UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

CLER ANTÔNIA JANSEN DA SILVA

Metabolomic kinetics investigation of *Camellia sinensis* kombucha using Mass Spectrometry and Bioinformatics approaches

Maringá
2021

CLER ANTÔNIA JANSEN DA SILVA

Metabolomic kinetics investigation of *Camellia sinensis* kombucha using Mass Spectrometry and Bioinformatics approaches

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Eduardo Jorge Pilau

Maringá
2021

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá - PR, Brasil)

S586m

Silva, Cler Antônia Jansen da

Metabolomic kinetics investigation of *Camellia sinensis* kombucha using Mass Spectrometry and Bioinformatics approaches / Cler Antônia Jansen da Silva. -- Maringá, PR, 2021.

65 f.: il. color., tabs.

Orientador: Prof. Dr. Eduardo Jorge Pilau.

Dissertação (Mestrado) - Universidade Estadual de Maringá, Centro de Ciências Biológicas, Programa de Pós-Graduação em Ciências Biológicas (Biologia Celular), 2021.

1. Chá fermentado. 2. Espectrometria de massas . 3. Metabolômica não direcionada. 4. Simbiose . I. Pilau, Eduardo Jorge, orient. II. Universidade Estadual de Maringá. Centro de Ciências Biológicas. Programa de Pós-Graduação em Ciências Biológicas (Biologia Celular). III. Título.

CDD 23.ed. 572.49

Síntique Raquel Eleutério - CRB 9/1641

CLER ANTÔNIA JANSEN DA SILVA

Metabolomic kinetics investigation of *Camellia sinensis* kombucha using Mass Spectrometry and Bioinformatics approaches

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas, sob apreciação da seguinte banca examinadora:

Aprovado em: 30/08/2021.

COMISSÃO JULGADORA

Prof. Dr. Eduardo Jorge Pilau
(Presidente)

Prof. Dr. Stanislau Bogusz Junior
(USP)

Profª. Drª. Cristina Giatti Marques de Souza
(UEM)

BIOGRAFIA

Cler Antônia Jansen da Silva, filha de Inelore Jansen e Eliezer da Silva, nasceu em 10 de abril de 1996, na cidade de Joinville, estado de Santa Catarina. Cresceu em Joinville/SC, onde concluiu o no ensino médio no Colégio da Univille em 2013.

Em 2015, ingressou na Universidade Estadual de Maringá e iniciou a graduação em Bioquímica, campus Maringá, estado do Paraná. Entre 2016 e 2018, realizou projetos de Iniciação em Desenvolvimento Tecnológico e Inovação (PIBITI), no Núcleo de Estudos em Produtos Naturais, no Departamento de Bioquímica. Em 2018, defendeu o Trabalho de Conclusão de Curso intitulado "Obtenção de transglicosilado e avaliação bioquímica e fisiológica comparada a adoçantes naturais e artificiais" e obteve o diploma de Bacharel em Bioquímica, pela Universidade Estadual de Maringá.

Em 2019, iniciou o mestrado em Ciências Biológicas, com área de concentração em Biologia Celular e Molecular, na Universidade Estadual de Maringá, e foi contemplada com bolsa CAPES. Desenvolveu pesquisa no Laboratório de Biomoléculas e Espectrometria de Massas, atuando principalmente nas áreas de Bioquímica de alimentos, Bioquímica de microrganismos e Espectrometria de Massas.

No dia 30 de agosto de 2021, se submeteu à defesa de dissertação para obtenção do título de Mestre em Ciências Biológicas pelo Programa de Pós-Graduação em Ciências Biológicas, da Universidade Estadual de Maringá.

AGRADECIMENTOS

Aos meus pais e irmãos por todo o apoio incondicional, por me receberem sempre de braços abertos e me confortarem nas horas mais difíceis. Serão sempre o exemplo de que nada se consegue sem esforço e coragem e de que as quedas também fazem parte do caminho. Nunca conseguirei compensar devidamente toda dedicação e sacrifícios que fizeram para tornar isto possível. Obrigada por todo o amor.

À minha família por me lembrarem de que o amor transcende a distância. Obrigada por todo carinho e incentivo.

Aos meus amigos pela amizade, atenção e ajuda em todos os momentos. Por nunca largarem minha mão ou me deixar desistir, por acreditaram sempre que eu era capaz e por desejarem sempre o melhor para mim. Obrigada por estarem sempre ao meu lado.

Aos integrantes do Laboratório de Biomoléculas e Espectrometria de Massas (LaBioMass) pelas contribuições, apoio e assistência durante todo o percurso da pesquisa. Obrigada pela amizade que fica.

Ao meu orientador pela transmissão de conhecimentos e valores, pela paciência e dedicação, por tudo.

À Companhia dos Fermentados que confiou em mim e na ciência.

À Direção, à Secretaria e aos Professores do Programa de Pós-Graduação em Ciências Biológicas.

À CAPES, pelo apoio financeiro que possibilitou essa pesquisa. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

À UEM pelo ensino público, gratuito e de qualidade.

APRESENTAÇÃO

Em concordância com as normas fixadas pelo Programa de Pós-graduação em Ciências Biológicas, a dissertação foi redigida na forma de um artigo a ser submetido ao periódico *Food Chemistry* (ISSN-0308-8146), Qualis A2 para Ciências Biológicas I, com modificações para facilitar o entendimento do texto e para obedecer aos critérios estabelecidos pelas resoluções vigentes do programa de pós-graduação.

RESUMO GERAL

Kombucha é produzido pela fermentação do chá da *Camellia sinensis*, combinado com a adição de sacarose e um sistema simbiótico de bactérias e leveduras. Entretanto, informações sobre a classe dos metabólitos presentes no kombucha ainda são vagas. Logo, a abordagem metabolômica não direcionada foi utilizada para investigar a cinética de fermentação de metabólitos da kombucha. No desenho experimental foi avaliada a influência de duas concentrações de sacarose e duas temperaturas de fermentação durante 10 dias de fermentação do kombucha. O chá não fermentado foi usado como controle. As amostras foram analisadas em um sistema de cromatografia líquida de ultra-alto desempenho acoplado à espectrometria de massas de alta definição com ionização por electrospray. Os metabólitos foram putativamente identificados e classificados utilizando a plataforma Global Natural Products Social Molecular Networking. Um perfil metabolômico diferente em kombucha foi observado. A concentração das classes de compostos foi influenciada pela temperatura e substrato e vias metabólicas de biotransformação de flavonoides estão intensamente presentes. Os dados do nosso trabalho reforçam a importância do SCOBY na produção de metabólitos e do potencial bioativo da kombucha.

PALAVRAS-CHAVE: chá fermentado, espectrometria de massas, GNPS, metabolômica não-direcionada, simbiose

ABSTRACT

Kombucha is produced through fermentation of *Camellia sinensis* tea leaves, combined with sucrose and a symbiotic system of bacteria and yeast cultures. However, there is a lack of information on the classes of metabolites and the untargeted metabolomics approach was used to investigate their modulation on fermentation kinetics. An experimental design was performed to evaluate the influence of two sucrose concentrations and two fermentation temperatures during 10 days of kombucha fermentation. A non-fermented tea was used as control. The samples were analyzed on an ultra-high performance liquid chromatography system coupled to high-definition electrospray ionization-mass spectrometry. The metabolites were putatively identified and classified using the Global Natural Products Social Molecular Networking platform. A distinct metabolomic profile in kombucha was observed. There were differences in concentration on compound classes due to fermentation conditions and intensive metabolic pathways of flavonoid biotransformation could be observed. Our data provides insights into the importance of SCOBY in metabolite production and the potential kombucha bioactivity.

KEYWORDS: fermented tea, mass spectrometry, GNPS, untargeted metabolomics, symbiosis

1. Introduction

Fermentation of food has been part of human history for millennia. There is evidence of fermented alcoholic beverages made from fruits, honey and rice in Neolithic China dating back to 7,000 B.C. (McGovern et al., 2004). Even before that, around 10,000 B.C., humans already had experience with fermenting milk for the production of yogurt (Tamime, 2002). The fermentation technique was, at first, used only to conserve food and enhance its flavor. However, in recent years, research has revealed that fermentation is an important step in the development of several unique health benefits from various products (Arora et al., 2013; Dimidi et al., 2019; Pessione & Cirrincione, 2016; Şanlier et al., 2017). The extensive information of benefits includes promotion of healthy gut microbiota and digestion, reduction of irritable bowel syndrome (Bell et al., 2018; Derrien & van Hylckama Vlieg, 2015; Laatikainen et al., 2016), and positive effects on heart health and immunity status (Wastyk et al., 2020). Thus, fermentations products, such as kombucha, kefir and tempeh, are in great demand and increasingly present on restaurant menus and the shelves of specialized markets as an alternative of new and healthy food products, which have the basics requirements for minimally processed products, high nutritional value and with health benefits (Coelho et al., 2020). In this actual food trends, a new report published by Grand View Research, 2020 (https://www.grandviewresearch.com/press-release/global-kombucha-market), the global kombucha market is estimated to be $7.05 billion by 2027, reflecting on a rapid expansion in the Brazilian market as well, supporting interest in their production and research into other applications.

Overall, the production of kombucha consists in the preparation of sweetened green or black tea from *Camellia sinensis*, fermented by a symbiotic culture of bacteria and yeasts (SCOBY), which is formed from a symbiosis of acetic bacteria (AAB), lactic acid bacteria (LAB), and yeasts embedded in a cellulose matrix (Coton et al., 2017; Jayabalan et al., 2014). 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 (Jayabalan et al., 2007; Sinir et al., 2019; Vázquez-Cabral et al., 2017; Villarreal-Soto et al., 2018).

Several studies have investigated the properties of kombucha (Chakravorty et al., 2019) There are evidence of kombucha components in the prevention of oxidative stress, neurodegenerative and cardiovascular diseases (Vázquez-Cabral et al., 2017; Villarreal-Soto, et al. 2019), and diabetes (Aloulou et al., 2012). Moreover, the beneficial activity of kombucha

seems to be related to the phenolics and secondary metabolites linked to the microbial metabolism of such components (Bhattacharya et al., 2013).

The characterization of the kombucha composition has become a concern and data about their characterization of its active components, the chemical classes to which these substances belong, and their evolution during fermentation are still scarce (Aloulou et al., 2012; Bhattacharya et al., 2013; Jayabalan et al., 2014). Several factors are involved in the biotransformation process, such as geographical regions from which the *Camellia sinensis* was produced, the bacterial and yeast genetic strains of the SCOBY, the bioavailability of substrates, the environmental conditions, and the method of preparation, manufacturing, or manipulation (Cardoso et al., 2020; Villarreal-Soto et al., 2018). Thus, in-depth understanding of the influence of certain factors can be of substantial importance for optimizing the fermentation process and its functionality.

Comprehension of such complex interactions is important for fermented foods to achieve desired end products and, consequently, promote health. The use of high-performance omics approaches in the last decade has allowed a better level of metabolic discovery that has the potential to assess the composition of fermented foods and functional evaluations (Wilburn & Ryan, 2017). Non-targeted metabolomics promotes the investigation of metabolites with a comprehensive approach to represent the molecular profile present in a sample. Indeed, metabolomics has been successfully applied in food and tea studies (Tan et al., 2016; Yang et al., 2018). Molecular Networking (MN), based on mass spectrometry (MS) data, and a data-sharing platform, the Global Natural Products Social Molecular Networking (GNPS), allows the assessment of related structural metabolites via similar fragmentation patterns, enabling their arrangement, visualization and identification when comparing to spectral libraries in the literature (Wang et al., 2016; Yang et al., 2013). In addition, molecular families can be grouped through fragmentation similarities on a chemical map, assisting on the interpretation of the large sets of metabolomic data and on exploring the chemical entities present (Wang et al., 2016).

The objective of this study was to provide an overview of the metabolite profile obtained by microbial fermentation from *Camellia sinensis* material 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 as well the possible metabolic pathways to new conversion products were also discussed. This information is desired to provide information of the role of these parameters on fermented beverages in order to develop better food products and in achieving food safety in Brazil or even worldwide.

2. Material and methods

2.1. Green tea and kombucha samples

The *Camellia sinensis* tea and kombuchas were prepared and collected from a bioreactor from the Companhia dos Fermentados (São Paulo, SP, Brazil).

2.2. Chemicals and reagents

Water with 0.1 % formic acid (v:v; hypergrade for LC-MS) and acetonitrile (hypergrade 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. Initial study: metabolomic profile of kombucha

An initial study was developed to evaluate differences in the metabolic profile between *Camellia sinensis* green tea and its respective kombucha. This study consisted of fresh tea (green tea of *Camellia sinensis* leaves; GT) and kombucha (*Camellia sinensis* green tea fermented with SCOBY for 7 days; GTK). To prepare the kombucha, 8 g of green tea leaves from *Camellia sinensis* was infused at 80 ºC for 10 minutes. Then, the green tea was cooled to room temperature, enough sucrose was added until obtaining an initial 5 brix degree of sucrose (5 ºBrix) and SCOBY was added to the fermentation process. Brix degree was measured by a Densimeter Saccharimeter.

2.3.1. Optimization of Sample Preparation and Non-Targeted Metabolomics Analysis

A non-extraction method was used due to the fact that it evaluates more faithfully about what is consumed by the population. Thereby, the sample preparation consisted of homogenizing the total 600 mL volume of samples and aliquoting 2 mL. Then, the sample was sonicated for 10 sec in an ultrasonic bath (Eco-Sonics) and was centrifuged at 6,400 rpm for 10 minutes. The supernatant was collected and filtered through a 0.22 µm filter (Merck Millipore; Burlington, MA, USA). The samples were stored at -4 ºC until further analysis.

Samples were analyzed by UHPLC-ESI-MS/MS using an Ultra high performance liquid chromatography (Nexera X2 Shimadzu, Japan) coupled to an Impact II mass spectrometry (quadrupole-Time-of-Flight geometry; Bruker Daltonics Corporation, Germany).

In addition, conditions of chromatographic separation are described next: an Acquity UPLC® CSH C18 column (100 x 2.1 mm x 1.7 μm) with gradient elution of water and formic acid (0.1 %; v:v) (A) and acetonitrile with formic acid (0.1 %; v:v) (B) as a mobile phase were used in the positive mode. 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. An Acquity UPLC® BEH C8 column (100 x 2.1 mm x 1.7 μm) with gradient elution of A and acetonitrile as mobile phase were used in the negative mode. Elution conditions were similar for positive and negative modes. The flow was maintained for 0.20 $mL.min^{-1}$ at 40 ºC during the chromatographic separation. The electrospray ionization source was operated with capillary voltage set to 4.0 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 to 1500 with an acquisition rate of 5 Hz, with the 3 and 5 most intense ions selected for automatic fragmentation (Auto MS/MS) in positive and negative modes, respectively. The fragmentation spectra were obtained using collision-induced dissociation with a collision energy ramp of 20-45 eV range. All analyses were carried out in triplicate. The negative mode was chosen to perform the following studies about kinetics.

2.3.2. Molecular Networking (MN)

The data were 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 (Wang et al., 2016).

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 was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor *m/z*. 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 0.02 *m/z* units and a MS/MS product ion tolerance of 0.02 *m/z* 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 (Forsythe & Wishart, 2009; Horai et al., 2010; Sawada et al., 2012). 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: b075eaa6b0bd450b85446c1f4bf7c230 (MN negative) and b0bc13953541443781eef3396cc387c9 (MN positive).

The MN data were exported to Cytoscape 3.7.1 software (Shannon et al., 2003) 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 less than 10 ppm. These annotations are considered putative annotation based on spectral library similarity or putatively characterized compound class based on spectral similarity to known compounds of a chemical class (Sumner et al., 2007).

