Etude d'un procédé de fermentation de Kombucha : caractérisation chimique, microbienne et activités biologiques

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Résumé

Le Kombucha est une boisson fermentée symbiotique. Il s'agit d'un thé traditionnel fermenté par un consortium de plusieurs bactéries (*Komagataeibacter* sp, *Gluconacetobacter* sp, *Gluconobacter* sp, *Acetobacter* sp…) et levures (*Brettanomyces* sp, *Schizosaccharomyces* sp, *Zygosaccharomyces* sp…) dont la fermentation conduit à la production de métabolites d'intérêt. La conception d'un procédé de fermentation optimal pour cette boisson est un défi réel en raison de la complexité de son microbiote. L'objectif de ce projet de recherche est d'identifier les paramètres clés susceptibles d'améliorer la production de molécules bioactives et d'établir une base pour sa mise à l'échelle industrielle. Il a été démontré que la géométrie de la cuve de fermentation et le transfert d'oxygène impactent la production de différents métabolites et composés bioactifs. Des extraits ont été préparés par extraction liquide/liquide suivi d'analyses des activités biologiques *in-vitro* (activités antioxydante, anti-15-lipoxygénase, anti-acétylcholinestérase et anti-prolifération des cellules cancéreuses) pour étudier l'influence du procédé sur les propriétés des métabolites. En parallèle, une identification et quantification chimique des différents extraits a été réalisée par HPLC-DAD et GC-MS. Une analyse de séquençage à haut débit a été réalisée, permettant l'identification des microorganismes dominants, de leurs gènes fonctionnels et de leurs principales voies métaboliques. L'influence de l'origine du consortium microbien a été évaluée, la cinétique de fermentation optimale a été établie et la caractérisation de la cellulose bactérienne produite a été effectuée. Nos études ont mis en évidence un lien entre le processus, le microbiote, les composés chimiques produits lors de la fermentation et le profil biologique du produit final. Ce travail représente une avancée significative dans l'établissement de certains paramètres clés pour l'optimisation de la fermentation de Kombucha et de la production de métabolites.

Mots-clés: Symbiose, *Komagataeibacter*, *Brettanomyces*, Thé Kombucha, Analyse de métabarcodage, Transfert d'oxygène, Cellulose bactérienne

Abstract

Kombucha, is a traditional tea fermented by a consortium of several bacteria (*Komagataeibacter* sp, *Gluconacetobacter* sp, *Gluconobacter* sp, *Acetobacter* sp…) and yeasts (*Brettanomyces* sp, *Schizosaccharomyces* sp, *Zygosaccharomyces* sp, *Saccharomyces* sp …) whose fermentation leads to the production of metabolites of interest. The design of an optimal fermentation process for this beverage is a big challenge due to its complex microbiota. The objective of this research project is to identify the key parameters that may improve the production of bioactive molecules and further settle a base for its industrial scale-up. It was demonstrated that the geometry of the fermentation vessel and the oxygen transfer have an influence on the production of metabolites and bioactive compounds. Extracts were prepared by liquid/liquid extractions followed by analysis of their *in-vitro* biological activities (antioxidant, anti-15lipoxygenase, anti-acetylcholinesterase and anti-proliferation activity of cancer cells) to study the influence of the process on the properties of the metabolites. In parallel, an identification and chemical quantification of the different extracts was carried out by HPLC-DAD and GC-MS. High throughput sequencing analysis was performed allowing the identification of the dominant microorganisms, their functional genes and main metabolic pathways. The influence of the origin of the microbial consortium was evaluated, the kinetics of fermentation and the characterization of the produced bacterial cellulose was also studied. Our studies revealed a link between the process, the microbiota, the chemical compounds produced during fermentation and the biological profile of the final product. This work represents a significant advancement in establishing some of the key parameters for the optimization of Kombucha fermentation and metabolites production.

Keywords: Symbiosis, *Komagataeibacter*, *Brettanomyces*, Kombucha tea, Metabarcoding analysis, Oxygen transfer, Bacterial cellulose

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BSTFA	N,O-Bis(triméthylsilyl)trifluoroacétamide
BHB	Acide 3-tert-butyl-4-hydroxybenzoïque
CAFF	Caféine
CAA	Acide caféique
C	Catéchine
CGA	Acide chlorogénique
CA	Acide p-coumarique
DMSO	Diméthylsulfoxyde
DPPH	2,2-diphényl-1-picrylhydrazyle
EC	(−)-Epicatechin
ECG	Gallate d'épicatéchine
EGCG	Gallate d'épigallocatéchine
FA	Acide férulique
GA	Acide gallique
GC-MS	Chromatographie en phase gazeuse couplée à la spectrométrie de masse
HCT-116	Lignée cellulaire du cancer du côlon humain
HDMS	Hexaméthyldisilazane
HPLC	Chromatographie en phase liquide à haute performance
LOD	Limite de détection
LOQ	Limite de quantification
LOX	Lipoxygénase
MCF-7	Lignée cellulaire humaine du cancer du sein
MTT	Bromure de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
NDGA	Acide nordihydroguaïarétique
NF	Thé noir non-fermenté
NSAID	Anti-inflammatoires non stéroïdiens
R2	Coefficient de corrélation
RI	Indice de réfraction
RUT	Rutine
s/h	surface/hauteur
s/v	surface/volume, zone interfaciale spécifique

TAX	Taxifoline
TB	Théobromine
TCA	Acide trans-cinnamique
TMCS	Chlorotriméthylsilane
TPC	Teneur en phénols totaux
UPLC	Chromatographie liquide ultra performante
VVM	Volume d'air par volume de milieu par minute

Valorisation de la thèse

Publications

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

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Communication par affiche

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Diffusion

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Introduction générale

Anton de Bary a été la première personne à décrire le terme « symbiose » comme « le fait de vivre ensemble d'organismes différents » en 1879. Cette définition s'applique à différentes associations interspécifiques (parasitisme, commensalisme ou mutualisme) et ces interactions microbes-microbes peuvent affecter négativement ou être bénéfique pour les partenaires selon le cas. Dans les fermentations alimentaires, les relations mutualistes entre plusieurs levures et bactéries, qui se sont révélées capables de développer une harmonie entre elles et leur environnement, ont été associées à l'enrichissement et à la fonctionnalité du produit final.

L'étude de la symbiose est encore assez complexe et n'a pas été complètement comprise jusqu'à aujourd'hui et pourtant, les êtres humains ont profité de ces interactions microbiennes pour produire divers aliments fermentés. Cependant, on a peu d'information sur le rôle spécifique des micro-organismes dans l'échange de métabolites ou sur leur capacité à se développer dans certaines conditions.

Un des plus anciens exemples des consortia mutualistes s'appelle Kombucha et sa consommation a été observée pour la première fois vers l'année 220 av. J.-C. en Mandchourie avant d'être étendue en Europe et dans le reste du monde. Cette ancienne boisson fermentée est le résultat de différents processus biologiques et/ou biochimiques qui transforment un simple thé noir sucré en une culture riche en micro-organismes et en saveurs acidulées, produisant simultanément une structure gélatineuse à l'interface air/liquide, qui se développe et s'adapte continuellement aux conditions dans lesquelles elle vit et qui est aussi appelé SCOBY dû à son acronyme en anglais (Symbiotic culture of bacteria and yeasts).

La composition microbienne spécifique du consortium microbien du Kombucha peut varier en fonction de la géographie, du climat et des conditions de fermentation. Cependant, les principaux partenaires symbiotiques appartiennent à l'ordre des *Saccharomycetales* dans le cas des levures, responsables de la transformation des sucres en alcool et en dioxyde de carbone, et au genre *Komagataeibacter* dans le cas des bactéries productrices de cellulose et d'acide acétique. Cet écosystème microbien, évoluant dans des zones plus ou moins aérobies dans le réacteur, crée un espace–hétérogène pour différentes réactions métaboliques utilisant les substrats tels que le saccharose, l'oxygène et les composés naturels du thé, menant à l'excrétion de métabolites dans le milieu, ou des modifications biochimiques des différentes molécules en présence.

Le Kombucha est donc un petit écosystème très réactif offrant de nombreuses possibilités d'interaction et de production de composés bioactifs avec des propriétés potentiellement

intéressantes contre l'oxydation, l'inflammation et le cancer. Cependant, les conditions de fermentation doivent être étudiées afin de mieux comprendre leur impact et ainsi améliorer ces propriétés.

Un réel challenge actuel concerne la montée en échelle des réacteurs de production du Kombucha. En effet, cette boisson est fabriquée de manière artisanale depuis près de 2000 ans, principalement en petites quantités. Aujourd'hui l'attrait pour cette boisson et sa consommation ne cessent d'augmenter, de façon différente selon les continents, mais induisant la nécessité d'accroissement des volumes de sa production. La montée en échelle d'une fermentation symbiotique nécessite cependant une bonne compréhension du fonctionnement de l'écosystème microbien très complexe du Kombucha, chaque population ayant sa propre capacité à croître et à s'adapter à un nouvel environnement tout en interagissant avec les autres populations. Plusieurs paramètres doivent être pris en compte à l'échelle laboratoire avant de faire le scale-up de cette boisson complexe et pétillante.

Plusieurs auteurs ont étudié et rapporté les effets bénéfiques du Kombucha, mais de nombreux verrous restent à lever concernant l'impact des conditions du procédé sur le déroulement de la fermentation, ainsi que la caractérisation de ses composés actifs et leur évolution au cours de la fermentation, ceux-ci pouvant être liés à des activités bénéfiques sur la santé humaine. Comprendre les relations entre les microorganismes, les conditions du procédé et la composition chimique de la boisson est primordial. L'influence de la géométrie de réacteur sur l'activité des populations doit notamment être étudiée, puisque ce sont ces activités microbiennes qui produiront des métabolites d'intérêts. Des approches scientifiques et techniques complémentaires sont donc nécessaires et doivent être menés en parallèle pour améliorer les qualités organoleptiques ou fonctionnelles et la cinétique de fermentation du thé Kombucha.

La richesse microbienne qui caractérise ce thé fermenté pose un défi en termes d'identification et de quantification correctes, car les méthodes conventionnelles de microbiologie sont insuffisantes pour une caractérisation précise, des méthodes de biologie moléculaire de PCR quantitative et/ou de séquençage du génome complet ont donc pu être utilisées dans ce projet de thèse.

Développer un procédé de fermentation lors de mise en œuvre de consortium mixte n'est pas une tâche facile, il n'existe pas un seul paramètre global capable de fournir les conditions de croissance optimales pour tous les microorganismes présents dans le consortium. La première

question serait donc : quels sont les objectifs en termes de produits finis ? De procédé de fermentation ? Comment choisir les paramètres clés de la fermentation en fonction de l'objectif?

Il reste ainsi encore plusieurs difficultés et défis à surmonter pour parvenir à une compréhension complète de cette fermentation complexe. Les recherches scientifiques effectuées à ce jour n'ont pas fini de découvrir tous les facteurs possibles pouvant affecter la population microbienne ni ses métabolites produits. Plusieurs questions doivent encore être résolues pour assurer un contrôle de qualité et obtenir un procédé de fermentation robuste.

Notre défi dans cette recherche sera de contribuer à la compréhension de cette symbiose particulière appelée «Kombucha». Grâce à ces travaux de thèse, nous espérons contribuer à l'avancement des connaissances :

- Sur la montée en échelle de la production industrielle du Kombucha à travers une production standardisée, d'abord au niveau laboratoire
- Sur la production des extraits qui pourraient avoir une utilisation potentielle en tant qu'additifs, compléments alimentaires ou être destinés à la préparation d'autres aliments fonctionnels
- Sur la qualité et la quantité de la cellulose.

Notre projet de thèse a pour objectif principal la conception d'un procédé favorisant la production de molécules bioactives dans un milieu de fermentation complexe.

Une étape cruciale pour la détermination des conditions du procédé dans cet objectif est la caractérisation biologique du produit obtenu. Pour ce faire, notre choix de méthodologie s'est porté sur la réalisation d'extractions des composés bioactifs suivie d'une caractérisation chimique et d'une quantification des molécules produites. Le fait de prendre en compte les variations des composés extraits dans chaque condition nous permettra de relier les profils les plus intéressants aux paramètres de procédé.

La deuxième phase consiste à évaluer les propriétés fonctionnelles des extraits obtenus contre des enzymes clés liées à certaines des principales maladies chroniques et à établir une corrélation entre la bioactivité et les composés responsables respectifs, et enfin chercher à le relier aux microorganisme(s) qui les a produits ou transformés. Et c'est à travers de toutes les méthodologies complémentaires mentionnées ci-dessus que l'on souhaite arriver à mieux

comprendre le comportement du consortium, son potentiel biologique et à établir certains des paramètres clés nécessaires à la mise à l'échelle du thé Kombucha.

Afin de pouvoir établir une méthode scientifique appropriée pour notre recherche, une étude bibliographique approfondie a été menée, qui nous a permis d'avoir une vision globale des paramètres clés à prendre en compte.

Les résultats obtenus seront présentés en cinq chapitres. Dans le premier chapitre de résultats (chapitre III) nous allons exposer l'impact de différentes géométries sur la cinétique de fermentation et la production de métabolites. Les résultats de cette étude contribueront à définir les bases pour optimiser la mise à l'échelle industrielle de la Kombucha.

Nous tenterons alors, dans le deuxième chapitre de résultats (chapitre IV), de changer la méthode traditionnelle de fermentation statique du Kombucha en ajoutant deux sources d'aération afin de «booster » la production de molécules bioactives et observer l'impact de ce paramètre dans la population microbienne.

Le fait que le thé Kombucha soit produit dans le monde entier rend important le fait de considérer l'impact de l'origine de son inoculum. Pour cela dans le troisième chapitre de résultats (chapitre V) nous présenterons une étude réalisée avec trois inoculums différents. Les cinétiques obtenues ainsi que leurs caractérisations chimiques et biologiques seront aussi décrites et analysées.

Nous nous intéressons ensuite dans le chapitre VI, quatrième chapitre de résultats, à l'effet de la durée de fermentation sur la composition biochimique du Kombucha ainsi que les différentes activités biologiques en découlant. En effet, ces durées de fermentation peuvent être variables. Les résultats obtenus nous permettront de mieux définir les temps de fermentation en fonction des activités souhaitées, tout en réduisant les coûts de production pouvant augmenter lors de fermentations longues.

Ensuite, le dernier chapitre (chapitre VII) est consacré à la caractérisation de la cellulose microbienne obtenue, afin de voir l'impact du procédé sur sa production et ses propriétés physico-chimiques.

Et finalement les conclusions générales ainsi que les perspectives de ces travaux seront données.

Chapitre I : Étude bibliographique

1.1 Kombucha, un thé fermenté ancestral devenu une tendance émergente

La tradition de la fabrication du Kombucha remonte à de nombreux pays asiatiques, en particulier la Chine où il est élaboré depuis plus de 2000 ans (Marsh *et al*., 2014). Aujourd'hui sa demande est très élevée sur les marchés des pays développés, en particulier aux États-Unis et dans d'autres pays d'Europe. L'Amérique a représenté environ 51,16% de l'ensemble des ventes mondiales en 2016, où le Kombucha a été classé parmi les boissons fermentées les plus vendues aux États-Unis (Jayabalan & Viduranga, 2019). Le marché mondial du Kombucha était évalué à environ 1062 millions USD en 2016 et devrait atteindre environ 2457 milliards USD en 2022, avec un taux de croissance annuel d'environ 25% entre 2017 et 2022 (Zion Market Research, 2017).

1.2 Preuves scientifiques de ses bienfaits pour la santé

La popularité de ce thé fermenté est due à ses bienfaits supposés sur la santé, et dans le passé le simple avis des consommateurs était suffisant. Néanmoins, la recherche scientifique a permis de confirmer certains aspects dans les dernières années par des études *in-vivo* et *in-vitro* (Villarreal-Soto *et al*., 2018). L'effet du Kombucha a récemment été étudié sur le microbiote intestinal chez les rats et les résultats ont montré que l'accumulation de graisse dans le foie a été significativement diminué par le traitement avec la boisson (Jung *et al*., 2019). Son activité antimicrobienne a été testée contre *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* et *Serratia marcescens* et il a montré une inhibition à partir du 14ème jour de fermentation laquelle a ensuite été augmenté jusqu'au 60ème jour, inhibant efficacement les cinq souches de bactéries (Vohra *et al*., 2019). D'autres études ont montré que le consortium bactérien est capable de fermenter différents substrats produisant des boissons homologues présentant des propriétés bénéfiques intéressantes. Différents cultivars de salak (*Salacca zalacca*) ou fruit de serpent ont été fermentés avec le consortium du Kombucha. Lyrawati *et al*., (2018) ont fermenté des jus des fruits du serpent des cultivars Suwaru, Madura, Pondoh et Bali pendant 14 jours et les ont administrés par voie orale à des rats diabétiques induits par la streptozotocine à raison de 5 mL/kg de poids corporel par jour au cours de 28 jours. Les résultats ont montré que le glucose plasmatique à jeun était significativement plus faible que pour le groupe témoin. Par la suite, Zubaidah et al., (2019), ont comparé l'activité antidiabétique *in-vivo* du Kombucha de salak contre celle de la metformine et ont observé que les deux étaient aussi efficaces pour la gestion du diabète induit, ce qui suggère que la boisson

pourrait potentiellement remplacer la metformine en tant qu'agent de traitement du diabète. Elkhtab *et al*. (2017), cherchaient des composés naturels capables de traiter l'hypertension humaine et ont observé que le lait fermenté avec le consortium de Kombucha contenait également plusieurs peptides inhibiteurs d'une des enzymes responsables de cette pathologie. Vitas *et al*., (2018), ont étudié la composition chimique et l'activité biologique de nouveaux types de boissons à base d'achillée fermenté par le consortium et ont trouvé une bonne activité antimicrobienne contre *Staphylococcus aureus, Klebsiella pneumoniae, E. coli, Proteus vulgaris, Proteus mirabilis* et *B. subtilis*. Rahmani *et al*., (2019), ont pu proposer une valeur nutraceutique supplémentaire à un légume comestible consommé par les populations nord-africaines (*Brassica tournefortii*) en utilisant un processus de fermentation de deux semaines avec le consortium de Kombucha. Et récemment, Zhou *et al*., (2019) ont étudié les effets du Kombucha sur la conservation fraîche du raisin de table après la récolte (*Vitis vinifera* cv. Fujiminori) en trempant les raisins dans le Kombucha pendant 15 min, puis en les conservant à 4 °C. Les résultats ont montré que l'application de Kombucha a permis de réduire la détérioration des fruits de table pendant la conservation par le froid et a également retardé la diminution de la dureté du fruit, des teneurs en solides solubles et en Vitamine C.

1.3 Révélant le mystère des microorganismes responsables de la bioactivité … Qui fait quoi ?

La population microbienne présente dans les consortia du Kombucha a été largement étudiée par divers auteurs (Teoh *et al*., 2004; Marsh *et al*., 2014; Reva *et al*., 2015; Chakravorty *et al*., 2016; Coton *et al*., 2017). Cependant, des études ont montré que divers paramètres tels que la température (De Filippis *et al*., 2018), le temps de fermentation (Muhialdin *et al*., 2019), les dimensions du fermenteur (Villarreal-soto *et al*., 2019), les substrats utilisés (Rahmani *et al*., 2019), l'agitation (Chapitre IV), etc., peuvent impacter la population présente et leurs métabolites respectifs.

Afin de pouvoir établir des liens entre la population microbienne et la composition chimique, les activités biologiques et les procédés utilisés lors de la fermentation du thé de Kombucha, une analyse de séquençage globale « shotgun » a été fait (Chapitres IV et V), et les microorganismes dominants qui ont été identifiés seront décrits ci-dessous :

1.3.1 Levures

- ***Brettanomyces bruxellensis* « le spécialiste de l'adaptation »**

Taxonomie

Cette levure a été décrite pour la première fois en 1904 par Niels Hjelte Claussen, déduit des mots grecs Brettano (brasseur britannique) et Myces (champignon). Plus tard, Van Laer a isolé une souche de levure de bières belges présentant les mêmes caractéristiques que celles décrites par Claussen et l'a classée comme *Brettanomyces bruxellensis* (Custers, 1940). *Brettanomyces* spp. sont les contreparties du genre *Dekkera* qui ne forment pas de spores.

Besoins nutritionnels

B. bruxellensis est tolérant à l'éthanol, anaérobie facultative, Crabtree positif et facilement adaptable à diverses combinaisons de stress environnementaux, tels que : concentrations élevées en éthanol, pH bas, absence de sources de carbone et d'azote facilement fermentables, taux d'oxygène bas ou d'haute stress osmotique (Steensels *et al*., 2015). Ses taux de croissance optimaux se situent généralement entre 25 et 28 °C, cependant elle peut pousser jusqu'à 35 °C, même à des valeurs de pH variables. Est capable d'utiliser de nombreuses sources de carbone, notamment le glucose, le fructose, le maltose, le mannose, l'éthanol, l'acide acétique et le glycérol (Smith & Divol, 2016).

Metabolites

Les composés phénoliques volatils sont les molécules clés responsables de certaines des caractéristiques aromatiques les plus reconnues associées aux espèces de *Brettanomyces* et cela implique l'action séquentielle de trois enzymes sur un substrat d'acide hydroxycinnamique (Suárez *et al*., 2007), qui sont montrés dans la **Figure 1**. Néanmoins, la production d'autres métabolites comme les acides gras ou les esters n'est pas négligeable (Tubia *et al*., 2018). Sa capacité de production de phénols volatils augmente lorsque les concentrations en alcool sont plus faibles et les températures plus élevées. Environ 80% des *Brettanomyces* peuvent produire ces phénols.

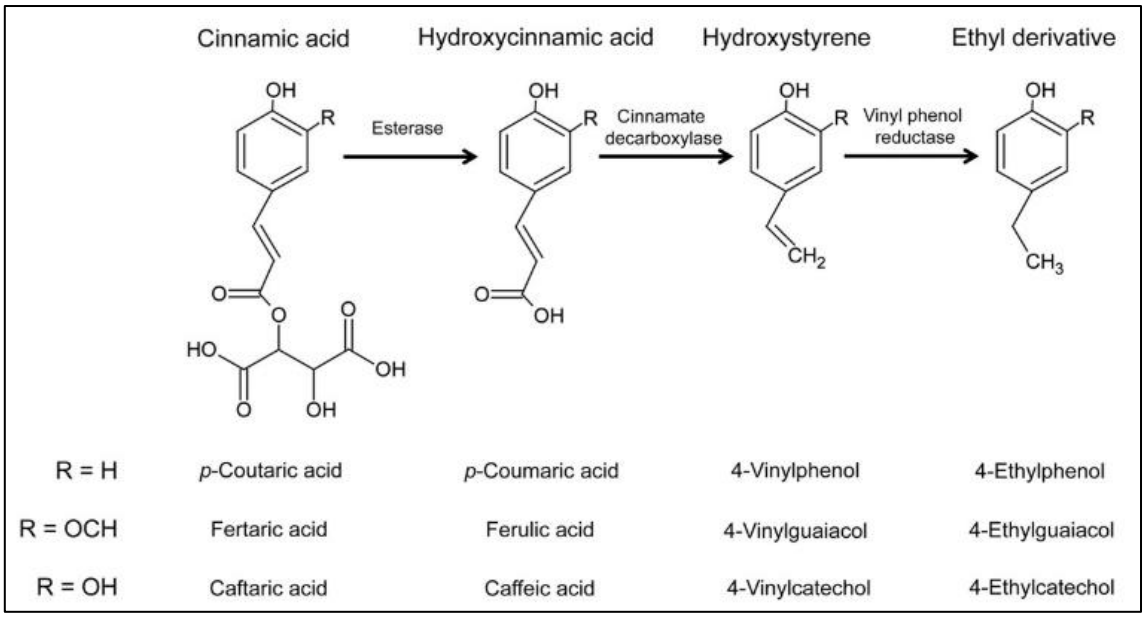


Figure 1. Formation de phénols volatils par *Brettanomyces/Dekkera* spp. (Steensels *et al*., 2015).

- ***Schizosaccharomyces pombe* « l'agent de désacidification »**

Taxonomie

Schizosaccharomyces pombe, également connue sous le nom de levure à fission, a été découverte par Lindner en 1983. Le genre comprend trois espèces principales: *S. pombe*, *S. japonicus* et *S. octosporus.* Cependant, l'espèce la plus utilisée dans les industries de fermentation y compris cela du Kombucha c'est *S. pombe* (Loira *et al*., 2018).

Besoins nutritionnels

Son taux de croissance est très lent, avec une longue phase de latence et un besoin élevé en vitamines, cependant, elle a un faible besoin en azote. En ce qui concerne les sources de carbone en plus du glucose, *S. pombe* peut également utiliser du glycérol, du saccharose, de la raffinose et du maltose. Les environnements de croissance de cette levure peuvent avoir une faible activité de l'eau, une forte teneur en sucre, très faible pH et une grande plage de températures (Palomero *et al*., 2012).

Metabolites

La principale caractéristique qui distingue *Schizosaccharomyces* des autres genres de levure est sa capacité à métaboliser l'acide malique en éthanol et en CO_2. Ou l'acide malique est décarboxylé en acide pyruvique par l'action de l'enzyme acide malique décarboxylase et des

ions Mn^{2+}/Mn^{3+}. Et ensuite, l'acide pyruvique suit la voie de la fermentation alcoolique, étant décarboxylé en acétaldéhyde puis réduit en éthanol (**Figure 2**).

La production élevée d'acide pyruvique et de pyranoanthocyanines a été rapporté en étant environ cinq fois plus que celle du genre *Saccharomyces* (Benito *et al*., 2014).

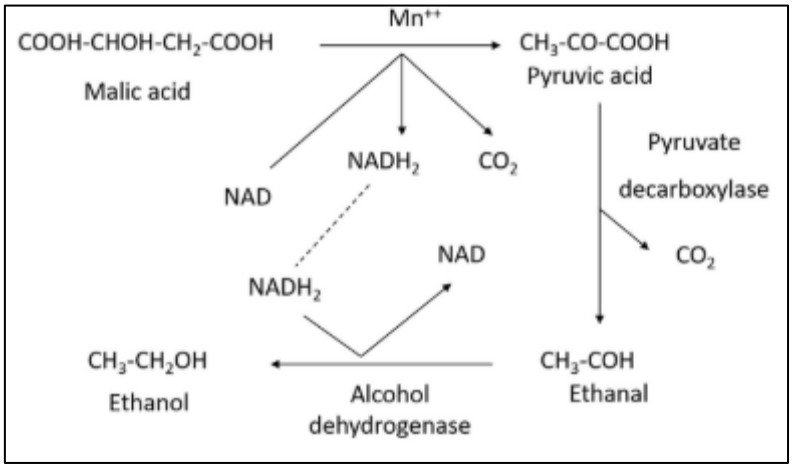


Figure 2. Fermentation maloalcoolique par *S. pombe* (Santiago Benito, 2019).

- ***Zygosaccharomyces bailii* « une levure d'altération aux caractéristiques intéressantes »**

Taxonomie

La description originale de l'espèce a été fournie par Paul Lindner (1895) et par la suite Guilliermond (1912) a transféré l'espèce au genre *Zygosaccharomyces* et l'a renommée *Z. bailii* (James & Stratford, 2011).

Besoins nutritionnels

Ce genre comprend le plus grand nombre de levures tolérantes au sel et au sucre, en ayant une préférence pour le fructose. Elle peut facilement croitre dans de milieux avec faible pH, faible activité de l'eau, des quantités suffisantes de sucres fermentables, environnements acides, à des températures relativement élevées et peuvent survivre en présence de fortes concentrations de conservateurs chimiques (Dakal *et al*., 2014).

Metabolites

Divers composés aromatiques tels que les alcools, les acides et les esters peuvent être produits lors de la fermentation. Les alcools et acides sont générés par les levures à partir des acides

aminés par la voie d'Ehrlich et à partir de sucres par la voie de Harris (**Figure 3**) (Xu *et al*., 2017). De meilleurs rendements de production de protéines ont été rapportés par rapport à *S. cerevisiae* (Vigentini *et al*., 2005), et son extrême tolérance aux environnements acides représente le rend le candidat idéal pour la production d'acides organiques.

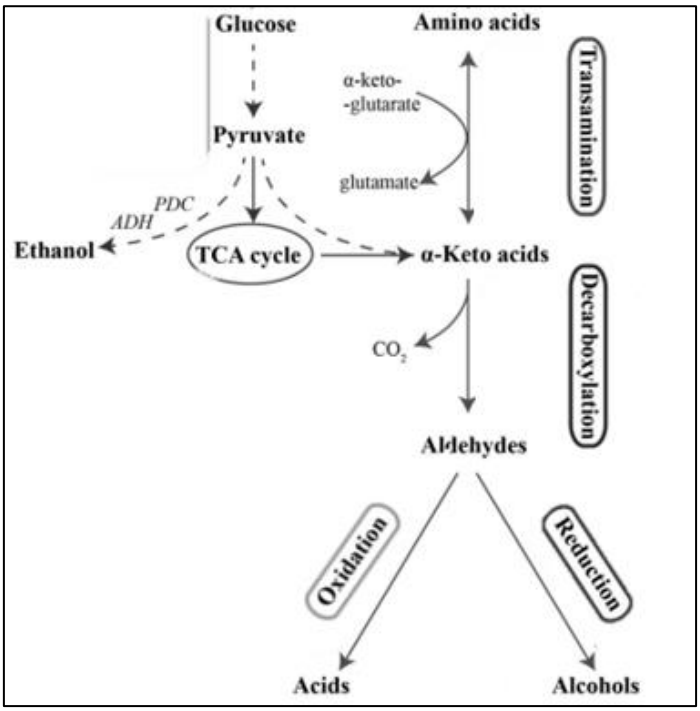


Figure 3. Mécanismes de formation de divers composés aromatiques par *Z. bailii* (Xu *et al*., 2017 avec modifications).

1.3.2 Bactéries acétiques « les productrices de cellulose par excellence »

- ***Komagataeibacter* spp**

Taxonomie

Le genre *Komagataeibacter* a récemment été proposé par Yamada, (2014), et regroupe 14 espèces précédemment classés dans le genre *Gluconacetobacter* tels que, *K. europaeus, K. intermedius, K. oboediens, K. kakiaceti, K. hansenii, K. medellinensis, K. rhaeticus et K. xylinus*, étant les deux derniers, le plus représentatives du consortium de Kombucha. Ce genre a été nommé d'après un microbiologiste japonais, le professeur Kazuo Komagata, en reconnaissance de sa contribution à la systématique de bactéries acétiques.

Metabolites

La biologie moléculaire de *Komagataeibacter* a été étudiée de manière limitée jusqu'ici et s'est concentrée principalement sur la voie métabolique de la production de cellulose à partir de glucose. Cependant, *Gluconacetobacter* est le genre avec le plus d'espèces, et déploie la plus grande variété de capacités physiologiques, telles que la production d'acide organique, la fixation de l'azote, la synthèse de polymères glucidiques, entre autres (Velasco-Bedran & Lopez-Isunza, 2007). La **Figure 4** présente un schéma du métabolisme structuré de la physiologie complexe de *G. xylinus*.

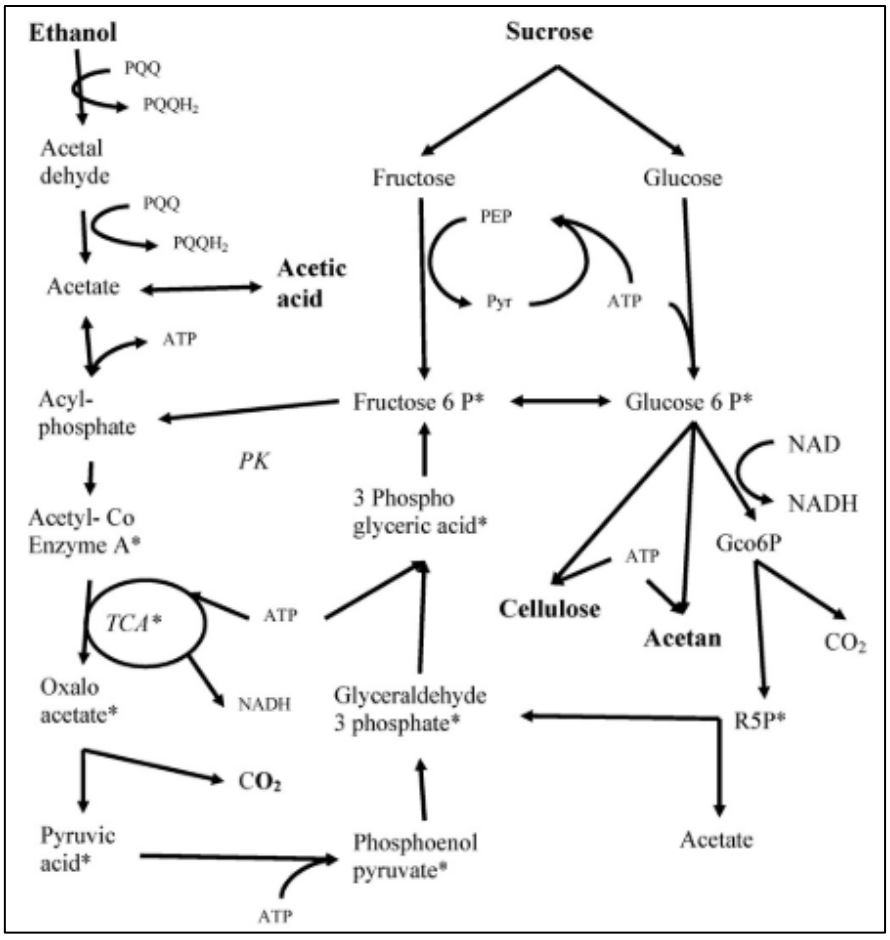


Figure 4. Catabolismes de l'éthanol et du glucose/fructose en lien avec les voies métaboliques centrales. Gco6P: acide gluconique 6-phosphate ; R5P: ribose 5 phosphate ; PK: phosphocétolase voie (Velasco-Bedran & Lopez-Isunza, 2007).

- ***Gluconobacter oxydans***

Taxonomie

Un groupe de bactéries gram-négatives apparentées à *Acetobacter* a été renommé et placé sous le genre *Gluconobacter* par Asai & Shoda, (1958).

Metabolites

L'oxydation incomplète des sucres, des alcools et des acides par souches de *Gluconobacter* peut conduire à la production L-sorbose à partir de D-sorbitol, de l'acide D-gluconique, les acides 5-céto et 2-cétogluconiques à partir de D-glucose et la dihydroxyacétone à partir de glycérol (Gupta *et al*., 2001). *Gluconobacter* sp. oxyde le glucose par deux voies alternatives, l'un est à l'intérieur de la cellule, suivi d'une oxydation via la voie du pentose phosphate et l'autre opère à l'extérieur de la cellule et implique la formation d'acide gluconique (**Figure 5**) (Gupta *et al*., 2001).

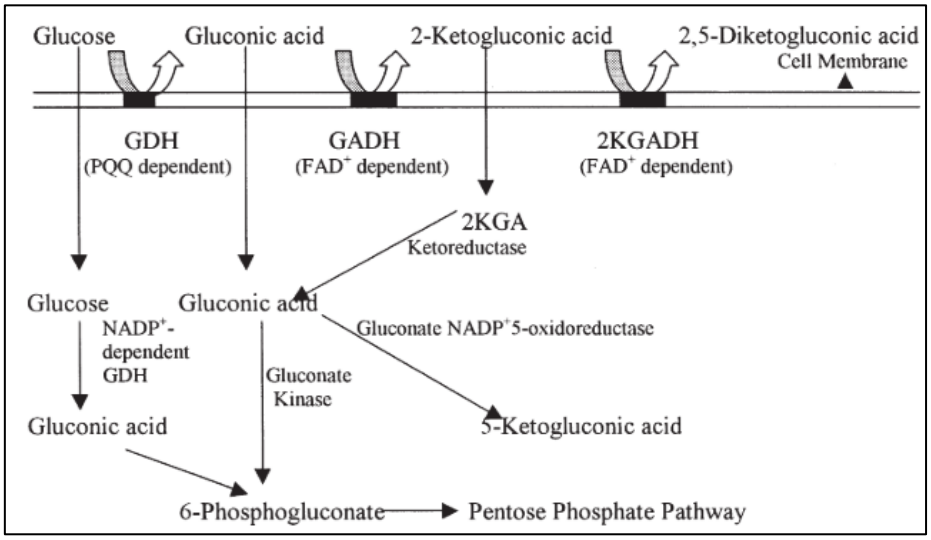


Figure 5. Métabolisme de l'oxydation du glucose par *Gluconobacter oxydans* (Gupta *et al*., 2001).

Besoins nutritionnels

Les bactéries de ce genre se caractérisent par les éléments suivants : aérobies obligatoires et gram-négatives, manque de flagelle, production d'acide acétique à partir d'éthanol et croissance en présence de 0,35% d'acide acétique (v/v) (Ryngajłło *et al*., 2018). Leur croissance optimale se situe entre un pH de 5 et 6,5, mais peut également se produire à des valeurs très acides de 3-4. Elles sont généralement considérés comme mésophiles, en ayant sa température optimale de croissance autour de 30 °C (Lynch *et al*., 2019).

La suite de l'étude bibliographique sera présentée sous forme d'une revue qui a déjà été publiée :

- " Understanding Kombucha Tea Fermentation: A review" publié dans le journal *Food Science*. 2018 https://doi.org/10.1111/1750-3841.14068.

Understanding Kombucha Tea Fermentation: A Review

Silvia Alejandra Villarreal-Soto, Sandra Beaufort, Jalloul Bouajila, Jean-Pierre Souchard, and Patricia Taillandier

Journal of Food Science A Publication of the Institute of Food Technologists

Abstract: Kombucha is a beverage of probable Manchurian origins obtained from fermented tea by a microbial consortium composed of several bacteria and yeasts. This mixed consortium forms a powerful symbiosis capable of inhibiting the growth of potentially contaminating bacteria. The fermentation process also leads to the formation of a polymeric cellulose pellicle due to the activity of certain strains of *Acetobacter sp*. The tea fermentation process by the microbial consortium was able to show an increase in certain biological activities which have been already studied; however, little information is available on the characterization of its active components and their evolution during fermentation. Studies have also reported that the use of infusions from other plants may be a promising alternative.

Keywords: bioactivity, fermentation, kombucha tea, microbial cellulose, symbiosis

Practical Application: Kombucha is a traditional fermented tea whose consumption has increased in the recent years due to its multiple functional properties such as anti-inflammatory potential and antioxidant activity. The microbiological composition of this beverage is quite complex and still more research is needed in order to fully understand its behavior. This study comprises the chemical and microbiological composition of the tea and the main factors that may affect its production.

Fermentation is one of the most antique methods of food preservation. It is also a low cost energy conservation system, which is essential to ensure the life and safety of food. Many biochemical changes occur during fermentation and may affect the nutrient compounds and consequently the properties of the final product, like the bioactivity and digestibility. Recently, this bioprocess has been applied for the production and extraction of bioactive compounds from plants in food and beverage industries (Hur and others 2014). Kombucha tea is obtained from a symbiotic culture of acetic acid bacteria (*Komagataeibacter, Gluconobacter* and *Acetobacter* species) (Roos and Vuyst 2018), lactic acid bacteria (*Lactobacillus, Lactococcus*) (Marsh and others 2014), and yeasts *(Schizosaccharomyces pombe, Saccharomycodes ludwigii, Kloeckera apiculata, Saccharomyces cerevisiae, Zygosaccharomyces bailii, Torulaspora delbrueckii, Brettanomyces bruxellensis*) (Coton and others 2017) in a sweet medium, generally black tea. Its fermentation process also leads to the formation of a floating biofilm on the surface of the growth medium due to the activity of certain strains of acetic acid bacteria (Watawana and others 2016). The main acids present are acetic, gluconic, tartaric, malic and in less proportion citric acid. All these acids are responsible for its characteristic sour taste (Jayabalan and others 2007). Actual food trends towards minimally processed products, without additives, high nutritional value and with health benefits have increased with consumer awareness. In this context, the traditional Kombucha tea has recently captured the attention of researchers and consumers because of its probiotic characteristics. However, the manufacturing technology, its microbiota, byproducts and physicochemical properties are important facts to consider for industrial production. There are several types of fermentation and obtained products depending on the metabolic pathway followed. Kombucha fermentation is a combination of three of them: alcoholic, lactic and acetic one, this because of the presence of several yeasts and bacteria coexisting in the medium. Being initiated by osmotolerant microorganisms and ultimately dominated by acid-tolerant species. Several authors have studied the benefits of Kombucha tea, however there is little information on the characterization of its active components, their evolution during fermentation and their pharmacological activities. Moreover, the influence of fermenters, substrates, metabolites and their improvements on the organoleptic qualities and fermentation kinetics should be also evaluated.

