacial area on bacterial cellulose production as well as on the microbial kinetics, and different theories have been proposed [10,11,14]. Although these studies propose various interesting theories, little information is available regarding the influence of these parameters on the kinetics and biological activities of kombucha tea. Therefore, the aim of this study was to evaluate the impact of the vessel geometry by examining the dynamics and bioactive properties of fermentation products from two vessels with different surface/height ratios but the same surface/volume ratio.

2. Materials and methods

2.1. Starter culture

The kombucha SCOBY used in this study was obtained from the Laboratory Symbiotec, Villeneuve-Tolosane, France. The culture was produced using a previous backslipping method.

2.2. Chemicals and reagents

The analytical standards used for the identification and quantification of the main phenolic compounds found in the tea were: gallic acid, theobromine, caffeine, chlorogenic acid, catechin, (-)-epicatechin, caffeic acid, coumaric acid, ferulic acid, rutin hydrate, taxifolin, trans-cinnamic acid and 3-tert-butyl-4-hydroxybenzoic acid, all of which were obtained from Sigma-Aldrich (USA).

2.3. Preparation of standard solutions

Standard stock solutions of gallic acid, theobromine, caffeine, chlorogenic acid, catechin, (-)-epicatechin, caffeic acid, coumaric acid, ferulic acid, rutin hydrate, taxifolin, trans-cinnamic acid and 3-tert-butyl-4-hydroxybenzoic acid, were individually prepared at concentrations of 50, 20, 10, 5, and 1 mg/L in water/acetonitrile (80:20 v/v). Quantification was performed using the method described in Section 2.9. All of the solutions were stored at -20°C to prevent degradation.

2.4. Preparation of the tea infusion and inoculation

Ten grams of black tea leaves obtained from the laboratory Symbiotec were added to 1 L of boiling water and allowed to infuse for 15 min at 80°C . Afterwards, the tea leaves were removed, and sucrose was added at a concentration of 70 g/L. After the infusion was cooled to room temperature (25°C), two different volumes 1 L (vessel A) and 6 L (vessel B), were inoculated with 40 g/L of the starter culture provided by Laboratory Symbiotec. The inoculated black tea was poured into the different sized fermentation vessels, resulting in the same specific interfacial area s/v of 0.066 cm^{-1} but two different s/h ratios (Table 1). The vessels were covered with cheesecloth and incubated at 25°C for 21 days. All fermentations were performed in duplicate. The final biofilm was weighed and the yield was calculated according to the following

Table 1
Characteristics of the fermentation vessels.

Vessel	Diameter (cm)	Area (cm ²)	Volume (cm ³)	s/v (cm ⁻¹)	Height (cm)	s/h (cm)
A	9	63.6	963	0.066	15.15	4.19
B	23	415.2	6291	0.066	15.15	27.41

s/h : surface/height. s/v : surface/volume, specific interfacial area.

formula:

Yield (%) = Dry weight of bacterial cellulose (g/L) / Sucrose concentration (g/L).

2.5. pH determination

The pH of the fermented tea was measured using an electronic pH-meter (Eutech Instruments, model pH 700).

2.6. Sugars, ethanol and acetic acid quantification (UPLC-RI)

One milliliter of the fermented medium was centrifuged at 10 000 rpm for 5 min (Fisherbrand Centrifuge, Illinois, USA). The supernatant was then filtered through a membrane filter (Fisherbrand, PTFE) ($0.45\text{ }\mu\text{m}$) into UPLC vials. The resulting filtrate was subjected to quantitative analyses of sucrose, glucose, fructose, acetic acid and ethanol using HPLC (Thermo Scientific, Dardilly, France). Samples were injected into the UPLC system equipped with a refractive index detector and a Rezex ROA-Organic acid H+ (8%), 250 x 4.6 mm ion exclusion column (Phenomenex, Le Pecq, France) thermostated at 30°C . The elution was performed at $170\text{ }\mu\text{L}/\text{min}$ with a 10 mM sulfuric acid solution (pH 2.2). Twenty-five microliters of injection volume were automatically analyzed. The concentration of each compound was quantified using standard curves and expressed as (g/L). Standard solutions were prepared with an analysis range of 1.25–10.00 g/L for the sucrose, glucose, fructose and ethanol quantifications and 0.25–2.00 g/L for the glycerol and acetic acid quantifications (Table 2). All the standards and sample dilutions were prepared using high quality deionized water.

2.7. Sample preparation and extraction

Kombucha fermented tea was extracted by using a differential liquid-liquid method with immiscible organic solvents of increasing polarity (ethyl acetate and butanol). The remaining aqueous phase was also characterized. All the extractions were performed at room temperature with 500 mL of solvent and 500 mL of fermented medium (1:1), and were performed twice to maximize the extraction process. Mechanical agitation was applied to increase the interfacial area between the two phases followed by a settling stage that allowed the phases to separate. The solvents were then evaporated using a vacuum rotary evaporator at 35°C (Heidolph, Germany) to obtain a dry extract.

Table 2
LOD, LOQ, RT and R^2 of standards for UPLC analysis.

Metabolite	RT (min)	LOD (mg/L)	LOQ (mg/L)	R^2
Sucrose	10.97	5	10	0.99
Glucose	12.57	5	10	0.99
Fructose	13.74	5	10	1.00
Glycerol	17.29	5	10	0.99
Acetic acid	19.28	5	10	0.99
Ethanol	24.02	10	20	0.99

RT: retention time. LOD: limit of detection. LOQ: limit of quantification. R^2 : coefficient of correlation.

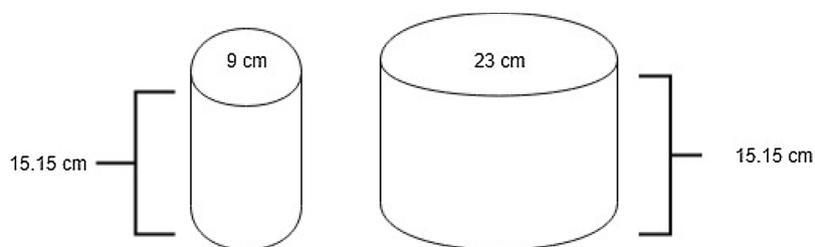


Fig. 1. Dimensions of vessels used for kombucha tea fermentation.



Fig. 2. Scanning electron micrograph of bacteria embedded within the cellulose microfibrils from kombucha tea.

2.8. Chemical composition (GC–MS)

Gas chromatography-mass spectrometry detection (GC–MS) analysis was carried out with a Varian Saturn 2000 (Les Ulis, France) ion trap GC/MS with a CP-3800 GC system fitted with a fused silica capillary DB-5MS column (5% phenylmethylpolysiloxane, 30 x 0.25 mm, film thickness 0.25 μm). Chromatographic conditions were a 60–260 °C temperature increase at a gradient of 5 °C/min and 15 min of isothermal conditions at 260 °C. A second gradient was applied to reach 340 °C at 40 °C/min. The trap temperature was 250 °C and that of the transfer line was 270 °C. Mass scanning was performed from 40 to 650 *m/z*. Extracts were solubilized in their solvents of extraction (except for water extract, where methanol was used) at 3 mg/mL and 2 μL were injected. Compounds were identified by (i) comparison of their retention index (RI) relative to C5–C24 *n*-alkanes obtained on a nonpolar DB-5MS column with those provided in the literature and (ii) by comparison of their mass spectra with those recorded in NIST 08 (National Institute of Standards and Technology).

