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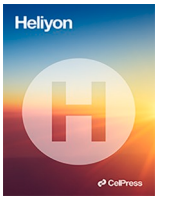

Research article

Metabolomic kinetics investigation of *Camellia sinensis* kombucha using mass spectrometry and bioinformatics approaches

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ABSTRACT

Kombucha is created through the fermentation of *Camellia sinensis* tea leaves, along with sucrose, utilizing a symbiotic consortium of bacteria and yeast cultures. Nonetheless, there exists a dearth of comprehensive information regarding the spectrum of metabolites that constitute this beverage. To explore this intricate system, metabolomics was used to investigate fermentation kinetics of Kombucha. For that, an experimental framework was devised to assess the impact of varying sucrose concentrations and fermentation temperatures over a ten-day period of kombucha fermentation. Following fermentation, samples were analyzed using an LC-QTOF-MS system and a distinctive metabolomic profile was observed. Principal component analysis was used to discriminate between metabolite profiles. Moreover, the identified compounds were subjected to classification using the GNPS platform. The findings underscore notable differences in compound class concentrations attributable to distinct fermentation conditions. Furthermore, distinct metabolic pathways were identified, specially some related to the biotransformation of flavonoids. This comprehensive investigation offers valuable insights into the pivotal role of SCOBY in driving metabolite production and underscores the potential bioactivity harbored within Kombucha.

1. Introduction

For millennia, the art of food fermentation has woven itself into the fabric of human history. Traces of fermented alcoholic elixirs crafted from fruits, honey, and rice have been unearthed in Neolithic China, harkening back to 7,000 BCE [1]. Millennia earlier, around 10,000 BCE, humans had already unlocked the secrets of milk fermentation, birthing the luscious creation known as yogurt [2]. In its initial stages, fermentation was harnessed to preserve and enrich the flavors of sustenance.

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Nonetheless, the landscape has shifted markedly in recent times, as there is evidence of health advantages promoted by fermentation [3,4]. This compendium of benefits encompasses fostering a harmonious gut microbiota and optimizing digestion, mitigating symptoms of irritable bowel syndrome [5,6], and promoting cardiovascular health and the immune system [7].

Fermented foods, such as Kombucha, kefir, and tempeh have surged in demand, as they are minimally processed and have elevated nutritional content [8]. A recent report by Grand View Research [9] forecasts the global kombucha market to burgeon to $7.05 billion by 2027. This increasing interest has promoted not only production but also in exploration of novel applications.

The production of Kombucha is a meticulous process, commencing with the crafting of sweetened green or black tea sourced from the leaves of *Camellia sinensis*. Then, this beverage is inoculated with symbiotic culture of bacteria and yeasts, known as SCOBY. This consortium is composed by acetic bacteria (AAB), lactic acid bacteria (LAB), and yeasts, concealed within a matrix of cellulose [10,11]. During fermentation, tea compounds undergo a transformation by this complex microbial consortium and some key substances described in the literature are organic acids, carbohydrates, vitamins, proteins, polyphenols, minerals [12,13].

Several studies have investigated the properties of Kombucha [14]. There is evidence of Kombucha components preventing oxidative stress, neurodegenerative and cardiovascular diseases and diabetes [15]. Moreover, the beneficial activity of Kombucha seems to be related to phenols and secondary metabolites resulting from microbial metabolism of these components [16].

The characterization of the Kombucha composition has emerged as a focal point of concern. Data on the characterization of its active components, the chemical classes to which these substances belong, and their modifications during fermentation remain limited [11,16]. Several factors are involved in the biotransformation process, such as geographical regions from which the *Camellia sinensis* was produced, the bacterial and yeast strains of the SCOBY, the bioavailability of substrates, the environmental conditions, and the methods of product preparation, manufacturing, or manipulation [13,17]. Therefore, a comprehensive understanding of the influence of specific factors holds significant importance in optimizing the fermentation process and its functionality.

Understanding these intricate interactions is crucial for the production of desired end products and the promotion of health by fermented foods [18]. The use of high-performance omics approaches over the past decade has improved the capacity for metabolic discovery, offering enhanced capabilities in assessing the composition of fermented foods and conducting functional evaluations. Non-targeted metabolomics promotes the investigation of metabolites with a comprehensive approach, aiming to capture the complete molecular profile present in a given sample. Indeed, metabolomics has been successfully applied in food and tea studies [19]. Molecular Networking (MN), based on mass spectrometry (MS) data, and a data-sharing platform, the Global Natural Products Social Molecular Networking (GNPS), enables the assessment of related structural metabolites via similar fragmentation patterns, enabling their arrangement, visualization, and identification when comparing to spectral libraries in the literature [20]. In addition, molecular families can be grouped through fragmentation similarities on a chemical map, assisting in the interpretation of the large sets of metabolomic data and in exploring the chemical entities present [20].

The objective of this study was to provide an overview of the metabolite profile of various Kombuchas obtained from the microbial fermentation of *Camellia sinensis* infusion using a non-targeted metabolomics approach with MS and MN, and to investigate alterations in the kinetics Kombucha. The effects of temperature and substrate concentration in the fermentation process and the possible metabolic pathways to new conversion products were also discussed.

2. Material and methods

2.1. Green tea and kombucha samples

The *Camellia sinensis* tea and kombuchas were prepared and collected from the Companhia dos Fermentados (São Paulo, SP, Brazil). Soluble leaves of *Camellia sinensis* green tea (Vemat - L. 19343), at a final concentration of 6 g/L, were used to prepare 40 L of a green tea base. Then, 4 L of backslopping inoculum was added and mixed with a mixer to dissolve oxygen (approximately 8 ppm). Prior to fermentation, an aliquot was collected to serve as a control (*Camellia sinensis* green tea).

2.2. Chemicals and reagents

Water with 0.1 % formic acid (v:v; hyper grade for LC-MS) and acetonitrile (hyper grade for LC-MS) were obtained from Merck (Billerica, MA, USA). Ultra-pure water was produced using an EMD Millipore Direct-Q™ 3 system (Merck Millipore; Burlington, MA, USA). Syringe filters (0.22 μm, PTFE, 4 mm) were obtained from Merck Millipore (Burlington, MA, USA).

2.3. Kinetics study: kombucha preparation and fermentation conditions

Four treatments with different conditions were used to evaluate the effect of temperature and sucrose concentration on Kombucha fermentation kinetics. Table 1 summarizes the fermentation conditions used for treatments A, B, C, and D. To prepare the Kombuchas,

Table 1
Kombucha treatments (A, B, C and D) and fermentation conditions.

Treatments	A	B	C	D
Temperature (°C)	20	20	30	30
Brix degree (°Brix)	5	10	5	10

the *Camellia sinensis* green tea base was divided into two equal parts. At each one, enough sucrose was added to obtain the desired brix degree, expressed in Brix scale (°Brix): either 5 °Brix or 10 °Brix. Brix degree was measured by a Densimeter Saccharimeter. Later, each respective concentration was again subdivided in two, and each was held at 20 or 30 °C to handle the fermentation. Then, a SCOBY was added and during the entire fermentation process the temperature in the refrigeration chambers were similar. To minimize fermentation variations, all the used SCOBYs were obtained from the same microbial consortium.