1.4 Kombucha tea: A complex fermented beverage

Kombucha is a popular drink among many traditional fermented foods. Bacteria and yeasts present in the medium create a powerful symbiosis capable of inhibiting the growth of

contaminating microorganisms (Vitas and others 2013). It is composed of two phases: a floating biofilm and a sour liquid phase. Acetic acid, gluconic acid and ethanol are the main components in the liquid, but also in the biofilm due to its great water absorption capacity (Czaja and others 2006). Under aerobic conditions the symbiotic consortium of Kombucha is able to convert sugar and tea in a period from 7 to 10 days in a lightly carbonated, slightly sour and refreshing drink, which is composed of several acids, 14 amino acids, vitamins and some hydrolytic enzymes (Malbaša and others 2011).

1.4.1 Chemical composition

Detailed knowledge of the composition and properties of Kombucha tea is crucial for better understanding its kinetics. However, the composition and metabolite concentration are dependent on the inoculum source (Nguyen and others 2015), the sugar and tea concentration (Fu and others 2014; Watawana and others 2017), the fermentation time (Chen and Liu 2000), and the temperature used (Lončar and others 2006; Jayabalan and others 2008). Any change in the fermentation conditions might affect the final product. Nevertheless, the main components and some key metabolites produced in the fermented tea are present below in **Table 1.** Final sugar concentrations can differ from one fermentation to another, which indicates that the metabolic pathway doesn't always occur in the same way (Chen and Liu, 2000). Regarding the production of organic acids, Jayabalan et al., (2007) observed the increasing production of acetic acid through the fermentation until a maximum of 9.5 g/L after 15 days. In the case of D-glucuronic acid, it reached a maximum concentration of 2.3 g/L on the 12^{th} day and in less quantity 0.54 g/L of lactic acid were detected in the third day. As for the anionic concentration, it remained at low values, ranging between 0.04 to 3.20 mg/g, being F^- and Cl^- the most present anions (Watawana and others 2015) . The chemical composition as well as the concentration of each metabolite produced in Kombucha will always depend on the inoculum, initial sugar concentration, etc. However, among the main constituents of Kombucha tea, glucuronic acid is considered to be the main therapeutic agent (Kumar and Joshi 2016).

Table 1. General chemical composition of Kombucha.

	Compound	Average composition	Initial sucrose	Fermentation time	References
Organic acids	Acetic acid	5.6 g/L	70 g/L	15 days	Blanc (1996)
	Acetic acid	8.36 g/L	100 g/L	18 days	Jayabalan and others (2007)
	Acetic acid	11 g/L	100 g/L	30 days	Chen and Liu (2000)
	Gluconic acid	39 g/L	100 g/L	60 days	Chen and Liu (2000)
	Glucuronic acid	0.0160 g/L	70 g/L	21 days	Lončar and others (2000)
	Lactic acid	0.18 g/L	100 g/L	18 days	Jayabalan and others (2007)
Vitamins	Vitamin B1	0.74 mg/mL	70 g/L	15 days	Bauer-petrovska (2000)
	Vitamin B2	8 mg/100 mL	70 g/L	10 days	Malbaša and others (2011)
	Vitamin B6	0.52 mg/mL	70 g/L	15 days	Bauer-petrovska (2000)
	Vitamin B12	0.84 mg/mL	70 g/L	15 days	Bauer-petrovska (2000)
	Vitamin C	25 mg/L	70 g/L	10 days	Malbaša and others (2011)
General composites	Ethanol	5.5 g/L	100 g/L	20 days	Chen and Liu (2000)
	Proteins	3 mg/mL	100 g/L	12 days	Jayabalan and others (2007)
	Tea polyphenols	7.8 mM gallic acid equivalents (GAE)	100 g/L	15 days	Chu and Chen (2006)
Minerals	Cu, Fe, Mn, Ni, Zn	0.1 - 0.4 µg/mL	70 g/L	15 days	Bauer-petrovska (2000)
Anions	F^-, CI^-, Br^-, I^-, NO_3^-, HPO_4^- SO_4^-	0.04 - 3.20 mg/g	100 g/L	7 days	Kumar and others (2008)

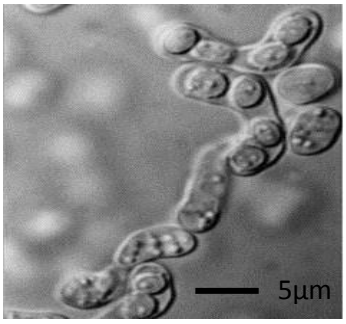
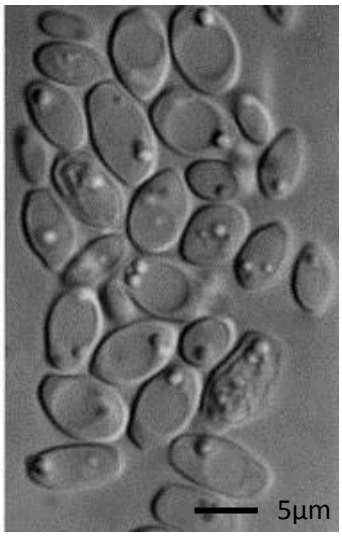
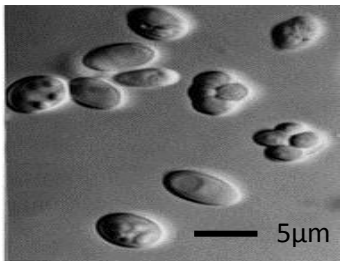
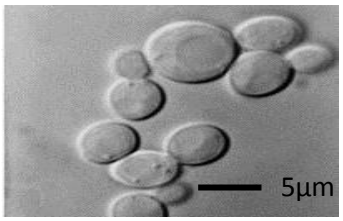
1.4.2 Microbiological composition

Several studies have shown that the microbial spectrum of Kombucha consortium, also called SCOBY or tea fungus, may vary between fermentations (Reva and others 2015; Chakravorty and others 2016; Coton and others 2017). However, there is a number of species that remains in most of Kombucha cultures, which are described next.

1.4.2.1 Yeasts

Most yeasts species can ferment sugar to ethanol, yet many modern alcoholic fermentation processes are initiated by a single starter culture, commonly being *Saccharomyces cerevisiae* due to its high efficiency. However non-*Saccharomyces* yeasts are becoming increasingly used in the industry in mixed fermentations (wine, tequila, etc.) in order to enrich the aromatic profile, and to enhance the complexity and the kinetics of the final product (Nehme and others 2008; Lopez and others 2014). Microbial interactions between *Saccharomyces* and non-*Saccharomyces* yeasts seems to be an advantageous option in mixed fermentation processing, having several benefits like avoiding the risks of stuck fermentation, the addition of aromas and flavors, allows the modification of undesired parameters, between others (Sun and others 2014). And in this sense, Kombucha's yeasts interaction has proven to be a consortium that generates final desirable characteristics. There are many yeasts genus and species in Kombucha culture, a broad spectrum has been reported including species of *Zygosaccharomyces, Candida, Kloeckera/Hanseniaspora, Torulaspora, Pichia, Brettanomyces/Dekkera, Saccharomyces, Lachancea, Saccharomycoides, Schizosaccharomyces,* et *Kluyveromyces* (Marsh and others 2014*;* Chakravorty and others 2016*;* Coton and others 2017). Despite of its variability, some of the predominant species are presented in **Table 2.**

Table 2. Some yeasts species present in Kombucha culture.

Species	Morphology	Characteristics
Schizosaccharomyces pombe		• High fermentative power • Ability to convert malic acid to ethanol • Release of high quantities of polysaccharides (Domizio and others 2017)
Brettanomyces bruxellensis		• High resistance to osmotic and ethanol stress • Higher efficiency to utilize the available N sources than *Saccharomyces cerevisiae* • Tendency to ferment sugars to ethanol, and produce high concentrations of acetic acid in aerobic conditions (Steensels and others 2015)
Saccharomyces cerevisiae		• High ethanol tolerance • Rapid fermentation rates • Insensitivity to temperature and substrate concentration (Choonut and others 2014)
Zygosaccharomyces rouxii		• Highly osmo and halotolerant • Counteract better sugar and salt stress than *S. cerevisiae* (Dakal and others 2014)

Only images from Pitt and Hocking 2009 with modifications.

In addition to those already mentioned, several authors have quantified some other yeasts present in the Kombucha culture, Watawana and others (2016) reported *Zygosaccharomyces* as the predominant yeast with 84.1% of relative percentage of abundance and *Dekkera* and *Pichia* species with 6 and 5% respectively. Mayser, P., (1995), revealed biofilm-forming yeasts such

as *Candida krusei* or *Issatchenkiaorientalis* as well as species of the apiculatus yeasts (*Kloeckera, Hanseniaspora*). A new ascosporogenous yeast called *Zygosaccharomyces kombuchaensis* was isolated from Kombucha tea by (Kurtzman and others 2001).

1.4.2.2 Bacteria

The dominant bacteria of Kombucha tea culture are acetic acid bacteria, which are aerobic bacteria able to use alcohol as a substrate to form acetic acid. These bacteria, in contrast to yeast, require large amounts of oxygen for their growth and activity. The metabolic process is based on the conversion of acetaldehyde into ethanol and acetaldehyde hydrate into acetic acid by the enzyme acetaldehyde dehydrogenase (Jayabalan and others 2007). Several acetic acid bacteria are present in the tea fungus, including: *Acetobacter xylinoides, Bacterium gluconicum, Acetobacter aceti, Acetobacter pasteurianus, Gluconobacter oxydans* (Jayabalan and others 2014). Marsh and others (2014) worked with rRNA sequence analysis and found between 86 and 99% relative abundance of *Gluconacetobacter* through all the fermentation both in the liquid medium and in the biofilm. Similar results were obtained by Watawana and others (2016) who fermented coconut water with the tea fungus and found *Gluconacetobacter* as the main one with a relative percentage of 85.6 and in less proportion *Acetobacter, Lactobacillus, Leuconostoc,* and *Bifidobacterium* species.

1.4.2.2.1 Cellulose Production

There are several types of bacteria that can produce microbial cellulose, such as: *Aerobacter, Agrobacterium, Azotobacter, Rhizobium, Salmonella* and *Gluconacetobacter* (Mohite and Patil 2014). Among the *Acetobacter* gender, the dominant specie is *Acetobacter xylinum,* which was reclassified as *Gluconacetobacter xylinus* and more recently to *Komagataeibacter xylinus* (Yamada and others 2012). A specific biochemical activity of this bacterium is the oxidation of glucose to gluconic acid, which is found in the liquid phase, then another specific metabolism leads to the synthesis of microbial cellulose forming the biofilm that remains in the liquid surface. The process includes the synthesis of uridine diphospho-glucose (UDPGlc) (**Figure 6**), which is the cellulose precursor, then each single cell of *Acetobacter* may polymerize up to 200, 000 glucose residues per second into β-1,4-glucan chains. The advantage of this form of cellulose production is that the bacterium grows rapidly under controlled conditions and can produce cellulose from a variety of carbon sources including glucose, ethanol, sucrose, and glycerol.

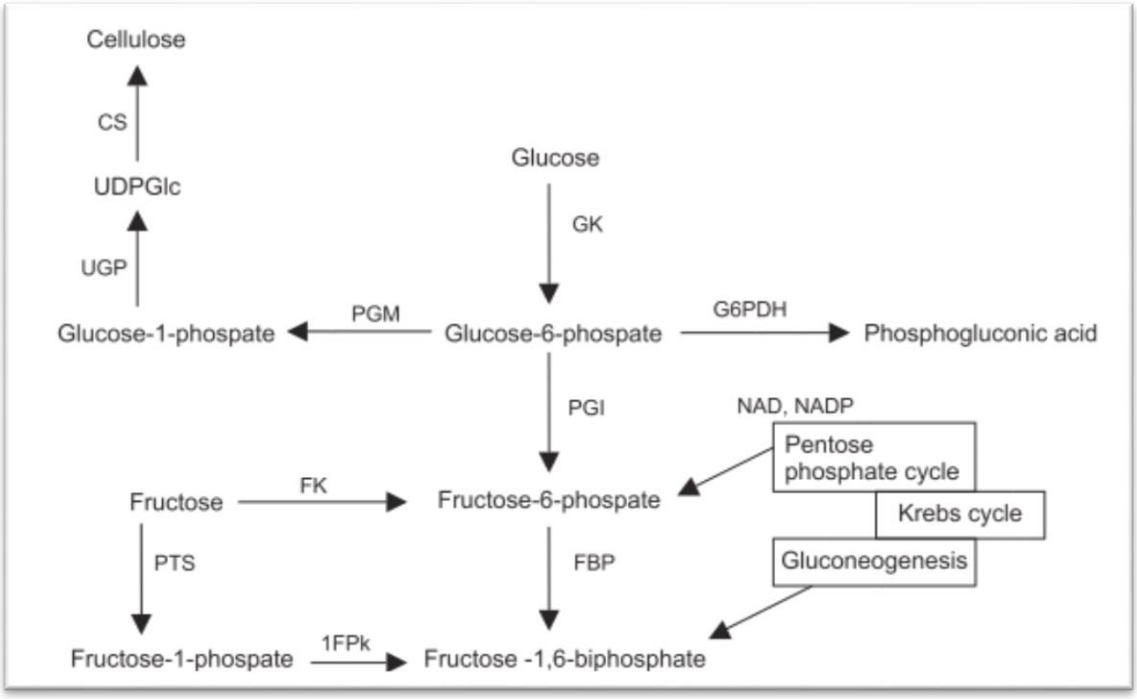


Figure 6. Biochemical pathway for cellulose synthesis by *A. xylinum*. CS cellulose synthase, GK glucokinase, FBP fructose-1,6-biphosphate phosphatase, FK fructokinase, 1FPk fructose-1-phosphate kinase, PGI phosphoglucoisomerase, PMG phosphoglucomutase, PTS system of phosphotransferases, UGP pyrophosphorylase uridine diphosphoglucose, UDPG uridine diphosphoglucose, G6PDH glucose-6-phosphate dehydrogenase, NAD nicotinamide adenine dinucleotide, NADP nicotinamide adenine dinucleotide phosphate (Chawla and others 2009).

The microbial cellulose is produced extracellularly in the form of fibrils that are attached to the bacterial cell. Each single cell has between 50 and 80 pores or complex terminals (CTs) with a diameter of 3.5nm for extruding cellulose out of their membrane (**Figure 7)**. These chains are later assembled forming thicker fibrils called macrofibrils creating a 3-D structure of about 1000 individual glucan chains which can hold up to 200 times more water of its dry mass and possess high conformability and great elasticity. Bacteria produce two forms of cellulose, cellulose I and cellulose II. Cellulose I is a ribbon-like polymer composed of bundles of microfibrils, while cellulose II is an amorphous polymer that is thermodynamically more stable than cellulose I (Podolich and others 2016).

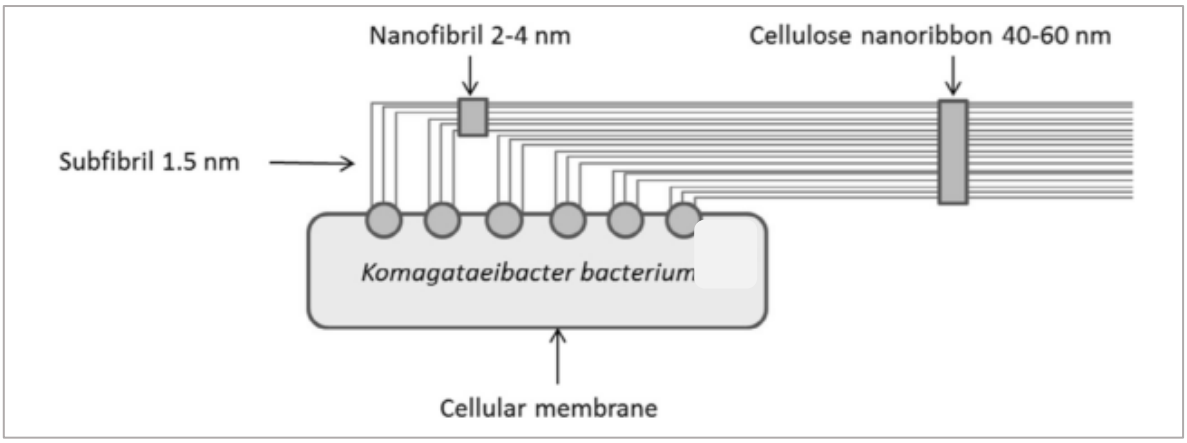


Figure 7. Assembly of cellulose microfibrils by *K. xylinum* (Cacicedo and others 2015 after modifications).

In the first state, the producing cellulose bacteria increases its population through the consumption of the dissolved oxygen. During this time the microorganism synthesizes certain amount of cellulose in the liquid medium and just the bacteria that are in the air/medium interface can maintain its activity and produce cellulose, which is formed by superimposed layers. As fermentation time advances, the membrane thickness is increased by the generation of new layers on the surface, forming a suspended structure in the culture medium. The development of the biofilm along with hydrogen and C-H bonding will continue through all fermentation, its synthesis will reach its limit when it grows downward entrapping all bacteria, which then will become inactive due to insufficient oxygen supply (Esa and others 2014). The bacteria remaining in the liquid phase of the culture medium are in a dormant state and can be reactivated and used as inoculum in a later fermentation (Ruka and others 2012). This biofilm possesses high crystallinity, high tensile strength, extreme insolubility in most of the solvents, moldability, high degree of polymerization, it is one hundred times thinner than that of cellulose fibrils obtained from plants and its water holding capacity is over 100 times higher (Chawla and others 2009). One of its main characteristics is its purity, which distinguish it from the plant one that contains hemicellulose and lignin (Sulaeva and others 2015), but also its high degree of crystallinity (>60%) where the crystals formed are composed of cellulose type Iα and Iβ. These unique properties, as well as its purity have allowed many successful applications in the field of biomedical materials (Kuo and others 2015). The biofilm may vary depending on the used strains, culture time and chemical additives present in the culture media (Lee and others 2015), but it is also hypothesized that microbial cellulose obtained from a Kombucha culture may produce a biofilm with different characteristics than those form typical sources (Nguyen and others 2008). There are several factors to consider in order to maximize the yield of

microbial cellulose and optimize the process, for example: the volume of the inoculated media, the incubation time, the surface area and surface height conditions (Cacicedo and others 2015). The removal of the amorphous parts of the nanofibrils through acid hydrolysis can produce nanocrystals, which can be used for different purposes in several areas as food packaging or medical applications (Campano and others 2016).

1.5 Microorganisms interactions

The complexity of understanding Kombucha fermentation kinetics are mainly due to the important number of microorganisms present and the interactions between them (Markov and others 2003), which are considered to have inhibitory effects on the ethanol production. However, the death and autolysis of yeast cells releases also vitamins and other nutrients that stimulate the growth of important bacteria. Most microbial species excrete metabolic products that can either stimulate or inhibit the specific growth rate of the other species, establishing commensalistic or amensalistic interactions which have to be extensively analyzed to achieve the comprehension of this phenomenon of coexistence. Some bacteria groups such as lactic acid bacteria (LAB) and acetic acid bacteria (AAB), as well as yeasts species such as *Saccharomyces cerevisiae*, have well established roles in the fermentation. However, until today, there are many other species whose roles have not been extensively characterized, nor the interactions between them. There are a number of obstacles in understanding microbial ecosystems, the first one is the enormous diversity and complexity of most of the microbial communities, for example certain microorganisms can participate in parallel, while others act in a sequential manner with a dominant evolution during fermentation (Chakravorty and others 2016). In the case of Kombucha, the different yeasts and bacteria species act in parallel producing two different final products: the fermented tea and the biofilm. At the beginning of the fermentation yeast hydrolyze sucrose into glucose and fructose, formerly the ethanol is produced and finally acetic acid bacteria transform ethanol into acetic acid, nonetheless the production of gluconic and glucuronic acids is also remarkable (**Figure 8**).

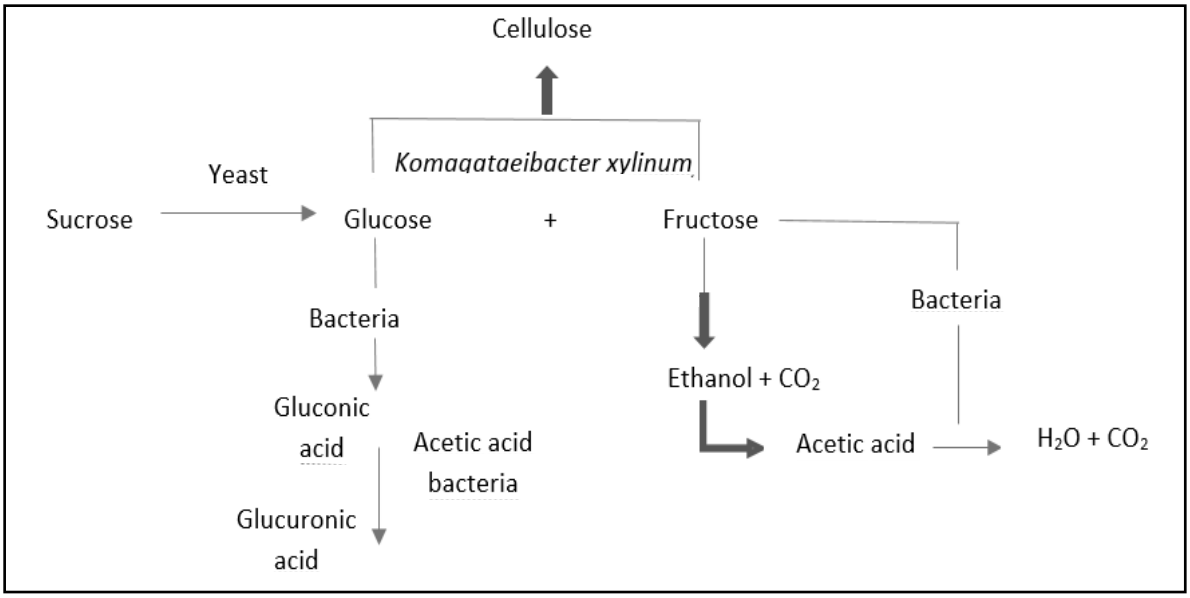


Figure 8. Main metabolic activity of Kombucha tea (Markov and others 2003).

1.6 Microbial identification methodologies

Traditionally microorganisms have been classified and identified mainly by morphological and physiological criteria, nevertheless in addition to these standard tests, biochemical methods provide important data for the characterization, however, the result of these tests sometimes lead to erroneous characterizations because these functions are controlled by one or few genes. Besides, all these tests take time and sometimes their determination and classification is ambiguous due to the variability of the species. That is why, it is recommended to complement the conventional techniques with molecular techniques to elucidate not only the degree of relationship, but also to reveal the connections between evolutionary mechanisms (Kurtzman and others 2001).Chen and Liu, (2000) reported a technique for enumeration of acetic acid bacteria and yeast in the liquid and in the biofilm. They declared the number of viable cells and were also able to isolate some of the yeasts and main acetic acid bacteria throughout the fermentation, where they noted that the number of viable cells was greater in the liquid broth than in the biofilm. The protocol was based on the use of potato dextrose and GYCA agar medium to numerate yeasts and acetic acid bacteria from 20 g of sample homogenized in a blender for 9 min then incubated at 30°C for 3 days. Cell counts were expressed as colony-forming units per milliliter. Hidalgo and others (2012) worked with yeasts and bacteria identification methods and obtained 453 yeasts and 270 bacteria isolates. In the case of yeasts, YPD agar was used (Yeast extract 10 g/L, Bacteriological Peptone 20 g/L, D-glucose 20 g/L and Agar 20 g/L) and incubated for 48 h at 28 °C. Then about 25 and 30 colonies were randomly

picked and plated on a selective Lysine medium to differentiate *Saccharomyces* and non-*Saccharomyces* yeasts, and finally a PCR reaction mix and RFLP- PCR of rDNA was performed to identify the yeasts species. For the bacteria isolation method GYCA medium was used (10% Glucose, 1% Yeast extract, 2% $CaCO_3$, and 1.5% agar supplemented with 100 mg/L of natamicine). Then total DNA was extracted using the CTAB method (cetyltrimethylammonium bromide), all acetic acid bacteria were genotyped using ERIC and (GTG) 5-rep-PCR fingerprinting techniques and the electrophoresis was analyzed in 1.5% (w/v) agarose gels. Colonies with a halo around them were subjected to Gram staining and catalase test, which verified their identity as AAB. Enterobacterial Repetitive Intergenic Consensus-PCR and Repetitive Palindromic-PCR have been proposed as suitably accurate techniques for identifying bacteria strains and for determining taxonomic relationships between bacterial species because of their high degree of polymorphism. It is recommended to use combined techniques such as RFLP because it seems to provide additional information to establish a phylogenetic placement of several real yeasts. This was demonstrated by (Kurtzman and others 2001), where the combination of these techniques allowed to distinguish the different species within the genus *Zygosaccharomyces*, obtained from Kombucha isolates. According to Meersman and others (2013) the use of a traditional method combined with one of accurate identification such as ribosomal DNA, sequence-based PCR or fragment length polymorphism will led to a more precise quantification of the microbiota and a further isolation of potential starter cultures. However, the first culture-independent, high throughput sequencing analysis of the Kombucha biofilm was done by Marsh and others (2014) with which they were able to identify the major bacterial and yeast populations present both in the biofilm and in the liquid broth, being *Gluconacetobacter* and *Zygosaccharomyces*, respectively.

1.7 Factors influencing Kombucha fermentation

Fermentation is influenced by many factors such as, temperature, pH, the amount of oxygen, the CO_2 dissolved, the operating system, the supply of precursors, the shear rate in the fermenter as well as the nature and composition of the medium (Marsh and others 2014). Any variation in these factors can affect the rate of fermentation, the spectrum, the performance, the organoleptic properties, the nutritional quality and other physicochemical properties of the product. Different plant varieties, sugar concentrations, fermentation time, and composition of tea fungus may account for differences in composition and therefore the biological activities would also be affected (Wolfe and Dutton 2015).

1.7.1 Substrate

Usually Kombucha beverage is obtained from the fermentation of sweetened green or black teas, but some authors (Battikh and others 2012; Watawana and others 2016) have studied other substrates as an alternative for its production obtaining interesting results. Battikh and others (2012) tested the antimicrobial activity of several Kombucha tea analogues finding improved inhibition values than with the traditional beverage, mostly against *Candida* species. Velićanski and others (2013) demonstrated that sweetened Echinacea (*Echinacea purpurea L.*) and winter savory (*Satureja montana L.*) can be used as alternative nitrogen sources, reducing the fermentation time and obtaining comparable characteristics to the traditional beverage. Watawana and others (2015) fermented coconut water (*Cocos nucifera var. aurantiaca*) with the Kombucha consortium and observed an enhancement of some interesting biological activities. And recently, Ayed and others (2016) developed a Kombucha beverage from grape juice with improved sensorial and functional properties after just six days of fermentation. According to these studies, it can be concluded that investigating the therapeutic potential of Kombucha beverages prepared from different substrates would be an interesting approach.

1.7.2 Time effect

Kombucha tea fermentation normally ranges from 7 to 20 days and the biological activities may increase during this process, however the best results have been obtained in an average of 15 days (Chu and Chen, 2006). Although most of the antioxidant activities that have been obtained have increased with the incubation time, prolonged fermentation it is not recommended due to the accumulation of organic acids, which could reach damaging levels for direct consumption. Furthermore, the CO_2 generated can start to be accumulated at the interface between the biofilm and the broth and may block the transfer of nutrients creating a starved environment (Chu and Chen 2006). Selecting the duration of the fermentation period also depends on the expected sensory attributes. Reiss (1994) reported that within 6 to 10 days of fermentation a fruit-like refreshing beverage was obtained, contrary to the vinegar taste that is obtained with a prolonged period. According to the Food and Drug Administration Model Food Code for Kombucha brewing (Nummer 2013) no more of 10 days of fermentation are recommended if produced for human consumption. Coton and others (2017) studied the evolution of the microbial populations of Kombucha tea from industrial production throughout time (0, 2, 4 and 8 days). They observed that most of the AAB where more abundant in the biofilms than in the liquid broth at day 0 and that after 8 days they arrived to an equilibrium, compared to yeast species that seemed to be quite stable in both phases during the whole fermentation. Chakravorty and

others (2016) evaluated the polyphenol content and the antioxidant activity of Kombucha tea during the course of its fermentation (0,7,14 and 21 days) and observed a high tendency to increase specially after the 7th day, which may be due to the higher microbial diversity achieved by that time.

1.7.3 Temperature effect

Maintaining the optimum temperature throughout the fermentation results in a better microbial growth and enzyme activity, therefore, the fermentation benefits are improved. In addition, the antioxidant activity of foods with plant origins can be influenced by temperature variations, for example, the production of phenolic compounds (Hur and others 2014). Generally, the temperature values of Kombucha fermentation ranges between 22-30°C. However, Vitas and others (2013) carried out the fermentation of milk products with the tea fungus at temperature values of: 37, 40 and 43°C using optimization models, according to their results the temperature was the most significant factor for the duration of fermentation, and the highest values of antioxidant activity were obtained with temperature values between 37-42°C. According to Lončar and others (2006) quantities of generated acids and metabolites, as well as Vitamin C, were greater in samples obtained at higher temperatures.

1.7.4 pH

The pH is one of the most important environmental parameters affecting the fermentation of Kombucha, because some of the acids formed as acetic and gluconic, could be responsible of the biological activities of the resulting beverages. It is also closely related to the microbial growth and the structural changes of the phytochemical compounds which may influence the antioxidant activity (Hur and others 2014). However, the lowest acceptable pH value should not decrease below three, which is the one of digestive tract (Lončar and others 2006). Also, in accordance with Šaponjac (2014), to obtain a pleasant sour beverage, the fermentation should be ended when the total acidity reaches the optimum value of 4-5 g/L. However, the period of time to obtain this value may differ depending on the origin of the culture medium and fermentation conditions.

1.8 Oxygen transfer rate and scaling-up process

Most fermentation processes are aerobic and, therefore, require the provision of oxygen. If the stoichiometry of respiration is considered, then the oxidation of glucose may be represented as:

$$C_6H_{12}O_6 + 6O_2 = 6H_2O + 6CO_2$$

Where 192 grams of oxygen are required for the complete oxidation of 180 grams of glucose. However, both components must be in solution before they are available to a microorganism and oxygen is approximately 6000 times less soluble in water than is glucose, thus, it is not possible to provide a microbial culture with the necessary amount of oxygen for completing the oxidation of the glucose or any other carbon source, in one addition. At the beginning of the process, significant amounts of ethanol and monosaccharides required for acetic acid bacteria are provided by Kombucha yeasts. The oxidation of ethanol into acetic acid requires one mole of oxygen (32g) to completely oxidize one mole of ethanol (46g), therefore, the activity of acetic acid bacteria as strict aerobic organisms depends on the transfer of oxygen from the air into the fermentation broth. For that reason, a microbial culture must be supplied with oxygen during growth at a sufficient rate to satisfy the organisms demand (Stanbury 2013). As being a beverage in constant study and evolution, Kombucha has mainly been studied at lab-scale, from 200 mL to 2 L. However, few researchers have studied its fermentation in higher volumes. Malbaša and others (2006) applied a regression analysis method to a batch process of 8 L and concluded that the pH is a variable that can allow scale-up estimation. Later, Cvetković and others (2008) studied the impact of the specific interfacial area as a variable that could control the production of Kombucha tea, using reactors of 90 L and concluded that reactors having the same interfacial area, although being different in size could provide similar mass transfer conditions. And recently, Coton and others (2017) worked with volumes of 1000 L and studied the microbial ecology of the produced tea by metabarcoding and culture-based methods. They observed that the microbial population seemed not to be affected by the industrial-scale microbial stress factors, which could lead to the standardization of Kombucha tea for industrial production. Besides of the volume, there are several parameters for bioprocess development to be taken into account, where the most important include the vessels geometry and the agitation type (Junker 2004). In Kombucha fermentation according to some authors, the agitation processing affects the structure of the biofilm due to the reported loss of mechanical strength (Chawla and others 2009). In static cultures, substrates have to be entirely transported by diffusion and the oxygen availability might become the limiting factor for cell metabolism, which could have a negative effect on the production and quality of cellulose. The kinetic factor that express the relationship between the dissolved oxygen and the surface/volume of the medium is the specific interfacial area, which is directly related to other factors, such as the reactor cross-section and the mass transfer coefficient (Cvetković and others 2008). This means that the rate of Kombucha batch fermentation without agitation and without introducing gas depends on the specific interfacial area. Cvetković (2008) developed a mathematical model to

scale Kombucha tea fermentation based on several specific interface areas. The verification of the model was made in reactors of large volume (90L) and very small vessels of 0.33L. The model standardizes the optimal conditions as: 70 g/L of initial substrate (sucrose), interfacial area of 0.0231-0.0642 cm^{-1} and 14 days of fermentation. They concluded that regardless of the vessel size or volume, if the value of the interfacial area is constant they could ensure the production of Kombucha tea with similar properties. In the specific case of batch fermentation of Kombucha tea, several biological factors should be taken into account. Especially in the absence of agitation, where a microbial disintegration may occur between the aerobic acetic bacteria which will tend to occupy the surface layer and the yeast which may precipitate to the bottom of the vessel (Lončar and others 2006), and this might have negative effects in the fermentation process. Besides the fact that microbial cellulose has been already well studied by some authors (Czaja and others 2006; Campano and others 2016), the available information defining the optimal reactor conditions for its development such as, surface/volume or surface/height are limited. In order to investigate the influence of the volume in the processing, Lončar and others (2006) worked with several conditions and found that the best geometric conditions for scaling-up the fermentation were obtained with a reactor of 4L and a diameter of 17 cm. Goh and others (2012) investigated the relationship between the yield, the properties of the biofilm produced from Kombucha fermentation and the surface area, and found that the production of the biofilm was increased with an intensification of surface area, and was decreased with a broader depth. This can be explained because the metabolic process is completely aerobic and it is constantly generating carbon dioxide, which might be trapped in the pellicle and end up being accumulated in greater quantities especially in the deeper mediums. However, Caicedo and others (2001) found that even though the surface area is determinant, the height is not unimportant, since it was observed that a minimal height is needed for the development of the pellicle, this taking into account the production of several layers of cellulose throughout the fermentation which will occupy part of the initial volume. Beside all the previously mentioned parameters, Kombucha's elaboration process may also affect its final properties. The process still remains quite artisanal and the exact proportion of components may vary depending on the expected product. However, it generally follows the next order: Tea leaves or plant extracts are added to boiling water and allowed to infuse for about 10 to 15 min after which the leaves are removed. Sucrose is next dissolved in the hot tea and after the infusion is left to cool. The sweetened beverage is subsequent poured into a container and inoculated with about 3% w/v of already prepared Kombucha biofilm, afterwards the container is covered with a clean cloth and incubated at room temperature.

Nevertheless, in order to optimize the industrial production of Kombucha tea, as being a functional beverage, a complete study including high-volume production, microbiological identification and biological assays should be performed.

1.9 Biological activities

Tea is consumed in China since 5000 years ago, it is the second most popular beverage after water, and it is even considered as the oldest known medicine because of its health benefits. Torino and others (2013) reported that the bioconversion from conjugated forms of phenolic compounds into their free form during fermentation improves their healthy function. Several investigations have been done in order to understand the impact of tea fermentation with the microbial consortia and some of its biological activities have seen to be improved (**Table 3**).

Even though most of the biological assays in Kombucha tea have been done *in-vitro*, several authors have done *in-vivo* studies using rats and have found interesting results. Srihari and others (2013) evaluated the antihyperglycaemic efficacy of the fermented tea in diabetic rats by testing different concentrations of Kombucha extracts during 45 days. They observed that with the daily administration of 6 mg/kg bw the glycosylated haemoglobin was decreased and the plasma insulin was increased. Bhattacharya and others (2013) studied the protective effect of Kombucha in different organs including pancreas, liver, kidney and heart of diabetic rats models and the obtained results showed significant antidiabetic potential which allowed the restoration of the induced pathophysiological changes. Later, Gharib (2014) subjected a group of rats to electromagnetic waves increasing the iron copper levels and decreasing the zinc content. The rats were then administered with Kombucha tea during 9 weeks and a decrease in the iron content was observed, going from 99.5 to 65 µg/g. This allow to conclude that Kombucha could have ameliorative signs against the effects of electromagnetic radiation. And recently, Bellassoued and others (2015) worked with rats fed with cholesterol-rich diets and high thiobarbituric acid reactive substances (TBARS) levels, and found that the TBARS concentration was significantly reduced in the liver and kidney after the treatment with the fermented tea. Their results showed that Kombucha tea could be considered as a therapeutic agent against liver and kidney toxicities. Although several in-vitro biological activities have been studied for Kombucha tea, still clinical investigations and more in-vivo evaluations should be performed in order to confirm the claimed health benefits of the beverage.

Table 3. *In-vitro* biological activities of Kombucha tea.

Biological assays	Conditions of the tested Kombucha	Results	Authors
Antimicrobial activity	14 fermentation days	Growth inhibition of: *Shigellasonnei, Escherichia coli, Salmonella enteritidis* and *Salmonella typhimurium*	(Sreeramulu and others 2000)
	9 fermentation days	*Helicobacter pylori, Escherichia coli, Staphylococcus aureus* and *Agrobacterium tumefaciens*	(Greenwalt and others 1998)
	21 fermentation days	*Candida glabrata, Candida tropicalis, Candida sake, Candida dubliniensis and Candida albicans*	(Battikh and others 2013)
Antioxidant activity (DPPH)	10 fermentation days with a mixed culture of acetic bacteria and *Saccharomyces cerevisiae*	60% of inhibition against the radical DPPH with 0.1mL of Kombucha tea	(Malbaša and others 2011)
	7 fermentation days of Kombucha from Rooibos tea	EC_{50} of 20 mg/kg	(Hoon and others 2014)
	21 fermentation days	IC_{50} of 0.92 mg/Ml	(Chakravorty and others 2016)

Anti-inflammatory activity	8 fermentation days of Oak infusion	Suppression of the proinflammatory cytokines TNF-alpha and IL-6	(Vázquez-Cabral and others 2017)
Anticarcinogenic potential	14 fermentation days with subsequent solvent fragmentation (chloroform, ethyl acetate and butanol)	Cytotoxic effect of 21.5% in 786-O and 93.45% of inhibition of U2OS cells with 100 µg/mL, reduced cell motility in A549	(Jayabalan and others 2011)

1.10 Potential toxicity

Kombucha fermentation is commonly homemade, therefore it is important to be cautious because pathogenic microorganisms can contaminate the tea throughout the preparation. Some cases of health disorders have been reported by some individuals with suspected dizziness and nausea, severe illness, allergic reactions and head pain, thus leading to its contraindication in pregnant and lactating women (Srinivasan and others 1997; Jayabalan and others 2014; Watawana and others 2015). On the other hand, Vijayaraghavan and others (2000) evaluated oral toxicity for 90 days in rats and any toxic signs were detected The U.S. Food and Drug Administration also carried out some tests and reported that Kombucha tea is safe for human consumption. However, because of the previously mentioned reasons and to the microbiological complexity of this beverage it is always important to produce it under the FDA Model Food Code (Nummer 2013).

1.11 Conclusion and perspectives

Even though nowadays Kombucha tea is known all over the world, its biological properties are not well understood. More research concerning Kombucha fermentation kinetics is needed in order to be able to identify the produced metabolites, especially those that may be potentially beneficial and to understand its relationship with the biological activities. Regarding the Kombucha substrates, plant extracts have attracted great interest because of their multiple applications. Furthermore, the extension of a fermentation process from a laboratory scale to a commercial product is a challenge because of the difficulty of evaluating the factors which may influence the scaling process during cultivation. More scientific research should be done to understand the links between the fermentation and the biological activities of Kombucha tea, establishing it as a functional beverage with a clear evidence in the advantages and disadvantages of its consumption. There are a number of parameters and variations to be measured, controlled and experienced in order to determine the optimum fermentation conditions.

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1.13 Conclusion de l'analyse bibliographique

Cette étude nous a permis d'obtenir une vision globale de tous les facteurs qui participent de manière importante à la production de thé de Kombucha.

Tout d'abord nous avons compris que le thé noir est une source importante de vitamines, minéraux, acides organiques et composés phénoliques qui peut enrichir ses propriétés lorsqu'il est fermenté avec le consortium du Kombucha. Néanmoins, l'étonnante versatilité métabolique du consortium lui donne aussi la capacité de fermenter plusieurs plantes et sources de carbone différentes.