2.8.1. Derivatization

The derivatization method used [15] consisted of dissolving 5 mg of each extract in 1 mL of acetonitrile and then adding 150 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) reagent and 1.5 μL of chlorotrimethylsilane (TMCS). After stirring, nitrogen was bubbled through the solution 20 s to eliminate the humidity from the air, and then the mixture homogenized via ultrasound for 10 s followed by an incubation in a heated bath at 40 °C, for 15 min. All samples (2 μL) were injected directly. The identification of derivatized compounds from the organic extracts was carried out with the same GC–MS equipment but with another gradient: 5 min at 60 °C, 60–270 °C at 15 °C/min, 6 min at 270 °C, 270–300 °C at 50 °C/min and finally 4.5 min at 300 °C. The entire chromatographic program lasted 30 min. The identification of

compounds was performed as described in Section 2.8.

2.9. Aromatic compound and polyphenol determination (HPLC-UV)

HPLC analyses were performed using a Thermo Scientific Dionex UltiMate 3000 pump and UV-150 model detector. Separation was achieved on a RP-C18 column (Phenomenex, Le Pecq, France), 25 cm x 4.6 mm and particle size 5 μm, at room temperature. Elution was performed at a flow rate of 1.2 mL/min, using a mobile phase consisting of acidified water (pH = 2.65) acidified with pure acetic acid and water/acetonitrile (20:80 v/v, pH = 2.65). To detect the majority of compounds, all samples were prepared at the same concentration (20 mg/mL). Twenty microliters were then injected, and the detection was performed at 280 nm.

2.10. Antioxidant activity (DPPH assay)

The free radical scavenging activity was measured according to a previously described method [16] with some modifications. Twenty microliters of the kombucha extracts were mixed with 180 μL of a 0.2 mM methanolic DPPH solution. The mixture was then incubated at 25 °C for 30 min. The reduction of the DPPH free radicals was assessed by reading the absorbance at 520 nm in a Multiskan Go spectrophotometer, and the results were recorded as $A_{\text{(sample)}}$. A blank experiment was performed using the same procedure without the extract, and the absorbance was measured as $A_{\text{(blank)}}$. Ascorbic acid was used as a standard. The free radical-scavenging activity of each solution was then calculated as the percent inhibition according to the following equation:

$$\% \text{ inhibition} = 100 (A_{\text{(blank)}} - A_{\text{(sample)}}) / A_{\text{(blank)}}$$

2.11. Determination of total phenolic concentration (TPC)

TPC of kombucha fermented tea was quantified using the Folin-Ciocalteu colorimetric method [16]. One hundred microliters of Folin-Ciocalteu reagent (0.2N) were added to 20 μL of each extract prepared at a concentration of 3 mg/mL. After 5 min of incubation at room temperature, 80 μL of sodium carbonate solution (75 g/L in water) were added. Then the mixture was incubated for 15 min and the absorbance was measured at 765 nm. A standard calibration curve was done using different concentrations of gallic acid (0–200 μg/mL). The results were expressed as mg of gallic acid equivalents (GAE)/g of dry weight (dw).

2.12. Anti-inflammatory activity

Anti-inflammatory evaluation was performed against the enzyme 5-LOX using a previously described method [16]. The activity measurement was tested in a 96-well plate containing 150 μL of 100 mM phosphate buffer (pH 7.4), 20 μL of the extract solution at a concentration of 3 mg/mL, 60 μL of linoleic acid (3.5 mmol/L), and 20 μL of 5-LOX (Soybean 500 U). The mixture was then incubated at 25 °C for 10 min in a Multiskan Go spectrophotometer and the absorbance was measured at 234 nm. The anti-inflammatory activity was defined as the

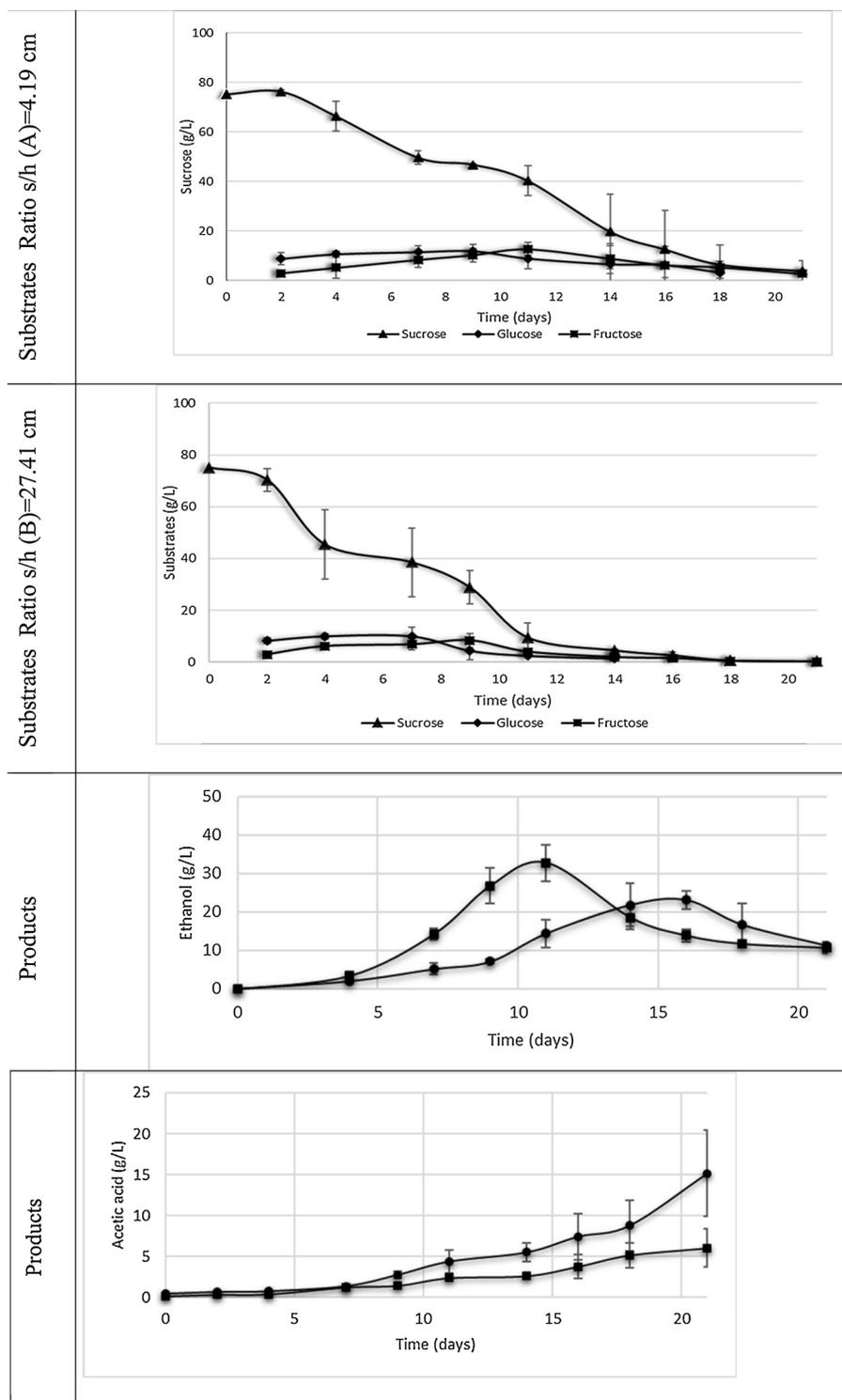


Fig. 3. Kinetics of kombucha fermentation : (●) Ratio s/h A = 4.19 cm (■) Ratio s/h B = 27.41 cm Data are mean ± S.D. (n = 2).

percentage of inhibition of the 5-LOX enzyme. Nordihydroguaiaretic acid (NDGA) was used as a standard.