2.3.1. Kombucha kinetics

For chemical composition analysis with kinetics fermentation, samples were collected every 24 h for 10 days for all treatments: A (0–10), B (0–10), C (0–10) and D (0–10). A total volume of 5 mL of each experiment at each fermentation time was collected from the middle of each jar and frozen at −4 °C until analysis.

2.3.2. Sample preparation and non-targeted metabolomics analysis

The total volume of each sample was homogenized, and a 2 mL aliquot sonicated for 10 s in an ultrasonic bath (Eco-Sonics, Brazil) and centrifuged at 6,400 rpm for 15 min. The supernatant was collected and filtered through a 0.22 μm PTFE syringe filter (Merck Millipore; Burlington, MA, USA) before analysis. Samples were stored at −20 °C until further analysis, and were analyzed using an Ultra high performance liquid chromatography (UHPLC-ESI-MS/MS; Nexera X2 Shimadzu, Japan) coupled to an Impact II mass spectrometer (quadrupole-Time-of-Flight geometry; Bruker Daltonics Corporation, Germany). An Acquity UPLC® CSH C18 column (100 × 2.1 mm x 1.7 μm) was used with the same gradient elution of water and formic acid (0.1 %; v:v) (A) and acetonitrile (B) as a mobile phase were used for negative mode. The analyses were also performed in positive mode, but were not used due to technical issues with the control board. Chromatographic separation was performed for 31 min using the gradient: 5 % B 0–3 min, 7 % B 3–5 min, 13 % B 5–15 min, 50 % B 15–20 min, 70 % B 20–23 min, 98 % B 23–26 min, 98 % B 26–28 min, 10 % B 28–31 min. The flow was maintained for 0.20 mL min^{-1} at 40 °C during the chromatographic separation. For the experiments, capillary voltage set to 3.5 kV, source temperature 200 °C, desolvation gas flow 8 L min^{-1}, and nebulization gas pressure at 4 bar. Data were collected between the *m/z* ranges of 50–1500 with an acquisition rate of 5 Hz, with the 3 most intense ions selected for automatic fragmentation (Auto MS/MS). The fragmentation spectra were obtained using collision-induced dissociation with a collision energy ramp in 20–45 eV range. All analyses were carried out in triplicate.

2.3.3. Chemical classification and annotation of metabolites by the GNPS platform

2.3.3.1. Molecular networking (MN). The data was acquired and converted to the mzXML extension. The fragmentation data (MS/MS) were transferred to the GNPS virtual platform server to generate the chemical maps, according to the platform documentation [20].

A molecular networking (MN) was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data were filtered by removing all MS/MS fragment ions within ± 17 Da of the precursor *m/z*, which is frequently observed in MS/MS spectra acquired on qTOFs. If no removal of MS/MS fragment ions within ± 17 Da of the precursor *m/z* is done, their presence can potentially interfere with the analysis and interpretation of the data, leading to inaccuracies or misinterpretations in downstream analyses such as metabolite identification or quantification. Therefore, removing these residual precursor ions helps to improve the quality and reliability of the data by reducing unwanted noise and artifacts. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the ± 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of *m/z* 0.02 units and a MS/MS product ion tolerance of *m/z* 0.02 units to create consensus spectra. Furthermore, consensus spectra that contained fewer than 2 spectra were discarded. A MN was then created where edges were filtered to have a cosine score above 0.65 and more than 4 matched peaks. Further, edges between two nodes were kept in the molecular network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the molecular network were then searched against GNPS spectral libraries, such as ReSpect, Massbank and HMDB [21–23]. The library spectra were filtered in the same manner as the input data. The chemical map was made with triplicate analysis and the injection blanks were subtracted to generate it. Files with all parameters available in GNPS ID: 3150157da42d4555a75cd88513ca6660 (A), 27b81d1478d4455cb9ab751d4c2bf6ea (B), d4a4c1a864a84c-b4aea9a9968ebd2a9c (C) and e9ea7466305040b7972202476443c113 (D).

The MN data was exported to Cytoscape 3.7.1 software [24] for visualization. Product ion spectra with similarities to those in the mass spectral libraries had these spectra manually verified and the mass error calculated, using a mass error tolerance of less than 10 ppm. These annotations are considered putative annotations based on spectral library similarity or putatively characterized compound class based on spectral similarity to known compounds of a chemical class [25].

MolNetEnhancer was used to combine complementary molecular mining tools with the MN network, such as the *in silico* annotation tools, such as network annotation propagation (NAP) and MS2LDA [26]. Chemical class annotations were performed using the ClassyFire chemical ontology.

2.3.4. Chemometric analysis

The UHPLC-ESI(-)-MS/MS analysis data were pre-processed using Profile Analysis 2.3 software to generate a matrix with the molecular features of the samples (retention time and *m/z*). The data matrices were evaluated in MATLAB R2017a® environment through the PLS Toolbox 7.8® to perform the Principal Component Analysis (PCA). The Matlab software is owned from Universidade

Tecnológica Federal do Paraná, while the PLS Toolbox is from Embrapa Solos, Rio de Janeiro, Brazil.

3. Results

3.1. Metabolomic kinetics investigation

The UHPLC-ESI(-)-MS/MS analysis was performed to evaluate the metabolite profile of Kombucha kinetics and distinguish them under different fermentation conditions. The MolNetEnhancer analysis was applied in MN to discover chemical annotation of the diversity of structures in molecular families [27]. MolNetEnhancer provided the putative chemical classification of compounds detected for each treatment (A, B, C and D) in *Camellia sinensis* green tea (control) and Kombucha samples on the MN (Fig. 1: A, B, C, and D, respectively).

The chemical composition of Kombuchas included similar chemical entities such as flavonoid class, organic oxygen compounds