Le fait que le consortium soit composé de certains microorganismes d'altération tels que les levures *Brettanomyces bruxellensis* ou *Zygosaccharomyces bailii* avec des capacités uniques, ainsi comme par de bactéries acétiques productrices de cellulose lui confère une richesse extraordinaire en métabolites secondaires et composés bioactifs. Cependant, seulement un bioprocédé suffisamment adapté pourrait créer un environnement adapté à leur production.

Le résultat d'études de longues périodes de fermentation a montré que 10 jours suffisent pour produire une boisson à haute teneur en composés bioactifs et avec une évaluation sensorielle acceptable. Les fermentations de plus de 15 jours semblent diminuer significativement le profil biologique du Kombucha et augmentent en même temps les coûts de production.

Le transfert d'oxygène c'est un élément clé à prendre en compte pour la mise à l'échelle de la production de thé Kombucha au niveau industriel, dans la mesure où une bonne oxygénation du milieu permet de réaliser l'oxydation complète des sources de carbone, générant ainsi les échanges métaboliques responsables de la formation de composés bioactifs.

La recherche scientifique a permis de corroborer le potentiel bénéfique du Kombucha au niveau *in-vivo* et *in-vitro*. Cependant, Kapp *et al*., (2019) ont recherché tous les études scientifiques dans les bases de données pour toutes les années disponibles et ont trouvé un seul étude réalisé sur de sujets humains. Pour cela, étudier les effets bénéfiques du Kombucha avec des essais cliniques humains reste l'un des plus grands défis à relever.

Chapitre II : Matériels et méthodes

Sommaire

2.1 Préparation du Kombucha et caractérisation du milieu fermenté

2.1.1 Inoculum

Les souches de Kombucha utilisées dans ce projet de recherche ont quatre origines différentes, la souche de départ a été obtenue auprès d'un laboratoire français (Laboratoire Symbiotec, Villeneuve-Tolosane, France) et qu'on désignera « Industriel ». Par la suite trois nouvelles souches ont été acquises : la première une artisanale fournie par un particulier (origine France), la deuxième achetée sur le site www.je-mange-vivant.com et désignée par la suite « Commercial » et la troisième reconstituée en inoculant 40 mL d'une Kombucha acheté dans le commerce de la marque « Germline » dans 950 mL d'infusion du thé noir et 10 mL de vinaigre et qu'on désignera par la suite « Boisson ». Les quatre souches ont été repiquées chaque 15 jours afin d'être conservées et maintenues actives et ainsi que pour standardiser la procédure. Les souches issues de la fermentation du Kombucha sont couramment appelées SCOBY.

2.1.2 Préparation du thé et inoculation

Un protocole a été optimisé au LGC pour la préparation de l'infusion du thé noir et est décrit ci-dessous. De l'eau du robinet est mise à chauffer dans une chaudière électrique de 19 L (Kitchen Chef Professional®, Zj-200T), une fois que l'eau atteint 40°C, du saccharose (Fisher Chemical UK®) est ajouté à une concentration de 70 g/L. Dès que la température a atteint les 80°C, 10 g/L de thé noir variété Ceylan sont insérés dans un sachet en tissu et sont laissés infuser pendant 15 minutes. Le sachet du thé est ensuite enlevé et on laisse refroidir l'infusion jusqu'à une température inférieure à 30°C. Afin de bien caractériser l'infusion, le pH et les dégrées Brix sont mesurés après chaque préparation. Pour l'inoculation, des béchers en verre de 2 L sont utilisés avec un diamètre de 6,5 cm. Ensuite, 970 mL du thé sucré, 20 mL du milieu issu d'une fermentation précédente, 10 mL du vinaigre de cidre (Tête Noire) et 20 g/L (poids humide) de SCOBY sont ajoutés. Les béchers sont recouverts avec une charlotte en tissu pour permettre un transfert d'oxygène par diffusion et finalement sont mis à incuber dans une étuve à 25°C à l'abri de la lumière pendant deux semaines, sans agitation.

2.1.3 Détermination du pH

Le pH du thé fermenté a été mesuré avec un pH-mètre électronique (Eutech pH 700, USA).

2.1.4 Mesure de la concentration en solides solubles par réfractométrie

La mesure de dégrées Brix a été réalisée avec un refractomètre (Atago, USA).

2.1.5 Suivi de la fermentation

Afin de pouvoir effectuer le suivi de la fermentation du thé, des prélèvements ont été réalisés trois fois par semaine après une agitation douce pour bien homogénéiser le milieu.

2.1.6 Quantification des sucres, d'éthanol et d'acide acétique (UPLC-IR)

La cinétique de fermentation a été suivie par chromatographie en phase liquide à ultra haute performance avec un détecteur d'indice de réfraction (UPLC-IR). En utilisant le protocol suivant : Un millilitre de milieu fermenté a été centrifugé à 10 000 rpm/min pendant 5 min, puis le surnageant a été filtré avec un filtre à membrane (0,45 µm) dans des vials de UPLC. Le filtrat résultant a été soumis à une analyse quantitative du saccharose, du glucose, du fructose, d'acide acétique et d'éthanol par UPLC-IR (Thermo Scientific, France), comme décrit par Brou et al, (2018). Les échantillons ont été injectés dans le système de chromatographie en phase liquide à haute performance équipé d'un détecteur d'indice de réfraction et d'une colonne Rezex ROA-H+ (8%), à exclusion d'ions 250 * 4,6 mm (Phenomenex, France) thermostatée à 30°C. L'élution a été effectuée à 170 µL/min avec une solution d'acide sulfurique à 10 mM (pH 2,2). Les concentrations de chaque composé ont été quantifiées à l'aide de courbes standard et exprimées en grammes par litre (g/L). Des solutions standard ont été préparées en allant de 1,25 à 10 g/L pour les quantifications de saccharose, glucose, fructose et d'éthanol et de 0,25 à 2 g/L pour les quantifications de glycérol et d'acide acétique. Tous les étalons et dilutions d'échantillons ont été préparés avec de l'eau déminéralisée de haute qualité.

2.1.7 Détermination des carbohydrates totaux

Le principe de la technique c'est d'hydrolyser les polysaccharides par de l'acide sulfurique à chaud, les monomères obtenus se complexent à l'anthrone pour donner un composé bleu-vert (Weiner, 1978). L'intensité de la coloration obtenue est proportionnelle à la quantité de sucres. Une gamme étalon de 0 à 0,1 g/L en sucre correspondant est préparée pour chaque série de dosages. Les échantillons à doser sont dilués de façon à obtenir une concentration finale en sucres totaux comprise entre 0,02 et 0,08 g/L. Les concentrations obtenues sont exprimées en équivalent g/L de sucre d'étalonnage (sucre majoritaire dans les sucres dosés). Le réactif d'anthrone doit se préparer le jour des analyses et ensuite le garder au frais. Le protocole est le suivant : 400 mg d'Anthrone sont dissous dans 200 mL d'acide sulfurique à 95%. Dans des tubes en verre de 10 à 20 mL plongés dans de la glace avec des billes de verre, mettre 1 mL d'échantillon dilué de façon à contenir moins de 0,1 g/L de sucres totaux et 1 mL de l'eau distillé pour le cas du blanc. Puis ajouter dans tous les tubes 2 mL de réactif et bien homogénéiser au vortex. Les tubes sont gardés au froid pendant le temps de manipulation. Les tubes sont ensuite placés dans un bain-marie à 95°C pendant 5 minutes, puis immédiatement remis dans la glace. Une fois refroidis à 20°C, les tubes sont homogénéisés et l'absorbance est lue à 625 nm avec un spectrophotomètre Jenway 7315 (Cambridge,UK).

2.2 Mesure du *k*La

Le coefficient volumétrique de transfert d'oxygène (*k*La) a été déterminé par la méthode de gazage dynamique (Tribe *et al*., 1995). Le liquide a été désoxygéné par entraînement à l'azote. Ensuite, l'azote a été remplacé par de l'air et la concentration en oxygène dissous dans le liquide a été mesurée à l'aide d'une électrode à oxygène dissous (Mettler Toledo) jusqu'à ce que l'équilibre soit atteint. Les valeurs de *k*La ont été déterminées à partir de la pente de la droite obtenue à partir d'un graphique de ln (C-CL) en fonction du temps.

2.3 Caractérisation des métabolites secondaires

Le thé fermenté et non fermenté, ont été caractérisés biochimiquement en réalisant des extractions chimiques avec des solvants organiques pour pouvoir ensuite évaluer leurs potentiels biologiques.

2.3.1 Extractions liquide-liquide à polarité croissante

Le thé fermenté et non fermenté de Kombucha a été extrait par un procédé liquide-liquide (**Figure 9**) avec du cyclohexane, de l'acétate d'éthyle suivi du butanol comme solvants, en partant du solvant avec la plus faible polarité vers une plus grande polarité ; la phase aqueuse résiduelle a aussi été caractérisée. Le cyclohexane n'a pas permis d'extraire des métabolites secondaires. Dans le cas du chapitre VI l'extrait sec total du thé fermenté a été obtenu en évaporant son volume directement sans fragmenter avec les solvants organiques. Toutes les autres extractions ont été effectuées à température ambiante avec 500 mL de solvant/milieu fermenté (1:1) et ont été effectuées deux fois afin de maximiser le processus d'extraction. Les solvants ont ensuite été évaporés en utilisant un évaporateur rotatif sous vide à 35 °C (RV 10 Auto VWR, IKA, Staufen, Germany).

Figure 9. Extractions liquide-liquide.

2.3.2 Détermination des composés polyphénoliques (HPLC-DAD)

L'analyse HPLC a été réalisée avec un détecteur Thermo Scientific UltiMate pompe 3000 et un détecteur Waters DAD-150. La séparation a été réalisée sur une colonne de RP-C18, 25

cm x 4,6 mm et une taille de particule de 5 µm, à température ambiante. L'élution a été effectuée à un débit de 1,2 mL/min, en utilisant une phase mobile constituée d'eau acidifiée (acide acétique, pH = 2,65) et d'un mélange eau/acétonitrile (20:80 v/v, pH= 2,65. Le gradient utilisé était de 0,1 à 30% de B de 0 à 35 minutes; 30 à 50% de B de 35 à 40 min; 50-99,9% de B de 40–45 min et 99,9 à 0,1% de B de 45 à 60 min. 20 mL ont été injectés à une concentration de 20 mg/mL et la détection a été effectuée à 280 nm.

2.3.3 Chromatographie en phase gazeuse-spectrométrie de masse (GC-MS)

Les analyses par chromatographie en phase gazeuse et spectrométrie de masse (CG-MS) (Les Ulis, France) ont été effectuées sur un système GC/MS HP6890 à piège à ions Varian Saturn 2000 avec un système CP-3800 équipé d'une colonne capillaire DB-5MS en silice (5% de phénylméthylpolysyloxane, 30 x 0,25 mm, épaisseur du film 0,25 µm). Les conditions chromatographiques étaient une élévation de température de 60 à 260 °C avec un gradient de 5 °C/min et une isotherme de 15 minutes à 260°C. Un deuxième gradient a été appliqué de 260 à 340°C à 40°C/min. La température de l'injecteur était de 250°C et celle de la ligne de transfert de 270°C. Le balayage de masse était de 40 à 650 amu. Les extraits ont été solubilisés dans leurs solvants d'extraction (à l'exception de l'extrait aqueux dans lequel du méthanol était utilisé) à 3 mg/mL et 2 µL ont été injectés. Les composés ont été identifiés par la comparaison de leurs spectres de masse avec ceux enregistré dans NIST 08 (Institut national des normes et de la technologie).

2.3.3.1 Dérivatisation

La méthode de dérivatisation utilisée (Tzing & Ding, 2010) a consisté à dissoudre 5 mg de chaque extrait dans 1 mL d'acétonitrile et à ajouter 150 µL de réactif N, O-bis (triméthylsilyl) trifluoroacétamide (BSTFA) et 1,5 µL de chlorotriméthylsilane (TMCS). Après agitation, chaque échantillon a été incubé pendant 15 min dans une étuve chauffée à 60 °C. L'identification des composés volatils des extraits organiques, sans ou avec dérivatisation, a été réalisée avec le même équipement (GC-MS), mais le gradient suivant a été appliqué : 5

min à 60 °C, 60–270 °C à 15 °C/min, 6 min à 270 °C, 270–300 °C à 50 °C/min et enfin 4,5 min à 300 °C. L'ensemble du programme chromatographique a duré 30 min.

2.3.4 Détermination des phénols totaux par la méthode de Folin-Ciocalteu

Le test de Folin-Ciocalteu repose sur le transfert d'électrons en milieu alcalin des composés phénoliques vers les complexes d'acide phosphomolybdique pour former des complexes bleus, déterminés par spectroscopie. Pour déterminer les polyphénols totaux dans les extraits de Kombucha, la procédure suivante a été utilisée :

Cent microlitres de réactif Folin-Ciocalteu (0,2 N) ont été ajoutés à 20 µL de chaque extrait préparé à une concentration de 3 mg/mL. Après 5 min d'incubation à la température ambiante, 80 µL de solution de carbonate de sodium (75 g/L dans de l'eau) ont été ajoutés. Ensuite, le mélange a été incubé pendant 15 min et l'absorbance a été mesurée à 765 nm. Une courbe d'étalonnage standard a été réalisée en utilisant différentes concentrations d'acide gallique (0–200 µg/mL). Les résultats ont été exprimés en mg d'équivalent acide gallique (GAE)/g de matière sèche.

2.4 Evaluation de l'activité antioxydante et des activités biologiques des extraits du thé de Kombucha

Afin de tester l'activité antioxydante (test du DPPH) et les activités biologiques (Anti-inflammatoire (15 lipo-oxygénase), Anti-cancéreuse (HCT-116, OVCAR et MCF-7)) des solutions mères des différents extraits obtenus à une concentration de 3 mg/mL (DMSO 100%) ont été préparées pour chaque extrait. Toutes les activités ont été testées à une concentration de 50 mg/L d'extrait.

2.4.1 Activité antioxydante

L'activité antioxydante a été étudiée en utilisant le radical libre 1,1-diphényl-2-picrylhydrazyle (DPPH). Vingt microlitres d'extraits de Kombucha ont été mélangés avec 180 µL d'une solution méthanolique du DPPH à 0,2 mM. Le mélange a ensuite été incubé à

25 °C pendant 30 min, selon la méthode décrite précédemment (Bekir *et al*., 2013). La réduction des radicaux libres DPPH a été évaluée en lisant l'absorbance à 520 nm dans un spectrophotomètre Multiskan Go et les résultats ont été enregistrés sous la forme de l'absorbance de l'échantillon A (échantillon). Une expérience témoin a été réalisée en utilisant la même procédure sans extrait et l'absorbance a été mesurée comme étant A (blanc). L'acide ascorbique a été utilisé comme standard à une concentration finale de 0.1 mg/mL. L'activité de piégeage des radicaux libres de chaque solution a ensuite été calculée en tant que pourcentage d'inhibition selon l'équation suivante :

$$\% \text{ d'inhibition} = 100 \, (A \text{ (blanc)} - A \text{ (échantillon)}) / A \text{ (blanc)}$$

La concentration inhibitrice médiane nécessaire de chaque extrait pour inhiber à moitié le radical (IC_{50}) a été mesurée pour les échantillons avec un pourcentage d'inhibition supérieur à 70% en testant des concentrations plus faibles que 50 mg/L.

2.4.2 Test Anti-inflammatoire

Cette activité a été testée contre l'enzyme 15 lipo-oxygénase (15-LOX) (Kammoun *et al*., 2015), qui est une enzyme essentiellement exprimée par les leucocytes, et responsable de la synthèse des leucotriènes, principaux médiateurs lipidiques de l'inflammation.

La mesure de l'activité a été testée sur une plaque à 96 puits contenant 150 µL de tampon phosphate 100 mM (pH 7,4), 20 µL de la solution d'extrait à une concentration de 3 mg/mL, 60 µL d'acide linoléique (3,5 mmol/L) et 20 µL de 15-LOX (soja 500 U). Pour le blanc 20 µL de DMSO ont été utilisés. La préparation de dilutions a été faite de sorte que le DMSO ne dépasse pas 1%. Le mélange a ensuite été incubé à 25 °C pendant 10 min dans un spectrophotomètre Multiskan Go et l'absorbance a été mesurée à 234 nm. L'activité anti-inflammatoire est présentée sous forme de pourcentage d'inhibition de l'enzyme 15-LOX. L'acide nordihydroguaïarétique (NDGA) a été utilisé comme standard à une concentration de 3 mg/mL.

2.4.3 Test d'antiproliferation de cellules cancéreuses humaines

Le test MTT est basé sur la réduction du bromure de 3-(4,5-diméthylthiazol-2-yl)-2,5-diphényltétrazolium par la déshydrogénase mitochondriale de cellules intactes en un produit de formazane violet (Kammoun *et al*., 2015). La cytotoxicité des extraits a été estimée sur les lignées cellulaires du cancer du côlon humain (HCT-116), cancer des ovaires humaines (OVCAR) et cancer du sein humain (MCF-7).

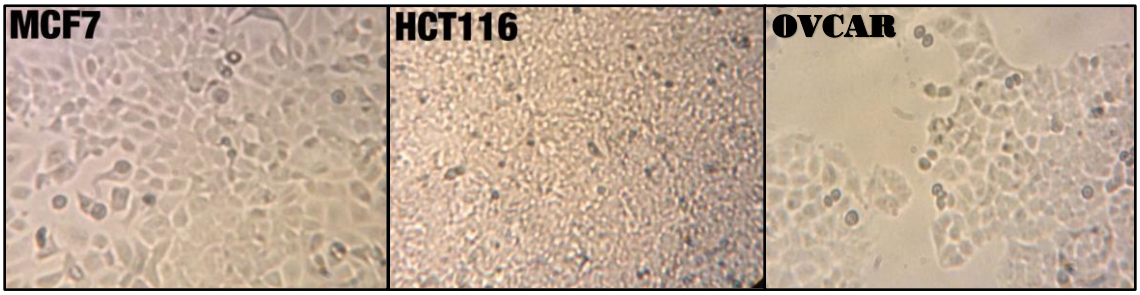

Figure 10. Lignes de cellules tumorales humaines (Rolland Ludovic, IMRCP, 2014).

Les cellules ont été maintenues à 37 °C dans un incubateur humidifié à 5% de CO_2 (Thermo Scientific, Allemagne) en utilisant le milieu d'Eagle modifié de Dulbecco (DMEM, Sigma Aldrich, Etats-Unis) pour la lignée cellulaire du cancer du sein humain et le RPMI-1640 (Sigma Aldrich, USA) pour la lignée cellulaire du cancer du côlon et des ovaires. Les cellules ont été ensemencées dans des plaques à 96 puits à une concentration de 3×10^4 cellules/puits dans 100 µL de milieu de culture, puis 100 µL de milieu de culture contenant l'échantillon à différentes concentrations ont été ajoutés après 24 h. Les cellules de contrôle non traitées ont également été conservées pour comparer l'inhibition de la croissance. Les plaques ont ensuite été incubées à 37 °C pendant 48 h. Le surnageant a ensuite été retiré et 50 µL de MTT ont été ajoutés puis incubés pendant 40 minutes. Après avoir éliminé le réactif MTT (Sigma, M-5655), 80 µL de DMSO ont été ajoutés pour solubiliser les cristaux de formazan. L'absorbance a été mesurée à 605 nm. Tous les extraits ont été remis en suspension dans du DMSO, puis dilués dans le tampon de sorte que le DMSO ne dépasse pas 1%. Le tamoxifène a été utilisé comme contrôle positif à trois concentrations différentes (0,05, 0,5 et 5 µM).

2.5 Caractérisation de la cellulose bactérienne

En raison de ses nombreuses propriétés intéressantes, la cellulose produite a aussi été caractérisée à la fin de la fermentation afin de connaitre son rendement de production et ses caractéristiques physicochimiques.

2.5.1 Calcul de rendement massique

Le biofilm final (**Figure 11**) a été pesé et le rendement a été calculé selon la formule suivante:

Rendement (%) = Poids sec de la cellulose bactérienne (g/L) /concentration en saccharose consommé (g /L) x 100

Figure 11. Cellulose bactérienne en poids humide (gauche) et poids sec (droite) (Villarreal-Soto, 2016).

2.5.2 Observation au microscope électronique à balayage (MEB)

Ce microscope permet d'observer la structure de la surface du biofilm en haute résolution pour comprendre son organisation. Cela s'est fait à partir de la production des images en haute résolution de la surface de l'échantillon en utilisant le principe des interactions électrons-matière. Les images ont été acquises en utilisant un MEB de paillasse, le TM 3000 (Keyene, France). Les biofilms étant essentiellement composés d'eau, ne peuvent pas être observés directement au microscope électronique sous peine de les dégrader lors de la mise sous vide et de ne pouvoir produire d'image puisqu'ils ne sont pas naturellement conducteurs (Priester *et al*., 2007). Une première étape de préparation est donc nécessaire pour maintenir l'échantillon dans un état morphologique aussi proche que possible de son état naturel. Pour

cela, il est nécessaire d'effectuer un traitement de fixation, préalable à l'étape de déshydratation, afin d'éviter les déformations de substances protéiniques ou lipidiques qui le composent. Dans la méthode "conventionnelle" de préparation d'échantillons pour l'observation en microscopie électronique, cette étape de fixation est réalisée chimiquement (fixation au glutaraldéhyde) suivie de la déshydratation par substitution à la température ambiante de solvants organiques (acétone et hexamethyldisilazane (HDMS)) à l'eau. Après cette étape, la métallisation de l'échantillon est nécessaire afin d'obtenir une image numérique.

1) Prélèvement

Couper le biofilm en petits morceaux de 1 $cm^{2.}$

2) *Fixation* (permet de conserver la structure du matériel biologique)

Fixateurs aldéhydiques :

– 2 volumes de glutaraldéhyde à 4% v/v dans de l'eau

– 1 volume de tampon phosphate (K_2HPO_4 / KH_2PO_4) (pH = 7,4) à 0,4 M

– 1 volume eau distillée

Le temps de fixation est de 2 heures pour les morceaux de SCOBY présentant une composition riche en eau.

Lavage : 2 bains successifs de 15 minutes

– 1 volume de tampon phosphate (K_2HPO_4 / KH_2PO_4) (pH 7,4) à 0,4 M

– 2 volumes de saccharose à 0,4 M

– 1 volume d'eau distillée

3) Déshydratation :

Le matériel biologique doit être séché avant d'être introduit dans l'enceinte sous vide secondaire du MEB par des passages successifs des échantillons dans des bains d'acétone de concentration croissante.

Acétone-eau [50%-50%], 5 minutes,

Acétone-eau [70%-30%], 5 minutes,

Acétone 100%, 30 minutes,

Acétone – HMDS [50%-50%] 5 minutes,

HMDS 100% jusqu'à évaporation (Environ 12h).

Recouvrir l'échantillon avec le produit et laisser évaporer sous la hotte.

4) Métallisation : La métallisation a pour but de rendre l'échantillon conducteur aux électrons évitant ainsi l'accumulation des charges parasites négatives à la surface de l'échantillon au cours de son observation. Les échantillons sont collés sur des supports métalliques**,** à l'aide de ruban adhésif double face en carbone, pour être ensuite introduits dans une enceinte sous vide (scan coat six – Edwards) pour subir une pulvérisation cathodique (**Figure 12**). Cette pulvérisation cathodique consiste à recouvrir les échantillons d'une fine couche d'or (entre 20 et 40 nm) tout en respectant et en conservant leur topographie.

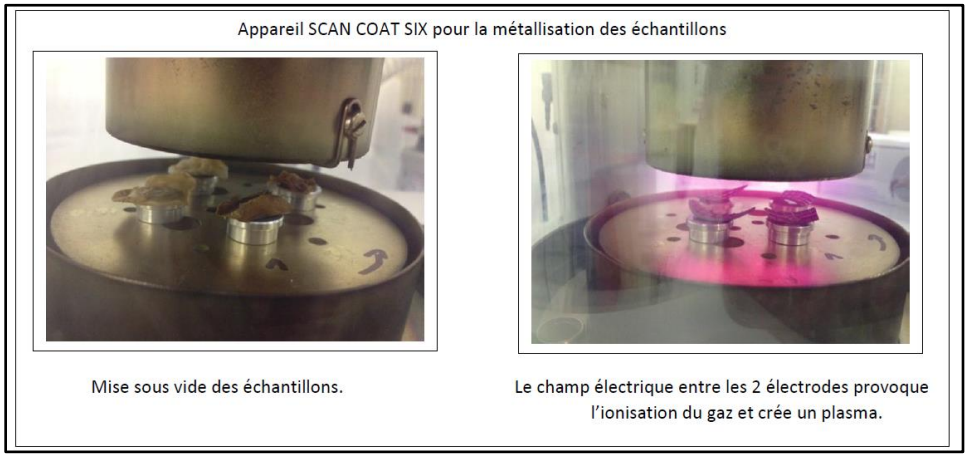

Figure 12. Métallisation des échantillons.

Afin de mieux observer la surface de biofilms, une observation a aussi été réalisée sur le centre de Microcaractérisation Raimond Castain UMS 3623. Les images ont été réalisées avec un instrument JEOL-JSM-6700F. La plage de grossissement utilisée sur les images SEM était X100 - X25000.

2.5.3 Analyse thermogravimétrique (TG) et dérivée par thermogravimétrie (DTG)

Les deux balayages ont été effectués dans des conditions contrôlées (atmosphère inerte) par un analyseur TGA Q600 de TA Instruments (USA) au sein de la SAP du LGC UMR 5503 (Bouajila *et al*, 2003). Environ 8 mg ont été introduits dans un creuset en alumine pour chaque test. Les températures ont été testées dans la plage de 25 à 1000 °C à 10°C/min. Les analyses TG et DTG ont été utilisées pour déterminer les propriétés thermiques des biofilms de Kombucha telles que la température initiale de la dégradation (T_{on}) ou la température de la vitesse maximale de la dégradation (T_d). Cette technique permet de repérer les températures des pertes de l'humidité et/ou d'autres substances volatiles.

2.5.4 Calorimétrie à balayage différentiel (DSC)

Les analyses ont été effectuées avec un instrument Q2000 de TA Instruments (USA) au sein de la SAP du LGC UMR 5503 (Bouajila *et al*, 2003). 5 mg d'échantillon ont été placés dans un support DSC en aluminium avec un couvercle en aluminium standard. Les échantillons ont été testés sous un débit d'azote de 50 mL/min. La rampe de température utilisé était de 10 °C/min de -50 °C à 400 °C. L'analyse DSC a été utilisée pour étudier les propriétés thermiques des biofilms de Kombucha en tant que la température de transition vitreuse (T_g), la température de transition de phase cristalline (T_c) et les températures de dégradation.

2.6 Extraction et quantification d'ADN microbienne

Cette méthode a été réalisée en utilisant le kit d'extraction Geneall exgene ™ avec un protocole pour la phase solide et un autre pour la phase liquide.

2.6.1 Protocole d'extraction (phase solide)

1. Laver la SCOBY avec de l'eau stérile.
2. Couper la SCOBY en petits morceaux.
3. Ajouter environ 800 mg de SCOBY dans un tube Powerbead.
4. Ajouter 550 µL of tampon SL.

5. Ajouter 90 µL de lysozyme (50 mg/mL) et 50 µL de mutanolysin (100 U/mL).
6. Incuber à 60°C pendant 15 minutes en agitant de temps en temps.
7. Ajouter 28 µL de Proteinase K et incuber pendant 15 minutes à 60°C en agitant de temps en temps.
8. Insérer le powerbead tube dans le Qiagen Tissue lyser 2 pendant 10 minutes à 20/s.
9. Centrifuger à 10000 rpm pendant 10 minutes à température ambiante et ensuite transférer soigneusement le surnageant à un tube à centrifuger de 1.5 mL.
10. Ajouter 50 µL de tampon RH.
11. Ajouter 300 µL de tampon PD et bien mélanger au vortex.
12. Centrifuger à 10000 rpm pendant 5 minutes à température ambiante et ensuite transférer soigneusement le surnageant à un tube à centrifuger de 2 mL.
13. Ajouter 900 µL de tampon TB et bien mélanger au vortex. (En cas de précipitation préchauffer dans un bain-marie à 56 °C pour dissoudre complètement).
14. Transférer jusqu'à 700 µL du mélange sur une mini colonne de centrifugation.
15. Centrifuger à 10000 rpm pendant 30 secondes à température ambiante. Jeter le passe-partout et réinsérer la mini-colonne dans le même tube.
16. Répéter deux fois les étapes 14 et 15.
17. Ajouter 500 µL de tampon NW à la mini-colonne.
18. Centrifuger à 10000 rpm pendant 30 secondes à température ambiante. Jeter le passe-partout et réinsérer la mini-colonne dans le même tube.
19. Centrifuger à vitesse maximale pendant 1 minute à température ambiante pour enlever le buffer résiduel. Et ensuite transférer à un tube à centrifuger de 1.5 mL.
20. Ajouter 30 µL de tampon EB au centre de la membrane dans la mini colonne de centrifugation. Incuber pendant 1 minute à température ambiante et centrifuger à 10000 rpm pendant 1 minute à température ambiante.

2.6.2 Protocole d'extraction (phase liquide)

1. Mélanger la phase liquide du Kombucha avant le prélèvement.
2. Verser 50 mL de liquide dans un tube falcon.
3. Centrifuger pendant 10 minutes à 5000 rpm à température ambiante. Jeter le surnageant.

4. Remettre en suspension le culot dans 550 µL de tampon SL.
5. Ajouter 33 µL de Proteinase K et incuber à 55°C pendant 30 minutes et couvrir avec papier aluminium.

La suite du protocole c'est la même que pour la phase solide à partir de l'étape 8.

2.6.3 Protocole de quantification

La quantification d'ADN a été réalisée en utilisant le Kit de dosage Qubit ™ (Thermo Fisher Scientific, USA).

1. Préparer la solution de travail en diluant le réactif Qubit dsDNA HS 1:200 dans le tampon dsDNA HS.
2. Définir le nombre de tubes de 0,5 mL à utiliser pour les étalons et les échantillons. Le test dsDNA HS requiert 2 standards. Le volume final dans chaque tube doit être de 200 µL. Chaque tube standard nécessitera : 190 µL de solution de travail Qubit ™, et chaque tube à échantillon nécessitera 198 µL. Il faut préparer suffisamment de solution de travail avant de commencer.
3. Ajouter 190 µL de solution de travail Qubit ™ dans chacun des tubes utilisés pour les étalons.
4. Ajouter 10 µL de chaque standard Qubit ™ dans le tube approprié et mélanger au vortex pendant 2 à 3 secondes, en faisant attention de ne pas créer de bulles.
5. Ajouter 198 µL de solution de travail Qubit ™ dans chacun des tubes utilisés pour les échantillons.
6. Ajouter 2 µL d'échantillon dans le tube approprié et mélanger au vortex pendant 2 à 3 secondes, en faisant attention de ne pas créer de bulles.
7. Laisser tous les tubes incuber à température ambiante pendant 2 minutes. Mettre à l'abri de la lumière.
8. Réaliser la lecture dans le Fluorimètre Qubit® 2.0 (Thermo Fisher Scientific, USA). Il faut d'abord insérer les deux standards et ensuite les échantillons. La quantité d'ADN est exprimée en ng/µL.

2.7 qPCR des levures et bactéries totales

Le protocole de qPCR utilisé est celui de Park *et al*., (2009) avec modifications et a été réalisé dans l'installation de séquençage de Teagasc (Moorepark, County Cork, Irlande). Les réactions qPCR (12 µL) contenaient 2 µL d'ADN matrice, 3,6 µL de solution d'amorces mélangée à 1 µM chacune (300 nM final), 6,4 µL de mélange maître SyBr Biorad (Thermo Fisher) contenant l'ADN polymérase, SYBR Green I et un mélange de dNTP dans un tampon. Les paires d'amorces de PCR utilisées pour amplifier les gènes d'ARNr de chaque groupe de micro-organismes utilisés dans cette étude sont ceux énumérés dans Park *et al*., (2009).

Les conditions de qPCR suivantes ont été utilisées: dénaturation initiale, 15 min à 95 ° C; suivis de 60 cycles à 94 °C pendant 20 secondes, à 58 °C pendant 30 secondes et à 72 °C pendant 45 secondes; et une extension finale de 5 min à 72 °C. L'amplification PCR a été suivie par une analyse de la courbe de fusion en procédant comme suit: 20 secondes à 95 °C et la température a diminué de 95 à 65 °C à une vitesse de 0,11 °C/s, et une longueur d'onde d'excitation de 485 nm.

2.8 Analyse de séquençage à haut débit

Les échantillons de Kombucha ont été séquencés sur la plate-forme de séquençage NextSeq de l'installation de séquençage Teagasc (Moorepark, County Cork, Irlande). La préparation de la bibliothèque a été effectuée selon le protocole Illumina Nextera XT. La quantification de l'ADN a été effectuée en utilisant le test ADNdb haute sensibilité Qubit. La qualité finale de la bibliothèque a été évaluée en effectuant une analyse sur une puce à ADN Agilent High Sensitivity et une quantification par qPCR à l'aide du kit de quantification de bibliothèque KAPA pour Illumina (Roche). Le séquençage a été effectué à l'aide d'un kit High Output V2 de 300 cycles, conformément aux protocoles de séquençage Illumina standard. Comme décrit par Doyle *et al*., (2017).

Les lectures de séquence ont été obtenues à partir du séquençage Nextseq exécuté sous la forme de fichiers Bcl. Ces fichiers ont été convertis au format fastq à l'aide du logiciel bcl2fastq. En utilisant Picard, fastq a été converti au format Sam. Picard a également été

utilisé pour éliminer les doublons. Les séquences ont ensuite été vérifiées et ajustées. Les lectures avant et arrière ont été combinées dans un seul fichier fasta pour chaque échantillon.

Kaiju (Menzel *et al*., 2016) a été utilisée pour attribuer une taxonomie aux lectures, écartant les taxa d'abondance relative inférieure à 0,1%. Ce réglage a été choisi car d'autres études ont montré un taux de découverte élevé de faux positifs inférieur à ce seuil. Superfocus (Silva *et al*., 2015) a été utilisé pour attribuer une fonctionnalité aux lectures. Tous les pourcentages rapportés à tous les niveaux taxonomiques sont des pourcentages des lectures attribuées uniquement. Les tracés récapitulatifs ont été créés dans la version R 3.6.0 en utilisant le package ggplot2 (Wickham *et al*., 2009).

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Chapitre III: Impact de la géométrie du fermenteur sur les propriétés chimiques et fonctionnelles du thé Kombucha

Sommaire

Problématique de recherche et résumé du troisième chapitre

Le principe de la production du thé de Kombucha est assez simple et pendant de nombreuses années, sa production n'a été réalisée que de manière artisanale et en petits volumes. Cependant, l'augmentation progressive de sa consommation partout dans le monde a suscité l'intérêt des industriels et des chercheurs depuis quelques années. Pourtant il y a encore peu d'informations concernant les preuves scientifiques de leurs propriétés bénéfiques pour la santé ainsi comment de la maîtrise de son procédé. D'après la revue bibliographique qui a été faite, l'étude réalisé par Cvetković *et al*., (2008) avait apporté jusqu'à présent la seule piste concernant un paramètre clé pour l'optimisation de la Kombucha (s/v = 0.0231 – 0.0642 cm^{-1}). Selon ses observations le rapport surface/volume est une variable qui définit et contrôle la fermentation quelle que soit la taille du récipient. Néanmoins, des études préliminaires réalisées dans notre laboratoire ont montré des comportements différents entre les échantillons lors de la variation du volume ou de l'ajout d'agitation, aussi en gardant la même valeur du ratio s/v. Donc, afin de vérifier la théorie on a décidé de travailler avec deux fermenteurs de géométrie différente mais en gardant le même ratio s/v **(**Erreur ! Source du envoi introuvable.). En ce qui concerne le protocole d'inoculation et de préparation de l'infusion, nous avons fait selon les conditions préétablies par le laboratoire Symbiotec (Villeneuve-Tolosane, France).

Les conditions opératoires choisies ont été les suivantes :

Tableau 4. Caractéristiques des fermenteurs utilisés.

Fermenteur	***Diamètre (cm)***	***Aire (cm)***	***Volume (cm^3)***	***s/v (cm^{-1})***	***Hauteur (cm)***	***s/h (cm)***
A	9	63,6	963	0,066	15,15	4,19
B	23	415,2	6291	0,066	15,15	27,41

Les cinétiques de fermentation ont été suivies pendant 21 jours de fermentation et des différences nettes dans la consommation de saccharose et la production d'éthanol ont été observés. Ensuite une analyse de sa composition chimique par des outils chromatographiques a été faite et quatorze composés ont été identifiés et quantifiés par HPLC-DAD, un dosage de polyphénols par la méthode de Folin-Ciocalteu a aussi été réalisé. Une forte augmentation

de certains composés a été obtenue après fermentation avec le consortium dans les deux ratios étudiés. En parallèle une analyse GC-MS avec dérivatisation nous a permis d'identifier 47 composés appartenant à plusieurs familles chimiques telles que les acides organiques, les alcools, les sucres et les composés phénoliques. Afin de corréler les schémas de production de ces molécules avec les différentes géométries, une analyse en composantes principales a été réalisée. Les résultats ont montré une corrélation négative entre le thé avant et après fermentation suggérant que ces molécules identifiées par GC-MS ont eu des comportements opposés en ce qui concerne leur production. Finalement, le profil biologique des thés fermentés a été déterminé par des techniques *in-vitro* et des résultats intéressants ont été obtenus surtout dans la condition avec le ratio s/h plus grand (B). Ce premier chapitre montre comment la détermination d'un des paramètres clés pour l'optimisation de la production du thé de Kombucha a été effectuée. Les résultants ont fait l'objet d'une publication intitulé « Impact of fermentation conditions on the production of bioactive compounds with anticancer, anti-inflammatory and antioxidant properties in Kombucha tea extracts » publié dans le journal Process Biochemistry.

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

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Abstract

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

Key words: Fermentation, Kombucha, Bioactive compounds, Black tea.

3.1 Introduction

Kombucha is a beverage of Manchurian origin obtained from sweet tea infusions which are fermented by a mixed microbial consortium composed of bacteria (e.g., *Komagataeibacter xylinum, Acetobacter xylinoides, Gluconobacter oxydans, , Gluconacetobacter hansenii, Oenococcus oeni,Komagataeibacter europaeus, Lactobacillus* sp…) and yeasts (e.g., *Saccharomyces* sp., *Schizosaccharomyces pombe, Zygosaccharomyces kombuchaensis Torulaspora delbrueckii*, *Brettanomyces* sp...) (Teoh *et al*., 2004; Jayabalan *et al*., 2010; Marsh *et al*., 2014*;* Coton *et al*., 2017). During Kombucha fermentation, black tea compounds such as flavonoids, amino acids and phenolic acids (Naveed *et al*., 2018) together with sucrose undergo a transformation by the action of yeasts and bacteria. Many metabolites, such as organic acids (glucuronic, acetic), vitamins (C, B_1, B_2, B_{12}) and ethanol, are produced by this complex microbial consortium (Lin *et al*.,1998; Malbaša *et al*., 2006; Coton *et al*., 2017). Among them, some are able to inhibit the growth of potentially contaminating bacteria. Kombucha tea is also known for being a functional food with several benefits (Watawana *et al*., 2015), such as neurodegenerative disease prevention, blood pressure reduction, antioxidant activity, hypoglycemic effect, detoxification activity and anticancer properties, which are attributed to the produced metabolites during fermentation. This fermentation also leads to the formation of a solid polymeric structure ("bacterial cellulose") that floats on the surface of the culture medium because of the activity of certain strains such as *Komagataeibacter xylinus* or *Gluconacetobacter* spp. Moreover, other species that are not part of the Kombucha consortium are also capable to produce this cellulosic biofilm, such as *Aerobacter, Agrobacterium, Azotobacter*, and *Rhizobium, Salmonella* (Mohite & Patil, 2014). As the fermentation advances, this cellulosic biofilm keeps widening and forming new layers that accumulate one over the other, and after 7 to 14 days, the cellulosic biofilm may reach a thickness between 8 and 12 mm. Its formation as well as fermentation kinetics can be influenced by several parameters such as, temperature, pH, dissolved oxygen, type of sugars, fermentation time, and vessel geometry (Phunsri *et al*., 2003; Hornung, 2006; Rosma *et al*., 2012). The last parameter is especially important in Kombucha fermentation because it is performed in static conditions. Therefore, the activity of acetic acid bacteria, which are strictly aerobic, will highly depend on the transfer of oxygen from the air into the fermentation broth (Phunsri *et al*., 2003). Several studies have shown the impact of the interfacial area on bacterial cellulose production as well as on the microbial kinetics, and different theories have been proposed (Schramm & Hestrin,

1954; Phunsri *et al*., 2003; Hornung, 2006). Although these studies propose various interesting theories, little information is available regarding the influence of these parameters on the kinetics and biological activities of Kombucha tea. Therefore, the aim of this study was to evaluate the impact of the vessel geometry by examining the dynamics and bioactive properties of fermentation products from two vessels with different surface/height ratios but the same surface/volume ratio.