2.4. Kinetics study: kombucha preparation and fermentation conditions

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 green tea base. Then, 4 L of backslopping inoculum was added and mixed with a mixer to dissolve oxygen (approximately, 8 ppm). An aliquot was collected to serve as a control (*Camellia sinensis* green tea).

To evaluate the effect of temperature and sucrose concentration on fermentation kinetics kombucha four treatments with different conditions were applied. Table 1 summarizes the fermentation conditions used for treatments A, B C and D. To prepare the kombuchas, the *Camellia sinensis* green tea base was divided in two equal parts. At each one of them, enough sucrose was added to obtain the desired brix degree, expressed in Brix scale (ºBrix): either 5 ºBrix and 10 ºBrix. Brix degree was measured by a Densimeter Saccharimeter. Later than, each respective concentration was again subdivided in two, each was held at 20 or 30 ºC to handle the fermentation. Then, a SCOBY was added and during the entire fermentation process the temperature was maintaining the same in a refrigeration chamber. To minimize variations of fermentation, all of the used SCOBYs were SCOBYs-brothers obtained from the same microbial consortium.

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

Treatments	**A**	**B**	**C**	**D**
Temperature (ºC)	20	20	30	30
Brix degree (ºBrix)	5	10	5	10

2.4.1. Kombucha kinetics

For chemical composition analysis with kinetics fermentation, samples were collected every 24 hours 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 ºC until analysis.

2.4.2. Sample preparation and non-targeted metabolomics analysis

The total volume of each sample was homogenized and a 2 mL aliquot sonicated for 10 sec in an ultrasonic bath (Eco-Sonics) 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) prior to analysis. The samples were stored at -4 ºC until further analysis. Samples were analyzed on the same equipment as in the initial study by UHPLC-ESI-MS/MS using an Ultra high performance liquid chromatography (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 x 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 used in the negative mode. 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 ºC during the chromatographic separation.

For experiment A, the electrospray ionization source was operated in negative mode with capillary voltage set to 4.0 kV and for the others 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 to 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 of 20-45 eV range. All analyses were carried out in triplicate.

2.4.3. Chemical classification and annotation of metabolites by the GNPS Platform

2.4.3.1. *Molecular Networking (MN).* The data were 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 (Wang et al., 2016).

The Molecular Networking (MN) was generated by applying the same data parameters described in item 2.2.2, using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The spectra in the MN were then searched against GNPS spectral libraries, such as ReSpect, Massbank and HMDB (Forsythe & Wishart, 2009; Horai et al., 2010; Sawada et al., 2012). The library spectra were filtered in the same manner as the input data. The chemical map was made with triplicate analysis. Injection blanks and the water used to prepare the green tea were subtracted to generate the chemical map and the control (*Camellia sinensis* green tea) was the same for all treatments. The parameter to consider a minimum number of MS/MS spectra in a consensus MS/MS spectra to be considered for MN was set to 5 in treatment A and 3 to the other ones. Files with all parameters available in GNPS ID: 3150157da42d4555a75cd88513ca6660 (A), 27b81d1478d4455cb9ab751d4c2bf6ea (B), d4a4c1a864a84cb4aea9a9968ebd2a9c (C) and e9ea7466305040b7972202476443c113 (D).

The MN data were exported to Cytoscape 3.7.1 software (Shannon et al., 2003) 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 less than 10 ppm. These annotations are considered putative annotation based on spectral library similarity or putatively characterized compound class based on spectral similarity to known compounds of a chemical class (Sumner et al., 2007).

2.4.3.2. MolNetEnhancer workflow description for chemical class annotation of Molecular Networks. To enhance chemical structural information within the MN, information from in silico structure annotations from GNPS Library Search, Network Annotation Propagation were incorporated into the network using the GNPS MolNetEnhancer workflow (https://ccms-ucsd.github.io/GNPSDocumentation/molnetenhancer/) on the GNPS website (http://gnps.ucsd.edu). Injection blanks and the water used to prepare the tea were subtracted to generate the chemical map and the control (*Camellia sinensis* green tea) was the same for all treatments.

MolNetEnhancer combines complementary molecular mining tools with the MN network, such as the *in silico* annotation tool, such as network annotation propagation (NAP)

and MS2LDA (da Silva et al., 2018; Wandy et al., 2018). Chemical class annotations were performed using the ClassyFire chemical ontology.

2.4.4. Chemometric analysis

The UHPLC-ESI(-)-MS/MS analysis data were pre-processing 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 by MATLAB R2017a® software using the PLS Toolbox 7.8® to perform Principal Component Analysis (PCA).

3. Results

3.1. Metabolomic profile initial study

The UHPLC-ESI-MS/MS analysis was performed to evaluate the metabolite profile differences between kombucha and control samples. The GNPS MN was used to organize the UHPLC-ESI-MS/MS data. Therefore, chemical maps of *Camellia sinensis* green tea (GT) and kombucha (GTK) samples generated by MN summarize the number of chemical entities obtained in the analysis, represented by a node, which are grouped into molecular families through similarities of their fragmentation spectra (Appendix A).

A total of 189 chemical entities were obtained by MN in both ionization modes. The chemical map obtained using the positive mode (Figure A.1) is composed by 73 chemical entities. Of these, 28 (38.36 %) are associated exclusively with GTK, 16 exclusively with GT and 29 (21.92 %) were associated with both samples. Sixteen chemical entities had a match with the GNPS database (21.92 %). For the negative mode (Figure A.2), 117 chemical entities were observed. Forty-eight chemical entities were associated with GTK (41.03 %); 23 were associated with GT (19.66 %) and 46 were associated between the two samples (39.32 %). Twenty-three chemical entities had a match with the GNPS database (19.66 %).

As expected, a distinct metabolic profile from GT and GTK can be observed, both in positive (Figure A.1) or negative modes (Figure A.2). Moreover, the distinct metabolic profile can also be observed in the base peak chromatograms (BPC) obtained from positive and negative ionization modes (Appendix B: Figure B.1 and B.2, respectively). BPC showed differences in chromatographic peaks (absence/ presence and intensities) between the retention time interval. However, only the visual inspection of BPC is not enough to discriminate changes on the biotransformation of chemical entities after fermentation by SCOBY.

All the chemical entities were summarized in Table C.1 (Appendix C). After manually checking the raw data, product ion spectra, mass accuracy and differences between putative compounds, the ions, $[M-H]^-$ *m/z* 447.09, $[M-H]^-$, $[M-H]^-$ *m/z* 447.09, $[M-H]^-$ *m/z* 463.08, $[M-H]^-$ *m/z* 479,08, $[M-H]^-$ *m/z* 739.20, were putatively identified as isoorientin, kaempferol-3-glucoside, quercetin-3-glucoside, myricetin-3-glucoside and kaempferol-3-glucoside-2-rhamnoside-7-rhamnoside, respectively. These formed a cluster of compounds of the same chemical family. The analogs of this class of compounds were separated by 15.996 Da, 308.11 Da mass shifts, usually attributed to differences of O and $C_{12}H_{20}O_9$, respectively. Putative molecular formulas and charge-to-mass ratio are $C_{33}H_{40}O_{20}$ with $[M-H]^-$ *m/z* 755.20 (quercetin 3-rhamnoside, mass neutra 756.21); and $C_{33}H_{40}O_{21}$ with $[M-H]^-$ *m/z* 771.19 (kaempferol 3-sophorotrioside; mass neutra 772.20), respectively.

Intensity alterations of some phenolic compounds before and after fermentation were observed (Appendix C) corroborating the distinct characterization of the green tea and the fermented beverage. Kaempferol 3-O-rutinoside, epiafzelechin 3-gallate and catechin 3-O-gallate were observed only after the fermentation process. Besides that, saccharic acid-1,4-lactone (DSL) was also exclusively found after fermentation.

Many of the phenolic compounds were observed in their glycosidic forms, with glycosides, galactosides and rutinosides attached (Appendix C). There was an increase in glycosylated compounds compared to their precursors (Appendix D). For instance, rutin, epicatechin gallate, epigallocatechin gallate and myricetin-3-glucoside intensities increased following the fermentation process. On the other hand, quercetin, afzeclin, catechin and gallic acid intensities decreased following the fermentation process. Other compounds such as caffeine and theobromine also decreased with fermentation.

In the large class of phenylpropanoids and polyketides, a diversity of unknown chemical entities were found, with the attribution of a molecular formula and subclass of the flavonoids to most of them. These compounds are in a wide range of precursor ion mass variation, from *m/z* 266 to *m/z* 1367.

3.2. Metabolomic kinetics investigation

The UHPLC-ESI(-)-MS/MS analysis was performed to evaluate the metabolite profile of kombucha kinetics and differentiate them under different fermentation conditions. The MolNetEnhancer analysis was applied in MN to discover chemical annotation of the diversity of structures in molecular families (Ernst et al., 2019). 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 (Figure 1: A, B, C, and D, respectively).

The chemical composition of kombuchas includes similar chemical entities such as flavonoid class, organic oxygen compounds (such as carboxylic acids), as well as other classes which are yet to be classified (no matches) for the all treatments. The chemical map obtained from treatment A (20 ºC; 5 ºBrix; Figure 1A) is composed by 234 chemical entities. Of these, 66.24 % are chemical entities belonging to the flavonoid class. It also has 2.14 % 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 ºC; 10 ºBrix; Figure 1B), is composed by 215 chemical entities. Of these, 76.74 % are chemical entities belonging to the flavonoid class. It also has 2.79 % as carboxylic acids, 4.65 % as organic oxygen compounds and 14.42 % were undefined. 79 (36.74 %) were associated with treatment B and control. The C (30 ºC; 5 ºBrix) chemical map obtained (Figure 1C) is composed by 200 chemical entities. Of these, 76.00 % are chemical entities belonging to the flavonoid class. It also has 3.5 % 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 ºC; 10 ºBrix) chemical map obtained (Figure 1D) is composed of 239 chemical entities. Of these, 69.46 % are chemical entities belonging to the flavonoid class. It also has 2.51 % 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, changes in the content of compounds 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 belongs exclusively for 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 Figure 2. Comparing the treatments containing similar temperatures, A (20 ºC; 5 ºBrix) and B (20 ºC; 10 ºBrix), a higher sucrose concentration in B resulted in increase of flavonoid components. On the other hand, the treatments performed under the high temperature, C (30 ºC; 5 ºBrix) and D (30 ºC; 10 ºBrix), exhibited the opposite result, having the D a decrease in flavonoids. Similarly,

comparing the treatments under equal sucrose concentration, A (20 ºC; 5 ºBrix) and C (30 ºC; 5 ºBrix), a higher temperature in C has increased the flavonoids ratio. Again, an opposite effect was observed by comparing B (20 ºC; 10 ºBrix) and D (30 ºC; 10 ºBrix), when the highest temperature resulted on lower value in flavonoid class. In brief, when evaluated by the techniques used in this study, the temperature and substrate concentration has a different influence on the chemical class of compound production.

The significant metabolites putatively discovered in control and kombucha samples of the previous figures are summarized in Table 2. They were assigned to the chemical classification in which they belong. It had repeatability of some chemical entities compounds in all experiments, but few ones were exclusively for a particular experiment condition. Of all detected compounds, many of them have been attributed to a molecular formula, but not all were identified. Other chemical entities such as polyphenols, organic acids, flavones, and carbohydrates compounds were putatively identified in 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 ºC; 10 ºBrix) and D (30 ºC; 10 ºBrix), respectively. These values were greater when compared to treatments A (20 ºC; 5 ºBrix) and C (30 ºC; 5 ºBrix), which were 155 and 152, respectively. However, kombucha A showed a greater diversity of identified and non-identified flavonoid compounds (Table 2). Nonetheless, most of 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. In kombuchas, it also had an increase in the glycosylated compounds quantities when compared to the non-fermented tea. These compounds are observed having a wide range of precursor ion mass variation, from *m/z* 273.00 to *m/z* 879.00 and they were associated to the microbial production during the fermentation process.

As the flavonoid class contained the majority of novel compounds when fresh *Camellia sinensis* green tea and their kombuchas were compared, a cluster will be highlighted to discuss the fermentation kinetics (Figure 3). Furthermore, since the experiments B and C had higher concentration of flavonoids, the cluster shown in Figure 3 belong to the treatment C. The fermentation did not significantly modify most of the metabolites belonging to *Camellia sinensis* green tea, which includes epicatechin gallate, epigallocatechin gallate, epicatechin-3-(3-methyl-gallate) (Figure 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, product ion spectra, mass accuracy and 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 of 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 clusterized with relatively high cosine scores. Using this approach, other ions belonging to the same cluster could be putatively identified. Furthermore, a pie chart layout was generated using the peak ion area in each fermentation time of kombucha group (0-10) for qualitative evaluation (Figure 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.

3.2.1. Principal Component Analysis (PCA)

The data matrices from the four experiments were analyzed by PCA, an unsupervised method employed to determine patterns between multivariate samples. Samples with high correlation group together, and the difference between any points corresponding to samples is an approximation of their similarity with respect to the variables.

The PCA analysis (Figure 4) showed a clear tendency of separation between the data set of 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 when compared to the others. The samples on day 0 have similar performance. The other fermentation days are more clustered, however, a tendency to segregate as the fermentation process progresses was observed. 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.

In the PCA of treatment A, the first main component (PC1) explained 66.15 % of the total variability of the data set, while the second main component (PC2) explained 8.67 % of the total variability of the data set. 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 variability of the data set while PC2 explained 16.92 %. Finally, in treatment D, PC1

explained 54.68 % of the total variability of the data set, while PC2 explained 20.07 % of the total variability of the data set.

4. Discussion

4.1. Metabolomic profile initial study

A distinct metabolite profile kombucha (Figure A.1 and A.2; Appendix A) was expected and it can be explained for the food fermentation processing. Microbial composition and its development are important factors influencing fermentation and end products quality. The microbiota of the SCOBY cooperates in symbiosis, and in a not fully understood and elucidated way. Studies reported that it can be explained by microbial reactions of biotransformations, enzymatic actions and changes in pH when in the fermentative process (Chu & Chen, 2006; Ivanišová et al., 2019; Jayabalan et al., 2007; Kallel et al., 2012). The most dominant microorganisms in SCOBY are *Acetobacter*, *Gluconobacter* and *Oenococcus bacteria*, *Brettanomyces* and *Zygosaccharomyces* yeasts, which can be involved in the biotransformations reactions (Chakravorty et al., 2019). Moreover, product processing, such as the storage, handling and methodology itself affects the chemical composition of foods (Gauglitz et al., 2020).

During the fermentation, the process leads, especially, to an increase of several phenolic compounds, contributing to the diversity of kombucha compounds (Chakravorty et al., 2019). Hence, phenolic compounds have greater differences (Table C.1; Appendix C) and with many not identified from modifications that unknown yeasts and bacteria perform. This conversion changes the original structures and modulates the bioavailability of these dietary compounds (Wang et al., 2018). One of the reactions by fermentative microbiota is glycosylation. An increase observed in these glycosylated compounds (Table D.1; Appendix D) might be positive for the fermented beverage. The biotransformation of flavonoids affects how these compounds behave in solution, increasing their solubility and stability compared to non-agglutinated flavonoids. However, if bioactivity and bioavailability improve human health effects due to the following glycosylation, it has yet to be determined (Xiao et al., 2014). The evidence of increased glycosylated and conjugated phenolics compounds formation after the fermentation process can also be probably due to acidic fermentation conditions (Tu et al., 2019). On the other hand, another study has reported that there is a degradation of the conjugated or glycosylated phenolic compounds to their free forms (Acosta-Estrada et al., 2014). Thus, their biotransformation during fermentation is also controversial in the literature.