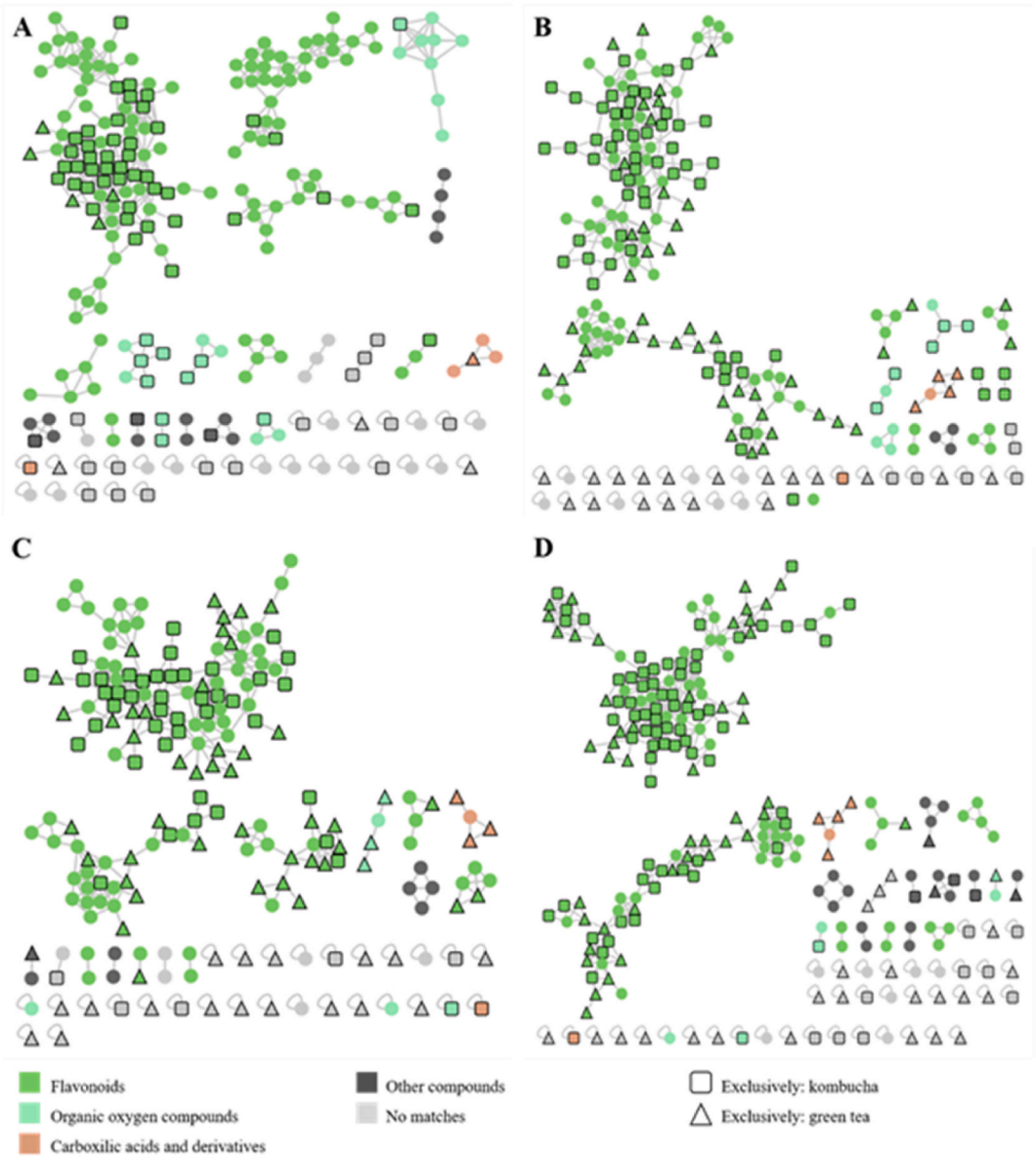

Fig. 1. Molecular network of the MS/MS spectra obtained by analysis of the control and Kombucha samples from treatments A, B, C and D, (1A, 1B, 1C and 1D, respectively) colored by chemical classes terms selected as a indicated in the legend annotated on the molecular network using the MolNetEnhancer. Square nodes were associated with compounds found exclusively in the Kombucha group and triangle nodes were associated with compounds found exclusively in control group.

(such as carboxylic acids), as well as other classes which were not classified (no matches) for all treatments. The chemical map obtained from treatment A (20 °C; 5 °Brix; Fig. 1A) was composed by 234 chemical entities. Of these, 66.24 % were chemical entities belonging to the flavonoid class. It also had 2.14 % classified as carboxylic acids, 10.26 % as organic oxygen compounds, and 14.96 % were undefined. Of all these compounds, 152 (64.96 %) were associated with treatment A and control. The chemical map obtained from treatment B (20 °C; 10 °Brix; Fig. 1B) comprised 215 chemical entities. Of these, 76.74 % were chemical entities belonging to the flavonoid class. It also had 2.79 % chemical entities classified as carboxylic acids, 4.65 % as organic oxygen compounds, and 14.42 % were undefined. 79 (36.74 %) chemical entities were associated with treatment B and control. The C (30 °C; 5 °Brix) chemical map obtained (Fig. 1C) comprised 200 chemical entities. Of these, 76.00 % were chemical entities belonging to the flavonoid class. It also had 3.5 % chemical entities classified as carboxylic acids, 2.5 % as organic oxygen compounds, and 14.00 % as undefined. 84 (42.00 %) were associated with treatment C and control. Finally, the D (30 °C; 10 °Brix) chemical map obtained (Fig. 1D) comprised 239 chemical entities. Of these, 69.46 % were chemical entities belonging to the flavonoid class. It also has 2.51 % chemical entities classified as carboxylic acids, 2.51 % as organic oxygen compounds, and 16.32 % were undefined. 78 (32.64 %) were associated with treatment D and control.

The profile of the *Camellia sinensis* green tea was also explored to ascertain the differences in the unique metabolites synthesized by the microbial consortium and to evaluate the different experimental conditions with the respective Kombuchas. Again, compounds changes were observed between the control and Kombuchas samples. In total, 73(31.20 %), 70 (32.56 %), 49 (24.50 %), and 84 (35.15 %) new chemical compounds were putatively uncovered belonging exclusively to Kombuchas due to the fermentation process in the MN for experiments A, B, C, and D, respectively.

Pie charts of the compound classes from the 4 treatments are presented in Fig. 2 (A-D). Comparing the treatments fermented with similar temperatures, i.e. A (20 °C; 5 °Brix) and B (20 °C; 10 °Brix), a higher sucrose concentration in B resulted in increased flavonoid components. On the other hand, the Kombuchas fermented at the highest temperature, i.e. C (30 °C; 5 °Brix) and D (30 °C; 10 °Brix), had the opposite result, in which the D had a decrease in flavonoids. Similarly, comparing the treatments under equal sucrose concentration, i.e. A (20 °C; 5 °Brix) and C (30 °C; 5 °Brix), a higher temperature in C has increased the flavonoid ratio. Finally, the opposite effect was observed by comparing B (20 °C; 10 °Brix) and D (30 °C; 10 °Brix), when the highest temperature resulted in a lower value in the flavonoids class. In brief, when evaluated by the techniques used in this study, the temperature and substrate concentration have a different influence on the chemical type of compound production.

The significant metabolites putatively discovered in control and Kombucha samples presented previously are summarized in Table 2. They were assigned to the chemical classification to which they belonged. We observed repeatability of some chemical entity's compounds across all experiments, but few were exclusively for a particular experiment condition. Among all the detected compounds, many have been assigned a molecular formula, but not all have been identified. Other chemical entities such as polyphenols, organic acids, flavones, and carbohydrates compounds were putatively identified (Table 2). Mainly quercetin, kaempferol, myricetin, and their glycosides were available in green tea.

A total of 165 and 166 compounds were detected in experiments B (20 °C; 10 °Brix) and D (30 °C; 10 °Brix), respectively. These values were higher when compared to treatments A (20 °C; 5 °Brix) and C (30 °C; 5 °Brix), which were 155 and 152, respectively. However, Kombucha A had a greater diversity of identified and non-identified flavonoid compounds (Table 2). Nonetheless, most of