3.2 Materials and Methods

3.2.1 Starter culture

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

3.2.2 Chemicals and reagents

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

3.2.3 Preparation of standard solutions

Standard stock solutions were individually prepared at concentrations of 50, 20, 10, 5, and 1 mg/L in water/acetonitrile (80:20 v/v). Quantification was performed using the method described in section 3.2.9. All of the solutions were stored at -20°C to prevent degradation.

3.2.4 Preparation of the tea infusion and inoculation

Ten grams of black tea leaves obtained from the laboratory Symbiotec were added to 1 L of boiling water and allowed to infuse for 15 min at 80°C. Afterwards, the tea leaves were removed, and sucrose was added at a concentration of 70 g/L. After the infusion was cooled to room temperature (25 °C), two different volumes 1 L (vessel A) and 6 L (vessel B), were inoculated with 40 g/L of

the starter culture provided by Laboratory Symbiotec. The inoculated black tea was poured into the different sized fermentation vessels, resulting in the same specific interfacial area s/v of 0.066 cm^{-1} but two different s/h ratios (**Table 5**). The vessels were covered with cheesecloth and incubated at 25°C for 21 days. All fermentations were performed in duplicate. The final biofilm was weighed and the yield was calculated according to the following formula:

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

Table 5. Characteristics of the fermentation vessels.

Vessel	Diameter (cm)	Area (cm^2)	Volume (cm^3)	s\v (cm^{-1})	Height (cm)	s\h (cm)
A	9	63.6	963	0.066	15.15	4.19
B	23	415.2	6291	0.066	15.15	27.41

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

3.2.5 pH determination

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

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

One milliliter of the fermented medium was centrifuged at 10 000 rpm for 5 min (Fisherbrand Centrifuge, Illinois, USA). The supernatant was then filtered through a membrane filter (Fisherbrand, PTFE) (0.45 µm) into UPLC vials. The resulting filtrate was subjected to quantitative analyses of sucrose, glucose, fructose, acetic acid and ethanol using HPLC (Thermo Scientific, Dardilly, France). Samples were injected into the UPLC system equipped with a refractive index detector and a Rezex ROA-Organic acid H+ (8%), 250x4.6 mm ion exclusion column (Phenomenex, Le Pecq, France) thermostated at 30°C. The elution was performed at 170 µL/min with a 10 mM sulfuric acid solution (pH 2.2). Twenty-five microliters of injection volume were automatically analyzed. The concentration of each compound was quantified using standard curves

and expressed as (g/L). Standard solutions were prepared with an analysis range of 1.25-10.00 g/L for the sucrose, glucose, fructose and ethanol quantifications and 0.25-2.00 g/L for the glycerol and acetic acid quantifications (**Table 6**). All the standards and sample dilutions were prepared using high quality deionized water.

Table 6. LOD, LOQ, RT and R^2 of standards for UPLC analysis.

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

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

3.2.7 Sample preparation and extraction

Kombucha fermented tea was extracted by using a differential liquid-liquid method with immiscible organic solvents of increasing polarity (ethyl acetate and butanol). The remaining aqueous phase was also characterized. All the extractions were performed at room temperature with 500 mL of solvent and 500 mL of fermented medium (1:1), and were performed twice to maximize the extraction process. Mechanical agitation was applied to increase the interfacial area between the two phases followed by a settling stage that allowed the phases to separate. The solvents were then evaporated using a vacuum rotary evaporator at 35°C (RV 10 Auto VWR, IKA, Staufen, Germany) to obtain a dry extract.

3.2.8 Chemical composition (GC-MS)

Gas chromatography-mass spectrometry detection (GC-MS) analysis was carried out with a Varian Saturn 2000 (Les Ulis, France) ion trap GC/MS HP6890 with a CP-3800 GC system fitted with a fused silica capillary DB-5MS column (5% phenylmethylpolysyloxane, 30 x 0.25 mm, film thickness 0.25 µm). Chromatographic conditions were a 60–260°C temperature increase at a gradient of 5°C/min and 15 min of isothermal conditions at 260°C. A second gradient was applied to reach 340°C at 40°C/min. The trap temperature was 250°C and that of the transfer line was 270°C. Mass scanning was performed from 40 to 650 m/z. Extracts were solubilized in their solvents of extraction (except for water extract, where methanol was used) at 3 mg/mL and 2 µL were injected. Compounds were identified by comparison of their mass spectra with those recorded in NIST 08 (National Institute of Standards and Technology).

3.2.8.1 Derivatization

The derivatization method used consisted of dissolving 5 mg of each extract in 1 mL of acetonitrile and then adding 150 µL of N,O-Bis (trimethylsilyl)trifluoro-acetamide with trimethylchlorosilane (Sigma-Aldrich). After stirring, the mixture was homogenized via ultrasound for 10 s followed by an incubation in a heated bath at 40 °C, for 15 min. All samples (2 µL) were injected directly. The identification of derivatized compounds from the organic extracts was carried out with the same GC-MS equipment but with another gradient: 5 min at 60°C, 60–270°C at 15°C/min, 6 min at 270°C, 270–300°C at 50°C/min and finally 4.5 min at 300°C. The entire chromatographic program lasted 30 min. Compounds were identified by comparison of their mass spectra with those recorded in NIST 08 (National Institute of Standards and Technology).

3.2.9 Aromatic compound and polyphenol determination (HPLC-DAD)

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

compounds, all samples were prepared at the same concentration (20 mg/mL). Twenty microliters were then injected, and the detection was performed at 280 nm.

3.2.10 Antioxidant activity (DPPH assay)

The free radical scavenging activity was measured according to a previously described method (Bekir *et al*., 2013) with some modifications. Twenty microliters of the Kombucha extracts were mixed with 180 µL of a 0.2 mM methanolic DPPH solution. The mixture was then incubated at 25°C for 30 min. The reduction of the DPPH free radicals was assessed by reading the absorbance at 520 nm in a Multiskan Go spectrophotometer, and the results were recorded as A(sample). A blank experiment was performed using the same procedure without the extract, and the absorbance was measured as A(blank). Ascorbic acid was used as a standard. The free radical-scavenging activity of each solution was then calculated as the percent inhibition according to the following equation:

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

3.2.11 Determination of total phenolic concentration (TPC)

TPC of Kombucha fermented tea was quantified using the Folin-Ciocalteu colorimetric method (Bekir et al., 2013). One hundred microliters of Folin-Ciocalteu reagent (0.2 N) were added to 20 µL of each extract prepared at a concentration of 3 mg/mL. After 5 min of incubation at room temperature, 80 µL of sodium carbonate solution (75 g/L in water) were added. Then the mixture was incubated for 15 min and the absorbance was measured at 765 nm. A standard calibration curve was done using different concentrations of gallic acid (0-200 µg/mL). The results were expressed as mg of gallic acid equivalents (GAE)/g of dry weight (dw).

3.2.12 Anti-inflammatory activity

Anti-inflammatory evaluation was performed against the enzyme 15-LOX using a previously described method (Bekir *et al*., 2013). The activity measurement was tested in a 96-well plate containing 150 µL of 100 mM phosphate buffer (pH 7.4), 20 µL of the extract solution at a concentration of 3 mg/mL, 60 µL of linoleic acid (3.5 mmol/L), and 20 µL of 5-LOX (Soybean

500 U). The mixture was then incubated at 25°C for 10 min in a Multiskan Go spectrophotometer and the absorbance was measured at 234 nm. The anti-inflammatory activity was defined as the percentage of inhibition of the 15-LOX enzyme. Nordihydroguaiaretic acid (NDGA) was used as a standard.

3.2.13 Antiproliferation evaluation on human cancer cells

This assay is based on the reduction of 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Antiproliferation of extracts on human colon cancer (HCT-116) and human breast cancer (MCF-7) cell lines was estimated as described previously (Bekir *et al*., 2013) with modifications. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany) using Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, USA) for the human breast cancer cell line and RPMI-1640 (Sigma Aldrich, USA) for the colon cancer cell line. Cells were seeded in 96-well plates at a concentration of 3×10^4 cells/well in 100 µL of culture medium, and then 100 µL of culture medium containing a sample at various concentrations were added. Non-treated control cells were also maintained for comparing growth inhibition. The plates were then incubated at 37 °C for 48 h. The supernatant was then removed, and 50 µL of MTT solution was added followed by incubation, for 40 min. After removing the MTT reagent (Sigma, M-5655), 80 µL of DMSO were added to solubilize the formazan crystals. The absorbance was measured at 605 nm. All extracts were re-suspended in DMSO followed by dilution in the buffer so that the DMSO did not exceed 1%. Tamoxifen was used as a positive control.

3.2.14 Statistical analysis

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

3.3 Results and discussion

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

Cvetković *et al*., (2008) developed a mathematical model to ensure the scaling-up process of Kombucha tea fermentation, which can be quite complex and should take into account several variables. In their study, they proposed that the specific interfacial area was the key variable that would ensure the scaling-up procedure. They concluded that regardless the vessel size, if the specific interfacial area (s/v) is the same, the final Kombucha tea may had the same characteristics. To prove this theory, two vessels (**Figure 13)** with different s/h ratio (4.19 and 27.41 cm) and the same specific interfacial area s/v (0.066 cm^{-1}) were used to determine which parameter had a bigger impact on the processing.

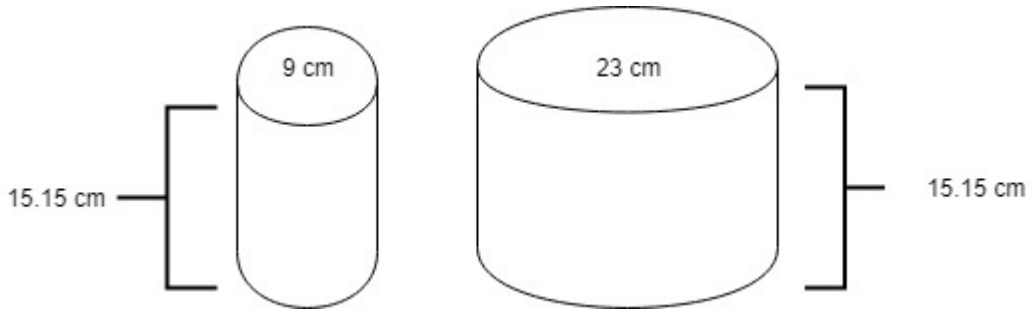


Figure 13. Dimensions of vessels used for Kombucha tea fermentation.

3.3.2 Cellulose production and scaling-up process

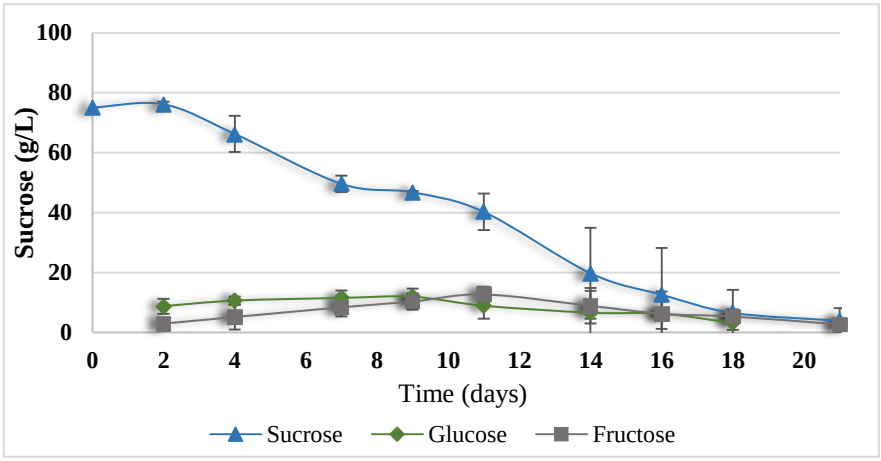
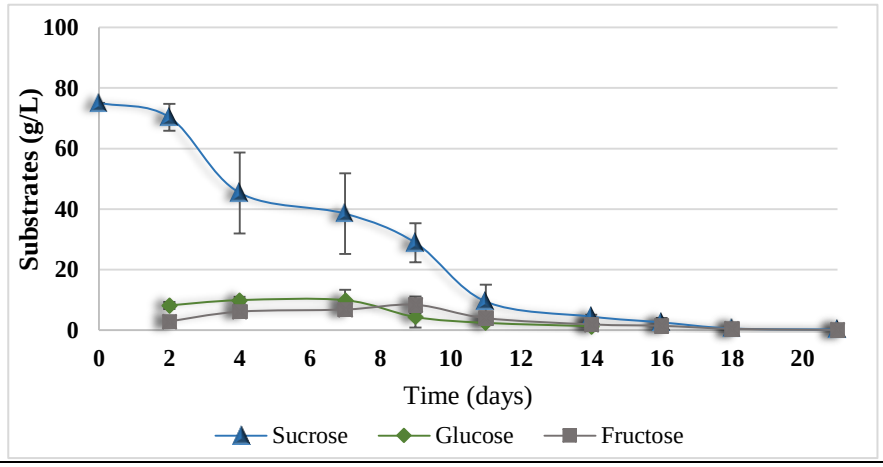
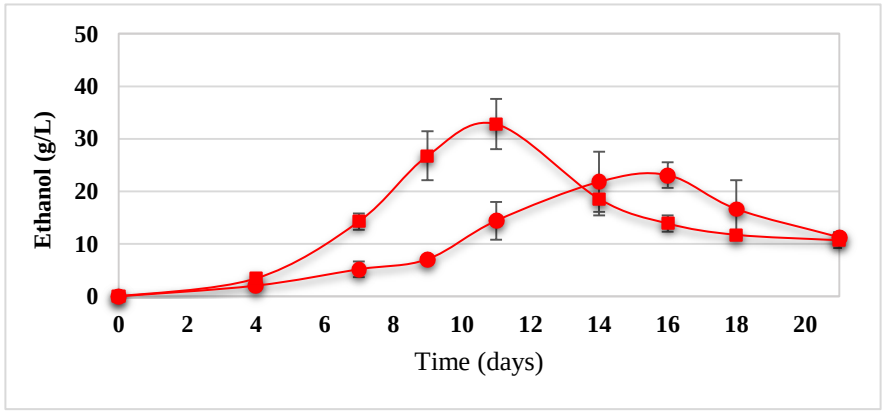
Cellulose production values were similar for both vessels reaching a value of 23 g/L with a depth of 15 cm and a final yield percentage of 1.1 and 0.7 for vessels A and B, respectively, after 21 days of fermentation. This similarity in the cellulose production, despite different vessel depths could be due to the fact that bacteria form the biofilm at the air-liquid surface. According to Hornung *et al*., (2006), this biofilm represents approximately 10% of the total bacterial cells that may have been trapped in the cellulose matrix (**Figure 14**) contributing in this way to its formation. This can be a key information regarding Kombucha fermentation, because the biofilm development could be improved and because larger volumes of fermented tea could be obtained at the same time. Thus, it was concluded that the depth was a determining parameter for the optimization of

cellulose production because it can reduce the fermentation time and therefore the cost of the process.

Figure 14. Scanning electron micrograph of bacteria embedded within the cellulose microfibrils from Kombucha tea.

3.3.3 Sugars consumption

The results of the fermentation kinetics (**Figure 15**) revealed that the parameter s/h had a clear impact, both on substrates consumption and on metabolite production. Sucrose was hydrolyzed into glucose and fructose in both cases and occurred linearly with time, reaching final values of 3.8 and 0.2 g/L in vessels A and B, respectively. After 15 days of fermentation, the sucrose concentration was around 12 and 2 g/L, for vessels A and B, respectively, similar to the values obtained by Kallel *et al*., (2012) with a concentration of 100 g/L sucrose at the beginning of fermentation. Regarding the consumption of reducing sugars, glucose is typically preferred by the microbial consortium over fructose, which normally results in a difference between the consumption of the two sugars, leading to a sluggish fermentation and therefore, an increased risk of potential contamination (Tronchoni *et al*., 2009). However, in the case of Kombucha fermentation, both sugars were consumed without accumulating in the fermented medium because of the multiple microorganisms and biochemical pathways that were occurring simultaneously. Nevertheless, in vessel B, which was the one with the higher surface area, sucrose began to be consumed in the first days of incubation. However, sucrose consumption was much slower vessel A, showing that a higher s/h ratio may accelerate the fermentation kinetics.

Substrates Ratio s/h (A)=4.19 cm	
Substrates Ratio s/h (B)=27.41 cm	
Products	

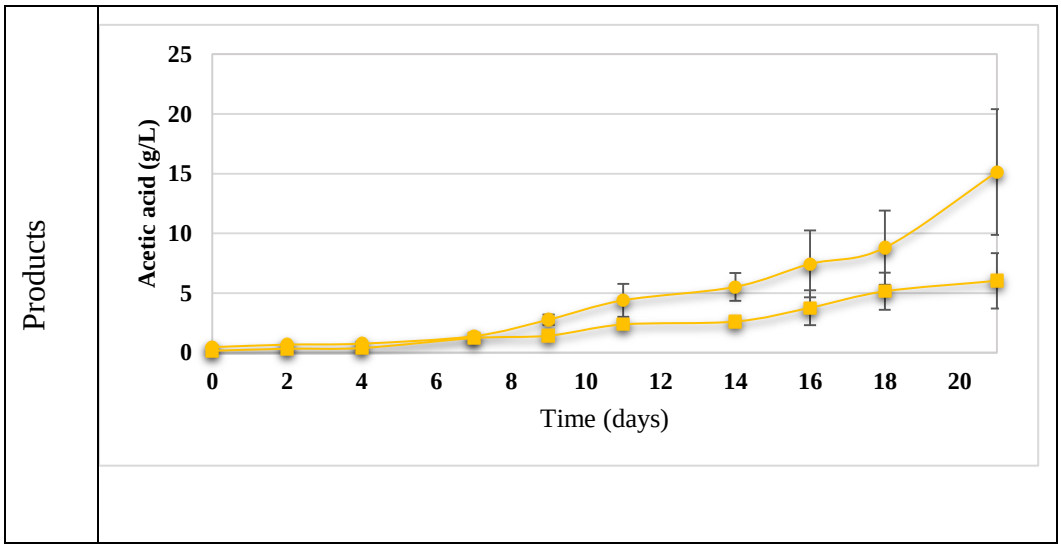


Figure 15. Kinetics of Kombucha fermentation : (◉) Ratio s/h A= 4.19 cm (■) Ratio s/h B= 27.41 cm Data are mean ± S.D. (n=2).

3.3.4 Ethanol content

The production of ethanol was faster in the vessel with the higher surface area, with a maximum production of 32.8±4.7 g/L (4.1% v/v) after 11 days, compared to a production of 23.1±2.4 g/L (2.9% v/v) after 16 days in the vessel with the smaller surface area. However, notably despite this fact, the final ethanol concentration was almost the same in both vessels after 21 days of fermentation, of around 11 g/L (1.4% v/v), although in the vessel with the higher surface area it was rapidly produced and rapidly re-consumed. The obtained ethanol concentrations are higher than those reported in other studies(Reiss, 1994; Velićanski *et al*., 2013) which were in the range of 2-8 g/L with 70 and 100 g/L initial sucrose, compared to 11.2 g/L. Differences between several Kombucha fermentations may be caused by the origin of the inoculum as well as the fermentation conditions.

3.3.5 pH values and acidity

The pH value of the initial black tea infusion was 6.4, which dropped nearly three units after 1 h of incubation with the consortium and continued to decrease during the first 15 days until it became stable, reaching a final value of 2.7. Several authors have reported the same behavior (Malbaša *et al*., 2006; Kallel *et al*., 2012; Velićanski *et al*., 2013) which is normal because of the organic acids

produced, mainly acetic acid. Cvetković *et al*., (2008) found the same tendency in terms of titratable acidity, where the specific area ratios had a huge impact. The final concentrations of acetic acid after 21 days were different between the two ratios, 15.1 in A and 6.0 g/L in B. Usually acetic acid bacteria oxidize ethanol to acetaldehyde and then to acetic acid (Jayabalan *et al*., 2014), but this was not the case in vessel B, in which the concentration of acetic acid was much lower than that of ethanol. This can be explained either by the cell concentration of acetic acid bacteria or by the metabolic path followed in each culture media, considering the hypothesis that some bacteria can produce ethanol and others can consume it and produce acetic acid. This suggests that important interactions occur between yeast and bacteria that are present both in the liquid broth and in the cellulose pellicle (Marsh *et al*., 2014; Nguyen *et al*., 2015; Chakravorty *et al*., 2016).

3.3.6 Chemical composition of Kombucha extracts

3.3.6.1 HPLC analysis

HPLC analysis was performed and several compounds were detected at 280 nm in all Kombucha extracts. **Figure 16** shows the high-resolution separation of 14 compounds in the ethyl acetate extract of the non-fermented tea samples compared to 8 compounds in the fermented samples. This decrease may be due to the microbial transformation, or to the nature of the phenolic compounds (Kallithraka *et al*., 2009). It can be observed that compound **a** increased in concentration from trace amounts to a high intensity peak in vessel B after fermentation. Compounds **e**, **f,** and **g** were transformed during fermentation and **b**, **c** and **d** were produced in vessels B and A, respectively. A similar profile was obtained with butanol extracts (**Figure 17**), in which compound **a** showed the same behavior of increased intensity together with ethyl acetate and the aqueous fraction. Regarding the aqueous fraction (**Figure 18**), the compounds obtained after fermentation were visibly of higher intensity with the ratio of vessel B compared to the trace amounts found in the non-fermented tea. This analysis allowed to observe the highest impact of the s/h ratio and the fermentation on the compounds formation or biotransformation process.

3.3.6.2 Identification and quantification of the main phenolic compounds

Fourteen components were identified and quantified in the black tea and Kombucha samples using a gradient HPLC method based in their retention times compared with those of standards. The content of each component was calculated as milligrams per kilogram of Kombucha tea extract (**Table 7**). It was observed in the ethyl acetate extracts (**Figure 16**) that the highest content of phenolic compounds was in the non-fermented tea and then it gradually decreased after fermentation.

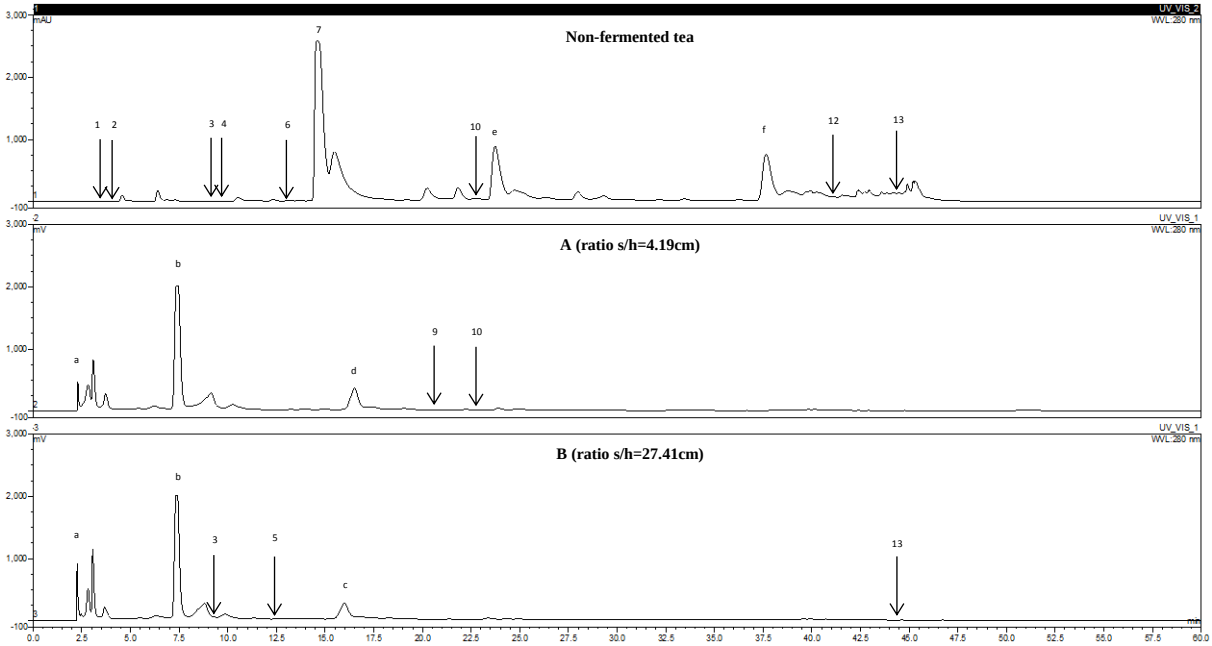


Figure 16. HPLC chromatograms of ethyl acetate Kombucha extracts. Peaks: 1-gallic acid (GA); 2-theobromine (TB); 3-catechin (C); 4-chlorogenic acid (CGA); 5-(-)-epicatechin (EC); 6-caffeic acid (CAA); 7-caffeine (CAFF); 9-ferulic acid (FA); 10-rutin hydrate (RUT); 12-trans-cinnamic acid (TCA); 13-3-tert-butyl-4-hydroxybenzoic acid (BHB).

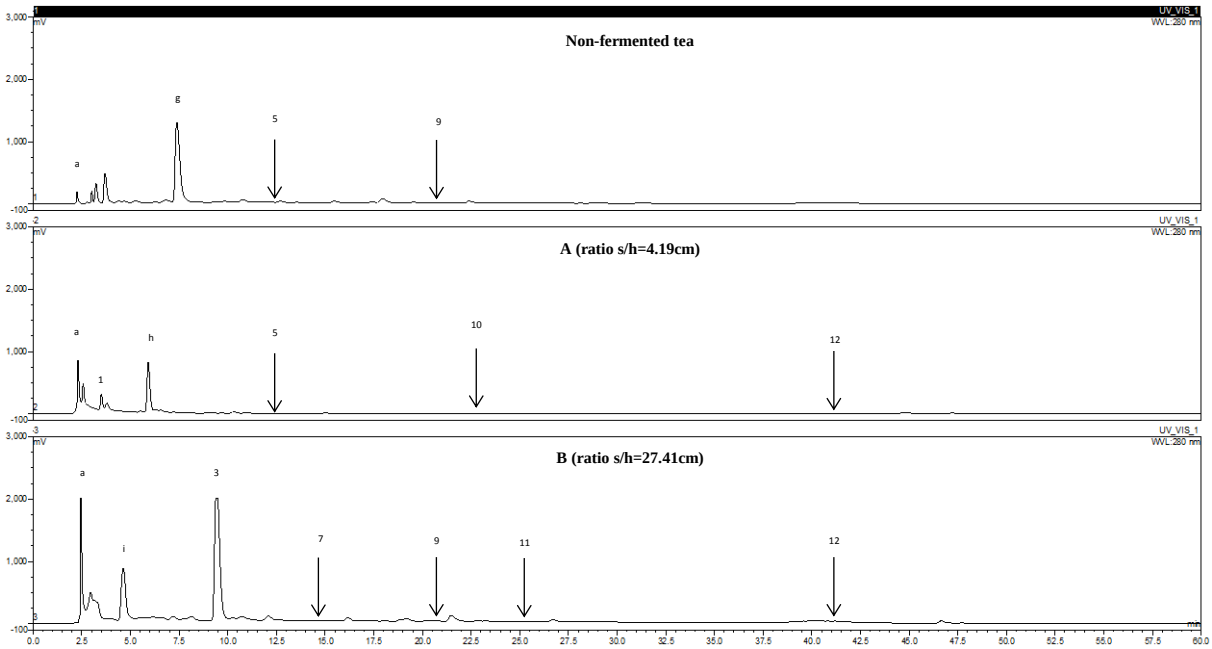


Figure 17. HPLC chromatograms of butanol Kombucha extracts. Peaks: 1-gallic acid (GA); 3-Catechin (C); 5-(-)-epicatechin (EC); 7-caffeine (CAFF); 9-ferulic acid (FA); 10-rutin hydrate (RUT); 11-taxifolin (TAX); 12-trans-cinnamic acid (TCA).

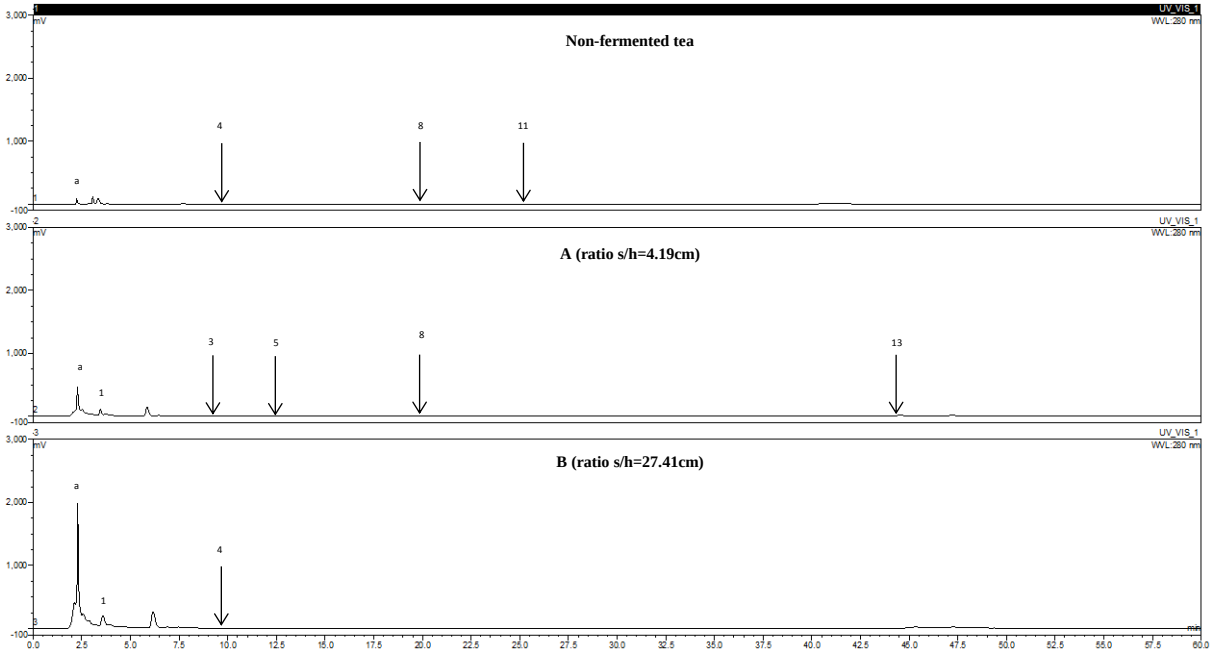


Figure 18. HPLC chromatograms of aqueous Kombucha extracts. Peaks: 1-gallic acid (GA); 3-catechin (C); 4-chlorogenic acid (CGA); 5-(-)-epicatechin (EC); 8-coumaric acid (CA); 11-taxifolin (TAX); 13-3-tert-butyl-4-hydroxybenzoic acid (BHB).

Black tea contains several phenolic compounds that may be subjected to biotransformation during processing, increasing or reducing their concentration. Yeast and bacteria are also known to play a crucial role in the metabolism of these compounds (Wang *et al*., 2018). For the identified compounds, TB and CAA were completely transformed, and CAFF was nearly depleted decreasing from a concentration of 35142.4 to 41.1 mg/kg after 21 days of fermentation. The release of catechins from microbial cells and the oxidation of EGCG or ECG by oxidative degallation (Muthumani & Kumar, 2007) could explain the amount determined in this study of 3161.6 mg/kg in the case of the vessel B compared to the one that Lin et al., (1998) found (50 mg/kg) in non-fermented black tea samples. The increased concentrations (~80%) of GA in both fermented ratios may be due to the de-esterification of the 3-galloyl substituted catechins by the enzymes secreted during the fermentation (Sang et al., 2011). On the other hand, in the butanolic extract of the fermented tea the concentration of many phenolic compounds (C, FA, TAX) increased with the high s/h ratio as did the GA content in the aqueous residual phase compared to the values in the non-fermented tea. Other compounds such as, EC and CA were considerably reduced during the tea fermentation. Similar concentrations as those reported by Souza et al (Souza *et al*., 2019) were obtained in the range of 0.1-0.3 mg g^{-1} for CA. Rutin hydrate and 3-tert-butyl-4-hydroxybenzoic acid were present before and after the fermentation, differing from TAX which seems to be produced during fermentation especially in well aerated vessels.

3.3.6.3 GC-MS analysis

The compounds identified in the GC-MS analysis are summarized in **Table 8**. Caffeine and 2,5-di-tert-butylphenol were detected in the non-fermented tea samples as well as in the fermented samples. The samples were then derivatized by silylation to increase their detectability and 47 different compounds were detected including several organic and phenolic acids, alcohols and sugars. Glycerol was found in all samples. It can be produced by *Saccharomyces cerevisiae* during the hydrolysis of glucose to ethanol (Wang & Helliwell, 2001). It can also be produced by several osmotolerant yeasts belonging to the genera *Candida, Pichia, Schizosaccharomyces, Torulaspora and Zygosaccharomyces,* that are normally present in the Kombucha consortium. Around 50% of the detected compounds were found in the ethyl acetate extracts, and these were also the compounds with the highest biological activities. This may be attributed to the presence of the

organic acids that have been reported to be active compounds in Kombucha tea (Jayabalan *et al*., 2007).

Table 7. Content of phenolic compounds, theobromine and caffeine from black tea and Kombucha extracts by HPLC (mg/kg of dry extract).

N°	RT	Compound	Non-fermented tea			A (ratio s/h=4.19cm)			B (ratio s/h=27.41cm)		
			EtOAc	BuOH	H_2O	EtOAc	BuOH	H_2O	EtOAc	BuOH	H_2O
1	**3.45**	Gallic acid (GA)	26.0±3.0				2969.0±486.6	1359.5±499.7			2859.4±76.3
2	**4.26**	Theobromine (TB)	50.2±0.8								
3	**9.56**	Catechin (C)	12.4±0.3					0.2±0.1	575.0±0.8	3161.7±7.0	
4	**9.93**	Chlorogenic acid (CGA)	8.3±0.4		4.0±0.5						2.7±0.1
5	**11.93**	(−)-Epicatechin (EC)		87.5±3.4			2.6±0.8	0.3±0.0	1.6±0.5		
6	**12.99**	Caffeic acid (CAA)	179.1±10.6								
7	**14.6**	Caffeine (CAFF)	35142.4±555.8							41.1±0.7	
8	**19.33**	P-Coumaric acid (CA)			21.3±0.1			0.5±0.3			
9	**20.60**	Ferulic acid (FA)		4.7±0.9		7.6±0.3				88.0±1.6	
10	**22.69**	Rutin hydrate (RUT)	56.4±3.1			7.13±2.8	2.3±0.1				
11	**25.3**	Taxifolin (TAX)			0.3±0.0					3.1±0.2	
12	**41.54**	Trans-Cinnamic Acid (TCA)	852.2±5.4				0.4±0.0			0.6±0.2	
13	**44.56**	3-tert-Butyl-4-hydroxybenzoic acid (BHB)	16.2±2.0					279.0±22.7	13.9±1.3		

Table 8. GC-MS analysis (area x10^6) of Kombucha tea extracts without and with derivatization.

Without derivatization											
N°	**RT**	**Compound**	**Non-fermented tea**			**A (ratio s/h=4.19cm)**			**B (ratio s/h=27.41cm)**		
	(min)		**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**
1	13.88	2,5-Di-tert-butylphenol				0.8	0.1		1.6	0.2	
2	16.84	Caffeine	9.1	0.06		5.3	14.2			7.0	
With derivatization											
1	7.02	Isovaleric acid				0.6			3.0	1.4	
2	7.59	Carbonic acid				1.3					
3	7.99	Pyruvic acid				16.5	19.1	30.3	20.0	19.3	
4	8.28	Formic acid				2.2					
5	8.42	2,3 Butanediol				41.6		11.8	48.4	49.5	7.0
6	8.57	2,3 Butanediol (isomer 2)					27.9	0.48			
7	8.91	Lactic acid				66.8	25.9				
8	9.03	Furan, tetrahydro-2-peroxy	26.0	12.7		143.7	89.0	305.5	245.1	38.4	147.0
9	9.17	Caproic acid (hexanoic)				0.8					
10	10.03	3-Hydroxypropionic acid						3.9			

11	10.12	(2E)-2-Butene-1,4-diol						7.6
12	10.18	α-Hydroxy isovaleric acid	8.8			7.9		
13	10.98	2-Hydroxy-3-methylpentanoic acid				2.9		
14	11.07	Butyric acid						5.0
15	11.10	2-Phenylethanol	4.7			11.2		
16	11.33	Glycerol	222.0	3338.4	1605.3	244.7	3219.6	3791.1
17	11.41	Octanoic acid	0.8			0.3		
18	11.70	Propanoic acid		69.2		1.5		
19	11.90	Succinic acid	923.6	443.7	56.6	1185.5	559.5	23.6
20	12.22	Z-Fumaric acid				9.6		
21	13.37	Malic acid	12.2	18.9		17.0	21.7	41.0
22	13.71	Salicylic acid	2.4					
23	13.74	Erythronic acid			9.3			
24	13.85	Threonic acid			1.4			
25	13.94	3,5-Di-t-butyl-4-hydroxybenzoic acid ethyl ester	13.0			19.7	2.4	

26	14.08	2-Hydroxyglutaric acid					17.2		12.9	4.9
27	14.2	4-Hydroxyphenyl ethanol			27.9			38.7		
28	14.31	2-Hydroxy-2-phenyl-propanoic acid			32.5					
29	14.37	2-Ketoglutaric acid					2.0		20.7	
30	14.63	Beta-D-Arabinopyranose							1.2	
31	14.86	2-Hydroxyadipic acid			0.3					
32	15.06	L-(-)-Arabitol					40.0		18.3	76.8
33	15.12	Erythritol					3.0			
34	15.26	2-Deoxyribonic acid					0.5			
35	15.55	D-(-)-Tagatofuranose							150.3	
36	15.75	L-sorbofuranose	0.8	7.5	13.1	1195.7	2976.0	10.3	424.6	
37	15.89	Citric acid						21.5		781.5
38	15.92	Mannopyranoside							4.0	
39	15.95	L-(-)-Arabitol (isomer)							110.1	
40	16.32	Lactulose					5076.2			
41	16.47	Gluconic acid				77.2	638.6	13.7	42.2	288.1

42	16.55	2-Hydroxy-3-(4-hydroxyphenyl)propanoic acid				17.2					
43	16.9	Gallic acid	159.0			460.7	85.8		651.7	73.1	
44	17.04	Ribonic acid						56.0			
45	20.23	Sucrose	8.2	1025.2				20.9			106.5
46	20.81	D-(+)-Turanose	4.7				12.3	61.5		7.1	
47	24.54	Catechin				11.5			10.0		

Lactic acid is known to function as a natural preservative in foods and to have beneficial effects on the digestive system (Malbaša *et al*., 2008). The obtained results agree with those found in a previous study (Jayabalan *et al*., 2007), in which the presence of lactic acid was observed after three days of fermentation in both green and black teas, but not in non-fermented sample, as this acid is formed by some yeasts and mainly by lactic acid bacteria. Pyruvic, malic, salicylic, succinic, citric and gluconic acids were detected in all samples, and these acids have been reported to be the most abundant acids found in Kombucha tea (Watawana *et al*., 2015).

3.3.6.3.1 Principal component analysis (PCA)

The molecules production patterns were investigated using PCA (**Figure 19**). Principal components 1 and 2 explained 59.9 and 21.7 of the total variance, respectively. This analysis was done in order to investigate the relationship between the produced metabolites in the Kombucha samples at the end of fermentation (two different ratios: A and B) and the non-fermented tea. A clear difference in the diversity of detected molecules can be observed between the NF and the A and B, showing the high impact of the fermentation (**Figure 19a**). However, a negative correlation was observed specifically in the case of the phenolic compounds (catechin, 2,5-di-tert-butylphenol) and organic acids (isovaleric, pyruvic, lactic, citric, gallic, gluconic) (**Figure 19b**). Suggesting that these molecules had opposite behaviors regarding their production, either by increasing (gallic acid) or by being produced just after 21 days of fermentation (organic acids) when compared to the NF tea. The low positive correlation observed in acids and alcohols, revealed that these compounds were present in both ratios but did not follow exactly the same production pattern.