2.13. Antiproliferation activity

This assay is based on the reduction of 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Antiproliferation of extracts on human colon cancer (HCT-116) and human breast cancer (MCF-7) cell lines was estimated as described

previously [16] with modifications. Cells were maintained at 37 °C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany) using Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, USA) for the human breast cancer cell line and RPMI-1640 (Sigma Aldrich, USA) for the colon cancer cell line. Cells were seeded in 96-well plates at a concentration of 3 × 10⁴ cells/well in 100 μL of culture medium, and then 100 μL of culture medium containing a sample at various concentrations were added. Non-treated control cells were also maintained for comparing growth inhibition. The plates were then incubated at 37 °C for 48 h. The supernatant was then removed, and 50 μL of MTT

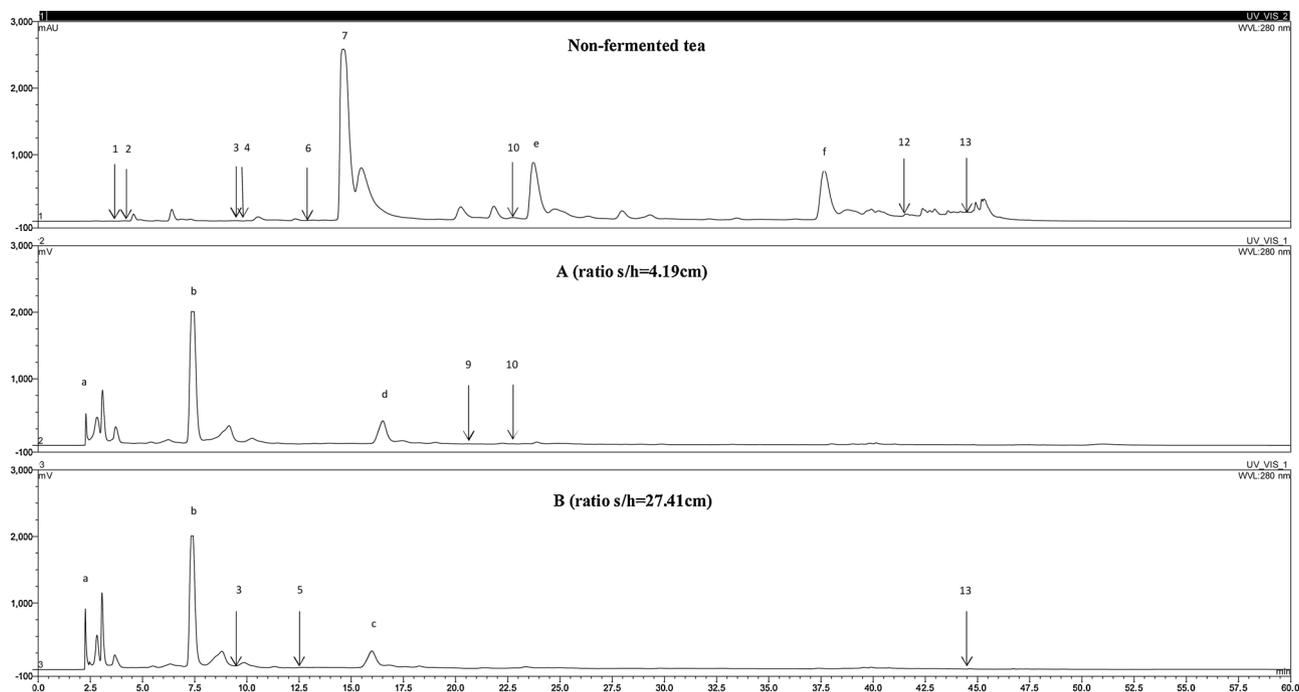


Fig. 4. HPLC chromatograms of ethyl acetate kombucha extracts. Peaks: 1-gallic acid (GA); 2-theobromine (TB); 3-catechin (C); 4-chlorogenic acid (CGA); 5-(-)-epicatechin (EC); 6-caffeic acid (CAA); 7-caffeine (CAFF); 9-ferulic acid (FA); 10-rutin hydrate (RUT); 12-trans-cinnamic acid (TCA); 13-3-tert-butyl-4-hydroxybenzoic acid (BHB).