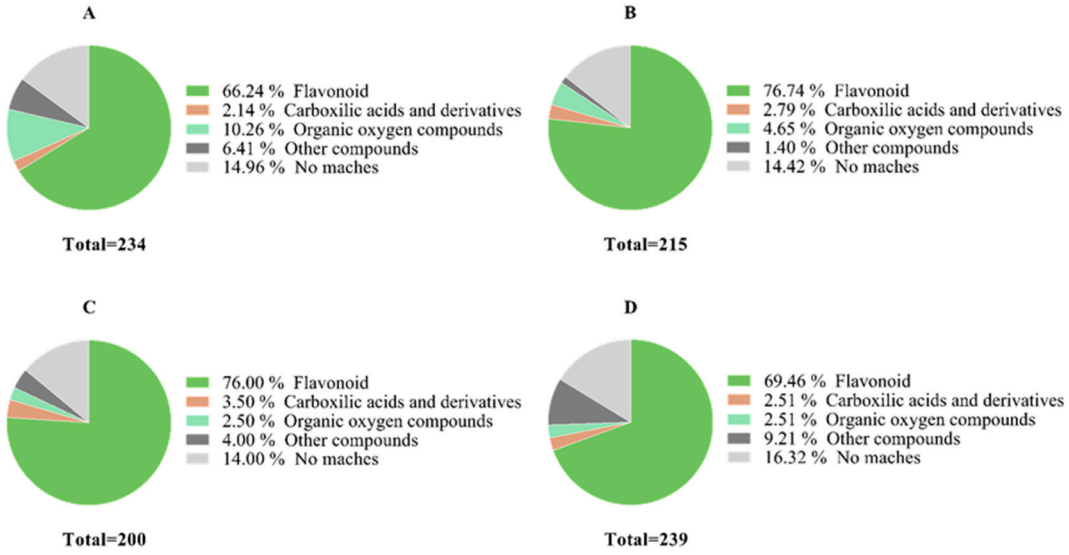

Fig. 2. Pie chart obtained of molecular network of the MS/MS spectra obtained by analysis of the Kombucha samples from experiments A, B, C and D, (2A, 2B, 2C and 2D, respectively) colored by chemical classes terms selected as a indicated in the legend annotated on the molecular network using the MolNetEnhancer. The chemical entities detected only in the control analyses were not considered for the total percentage shown in these images.

Table 2
Metabolites putatively identified in all kombucha treatments (A, B, C and D).

Putative Metabolite Identification	Molecular Formula	$[M-H]^-$ Theoretical	$[M-H]^-$ Measured	Mass accuracy (ppm)	Control	Treatments			
						A (20 °C; 5 °Brix)	B (20 °C; 10 °Brix)	C (30 °C; 5 °Brix)	D (30 °C; 10 °Brix)
Flavonoid									
Afzelechin	$C_{15}H_{14}O_5$	273.0757	273.0759	0.55	X	X	X	X	X
Eriodictyol	$C_{15}H_{12}O_6$	287.0550	287.0551	0.30	X	X			
Catechin	$C_{15}H_{14}O_6$	289.0707	289.0698	−2.99	X	X	X	X	X
Epigallocatechin	$C_{15}H_{14}O_7$	305.0656	305.0647	−2.88	X	X	X	X	X
Theogallin	$C_{14}H_{16}O_{10}$	343.0660	343.0651	−2.54	X	X	X	X	X
Epiafzelechin 3-gallate	$C_{22}H_{18}O_9$	425.0867	425.0857	−2.37	X	X	X	X	
Apigenin-8-glucoside	$C_{21}H_{20}O_{10}$	431.0973	431.0981	1.92	X				X
Epicatechin gallate	$C_{22}H_{18}O_{10}$	441.0816	441.0810	−1.41	X	X	X	X	X
Kaempferol-3-glucoside	$C_{21}H_{20}O_{11}$	447.0922	447.0916	−1.32	X		X		X
7-Galloyltaxifolin	$C_{22}H_{16}O_{11}$	455.0609	455.0595	−3.05	X	X			
Epicatechin 3-(3-methylgallate)	$C_{23}H_{20}O_{10}$	455.0973	455.0957	−3.46	X	X	X	X	
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	457.0765	457.0753	−2.71	X	X	X	X	X
Quercetin-3-glucoside	$C_{21}H_{20}O_{12}$	463.0871	463.0867	−0.87	X		X		X
Epigallocatechin 3-(3-methyl-gallate)	$C_{23}H_{20}O_{11}$	471.0922	471.0905	−3.58	X	X	X	X	
Myricetin-3-glucoside	$C_{21}H_{20}O_{13}$	479.0820	479.0820	−0.04	X		X		
Procyanidin	$C_{30}H_{26}O_{12}$	577.1341	577.1323	−3.04	X	X			
Rutin	$C_{27}H_{30}O_{16}$	609.1450	609.1434	−2.64	X	X	X	X	X
Myricetin 3-neohesperidoside	$C_{27}H_{30}O_{17}$	625.1399	625.1385	−2.28	X	X	X	X	X
Unknown compound	$C_{11}H_{20}O_{11}$	327.0922	327.0918	−1.19	X				
Unknown compound	$C_{12}H_{22}O_{11}$	341.1078	341.1084	1.65					X
Unknown compound	$C_{13}H_{24}O_{12}$	371.1184	371.1189	1.34					X
Unknown compound	$C_{12}H_{22}O_{13}$	373.0977	373.0978	0.36		X			
Unknown compound	$C_{12}H_{22}O_{13}$	373.0977	373.0980	0.89			X		
Unknown compound	$C_{14}H_{24}O_{12}$	383.1184	383.1189	1.30	X		X		
Unknown compound	$C_{13}H_{24}O_{13}$	387.1133	387.1135	0.47	X		X		
Unknown compound	$C_{12}H_{24}O_{14}$	391.1082	391.1083	0.17		X			
Unknown compound	$C_{18}H_{25}O_{10}$	400.1364	400.1383	4.75	X	X			
Unknown compound	$C_{18}H_{25}O_{11}$	416.1313	416.1334	5.02	X	X			
Unknown compound	$C_{15}H_{28}O_{14}$	431.1395	431.1403	1.78	X		X		
Unknown compound	$C_{20}H_{34}O_{10}$	433.2068	433.2068	−0.06	X		X		
Unknown compound	$C_{18}H_{13}O_{13}$	436.0272	436.0285	2.89	X				
Unknown compound	$C_{16}H_{28}O_{15}$	459.1344	459.1344	−0.10		X			
Unknown compound	$C_{17}H_{30}O_{15}$	473.1501	473.1490	−2.32	X				
Unknown compound	$C_{16}H_{28}O_{16}$	475.1294	475.1289	−0.97	X	X	X		
Unknown compound	$C_{16}H_{30}O_{16}$	477.1450	477.1451	0.19		X			
Unknown compound	$C_{17}H_{30}O_{16}$	489.1450	489.1439	−2.27		X			
Unknown compound	$C_{17}H_{32}O_{17}$	507.5560	507.1558	0.44	X	X	X		
Unknown compound	$C_{26}H_{24}O_{11}$	511.1235	511.1272	7.26		X			
Unknown compound	$C_{19}H_{28}O_{16}$	511.1294	511.1302	1.64					X
Unknown compound	$C_{31}H_{32}O_7$	515.2064	515.2078	2.66	X		X		
Unknown compound	$C_{18}H_{32}O_{17}$	519.1556	519.1561	1.01			X		
Unknown compound	$C_{18}H_{34}O_{17}$	521.1712	521.1709	−0.63		X			
Unknown compound	$C_{18}H_{30}O_{18}$	533.1348	533.1344	−0.83			X		
Unknown compound	$C_{18}H_{32}O_{18}$	535.1505	535.1505	0.02			X		
Unknown compound	$C_{27}H_{30}O_{14}$	577.1552	577.1540	−2.05	X	X			
Unknown compound	$C_{20}H_{34}O_{19}$	577.1587	577.1601	2.44		X			
Unknown compound	$C_{21}H_{38}O_{20}$	609.1873	609.1861	−1.92		X			
Unknown compound	$C_{39}H_{26}O_9$	637.1493	637.1519	4.07		X			
Unknown compound	$C_{24}H_{44}O_{23}$	699.2190	699.2174	−2.24		X			
Unknown compound	$C_{30}H_{54}O_{29}$	877.2667	877.2667	0.00			X		
Unknown compound	$C_{30}H_{56}O_{29}$	879.2824	879.2812	−1.31			X		
Organooxygen compounds									
Glucuronic acid	$C_6H_{10}O_7$	193.0343	193.0354	5.81	X	X	X	X	X
Gluconic acid	$C_6H_{12}O_7$	195.0499	195.0506	3.42	X	X	X	X	X
Maltopentaose	$C_{30}H_{52}O_{26}$	827.2663	827.2651	−1.46	X	X	X	X	X
Maltohexaose	$C_{36}H_{62}O_{31}$	989.3191	989.3181	−1.04	X	X	X	X	X
Maltoheptaose	$C_{42}H_{72}O_{36}$	1151.3720	1151.3703	−1.44	X	X	X	X	X
Carboxylic acids and derivatives									
Citric acid	$C_6H_8O_7$	191.0186	191.0188	0.90		X	X	X	X
Fructosyl pyroglutamate	$C_{11}H_{17}NO_8$	290.0870	290.0870	−0.15	X	X	X	X	X