3.3.7 Phenolic composition and antioxidant activity

The total phenolic composition present in Kombucha extracts was determined by the Folin-Ciocalteu method obtaining concentrations in the range of 36.0-221.6 mg eq AG/g for all samples (**Table 8**). Similar concentrations were found by Turkmen *et al*., (2006) who obtained values in the range of 29.1-80.4 mg eq AG/g in extracts of non-fermented black tea, showing that tea phenolic composition is fairly constant despite tea processing. However its chemical structure may change or be polymerized into molecules with higher molecular weight (Chu & Chen, 2006). This might have happened during the fermentation as the total phenolic composition in the butanol and water extracts was twice that in the non-fermented tea.

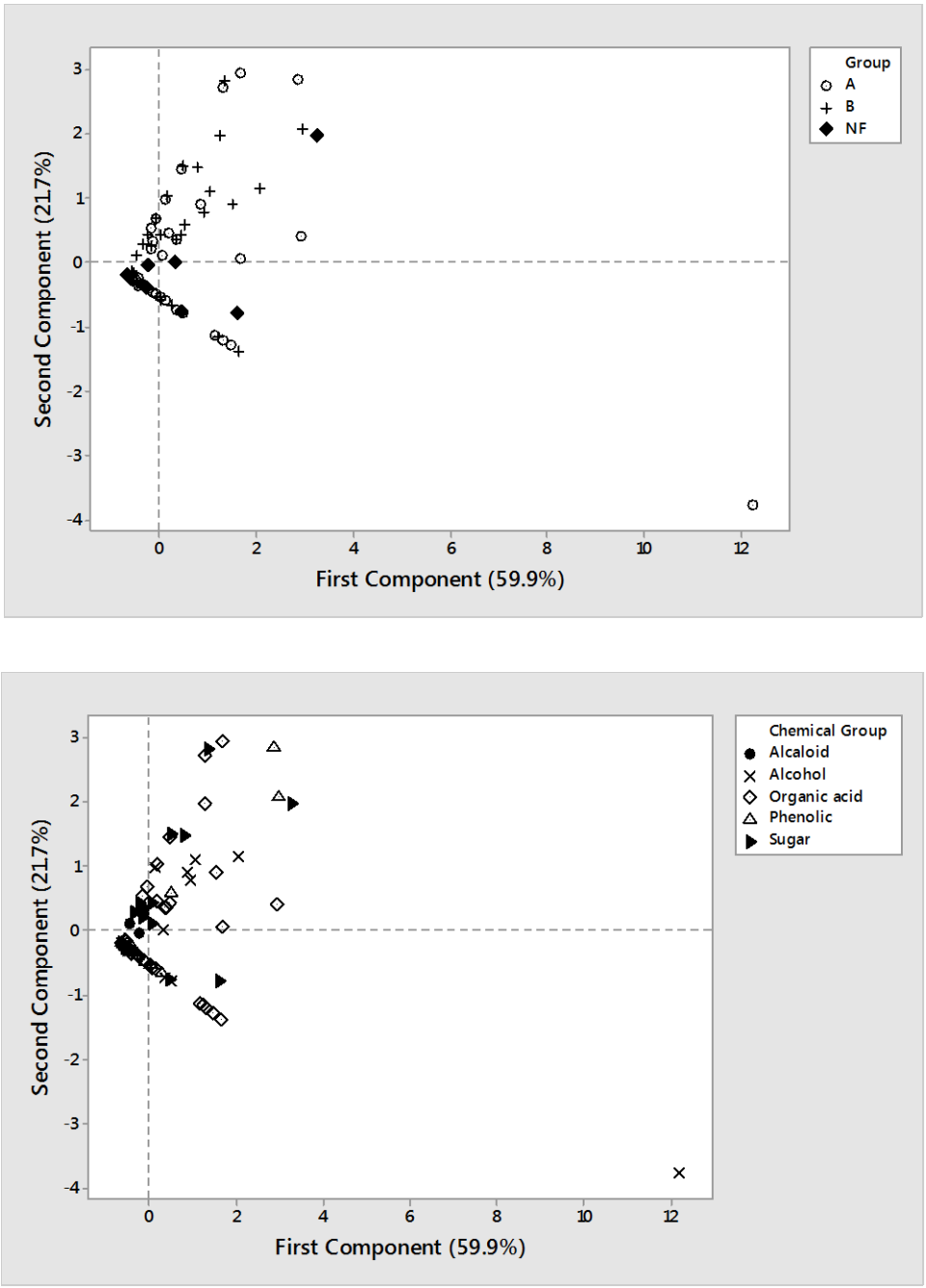


Figure 19. PCA score plots of the GC-MS obtained molecules. (a) three different samples where NF: non-fermented tea and at the end of the fermentation for the A and B conditions (ratios s/h= 4.19 cm and s/h=27.41 cm) and (b) chemical groups distribution.

The antioxidant capacity of the extracts was measured by the DPPH method in the final solvent extracts. **Table 9** shows the antioxidant activities of the tested samples (ethyl acetate, butanol and aqueous fractions), which were high in both s/h ratio conditions, indicating that the activity it is not affected by the vessel geometry or the fermented volume. The enhancement of antioxidant activity has been reported in others studies (Jayabalan *et al*., 2008; Kallithraka *et al*., 2009), which can be due to the interactions between microbial enzymes and phytochemicals

in the fermented media. In the case of the two tested ratios, the increase in the phenolic content as well as in the antioxidant activity were higher with the highest vessel ratio, increasing from 70.9% inhibition in the non-fermented tea to 96.2% in the butanol extracts and from 14.6 to 23.5% in the water extracts. The half-maximal inhibitory concentration (IC_{50}) was also calculated. The highest value was in the ethyl acetate extracts, which probably means that most of the antioxidants compounds in the tested samples were non-polar.

Table 9. Total polyphenols, DPPH and anti-inflammatory activities of Kombucha tea.

Sample	Extract	Total polyphenols (mg eq AG/g)	DPPH		Anti-inflammatory activity	
			% at 50 µg/mL	IC_{50} (µg/mL)	% at 50 µg/Ml	IC_{50} (µg/mL)
Non-fermented tea	EtOAc	215.0±0.9^{a}	98.1±0.3^{a}	9.5±0.3^{a}	66.0±1.7^{a}	>50^{a}
	BuOH	35.9±0.8^{b}	70.9±1.7^{b}	28.0±0.5^{b}	0^{b}	>50^{a}
	H_2O	3.0±0.2 ^{c}	14.6±1.1^{c}	>50^{c}	0^{b}	>50^{a}
A	EtOAc	221.6±6.8^{a}	96.4±0.0^{a}	9.0±0.1^{a}	87.7±0.4^{c}	24.3±0.2^{b}
	BuOH	43.4±2.3^{b}	77.6±2.0^{d}	26.0±0.0^{d}	36.9±0.5^{d}	>50^{a}
	H_2O	8.9±0.2^{c,d}	18.2±0.0^{e}	>50^{c}	19.0±0.9^{e}	>50^{a}
B	EtOAc	212.4±6.1^{a}	96.3±0.3^{a}	9±0.0^{a}	91.47±0.7^{c}	9.0±0.0^{c}
	BuOH	92.2±1.7^{e}	96.2±0.4^{a}	16±0.5^{e}	81.8±1.6^{f}	16.0±0.5^{d}
	H_2O	11.8±1.0^{d}	23.5±0.6^{e}	>50^{c}	20.5±0.9^{e}	>50^{a}

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

3.3.8 Anti-inflammatory activity

No studies on 15-LOX inhibition with Kombucha tea extracts have been published in the literature. Our results (**Table 9**) indicated an improvement in this activity after fermentation with the Kombucha consortium. The non-fermented tea obtained 66% or even 0% of inhibition compared to 87-91% after 21 days of fermentation. An IC_{50} value of 9.0±0.00 µg/mL was

obtained for vessel B, with ethyl acetate extract, which is close to the maximal inhibitory concentration of 7.0 ± 0.2 µg/mL for the natural LOX inhibitor Nordihydroguaiaretic acid (NDGA). These results indicated that Kombucha extracts can potentially be effective as 15-LOX inhibitors and consequently become an alternative for the development of non-steroidal drugs (NSAIDs).

3.3.9 Antiproliferation evaluation on human cancer cells

Kombucha tea extracts were tested against human breast cancer (MCF-7) and human colon cancer (HCT-116) cell lines and an enhancement of anticarcinogenic activity after fermentation in all vessels and for both cell lines was observed (**Figure 20**). The Kombucha consortium was able to produce bioactive compounds with different polarities, obtaining an inhibition increase in all the tested extracts compared to the non-fermented tea. However, the highest inhibition percentage was obtained with vessel B, especially against the HCT-116 cell line. These results showed a good similarity with those obtained by Jayabalan *et al*., (2008) where the highest inhibition percentage was found with the ethyl acetate extract (55.3%) against HCT-116 cell line. However, this was significantly different from the rest of the samples at a concentration of 50 µg/mL. Regarding the human breast cancer cells, the fermentation was found to have the largest effect, increasing the inhibition percentages in both vessels.

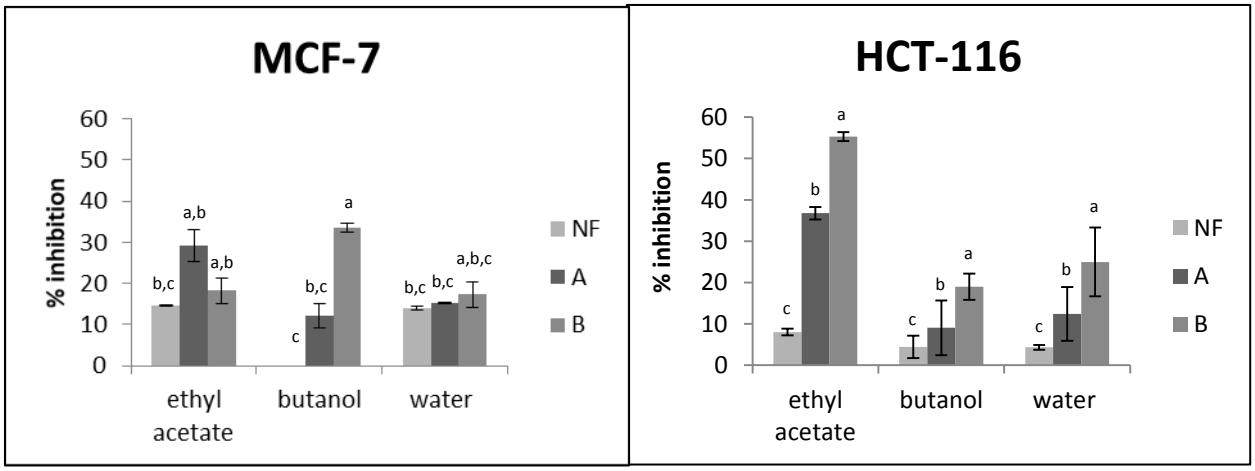


Figure 20. Effect of Kombucha tea extracts (50 µg/mL) on human breast cancer (MCF-7) and human colon cancer (HCT-116) cells. NF: non-fermented tea; A: ratio s/h= 4.19 cm; B: ratio s/h=27.41 cm. Bars that are labeled with different letters (a−c) differ significantly ($p < 0.05$).

3.4 Conclusion

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

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3.5 References

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3.6 Analyse en composantes principales

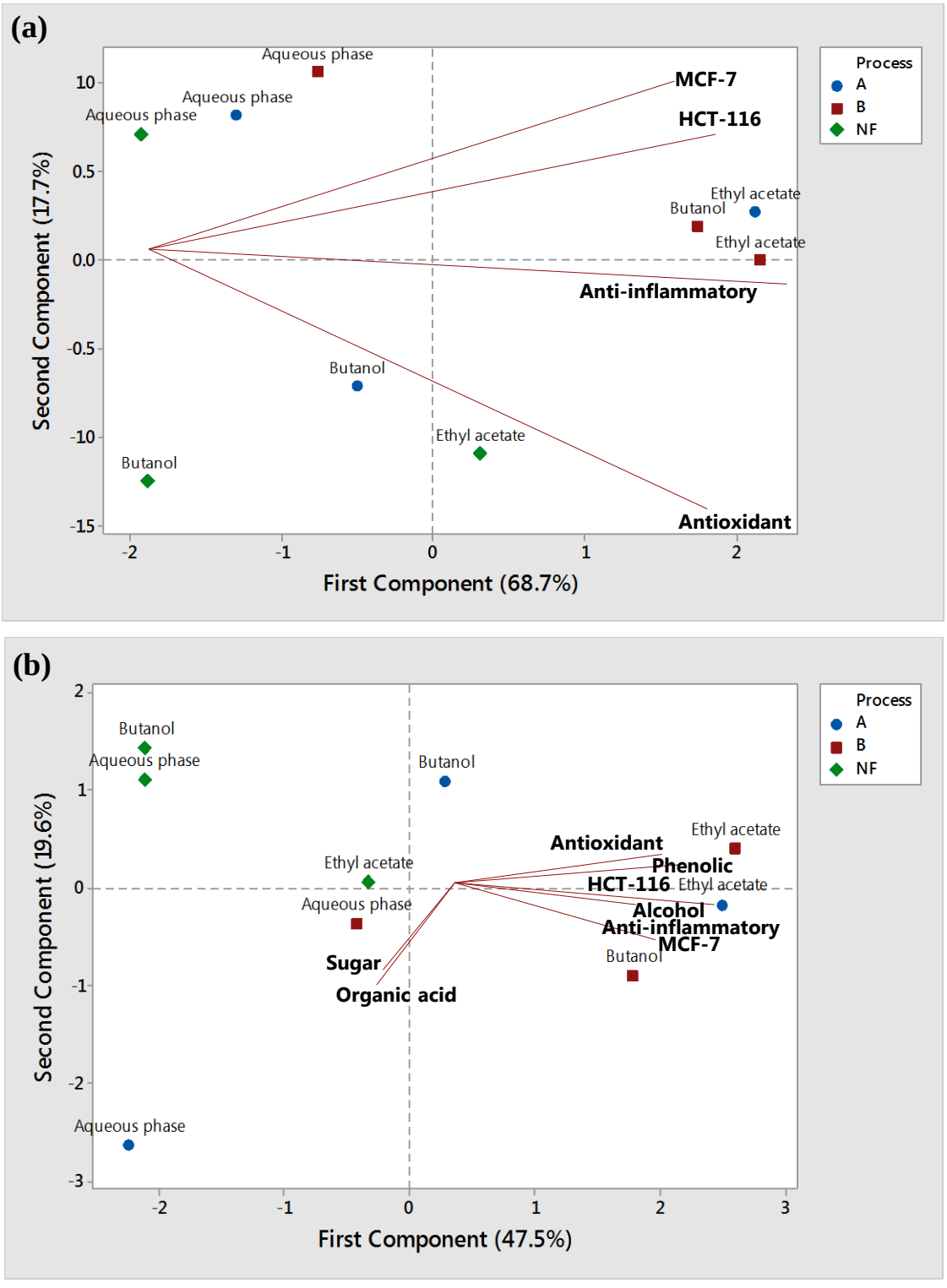

Figure 21. Analyse en composantes principales des activités biologiques (a) et familles chimiques (b). NF: thé non-fermenté ; A: ratio s/h=4.19 cm ; B: ratio s/h=27.41 cm.

Le PCA a été réalisée pour évaluer la corrélation entre les variables analysées pendant les fermentations (activités biologiques) et la proximité entre les échantillons (composition chimique). Les échantillons ont été différenciés en trois groupes principaux en fonction du solvant d'extraction **(Figure 21a)**. Aucune des phases aqueuses n'a pas été active, contrairement aux extraits fermentés d'acétate d'éthyle qui ont été fortement corrélés de façon positive aux activités cytotoxique et anti-inflammatoire. Les extraits fermentés d'acétate d'éthyle ont montré une bonne corrélation avec l'activité antioxydante. Cependant, parmi les trois échantillons étudiés, la condition B semble être la plus cytotoxique et anti-inflammatoire en gardant aussi un profil antioxydant élevé. L'analyse de corrélation avec les familles chimiques **(Figure 21b)** a montré que la présence de composés phénoliques et les alcools ont une forte corrélation avec les activités biologiques étudiées, contrairement aux sucres et acides organiques qui semblent porter aucune activité.

La classification obtenue à partir de cette analyse a permis d'identifier le procédé porteur du meilleur profil biologique ainsi comment corréler les activités avec des groupes chimiques présentes, ce qui pourrait permettre de cibler ces composés dans des études ultérieures afin d'optimiser sa production.

3.7 Conclusion du chapitre

Dans ce premier chapitre expérimental nous nous sommes intéressés à la géométrie du récipient comme facteur d'influence sur la cinétique de fermentation et la production de composés bioactifs. Le design du fermenteur semblait être un paramètre clé pour la production du thé de Kombucha.

L'élection de deux géométries différentes nous a permis d'étudier l'effet du ratio surface/hauteur sur les propriétés chimiques et biologiques du produit final. Des différences nettes ont été observées dans les cinétiques de fermentation. C'était possible de constater dans les conditions testées, qu'une surface plus grande accélère la consommation de sucres et donc la production d'éthanol.

La fermentation du thé avec le consortium a été révélé être une source potentielle de certains métabolites d'intérêt. Cependant, la géométrie choisie a eu un impact direct sur sa production. Le ratio surface/hauteur a été proposé comment un paramètre que peut améliorer le profil biologique du thé fermenté en conditions statiques. Les résultats obtenus suggèrent que

l'augmentation du contact entre le milieu de culture et l'oxygène augmente la production de molécules bioactives tels que la catéchine, l'acide férulique ou l'acide succinique.

Chapitre IV: Impact du transfert d’oxygène et analyse du métabarcodage

Sommaire

Problématique de recherche et résumé du quatrième chapitre

La formation de métabolites secondaires est contrôlée par les conditions de croissance des cellules, la composition du milieu et le procédé utilisé (Nigam, 2009). De manière générale dans les fermentations aérobies, la considération la plus importante est souvent l'apport suffisant en oxygène aux cellules. Le thé de Kombucha est une source potentielle de métabolites bioactifs et sa fermentation aérobie est réalisée classiquement de manière statique. Cependant, une étude précédente (Chapitre III, Villarreal-soto *et al*., 2019) a montré que le fait d'augmenter la surface en contact avec l'oxygène par le ratio surface/hauteur (s/h) augmente la production de certains métabolites, ainsi que son profil bioactif, dans les conditions testés. De ce fait, deux conditions ont été proposées en ajoutant l'agitation et l'aération afin d'augmenter le transfert d'oxygène afin de les comparer avec la fermentation dans la condition standard statique. Un nouveau protocole et un nouveau SCOBY ont été utilisés : s/h= 9.02 cm et s/v=0,132 cm^{-1}. Les conditions testées pour cette expérience ont été les suivantes:

1. Fermentation statique: Contrôle (C)

2. Agitation orbitale: 200 rpm (Lab-Mix 20, Fischer Scientific) (D). Dans ce cas le SCOBY a été coupé en morceaux de 1 cm^2 puis ajoutés au liquide.

3. Aération avec des bulles d'air par un diffuseur d'aquarium à un débit de Q=0,028 L/s (soit 1.68 VVM) (E).

Les valeurs de *k*La (coefficient volumétrique de transfert d'oxygène) ont été mesurées dans toutes les conditions afin de mieux caractériser les procédés mis en place. La qPCR nous a permis de suivre la population de bactéries et levures au cours de 20 jours de fermentation pour pouvoir analyser les cinétiques et l'évolution de ses métabolites. Afin de mieux évaluer l'impact du transfert d'oxygène sur les populations microbiennes, une analyse de métaséquençage a été faite révélant les espèces dominantes ainsi que leur évolution entre le début et la fin de la fermentation. Les analyses chromatographiques ont montré des différences dans les concentrations de composés phénoliques, surtout de l'acide gallique, ayant obtenu sa concentration maximale dans la condition (E). Ensuite, la diversité fonctionnelle des gènes prédits au sein du consortium microbien a été étudiée, ce qui nous a conduits à proposer les principales voies métaboliques liées à la production de certains composés avec des propriétés intéressantes tels que des acides organiques ou des composés phénoliques. Et finalement les profils biologiques ont été évalués pour les trois conditions. Autant que nous sachions, il s'agit de la première étude de la fermentation du thé de Kombucha utilisant l'agitation et/ou l'aération.

Alternative processes for the production of Kombucha tea: metabarcoding analysis, biological activities and chemical profile

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Abstract

Oxygen transfer is a key parameter for microbial growth and metabolite production in fermented beverages. Two fermentation processes with agitation (200 rpm) and aeration (Q=0.028 L/s) were tested for the production of Kombucha tea. The *k*La obtained values varied between 0.12 and 7.44 h^{-1}, changing the final concentrations of ethanol and acetic acid. The microbial diversity and relative abundance varied between the two unconventional methods and their respective control in static condition. Dominant yeasts and bacteria species where *Brettanomyces bruxellensis* and *Komagataeibacter rhaeticus*, respectively. Gallic acid production was improved after fermentation, going from 6485.9 mg/kg in the static condition to 21924.3 mg/kg in the aerated one. A significant correlation was obtained between the phenolic composition of the samples and their antioxidant activity and antiproliferation against human colon cancer cells (HCT-116). This study reveales the impact of the oxygen transfer on the functional and chemical properties of the produced Kombucha teas.

Keywords: Oxygen transfer, Kombucha tea, High throughput sequencing, Fermentation, HPLC, GC-MS, Phenolic.

4.1 Introduction

Kombucha fermentation process has been a subject of research for about 20 years, and several studies regarding its process (Malbaša *et al*., 2006; Lončar *et al*., 2006; Cvetković *et al*., 2008) and its microbiology (Marsh *et al*., 2014; Chakravorty *et al*., 2016; Coton *et al*., 2017) have been reported. However, its complex microbiota composed of different species of yeasts and bacteria propitiates a separation, as yeasts tend to precipitate in the bottom of the vessel while bacteria, being strictly aerobic microorganisms tend to occupy the surface layer of the medium by being entrapped within the floating cellulose matrix (Chen & Liu, 2000). This lack of microbial homogeneity could affect Kombucha fermentation kinetics and hence its metabolites production. One physical parameter that can help to ensure consistency and more homogenous reaction conditions is the oxygen transfer rate (Schmidt, 2005) which is an important factor for the optimization and scale-up of fermented beverages. The production of Kombucha tea is done in static conditions, having the cellulose matrix floating at the liquid-air interface. The aforementioned could result in detrimental changes for the microbiota by blocking the transfer of nutrients due to the accumulation of microbially produced carbon dioxide below the cellulose matrix and inhibiting the transfer of oxygen to the liquid broth (Chen & Liu, 2000). In a fermentation system, the formation of secondary metabolites is controlled by the microbial growth conditions, and some authors (Pfefferle *et al*., 2000; Alonso *et al*., 2005) have already observed that aeration and agitation improve its production. Moreover, regarding the scale-up, it is important to conserve the parameters that are essential for the microorganisms, and so far, the pH, interfacial area and microbial ecology have been studied from eight up to a thousand liters by different authors (Malbaša *et al*., 2006; Cvetković *et al*., 2008; Coton *et al*., 2017). A previous study (Villarreal-soto *et al*., 2019), also showed that increasing the surface of the liquid-air interface and the surface/height (s/h) ratio improved the functional profile of the Kombucha tea. Nevertheless, all those studies were done in static conditions and the oxygen transfer, which is a critical limiting factor on microbial metabolism is greatly impacted by the aeration and agitation (Weber & Agblevor, 2005; Rahbani *et al*., 2015). Therefore, the aim of this study was to carry out two unconventional fermentation methods for this complex beverage using aeration and agitation in order to see if these parameters could lead to the production of a Kombucha tea with improved characteristics. As far as we know, this is the first time that Kombucha fermentation is carried out using the above-mentioned conditions.

4.2 Materials and Methods

4.2.1 Starter culture

The Kombucha SCOBY used in this study was obtained from the website www.jemangevivant.com. The culture was produced using a backslopping method.

4.2.2 Preparation of Kombucha tea and set-up of the alternative fermentation conditions

Black tea was prepared according to the following procedure: 70 g of sugar were added to 1 L of water at 40°C. Once the water reached 80 °C, 10 g of black tea (Royal Ceylan, Lipton ®) were also incorporated and allowed to infuse for 15 minutes. Then the tea was removed and the infusion was left to cool. After the infusion was cooled to room temperature (25 °C), tea was poured into 2 L glass containers (ratios: s/h=9.02 cm, s/v=0.132 cm^{-1}) and 20 mL/L of the previous fermented liquid media, 10 mL/L of cider vinegar and 20 g/L of SCOBY were added. Three different conditions were tested for this experiment:

1. Static fermentation/Control (C)
2. Orbital shaking: 200 rpm (Lab-Mix 20, Fischer Scientific) (D)
3. Aerated with air bubbles (Air bubble aquarium) (Q=0.028 L/s) (E)

In the case of the agitated condition the SCOBY was cut in pieces of 1 cm^2 and then added to the liquid. The vessels were covered with cheesecloth and incubated at 25°C for 20 days. All fermentations were performed in triplicate.

4.2.3 *k*La measurement

The volumetric coefficient of oxygen transfer (*k*La) was determined by the dynamic gassing out method (Tribe *et al*., 1995). The liquid was deoxygenated by stripping with nitrogen. Then, nitrogen was replaced by air and the dissolved oxygen concentration in the liquid was measured with a dissolved oxygen electrode (Mettler Toledo) until equilibrium was reached. The *k*La values were determined from the slope of the straight line obtained from a plot of ln (C-CL) against the time.

4.2.4 Quantitative PCR analysis

The qPCR protocol used is that of Park *et al.*, (2009) with modifications .The qPCR reactions (12 µL) contained 2 µL of template DNA, 3.6 µL of primer solution mixed with 1 µM each (300 nM final), 6.4 µL of SyBr Biorad masterbatch (Thermo Fisher) containing the DNA polymerase, SYBR Green I and a mixture of dNTPs in a buffer. The pairs of PCR primers used to amplify the rRNA genes of each group of microorganisms used in this study are those listed in Park et al., (2009). The following qPCR conditions were used: initial denaturation, 15 min at 95 °C; followed by 60 cycles at 94 °C for 20 seconds, at 58 °C for 30 seconds, at 72 °C for 45 seconds; and a final extension of 5 min at 72 °C. The PCR amplification was followed by the analysis of the melting curve as follows: 20 seconds at 95 °C and the temperature decreased from 95 to 65 °C at a rate of 0.11 °C/sec.

4.2.5 High throughput sequencing analysis

Kombucha liquid samples (Start: day 1 and End: day 20) from the three conditions (C, D and E) were sequenced on the NextSeq sequencing platform in the Teagasc sequencing facility (Moorepark, County Cork, Ireland). Library preparation was carried out according to the Illumina Nextera XT protocol. Quantification of DNA was done using Qubit High Sensitivity dsDNA assay. Final library quality was assessed by running on an Agilent High Sensitivity DNA chip, and quantification by qPCR using the KAPA Library Quantification Kit for Illumina (Roche). Sequencing was carried out using a 300 cycle High Output V2 kit, following standard Illumina sequencing protocols. As described by Doyle *et al*., (2017). Sequence reads were obtained from the Nextseq sequencing run in the form of Bcl files. These files were converted to fastq format using bcl2fastq software. Using Picard, fastq was converted to Sam format. Picard was also used to remove duplicates. The sequences were then quality checked and trimmed. Forward and reverse reads were combined into a single fasta file for each sample. Kaiju (Menzel *et al*.,2016) was used to assign taxonomy to the reads, discarding taxa with relative abundance of less than 0.1%.This setting was chosen as other studies have shown a high false positive discovery rate below this threshold. Superfocus (Silva *et al*.,2016) was used to assign functionality to the reads. All percentages reported at all taxonomic levels are percentages of the assigned reads only. Summary plots were created in R version 3.6.0 using the package ggplot2 (Wickham *et al*., 2009).

4.2.6 Sugars, ethanol and acetic acid quantification

Sugars consumption and metabolites production were analyzed in the liquid phase by ultra performance liquid chromatography (UPLC-RI) (Thermo Scientific, Dardilly, France). According to the method described by Villarreal-soto *et al*., (2019).

4.2.7 Extraction methodology and biological evaluation

Sequential liquid-liquid extractions with organic solvents (ethyl acetate and butanol) were done with the Kombucha samples at the end of the fermentation. The solvents were then evaporated using a vacuum rotary evaporator at 35 °C (RV 10 Auto VWR, IKA, Staufen, Germany) in order to obtain a dry extract.

Stock solutions were prepared from each dry extract in a concentration of 3 mg/mL, using DMSO 100% as solvent. Then, for all the biological evaluations the stock solutions were tested initially at 50 µg/mL and at 25 or 5 µg/mL in the case of high inhibition percentages (>70%). Then the antioxidant, anti-inflammatory and antiproliferation activities were evaluated according to the methods previously described (Villarreal-soto *et al*., 2019). The non-fermented tea (NF) was also evaluated using the same protocol.

4.2.7.1 Phenolic and aromatic compounds determination

The dried extracts were injected in a concentration of 20 mg/mL of acetonitrile and water (20:80 v/v) and then phenolic and aromatic compounds were identified and quantified by HPLC-DAD (Thermo Scientific Dionex Ultimate 3000). Chemical composition was performed by gas chromatography-mass spectrometry (GC-MS) Varian Saturn 2000 (Les Ulis, France). Total phenolic composition was quantified using the Folin-Ciocalteu assay.

4.2.8 Statistical analysis

The programs Minitab 17 and Microsoft Excel were used for the statistical treatment of the data. A Pearson's product-moment correlation and its statistical significance ($p < 0.05$) were run to assess the relationship between the total phenolic composition (TPC) and the biological activities for all the samples studied (12). All data are expressed as the mean ± standard deviation (SD) of triplicate measurements.

4.3 Results and discussion

4.3.1 Oxygen transfer coefficient and microbial dynamics

The obtained *k*La values are remarkably lower than those of other research studies done in bioreactors (Weber & Agblevor, 2005; Rahbani *et al*., 2015). However, a significant impact was observed in the three tested conditions, obtaining values from 0.12 h^{-1} in the static control, 1.5 h^{-1} in the agitated condition and 7.44 h^{-1} in the case of the aerated one. **Figure 22** shows the fermentation kinetics of each condition and the evolution of its respective microbial population in the liquid phase during 20 days of fermentation. It can be observed that sucrose was consumed slower with the oxygen transfer coefficient of 0.12 h^{-1} compared to the one of 1.5 h^{-1}, which can be explained by the fact that agitation allowed the homogenization of the yeasts avoiding their precipitation at the bottom of the vessel, thus accelerating its activity. Regarding the ethanol production, similar concentrations of around 25 g/L were reached with the lower *k*La values. Both conditions created a semi-aerobic environment for the facultative anaerobes yeasts, facilitating a stable population throughout the fermentation. Conversely, nearly no ethanol was produced in the aerated condition with the higher coefficient of 7.44 h^{-1}, where the hydrolyzed sugars where directly synthetized towards the production of bacterial cellulose and acetic acid. The metabolism of yeasts has been reported to be inhibited by high concentrations of acetic acid (4.5-6 g/L) (Aguilar *et al*., 2003). This could explain the cell decrease from day 7 when the acid concentration started to increase from 9.15 to 37 g/L. Regarding the yeasts population, the agitated condition seemed to increase the amount of cells compared to the other conditions (**Figure 22D**). This could be due to their better distribution in the media, or to the fact that the SCOBY matrix was disaggregated for the inoculation, liberating the entrapped yeasts into the fermented liquid. Acetic acid bacteria are obligate aerobes, which can explain their lower concentration in the static condition (**Figure 22C),** where the SCOBY covered the liquid-air interface decreasing the oxygen transfer. According to Hornung *et al.*, (2006) only around 10% of the total bacterial cells are active in the oxygen rich air space and the rest remains constant, congruent with the results of this condition. Kombucha fermentation is done with the traditional method of surface culture, which means that most of the bacteria are embedded into the SCOBY allowing them to stay closer to the air. However, in the case of the agitated condition the cellulose matrix was cut and introduced into the liquid, changing the fermentation into a submerged process (Barja *et al*., 2016). The fact that the bacteria population remained mainly constant and that at the same time the ethanol concentration tended to increase instead of being transformed into acetic acid, could mean that the bacteria where alive but in an inactive

state due to the lack of contact with the atmospheric oxygen (Hornung, 2006). Acetic acid ability to inhibit growth of microorganisms includes bacteria. Nevertheless, some species such as *Komagataeibacter* are well known for their high resistance to this lipophilic acid and this specie has been reported in the Kombucha consortium (Coton *et al*., 2017). This could explain the abundant concentration of bacteria despite the high acetic acid concentration, which reached around 40 g/L in the aerated condition (**Figure 22E**). Moreover, the decrease of the acetic acid concentration around seven days could be due to the formation of a sphere of cellulose in the media, which could have trapped part of the microbial population, reducing its concentration.

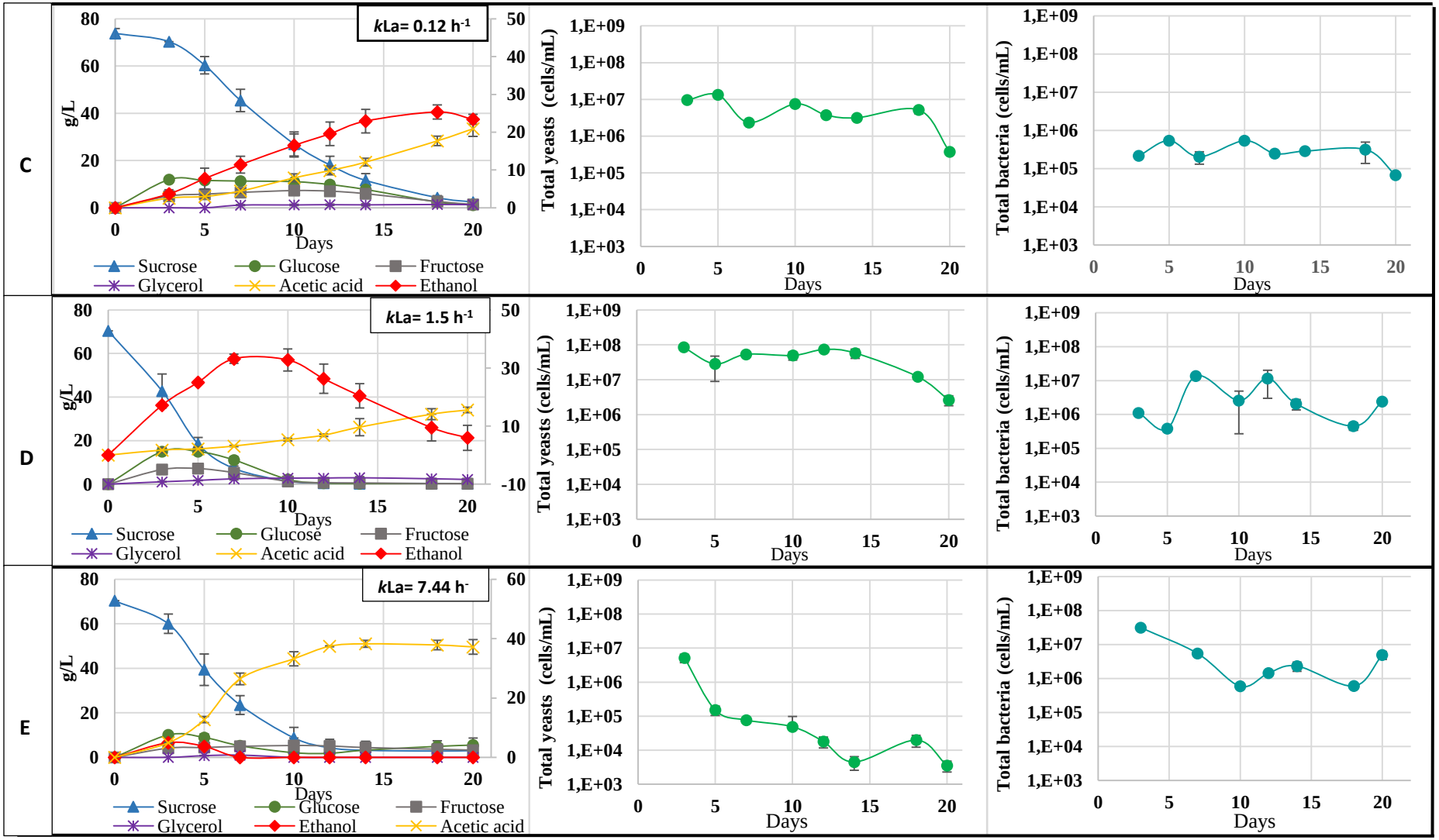

Figure 22. Fermentation kinetics of some metabolites by UPLC-RI and quantitative, Sucrose left Y-axis, Glucose, Fructose, Glycerol, Ethanol and Acetic acid, right X-axis and qPCR of yeasts and bacteria in the liquid phase. Where C: static media, D: 200 rpm, E: Q=0.028 L/s.

4.3.2 Metabarcoding analysis

The relative abundance and dynamics of the microbial population in Kombucha liquid samples was evaluated by high throughput sequencing. In order to compare its composition, samples were taken at the beginning and end of the fermentation (20 days). The analysis revealed the presence of different yeasts and bacteria species and a different distribution of the identified genus according to each condition (**Figure 23**).

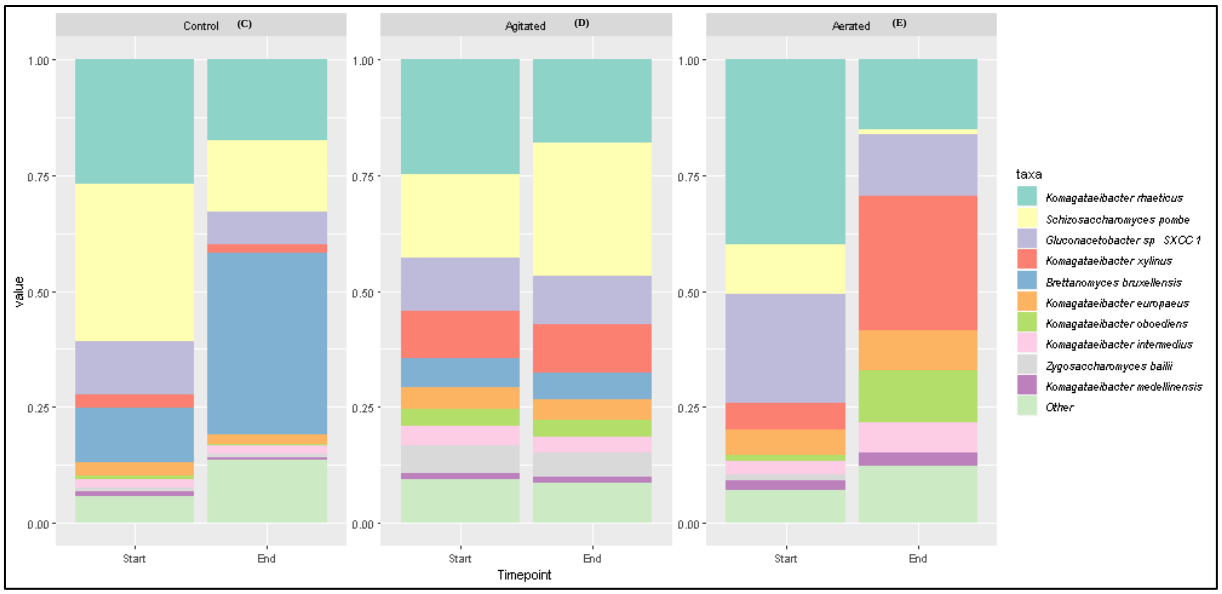

Figure 23. Bacterial and yeasts species diversity of Kombucha samples using high throughput sequencing analysis. Where C: static media, D: 200 rpm, E: 0.028 L/s and Start: day 1, End: day 20.