Green tea contains certain compounds that can undergo the process of biotransformation during fermentation, increasing or reducing its concentration (Table D.1; Appendix D). A decrease in catechin content was found by Jayabalan et al. (2007), attributing this process to the secretion of fungal and bacterial enzymes. Catechin was not completely depleted even after seven days of fermentation of black tea, which can be due to the inactivation of the enzyme polyphenol oxidase (Muthumani & Kumar, 2007). Also, a decrease in the intensity of gallic acid can be related to the formation of epiafzelechin 3-gallate, found only after the fermentation process (Tounekti et al., 2013).

Caffeine and theobromine alkaloids are metabolites found abundantly in tea. Caffeine acts as a central nervous system stimulant due to its action as an adenosine A2A receptor antagonist (Chu et al., 2012). The degradation of caffeine during the fermentation process was also reported by Wang et al. (2008) and Chakravorty et al. (2016). A more recent study addresses that the symbolic consortium of kombucha uses caffeine as a source of nitrogen and stimulates cellulose synthesis by bacteria (Chakravorty et al., 2019). In this same way, the decrease in theobromine is explained, since it is part of the same catabolic pathway as caffeine (Zhou et al., 2020).

The DSL was found exclusively in kombucha samples corroborating with previous studies that have also reported the presence of this chemical metabolite in kombucha (Chakravorty et al., 2016). One of DSL's characteristics is the important factor in boosting the human immune system. It has excellent antioxidant properties, and this compound is not found in unfermented teas. DSL's production was increased when there was symbiosis of lactic acid bacteria (LAB) with *Gluconobacter bacteria*, usually present in SCOBY (Martínez-Leal et al., 2018).

Substrate, carbon source, and other parameters established in fermentation also influence the chemical composition of the beverage (Chakravorty et al., 2019; Villarreal-Soto et al., 2020). This initial study provided an insight into the applicability of these bioinformatics tools in analyzing the metabolic profile of kombucha, distinguishing it from *Camellia sinensis* green tea. It was pointed out by the intensity that the fermentation induced structural modifications and breakdowns, especially the phenolics' biotransformation reactions. Along with the growing attention to kombucha to their positive benefits, and as SCOBY plays a useful method for increasing the supply of these dietary compounds, the coming kinetics study will approach how temperature and substrate concentration could affect these metabolites production.

4.2. Metabolomic kinetics investigation

In general, the chemical composition of kombucha includes mainly the compounds found in tea leaves as phenolic compounds and their derivatives, organic oxygen compounds and others that were shown in Figure 1 (Zhu et al., 2020). The majority observed in Figure 2, belong to the flavonoids class. Flavonoids are secondary metabolites that are synthesized by plants (Pandey et al., 2016) and fungus (Jia et al., 2016). In humans, these compounds are known for their antioxidant properties, as free radical scavengers and even as metal chelators. In some diseases, associates with reactive oxygen species, such as cancer, cardiovascular and neurodegenerative diseases, phenolic compounds display forward applications (Gopal et al., 2016), with anti-inflammatory (Kim et al., 2004) and antibacterial (Cushnie & Lamb, 2011) activities. In plants, they are known as UV protectors (Kootstra, 1994), symbiotic nitrogen fixation (Fox et al., 2001), and antimicrobial agents (Winkel-Shirley, 2001), while in fungus the biological functions are unclear.

Camellia sinensis polyphenols, especially the catechins, namely, epicatechin, gallocatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, are reported to be responsible for the most beneficial effects of green tea. A study describes that epigallocatechin gallate is effective in cardiovascular disease in lowering low-density lipoprotein cholesterol levels and inhibiting the formation of blood clots, some of its risk factors (Sinija & Mishra, 2009). These compounds were also found in the kombucha after fermentation, pointing to further positive properties of the fermented tea.

Studies have shown that the antioxidant capacity of kombucha can be affected by the temperature used in the beverage processing (Jayabalan et al., 2008) and by the fermentation time (Chu & Chen, 2006). In theory, 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 (Villarreal-Soto et al., 2018). Another study revealed that the amounts of metabolites produced were greater in the samples obtained at higher temperatures (Lončar et al., 2006). However, in this study, when comparing the treatments with the same ºBrix, the effect of the temperature on fermentation had opposite influences on the proportion of the compound class. One of the suppositions 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 in how the multiple parameters modulate the biotransformation, which that are not well known. 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 microorganisms involved, but more detailed and well-controlled studies are required to better understand this subject. The same is applied to substrate concentration.

From the techniques used in our work, the company could consider producing the beverage under the same fermentation conditions in B (20 ºC; 10 ºBrix) or C (30 ºC; 5 ºBrix). 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. Another finding in this study is that in apart from having higher production costs, such as heating and substrate concentration, the D treatment (10 ºBrix; 30 ºC) produced fewer chemical entities with a lower ratio of phenolic compounds. Nevertheless, other conditions should be evaluated, including company 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 (Jayabalan et al., 2014). Fermentation is followed by the production of organic acids, such as acetic, gluconic, and glucuronic acid. Some of the organic acids were observed in Figure 1 and are described in Table 2. Organic acids are vital components that affect the sensory, chemical, and microbiological stability of foodstuffs and beverages (Vitas et al., 2018). The organic acids are fermentation end products by SCOBY metabolism fermentation while the different yeasts and bacteria species are acting in parallel symbiosis. While the microorganisms ferment the substrate, the enzymes produced by the yeasts community metabolizes the sucrose into glucose and fructose and then produces ethanol and CO_2. Then, through oxidation reactions, the bacterial enzymes convert the ethanol into acetic acid, which results in the 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 (Jayabalan et al., 2014; Villarreal-Soto et al., 2018).

Hence, glucuronic acid derives from the microbiological process of glucose oxidation and with other organic acids are among the biggest factors responsible for the acidity of kombucha in green tea compared to black tea (Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Once again, a compound from fermentation shows positive activity, this time with

detoxifying properties. Glucuronic acid has the capabilities to enable the excretion of xenobiotic compounds in a way that binds to it (Nguyen, Nguyen, et al., 2015). 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. It is further evidence of the potential health benefits of kombucha.

Of all the existing microbial species in the beverage, the dominant belongs to the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* as mentioned before. Bacterias such as *Acetobacter aceti*, *Acetobacter pasteurianusn*, *Gluconobacter oxydans* and *Komagataeibacter xylinus* are found frequently in the probiotic tea culture (Jayabalan et al., 2016; Villarreal-Soto et al., 2018; Zhang et al., 2018). An alternative metabolic route occurs in some bacteria that induce the Entner-Doudoroff pathway to the degradation of other substrates, like in the gluconate catabolism (Vegge et al., 2016). This pathway occurs in *Gluconacetobacter* strains and in *Komagataeibacter xylinus* in the cellulose matrix synthesizing process to embed the kombucha microbiota (Ryngajłło et al., 2019; Shimizu, 2013). In this non-phosphorylative pathway, 2-keto-3-deoxy-gluconic acid is an intermediate of this 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 is taking place with the intermediate 2-keto-3-deoxy-gluconic acid is the pentose and glucuronate interconversions (KEGG: map00040).

Regarding the production of compounds over the fermentation days and the greater diversity (Table 2), the phenolic compounds are bio-transformed with the fermentation process. The symbiotic culture of yeasts and bacteria modify the original chemical compounds in green tea, especially the polyphenols (Chakravorty et al., 2019). Bioaccessible phenolics can interact with other food components and suffer other reactions as glycosylation, deglycosylation, methylation, glucuronidation (Huynh et al., 2014). Moreover, other microbes are part of the SCOBY microbial composition (Nguyen, Dong, et al., 2015). The genus *Lactobacillus* is most likely responsible for the formation and degradation of polymeric phenolic compounds, and the production of free phenolic compounds, which can act as a detoxification mechanism in the oxidative environment (Shetty & Sarkar, 2020).

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 (Yildiz et al., 2021), the microbial population and

their interactions (May et al., 2019). As companies have distinct SCOBYs, the fermentation kinetics of each one would be different. In spite of the greater diversity corroborates the initial study, the differences observed in flavonoids compounds compared to the other investigators include all the factors explained before and even the sample preparation method used.

The combination of untargeted metabolomics and with the GNPS was used to prospect possible metabolites involved in the flavonoid modulation of tea fermentation. An expressive content of flavonoids compounds was expected (Figure 3). This class represents the main group of antioxidants present in kombucha, which have important bioactive effects (Villarreal-Soto et al. 2019). An increase in the flavonoids compounds is related to changes in the fermentation environment as mentioned before, in which an acid level can release the combined one to their free forms (Tu et al, 2019). Another reason can be taken by the enzymatic activity, such as β-glucosidase (Xiao et al., 2014), promoting the degradation of complex tea flavonoids to smaller molecules released by the inhabitants of the kombucha consortium (Villarreal-Soto et al., 2019).

Ivanišová et al. (2019) reported that the kombucha has significant antioxidative potential due to their total phenolic and flavonoid contents. Despite these compounds (Figure 3) potentially having high antioxidant abilities, their capacity was not evaluated in this study. After the 7th day of fermentation, a couple more chemical entities were described and Chakravorty et al. (2016), observed that after the 7th day, the higher tendency in increasing the antioxidant activity might be caused to the higher microbial diversity achieved by that time. Similarly, another study reported an enhancement of antioxidant activity (Wang et al., 2018).

Glycosidic secondary modifications in the flavonoid structure were largely observed (Figure 3). Of the discovered chemical entities, most of them have not been putatively characterized and others are still unknown. It becomes clear that different microorganisms can produce a whole range of metabolites. 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 (Le Roy et al., 2016; Sordon et al., 2016). It is known, however, that the type of sugar residues attached in 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 individually are required (Xiao et al., 2014). However, some functions and effects can only be observed in additive and/or synergistic ways.

After the beginning of fermentation, the difference between the flavonoid profile 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 accomplished to get a more comprehensive view of the kombucha kinetics and the effects of these fermentation conditions (Chan et al., 2020; Zhang et al., 2021). The flavonoids biosynthetic pathway involves lengthy unknown reaction steps and regulations that significantly affect the metabolites production. The difficulty in keeping the SCOBY system stable is a challenge for a widespread acknowledgment of the biotransformation reactions and its metabolites. Then, mapping the microorganisms consortium would probably assist on the pathway elucidation.

Finally, considering food trends with high added value products, kombucha with higher concentration of flavonoids compounds and potentially great antioxidant activities should be pursued.

4.2.1. Principal Component Analysis (PCA)

The separation between *Camellia sinensis* green tea and kombucha in Figure 4 reflects on differentials metabolic characteristics in these two groups, corroborating to the previous analysis. In addition, according to the tendency of separation of kombucha samples along the days of kinetics, could be an indication that the microorganisms in SCOBY modify the structures present over time, showing different ions that have influenced the separation. The ions outside the confidence limit could have been caused by analytical error, since the other samples are grouped together. Finally, the PCA analysis were validate by the ions blanks which indicates that the ions produced are signals from the samples

The PCA analysis was reproducible for each sample group, since the replicates are grouped together. It is also observed that although the data in each of the PCA plots refer to four different treatments, the distribution of the samples in the quadrants present similarities, indicating that reproducibility also occurred between treatments, which consolidates the data obtained in this study.

5. Conclusions and future perspectives

The combination of mass spectrometry and the Global Natural Products Social Molecular network was successful to identify 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 to our partnership company. *Camellia sinensis* green tea fermentation by SCOBY could be considered as a potential process for releasing chemical entities from natural resources and in 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 quite important for further acknowledgment keeping the SCOBY system stable and even look for novel strategies to overcome this issue. There is still a lack of the cooperation in complex multispecies systems, the fully metabolic pathways as well as for its metabolites identification, which are some directions for future research. Other studies involving *in vitro* and *in vivo* studies of the bioactivity, bioaccessibility and even microbiota modulation of bioactive compounds are required to validate its potential and to ensure food safety.

Appendix A – Molecular Networking from the initial study

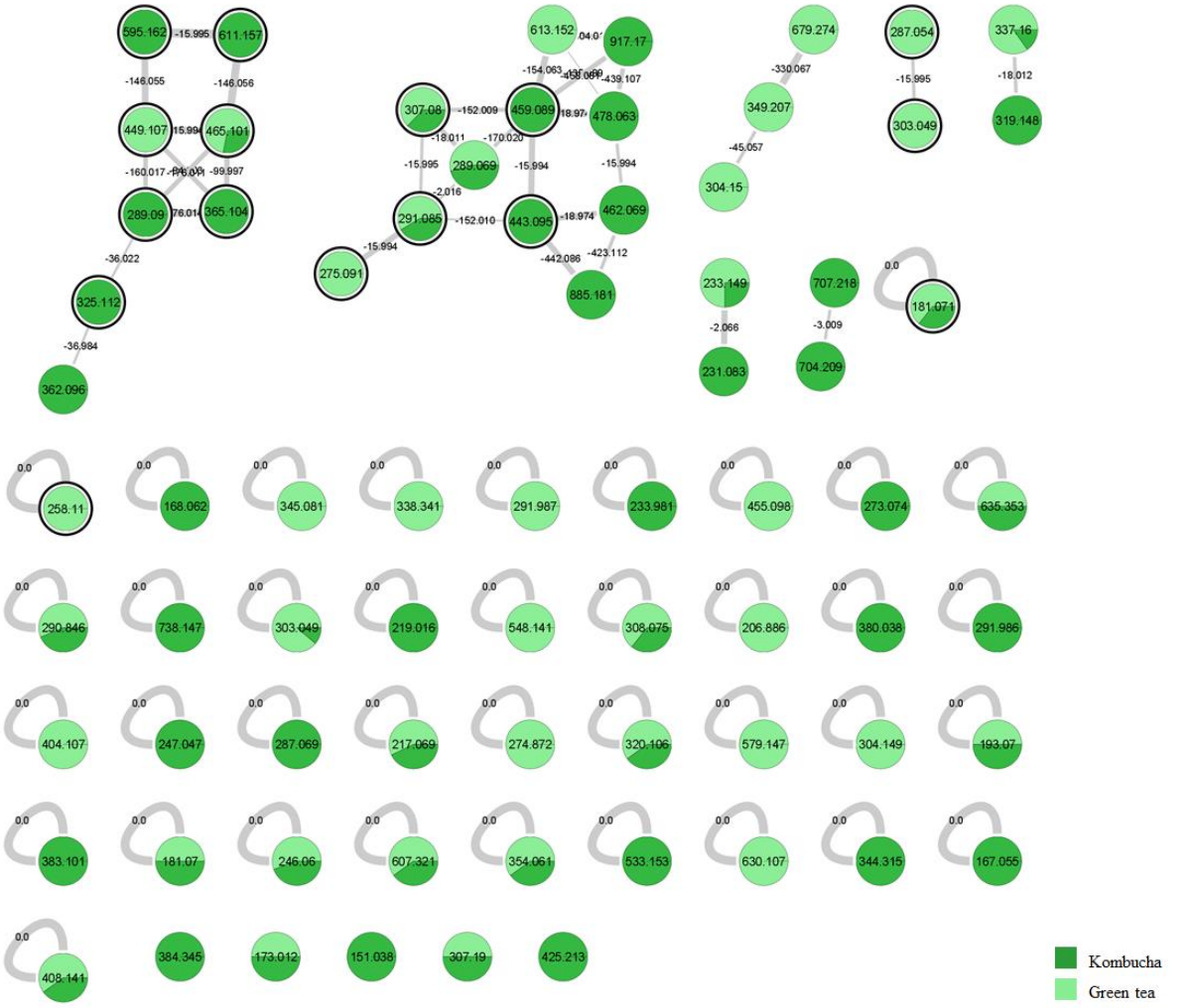


Figure A.1
Molecular Network of the MS/MS spectra obtained by analysis of the control and kombucha samples in the positive mode of ionization. The pie chart within each node corresponds to the percentage relative of the metabolite in the sample as indicated in the legend annotated on the molecular network. The light green ones represents the chemical entities found exclusively in control (*Camellia sinensis* green tea) and the dark green 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 nodes with black bold borders represent the MS/MS that had hit with spectra on the GNPS libraries.