(*continued on next page*)

Table 2 (*continued*)

Putative Metabolite Identification	Molecular Formula	$[M-H]^-$ Theoretical	$[M-H]^-$ Measured	Mass accuracy (ppm)	Control	Treatments A (20 °C; 5 °Brix)	B (20 °C; 10 °Brix)	C (30 °C; 5 °Brix)	D (30 °C; 10 °Brix)
Others									
2-keto-3-deoxy-gluconic acid	$C_6H_{10}O_6$	177.0394	177.0398	2.46		X	X	X	X
Ketodeoxyheptonate	$C_7H_{12}O_8$	223.0448	223.0450	0.70		X			
Ketodeoxyoctonate	$C_8H_{14}O_8$	237.0605	237.0604	−0.40		X			
Turanose	$C_{12}H_{22}O_{11}$	341.1078	341.1078	−0.11	X	X	X	X	X
Sucrose	$C_{12}H_{22}O_{11}$	341.1078	341.1071	−2.16		X	X		X

the unknown chemical entities had the attribution of a molecular formula. Similar to the initial study, various phenolic compounds were detected in their glycosidic forms. Kombuchas also had an increase in the quantities of glycosylated compounds when compared to non-fermented tea. These compounds were observed to have a wide range of precursor ion mass variation, from *m/z* 273.00 to *m/z* 879.00, and they were associated with microbial production during fermentation.

When comparing fresh *Camellia sinensis* green tea and their Kombuchas, the flavonoid class was found to contain the majority of novel compounds. A cluster will be highlighted to discuss the fermentation kinetics. (Fig. 3). Furthermore, since experiments B and C had a higher concentration of flavonoids, the cluster shown in Fig. 3 belongs to treatment C. The fermentation did not significantly modify most of the metabolites belonging to *Camellia sinensis* green tea, which includes epicatechin gallate, epigallocatechin gallate,

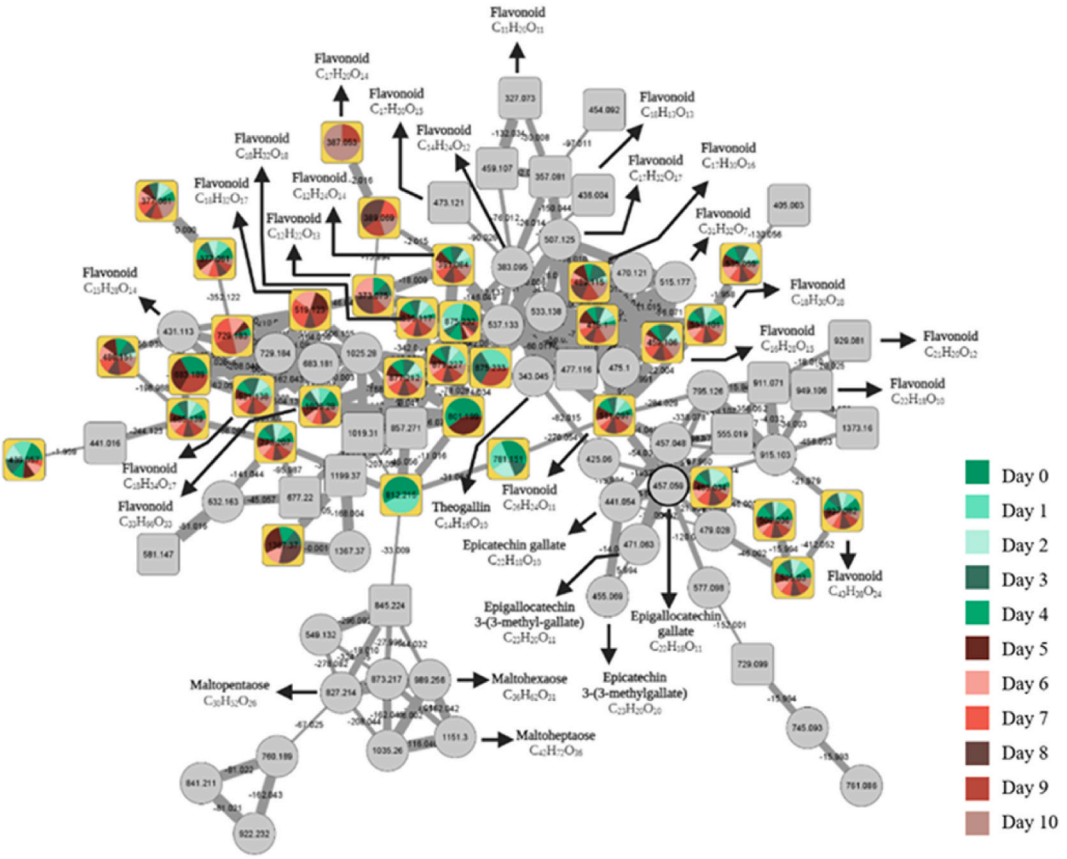

Fig. 3. Cluster of flavonoids containing metabolites putatively identified characterized by molecular network obtained from MS/MS data from control and Kombucha samples of experiment C. The square grey nodes represent the chemical entities found exclusively in control (*Camellia sinensis* green tea) and the square yellow nodes represents the chemical entities found exclusively in Kombucha samples. The edge represents the cosine score (0.65–0.99). The edge label represents the mass difference between nodes. The black bold borders nodes represent the MS/MS that had hit with spectra on the GNPS libraries. The pie chart within each node corresponds to the percentage relative of the metabolite in the sample by a day of fermentation as indicated in the legend annotated on the molecular network. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

epicatechin-3-(3-methyl-gallate) (Fig. 3) and afzeleclin, myricetin 3-neohesperidoside, multiflorin B (not shown in this cluster). There were 34 chemical entities produced after the tea fermentation. After manually checking the raw data, the product ion spectra, the mass accuracy, and the differences between putative compounds, the ions, $[M-H]^-$ *m/z* 441.066, $[M-H]^-$ *m/z* 455.08, and $[M-H]^-$ *m/z* 471.06 were putatively identified as epicatechin gallate, epicatechin 3-(3-methylgallate), and epigallocatechin 3-(3-methyl-gallate), respectively. The analogs of this class of compounds were separated by 15.996 Da and 14.014 Da mass shifts, usually attributed to differences in O and CH_2, respectively. The putative molecular formula and charge-to-mass ratio are $C_{14}H_{16}O_{10}$ with $[M-H]^-$ *m/z* 343.05 (theogallin, neutral mass 344.07). All the nodes had higher spectral similarity since they were clustered with relatively high cosine scores. Through this approach, other ions belonging to the same cluster could be putatively identified. Furthermore, a pie chart layout was generated using the Kombucha group's peak ion area in each fermentation time (0–10) for qualitative evaluation (Fig. 3). Two chemical entities were found only at the beginning of fermentation (not annotated for both cases) and the other two chemical entities were found only after day 7 of tea fermentation ($C_{12}H_{20}O_{14}$; $C_{12}H_{22}O_{14}$). Further compounds exclusively from Kombucha follow all the fermentation time points.