4.3.2.1 Bacterial ecology

Bacterial population of the Kombucha samples belongs to the Proteobacteria phylum. The genus *Komagataeibacter* was found to be the dominant one, with species such as, *K. rhaeticus*, *K. europaeus*, *K. hansenii*, *K. intermedius*, *K. oboediens* and *K. xylinus*, which have been already reported to be present in the Kombucha consortium by Coton *et al*., (2017). However, the species *K. kakiaceti* and *K. medellinensis* were also identified in this study. These two species have already been studied for their ability to oxidize ethanol into acetic acid with high efficiency (Barja *et al*., 2016) nevertheless they have not been reported in the Kombucha consortium before. The genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* were also present in Kombucha samples. *Komagataeibacter rhaeticus* was the most representative species of the bacterial population, with its highest abundance in the aerated condition (E), which was the one with the highest concentration of acetic acid (**Figure 22E**). This species is known for

its high acetic acid resistance due in part, to the presence of the phosphatidylcholine. This phospholipid increases its concentration in acidic conditions changing the permeability properties of the outer membrane, and decreasing the transport of some lipophilic molecules such as acetic acid (Barja *et al*., 2016).

4.3.2.2 Yeasts ecology

Regarding the yeasts population, Ascomycota was the main fungal phylum, which is similar to the results obtained by Marsh *et al*., (2014) who did a sequence-based analysis of some tea fungus samples and found *Zygosaccahromyces* as the dominant genus. *B. bruxellensis* appeared to be the dominant species in this study being present in all samples, with a highest proportion in the static condition (C). Coton *et al*., (2017) found similar results, obtaining *Dekkera bruxellensis* and *Dekkera anomala* as the most representative yeasts species in black and green tea, respectively. Liu *et al*., (1996) identified *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Brettanomyces bruxellensis* as the representative yeast isolates. Later Teoh *et al*., (2004) found *Z. bailii*, *Schizosaccharomyces pombe* and *Torulospora delbreuckii* as the predominant yeast species isolated from four Kombucha cultures. The identification of *Saccharomyces cerevisiae* seems to be consistent in most of the previously mentioned studies; however, this specie was not identified in the Kombucha consortium from this study. This shows that not negligible differences are present in the microbial population of several Kombucha teas, which may also impact the biological properties of the final product. *Brettanomyces bruxellensis* was the dominant yeast species, which reached its highest proportion in the static condition (C), followed by the agitated condition (D). This yeast is known for its high resistance to ethanol stress and ability to grow in low pH values, as well as for its tendency to ferment sugars to ethanol and acetic acid in aerobic conditions (Steensels *et al*., 2015). This may explain its high abundance in these conditions (D,C), which reached the highest ethanol concentrations of around 35 g/L after 10 days of fermentation (**Figure 22**) and 25g/L after 21 days (**Figure 22C).** Besides this aforementioned characteristics *Brettanomyces* spp. are also responsible for the production of several volatile phenols such as, 4-ethylphenol, which was also detected in this sample (**Table 11**). Metabarcoding analysis revealed a higher homogeneity of the microbiome taxonomy in the static condition (C). Contrary to the two unconventional conditions, which increased the population of several species, such as, *Z. bailii* and *Komagataeibacter* sp. in the case of the agitated (D) and aerated conditions (E), respectively.

4.3.3 HPLC analysis of main phenolic compounds

The obtained extracts were analyzed by a HPLC-DAD at 280 nm, which allowed the identification and quantification of 12 compounds (**Table 10**). Gallic acid, caffeine, caffeic acid and (-)-epicatechin appeared to be the most representative compounds in all samples. Regarding the extraction yields, similar values were obtained for the ethyl acetate and butanol extracts in the static (C) and agitated (D) conditions, going from 1.22 to 1.6 g ext/L. However, the aerated condition (E) obtained bigger differences between solvents, going from 0.82 g ext/L with the ethyl acetate extracts till 5.90 g ext/L with the butanolic ones (**Figure 24**).

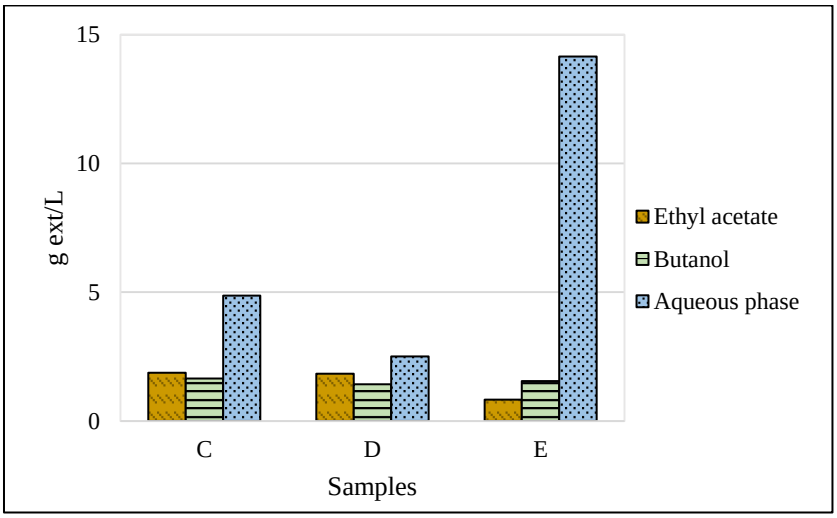

Figure 24. Extraction yields of different solvents and residual aqueous phases. Where C: static media, D: 200 rpm, E: 0.028 L/s

The obtained results (**Table 10**) showed that the applied processing could also influence the presence of caffeine, which had a tendency to increase, going from 114.53 to 1308.5 mg/kg after fermentation in the case of the aerated condition. Probably due to the inability of yeasts to adjust to high aeration rates, diminishing therefore its ability to degrade caffeine (Gummadi & Sucharita, 2009). However, a decrease of around 50% was observed in the case of the static one, which could be explained by the fact that yeasts can utilize caffeine as a nitrogen source decreasing its concentration. The same behavior of caffeine decrease was observed in a previous study (Villarreal-soto *et al*., 2019) done using a different Kombucha consortium. Caffeic acid seemed to be quite stable despite the processing, which may be due to its chemical structure of having two hydroxyl groups in the *ortho* and *para* positions, which increases its stability (Magnani *et al*., 2014). Epicatechin and catechin increased after fermentation, Jayabalan *et al*., (2007) studied the changes in tea polyphenols during Kombucha fermentation and observed an

increase of both compounds after 12 days, which could be due to the biotransformation of some isomers by the enzymes excreted by the microorganisms. The presence of ρ-Coumaric acid may be due to the action of the esterase activity in yeasts (Salameh *et al*., 2008). This acid has a great solubility in ethanol, which may explain that its higher concentration was found in the extracts of the aerated condition, which had also the maximum concentration of ethanol. Gallic acid is produced by the breakdown of tannic acid by a glycoprotein esterase from the microorganisms during fermentation (Alencar & Nunes, 2016). It is interesting to observe that its concentration increased with the aerated conditions, which could mean that tannase production was enhanced at higher concentrations of oxygen in the fermentation media. Banerjee *et al*., (2007) observed the same tendency when studying gallic acid production from *Aspergillus aculeatus*.

The **Figure 25** shows the chromatograms of the produced compounds (including non-identified peaks) before and after fermentation with the three conditions. It can be observed that several compounds were transformed after fermentation (4, 7, 19, and 20). The peak 7 was only produced in the samples (D) and (E) and not in the static condition. Moreover, the aerated condition obtained the higher increase of the peak 3 compared to the other conditions and compounds 10 and 12 were only produced in this sample. The following compounds have been identified: 3-gallic acid; 5-theobromine; 10-chlorogenic acid; 14-epicatechin; 18-ferulic acid and 20-trans-cinnamic acid.

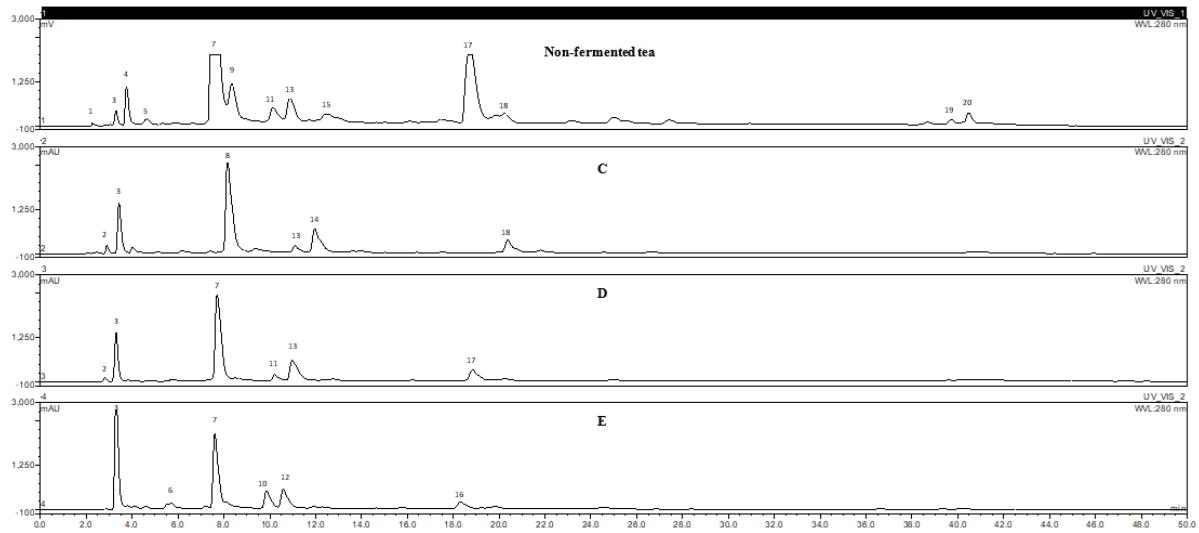

Figure 25. HPLC chromatograms of ethyl acetate kombucha extracts. Where C: static media, D: 200 rpm, E: 0.028 L/s.

Regarding the butanolic extracts it can be observed in **Figure 26** that the compound 3 increased, conversely to the compound 6 that was transformed after fermentation. Additionally, compound 8 was only produced in the static condition in both ethyl acetate and butanol extracts, suggesting that the production of this compound could be inhibited by a higher presence of oxygen

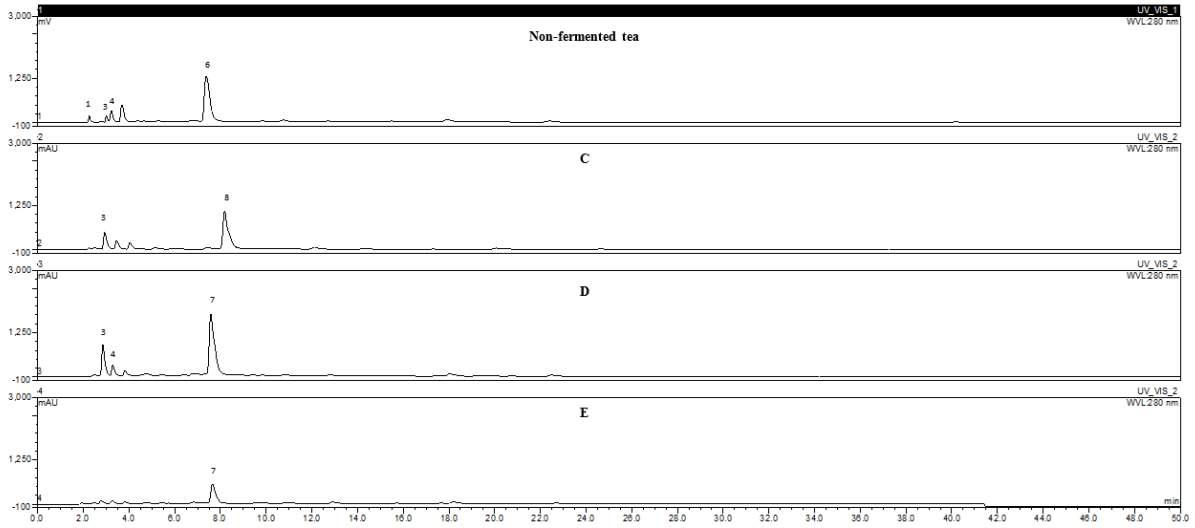

Figure 26. HPLC chromatograms of butanol kombucha extracts. Where C: static media, D: 200 rpm, E: 0.028 L/s

Table 10. Content of phenolic compounds, theobromine and caffeine from black tea (NF) and Kombucha extracts by HPLC (mg/kg of dry extract). Where C: static media, D: 200 rpm, E: 0.028 L/s.

Compound / Sample	Gallic acid	Theobromine	Catechin	Chlorogenic acid	(-)-Epicatechin	Caffeic acid	Caffeine	ρ-Coumaric acid	Ferulic acid	Rutin	Taxifolin	Trans-Cinnamic acid
NF-EtOAc	50±1.8				337.8±1.8		205.3±1.6				44.4±0.5	27.2±0.3
NF- BuOH					25.9±0.0		114.5±0.7					
NF- H_2O			5.1±0.0		1.0±0.1		1.4±0.5			3.3±0.0		
C- EtOAc	6485.9±553.8				1289.9±91.4	2577.6±465.0					9.4±0.4	
C- BuOH	1500.8±32.0		73.5±0.0		94.0±0.7	1286.0±59.2	69.4±1.3		218.1±49.9		2.1±0.0	
C- H_2O	332.7±19.7		13.5±0.8	15.0±0.4		81.6±0.0	0.1±0.0	0.5±0.0	0.1±0.0			
D- EtOAc	7734.5±199.0		1.5±0.0			10.3±0.0	34.0±1.9	114.5±0.0				5.0±0.0
D- BuOH	1649.8±32.4		219.0±0.4	289.0±21.9	107.8±25.7	3029.0±100.4	1308.5±211.3	430.2±26.8		485.1±5.3		2.6±0.3
D- H_2O	396.8±28.1	5.1±0.0	57.0±14.5	0.6±0.0	10.5±4.9	108.1±1.0	0.1±0.0	0.6±0.0				
E- EtOAc	21924.3±3312.6			2791.4±483.1	574.6±70.0	13.9±1.7	1182.4±195.0	82.0±14.8		384.2±24.4		
E- BuOH	1180.2±63.9		85.0±8.8	186.4±22.9	2.0±0.0	565.0±2.0	34.9±1.6	280.8±53.3		210.1±92.7		
E- H_2O	38.63±7.9		1.6±0.9	7.1±2.1		20.4±0.5	0.1±0.0	8.0±0.0		0.6±0.0	0.1±0.0	

4.3.4 GC-MS analysis

The GC-MS analysis results are summarized in **Table 11**. Clear differences were observed between samples. It can be seen that most compounds were produced in the static condition. Without derivation, no compounds were detected. After silylation, twenty-one compounds were identified and most of them were produced after fermentation. The results are presented in the form of peak areas for comparison between the fermentation conditions.

Carbonic, propanoic and ribonic acids were detected in the ethyl acetate extracts of the three tested conditions. Isovaleric acid highest area was obtained in the agitated condition compared to the traces found in the static and the aerated ones. The same tendency was observed regarding catechin production. Succinic and gallic acids were present in all the samples. The degradation of tannic acid by the action of the enzyme tannase releases gallic acid and glucose (Seth & Chand, 2000). This process can be either aerobic or anaerobic and may follow different pathways (Bhat *et al.*, 1998), leading to its oxidation to other compounds by the microorganisms which could explain the difference in the amount present in each sample. Regarding the gluconic acid production, it was just produced in the static condition, and in our previous study (Villarreal-soto *et al*., 2019) its highest production was obtained with the smallest vessel ratio (s/h). The production of gluconic acid by bacteria is carried out by the enzyme glucose dehydrogenase (Ramachandran *et al*., 2006), and oxygen supply seems to improve its production (Pal *et al*., 2016). However, Oosterhuis *et al*., (1985) performed some studies for the optimization of the gluconic acid production by *Gluconobacter oxydans* and obtained the highest concentration in the normally aerated condition compared to the oxygen enriched ones. These results suggest that at high oxygen concentrations the gluconic acid production and cell growth could be reduced since it could exceed the bacterial maximal oxidation capacity. Therefore, optimization experiments should be carried out in order to find the most advantageous oxygen transfer conditions for that purpose.

Table 11. GC-MS analysis (area x10^6) of Kombucha tea samples with derivatization. Where NF: black tea, C: static media, D: 200 rpm, E: 0.028 L/s.

Chemical group	Compound	NF			C			D			E		
		EtOAc	BuOH	H_2O	EtOAc	BuOH	H_2O	EtOAc	BuOH	H_2O	EtOAc	BuOH	H_2O
With derivatization													
Organic acids	Carbonic acid				20			24			35		
	Propanoic acid				69			63			24		
	Isovaleric acid				18			114			15		
	Pentanoic acid							2					
	Succinic acid				651			392			238		
	Malic acid				40			41			38		
	2-(Hydroxymethyl)Benzoic Acid				12			41					
	4-Methoxybenzoic acid										32		
	Pentanedioic acid				40			18					
	L-Phenyl lactate						346						
	Ribonic acid						290						
	D-Gluconic acid						1494						
Sugars	D-(-)-Ribofuranose			1854	9		350	11					
	(+)-Xylose						16						
	L-(-)-Arabitol						18				32		
	D-Psicofuranose						40						
	D-Arabino-1,4-lactone										39		
	D-(-)-Tagatofuranose		200	377			684						
	D-(+)-Talofuranose		36	716			7249						
	α-D-Mannopyranose						18						
	Myo-Inositol						30				24		
	D-(+)-Turanose		135	1790	368								
Alcohols	2,3 Butanediol				29.7			74			24		
	2-Phenylethanol				12			42					
	Glycerol				321		4115	300					
Phenolics	4-Hydroxyphenylacetic acid				5			3			18		
	4-Ethylphenol				65			61			52		

4.3.5 Functional diversity of predicted genes within the microbial consortium

Metabolic analyses using the SEED database showed several categories among the level 1 (**Figure 27**), being the most representative the amino acids and derivatives (7.8%), carbohydrates (11.3%), cofactors, vitamins, prosthetic groups and pigments (9.7%), protein metabolism (7.3%) and virulence (8.6%). This was followed by the metabolism of aromatic compounds (0.89%) and secondary metabolism (0.09%). The higher abundance of genes were found in the carbohydrate metabolism, which could correspond to the production of cellulose, as well as the uses of sucrose, glucose and fructose for fermentation. Several metabolic pathways were found according to the type of energy-generating process involved in sugar metabolism (Rodrigues *et al*., 2006) such as, the glycolytic and the pentose phosphate ones. One of the key enzymes involved in the utilization of acetic acid is the acetyl-CoA synthetase, which was present in both static and agitated condition but not in the aerated one. The aerated condition had the highest acetic acid concentration of 37.2 g/L, which suggests that higher concentration of oxygen could repress the presence of this enzyme. Genes encoding enzymes of the shikimate pathway such as, chorismate synthase, phenylalanine permease, shikimate kinase and chlorogenate esterase were found in the microbiota from all the fermentation conditions. Caffeic, coumaric and ferulic acids are derived from the shikimic acid pathway and the highest concentration of these acids was found in the static condition (**Table 10**). Succinic, malic, pentanoic and propanoic acids are derived from the citric acid cycle and they were mainly produced in the agitated condition, where *Brettanomyces bruxellensis* was the predominant yeast. The mechanism of gallic acid biosynthesis in bacteria was studied by Muir *et al*., (2011), and they found that the enzyme shikimate dehydrogenase was required for gallic acid production. This enzyme was found in all the samples, however its relative abundance differed, being of 0.029, 0.033 and 0.052 for C, D and E, respectively, at the end of the fermentation. The proportion of the shikimate dehydrogenase pathways was 1.7-fold higher in the aerated condition and the same tendency was observed regarding the presence of *K. rhaeticus* in the samples, which could suggest that this bacterium may be involved in the synthesis of gallic acid. However, regarding the quantification of this acid by HPLC, the detected concentration was 2.5-fold higher than the static and agitated conditions, which could mean that other functional genes may be involved in the synthesis of this phenolic acid.

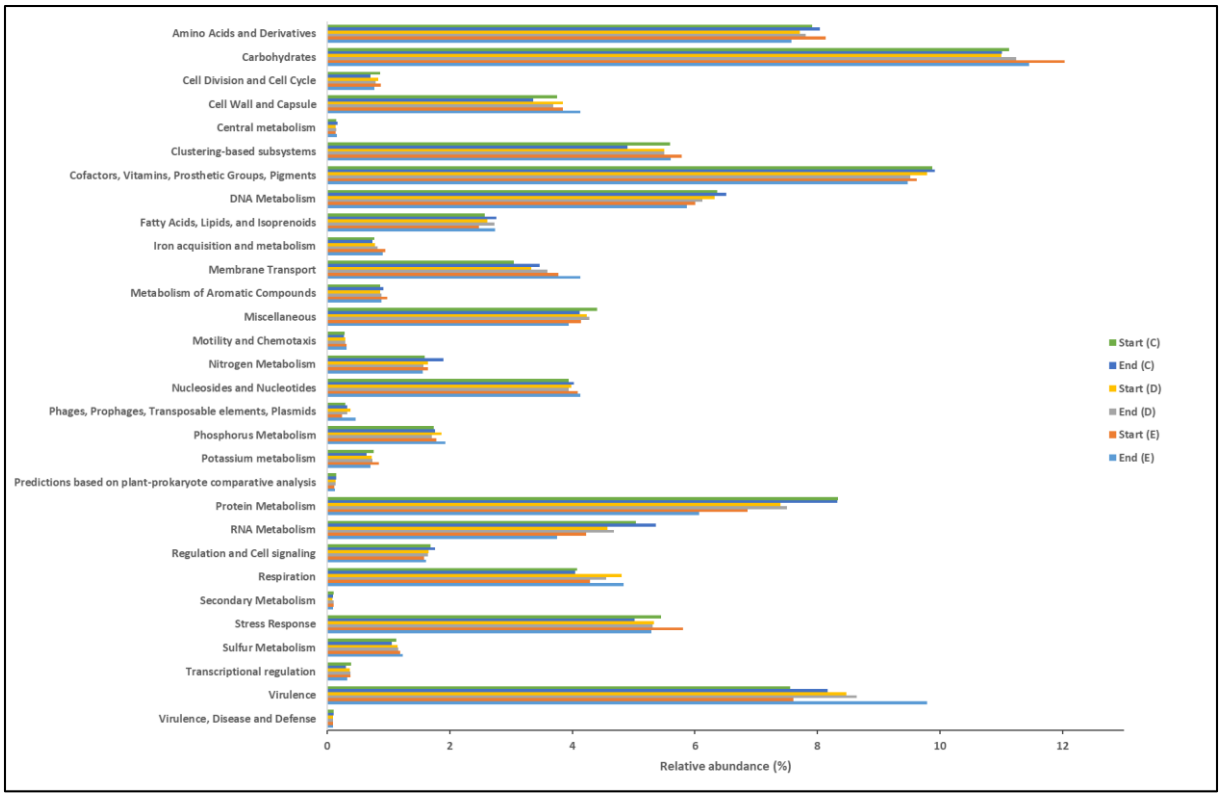

Figure 27. Relative abundance of functional genes subsystem level 1. Where C: static media, D: 200 rpm, E: 0.028 L/s.

4.3.6 Antioxidant capacity and phenolic composition

The polar extracts of ethyl acetate were able to scavenge the DPPH free radicals in all the conditions (**Figure 28**), obtaining around 96% of inhibition at a concentration of 50 µg/mL. Our results are higher than those obtained by Chu & Chen (2006) who worked with non-fragmented Kombucha tea samples (unknown concentration) and found inhibition percentages between 26.4 and 69.2% after 15 days of fermentation. But similar to those obtained by Jayabalan *et al*., (2008), who observed a scavenging activity of 88% on the 18th day of fermentation (unknown concentration) of green tea with the Kombucha consortium. The ethyl acetate and butanol extracts were effective antioxidants, showing an IC_{50} below 20 µg/mL (**Table 12**). **Figure 28** shows the total phenolic composition of Kombucha extracts, which presented the same tendency as the antioxidant activity, with a significant Pearson correlation of 0.927 ($p<0.05$), which means that the phenolic composition of around 20 to 130 mg eq AG/g may be responsible of this biological activity. It can be observed that that the butanolic extract (D) had a lower phenolic content but a high antioxidant activity, which could mean that this sample contains very potent antioxidants, compared to the others samples or a shorter presence of non-antioxidant compounds.

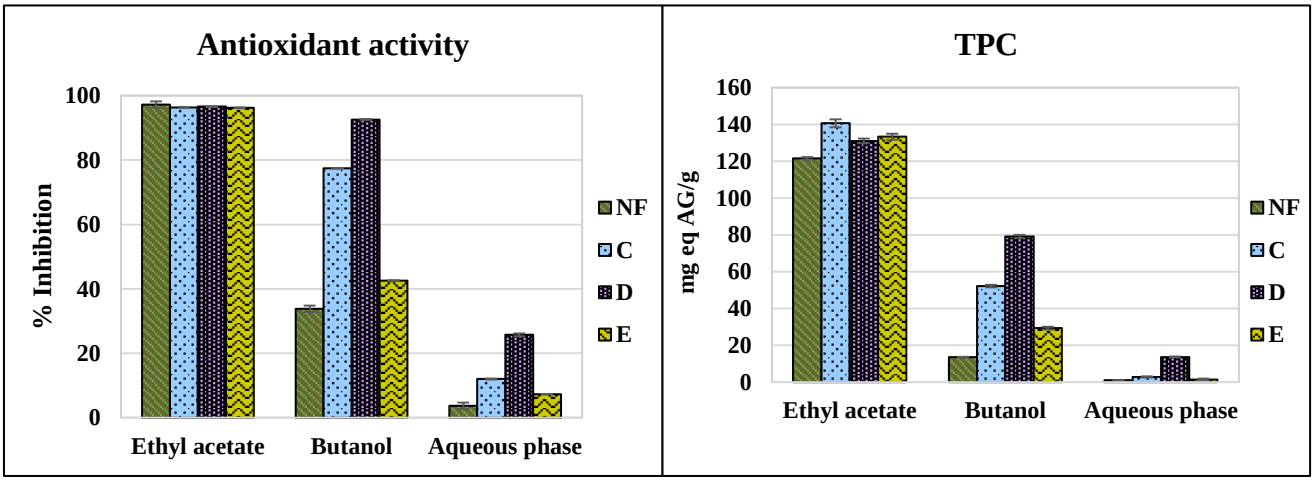

Figure 28. Antioxidant activity and total phenolic content (TPC) of of black tea (NF) and Kombucha samples. Where C: static media, D: 200 rpm, E: 0.028 L/s.

Regarding the chemical composition of the identified compounds, caffeine, chlorogenic acid, epicatechin and gallic acid seemed to be the representative ones in the ethyl acetate extracts. Phenolic acids are stable antioxidants which may be enhanced after fermentation (Jayabalan et al., 2008), but some of them are already present in the non-fermented tea (Souza et al., 2019), which could explain the fact that the infusion showed a good antioxidant activity before fermentation. Nevertheless, the agitated condition (D), showed the highest phenolic content and antioxidant activity with the butanol fractions compared to the other conditions, which suggest that aeration may improve the production of bioactive compounds such as caffeic acid, which reached its highest production of 3.029 mg/g in the agitated Kombucha sample of the butanolic extract (**Table 10**). Morevover, catechin, caffeine, ρ-coumaric acid and rutin also reached their higher concentration in this sample, which could explain the fact that this condition have obtained the best antioxidant profile and the higher phenolic composition of the fermented samples. However, the non-identified compounds before and after fermentation could play an important role in the antioxidant activity of the extracts.

Table 12. Half-maximal inhibitory concentration of the antioxidant activity of Kombucha samples and black tea (NF).

Solvents	Samples IC_{50} (µg/mL)			
	NF	**C**	**D**	**E**
EtOAc	7.6±0.5	11.0±0.5	9.4±0.2	8.4±0.2
BuOH	29.2±0.9	17.9±0.6	13.5±0.4	14.4±0.2
H_2O	>50	>50	>50	>50

IC_{50} values were calculated for inhibition percentages higher than 70% at 25 and 5 µg/mL ± standard deviations (n=3). Where C: static media, D: 200 rpm, E: 0.028 L/s.

4.3.7 Anti-inflammatory activity

The anti-inflammatory activity was studied against the enzyme 15-lipoxygenase and a decrease was observed after fermentation in all conditions in the case of the ethyl acetate extracts (**Figure 29**). However, among the fermented samples the agitated condition (D) showed an increase in the case of the ethyl acetate and butanol extracts compared to aerated condition (E). An opposite behavior was observed in a previous study (Villarreal-soto *et al*., 2019) where this activity was improved after the fermentation with the Kombucha consortium obtaining IC_{50} values from 9 to 24 µg/mL, meaning that the bioactive profile is directly influenced by the processing and the microbial population.

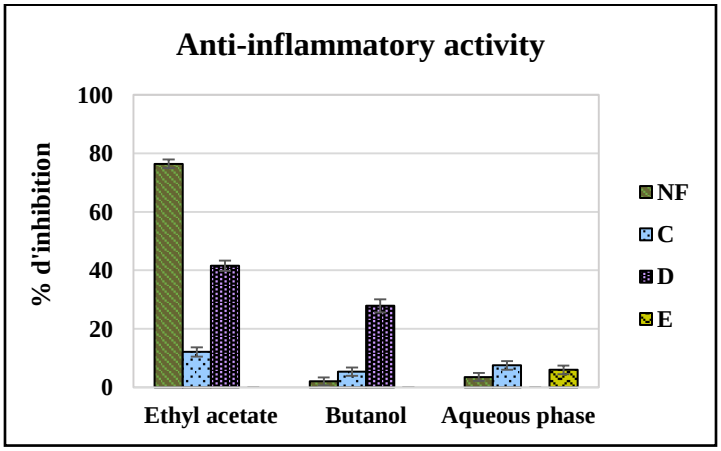

Figure 29. Anti-inflammatory activity of black tea (NF) Kombucha samples at 50 µg/mL. Where C: static media, D: 200 rpm, E: 0.028 L/s.

4.3.8 Antiproliferation evaluation on human tumor cell lines

Selective inhibition of human colon (HCT-116) and breast cancer (MCF-7) cells was obtained with the different tested extracts (**Table 13**). The highest inhibition percentage was reached with the ethyl acetate extract of the static condition, showing a high increase going from 9.0 to 73.7% after 21 days of fermentation (IC_{50}= 33 µg/mL), in the case of the HCT-116 cell line. Jayabalan *et al*., (2011) also obtained the highest inhibitions against 786-O cells with the ethyl acetate extracts at 100 µg/mL. The butanolic extracts of the same condition also increased their inhibition after fermentation going from 5.8 to 33% in the agitated condition. However, the antiproliferation effect decreased with the other conditions and in the case of the MCF-7 cell line, no increase was observed after fermentation with the consortium. Contrary to a previous study (Villarreal-Soto *et al*., 2018) where the inhibition against the breast cancer cells increased after fermentation. The Pearson correlation was done in order to see if there was a degree of association between the phenolic compounds and the antiproliferative activities. A significant value of 0.796 ($p<0.05$) was obtained in the case of the HCT-116 line, which suggest that the identified and non-identified phenolic compounds could take part of this inhibition effect. Caffeic acid has been reported as an inhibitor of colon and oral cancer cells (Magnani *et al*., 2014) and epicatechin is considered a potent inhibitor of cell signaling in different types of cancer (Prakash *et al*., 2019), both compounds were the main constituents of the tested extracts (**Table 10**).

Table 13. Antiproliferation activity against human colon cancer (HCT-116) and human breast cancer (MCF-7) cell lines.

Sample	Extract	Inhibition % at 50 µg/mL	
		HCT-116	**MCF-7**
NF	**EtOAc**	9.0±1.3	32.7±2.1
	BuOH	5.8±1.9	26.2±1.5
	H_2O	N.A	20.4±0.0
C	**EtOAc**	73.7±2.6	18.6±1.4
	BuOH	33.0±1.5	21.8±1.6
	H_2O	N.A	18.0±1.4
D	**EtOAc**	28.4±1.4	19.1±0.9
	BuOH	21.3±1.3	25.0±0.21
	H_2O	14±1.2	19.4±1.8
E	**EtOAc**	30.1±1.4	N.A
	BuOH	11.6±1.2	N.A
	H_2O	N.A	N.A

All the data are average values of triplicate analyses. Where NF: black tea, C: static media, D: 200 rpm, E: 0.028 L/s. N.A: not active.

4.4 Conclusion

The microbial population is very sensitive to the changes in fermentation media, such as, aeration, agitation or media acidification. The oxygen transfer seems to be a key parameter for the fermentation of Kombucha tea and should be taken into consideration especially for its scale-up production. The selected processes had a great impact on the metabolites production and their respective biological activities, as well as in their microbial population and their respective metabolic pathways. The butanolic extracts of the agitated condition (D) had the highest anti-inflammatory and antioxidant activities between the fermented samples. The aerated condition (E) caused a high increase in acetic acid production and showed a low biological profile. The aeration and agitation did not improve all the biological activities. However, they increased the production of several compounds of interest (identified and non-identified), which could be responsible of other biological activities or may be used for the development of new products in the cosmetic and therapeutic industries.

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4.6 Analyse en composantes principales

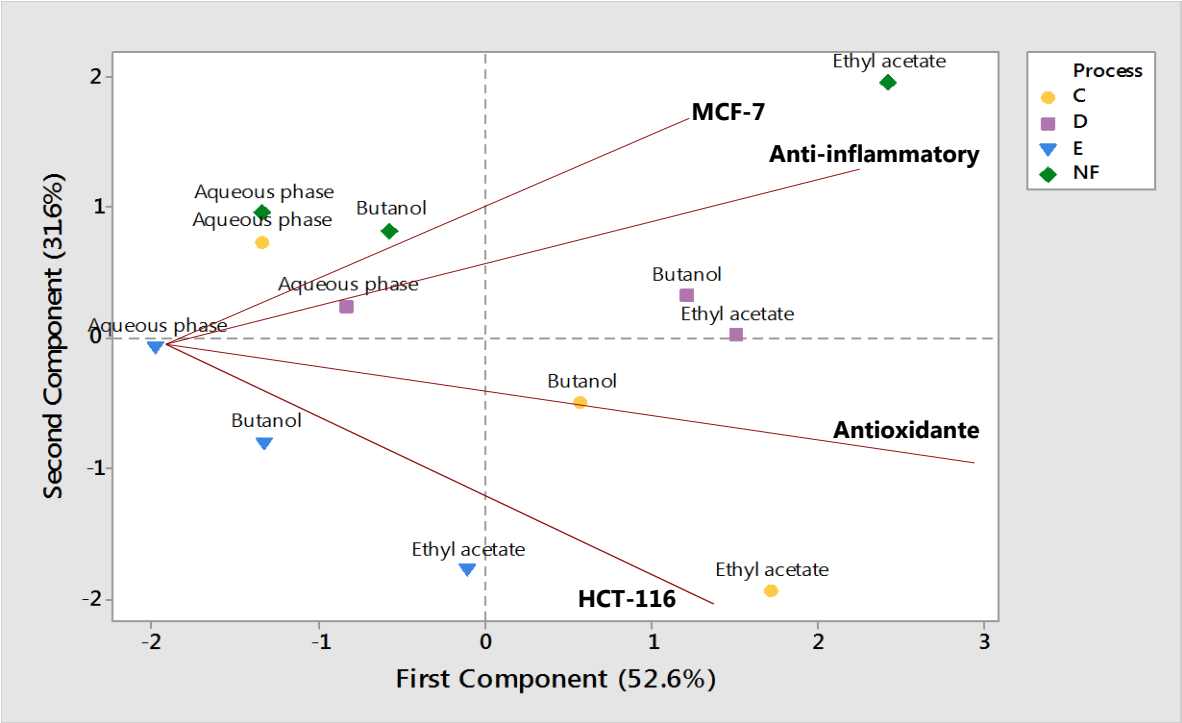

Figure 30. Analyse en composantes principales des activités biologiques et procédés. C: statique ; D: 200 rpm ; E: Q=0,028 L/s ; NF: thé non-fermenté.

L'analyse en composantes principales (ACP) a montré que les deux composantes principales expliquaient 84.2% de la variance, ce qui a permis de séparer les échantillons en fonction de sa corrélation avec les activités biologiques. À partir de l'analyse obtenue **(Figure 30)**, nous pouvons observer que les extraits de butanol et d'éthyle acétate de la condition agitée (D) sont les plus corrélés avec l'activité anti-inflammatoire et antioxydante. Suivis de la condition statique qui a montré une cytotoxicité contre la lignée cellulaire HCT-116 et une forte corrélation avec l'activité antioxydante. En général, ce sont les extraits fermentés qui ont montré un profil biologique plus élevé, par contre, en ce qui concerne l'activité anti-inflammatoire et la cytotoxicité contre la lignée MCF-7, c'est le thé non-fermenté qui a obtenu la corrélation plus forte, ce qui signifie que ces activités ont diminué après fermentation. Nous pouvons observer aussi une sélectivité des procédés contre les lignées cellulaires étudiés, de fait qu'elles sont apparues aux extrémités opposées du graphique. Cette sélectivité a déjà été observée dans des études antérieures menées par Schwartz & Shklar, (1992) qui ont étudié l'effet cytotoxique des caroténoïdes et observé une sélectivité vis-à-vis des cellules cancéreuses humaines mais pas vis-à-vis des cellules saines.

Avec cette analyse nous concluons que l'agitation (D) semble améliorer la plupart des activités étudiés et qu'ajouter une source d'aération semble avoir l'effet inverse. Cependant, le fait que la condition statique ait obtenu un profil biologique positivement corrélé avec des activités, notamment avec la cytotoxicité contre la lignée HCT-116, montre que différents procédés améliorent différentes activités, d'où l'importance du choix du protocole expérimental en fonction des objectifs recherchés. De plus, les extraits ayant de bonnes activités après fermentation restent à étudier pour identifier les composés formés et évaluer leurs activités pures.

4.7 Conclusion du chapitre

Dans le chapitre précèdent il a été démontré que la géométrie a un fort impact sur le déroulement de la fermentation dû principalement au contact avec l'oxygène. En conséquence et avec l'intérêt d'optimiser le procédé, ce deuxième chapitre de résultats a été consacré à étudier l'impact du transfert d'oxygène sur la population microbienne et leurs voies métaboliques.

Deux sources d'aération et agitation ont été choisis afin de stimuler l'oxygénation du milieu. Les coefficients de transfert d'oxygène (kLa) ont été mesurés et le suivi de fermentation a été fait en ciblant la population totale des levures at bactéries par de méthodes de qPCR.

Des comportements différents ont été obtenus selon chaque condition surtout en ce qui concerne la production d'éthanol et l'acide acétique en comparaison avec le témoin en condition statique.

La quantification chimique des composés phénoliques a révélé que l'aération augmente considérablement la production d'acide gallique, génère une production excessive d'acide acétique et présente une amélioration limitée des profils biologiques.

Les analyses de métabarcodage ont montré que l'effet d'aérer le milieu favorise la croissance de populations différentes et peut aussi mener à une expression distincte de gènes fonctionnels, impactant également sur le profil chimique et biologique final.

Parmi les trois conditions étudiées, l'agitation (D) semble être un paramètre important pour l'amélioration du profil biologique des extraits de Kombucha.

Chapitre V: Impact de l’origine du consortium sur le déroulement de la fermentation

Sommaire

Problématique de recherche et résumé du cinquième chapitre

Le consortium microbien du Kombucha est composé par des bactéries acétiques responsables de la production de la cellulose extracellulaire, et par des levures de nombreux genres qui se trouvent en partie intégrées dans le structure du polymère (Villarreal-Soto *et al*., 2018). Contrairement aux autres fermentations telles que le vin ou la bière, dans le mode d'inoculation pour la production du thé de Kombucha il y a souvent une variation significative de la quantité de liquide et de biofilm ou SCOBY utilisé. De plus, du fait des différentes origines et provenances du consortium la cinétique de croissance et la production de métabolites secondaires sont plus difficiles à contrôler. La population microbienne du Kombucha est répartie en deux phases principales, la phase solide (cellulose) et la phase liquide (thé fermenté). Bien que certains auteurs ont tenté de maitriser l'inoculation en créant leur propre consortium (Malbaša *et al*., 2011; Nguyen *et al*., 2015) la plupart des fermentations de Kombucha sont faites à partir des biofilms déjà constitues provenant d'une fermentation précédente. Afin d'étudier l'impact de l'origine du consortium sur le profil chimique et biologique du produit final, des fermentations ont été réalisées en utilisant trois consortia de Kombucha d'origine diverse néanmoins en gardant les mêmes conditions de fermentation.