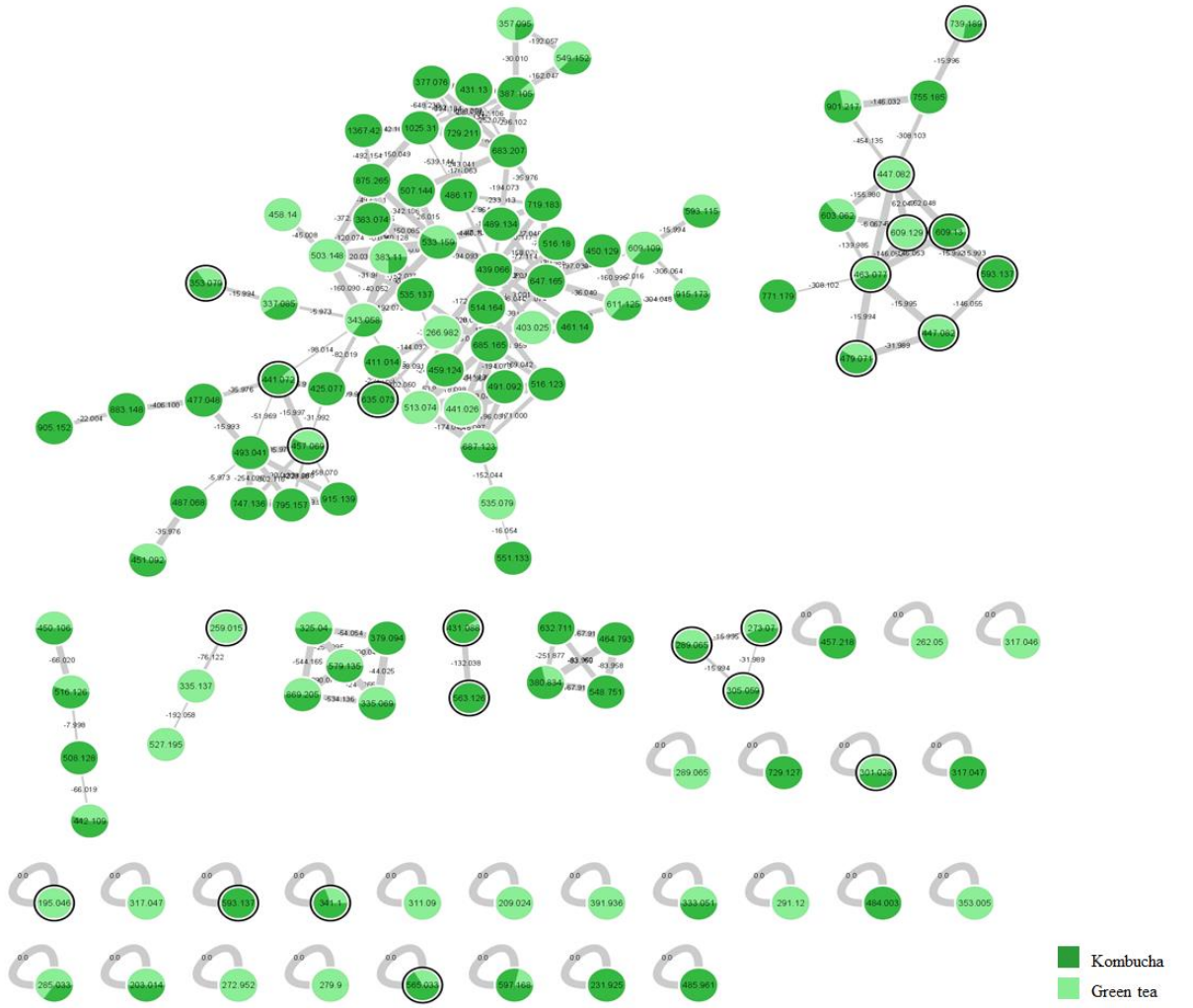


Figure A.2
Molecular Network of the MS/MS spectra obtained by analysis of the control and kombucha samples in the negative mode of ionization. The pie chart within each node corresponds to the percentage relative of the metabolite in the sample as indicated in the legend annotated on the molecular network. The light green ones represents the chemical entities found exclusively in control (*Camellia sinensis* green tea) and the dark green 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 nodes with black bold borders represent the MS/MS that had hit with spectra on the GNPS libraries.

Appendix B – Base Peak Chromatograms from the initial study

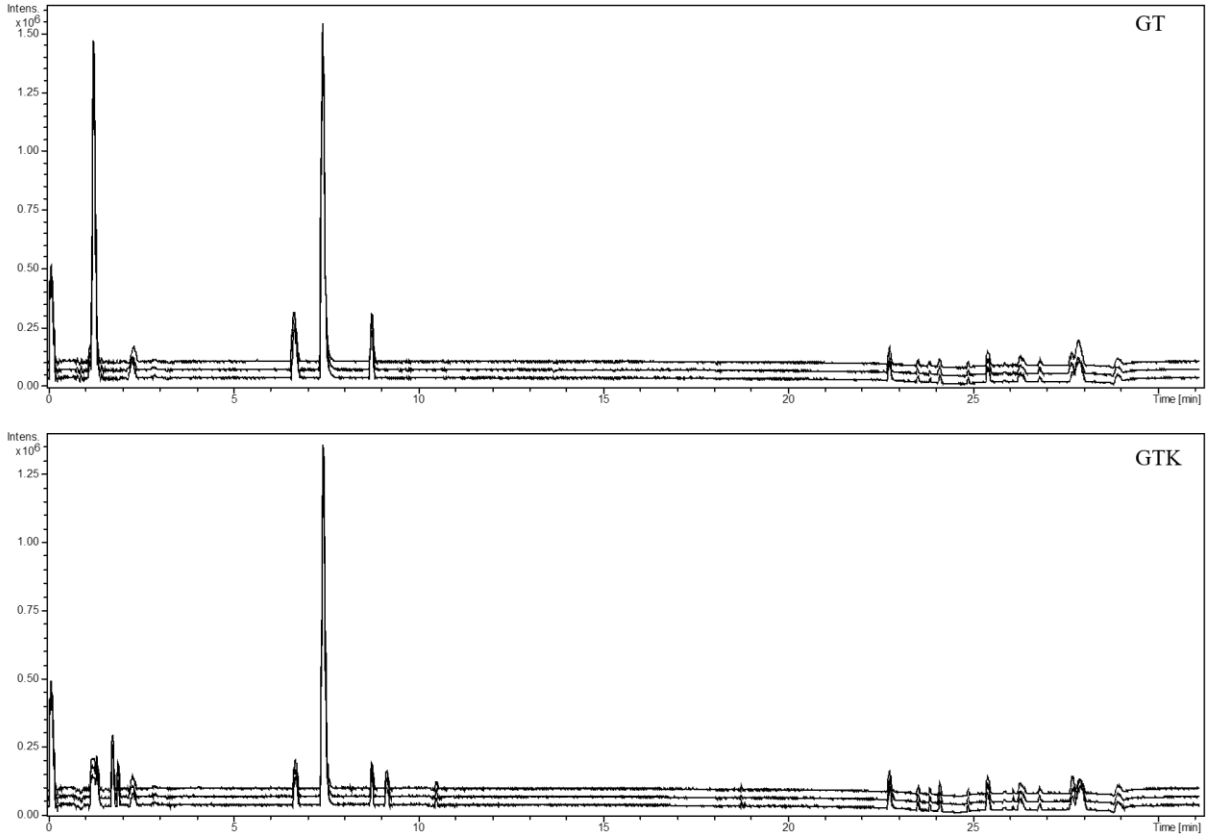


Figure B.1
Overlay of base peak chromatograms (BPC) of fresh *Camellia sinensis* green tea (GT) and kombucha (GTK) after analysis by UHPLC-ESI(+)-MS/MS

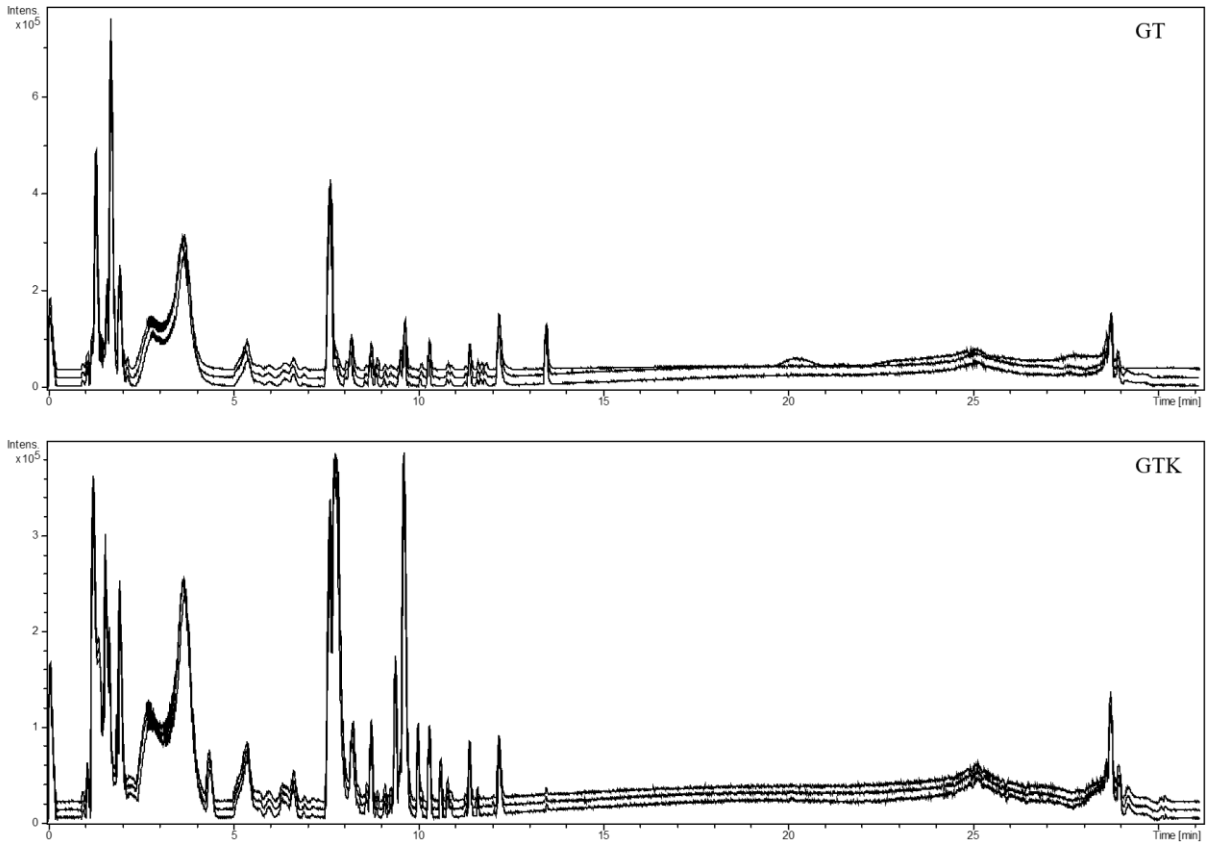


Figure B.2
Overlay of base peak chromatograms (BPC) of fresh *Camellia sinensis* green tea (GT) and kombucha (GTK) after analysis by UHPLC-ESI(-)-MS/MS

Appendix C – Identified metabolites from the initial study

Table C.1
Putatively identified metabolites extracted from *Camellia sinensis* green tea (GT) and kombucha (GTK) using the Molecular Networking tool and MS/MS spectra

Putative Metabolite Identification	Molecular formula	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Mass accuracy (ppm)	Adduct	GTK	GT
Flavonoid							
Afzelechin	$C_{15}H_{14}O_5$	275.0914	275.0923	3.27	$[M+H]^+$	X	X
Kaempferol	$C_{15}H_{10}O_6$	287.0550	287.0556	2.04	$[M+H]^+$	X	X
Eriodictyol	$C_{15}H_{12}O_6$	289.0707	289.0699	-2.65	$[M+H]^+$	X	X
Catechin	$C_{15}H_{14}O_6$	291.0863	291.0869	2.01	$[M+H]^+$	X	X
Quercetin	$C_{15}H_{10}O_7$	303.0499	303.0495	-1.42	$[M+H]^+$	X	X
Epigallocatechin	$C_{15}H_{14}O_7$	307.0812	307.0812	-0.09	$[M+H]^+$	X	X
Catechin gallate	$C_{22}H_{18}O_{10}$	443.0973	443.0963	-2.20	$[M+H]^+$	X	X
Luteolin 4'-glucoside	$C_{21}H_{20}O_{11}$	449.1078	449.1085	1.47	$[M+H]^+$	X	X
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	459.0922	459.0913	-1.93	$[M+H]^+$	X	X
Isoquercitin	$C_{21}H_{20}O_{12}$	465.1028	465.1037	2.04	$[M+H]^+$	X	X
Kaempferol 3-rutinoside	$C_{27}H_{30}O_{15}$	595.1657	595.1652	-0.92	$[M+H]^+$	X	

Putative Metabolite Identification	**Molecular formula**	**Theoretical mass (*m/z*)**	**Measured mass (*m/z*)**	**Mass accuracy (ppm)**	**Adduct**	**GTK**	**GT**
Rutin	$C_{27}H_{30}O_{16}$	611.1607	611.1594	-2.06	$[M+H]^+$	X	X
Unknown compound	$C_{15}H_{10}O_7$	303.0499	303.0510	3.53	$[M+H]^+$	X	X
Afzelechin	$C_{15}H_{14}O_5$	273.0757	273.0758	0.18	$[M-H]^-$	X	X
Kaempferol	$C_{15}H_{10}O_6$	285.0394	285.0398	1.53	$[M-H]^-$	X	X
Catechin	$C_{15}H_{14}O_6$	289.0707	289.0704	-0.92	$[M-H]^-$	X	X
Quercetin	$C_{15}H_{10}O_7$	301.0343	301.0344	0.40	$[M-H]^-$	X	X
Epigallocatechin	$C_{15}H_{14}O_7$	305.0656	305.0660	1.38	$[M-H]^-$	X	X
Epiafzelechin 3-gallate	$C_{22}H_{18}O_9$	425.0867	425.0885	4.21	$[M-H]^-$	X	
Apigenin-8-glucoside	$C_{21}H_{20}O_{10}$	431.0973	431.0968	-1.12	$[M-H]^-$	X	X
Epicatechin gallate	$C_{22}H_{18}O_{10}$	441.0816	441.0829	2.90	$[M-H]^-$	X	X
Catechin 3-gallate	$C_{22}H_{18}O_{10}$	441.0816	441.0829	2.90	$[M-H]^-$	X	
Catechin 5-gallate	$C_{22}H_{18}O_{10}$	441.0816	441.0829	2.90	$[M-H]^-$	X	X
Isoorientin	$C_{21}H_{20}O_{11}$	447.0922	447.0936	3.16	$[M-H]^-$		X
Kaempferol-3-glucoside	$C_{21}H_{20}O_{11}$	447.0922	447.0936	3.16	$[M-H]^-$	X	X
Epigallocatechin 3-p-coumaroate	$C_{24}H_{20}O_9$	451.1024	451.1037	2.97	$[M-H]^-$	X	X
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	457.0765	457.0766	0.14	$[M-H]^-$	X	X