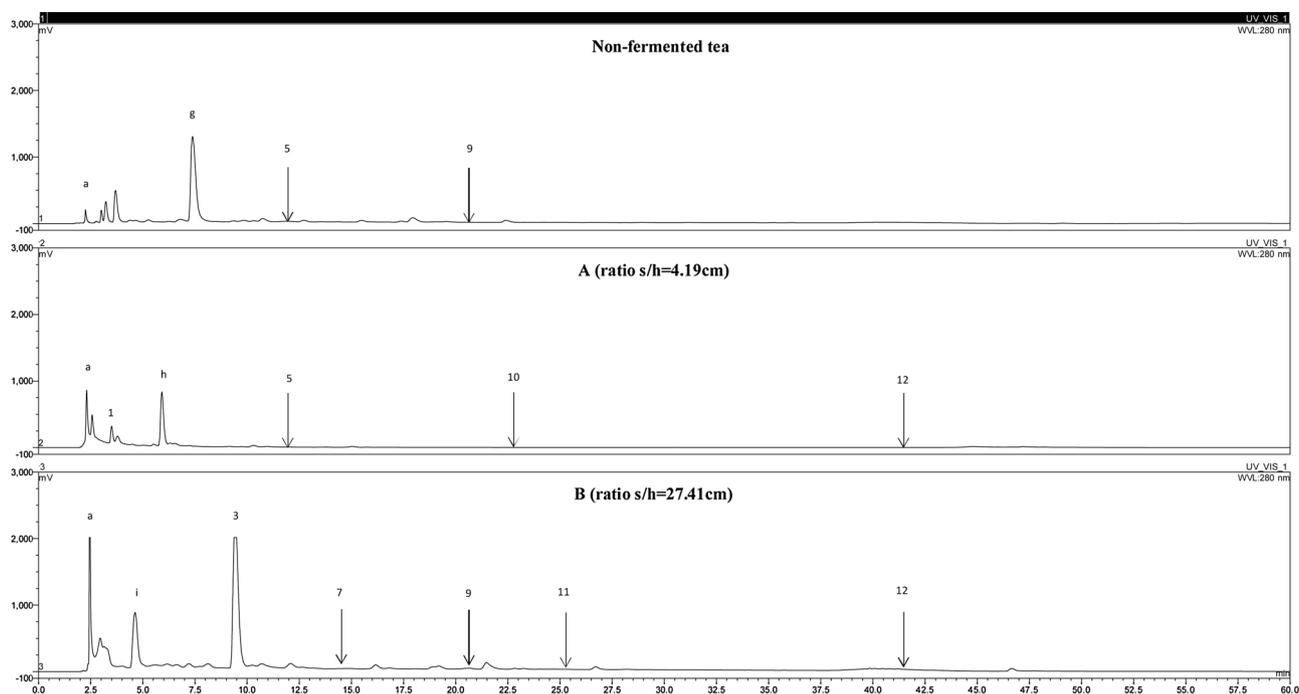


Fig. 5. HPLC chromatograms of butanol kombucha extracts. Peaks: 1-gallic acid (GA); 3-Catechin (C); 5-(-)-epicatechin (EC); 7-caffeine (CAFF); 9-ferulic acid (FA); 10-rutin hydrate (RUT); 11-taxifolin (TAX); 12-trans-cinnamic acid (TCA).

solution was added followed by incubation, for 40 min. After removing the MTT reagent (Sigma, M-5655), 80 μ L of DMSO were added to solubilize the formazan crystals. The absorbance was measured at 605 nm. All extracts were re-suspended in DMSO followed by dilution in the buffer so that the DMSO did not exceed 1%. Tamoxifen was used as a positive control.

2.14. Statistical analysis

The programs Minitab 17 and Microsoft Excel were used for the statistical treatment of the data. The means were compared by two-way analysis of variance (ANOVA) and the Tukey multiple comparison test ($p < 0.05$). All data are expressed as the mean \pm standard deviation (SD) of triplicate measurements.

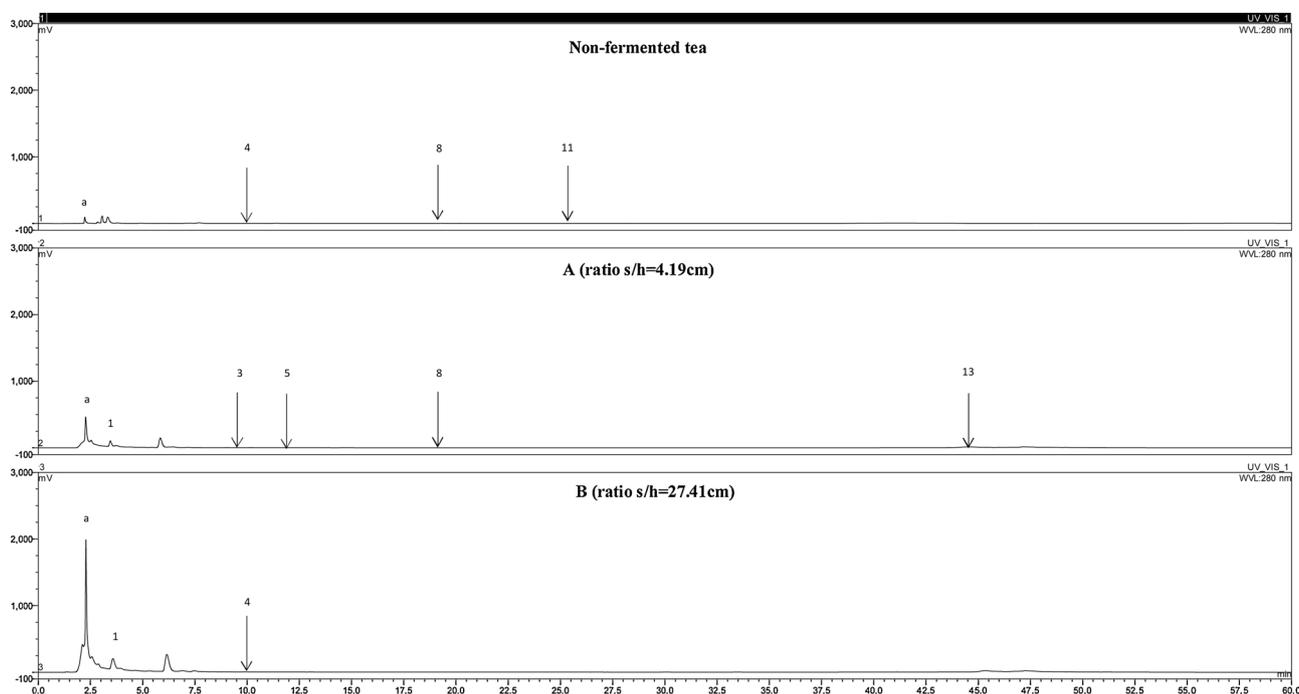


Fig. 6. HPLC chromatograms of aqueous kombucha extracts. Peaks: 1-gallic acid (GA); 3-catechin (C); 4-chlorogenic acid (CGA); 5-(-)-epicatechin (EC); 8-coumaric acid (CA); 11-taxifolin (TAX); 13-3-tert-butyl-4-hydroxybenzoic acid (BHB).

3. Results and discussion

3.1. Impact of the vessel geometry (s/h and s/v ratio)

Cvetković et al. [17] developed a mathematical model to ensure the scaling-up process of kombucha tea fermentation, which can be quite complex and should take into account several variables. In their study, they proposed that the specific interfacial area was the key variable that would ensure the scaling-up procedure. They concluded that regardless the vessel size, if the specific interfacial area (s/v) is the same, the final kombucha tea may have the same characteristics. To prove this theory, two vessels (Fig. 1) with different s/h ratio (4.19 and 27.41 cm) and the same specific interfacial area s/v (0.066 cm^{-1}) were used to determine which parameter had a bigger impact on the processing.

3.2. Cellulose production and scaling-up process

Cellulose production values were similar for both vessels reaching a value of 23 g/L with a depth of 15 cm and a final yield percentage of 1.1 and 0.7 for vessels A and B, respectively, after 21 days of fermentation. This similarity in the cellulose production, despite different vessel depths could be due to the fact that bacteria form the biofilm at the air-liquid surface. According to Hornung [11], this biofilm represents approximately 10% of the total bacterial cells that may have been trapped in the cellulose matrix (Fig. 2) contributing in this way to its formation. This can be a key information regarding kombucha fermentation, because the biofilm development could be improved and because larger volumes of fermented tea could be obtained at the same time. Thus, it was concluded that the depth was a determining parameter for the optimization of cellulose production because it can reduce the fermentation time and therefore the cost of the process.