Des différences nettes ont été observées entre les échantillons en ce qui concerne les vitesses de consommation de sucres. Le consortium (F) a été environ trois fois plus rapide que les deux autres, suggérant que la population de levures devait être plus élevée dans cet échantillon. Afin de corroborer cette hypothèse et de caractériser les trois consortia étudiés, une analyse métagénomique a été mise en œuvre. Les résultats ont révélé que les groupes dominants de bactéries et levures ainsi que leur distribution entre les phases étaient différents entre les trois SCOBY. La composition chimique des composés produits a aussi été impactée par la composition microbienne de chaque consortium, montrant des différences principalement dans la production d'acides organiques et de composés phénoliques. En ce qui concerne les profils fonctionnels obtenus, une activité antioxydante intéressante a été observée dans tous les consortia. Cette étude nous a permis d'observer que nonobstant les différentes origines, des espèces dominantes de bactéries et levures ont été présentes dans tous les échantillons, montrant ainsi une certaine stabilité et identité du consortium du Kombucha.

Metabolome-microbiome signatures in the fermented beverage, Kombucha

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Abstract

Kombucha is a fermented tea. Here we investigate the fermentation kinetics, metabolite production, microbiome and health promoting properties of three different consortia. Shotgun metagenomic sequencing revealed several dominant bacterial genera such as *Komagataeibacter*, *Gluconacetobacter* and *Gluconobacter*. *Brettanomyces* and *Schizosaccharomyces* were the most dominant yeasts identified. Species distribution reflected different patterns of sugar consumption, with *S. pombe* being present in samples with the highest sugar conversion. Liquid-liquid extractions were performed with organic solvents in order to obtain dried extracts, which were later characterized. HPLC-DAD and GC-MS analysis revealed differences in the production of organic acids, sugars, alcohols and phenolic compounds, where the presence of caffeine, propanoic acid and 2,3 butanediol differ greatly across the three kombuchas. Health assays were carried out in order to evaluate the biological profile of the fermented teas and an interesting IC_{50} value of 7 µg/mL was obtained against the DPPH radical. And an inhibition percentage of 44.5% was obtained with one of the consortia against the 15-lipoxygenase enzyme. Metabolomic analysis exhibited a link between the microbiota and the production of bioactive compounds in kombucha fermentation.

Keywords: Shotgun sequencing, Kombucha bioactivity, Chemical profile, Fermentation

5.1 Introduction

Successful interactions between bacteria and yeast species can lead to the generation of a wide range of metabolites with interesting bioactivities, including those form fermented beverage production. The microbial community responsible for Kombucha production is embedded in an extracellular polymeric matrix (pellicle) that is located at the liquid-air interphase (Nikolaev & Plakunov, 2007). The tea is also commonly inoculated with 10-20% of broth from a previous Kombucha fermentation (Malbaša *et al*., 2006; Četojević-Simin *et al*., 2012; Rosma *et al*., 2012), allowing the microbial population to disperse in the liquid media. The characteristic Kombucha microbiome includes several genera of acetic acid bacteria, yeasts and, to a lesser extent, lactic acid bacteria. Some studies (Marsh *et al*., 2014; Coton *et al*., 2017) have revealed *Komagataeibacter*, *Acetobacter* and *Gluconobacter* as the dominant bacteria genera, and *Brettanomyces* and *Zygosaccahromyces* as the dominant yeasts. These microorganisms interact through cooperative metabolism, contributing to the synthesis and organoleptic qualities of the final beverage. Several metabolic reactions occur during Kombucha fermentation leading to the production of intermediates and secondary metabolites, with potential applications in the pharmaceutical, cosmetic and food industries (Villarreal-Soto *et al*., 2018). Acetic acid bacteria are well known to convert sugars and alcohols into several organic acids, such as acetic-, gallic-, succinic- and malic acid (Gomes *et al*., 2018). The genus *Gluconacetobacter* and *Komagataeibacter* are cellulose producing bacteria which also participate in the production of gluconic and glucuronic acid by an oxidation reaction with the enzyme glucose dehydrogenase (Ramachandran *et al*., 2006). Yeasts are also important contributors due to their high fermentative capabilities, releasing high quantities of polysaccharides, great efficiency with respect to the utilization of available nitrogen sources and high level of resistance to osmotic and ethanol stress (Steensels *et al*., 2015; Domizio *et al*., 2017). Thus, the production of important compounds is directly related to the specific microbiota and the cross-feeding between them. In addition to the composition of the pellicle, the factors that will dictate the growth and activity of Kombucha consortia include the substrate (Četojević-Simin *et al*., 2012; Hoon *et al*., 2014; Watawana *et al*., 2015; Ayed *et al*., 2016; Rahmani *et al*., 2019), sources of carbon used (Reiss, 1994; Watawana *et al*., 2015; Vohra *et al*., 2019) and fermentation parameters (Cvetković *et al*., 2008; Villarreal-soto *et al*., 2019). As a result, the fermented teas can have quite different properties and chemical compositions. While all of these aforementioned parameters considerably influence the bioprocessing of Kombucha tea. These factors have been studied in isolation, and thus the relationship between bioactive molecules

production and microbial metabolism has, to date, not been fully understood. Developing a deeper understanding of the links between all of these elements can be of considerable value with respect to optimizing the fermentation process and its final functionality. In this study, three different Kombucha consortia were used to investigate their kinetics, microbial diversity via high-throughput sequencing, chemical composition and respective functional profile. This research is of relevance to Kombucha fermentation globally generating knowledge that will facilitate optimization of the process to yield desirable end products.

5.2 Materials and Methods

5.2.1 Starter cultures

The Kombucha pellicles used in this study were obtained from different suppliers. The first of them was obtained from a local house holding in France (F), the second was obtained from a commercial bottle of Kombucha tea and reconstituted as described in section 5.2.2 (G), and the third one was bought from the website www.jemangevivant.com (H). All the pellicles were fermented three times before experimental use in order to stabilize the microbial consortium.

5.2.2 Preparation of Kombucha tea

Black tea was prepared according to the following procedure: 70 g of sugar were added to 1 L of water at 40°C. Once the water reached 80 °C, 10 g of black tea (Royal Ceylan, Lipton ®) were also incorporated and allowed to infuse for 15 minutes. Then the tea was removed and the infusion was left to cool. After the infusion was cooled to room temperature (25 °C), tea was poured into 2 L glass containers (ratios: s/h=9.02cm, s/v=0.132 cm^{-1}) and 20 mL/L of the previous fermented liquid media, 10mL/L of cider vinegar and 20 g/L of SCOBY were added. The vessels were covered with cheesecloth and incubated at 25°C for 15 days. All fermentations were performed in duplicate.

5.2.3 Shotgun metagenomic analysis

The Geneall Exgene Soil SV extraction kit was used for all extractions, with some modifications. For the pellicle DNA extraction, 0.8g were removed and washed twice in 500 µL of sterile water, cut into smaller pieces and placed in a powerbead tube. Then, 550µl of SL

buffer, 90 µL of lysozyme (50 mg/mL) and 50 µL of mutanolysin (100 µL/mL) were added. The mixture was incubated at 60°C for 15 minutes with occasional vortexing. Twenty-eight µL of proteinase K were then added, with further 15 minute incubation, including occasional vortexing. The tube was then placed in a bead beater (Qiagen Tissue lyser 2) for 10 minutes at 20 oscillations/s. The manufacturer's instructions were then followed, including pelleting and washing steps, with a final volume of 30µl of elution buffer added at the final step.

For DNA extraction of the tea, kombucha tea was homogenized by stirring and 50 mL of tea were collected in a 50 mL falcon tube. Then the tube was centrifuged at 5000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 550 µL of SL buffer. The sample was then treated following the same protocol mentioned above.

Kombucha samples (liquid and solid phases from the end of the fermentation) were sequenced on the NextSeq sequencing platform in the Teagasc sequencing facility (Moorepark, Cork, Ireland). Library preparation was carried out according to the Illumina Nextera XT protocol. Quantification of DNA was performed using Qubit High Sensitivity dsDNA assay. Final library quality was assessed by running on an Agilent High Sensitivity DNA chip, and quantification by qPCR using the KAPA Library Quantification Kit for Illumina (Roche). Sequencing was carried out using a 300 cycle High Output V2 kit, following standard Illumina sequencing protocols. As described by Doyle *et al*., (2017).

Sequence reads were obtained from the Nextseq sequencing run in the form of Bcl files. These files were converted to fastq format using bcl2fastq software. Using Picard, fastq was converted to Sam format. Picard was also used to remove duplicates. The sequences were then quality checked and trimmed. Forward and reverse reads were combined into a single fasta file for each sample.

Kaiju (Menzel *et al*.,2016) was used to assign taxonomy to the reads, discarding taxa with relative abundance of less than 0.1%.This setting was chosen as other studies have shown a high false positive discovery rate below this threshold. All percentages reported at all taxonomic levels are percentages of the assigned reads only. Summary plots were created in R version 3.6.0 using the package ggplot2 (Wickham *et al*., 2009). Superfocus (Silva *et al*.,2016) and Humann2 (Species-level functional profiling of metagenomes and metatranscriptomes) was used to assign functionality to the reads.

5.2.4 Sugars, ethanol and acetic acid quantification

Sugars consumption and metabolites production were analyzed in the liquid phase by ultra performance liquid chromatography (UPLC-RI) (Thermo Scientific, Dardilly, France). According to the method described by Villarreal-soto *et al*., (2019).

5.2.5 Extraction methodology and biological evaluation

Sequential liquid-liquid extractions with organic solvents (ethyl acetate and butanol) were done with the Kombucha samples at the end of the fermentation. The solvents were then evaporated using a vacuum rotary evaporator at 35 °C (RV 10 Auto VWR, IKA, Staufen, Germany) in order to obtain a dry extract.

Stock solutions were prepared from each dry extract in a concentration of 3 mg/mL, using DMSO 100% as solvent. Then, for all the biological evaluations the stock solutions were tested initially at 50 µg/mL and at 25 or 5 µg/mL in the case of high inhibition percentages (>70%). Then the antioxidant, anti-inflammatory and antiproliferation activities were evaluated according to the methods previously described (Villarreal-soto *et al*., 2019). The non-fermented tea (NF) has also been evaluated.

5.2.5.1 Phenolic and aromatic compounds determination

The dried extracts were injected in a concentration of 20 mg/mL of acetonitrile and water (20:80 v/v) and then phenolic and aromatic compounds were identified and quantified by HPLC-DAD (Thermo Scientific Dionex Ultimate 3000). Chemical composition was performed by gas chromatography-mass spectrometry (GC-MS) Varian Saturn 2000 (Les Ulis, France). And total phenolic composition was quantified using the Folin-Ciocalteu assay.

5.3 Results and discussion

5.3.1 Fermentation kinetics of different consortia and their produced metabolites

Sugars consumption and the production of the primary metabolites across the respective Kombucha fermentations was analyzed. Despite the fermentation conditions remaining constant, differences in the specific microbial populations present had an impact on the kinetics

and evolution of the process. Differences in the levels of sugar conversion were observed between days 0 and 3 (**Figure 31**), where there was a 10 fold difference between the samples, when the same quantity of pellicle and fermented media were used for inoculation. The concentration of residual sugar was greatest for consortium (H) (~35 g/L), which may explain why ethanol production was lowest (6 g/L) in this sample after 15 days of fermentation. Sucrose was hydrolyzed into glucose and fructose in all cases. However, the manner in which they were consumed differed (**Figure 32**). In sample (F), sugars were completely depleted at 12 days, while for kombuchas (G) and (H), sugars remained in the media. As noted, Kombucha (H) was extreme in this regard, were fructose reached 12 g/L after 15 days. A similar sugar profile was observed by Chen & Liu (2000) and Kallel *et al*., (2012) were fructose was poorly metabolized compared to glucose, probably because the latter is used for the biosynthesis of cellulose and for the production of organic compounds, such as gluconic acid, or because is preferred as a carbon source by the specific Kombucha microbiota present. However, in another Kombucha study, Sievers *et al*., (1995) observed that fructose was metabolized prior to glucose, again possibly due to differences in the specific microbial composition and/or initial carbon source (Malbaša *et al*., 2008).

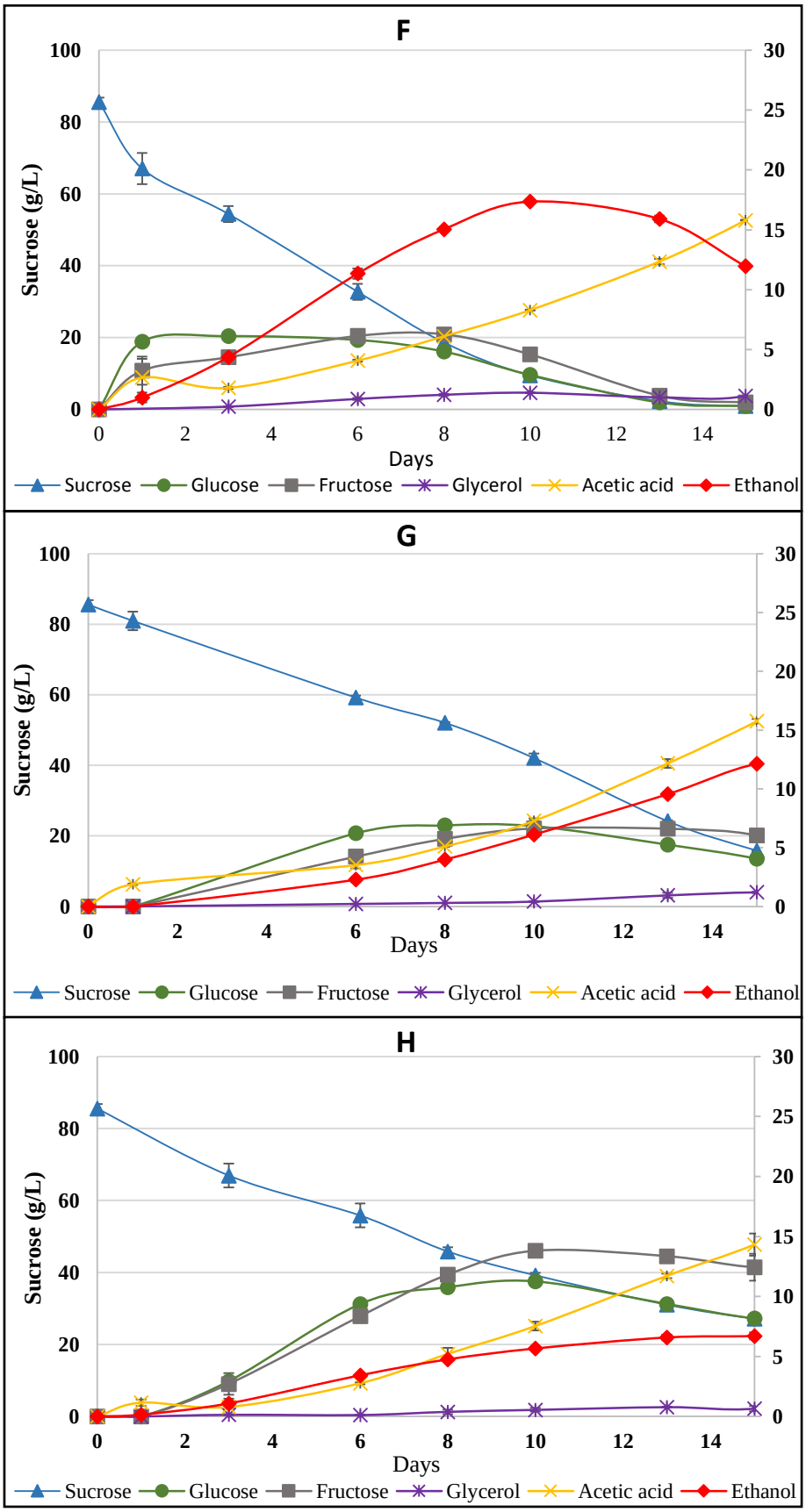

Figure 31. Evolution of the main compounds during Kombucha fermentations. All the data are average values of triplicate analysis. The scale on the left corresponds to the sucrose and the one on the right corresponds to all the other compounds.

Acetic acid concentration was similar for the three samples regardless of sugars consumption or ethanol production. This could be due to the bacterial population ability to use several metabolic pathways towards the acetic acid production (Gomes *et al*., 2018).

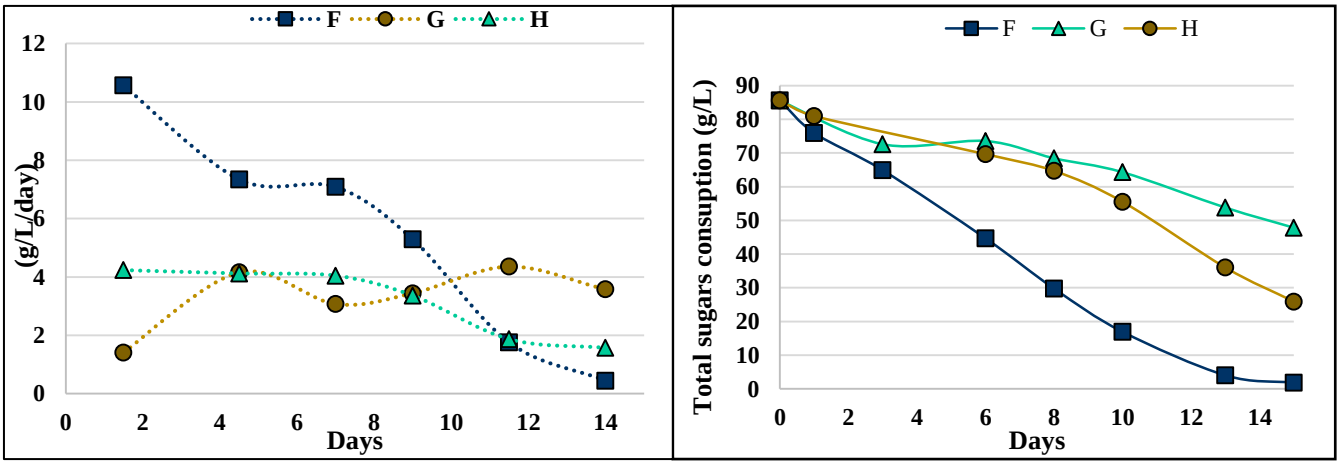

Figure 32. Evolution of average hydrolysis (left) and sugars consumption (right) rates for the three fermentations of Kombucha.

5.3.2 Metagenomics of solid and liquid phases

Shotgun metagenomics was used to examine the microbiota of these three Kombucha communities (solid and liquid phases) (**Figure 33**). Actinobacteria and Proteobacteria were the two bacterial phyla identified in all samples, with *Acetobacteraceae* being the dominant family. In the case of the yeasts, the dominant taxa belonged to the Ascomycota phylum of the *Saccharomycetaceae* and *Schizosaccharomycetaceae* families.

5.3.2.1 Microbial taxonomy

Almost 80% of the identified microbial diversity were *Acetobacteraceae*, with the dominant genera being, *Komagataeibacter*, *Gluconacetobacter* and *Gluconobacter* (**Figure 34**). Several species from the genus *Acetobacter* were also identified (*A. malorum*, *A. pasteurianus*, *A. pomorum*, and *A. tropicalis*). More specifically, at the species level, *Komagataeibacter rhaeticus* was the dominant bacterium in the samples (16-49%), followed by *Gluconacetobacter* sp SXCC1 (9-26%), both being present in greatest proportions in the solid phases (**Figure 33**). *Komagataeibacter xylinus* had a higher abundance in the liquid (16.89%) of Kombucha (G). Strains from this species have been proposed to have potential as probiotics

following investigations revealing an ability to survive gastric conditions, such as low oxygen pressure and the presence of bile salts and acidity (Lavasani *et al*., 2019). **Figure 34** also shows the quantity of sugars, acetic acid, glycerol and alcohol for the three consortia. The most notable difference between the three liquid phase compositions is the low levels of *Schizosaccharomycetaceae (S. pombe* at species level*)* in (H), which corresponds to the highest levels of all sugars left at the end of the experiment.

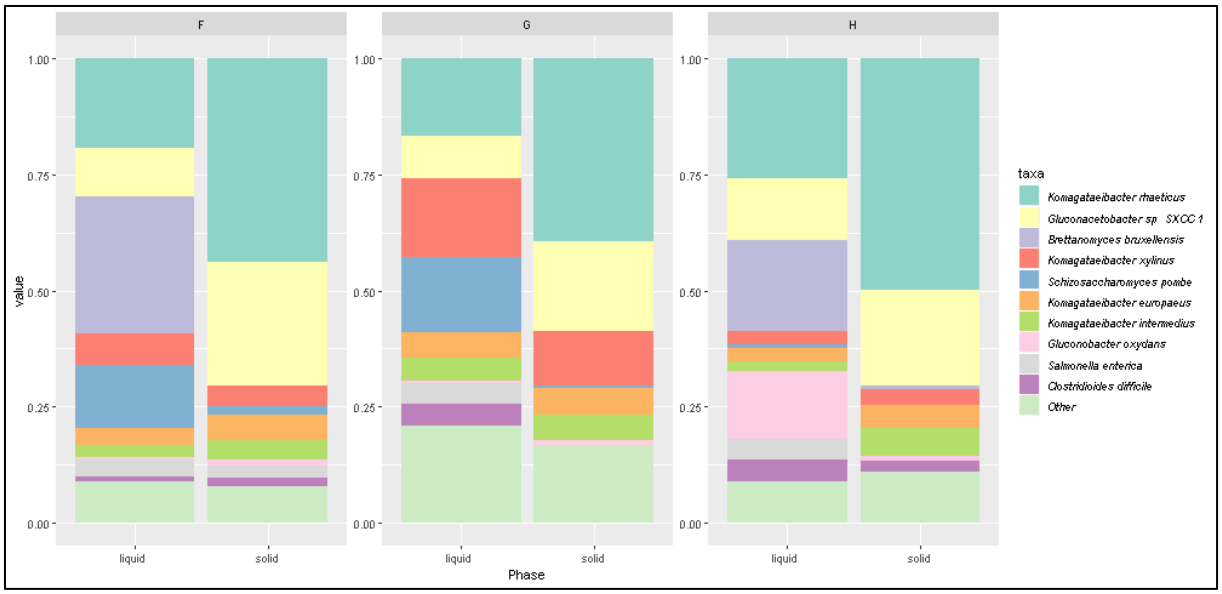

Figure 33. Taxonomic classification of microbial population in the solid and liquid phases of the three different consortia.

Four genera of yeasts, *Candida arabinofermentans*, *Brettanomyces bruxellensis*, *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii*, were identified across the three Kombuchas. The dominant fungal species, found primarly in the liquid phases, *B. bruxellensis* (29.56 %) and *S. pombe* (16.11 %). Previously, an amplicon-based analysis of Kombucha from green and black teas by Coton *et al*., (2017) revealed that *Dekkera bruxellensis* was the dominant yeast species and that its relative abundance changed with time between phases. At day cero, most of the yeasts were entrapped into the solid phase while at day 8 their abundance in the liquid increased. The same pattern was observed in this study, in that yeasts were not detected when shotgun metagenomic analysis was performed after 15 days of fermentation. From the perspective of individual Kombuchas, the sample (F) had the highest proportion of *S. pombe* and *B. bruxellensis* in the liquid phase. This may explain it having the fastest rate of sugar consumption (**Figure 32**) and highest ethanol production (17g/L). However, *B. bruxellensis* was not detected in the liquid or solid phase of sample (G), which had the slowest

rate of sugar consumption and hydrolysis. This is not surprising given that previous studies of the Kombucha microbiome (Teoh *et al*., 2004; Marsh *et al*., 2014; Reva *et al*., 2015; Coton *et al*., 2017) have highlighted considerable variability, even if *Gluconacetobacter* sp. and *Brettanomyces* sp. typically dominate.

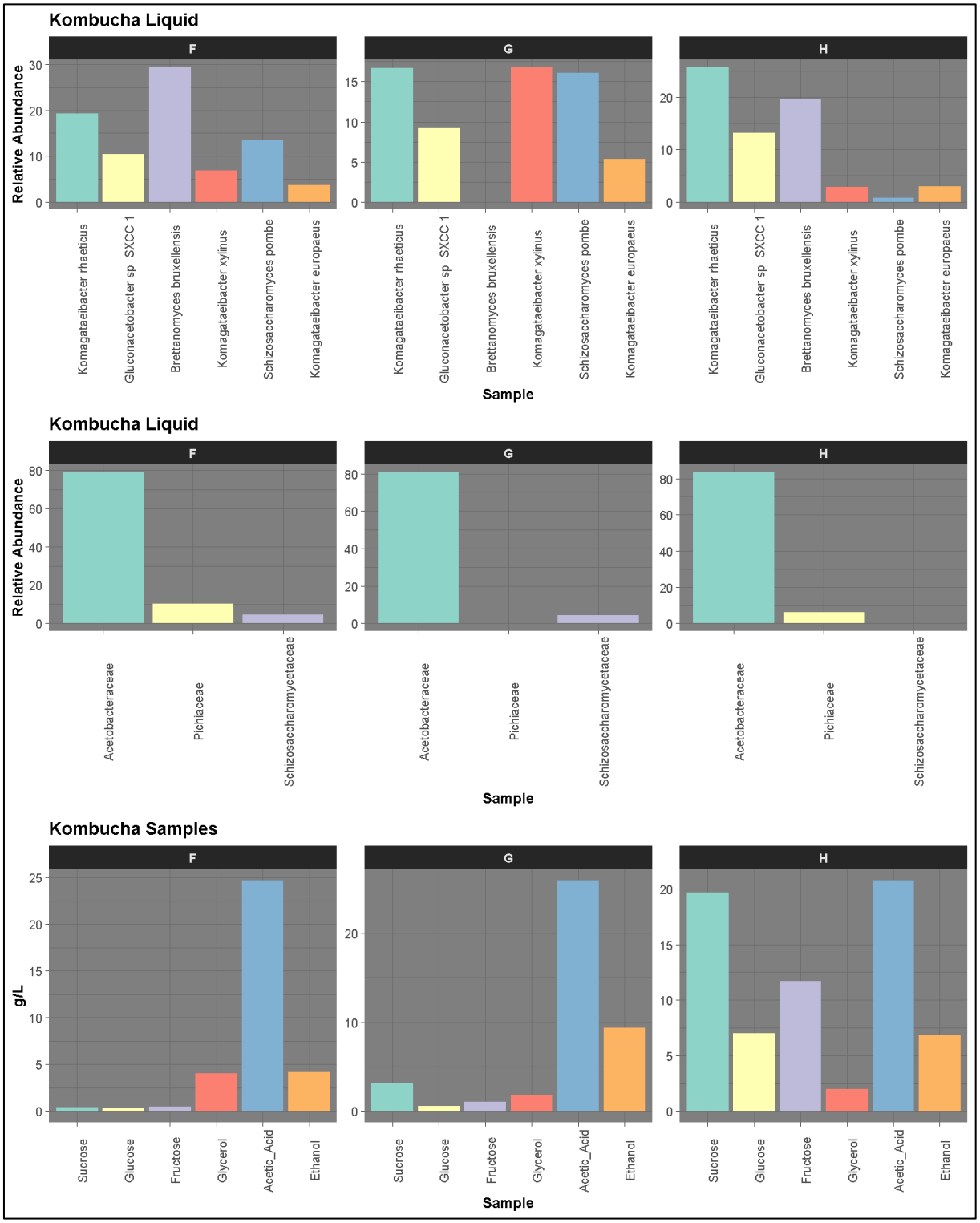

Figure 34. Most abundant species and families across F, G and H. Sugars, glycerol, acetic acid and ethanol concentrations at the endpoint of the three consortia.

Teoh *et al*., (2004) studied the yeasts population present in four Kombucha consortia during 14 days of fermentation and found *Z. baillii* to be the most representative species. The authors

found that the yeast population in the liquid grew exponentially before decreasing in the latter stages of fermentation, but that levels in the pellicle remained stable, probably due to a protective effect of the cellulose matrix. In the current study, the relative abundance of bacteria was 2-fold higher in the pellicle than in the liquid phase with the relative abundance of yeast being highest in the liquid phase. However, the yeast population was mainly detected in the liquid phases of the three studied samples. The consortia studied by Marsh *et al*., (2014) were dominated by the *Gluconacetobacter* and *Zygosaccharomyces* genera, corresponding to a relative abundance of 86-99% and 78-99% of the bacterium and yeast species, respectively, and were more homogenous than the Kombucha consortia identified in this study.

5.3.3 HPLC analysis of main phenolic compounds

Polyphenols are major plant secondary metabolites that exhibit a remarkably diverse range of bio-physicochemical properties, and are ubiquitous in plant extracts (Quideau *et al*., 2011). HPLC analysis was performed in order to identify the main phenolic compounds produced (**Table 14**). Gallic acid was produced with the three consortia in similar concentrations. This acid is produced by the enzyme shikimate dehydrogenase, an enzyme that was present across all three microbiomes and may explain the similarities in their production pattern. Catechin and epicatechin decreased after fermentation in all cases. Jayabalan et al., (2007) observed the same pattern when studying the changes in tea polyphenols during Kombucha fermentation and attributed the phenomenon to biodegradation by enzymes secreted by yeasts and bacteria. This decrease was also observed by Payne et al., (2010) when studying the fermentation of cacao beans but, in contrast, a previous Kombucha study (Villarreal-soto et al., 2019) the opposite behavior was observed in the case of the fermentation done with a higher surface/height ratio, where an increase in catechin concentration was obtained, going from 12.4 mg/kg of dry extract in the non-fermented tea till 3161.7 mg/kg after 21 days of fermentation. Caffeic acid, previously observed to be produced from the microbial conversion of other phenolic acids (Lin & Yan, 2012), increased after fermentation. Theobromine, rutin and chlorogenic acid production was favored by consortium (G). Only trace amounts of coumaric, ferulic and trans-cinnamic acids were found after fermentation with the three consortia. *Brettanomyces bruxellensis* catalyses the transformation of ρ-coumaric and ferulic acids into vinyl- and ethyl-phenols (Romano et al., 2008) and, thus, this could explain the lower concentration of these acids at the end of the fermentation. Vázquez-Cabral et al., (2017) fermented an oak infusion with the Kombucha consortium and detected lower concentrations of ρ-coumaric and ferulic

acids obtained in this study, being similar only regarding the rutin production of around 3 mg/L. Several pathways for the synthesis of aromatic compounds can be present in plant-based fermentations. In the specific case of bacteria, the biochemical pathways that are used to activate and cleave the aromatic ring depend on the availability of oxygen (Diaz et al., 2013). Yeasts metabolize aromatic compounds via the 3-oxoadipate pathway, but it has been demonstrated that the same yeasts may produce different secondary metabolites due to qualitative and quantitative differences of the fermented media (Romano et al., 2003). This suggests that microbial metabolism and the production of organic acids can be altered or inhibited depending on the fermentation conditions. **Figure 35** shows the high-resolution separation of 22 compounds in the ethyl acetate extracts of the three different consortia. A number of these compounds were identified as 3-gallic acid; 13-chlorogenic acid; 18-coumaric acid; 19-ferulic acid; 20-taxifolin and 22-trans-cinnamic acid. When comparing patterns to those of non-fermented tea, it was observed that several compounds were transformed. Moreover, compounds 2, 8 and 12 were produced after fermentation, but their presence varied depending on the consortium.

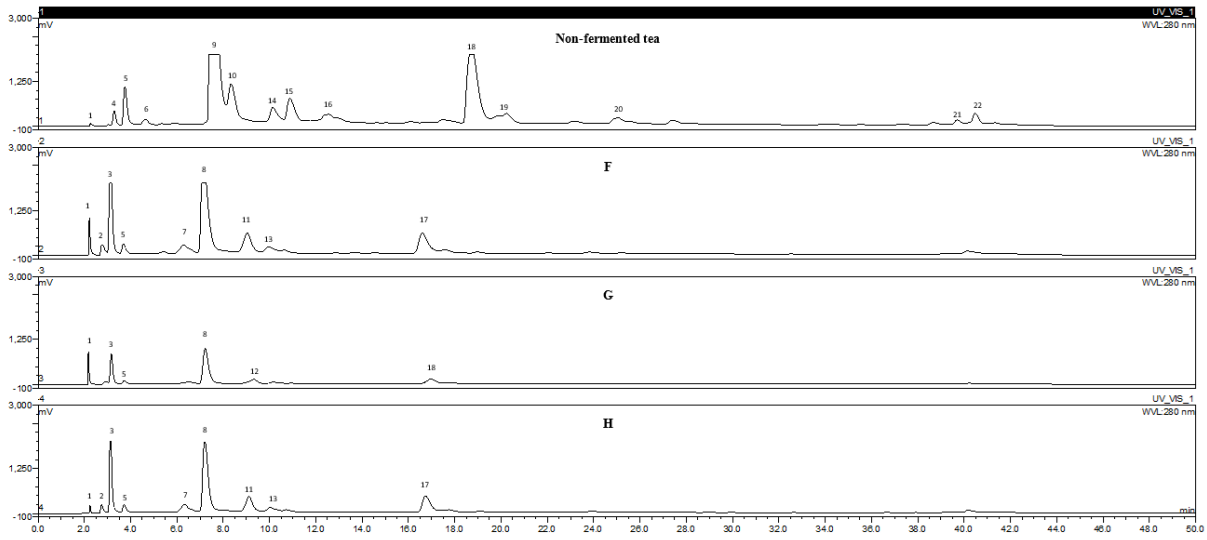

Figure 35. HPLC chromatograms of ethyl acetate kombucha extracts from the three consortia.

As depicted (**Figure 36**), a number of notable differences were observed in the case of Kombucha (F), with gallic acid and compounds 4 and 5 greatly increasing after fermentation and compound 9 being unique to this community. In addition, peak "a" was completely transformed after fermentation of Kombucha (F), but remained in the other two samples.

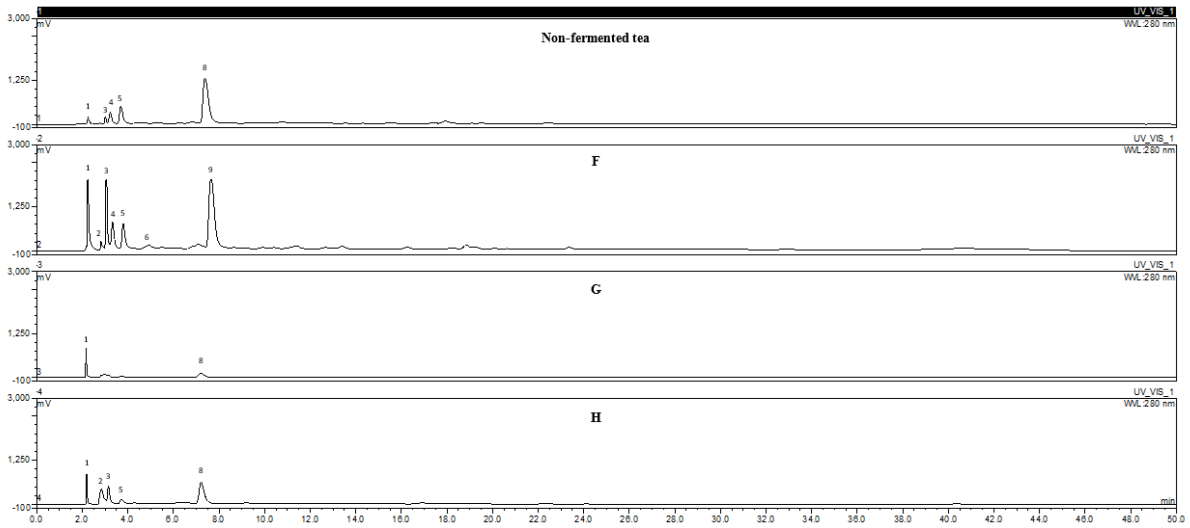

Figure 36. HPLC chromatograms of butanol kombucha extracts from the three consortia.

5.3.4 Gas chromatography-mass spectrometry analysis

GC-MS also contributed to metabolite analysis and led to the identification of several organic acids, sugars, alcohols and phenolic compounds (**Table 15**). Before derivatization, 2,2-Methylenebis(4-methyl-6-tert-butylphenol) was the only compound detected in the ethyl acetate extracts from the three Kombuchas and the non-fermented tea. In order to improve the identification and volatility of the produced compounds, a silylation was performed and twenty-six compounds were detected. Organic acids such as, isovaleric, succininc, malic, pentanoic and citric were predominantly found in the samples (F) and (H) in similar proportions, except from propanoic acid, which showed a 4- fold higher presence in the sample (H). Kombucha (G) had the lowest levels of organic acids, corresponding to the absence of *B. bruxellensis*. This yeast has been reported to be involved in the production of organic acids and volatile phenols (Andrade *et al*., 2007). Succinic acid is a dicarboxylic acid produced mainly as an intermediate of the tricarboxylic acid cycle during aerobic respiration and is also one of the fermentation end products of anaerobic metabolism (Chidi *et al*., 2018). The formation of organic acids, such as succinic acid, is reported to vary significantly with different fermentation conditions (Lamikanra, 1997). Its production also varies according to the microbial population, as was also observed in this study. L-(-)-Sorbofuranose was only detected in Kombucha (F), but several sugars were unique to kombucha H, which was also the kombucha with the highest concentration of residual sugars after fifteen days of fermentation. 2,3 butanediol, 2-Phenylethanol, Glycerol and 2-(4-Hydroxyphenyl) ethanol were detected in all the samples,

with the exception that 2-Phenylethanol was not detected in kombucha (G). The greatest proportions of these compounds was produced in Kombucha (F), which was also the fermented tea with the highest level of ethanol production (18 g/L). Among the phenolic compounds detected, all of those produced were present in the three kombuchas in similar proportions, except from catechin, which was only detected in sample (F) and (H). Results from a previous study (Villarreal-soto *et al*., 2019) showed higher areas of glycerol production compared to those observed with these consortia, possibly a consequence of the differences in the fermentation kinetics. Even if most of the yeasts species have similar central carbon metabolic pathways, differences in nutrient uptake and utilization, as well as in the regulation of fermentation and respiration, may yield different secondary metabolites (Chidi *et al*., 2018). Results from the previous study (Villarreal-soto *et al*., 2019) showed higher areas of glycerol production compared to the ones obtained with these consortia, which may be related to the differences in the fermentation kinetics. Even if most of the yeast species have similar central carbon metabolic pathways, differences in nutrient uptake and utilization, as well as in the regulation of fermentation and respiration, may produce different secondary metabolites (Chidi *et al*., 2018).

Table 14. Content of phenolic compounds, theobromine and caffeine from black tea (NF) and Kombucha extracts by HPLC (mg/kg of dry extract).

Compound / **Sample**	Gallic acid	Theobromine	Catechin	Chlorogenic acid	(-)-Epicatechin	Caffeic acid	Caffeine	ρ-Coumaric acid	Ferulic acid	Rutin	Taxifolin	Trans-Cinnamic acid
NF-EtOAc	50±1.8				337.8±1.8		205.3±1.6				44.4±0.5	27.2±0.3
NF- BuOH					25.9±0.0		114.5±0.7					
NF- H_2O			5.06±0.0		1.02±0.1		1.4±0.5			3.3±0.0		
F- EtOAc	11997.7±771.9						3.1±0.6					
F- BuOH	2407.7±73.6				0.5±0.3		3.1±0.2					
F- H_2O	54.3±4.5	54.3±5.6	1.7±0.0			11.3±0.0	0.1±0.0	0.2±0.0		0.1±0.0		
G- EtOAc	12966.5±285.7			493.6±0.0						155.6±0.0		
G- BuOH	490.0±142.5	414.5±11.3				18.7±0.5						
G- H_2O	178.0±61.8					25.5±2.1		0.1±0.0			0.1±0.0	
H- EtOAc	13020.9±66.8						20.7±1.3					
H- BuOH	1319.5±76.3						1.2±0.3					
H- H_2O	20.1±0.5		9.7±0.0						0.1±0.0	0.1±0.0		

Table 15. GC-MS analysis (area x10^6) of Kombucha tea samples and black tea (NF) without and with derivatization.

Without derivatization													
Chemical group	**Compound**	**NF**			**F**			**G**			**H**		
		EtOAc	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**
Phenolic	2,2-Methylenebis(4-methyl-6-tert-butylphenol)	550			505			127			500		
With derivatization													
Organic acids	Carbonic acid										41		
	Propanoic acid				80			87			344		
	Isovaleric acid				53						47		
	Pentanoic acid				83			18			65		
	Succinic acid				1115			990			1092		
	Malic acid							18			56		
	2-(Hydroxymethyl)Benzoic Acid				3			5			3		
	L-Phenyl lactate				26			5			18		
	L-propene-1,2,3-tricarboxylic acid				4						17		
	Citric acid				11						14		
Sugars	L-(-)-Sorbofuranose				63								
	D-Arabino-1,4-lactone										136		
	D-(-)-Fructofuranose		410										
	D-(-)-Tagatofuranose		200	377									
	α-D-Xylopyranose											4	
	Myo-Inositol											2	
	D-(+)-Turanose		135	1790								173	
	Lactulose											19	
Alcohols	2,3 Butanediol				183			25			36		
	2-Phenylethanol				23						12		
	Glycerol				136			293			105		
	2-(4-Hydroxyphenyl) etanol				134			45			78		
Phenolics	2-Tert-butyl-6-methylphenol	226			197			69			259		
	4-Hydroxyphenyllactic acid				95						10		
	4-Hydroxyphenylacetic acid				3								

5.3.5 Biological evaluation of Kombucha teas

In the interest of evaluating the beneficial potential of the fermented teas different *in-vitro* assays were performed and the results are discussed next.