Putative Metabolite Identification	**Molecular formula**	**Theoretical mass (*m/z*)**	**Measured mass (*m/z*)**	**Mass accuracy (ppm)**	**Adduct**	**GTK**	**GT**
Quercetin-3-glucoside	$C_{21}H_{20}O_{12}$	463.0871	463.0872	0.21	$[M-H]^-$	X	X
Myricetin-3-glucoside	$C_{21}H_{20}O_{13}$	479.0820	479.0820	-0.04	$[M-H]^-$	X	X
Proanthocyanidin	$C_{30}H_{26}O_{13}$	593.1290	593.1284	-0.96	$[M-H]^-$	X	X
Kaempferol-rutinoside	$C_{27}H_{30}O_{15}$	593.1501	593.1515	2.37	$[M-H]^-$	X	
Proanthocyanidin	$C_{30}H_{26}O_{14}$	609.1239	609.1227	-1.94	$[M-H]^-$	X	X
Rutin	$C_{27}H_{30}O_{16}$	609.1450	609.1446	-0.67	$[M-H]^-$	X	X
Kaempferol-3-glucoside-2-rhamnoside-7-rhamnoside	$C_{33}H_{40}O_{19}$	739.2080	739.2065	-2.04	$[M-H]^-$	X	X
Quercetin 3-rhamnoside	$C_{33}H_{40}O_{20}$	755.2029	755.2035	0.77	$[M-H]^-$	X	X
Kaempferol 3-sophorotrioside	$C_{33}H_{40}O_{21}$	771.1978	771.1983	0.60	$[M-H]^-$	X	
Assamicain	$C_{44}H_{36}O_{22}$	915.1614	915.1611	-0.38	$[M-H]^-$	X	X
Unknown compound	$C_{12}H_{22}O_{12}$	357.1028	357.1029	0.41	$[M-H]^-$	X	X
Unknown compound	$C_{13}H_{18}N_2O_{11}$	377.0832	377.0850	4.68	$[M-H]^-$	X	
Unknown compound	$C_{13}H_{20}O_{13}$	383.0826	383.0824	-0.43	$[M-H]^-$	X	
Unknown compound	$C_{14}H_{24}O_{12}$	383.1190	383.1189	-0.13	$[M-H]^-$	X	X
Unknown compound	$C_{13}H_{24}O_{13}$	387.1139	387.1138	-0.17	$[M-H]^-$	X	X

Putative Metabolite Identification	Molecular formula	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Mass accuracy (ppm)	Adduct	GTK	GT
Unknown compound	$C_{15}H_{28}O_{14}$	431.1401	431.1398	-0.65	$[M-H]^-$	X	
Unknown compound	$C_{21}H_{25}NO_{10}$	450.1400	450.1394	-1.38	$[M-H]^-$	X	
Unknown compound	$C_{16}H_{28}O_{15}$	459.1350	459.1345	-1.08	$[M-H]^-$	X	
Unknown compound	$C_{28}H_{14}O_{8}$	477.0610	477.0603	-1.56	$[M-H]^-$	X	
Unknown compound	$C_{18}H_{33}NO_{14}$	486.1823	486.1817	-1.19	$[M-H]^-$	X	
Unknown compound	$C_{30}H_{16}O_{7}$	487.0818	487.0798	-4.06	$[M-H]^-$	X	
Unknown compound	$C_{17}H_{30}O_{16}$	489.1456	489.1453	-0.53	$[M-H]^-$	X	
Unknown compound	$C_{19}H_{24}O_{15}$	491.1037	491.1036	-0.19	$[M-H]^-$	X	
Unknown compound	$C_{28}H_{14}O_{9}$	493.0560	493.0537	-4.58	$[M-H]^-$	X	
Unknown compound	$C_{18}H_{32}O_{16}$	503.1612	503.1607	-1.01	$[M-H]^-$		X
Unknown compound	$C_{19}H_{33}NO_{15}$	514.1772	514.1764	-1.54	$[M-H]^-$	X	
Unknown compound	$C_{21}H_{27}NO_{14}$	516.1353	516.1348	-1.03	$[M-H]^-$	X	
Unknown compound	$C_{19}H_{35}NO_{15}$	516.1928	516.1928	-1.63	$[M-H]^-$	X	
Unknown compound	$C_{19}H_{34}O_{17}$	533.1718	533.1716	-0.33	$[M-H]^-$	X	X
Unknown compound	$C_{18}H_{32}O_{18}$	535.1510	535.1502	-1.57	$[M-H]^-$	X	
Unknown compound	$C_{19}H_{34}O_{18}$	549.1667	549.1650	-3.08	$[M-H]^-$	X	X

Putative Metabolite Identification	Molecular formula	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Mass accuracy (ppm)	Adduct	GTK	GT
Unknown compound	$C_{26}H_{28}O_{14}$	563.1402	563.1411	1.64	$[M-H]^-$	X	X
Unknown compound	$C_{30}H_{28}O_{14}$	611.1401	611.1384	-2.75	$[M-H]^-$	X	X
Unknown compound	$C_{26}H_{38}O_{21}$	685.1827	685.1819	-1.22	$[M-H]^-$	X	
Unknown compound	$C_{28}H_{32}O_{20}$	687.1409	687.1396	-1.85	$[M-H]^-$	X	X
Unknown compound	$C_{37}H_{32}O_{17}$	747.1561	747.1552	-1.24	$[M-H]^-$	X	
Other phenylpropanoids and polyketides							
Gallic acid	$C_7H_6O_5$	171.0293	171.0292	-0.87	$[M+H]^+$		X
Phenyllactic acid	$C_9H_{10}O_3$	165.0552	165.0559	4.43	$[M-H]^-$	X	
Gallic acid	$C_7H_6O_5$	169.0137	169.0135	-1.17	$[M-H]^-$	X	X
5-Galloylquinic acid	$C_{14}H_{16}O_{10}$	343.0665	343.0662	-0.94	$[M-H]^-$	X	X
1,3,6-Tri-galloyl-glucopyranose	$C_{27}H_{24}O_{18}$	635.0884	635.0886	0.25	$[M-H]^-$	X	
7-[6-[[3,5-dihydroxy-6-(hydroxymethyl)-4-[3,4,5-trihydroxyoxan-2-yl]oxyoxan-2-yl]oxymethyl]-3,4,5-trihydroxyoxan-2-yl]oxy-6-methoxychromen-2-one	$C_{27}H_{36}O_{18}$	647.1823	647.1812	-1.76	$[M-H]^-$	X	
Unknown compound	$C_{15}H_{28}O_{14}$	431.1401	431.1398	-0.65	$[M-H]^-$	X	

Putative Metabolite Identification	Molecular formula	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Mass accuracy (ppm)	Adduct	GTK	GT
Unknown compound	$C_{26}H_{28}O_{14}$	563.1402	563.1411	1.64	$[M-H]^-$	X	X
Imidazopyrimidines							
Caffeine	$C_8H_{10}N_4O_2$	195.0882	195.0873	-4.62	$[M+H]^+$	X	X
Theobromine	$C_7H_8N_4O_2$	181.0726	181.0726	0.27	$[M+H]^+$	X	X
Aminoacids							
Glutamine	$C_5H_{10}N_2O_3$	147.0770	147.0763	-4.54	$[M+H]^+$		X
Glutamic acid	$C_5H_9NO_4$	148.0610	148.0605	-3.26	$[M+H]^+$		X
Theanine	$C_7H_{14}N_2O_3$	175.1083	175.1079	-2.10	$[M+H]^+$		X
Glutamine	$C_5H_{10}N_2O_3$	145.0613	145.0616	1.95	$[M-H]^-$	X	X
Nucleosides							
UDP-Galactopyranose	$C_{15}H_{24}N_2O_{17}P_2$	565.0472	565.0456	-2.82	$[M-H]^-$	X	X
Organic Acid							

Putative Metabolite Identification	Molecular formula	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Mass accuracy (ppm)	Adduct	GTK	GT
Lactic acid	$C_3H_6O_3$	89.0239	89.0241	2.60	$[M-H]^-$	X	
Malic acid	$C_4H_6O_5$	133.0137	133.0138	0.77	$[M-H]^-$	X	X
Citric acid	$C_6H_8O_7$	191.0192	191.0188	-1.98	$[M-H]^-$	X	X
Lipids and lipid-like molecules							
Glycerophosphocholine	$C_8H_{20}NO_6P$	258.1106	258.1104	-0.96	$[M+H]^+$		X
Unknown compound	$C_{22}H_{43}NO$	338.3423	338.3421	-0.59	$[M+H]^+$	X	X
Citramalic acid	$C_5H_8O_5$	147.0293	147.0297	2.39	$[M-H]^-$	X	
3-Hydroxymethylglutaric acid	$C_6H_{10}O_5$	161.0450	161.0450	0.01	$[M-H]^-$	X	
Glycerophosphoinositol	$C_9H_{19}O_{11}P$	333.0587	333.0588	0.38	$[M-H]^-$	X	X
Carbohydrates and carbohydrates conjugates							
Turanose	$C_{12}H_{22}O_{11}$	325.1135	325.1126	-2.68	$[M+H-H_2O]$	X	X
Sucrose	$C_{12}H_{22}O_{11}$	365.1060	365.1054	-1.59	$[M+Na]^+$	X	
Maltol glucoside	$C_{12}H_{16}O_8$	289.0923	289.0913	-3.60	$[M+H]^+$	X	

Putative Metabolite Identification	Molecular formula	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Mass accuracy (ppm)	Adduct	GTK	GT
Unknown compound	$C_{12}H_{22}O_{11}$	360.1506	360.1495	-3.02	$[M+NH_4]$	X	
Arabitol	$C_5H_{12}O_5$	151.0606	151.0607	0.34	$[M-H]^-$	X	X
Fructose	$C_6H_{12}O_6$	179.0556	179.0554	-0.91	$[M-H]^-$	X	X
Gluconic acid	$C_6H_{12}O_7$	195.0505	195.0504	-0.40	$[M-H]^-$	X	X
Glucuronic acid	$C_6H_{10}O_7$	193.0348	193.0349	0.37	$[M-H]^-$	X	
Glucose 6-phosphate	$C_6H_{13}O_9P$	259.0219	259.0212	-2.68	$[M-H]^-$		X
Trehalose	$C_{12}H_{22}O_{11}$	341.1084	341.1084	0.04	$[M-H]^-$	X	X
Alcohols and polyols							
Quinic acid	$C_7H_{12}O_6$	191.0556	191.0555	-0.33	$[M-H]^-$	X	X
3-O-Coumaroylquinic acid	$C_{16}H_{18}O_8$	337.0923	337.0935	3.43	$[M-H]^-$	X	X
Theogallin	$C_{14}H_{16}O_{10}$	343.0665	343.0662	0.94	$[M-H]^-$	X	X
5-Caffeoylquinic acid	$C_{16}H_{18}O_9$	353.0873	353.0862	-2.99	$[M-H]^-$	X	X
Caffeoylquinic acid isomer	$C_{16}H_{18}O_9$	353.0873	353.0880	2.10	$[M-H]^-$	X	X
Lactones							

Putative Metabolite Identification	**Molecular formula**	**Theoretical mass (*m/z*)**	**Measured mass (*m/z*)**	**Mass accuracy (ppm)**	**Adduct**	**GTK**	**GT**
Saccharic acid-1,4-lactone	$C_6H_8O_7$	191.0192	191.0194	1.16	$[M-H]^-$	X	

Note. GTK = kombucha. GT = *Camellia sinensis* green tea.

Appendix D – Relative abundance of metabolites putatively identified from the initial study

Table D.1
Relative abundance of putatively metabolites identified in Molecular Networking

Compound	GTK	GT
Afzelechin	16804 ± 223,5	**29747 ± 507,6***
Kaempferol	19431 ± 331,2	**110286 ± 1611***
Eriodictyol[a]	7184 ± 210,9	**11406 ± 216,7***
Catechin	317336 ± 2386	**397380 ± 921,6***
Quercetin	73924 ± 677,6	**125291 ± 4760***
Epigallocatechin	130576 ± 1904	**152404 ± 53,25***
Epicatechin gallate	**348030 ± 18567***	9278 ± 317,6
Luteolin 4'-O-glucoside[a]	11304 ± 455,3	12475 ± 368,4
Epigallocatechin gallate	**369355 ± 1510***	46259 ± 575,1
Isoquercitin[a]	21598 ± 331,4	**24162 ± 83,11***
Kaempferol 3-O-rutinoside	**8865 ± 372,2***	ND
Rutin	**157344 ± 144,8***	12864 ± 388,2
Epiafzelechin 3-gallate	**46753 ± 1169***	ND
Apigenin-8-glucoside	**16860 ± 341,2***	11334 ± 208,9

Compound	GTK	GT
Catechin 3-O-gallate	**18056 ± 5421***	ND
Catechin 5-O-gallate	**354060 ± 8288***	7785 ± 142
Isoorientin	ND	**10335 ± 1922***
Kaempferol-3-O-glucoside	57789 ± 25199	64595 ± 1287
Epigallocatechin 3-p-coumaroate	**27738 ± 62,87***	16185 ± 396,4
Quercetin 3-galactoside	56301 ± 1077	**109372 ± 1528***
Myricetin 3-galactoside	**89280 ± 769,1***	57729 ± 1040
Proanthocyanidin (EC or C) – (EGC ou GC)	**30667 ± 429,7***	25687 ± 403
Proanthocyanidin	15107 ± 264,5	13895 ± 486,4
Kaempferol 3-sophorotrioside	**8108 ± 1009***	ND
Assamicain	**247733 ± 21605***	ND
Gallic acid	46121 ± 249	**723439 ± 2880***
Caffeine[a]	1288941 ± 6117	**1480263 ± 13999***
Theobromine[a]	79372 ± 1113	**99616 ± 4297***

Note. Note. GTK = kombucha. GT = *Camellia sinensis* green tea. ND = no detected. [a] Means relative abundance from positive ionization. * Significantly different ($P < 0.05$).