3.1.1. Principal component analysis (PCA)

The data matrices from the four experiments were analyzed by PCA. This unsupervised method is employed to explore and find patterns between samples. Therefore, samples with a high correlation are grouped, and the difference between points corresponding to samples approximates their similarity concerning the variables. The PCA analysis (Fig. 4A–D) showed a tendency of separation between the samples from *Camellia sinensis* green tea and Kombucha. The graphics also reveal that the *Camellia sinensis* green tea samples are further away from the samples in the fermentation kinetics compared to the others. The samples on day 0 have similar performance, while other fermentation days are more clustered. However, a tendency to segregate samples was observed as the fermentation process advanced. The ions of the blank sample did not resemble any of the others. Further, in the graphics of treatments B, C, and D, it is visible that some replicates of the *Camellia sinensis* green tea appeared outside the 95 % confidence limit.

For the PCA applied to treatment A, the first principal component (PC1) explained 66.15 % of the total variance, while the second principal component (PC2) explained 8.67 % of the total variance. In treatment B, PC1 explained 66.22 % of the total variability of the data set, while PC2 explained 22.50 %. In treatment C, PC1 explained 49.11 % of the total variance, while PC2 explained 16.92 %. Finally, in treatment D, PC1 explained 54.68 % of the total variance, while PC2 explained 20.07 %. The higher the explained variance, the higher the cluster on that PC.

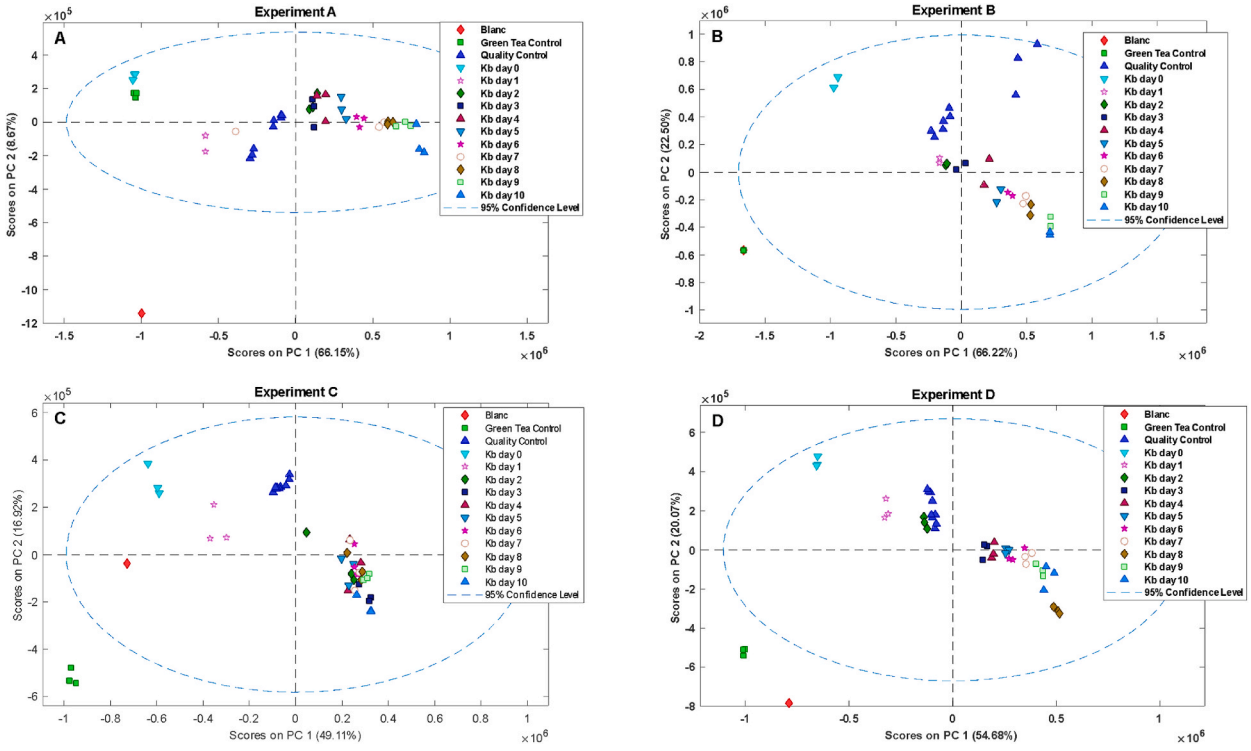

Fig. 4. Unsupervised chemometric modeling (UHPLC-ESI(-)-MS/MS data). PCA scores of the control and Kombucha samples from treatments A, B, C and D (4A, 4B, 4C and 4D, respectively) colored according to triplicate of a day of fermentation as indicated in the legend annotated.

4. Discussion

4.1. Metabolomic kinetics investigation

In general, the chemical composition of Kombucha includes mainly the compounds found in tea leaves, such as phenolic compounds and their derivatives, organic oxygen compounds and others [28] that were presented in Fig. 1. The majority of the chemical compounds observed in Fig. 2, belong to the flavonoids class. Flavonoids are secondary metabolites that are synthesized by plants [29] and fungus [30]. In humans, these compounds are known for their antioxidant properties, as free radical scavengers and even as metal chelators, preventing cancers, cardiovascular and neurodegenerative diseases [31], but also anti-inflammatory and antibacterial [32] activities. In plants, they are known as UV protectors, symbiotic nitrogen fixation [33], and antimicrobial agents [34], while in fungus the biological functions are unclear.

Polyphenols from *Camellia sinensis*, particularly the catechins (i.e. epicatechin, gallocatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate) have a myriad of benefits. For example, epigallocatechin gallate has activity against cardiovascular maladies by decreasing levels of low-density lipoprotein cholesterol and impeding the genesis of blood clots [35].

Studies have shown that the antioxidant capacity of Kombucha can be affected by the temperature used in the beverage processing [36] and by the fermentation time [37]. Overall, maintaining the fermentation temperature between 22 °C and 30 °C throughout the process results in better microbial growth and subsequently enzyme activity, boosting the fermentation benefits [13]. Another study revealed that the amounts of metabolites produced were greater in the samples obtained at higher temperatures [38]. However, in this study, when comparing the treatments with the same °Brix, the effect of the temperature on fermentation had opposite influences on the proportion of the compound class. One assumption is that these fermentation parameters can play different roles in modulating the enzymes present in the SCOBY. It could be related to the microbiota used to ferment green tea and to how the multiple parameters modulate the biotransformation, which are not well understood. Thus, when considering an isolated variable, its influence can have opposite results, which is not necessarily harmful. More research must be done on its potential antioxidant benefits. These variations will induce some disturbance in the metabolism of the involved microorganisms, but more detailed and well-controlled studies are required to better understand this issue. The same is applied to substrate concentration.