3.3. Sugars consumption

The results of the fermentation kinetics (Fig. 3) revealed that the parameter s/h had a clear impact, both on substrates consumption and on metabolite production. Sucrose was hydrolyzed into glucose and fructose in both cases and occurred linearly with time, reaching final

values of 3.8 and 0.2 g/L in vessels A and B, respectively. After 15 days of fermentation, the sucrose concentration was around 12 and 2 g/L, for vessels A and B, respectively, similar to the values obtained by Kallel et al [18] with a concentration of 100 g/L sucrose at the beginning of fermentation. Regarding the consumption of reducing sugars, glucose is typically preferred by the microbial consortium over fructose, which normally results in a difference between the consumption of the two sugars, leading to a sluggish fermentation and therefore, an increased risk of potential contamination [19]. However, in the case of kombucha fermentation, both sugars were consumed without accumulating in the fermented medium because of the multiple microorganisms and biochemical pathways that were occurring simultaneously. Nevertheless, in vessel B, which was the one with the higher surface area, sucrose began to be consumed in the first days of incubation. However, sucrose consumption was much slower in vessel A, showing that a higher s/h ratio may accelerate the fermentation kinetics.

3.4. Ethanol content

The production of ethanol was faster in the vessel with the higher surface area, with a maximum production of $32.8 \pm 4.7 \text{ g/L}$ (4.1% v/v) after 11 days, compared to a production of $23.1 \pm 2.4 \text{ g/L}$ (2.9% v/v) after 16 days in the vessel with the smaller surface area. However, notably despite this fact, the final ethanol concentration was almost the same in both vessels after 21 days of fermentation, of around 11 g/L (1.4% v/v), although in the vessel with the higher surface area it was rapidly produced and rapidly re-consumed. The obtained ethanol concentrations are higher than those reported in other studies [20,21] which were in the range of 2–8 g/L with 70 and 100 g/L initial sucrose, compared to 11.2 g/L. Differences between several kombucha fermentations may be caused by the origin of the inoculum as well as the fermentation conditions.

3.5. pH values and acidity

The pH value of the initial black tea infusion was 6.4, which dropped nearly three units after 1 h of incubation with the consortium and continued to decrease during the first 15 days until it became

Table 3
Content of phenolic compounds, theobromine and caffeine from black tea and kombucha extracts by HPLC (mg/kg).

N°	RT	Compound	InChI Key	Non-fermented tea				A (ratio s/h = 4.19 cm)				B (ratio s/h = 27.41 cm)			
				EtOAc	BuOH	H ₂ O	EtOAc	BuOH	H ₂ O	EtOAc	BuOH	H ₂ O	EtOAc	BuOH	H ₂ O
1	3.45	Gallic acid (GA)	LNTHITQWFMDLDM-UHFFFAOYSA-N	26.0 ± 3.0				2969.0 ± 486.6			1359.5 ± 499.7				2859.4 ± 76.3
2	4.26	Theobromine (TB)	YAPQBXYLJRXSA-UHFFFAOYSA-N	50.2 ± 0.8											
3	9.56	Catechin (C)	PFTAWBLQPZVEMU-HFRSBDPSA-N	12.4 ± 0.3											
4	9.93	Chlorogenic acid (CGA)	CWVRJTMFETXNAD-JUHZACGLSA-N	8.3 ± 0.4		4.0 ± 0.5									
5	11.93	(-)-Epicatechin (EC)	PFTAWBLQPZVEMU-UKRQHHQSA-N		87.5 ± 3.4										
6	12.99	Caffeic acid (CAA)	QAIPIRVGONGVQAS-DUXYPHPUSA-N	179.1 ± 10.6											
7	14.6	Caffeine (CAFF)	RYVYLZVUVJLVGH-UHFFFAOYSA-N	35142.4 ± 555.8											
8	19.33	p-Coumaric acid (CA)	NGSWKAQJWESNS-ZZKXWVIFSA-N		4.7 ± 0.9	21.3 ± 0.1									
9	20.60	Ferulic acid (FA)	KSEBMYQBYZTDHS-HWKANZROSA-N				7.6 ± 0.3								
10	22.69	Rutin hydrate (RUT)	PGHSKTKIQBATG-ZAAWVWGYSA-N	56.4 ± 3.1			7.13 ± 2.8	2.3 ± 0.1							
11	25.3	Taxifolin (TAX)	CXOWRCVTCMQVQX-LSDDHAIUSA-N			0.3 ± 0.0									
12	41.54	Trans-Cinnamic Acid (TCA)	WBYWAXJHAXXSJNLI-VOTSOKGWSA-N	852.2 ± 5.4											
13	44.56	3-tert-Butyl-4-hydroxybenzoic acid (BHB)	PVFDJRIEYDIEK-UHFFFAOYSA-N	16.2 ± 2.0											

All the data are average values of triplicate analyses.

stable, reaching a final value of 2.7. Several authors have reported the same behavior [6,18,21] which is normal because of the organic acids produced, mainly acetic acid. Cvetković [13] found the same tendency in terms of titratable acidity, where the specific area ratios had a huge impact. The final concentrations of acetic acid after 21 days were different between the two ratios, 15.1 in A and 6.0 g/L in B. Usually acetic acid bacteria oxidize ethanol to acetaldehyde and then to acetic acid [22,23], but this was not the case in vessel B, in which the concentration of acetic acid was much lower than that of ethanol. This can be explained either by the cell concentration of acetic acid bacteria or by the metabolic path followed in each culture media, considering the hypothesis that some bacteria can produce ethanol and others can consume it and produce acetic acid. This suggests that important interactions occur between yeast and bacteria that are present both in the liquid broth and in the cellulose pellicle [3,24–26].

3.6. Chemical composition of kombucha extracts

3.6.1. HPLC analysis

HPLC analysis was performed and several compounds were detected at 280 nm in all kombucha extracts. Fig. 4 shows the high-resolution separation of 14 compounds in the ethyl acetate extract of the non-fermented tea samples compared to 8 compounds in the fermented samples. This decrease may be due to the microbial transformation, or to the nature of the phenolic compounds [26]. It can be observed that compound **a** increased in concentration from trace amounts to a high intensity peak in vessel B after fermentation. Compounds **e**, **f**, and **g** were consumed during fermentation and **b**, **c** and **d** were produced in vessels B and A, respectively. A similar profile was obtained with butanol extracts (Fig. 5), in which compound **a** showed the same behavior of increased intensity together with ethyl acetate and the aqueous fraction. Regarding the aqueous fraction (Fig. 6), the compounds obtained after fermentation were visibly of higher intensity with the ratio of vessel B compared to the trace amounts found in the non-fermented tea. This analysis allowed to observe the highest impact of the s/h ratio and the fermentation on the compounds formation or biotransformation process.