REFERENCES

Acosta-Estrada, B. A., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bound phenolics in foods, a review. *Food Chemistry*, *152*, 46–55. https://doi.org/10.1016/j.foodchem.2013.11.093

Aloulou, A., Hamden, K., Elloumi, D., Ali, M. B., Hargafi, K., Jaouadi, B., Ayadi, F., Elfeki, A., & Ammar, E. (2012). Hypoglycemic and antilipidemic properties of kombucha tea in alloxan-induced diabetic rats. *BMC Complementary and Alternative Medicine*, *12*, 63. https://doi.org/10.1186/1472-6882-12-63

Arora, T., Singh, S., & Sharma, R. K. (2013). Probiotics: Interaction with gut microbiome and antiobesity potential. *Nutrition*, *29*(4), 591–596. https://doi.org/10.1016/j.nut.2012.07.017

Bell, V., Ferrão, J., Pimentel, L., Pintado, M., & Fernandes, T. (2018). One health, fermented foods, and gut microbiota. *Foods*, *7*(12), 195. https://doi.org/10.3390/foods7120195

Bhattacharya, S., Gachhui, R., & Sil, P. C. (2013). Effect of Kombucha, a fermented black tea in attenuating oxidative stress mediated tissue damage in alloxan induced diabetic rats. *Food and chemical toxicology*, 60, 328–340. https://doi.org/10.1016/j.fct.2013.07.051

Cardoso, R. R., Neto, R. O., Dos Santos D'Almeida, C. T., do Nascimento, T. P., Pressete, C. G., Azevedo, L., Martino, H. S. D., Cameron, L. C., Ferreira, M. S. L., & Barros, F. A. R. de. (2020). Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. *Food Research International*, *128*, 108782. https://doi.org/10.1016/j.foodres.2019.108782

Chakravorty, S., Bhattacharya, S., Bhattacharya, D., Sarkar, S., & Gachhui, R. (2019). 10 - Kombucha: A promising functional beverage prepared from tea. In *Non-Alcoholic Beverages* (pp. 285–327). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-815270-6.00010-4

Chakravorty, S., Bhattacharya, S., Chatzinotas, A., Chakraborty, W., Bhattacharya, D., & Gachhui, R. (2016). Kombucha tea fermentation: Microbial and biochemical dynamics. *International Journal of Food Microbiology*, *220*, 63–72. https://doi.org/10.1016/j.ijfoodmicro.2015.12.015

Chan, M., Sy, H., Finley, J., Robertson, J., & Brown, P. N. (2020). Determination of Ethanol Content in Kombucha Using Headspace Gas Chromatography with Mass Spectrometry Detection: Single-Laboratory Validation. *Journal of AOAC International*, *104*(1), 122–128. https://doi.org/10.1093/jaoacint/qsaa094

Chu, S. C., & Chen, C. (2006). Effects of origins and fermentation time on the antioxidant activities of kombucha. *Food Chemistry*, 98(3), 502-507. https://doi.org/10.1016/j.foodchem.2005.05.080

Chu, Y.-F., Chang, W.-H., Black, R. M., Liu, J.-R., Sompol, P., Chen, Y., Wei, H., Zhao, Q., & Cheng, I. H. (2012). Crude caffeine reduces memory impairment and amyloid β(1-42) levels in an Alzheimer's mouse model. *Food Chemistry*, *135*(3), 2095–2102. https://doi.org/10.1016/j.foodchem.2012.04.148

Coelho, R. M. D., Almeida, A. L. de, Amaral, R. Q. G. do, Mota, R. N. da, & Sousa, P. H. M. de. (2020). Kombucha: Review. *International Journal of Gastronomy and Food Science*, *22*, 100272. https://doi.org/10.1016/j.ijgfs.2020.100272

Coton, M., Pawtowski, A., Taminiau, B., Burgaud, G., Deniel, F., Coulloumme-Labarthe, L., Fall, A., Daube, G., & Coton, E. (2017). Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiology Ecology*, *93*(5). https://doi.org/10.1093/femsec/fix048

Cushnie, T. P. T., & Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents*, *38*(2), 99–107. https://doi.org/10.1016/j.ijantimicag.2011.02.014

da Silva, R. R., Wang, M., Nothias, L.-F., van der Hooft, J. J. J., Caraballo-Rodríguez, A. M., Fox, E., Balunas, M. J., Klassen, J. L., Lopes, N. P., & Dorrestein, P. C. (2018). Propagating annotations of molecular networks using in silico fragmentation. *PLoS Computational Biology*, *14*(4), e1006089. https://doi.org/10.1371/journal.pcbi.1006089

Derrien, M., & van Hylckama Vlieg, J. E. T. (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in Microbiology*, *23*(6), 354–366. https://doi.org/10.1016/j.tim.2015.03.002

Dimidi, E., Cox, S. R., Rossi, M., & Whelan, K. (2019). Fermented Foods: definitions and characteristics, impact on the gut microbiota and effects on gastrointestinal health and disease. *Nutrients*, *11*(8). https://doi.org/10.3390/nu11081806

Ernst, M., Kang, K. B., Caraballo-Rodríguez, A. M., Nothias, L.-F., Wandy, J., Chen, C., Wang, M., Rogers, S., Medema, M. H., Dorrestein, P. C., & van der Hooft, J. J. J. (2019). MolNetEnhancer: enhanced molecular networks by integrating metabolome mining and annotation tools. *Metabolites*, *9*(7). https://doi.org/10.3390/metabo9070144

Forsythe, I. J., & Wishart, D. S. (2009). Exploring human metabolites using the human metabolome database. *Current Protocols in Bioinformatics*, *25*(1), 14-8. https://doi.org/10.1002/0471250953.bi1408s25

Fox, J. E., Starcevic, M., Kow, K. Y., Burow, M. E., & McLachlan, J. A. (2001). Endocrine disrupters and flavonoid signalling. *Nature*, *413*(6852), 128–129. https://doi.org/10.1038/35093163

Gauglitz, J. M., Aceves, C. M., Aksenov, A. A., Aleti, G., Almaliti, J., Bouslimani, A., Brown, E. A., Campeau, A., Caraballo-Rodríguez, A. M., Chaar, R., da Silva, R. R., Demko, A. M., Di Ottavio, F., Elijah, E., Ernst, M., Ferguson, L. P., Holmes, X., Jarmusch, A. K., Jiang, L., … Dorrestein, P. C. (2020). Untargeted mass spectrometry-based metabolomics approach unveils molecular changes in raw and processed foods and beverages. *Food Chemistry*, *302*, 125290. https://doi.org/10.1016/j.foodchem.2019.125290

Grand View Research. (2020). *Kombucha Market Size Worth $7.05 Billion By 2027*. Retrieved from https://www.grandviewresearch.com/press-release/global-kombucha-market. Accessed July 29, 2021

Gopal, J., Muthu, M., Paul, D., Kim, D.-H., & Chun, S. (2016). Bactericidal activity of green tea extracts: the importance of catechin containing nano particles. *Scientific Reports*, *6*, 19710. https://doi.org/10.1038/srep19710

Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., Oda, Y., Kakazu, Y., Kusano, M., Tohge, T., Matsuda, F., Sawada, Y., Hirai, M. Y., Nakanishi, H., Ikeda, K., … Nishioka, T. (2010). MassBank: a public repository for sharing mass spectral data for life sciences. *Journal of Mass Spectrometry: JMS*, *45*(7), 703–714. https://doi.org/10.1002/jms.1777

Huynh, N. T., Van Camp, J., Smagghe, G., & Raes, K. (2014). Improved release and metabolism of flavonoids by steered fermentation processes: a review. *International Journal of Molecular Sciences*, *15*(11), 19369–19388. https://doi.org/10.3390/ijms151119369

Ivanišová, E., Meňhartová, K., Terentjeva, M., Godočíková, L., Árvay, J., & Kačániová, M. (2019). Kombucha tea beverage: Microbiological characteristic, antioxidant activity, and phytochemical composition. *Acta Alimentaria*, *48*(3), 324–331. https://doi.org/10.1556/066.2019.48.3.7

Jayabalan, R., Malbaša, R. V., & Sathishkumar, M. (2016). Kombucha. https://doi.org/10.1016/B978-0-08-100596-5.03032-8

Jayabalan, R., Marimuthu, S., & Swaminathan, K. (2007). Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chemistry*, *102*(1), 392–398. https://doi.org/10.1016/j.foodchem.2006.05.032

Jayabalan, R., Subathradevi, P., Marimuthu, S., Sathishkumar, M., & Swaminathan, K. (2008). Changes in free-radical scavenging ability of kombucha tea during fermentation. *Food Chemistry*, *109*(1), 227–234. https://doi.org/10.1016/j.foodchem.2007.12.037

Jayabalan, R., Malbaša, R. V., Lončar, E. S., Vitas, J. S., & Sathishkumar, M. (2014). A Review on Kombucha Tea-Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), 538–550. https://doi.org/10.1111/1541-4337.12073

Jia, M., Chen, L., Xin, H.-L., Zheng, C.-J., Rahman, K., Han, T., & Qin, L.-P. (2016). A Friendly Relationship between Endophytic Fungi and Medicinal Plants: A Systematic Review. *Frontiers in Microbiology*, *7*, 906. https://doi.org/10.3389/fmicb.2016.00906

Kallel, L., Desseaux, V., Hamdi, M., Stocker, P., & Ajandouz, E. H. (2012). Insights into the fermentation biochemistry of Kombucha teas and potential impacts of Kombucha drinking on starch digestion. *Food Research International*, *49*(1), 226–232. https://doi.org/10.1016/j.foodres.2012.08.018

Kim, H. P., Son, K. H., Chang, H. W., & Kang, S. S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *Journal of Pharmacological Sciences*, *96*(3), 229–245. https://doi.org/10.1254/jphs.crj04003x

Kootstra, A. (1994). Protection from UV-B-induced DNA damage by flavonoids. *Plant Molecular Biology*, *26*(2), 771–774. https://doi.org/10.1007/BF00013762

Laatikainen, R., Koskenpato, J., Hongisto, S.-M., Loponen, J., Poussa, T., Hillilä, M., & Korpela, R. (2016). Randomised clinical trial: low-FODMAP rye bread vs. regular rye bread to relieve the symptoms of irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics*, *44*(5), 460–470. https://doi.org/10.1111/apt.13726

Le Roy, J., Huss, B., Creach, A., Hawkins, S., & Neutelings, G. (2016). Glycosylation Is a Major Regulator of Phenylpropanoid Availability and Biological Activity in Plants. *Frontiers in Plant Science*, *7*, 735. https://doi.org/10.3389/fpls.2016.00735

Lončar, E., Djurić, M., Malbaša, R., Kolarov, L. J., & Klašnja, M. (2006). Influence of Working Conditions Upon Kombucha Conducted Fermentation of Black Tea. *Food and Bioproducts Processing*, *84*(3), 186–192. https://doi.org/10.1205/fbp.04306

Martínez-Leal, J., Valenzuela Suárez, L., Jayabalan, R., Huerta Oros, J., & Escalante-Aburto, A. (2018). A review on health benefits of kombucha nutritional compounds and metabolites. *CyTA - Journal of Food*, *16*(1), 390–399. https://doi.org/10.1080/19476337.2017.1410499

May, A., Narayanan, S., Alcock, J., Varsani, A., Maley, C., & Aktipis, A. (2019). Kombucha: a novel model system for cooperation and conflict in a complex multi-species microbial ecosystem. *PeerJ*, *7*, e7565. https://doi.org/10.7717/peerj.7565

McGovern, P. E., Zhang, J., Tang, J., Zhang, Z., Hall, G. R., Moreau, R. A., Nuñez, A., Butrym, E. D., Richards, M. P., Wang, C.-S., Cheng, G., Zhao, Z., & Wang, C. (2004). Fermented beverages of pre- and proto-historic China. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(51), 17593–17598. https://doi.org/10.1073/pnas.0407921102

Muthumani, T., & Kumar, R. S. S. (2007). Influence of fermentation time on the development of compounds responsible for quality in black tea. *Food Chemistry*, *101*(1), 98–102. https://doi.org/10.1016/j.foodchem.2006.01.008

Nguyen, N. K., Dong, N. T. N., Nguyen, H. T., & Le, P. H. (2015). Lactic acid bacteria: promising supplements for enhancing the biological activities of kombucha. *SpringerPlus*, *4*, 91. https://doi.org/10.1186/s40064-015-0872-3

Nguyen, N. K., Nguyen, P. B., Nguyen, H. T., & Le, P. H. (2015). Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. *LWT - Food Science and Technology*, *64*(2), 1149–1155. https://doi.org/10.1016/j.lwt.2015.07.018

Pandey, R. P., Parajuli, P., Koffas, M. A., & Sohng, J. K. (2016). Microbial production of natural and non-natural flavonoids: pathway engineering, directed evolution and systems/synthetic biology. *Biotechnology advances*, *34*(5), 634-662.

Pessione, E., & Cirrincione, S. (2016). Bioactive Molecules Released in Food by Lactic Acid Bacteria: Encrypted Peptides and Biogenic Amines. *Frontiers in Microbiology*, *7*, 876. https://doi.org/10.3389/fmicb.2016.00876

Ryngajłło, M., Jacek, P., Cielecka, I., Kalinowska, H., & Bielecki, S. (2019). Effect of ethanol supplementation on the transcriptional landscape of bionanocellulose producer Komagataeibacter xylinus E25. *Applied Microbiology and Biotechnology*, *103*(16), 6673–6688. https://doi.org/10.1007/s00253-019-09904-x

Sawada, Y., Nakabayashi, R., Yamada, Y., Suzuki, M., Sato, M., Sakata, A., Akiyama, K., Sakurai, T., Matsuda, F., Aoki, T., Hirai, M. Y., & Saito, K. (2012). RIKEN tandem mass spectral database (ReSpect) for phytochemicals: a plant-specific MS/MS-based data resource and database. *Phytochemistry*, *82*, 38–45. https://doi.org/10.1016/j.phytochem.2012.07.007

Şanlier, N., Gökcen, B. B., & Sezgin, A. C. (2019). Health benefits of fermented foods. *Critical Reviews in Food Science and Nutrition*, *59*(3), 506–527. https://doi.org/10.1080/10408398.2017.1383355

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., & Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research*, *13*(11), 2498–2504. https://doi.org/10.1101/gr.1239303

Shetty, K., & Sarkar, D. (Eds.). (2020). *Functional Foods and Biotechnology: Biotransformation and Analysis of Functional Foods and Ingredients*. CRC Press.

Shimizu, K. (2013). Metabolic regulation by global regulators in response to culture environment. In *Bacterial Cellular Metabolic Systems* (pp. 95–213). https://doi.org/10.1533/9781908818201.95

Sinija, V. R., & Mishra, H. N. (2008). Green tea: Health benefits. *Journal of Nutritional & Environmental Medicine*, *17*(4), 232–242. https://doi.org/10.1080/13590840802518785

Sinir, G. Ö., Tamer, C. E., & Suna, S. (2019). Kombucha tea: A promising fermented functional beverage. In *Fermented Beverages* (pp. 401-432). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-815271-3.00010-5

Sordon, S., Popłoński, J., & Huszcza, E. (2016). Microbial Glycosylation of Flavonoids. *Polish Journal of Microbiology*, *65*(2), 137–151. https://doi.org/10.5604/17331331.1204473

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., Fan, T. W.-M., Fiehn, O., Goodacre, R., Griffin, J. L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A. N., Lindon, J. C., Marriott, P., Nicholls, A. W., … Viant, M. R.