The fermentation process which was most suitable were from the experiments B (20 °C; 10 °Brix) and C (30 °C; 5 °Brix). Although the fermentation parameters are the extreme opposite of each other, they play similar roles in the production of the compounds. This means that with the higher temperature the lower sucrose concentration can be used to have almost the same proportion of phenolics compounds as with the lower temperature and higher sucrose concentration. Therefore, one must estimate which process is the most cost-effective. Another finding in this study is that in apart from having higher production costs, such as heating and substrate concentration, the D treatment (10 °Brix; 30 °C) produced fewer chemical entities with a lower ratio of phenolic compounds. Nevertheless, other conditions should be evaluated, including marketing and logistics, product safety, consumer acceptability, etc.

The chemical entities of the carbohydrate class and their conjugates (Table 2) are consumed by the acetic acid bacteria (AAB) that assimilate them to maintain their metabolism [11]. Fermentation is followed by the production of organic acids, such as acetic, gluconic, and glucuronic. Some of the organic acids were observed in Fig. 1 and are described in Table 2. Organic acids are vital components that affect the sensory, chemical, and microbiological stability of foodstuffs and beverages [39]. The organic acids are fermentation end products by SCOBY metabolism fermentation, while the different yeasts and bacteria species act in parallel symbiosis. While the microorganisms ferment the substrate, the enzymes produced by the yeast community metabolize the sucrose into glucose and fructose and then produce ethanol and CO_2. Then, through oxidation reactions, the bacterial enzymes convert the ethanol into acetic acid, which results in a low pH environment. Other steps can produce organic acids like gluconic, glucuronic, and other acids. Also, the bacteria produce the cellulose matrix, which leads to biofilm formation [11,13].

Glucuronic acid derives from the microbiological process of glucose oxidation and other organic acids are among the most prominent factors responsible for the acidity of Kombucha in green tea compared to black tea [11,13]. Glucuronic acid also has the capability to promote the excretion of xenobiotic compounds by binding to them [40]. Furthermore, it also has a role against lipid peroxidation by modulating the bioavailability of phenolic compounds with antioxidant properties, on the biotransformation of fatty acids reactions, providing further evidence of the potential health benefits of Kombucha.

The dominant genera in Kombucha are *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* and bacteria such as *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconobacter oxydans* and *Komagataeibacter xylinus* are frequently observed [13,41]. Some bacteria have an alternative metabolic route to Entner-Doudoroff to the degradation substrates like gluconate [42]. This pathway occurs in *Gluconacetobacter* strains and in *Komagataeibacter xylinus* during the cellulose matrix synthesizing process to embed the kombucha microbiota [43]. In this non-phosphorylative pathway, 2-keto-3-deoxy-gluconic acid is an intermediate of the gluconate catabolism, where the gluconic acid/galactonic acid produces glyceraldehyde and pyruvate (KEGG: M00309). 2-Keto-3-deoxy-gluconic acid was found only in Kombucha samples indicating that this pathway is activated probably by the presence of *Komagataeibacter xylinus* and the gluconic acid. Another metabolic pathway that occurs with the intermediate 2-keto-3-deoxy-gluconic acid is the pentose and glucuronate interconversions (KEGG: map00040).

The diversity of compounds is increased throughout the fermentation days (Table 2), during which phenolic compounds are biotransformed. The symbiotic culture of yeasts and bacteria modify the original chemical compounds in green tea, especially the polyphenols [14]. Bioaccessible phenolics can interact with other food components and suffer other reactions such as glycosylation, deglycosylation, methylation, glucuronidation [44]. Moreover, other microbes are part of the SCOBY microbial composition [45]. The genus *Lactobacillus* is most likely responsible for forming and degrading polymeric phenolic compounds, besides producing free phenolic compounds, which can act as a detoxification mechanism in the oxidative environment [46].

Bioaccessible phenolics are responsive to external conditions. The chemical composition variance across studies is expected from the type of tea, which changes according to seasonal differences, harvesting period [47], the microbial population and their interactions [48]. As industries have distinct SCOBYs, the fermentation kinetics of each one would be different. Despite the greater diversity supporting the initial study, the differences observed in flavonoid compounds compared to other investigations encompass all the factors explained previously, including the sample preparation method used.

The combination of untargeted metabolomics with the GNPS was used for prospecting possible metabolites involved in the flavonoid modulation of tea fermentation. An abundant content of flavonoid compounds was expected (Fig. 3). This class represents Kombucha's main group of antioxidants, which have important bioactive effects [14]. As mentioned, an increase in the flavonoid compounds is related to changes in the fermentation environment, in which an acid level can release the combined one to their free forms [49]. Another reason can be attributed to enzymatic activity, such as β-glucosidase, promoting the degradation of complex tea flavonoids to smaller molecules released by the Kombucha microbial consortium [14].

Ivanišová et al. [50] reported that Kombucha has significant antioxidative potential due to its total phenolic and flavonoid contents. Despite these compounds (Fig. 3) potentially having high antioxidant capacity, their potential was not evaluated in this study. Chakravorty et al. [51] observed that after the 7th day of fermentation, the greater antioxidant activity might be caused by the higher microbial diversity achieved by that time. Indeed, we observed further chemical entities after the 7th day of fermentation. Similarly, another study reported an enhancement of antioxidant activity [52].

Of the discovered chemical entities, most of them have not been putatively characterized and others are still unknown. Glycosidic secondary modifications in the flavonoid structure were largely observed (Fig. 3). Microbial glycosylation leads to an improvement of their water solubility. But it is not clear whether they are absorbed as aglycones, glycosides, or in both forms by the human body [53]. It is known, however, that the type of sugar residues attached to flavonoids influences their absorption, distribution and their metabolism (Xiao et al., 2014). To assess if the glycosylated reactions of flavonoids compounds affect positively or negatively in some potential health benefit, more experiments and studies about these compounds are required. However, some functions and effects can only be observed in additive and/or synergistic ways.