3.6.2. Identification and quantification of the main phenolic compounds

Fourteen components were identified and quantified in the black tea and kombucha samples using a gradient HPLC method based in their retention times compared with those of standards. The content of each component was calculated as milligrams per kilogram of kombucha tea extract (Table 3). It was observed in the ethyl acetate extracts (Fig. 4) that the highest content of phenolic compounds was in the non-fermented tea and then it gradually decreased after fermentation. Black tea contains several phenolic compounds that may be subjected to biotransformation during processing, increasing or reducing their concentration. Yeast and bacteria are also known to play a crucial role in the metabolism of these compounds [27]. For the identified compounds, TB and CAA were completely consumed, and CAFF was nearly depleted decreasing from a concentration of 35142.4 to 41.1 mg/kg after 21 days of fermentation. The release of catechins from microbial cells and the oxidation of EGCG or ECG by oxidative degallation [28] could explain the amount determined in this study of 3161.6 mg/kg in the case of the vessel B compared to that Lin [21] found (50 mg/kg) in non-fermented black tea samples. The increased concentrations (~80%) of GA in both fermented ratios may be due to the de-esterification of the 3-galloyl substituted catechins by the enzymes secreted during the fermentation [29]. On the other hand, in the butanolic extract of the fermented tea the concentration of many phenolic compounds (C, FA, TAX) increased with the high s/h ratio as did the GA content in the aqueous residual phase compared to the values in the non-fermented tea. Other compounds such as, EC and CA were considerably reduced during the tea fermentation. Similar concentrations as those reported by Souza et al [30] were obtained in the range of 0.1 – 0.3 mg g⁻¹ for

Table 4
GC–MS analysis (area x10⁶) of kombucha tea extracts without and with derivatization.

Without derivatization												
N°	RT (min)	Compound	InChI Key	Non-fermented tea			A (ratio s/h = 4.19 cm)			B (ratio s/h = 27.41 cm)		
				EtOAc	BuOH	H ₂ O	EtOAc	BuOH	H ₂ O	EtOAc	BuOH	H ₂ O
1	13.88	2,5-di-tert-butylphenol	KDBZVULQVCUNNA-UHFFFAOYSA-N				0.83	0.11		1.62	0.19	
2	16.84	Caffeine	RYYVLZVUVLJVGH-UHFFFAOYSA-N	9.14	0.06		5.25	14.19			6.97	
With derivatization												
1	7.02	Isovaleric acid	GWYFCOCPABKNJV-UHFFFAOYSA-N				0.64			2.96	1.39	
2	7.59	Carbonic acid	AOQORSDBSDUUBQ-UHFFFAOYSA-N				1.27					
3	7.99	Pyruvic acid	LCTONWCANYUPL-UHFFFAOYSA-N				16.53	19.11	30.25	20.09	19.27	
4	8.28	Formic acid	BDAGHXWWSANSR-UHFFFAOYSA-N				2.18					
5	8.42	2,3 Butanediol	OWBTYPJTUOEWEK-UHFFFAOYSA-N				41.57		11.75	48.36	49.47	6.97
6	8.57	2,3 Butanediol (isomer 2)	OWBTYPJTUOEWEK-UHFFFAOYSA-N					27.92				
7	8.91	Lactic acid	JVTAAEKCFNVCJ-UHFFFAOYSA-N				66.82	25.94				
8	9.03	Furan, tetrahydro-2-peroxy	JSZPBTUOIOMFMS-UHFFFAOYSA-N	26.06	12.66		143.67	89.03	305.54	245.10	38.41	147.06
9	9.17	Caproic acid (hexanoic)	FUZZWVXGSPDMH-UHFFFAOYSA-N				0.75					
10	10.03	3-Hydroxypropionic acid	ALRHLSYJTWAHJZ-UHFFFAOYSA-N						3.90			
11	10.12	(2E)-2-Butene-1,4-diol	ORTVZLZNOYNASJ-OWOJBTEDSA-N									7.58
12	10.18	α-Hydroxy isovaleric acid	NGEWQZIDQIYUNV-SCSAIBSYSYSA-N				8.84			7.85		
13	10.98	2-hydroxy-3-methylpentanoic acid	RILPIWOPNGRASR-UHFFFAOYSA-N							2.89		
14	11.07	Butyric acid	FERIUCNNQQJTOY-UHFFFAOYSA-N									4.97
15	11.10	2-Phenylethanol	WRMNZCZEMHIOC-P-UHFFFAOYSA-N				4.74			11.22		
16	11.33	Glycerol	PEDCQBHIVMGVHV-UHFFFAOYSA-N				221.99	3338.37	1605.27	244.68	3219.66	3791.11
17	11.41	Octanoic acid	WWZKQHOCKIZILMA-UHFFFAOYSA-N				0.82			0.28		
18	11.70	Propanoic acid	XBDQKXXYIPTUBI-ZDOIHCBSA-N					69.17		1.54		
19	11.90	Succinic acid	KDYFGRWQOYBRFD-UHFFFAOYSA-N				923.57	443.74	56.63	1185.47	559.50	23.59
20	12.22	Z-Fumaric acid	VZCYOOQTPCHFL-OWOJBTEDSA-N							9.60		
21	13.37	Malic acid	BJEPYKJPYRNKOW-UHFFFAOYSA-N				12.23	18.93		17.07	21.69	41.02
22	13.71	Salicylic acid	YGSDEFMSJLZEOE-UHFFFAOYSA-N				2.40					
23	13.74	Erythronic acid	JPLJQSOTBSSVTP-PWNYCUMCSA-N						9.36			
24	13.85	Threonic acid	JPLJQSOTBSSVTP-GBXIJSLDSA-N						1.40			
25	13.94	3,5-di-t-butyl-4-hydroxybenzoic acid ethyl ester	FJKROLUGYXJWQN-UHFFFAOYSA-N				13.07			19.68	2.43	
26	14.08	2-hydroxyglutaric acid	HWXBTNAVRSUOJR-UHFFFAOYSA-N						17.22		12.91	4.91
27	14.2	4-Hydroxyphenyl ethanol	YCCILVSKPBXVIP-UHFFFAOYSA-N				27.88			38.66		
28	14.31	2-hydroxy-2-phenylpropanoic acid	NWCHELUCVWRRS-UHFFFAOYSA-N				32.49					
29	14.37	2-Ketoglutaric acid	KPGXRSRHYNQIFN-UHFFFAOYSA-N						2.06		20.69	
30	14.63	Beta-D-Arabinopyranose	SRBFZHDQGSBBOR-SQOUGZDYSA-N								1.18	
31	14.86	2-hydroxyadipic acid	OTTXIFWBPRRYOG-UHFFFAOYSA-N				0.31					
32	15.06	L-(-)-Arabitol	HEBKCHPVOIAQTA-IMJSIDKUSA-N						40.08		18.31	76.76
33	15.12	Erythritol	UNXHWFMPMPAWVPI-ZXZARUISSA-N						3.06			
34	15.26	2-Deoxyribose	VBWUWJHOKCBQXNU-IUYQGCFFVSA-N						0.50			
35	15.55	D-(-)-Tagatofuranose	RFSUNEUAIZKAJO-OEXCPVAWSA-N								150.25	
36	15.75	L-sorbofuranose	RFSUNEUAIZKAJO-AMVSKUEXSA-N	0.76	7.46		13.11	1195.71	2976.05	10.33	424.60	
37	15.89	Citric acid	KRKNYBCHXYNGOX-UHFFFAOYSA-N							21.48		781.49
38	15.92	Mannopyranoside	SRBFZHDQGSBBOR-UHFFFAOYSA-N								4.05	
39	15.95	L-(-)-Arabitol (isomer)	HEBKCHPVOIAQTA-IMJSIDKUSA-N								110.11	
40	16.32	Lactulose	JCQLYHFGKNRPE-FCVZTGTOA-N						5076.23			
41	16.47	Gluconic acid	RGHNJXZEOKUKBD-SQOUGZDYSA-N					77.24	638.57	13.67	42.20	288.14
42	16.55	2-Hydroxy-3-(4-hydroxyphenyl)propanoic acid	JVGVDSUAVXRDY-UHFFFAOYSA-N				17.20					
43	16.9	Gallic acid	LNTHITQWFMADLM-UHFFFAOYSA-N	158.97			460.74	85.81		651.71	73.14	
44	17.04	Ribonic acid	QXKAIJAYHKCRRR-BXXZVTAOSA-N						55.95			
45	20.23	Sucrose	CZMRCDWAGMRECN-UGDNZRGBSA-N	8.22	1025.21				20.93			106.54
46	20.81	D-(+)-Turanose	SEWFWJUQVJHATO-RMDVNNDRSA-N	4.65				12.28	61.54		7.14	
47	24.54	Catechin	PFTAWBLQPZVEMU-HIFRSBDPSA-N				11.50			10.03		