(2007). Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics: Official Journal of the Metabolomic Society*, *3*(3), 211–221. https://doi.org/10.1007/s11306-007-0082-2

Tamime, A. Y. (2002). Fermented milks: a historical food with modern applications--a review. *European Journal of Clinical Nutrition*, *56*(4), S2–S15. https://doi.org/10.1038/sj.ejcn.1601657

Tan, J., Dai, W., Lu, M., Lv, H., Guo, L., Zhang, Y., Zhu, Y., Peng, Q., & Lin, Z. (2016). Study of the dynamic changes in the non-volatile chemical constituents of black tea during fermentation processing by a non-targeted metabolomics approach. *Food Research International*, *79*, 106–113. https://doi.org/10.1016/j.foodres.2015.11.018

Tounekti, T., Joubert, E., Hernández, I., & Munné-Bosch, S. (2013). Improving the Polyphenol Content of Tea. *Critical Reviews in Plant Sciences*, *32*(3), 192–215. https://doi.org/10.1080/07352689.2012.747384

Tu, C., Tang, S., Azi, F., Hu, W., & Dong, M. (2019). Use of kombucha consortium to transform soy whey into a novel functional beverage. *Journal of Functional Foods*, *52*, 81–89. https://doi.org/10.1016/j.jff.2018.10.024

Vegge, C. S., Jansen van Rensburg, M. J., Rasmussen, J. J., Maiden, M. C. J., Johnsen, L. G., Danielsen, M., MacIntyre, S., Ingmer, H., & Kelly, D. J. (2016). Glucose Metabolism via the Entner-Doudoroff Pathway in Campylobacter: A Rare Trait that Enhances Survival and Promotes Biofilm Formation in Some Isolates. *Frontiers in Microbiology*, *7*, 1877. https://doi.org/10.3389/fmicb.2016.01877

Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J.-P., Renard, T., Rollan, S., & Taillandier, P. (2019). Impact of fermentation conditions on the production of bioactive compounds with anticancer, anti-inflammatory and antioxidant properties in kombucha tea extracts. *Process Biochemistry*, *83*, 44–54. https://doi.org/10.1016/j.procbio.2019.05.004

Villarreal-Soto, S. A., Bouajila, J., Pace, M., Leech, J., Cotter, P. D., Souchard, J.-P., Taillandier, P., & Beaufort, S. (2020). Metabolome-microbiome signatures in the fermented beverage, Kombucha. *International Journal of Food Microbiology*, *333*, 108778. https://doi.org/10.1016/j.ijfoodmicro.2020.108778

Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J.-P., & Taillandier, P. (2018). Understanding Kombucha Tea Fermentation: A Review. *Journal of Food Science*, *83*(3), 580–588. https://doi.org/10.1111/1750-3841.14068

Vitas, J. S., Cvetanović, A. D., Mašković, P. Z., Švarc-Gajić, J. V., & Malbaša, R. V. (2018). Chemical composition and biological activity of novel types of kombucha beverages with yarrow. *Journal of Functional Foods*, *44*, 95–102. https://doi.org/10.1016/j.jff.2018.02.019

Vázquez-Cabral, B. D., Larrosa-Pérez, M., Gallegos-Infante, J. A., Moreno-Jiménez, M. R., González-Laredo, R. F., Rutiaga-Quiñones, J. G., Gamboa-Gómez, C. I., & Rocha-Guzmán, N. E. (2017). Oak kombucha protects against oxidative stress and inflammatory processes. *Chemico-Biological Interactions*, *272*, 1–9. https://doi.org/10.1016/j.cbi.2017.05.001

Wandy, J., Zhu, Y., van der Hooft, J. J. J., Daly, R., Barrett, M. P., & Rogers, S. (2018). Ms2lda.org: web-based topic modelling for substructure discovery in mass spectrometry. *Bioinformatics*, *34*(2), 317–318. https://doi.org/10.1093/bioinformatics/btx582

Wang, L., Luo, Y., Wu, Y., Liu, Y., & Wu, Z. (2018). Fermentation and complex enzyme hydrolysis for improving the total soluble phenolic contents, flavonoid aglycones contents and bio-activities of guava leaves tea. *Food Chemistry*, *264*, 189–198. https://doi.org/10.1016/j.foodchem.2018.05.035

Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., Nguyen, D. D., Watrous, J., Kapono, C. A., Luzzatto-Knaan, T., Porto, C., Bouslimani, A., Melnik, A. V., Meehan, M. J., Liu, W.-T., Crüsemann, M., Boudreau, P. D., Esquenazi, E., Sandoval-Calderón, M., … Bandeira, N. (2016). Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology*, *34*(8), 828–837. https://doi.org/10.1038/nbt.3597

Wang, X., Wan, X., Hu, S., & Pan, C. (2008). Study on the increase mechanism of the caffeine content during the fermentation of tea with microorganisms. *Food Chemistry*, *107*(3), 1086–1091. https://doi.org/10.1016/j.foodchem.2007.09.023

Wastyk, H. C., Fragiadakis, G. K., Perelman, D., Dahan, D., Merrill, B. D., Yu, F. B., Topf, M., Gonzalez, C. G., Van Treuren, W., Han, S., Robinson, J. L., Elias, J. E., Sonnenburg, E. D., Gardner, C. D., & Sonnenburg, J. L. (2021). Gut-microbiota-targeted diets modulate human immune status. *Cell*. https://doi.org/10.1016/j.cell.2021.06.019

Wilburn, J. R., & Ryan, E. P. (2017). Fermented Foods in Health Promotion and Disease Prevention: An Overview. *Fermented Foods in Health and Disease Prevention*, 3–19. Academic Press. https://doi.org/10.1016/B978-0-12-802309-9.00001-7

Winkel-Shirley, B. (2001). Flavonoid Biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiology*, *126*(2), 485–493. https://doi.org/10.1104/pp.126.2.485

Xiao, J., Chen, T., & Cao, H. (2014). WITHDRAWN: Flavonoid glycosylation and biological benefits. *Biotechnology Advances*. https://doi.org/10.1016/j.biotechadv.2014.05.004

Yang, C., Hu, Z., Lu, M., Li, P., Tan, J., Chen, M., Lv, H., Zhu, Y., Zhang, Y., Guo, L., Peng, Q., Dai, W., & Lin, Z. (2018). Application of metabolomics profiling in the analysis of metabolites and taste quality in different subtypes of white tea. *Food Research International*, *106*, 909–919. https://doi.org/10.1016/j.foodres.2018.01.069

Yang, J. Y., Sanchez, L. M., Rath, C. M., Liu, X., Boudreau, P. D., Bruns, N., Glukhov, E., Wodtke, A., de Felicio, R., Fenner, A., Wong, W. R., Linington, R. G., Zhang, L., Debonsi, H. M., Gerwick, W. H., & Dorrestein, P. C. (2013). Molecular networking as a dereplication strategy. *Journal of Natural Products*, *76*(9), 1686–1699. https://doi.org/10.1021/np400413s

Yildiz, E., Guldas, M., & Gurbuz, O. (2020). Determination of in-vitro phenolics, antioxidant capacity and bio-accessibility of Kombucha tea produced from black carrot varieties grown in Turkey. *Food Science and Technology*, *41*(1), 180–187. https://doi.org/10.1590/fst.00320

Zhang, J., Van Mullem, J., Dias, D. R., & Schwan, R. F. (2021). The chemistry and sensory characteristics of new herbal tea-based kombuchas. *Journal of Food Science*, *86*(3), 740–748. https://doi.org/10.1111/1750-3841.15613

Zhang, W., Wang, X., Qi, X., Ren, L., & Qiang, T. (2018). Isolation and identification of a bacterial cellulose synthesizing strain from kombucha in different conditions: Gluconacetobacter xylinus ZHCJ618. *Food Science and Biotechnology*, *27*(3), 705–713. https://doi.org/10.1007/s10068-018-0303-7

Zhou, B.-X., Ma, C., Xia, T., Li, X., Zheng, C., Wu, T., & Liu, X. (2019). Isolation, characterization and application of theophylline-degrading Aspergillus fungi. *Microbial cell factories*, *19*(1), 1-13. https://doi.org/10.21203/rs.2.18212/v1

Zhu, J., Xu, Q., Zhao, S., Xia, X., Yan, X., An, Y., Mi, X., Guo, L., Samarina, L., & Wei, C. (2020). Comprehensive co-expression analysis provides novel insights into temporal variation of flavonoids in fresh leaves of the tea plant (*Camellia sinensis*). *Plant Science: An International Journal of Experimental Plant Biology*, *290*, 110306. https://doi.org/10.1016/j.plantsci.2019.110306

FIGURES

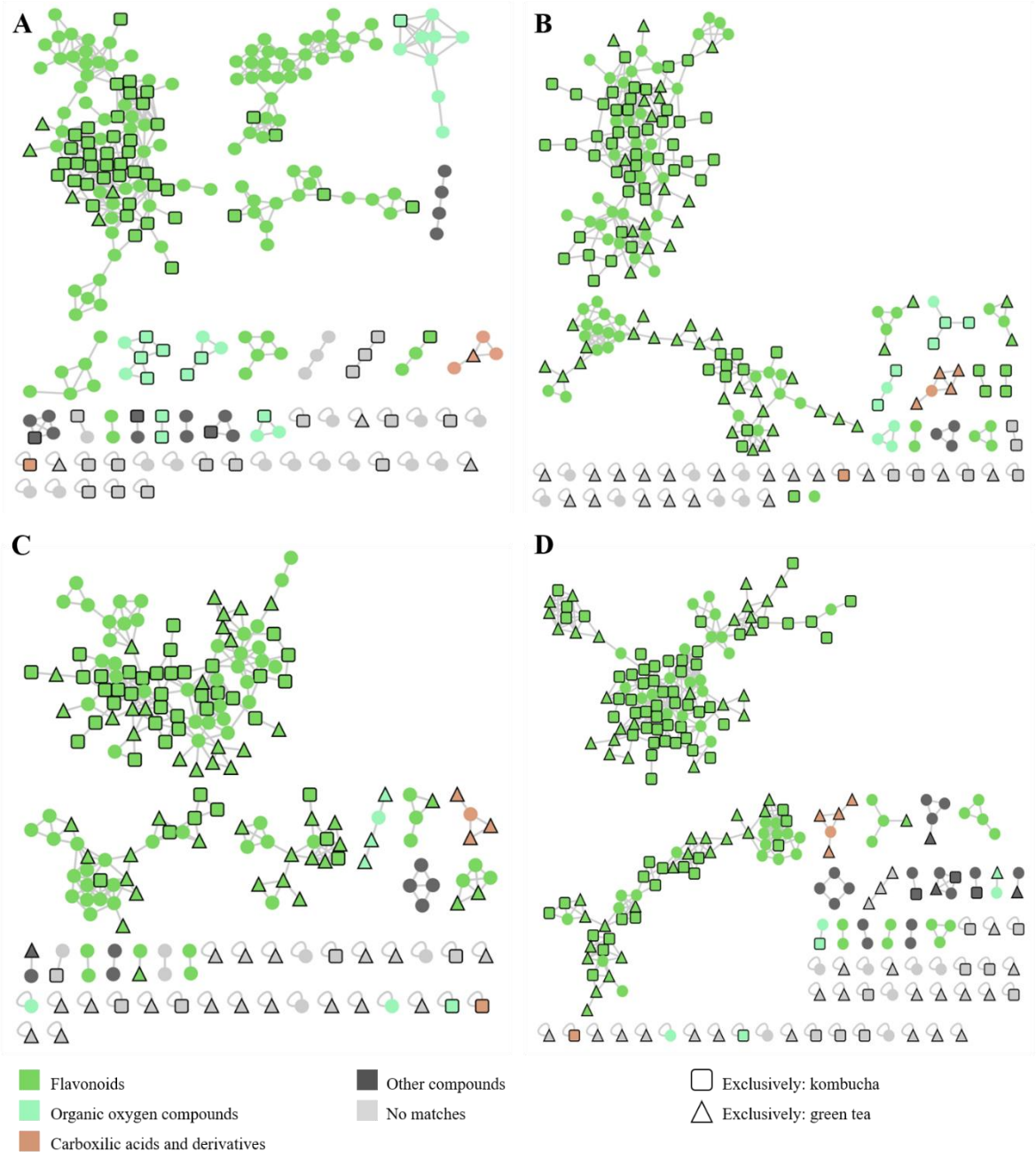


Figure 1
Molecular Network of the MS/MS spectra obtained by analysis of the control and kombucha samples from treatments A, B, C and D, (1A, 1B, 1C and 1D, respectively) colored by chemical classes terms selected as a indicated in the legend annotated on the molecular network using the MolNetEnhancer.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

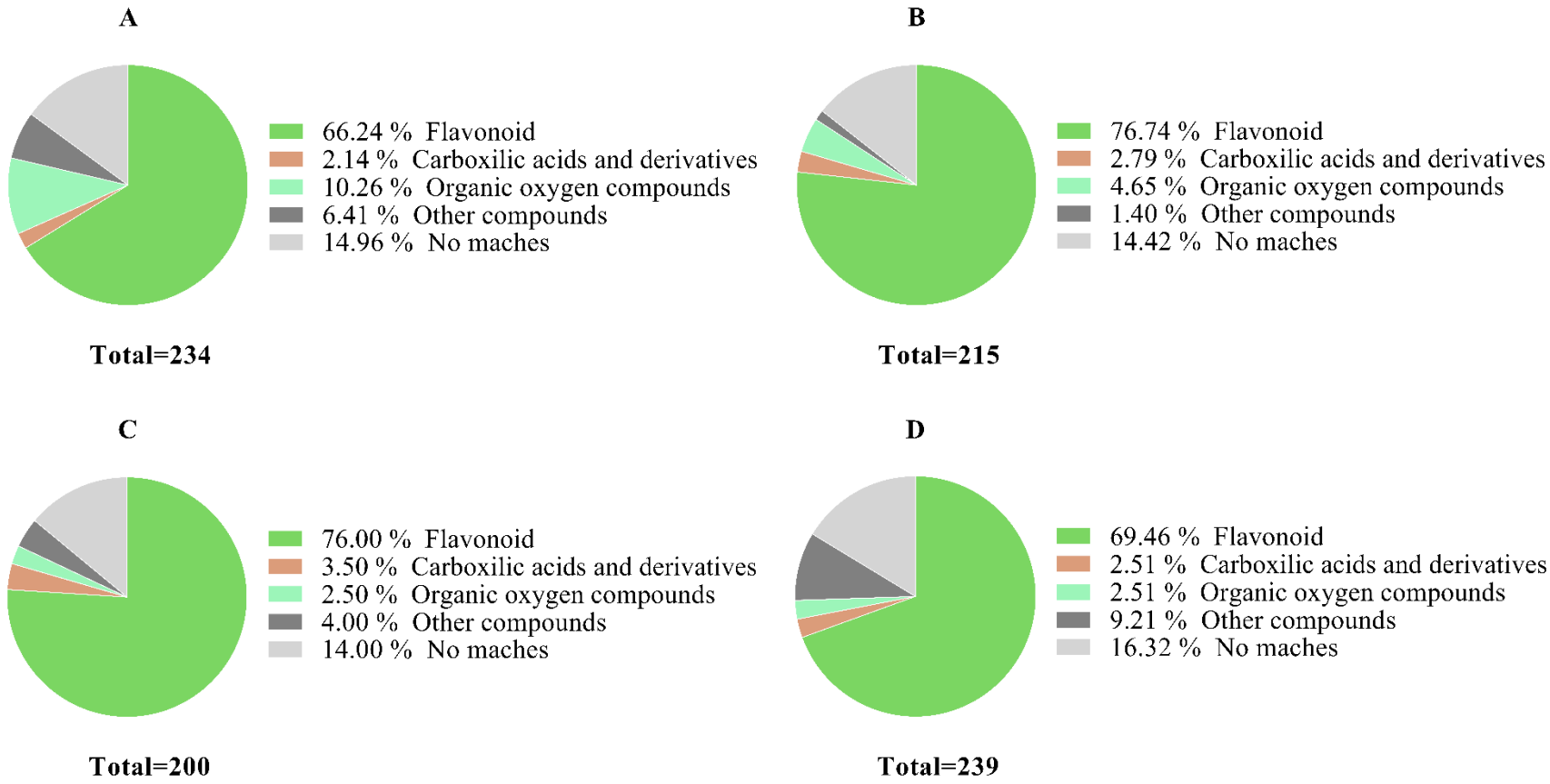


Figure 2
Pie chart obtained of Molecular Network of the MS/MS spectra obtained by analysis of the kombucha samples from experiments A, B, C and D, (1A, 1B, 1C and 1D, respectively) colored by chemical classes terms selected as a indicated in the legend annotated on the molecular network using the MolNetEnhancer. The chemical entities detected only in the control analyses were not considered for the total percentage shown in these images