After the initiation of fermentation process, the difference between the flavonoid profiles according to the kinetics days does not change drastically, except in particular cases as described above. Nevertheless, there must be the formation of more compounds not detected in this work, as volatile compounds, mostly alcohols, acids, and esters. Therefore, further studies using other techniques (for instance, gas chromatography coupled to mass spectrometry) should be undertaken to get a more comprehensive view of the Kombucha kinetics and the effects of these fermentation conditions [54]. The flavonoid biosynthetic pathway involves lengthy unknown reaction steps and regulations that significantly affect metabolite production. The difficulty in keeping the SCOBY system stable is a

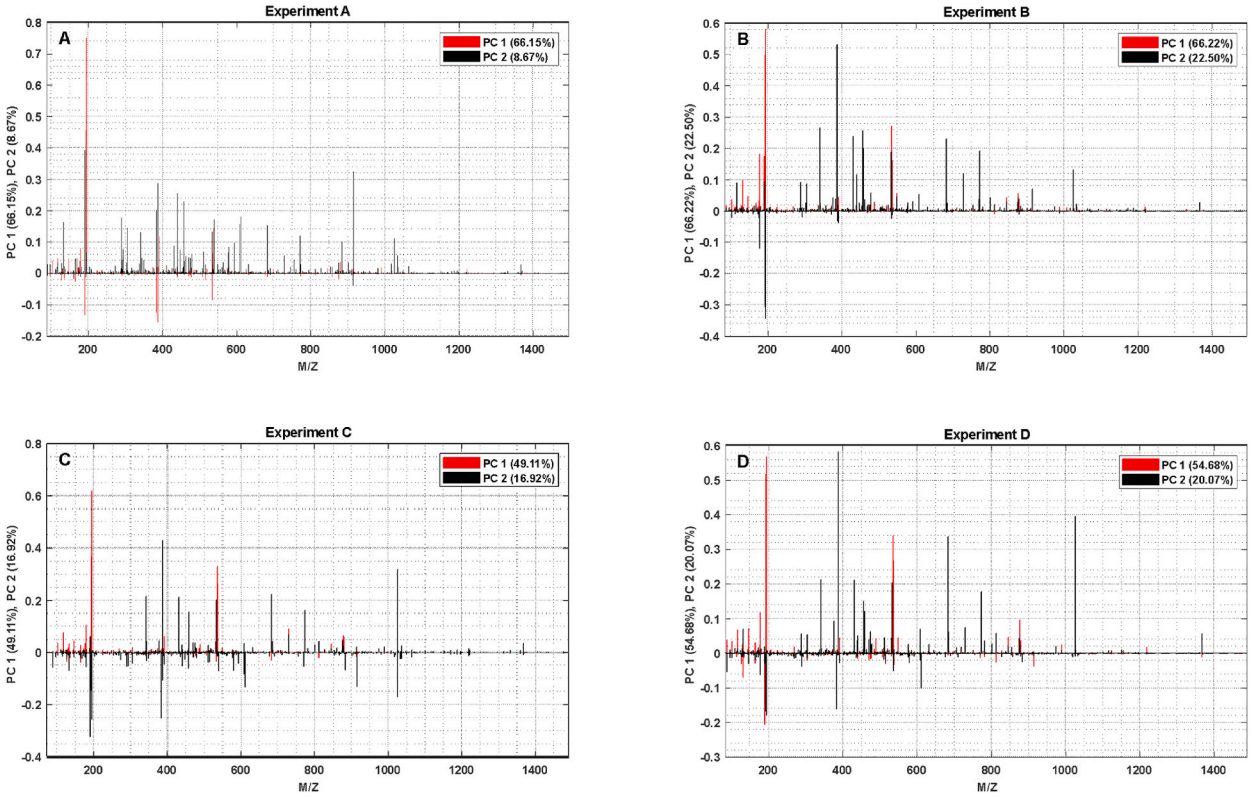

Fig. 5. PCA loadings for the Kombucha samples from experiments A, B, C, and D; (5A, 5B, 5C and 5D, respectively).

challenge for a widespread acknowledgment of the biotransformation reactions and their metabolites. Then, mapping the microorganism's consortium would probably assist in the pathway elucidation. Finally, considering food trends with high added value products, Kombucha with a higher concentration of flavonoid compounds and potentially significant antioxidant activities should be pursued.

4.2. Principal component analysis (PCA)

The PCA was applied to the metabolic data obtained from mass spectrometry, and Fig. 4 presents the PC1 vs PC2 for each experiment, while Fig. 5 (A-D) presents the respective loadings. The separation between *Camellia sinensis* green tea and Kombucha can be noticed in all the experiments. In experiment A, the metabolites obtained for the blank, the green tea control, the quality control, and the Kombucha samples on day 0 and day 1 of fermentation (Fig. 4A) were projected in PC1 negative values, being different from the fermentation days 2–10. In experiment B (Fig. 4B), besides the samples differentiated in experiment A, the kombucha metabolites obtained after 2 and 3 days of fermentation were also projected as being different from days 4–10. Nonetheless, the samples representing the metabolites obtained on days 2 and 3 were close to zero on PC1 and PC2. It means they have little importance on this projection (PC1 vs PC2). It can also be noticed that one of the samples of day 7 appears projected on PC1 negative. As it is a lonely sample, it can be suggested that this is a mis projected sample. Concerning the days of fermentation, the separation observed for experiment C was similar to the ones obtained in experiment A. It means that the blank, the green tea control, the quality control, and the Kombucha samples on day 0 and day 1 of fermentation were different from the others. For experiment D, the same separation was observed, plus the samples from day 2.

These projections reflect different metabolic characteristics due to fermentation, corroborating the previous analysis. In addition, the tendency of separation of kombucha samples along the days could indicate that the microorganisms in SCOBY modify the structures present over time. The PCA analysis was validated by the ions obtained in the blank, which indicates that the ions produced are signals from the samples.

5. Conclusions and perspectives

The combination of mass spectrometry and the Global Natural Products Social Molecular network successfully identified exclusive metabolites in Kombucha. The identification of metabolites and an increase in flavonoid glycosylated compounds content associated with Kombucha is powerful information on the development of value-added fermented food products. Although the fermentation processes are dependent on the fermentation parameters used, our work leads to certain improved conditions in substrate concentration and temperature applicable by the industry. Camellia sinensis green tea fermentation by the SCOBY could be considered a potential process for releasing chemical entities from natural resources and producing new bioactive compounds. The symbiotic complex can induce the biotransformation of flavonoids into their metabolites by different pathways, including glycosylation and others, according to bacteria and yeast strains. More studies are needed to elucidate the microbial pathways of flavonoid conversion. Therefore, it is important to acknowledge keeping the SCOBY system stable and look for novel strategies to overcome this issue. There is still a lack of cooperation in complex multispecies systems, the fully metabolic pathways, and its metabolites identification, which are some directions for future research. The fermentation process which was most suitable were from the experiments B (20 °C; 10 °Brix) and C (30 °C; 5 °Brix). From the techniques used in our work, the company could consider producing the beverage under the same fermentation conditions in B (20 °C; 10 °Bx) or C (30 °C; 5 °Bx). Although the fermentation parameters are the extreme opposite of each other, they play similar roles in the production of the compound classes. This means that with the higher temperature the lower sucrose concentration can be used to have almost the same proportion of phenolics as with the lower temperature and higher sucrose concentration. Therefore, the company must estimate which one has the most cost-effective process. The achievements of this study indicate that to validate the potential of Kombucha and to ensure food safety, there is a need for additional studies involving in vitro and in vivo experiments to determine the bioactivity, bioaccessibility, and even the microbiota modulation of the bioactive compounds.

Data availability statement

The data set has been submitted to Global Natural Products Social Molecular Networking (GNPS) and are available via GNPS ID: b075eaa6b0bd450b85446c1f4bf7c230.

CRediT authorship contribution statement

Cler Antônia Jansen: Writing – original draft, Methodology, Data curation. **Daniele Maria Zanzarin:** Writing – original draft, Investigation. **Paulo Henrique Março:** Data curation. **Carla Porto:** Supervision. **Rodolpho Martin do Prado:** Supervision. **Fernando Carvalhaes:** Supervision, Project administration. **Eduardo Jorge Pilau:** Writing – original draft, Supervision.

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.

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