CA. Rutin hydrate and 3-tert-butyl-4-hydroxybenzoic acid were present before and after the fermentation, differing from TAX which seems to be produced during fermentation especially in well aerated vessels.

3.6.3. GC–MS analysis

The compounds identified in the GC–MS analysis are summarized in Table 4. Caffeine and 2,5-di-tert-butylphenol were detected in the non-fermented tea samples as well as in the fermented samples. The samples were then derivatized by silylation to increase their detectability and 47 different compounds were detected including several organic and phenolic acids, alcohols and sugars. Glycerol was found in all samples.

It can be produced by *Saccharomyces cerevisiae* during the hydrolysis of glucose to ethanol [31]. It can also be produced by several osmotolerant yeasts belonging to the genera *Candida*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, that are normally present in the kombucha consortium. Around 50% of the detected compounds were found in the ethyl acetate extracts, and these were also the compounds with the highest biological activities. This may be attributed to the presence of the organic acids that have been reported to be active compounds in kombucha tea [32]. Lactic acid is known to function as a natural preservative in foods and to have beneficial effects on the digestive system [33]. The obtained results agree with those found in a

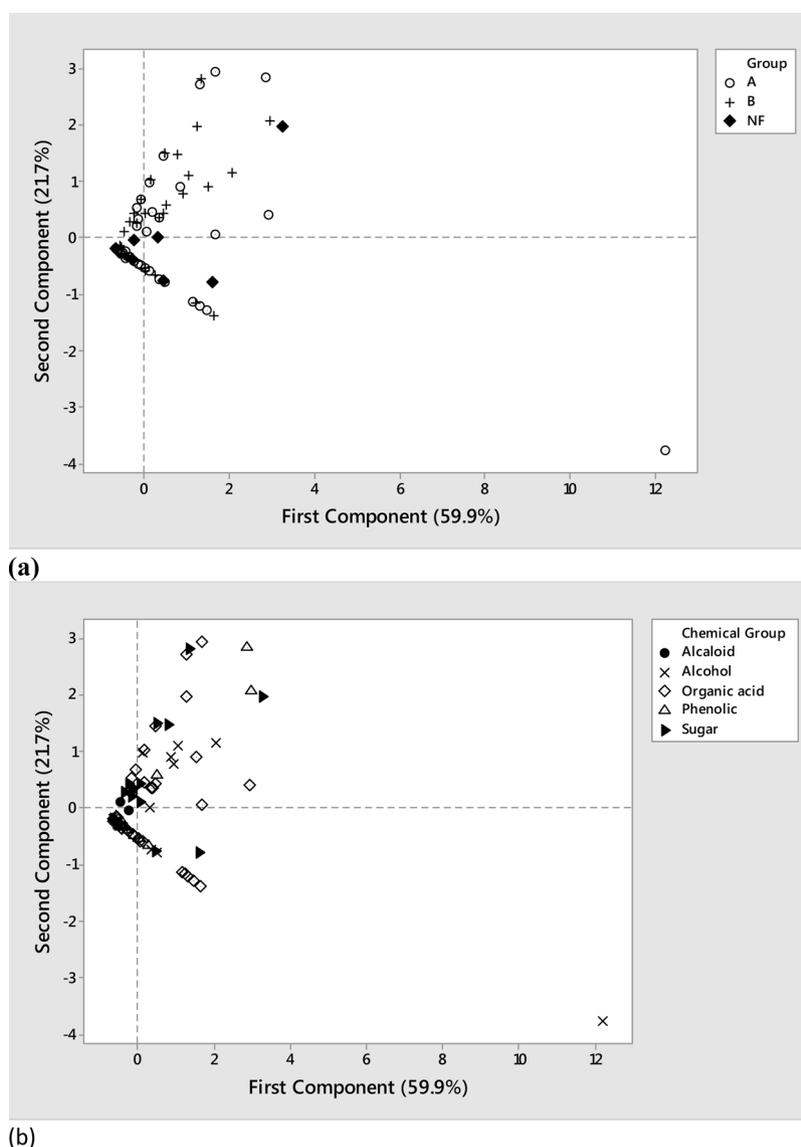


Fig. 7. PCA score plots of the GC-MS obtained molecules. (a) three different samples where NF: non-fermented tea and at the end of the fermentation for the A and B conditions (ratios $s/h = 4.19$ cm and $s/h = 27.41$ cm) and (b) chemical groups distribution.

Table 5
Total polyphenols, DPPH and anti-inflammatory activities of kombucha tea.

Sample	Extract	Total polyphenols (mg eq AG/g)	DPPH		Anti-inflammatory activity	
			% at 50 $\mu\text{g/mL}$	IC ₅₀ ($\mu\text{g/mL}$)	% at 50 $\mu\text{g/mL}$	IC ₅₀ ($\mu\text{g/mL}$)
Non-fermented tea	EtOAc	215.0 \pm 0.9 ^a	98.1 \pm 0.3 ^a	9.5 \pm 0.3 ^a	66.0 \pm 1.7 ^a	> 50 ^a
	BuOH	35.9 \pm 0.8 ^b	70.9 \pm 1.7 ^b	28.0 \pm 0.5 ^b	0 ^b	> 50 ^a
	H ₂ O	3.0 \pm 0.2 ^c	14.6 \pm 1.1 ^c	> 50 ^c	0 ^b	> 50 ^a
A	EtOAc	221.6 \pm 6.8 ^a	96.4 \pm 0.0 ^a	9.0 \pm 0.1 ^a	87.7 \pm 0.4 ^c	24.3 \pm 0.2 ^b
	BuOH	43.4 \pm 2.3 ^b	77.6 \pm 2.0 ^d	26.0 \pm 0.0 ^d	36.9 \pm 0.5 ^d	> 50 ^a
	H ₂ O	8.9 \pm 0.2 ^{c,d}	18.2 \pm 0.0 ^e	> 50 ^c	19.0 \pm 0.9 ^e	> 50 ^a
B	EtOAc	212.4 \pm 6.1 ^a	96.3 \pm 0.3 ^a	9 \pm 0.0 ^a	91.47 \pm 0.7 ^c	9.0 \pm 0.0 ^c
	BuOH	92.2 \pm 1.7 ^e	96.2 \pm 0.4 ^a	16 \pm 0.5 ^e	81.8 \pm 1.6 ^f	16.0 \pm 0.5 ^d
	H ₂ O	11.8 \pm 1.0 ^d	23.5 \pm 0.6 ^e	> 50 ^c	20.5 \pm 0.9 ^e	> 50 ^a

Values in the same column that are labeled with different letters (a–f) differ significantly ($p < 0.05$).

previous study [32], in which the presence of lactic acid was observed after three days of fermentation in both green and black teas, but not in non-fermented sample, as this acid is formed by some yeasts and mainly by lactic acid bacteria. Pyruvic, malic, salicylic, succinic, citric and gluconic acids were detected in all samples, and these acids have been reported to be the most abundant acids found in kombucha tea [8].