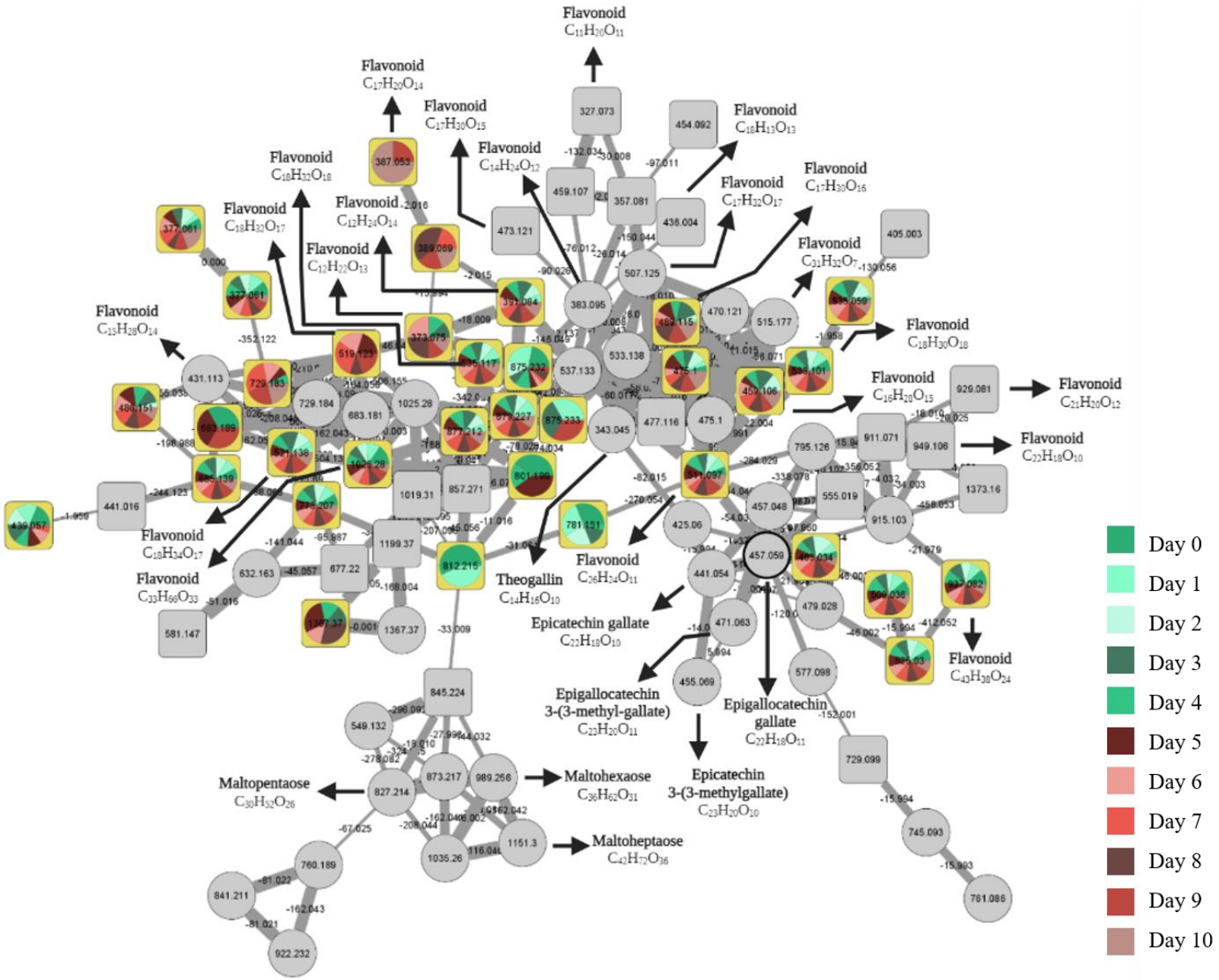
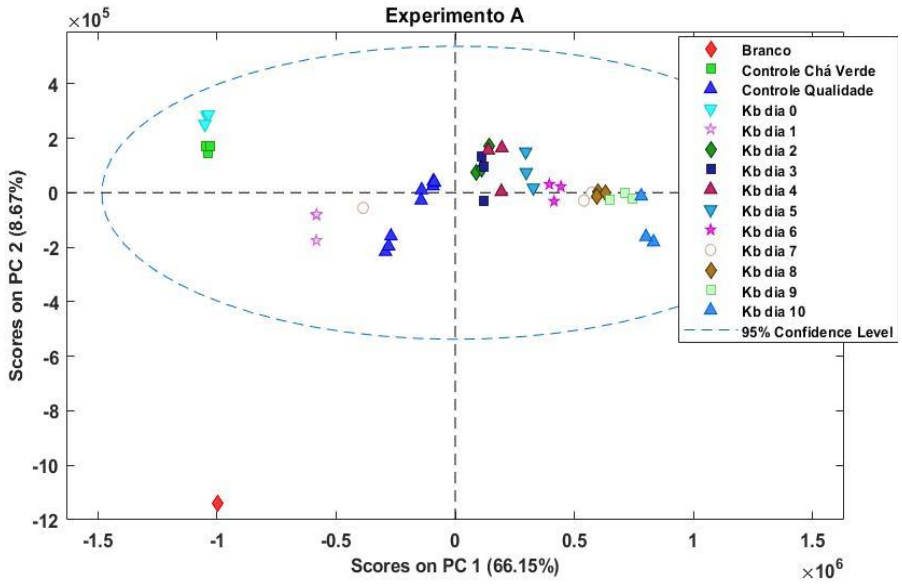
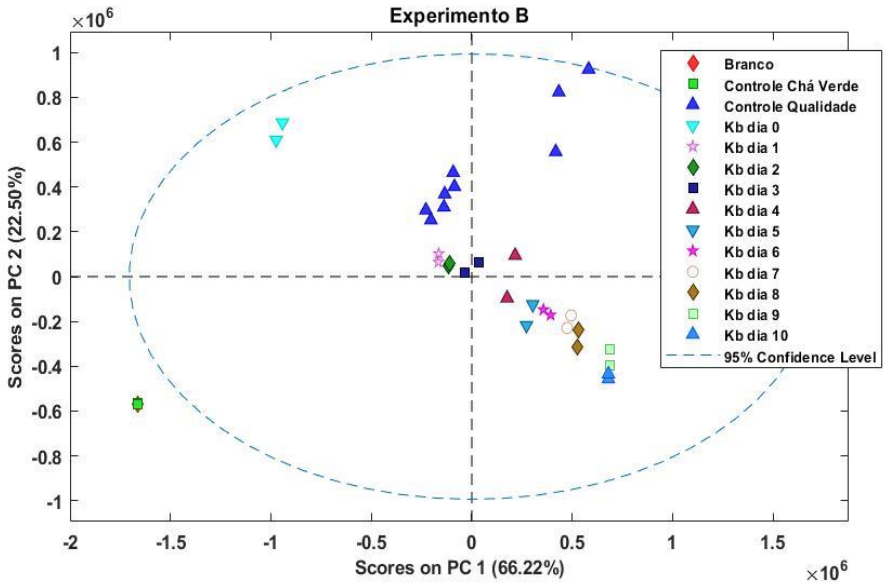
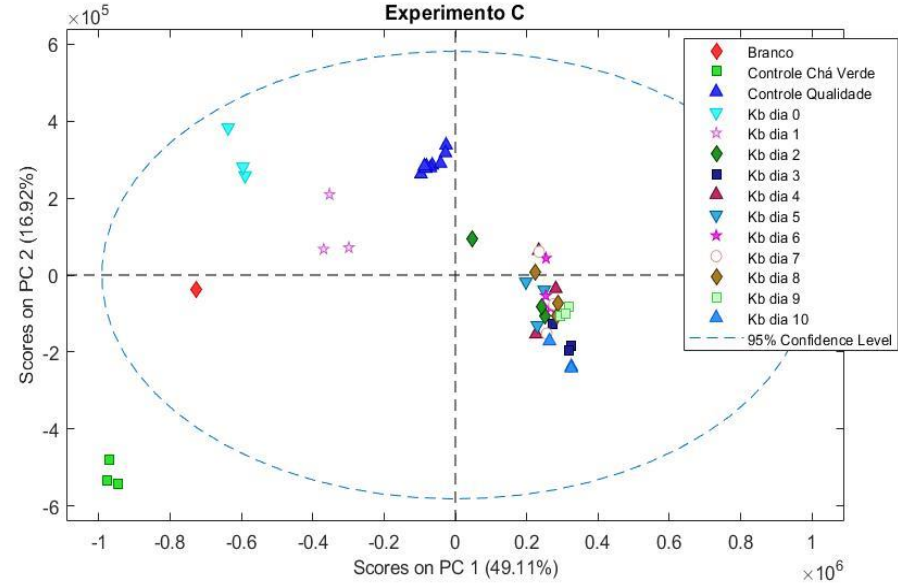

Flavonoid
C11H20O11
Flavonoid
C17H20O14
Flavonoid
C17H30O15
Flavonoid
C14H24O12
Flavonoid
C18H32O18
Flavonoid
C18H32O17
Flavonoid
C12H24O14
Flavonoid
C12H22O13
Flavonoid
C15H28O14
Flavonoid
C18H34O17
Flavonoid
C33H66O33
Flavonoid
C18H13O13
Flavonoid
C17H32O17
Flavonoid
C17H30O16
Flavonoid
C31H32O7
Flavonoid
C18H30O18
Flavonoid
C16H28O15
Flavonoid
C21H20O12
Flavonoid
C22H18O10
Flavonoid
C43H38O24
Theogallin
C14H18O10
Flavonoid
C26H24O11
Epicatechin gallate
C22H18O10
Epigallocatechin
3-(3-methyl-gallate)
C23H20O11
Epigallocatechin
gallate
C22H18O11
Epicatechin
3-(3-methylgallate)
C23H20O10
Maltopentaose
C30H52O26
Maltohexaose
C36H62O31
Maltoheptaose
C42H72O36
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10

Figure 3
Cluster of flavonoid 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 represents 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.


Experimento A
×10^5
Scores on PC 2 (8.67%)
Scores on PC 1 (66.15%)
×10^6
Branco
Controle Chá Verde
Controle Qualidade
Kb dia 0
Kb dia 1
Kb dia 2
Kb dia 3
Kb dia 4
Kb dia 5
Kb dia 6
Kb dia 7
Kb dia 8
Kb dia 9
Kb dia 10
95% Confidence Level


Experimento B
×10^6
Scores on PC 2 (22.50%)
Scores on PC 1 (66.22%)
×10^6
Branco
Controle Chá Verde
Controle Qualidade
Kb dia 0
Kb dia 1
Kb dia 2
Kb dia 3
Kb dia 4
Kb dia 5
Kb dia 6
Kb dia 7
Kb dia 8
Kb dia 9
Kb dia 10
95% Confidence Level


Experimento C
×10^5
Scores on PC 2 (16.92%)
Scores on PC 1 (49.11%)
×10^6
Branco
Controle Chá Verde
Controle Qualidade
Kb dia 0
Kb dia 1
Kb dia 2
Kb dia 3
Kb dia 4
Kb dia 5
Kb dia 6
Kb dia 7
Kb dia 8
Kb dia 9
Kb dia 10
95% Confidence Level

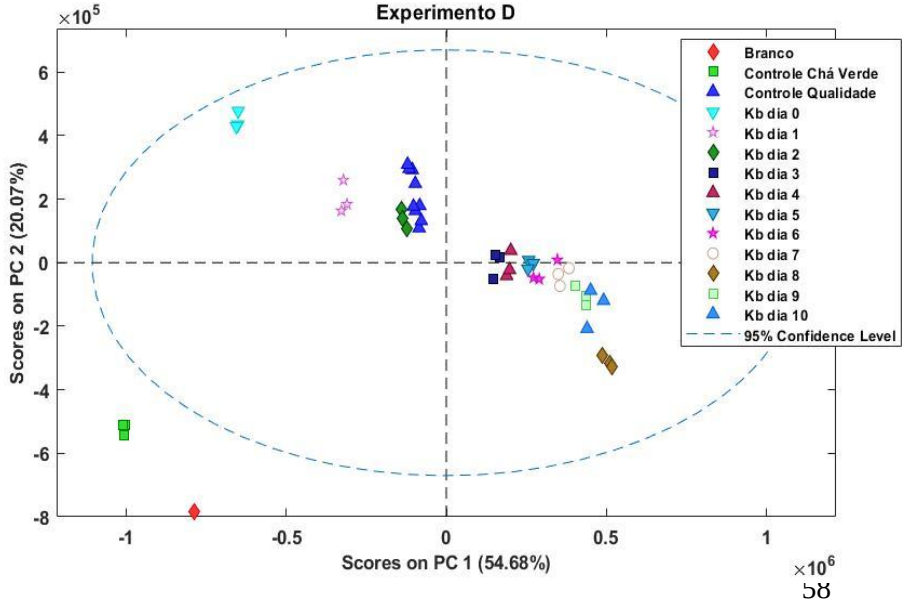

Experimento D
×10^5
Scores on PC 2 (20.07%)
Scores on PC 1 (54.68%)
×10^6
Branco
Controle Chá Verde
Controle Qualidade
Kb dia 0
Kb dia 1
Kb dia 2
Kb dia 3
Kb dia 4
Kb dia 5
Kb dia 6
Kb dia 7
Kb dia 8
Kb dia 9
Kb dia 10
95% Confidence Level

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

TABLE

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

					Treatments				
Putative Metabolite Identification	**Molecular Formula**	**$[M - H]^-$ Theoretical**	**$[M - H]^-$ Measured**	**Mass accuracy (ppm)**	**Control**	**A (20 ºC; 5 ºBrix)**	**B (20 ºC; 10 ºBrix)**	**C (30 ºC; 5 ºBrix)**	**D (30 ºC; 10 ºBrix)**
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	

					Treatments				
Putative Metabolite Identification	**Molecular Formula**	**$[M - H]^-$ Theoretical**	**$[M - H]^-$ Measured**	**Mass accuracy (ppm)**	**Control**	**A (20 ºC; 5 ºBrix)**	**B (20 ºC; 10 ºBrix)**	**C (30 ºC; 5 ºBrix)**	**D (30 ºC; 10 ºBrix)**
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		

					Treatments				
Putative Metabolite Identification	**Molecular Formula**	**$[M - H]^-$ Theoretical**	**$[M - H]^-$ Measured**	**Mass accuracy (ppm)**	**Control**	**A (20 ºC; 5 ºBrix)**	**B (20 ºC; 10 ºBrix)**	**C (30 ºC; 5 ºBrix)**	**D (30 ºC; 10 ºBrix)**
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		

					Treatments				
Putative Metabolite Identification	**Molecular Formula**	**$[M - H]^-$ Theoretical**	**$[M - H]^-$ Measured**	**Mass accuracy (ppm)**	**Control**	**A (20 ºC; 5 ºBrix)**	**B (20 ºC; 10 ºBrix)**	**C (30 ºC; 5 ºBrix)**	**D (30 ºC; 10 ºBrix)**
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	$C_{6}H_{10}O_{7}$	193.0343	193.0354	5.81	X	X	X	X	X

					Treatments				
Putative Metabolite Identification	**Molecular Formula**	**$[M - H]^-$ Theoretical**	**$[M - H]^-$ Measured**	**Mass accuracy (ppm)**	**Control**	**A (20 ºC; 5 ºBrix)**	**B (20 ºC; 10 ºBrix)**	**C (30 ºC; 5 ºBrix)**	**D (30 ºC; 10 ºBrix)**
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
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			

					Treatments				
Putative Metabolite Identification	**Molecular Formula**	**$[M - H]^-$ Theoretical**	**$[M - H]^-$ Measured**	**Mass accuracy (ppm)**	**Control**	**A (20 ºC; 5 ºBrix)**	**B (20 ºC; 10 ºBrix)**	**C (30 ºC; 5 ºBrix)**	**D (30 ºC; 10 ºBrix)**
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