3.6.3.1. Principal component analysis (PCA). The molecules production patterns were investigated using PCA (Fig. 7). Principal components 1 and 2 explained 59.9 and 21.7 of the total variance, respectively. This analysis was done in order to investigate the relationship between the produced metabolites in the Kombucha samples at the end of fermentation (two different ratios: A and B) and the non-fermented

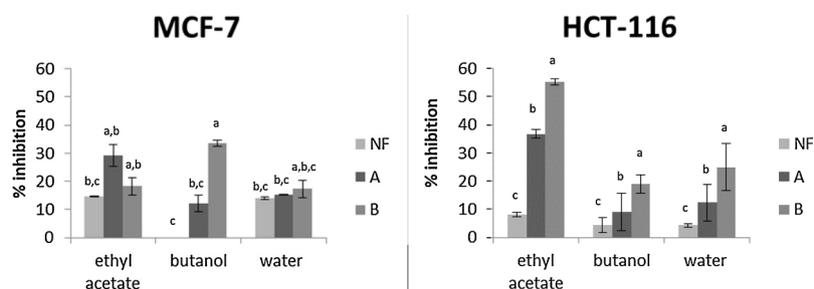


Fig. 8. Effect of kombucha tea extracts (50 µg/mL) on human breast cancer (MCF-7) and human colon cancer (HCT-116) cells. NF: non-fermented tea; A: ratio s/h = 4.19 cm; B: ratio s/h = 27.41 cm.

tea. A clear difference in the diversity of detected molecules can be observed between the NF and the A and B, showing the high impact of the fermentation (Fig. 7a). However, a negative correlation was observed specifically in the case of the phenolic compounds (catechin, 2,5-di-tert-butylphenol) and organic acids (isovaleric, pyruvic, lactic, citric, gallic, gluconic) (Fig. 7b), suggesting that these molecules had opposite behaviors regarding their production, either by increasing (gallic acid) or by being produced just after 21 days of fermentation (organic acids) when compared to the NF tea. The low positive correlation observed in acids and alcohols, revealed that these compounds were present in both ratios but did not follow exactly the same production pattern.

3.7. Phenolic composition and antioxidant activity

The total phenolic composition present in kombucha extracts was determined by the Folin-Ciocalteu method obtaining concentrations in the range of 36.0–221.6 mg eq AG/g for all samples (Table 5). Similar concentrations were found by Turkmen et al. [34] who obtained values in the range of 29.1–80.4 mg eq AG/g in extracts of non-fermented black tea, showing that tea phenolic composition is fairly constant despite tea processing. However its chemical structure may change or be polymerized into molecules with higher molecular weight [35]. This might have happened during the fermentation as the total phenolic composition in the butanol and water extracts was twice that in the non-fermented tea. The antioxidant capacity of the extracts, was measured by the DPPH method in the final solvent extracts. Table 5 shows the antioxidant activities of the tested samples (ethyl acetate, butanol and aqueous fractions), which were high in both s/h ratio conditions, indicating that the activity it is not affected by the vessel geometry or the fermented volume. The enhancement of antioxidant activity has been reported in others studies [26,27], which can be due to the interactions between microbial enzymes and phytochemicals in the fermented media. In the case of the two tested ratios, the increase in the phenolic content as well as in the antioxidant activity were higher with the highest vessel ratio, increasing from 70.9% inhibition in the non-fermented tea to 96.2% in the butanol extracts and from 14.6 to 23.5% in the water extracts. The half-maximal inhibitory concentration (IC₅₀) was also calculated. The highest value was in the ethyl acetate extracts, which probably means that most of the antioxidants compounds in the tested samples were non-polar.

3.8. Anti-inflammatory activity

No studies on 5-LOX inhibition with kombucha tea extracts have been published in the literature. Our results (Table 5) indicated an improvement in this activity after fermentation with the kombucha consortium. The non-fermented tea obtained 66% or even 0% of inhibition compared to 87–91% after 21 days of fermentation. An IC₅₀ value of 9.0 ± 0.00 µg/mL was obtained for vessel B, with ethyl acetate extract, which is close to the maximal inhibitory concentration of 7.0 ± 0.2 µg/mL for the natural LOX inhibitor Nordihydroguaiaretic

acid (NDGA). These results indicated that kombucha extracts can potentially be effective as 5-LOX inhibitors and consequently become an alternative for the development of non-steroidal drugs (NSAIDs).

3.9. Antiproliferation evaluation

Kombucha tea extracts were tested against human breast cancer (MCF-7) and human colon cancer (HCT-116) cell lines and an enhancement of anticarcinogenic activity after fermentation in all vessels and for both cell lines was observed (Fig. 8). The kombucha consortium was able to produce bioactive compounds with different polarities, obtaining an inhibition increase in all the tested extracts compared to the non-fermented tea. However, the highest inhibition percentage was obtained with vessel B, especially against the HCT-116 cell line. These results showed a good similarity with those obtained by Jayabalan et al. [36] where the highest inhibition percentage was found with the ethyl acetate extract (55.3%) against HCT-116 cell line. However, this was significantly different from the rest of the samples at a concentration of 50 µg/mL. Regarding the human breast cancer cells, the fermentation was found to have the largest effect, increasing the inhibition percentages in both vessels.

4. Conclusion

Several parameters should be taken into account to provide a suitable environment to the active consortium. Two different s/h ratios with the same interfacial area were compared to determine which parameter had more significant effect on the final product. The results showed that with a higher s/h ratio the fermentation kinetics were accelerated and the final anti-inflammatory and antiproliferation properties were improved. These results supports the conclusion that using different vessel geometries may produce different fermentation metabolites and bioactive compounds. A statistically significant difference (p < 0.05) was observed between all samples and extracts, also supporting this conclusion. Moreover, these findings suggested that fermentation of black tea with the kombucha consortium increases its bioactive potential and promotes the synergy between the fermentation metabolites and the microorganisms leading to the formation of interest compounds. These results represent preliminary findings that will aid in the evaluation of the bioactive potential of kombucha extracts.

Author contributions

Silvia Alejandra Villarreal-Soto performed the experiments and wrote the manuscript under the supervision of the other authors. Patricia Taillandier, Sandra Beaufort, Jalloul Bouajila, and Jean-Pierre Souchard validated the experiments and refined and revised the manuscript for publication. Thierry Renard and Serge Rollan provided the starting culture.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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