5.3.5.1 Antioxidant capacity and total phenolic composition

Similar concentrations of phenolic compounds were obtained in the non-polar extracts of all the samples (**Figure 38**). Ethyl acetate has been reported to extract the highest amount of total phenolic compounds and total flavonoids in some previous studies (Boussoussa *et al*., 2014; Rahmani *et al*., 2019). Gallic acid showed its highest concentration in the ethyl acetate extracts according to the HPLC-DAD analysis. This acid has been found to exhibit the greatest antioxidant capacity among various polyphenols (Badhani *et al*., 2015), which may explain that the highest inhibition percentage against the DPPH radical was obtained with these non-polar extracts. The obtained inhibition percentages are higher than those reported by other authors (Malbaša *et al*., 2011; Vohra *et al*., 2019) who obtained around 60% of inhibition after 7-10 days of fermentation. The antioxidant activity of the phenolic acids is affected by several factors such as the phenolic aromatic arrangement or the position of the hydroxyl group (Badhani *et al*., 2015). In addition, the extraction of the phenolic compounds depends on their polarity and the affinity for the organic solvent. This could explain the difference in the total phenolic compound composition and consequently in the antioxidant activity of the butanolic extracts and aqueous phases. It can be observed, (**Figure 38**) that the phenolic composition of the ethyl acetate extracts is 2-fold higher than that of the butanolic ones. Nevertheless, the extraction yields of the ethyl acetate extracts are lower than those of the butanolic ones (**Figure 37**). Both extracts exhibit similar inhibitions percentages against the free radicals at 50 µg/mL. A higher concentration of its IC_{50} value, of 16 µg/mL was obtained with the butanolic extracts compared to 7 µg/mL with the ethyl acetate ones (**Table 16**).

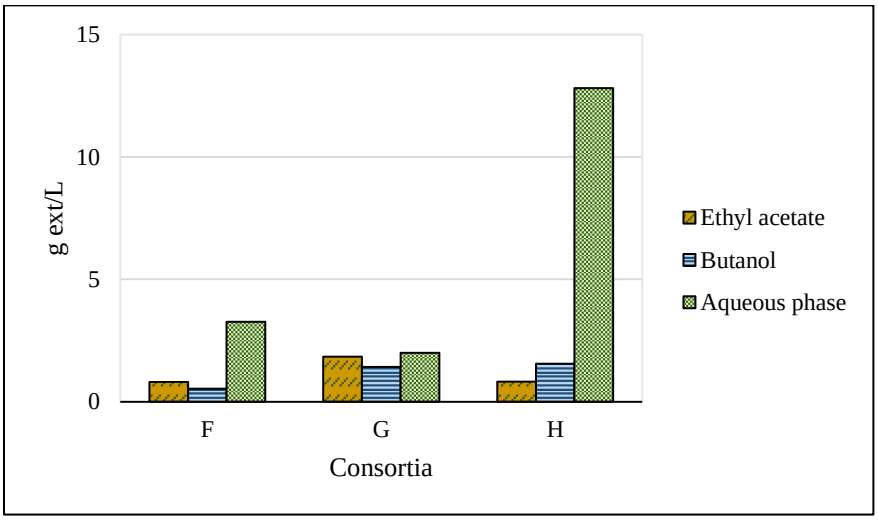

Figure 37. Extraction yields of different solvents and residual aqueous phases from the three different consortia.

Suggesting that the non-polar phenolic compounds are more stable and thus have a higher antioxidant activity. The higher antioxidant activity of the ethyl acetate extracts could be related to the higher presence of gallic acid in the three consortia (11997.7 -13020.9 mg/kg of dry extract), however no other compound was detected in similar concentrations in the butanolic extracts, which also showed a good antioxidant activity. This suggests that there may be other phenolic compounds present, responsible for preventing the oxidation, which were not quantified in this study.

Table 16. Half-maximal inhibitory concentration of the antioxidant activity of three Kombucha consortia and black tea (NF).

Solvents	**Samples IC_{50} (µg/mL)**			
	NF	**F**	**G**	**H**
EtOAc	7±0.2	7.0±0.4	6±0.3	8±0.9
BuOH	30±0.5	12±0.3	13.0.1	24±0.8
H_2O	>50	>50	>50	>50

IC_{50} values were calculated for inhibition percentages higher than 70% at 25 and 5 µg/mL ± standard deviations (n=3).

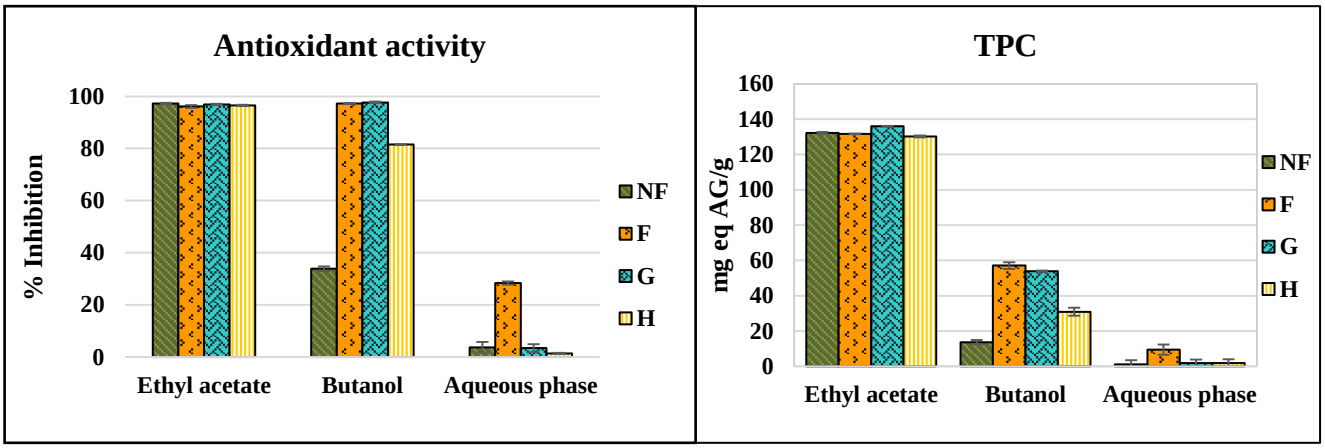

Figure 38. Antioxidant activity and total phenolic content (TPC) of black tea (NF) and Kombucha samples and from the three consortia.

5.3.5.2 Anti-inflammatory activity

The anti-inflammatory activity of Kombucha tea, as revealed by *in-vitro* assays, has been already reported (Vázquez-Cabral *et al*., 2017; Villarreal-soto *et al*., 2019) highlighting improved inhibition after fermentation with the consortia. The bioactivity of the final product is directly related to the processing technologies and its microbial and chemical composition (Michalska & Grzegorz, 2015). It can be observed (**Figure 39**) that the highest activity after fermentation was obtained with the consortium (G) with an inhibition percentage of 44.5%, followed by 28% with the consortium (H) in the ethyl acetate extracts. These results confirmed that Kombucha consortia have anti-inflammatory potential against the 15-lipoygenase enzyme.

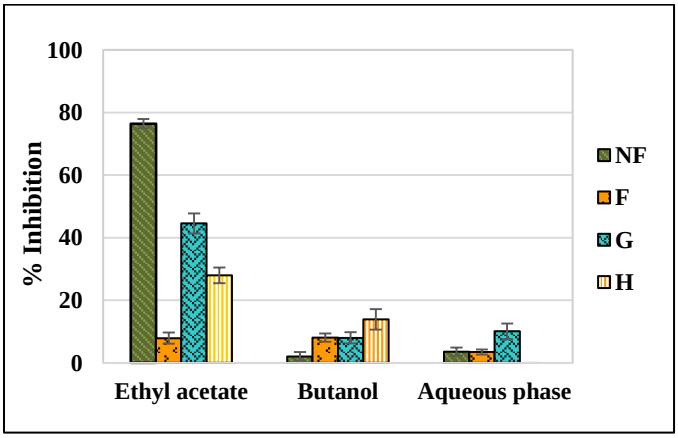

Figure 39. Anti-inflammatory activity of black tea (NF) and three Kombucha samples. All the data are average values of triplicate analysis.

5.3.5.3 Antiproliferative activity on human tumor cell lines

The anti-cancer effect of Kombucha extracts against the human cancer cell lines HCT-116, MCF-7 and OVCAR was investigated. The samples showed a higher selective antiproliferative activity against HCT-116 line in the case of the butanolic extracts compared to the non-fermented tea (**Table 17**). In particular, the consortium (G) seemed to best inhibit this cell line, with a 50% increase in inhibition being observed with the ethyl acetate and butanol extracts from the 21 day fermentation. This antiproliferative effect against the human colon cancer cells has been previously reported by Jayabalan *et al*., (2011) and Villarreal-soto *et al*., (2019), who found a higher increase of the inhibition after fermentation reaching around 60%. In the case of the MCF-7 cancer cell line, the consortium (F) showed a slight increase after 15 days of fermentation, however, the rest of the extracts did not show any increase compared to the control. However, Kombucha extracts did not show cytotoxic activity against the OVCAR cancer cells, where it can be observed that the inhibition found in the non-fermented tea decreased after fermentation with the consortia.

Table 17. Antiproliferation activity against human colon cancer (HCT-116), human breast cancer (MCF-7) and human ovarian cancer (OVCAR) cell lines.

Sample	Extract	Inhibition % at 50 µg/mL		
		HCT-116	**MCF-7**	**OVCAR**
NF	**EtOAc**	8.0±0.7	14.6±0.2	34.7±4.7
	BuOH	4.4±2.0	N.A	16.5±2.0
	H_2O	N.A	14.0±0.4	14.6±1.2
F	**EtOAc**	10.3±0.4	6.9±0.7	N.A
	BuOH	13.8±0.7	4.5±1.6	N.A
	H_2O	1.7±0.9	18.0±1.4	N.A
G	**EtOAc**	16.3±1.8	7.8±0.2	29.1±2.3
	BuOH	10.5±1.1	3.0±0.3	1.1±0.3
	H_2O	N.A	15.3±0.6	N.A
H	**EtOAc**	13.7±1.1	10.5±0.9	N.A
	BuOH	8.2±0.2	N.A	N.A
	H_2O	N.A	N.A	N.A

All the data are average values of triplicate analyses. Where NF: black tea, N.A: not active.

5.4 Conclusions

The study of metagenomic DNA from the solid and liquid phases of three kombucha consortia revealed differences across kombuchas and between the two phases. Fermentation kinetics showed an impact of the microbiota on sugar consumption and secondary metabolite production. It should be noted however, that some similarities were observed between the three different microbial populations. The most abundant species of bacteria were the same in all three samples, differing only in relative abundance. The yeast populations differed most considerably and, as a consequence, the sugar profile differed, in a manner that reflected the presence or absence of *S. pombe*. The specific microbiota revealed to have an impact on the biological profile of the obtained teas, where the consortium G seemed to have the highest inhibition percentages regarding its anti-inflammatory and antiproliferation activities. This consortium contained the highest relative abundance of *Komagataeibacter xylinus* in both solid and liquid phases and *Schizosaccharomyces pombe* in the liquid phase, which raises the possibility that these species could be responsible for the observed bioactive profile. Ultimately, this study exhibited a global approach of the factors influencing the production of kombucha tea and highlighted the main microbial population of its consortia.

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5.6 Analyse en composantes principales

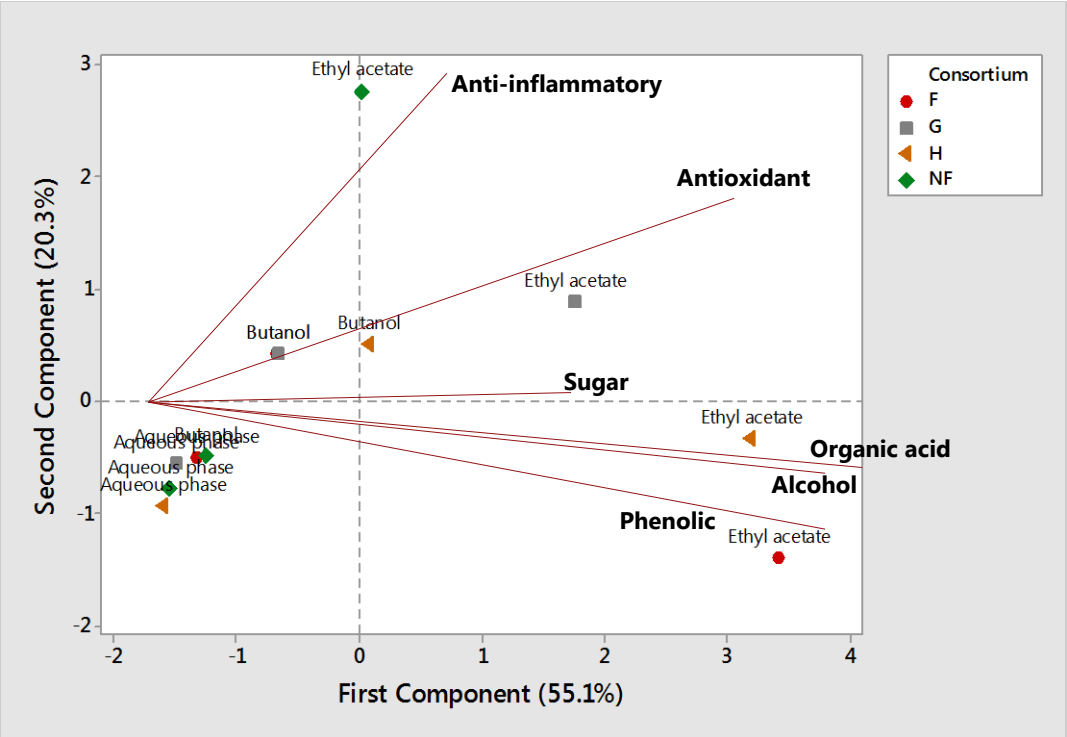

Figure 40. Analyse en composantes principales des activités biologiques et trois consortia différents. NF: thé non-fermenté.

La PCA a été réalisée sur la matrice de corrélation en tenant compte des trois consortia, leurs extraits et leurs activités biologiques. L'analyse a montré des différences marquées entre les extraits. Les phases aqueuses ont été séparées dû à leur inactivité, suivis d'extraits de butanol, jusqu'à atteindre les extraits non polaires d'acétate d'éthyle qui étaient ceux qui avaient une forte corrélation avec les activités biologiques et la présence de métabolites secondaires. Les trois consortia ont montré des corrélations avec les activités biologiques, cependant, c'est le consortium (G) celui qui se distingue comme le plus anti-inflammatoire. Cette analyse nous a permis de constater que la population microbienne de chaque consortium est directement liée à son profil biologique et à sa production de molécules après fermentation.

5.7 Conclusion du chapitre

La grande diversité microbienne présente dans le consortium de la Kombucha fait que la maitrise de sa fermentation soit complexe. Plusieurs facteurs peuvent intervenir dans la spécificité de chaque consortium tel que, l'origine géographique, la source de carbone utilisé ou les conditions de fermentation. De ce fait l'intérêt d'analyser différents consortia est apparu.

Cette partie a porté sur l'étude de trois consortia différents afin d'observer si la variation de la population microbienne avait un effet déterminant sur les activités biologiques du thé de Kombucha ainsi comment sur sa composition chimique.

Le séquençage de l'ADN a révélé une composition assez hétérogène entre les différentes biofilms étudies et leurs respectives phases liquides. Cette analyse nous a permis de conclure que malgré des origines différentes, les groupes dominants de bactéries et de levures semblent rester constants.

La distribution microbienne entre les deux phases (solide et liquide) a été très variable et cela a montré avoir un impact sur la cinétique de fermentation et les activités biologiques.

Le consortium avec l'abondance relative plus élevé en bactéries a obtenu les pourcentages d'inhibition les plus intéressantes.

Chapitre VI: Evolution des activités biologiques *in-vitro* et les métabolites produits au cours de la fermentation

Sommaire

Problématique de recherche et résumé du sixième chapitre

La détermination du temps de fermentation optimal est un paramètre important à prendre en compte pour la production de Kombucha. On sait déjà que cela ne devrait pas dépasser 10 jours pour avoir un goût agréable et que des périodes de fermentation plus longues ne sont pas recommandées en raison de la forte production d'acides (Chen & Liu, 2000). Certains auteurs ont étudié l'effet du temps sur la production des acides organiques et composés phénoliques, sur l'activité antioxydante du Kombucha et sur le rendement en cellulose bactérienne (Chu & Chen, 2006 ; Jayabalan *et al*., 2007; Jayabalan *et al*., 2008). Ils ont observé qu'aux alentours de 15 jours, les sucres ont été presque épuisés et les métabolites ont atteint la production maximale dans la plupart des cas étudiés. Cependant le thé de Kombucha est une source potentielle de molécules bioactives qui peuvent être responsables de plusieurs effets bénéfiques pour la santé (Leal *et al*., 2018). L'intérêt de cette chapitre est donc d'étudier l'évolution des métabolites et des activités biologiques au cours de la fermentation afin d'estimer la durée de fermentation optimale pour leur production indépendamment de l'aspect organoleptique. Cinq temps différents de fermentation ont été choisis: 0, 4, 8, 15 et 21 jours et les caractérisations chimique et biologique ont été réalisés.

Le suivi des cinétiques de fermentation a révélé un épuisement des sucres après 15 jours de fermentation coïncidant avec les productions maximales d'éthanol et d'acide acétique. La quantification de composés phénoliques par HPLC a montré qu'à l'exception de l'acide gallique qui est resté stable tout au long du processus, le reste des composés ont atteint leur production maximale entre 8 et 15 jours. Par contre, en ce qui concerne le profil fonctionnel, le temps optimal n'a pas été le même pour toutes les activités biologiques étudiées. Cette expérience nous a permis de comprendre qu'il n'y a pas de temps de fermentation qui améliore simultanément toutes les activités biologiques et en même temps la production de métabolites d'intérêt, d'où l'importance de la conception du procédé en fonction du produit final souhaité.

Evaluation of the *in-vitro* bioactivities and chemical composition of Kombucha tea during fermentation

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Abstract

In order to study the evolution of the biological activities of Kombucha tea sequential liquid-liquid extractions were performed during fermentations at 0, 4, 8, 15 and 21 days using solvents with different polarity. Those results were compared to similar analysis using total dried extracts of the same culture. The inhibition percentages against the DPPH radical were from 60 to 96% in the case of acetate and butanol extracts and from 15 to 75% in the case of the dried total dry extract, before and after fermentation, respectively. The cytotoxic activity was tested against human breast cancer (MCF-7) and human colon cancer (HCT-116) cell lines, finding an increase in the inhibition after 4 days of fermentation with the ethyl acetate extracts. Results showed that Kombucha's biological activities may vary depending on the polarity of the extracted compounds and the duration of fermentation. The extracted phenolic compounds and main components were also identified.

Keywords: biological activities, fermentation time, Kombucha tea, solvent extracts

6.1 Introduction

Bioactive compounds are extra nutritional constituents that are present in vegetables and fruits and have been related to the decrease in the incidence of some degenerative diseases as cancer, type 2 diabetes (Da Silva Pinto, 2013) or cardiovascular diseases (Biesalski *et al*., 2009). The first studies about Kombucha tea exerting anticancer properties in their consumers were done in the 60's (Dufresne & Farnworth, 2000). Subsequently, several authors have investigated the antiproliferative activity of the fermented beverage. Cetojevic-Simin *et al*., (2008) tested Kombucha beverages from black and winter savory (*Satureja montana L.*) teas against three human cell lines and found an IC_{20} value for inhibiting the cervix epithelioid carcinoma cell line with the winter savory Kombucha. Jayabalan *et al*., (2011) tested the cytotoxic and anti-invasive properties of chloroform, ethyl acetate, butanol and water extracts against three cancer cell lines (A549, U2OS and 786-O) and found an interesting inhibition percentage against the human osteosarcoma. Another disease that affects millions of persons around the world is inflammation, which is generally treated with the non-steroidal anti-inflammatory drugs (NSAIDs) that produce their therapeutic activities through the inhibition of the enzymes responsible of producing the prostaglandins, mainly the 15-lipoxygenase (15-LOX). However, these drugs possess several side effects and have limited efficacy. Therefore, 15-lipoxygenase inhibitors may be valuable for the treatment of the inflammatory disease and consequently become an alternative to the non-steroidal drugs (NSAIDs) (Dvorakova & Landa, 2017). Vázquez-Cabral *et al*., (2017) fermented oak leaves infusion with the Kombucha consortium and observed a significant anti-inflammatory activity by regarding the reduction in the level of the pro-inflammatory cytokines IL-6 and TNF-alpha. Tea is also known to possess beneficial effects, which are mainly due to the presence of some chemical compounds as polyphenols, alkaloids, volatile oils, polysaccharides, amino acids, lipids, vitamins, etc. (Nie & Xie, 2011). However, studies have shown (Chen & Liu, 2000; Aloulou *et al*., 2012) that fermentation of black tea with the Kombucha consortium produces several molecules that contribute to the potential therapeutic values of the fermented beverage, including pyruvic, lactic, succinic, malic, citric, acetic and glucuronic acid, between others (Villarreal-Soto *et al*., 2018). These bioactive molecules can be extracted either by various extractive techniques, or they can be arranged as they are in the fresh plant material once crushed or after a drying treatment. This would allow to obtain natural compounds with high added value for the food, cosmetic and/or pharmaceutical industry due to their functional, antimicrobial or antioxidant characteristics (Azmir *et al*., 2013). A common method is the serial exhaustive one, where extractions are carried out successively with solvents of increasing polarity in order to extract a wide range of

compounds (Ncube *et al.*, 2008). Nonetheless, it is also important to consider the impact of fermentation over the production of bioactive compounds, which could either increase or decrease their concentration in the media. Therefore, in order to study their evolution and biotransformation we measured the biological activities and produced phenolic compounds in kombucha tea during its fermentation time.

6.2 Materials and methods

6.2.1 Starter culture

The Kombucha consortium used in this study was obtained from www.je-mange-vivant.com and was backslopped at least three times on black tea before starting the experiment.

6.2.2 Preparation of Kombucha tea

Black tea was prepared according to the following procedure: 70 g of sugar were added to 1 L of water at 40°C. Once the water reached 80 °C, 10 g of black tea (Royal Ceylan, Lipton ®) were also incorporated and allowed to infuse for 15 minutes. Then the tea was removed and the infusion was left to cool. 970 mL of tea were poured into 2 L glass containers, and then it was inoculated with 20 mL of previous fermented liquid media and 20 g of the Kombucha consortium. Finally, in order to lower the pH 10 mL of commercial cider vinegar were also added.

Sampling and incubation

The cultivation method used for this study consisted in using several containers with a ratio s/h of 9.02 cm and s/v of 0.132 cm^{-1}, in order to obtain four different fermentation times of 4, 8, 15 and 21 days. The containers were then covered with cheesecloth and incubated at 25°C. All fermentations were done in duplicate.

6.2.3 Analytical methods

Sugars consumption and metabolites production were analyzed by ultra performance liquid chromatography (UPLC-RI) (Thermo Scientific, Dardilly, France). The dried extracts were

injected in a concentration of 20 mg/mL and then phenolic and aromatic compounds were identified and quantified by HPLC-DAD (Thermo Scientific Dionex Ultimate 3000). Chemical composition was performed by gas chromatography-mass spectrometry (GC-MS) Varian Saturn 2000 (Les Ulis, France). And total phenolic composition was quantified using the Folin-Ciocalteu assay. All according to the methods described by Villarreal-soto *et al*., (2019).

6.2.4 Extraction methodology and biological evaluation

Sequential liquid-liquid extractions with organic solvents (ethyl acetate and butanol) were done with the Kombucha samples at the end of the fermentation. The solvents were then evaporated using a vacuum rotary evaporator at 35 °C (RV 10 Auto VWR, IKA, Staufen, Germany) in order to obtain a dry extract. A total dry extract of fermented tea was obtained by evaporating its volume directly without fractionating it with organic solvents (Dried sample). Then the total phenol concentration and antioxidant, anti-inflammatory and antiproliferation activities were evaluated according to the methods previously described (Villarreal-soto *et al*., 2019). Stock solutions were prepared from each dry extract in a concentration of 3000 µg/mL, using DMSO 100% as solvent. Then, for all the biological evaluations the stock solutions were tested initially at 50 µg/mL and at 25 or 5 µg/mL in the case of high inhibition percentages (>70%).

6.3 Results and discussion

6.3.1 Extraction yields

Two different solvents of increased polarity (ethyl acetate and butanol) were used in order to extract the molecules of interest and the residual aqueous phase was treated as the extracts. The highest extraction yield was obtained with this aqueous phase, being of 68.0 mg ext/L. Regarding the non-polar extracts of ethyl acetate their values ranged from 0.6 to 0.9 mg ext/L, followed by the butanol ones going from 3.2 till 1.4 mg ext/L after 21 days of fermentation. **Figure 41** shows the behavior of the extracted compounds during the fermentation of Kombucha tea. It can be observed that the mass of the aqueous phase is decreasing progressively, this is normal as it corresponds mainly to the sugars concentration in the tea, which are consumed by yeasts and bacteria and transformed into other organic compounds. In contrast, the non-polar compounds extracted with ethyl acetate and butanol tended to increase

with time as they are mainly formed after the hydrolysis of sucrose, as for example, succinic, glucuronic or acetic acids (Jayabalan *et al*., 2007).

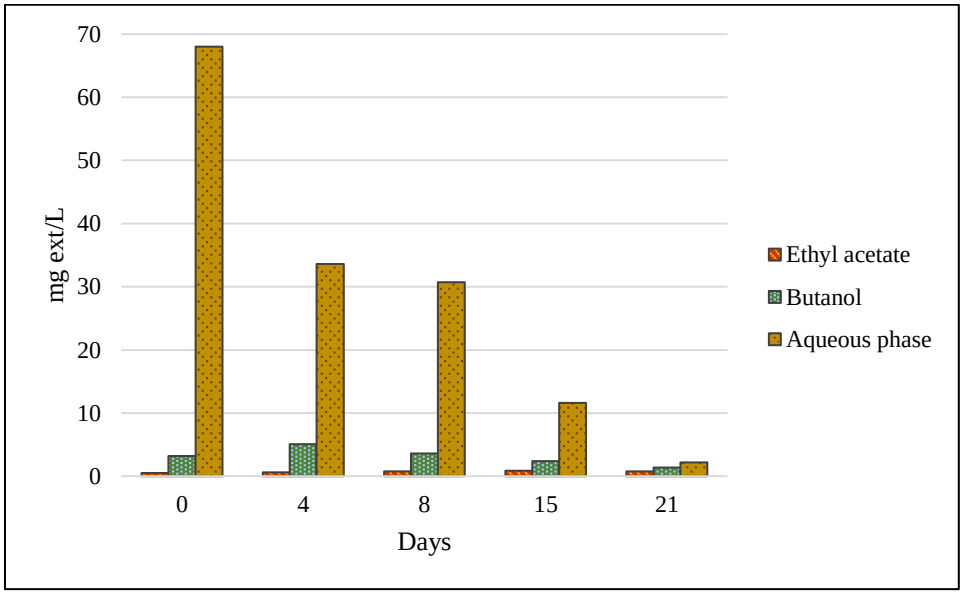

Figure 41. Evolution of the extraction yields of different solvents and residual aqueous phase during fermentation time.

6.3.2 Changes in Kombucha metabolites production and sugars consumption

A progressive reduction of sucrose can be observed between 0 and 8 days of fermentation (**Figure 42**), which was then hydrolyzed to glucose and fructose by the yeasts enzyme invertase. Acetic acid and ethanol increased progressively, going from 5-7 g/L to around 10-20 g/L after 21 days. Glycerol concentration increased during the first five days and then remained quite stable during the fermentation, reaching a maximum value of 1.05 g/L after fifteen days. The metabolites concentration observed in this fermentation kinetics differed from those found by other authors (Chen & Liu, 2000; Jayabalan *et al*., 2007; Chakravorty *et al*., 2016), which probably results from the different microbiota found in each Kombucha consortium. However, the acetic acid concentration was similar to the one obtained by Rahmani *et al*., (2019) who obtained 25 g/L after 14 days of fermentation of African mustard with the tea fungus.

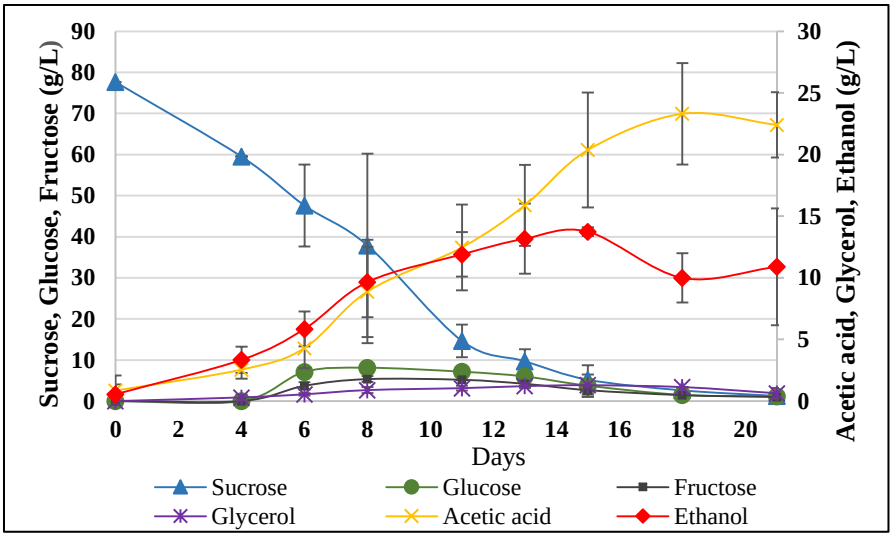

Figure 42. Changes in sugars consumption and acetic acid, glycerol and ethanol production.

6.3.3 Evolution of caffeine and phenolic compounds during fermentation

The identification and quantification of alkaloids and main phenolic compounds was done by ultra-performance liquid chromatography. The maximal concentration of caffeine found in Kombucha samples was of 1.0 mmol/kg (0.004 mg/mL) in the case of the acetate extract of non-fermented tea, which was nearly depleted after 5 days of fermentation with the Kombucha consortium. A caffeine concentration higher than 2.5 mg/mL may inhibit the bacterial population growth (Gokulakrishnan *et al*., 2005). Caffeine can be assimilated as a nitrogen source by yeasts and its degradation ability seems to be better at higher pH values, which may explain its decrease since the beginning of the fermentation. Moreover the addition of sucrose is supposed to enhance the decaffeination process as observed by Nanjundaiah *et al*., (2017). The microbial production of theobromine from caffeine was studied by Asano *et al*., (1993) and a similar behavior was observed in the 15th day of fermentation where caffeine concentration decreased while the theobromine one increased (**Figure 43**). The phenylpropanoid pathway was observed in the case of the phenolic acids production (Pyne *et al*., 2019), where ferulic acid derives from caffeic acid in the 5th day. ρ-Coumaric acid is the precursor of these phenolic acids, however, plant pathways may undergo some changes due to the enzymes compatibility of each microorganism (Lin & Yan, 2012). The fact that ρ-coumaric acid do not solubilizes in water but in alcohol, could also explain its detection around 5 days of fermentation when the alcohol concentration started to increase. But besides the several pathways of ρ-coumaric acid reduction during fermentation, the adsorption by yeasts cell wall, especially by the *Brettanomyces* sp., seems to be the reason of its major loss (Salameh *et al*., 2008). Chlorogenic acid is an ester of

caffeic acid, so this may explain the increase of its concentration after 5 days, when the one of caffeic acid started to decrease, respectively. The flavonols catechin and epicatechin are among the major polyphenolic compounds and tend to decrease during fermentation. Regarding the epicatechin formation which is a derivative from the oxidation of catechins (Tanaka *et al*., 2010), it was observed around the 8th day of fermentation. Jayabalan *et al*., (2007) studied the concentration changes of some epicatechin isomers and observed a considerable increase of epicatechin on the 12th day; a similar behavior was observed in our study around the 15th day, which may be due to their release from the microbial cells. Rutin hydrate, trans-cinnamic acid and taxifolin are considered as minor but functionally plant polyphenols, which were also detected in concentrations between 0.003, 0.09 and 0.002 mmol/kg, respectively. One of the aims of studying the evolution of metabolite production is to find its optimal fermentation time. The highest day of production varied among the detected compounds, being 4 days for theobromine and caffeic acid, 8 days for coumaric and ferulic acid and 15 days for epicatechin and gallic acid. In the case of the ethyl acetate extract, gallic acid was accumulated during fermentation, this is normal as it is normally oxidized by the o-quinones which are produced towards the end of the fermentation process (Huang *et al*., 2016) so unless fermentation is prolonged, they have no opportunity to oxidize it into other phenolic molecules. Gallic acid has been found to exhibit the highest antioxidant capacity among several phenolic compounds (Badhani *et al*., 2015). In the case of this study, it appeared as the most concentrated compound reaching its highest production of 75.0 mmol/kg of dry extract, after 8 days and remained quite stable through the fermentation. Ethyl acetate extract obtained the highest scavenging activity against the DPPH radical and showed the same behavior as the gallic acid concentration, which may prove the strong antioxidant capacity of this secondary metabolite.

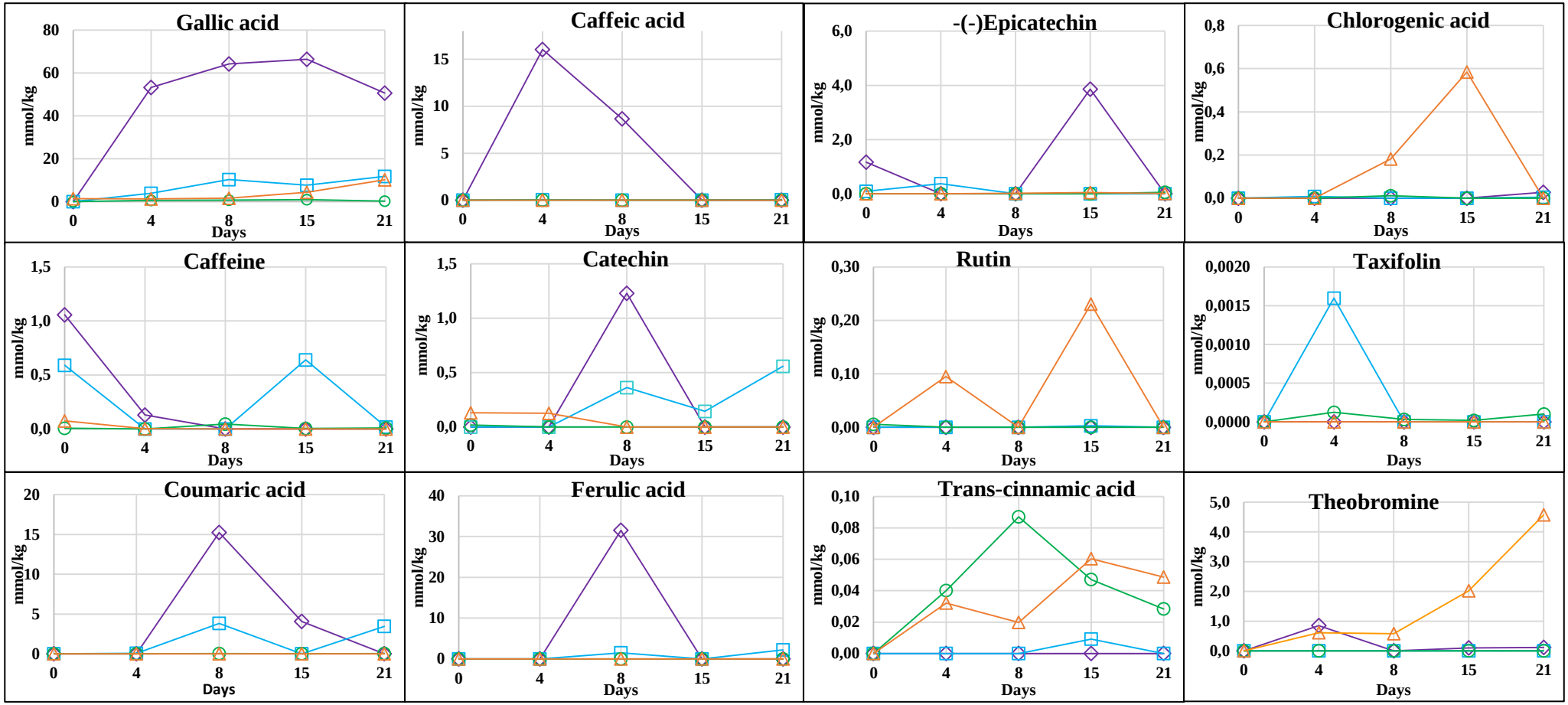

Figure 43. Evolution of phenolic compounds concentrations (mmol/kg) during 21 days of fermentation. (◇) Ethyl acetate, (□) Butanol, (○) Aqueous phase, (△) Dried sample.

The HPLC chromatogram (**Figure 44**) allows to observe the evolution of the compounds going from 0 day (non-fermented tea) until 21 days of fermentation. A great variability regarding the transformation and production of compounds was observed. Compound 3 was the only compound that remained stable and increased after fermentation. Compounds 4 and 12 were completely transformed after 4 days. It seems important to notice that many compounds were produced around 8 days and peaks 15 and 19 were only present between 8 and 15 days before being transformed. The following compounds have been identified: 3-gallic acid; 5-theobromine; 11-catechin; 17-coumaric acid; 19-ferulic acid; 22-trans-cinnamic acid.

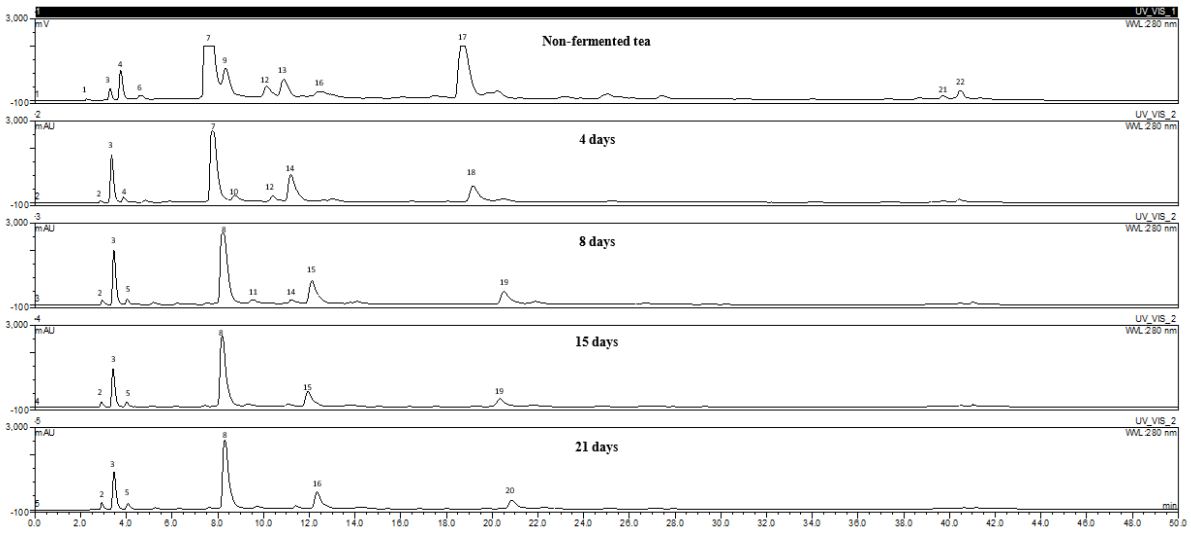

Figure 44. HPLC chromatograms of ethyl acetate kombucha extracts during 21 days of fermentation.

It can be observed in **Figure 45** that compounds 2 and 7 seemed to be the major ones during all the fermentation time. However, compound 2 was already present in the non-fermented tea and increase progressively after fermentation moreover, compound 7 was produced after fermentation with the consortium and remained constant. Compounds 3 and 4 were present before and after fermentation in trace amounts.

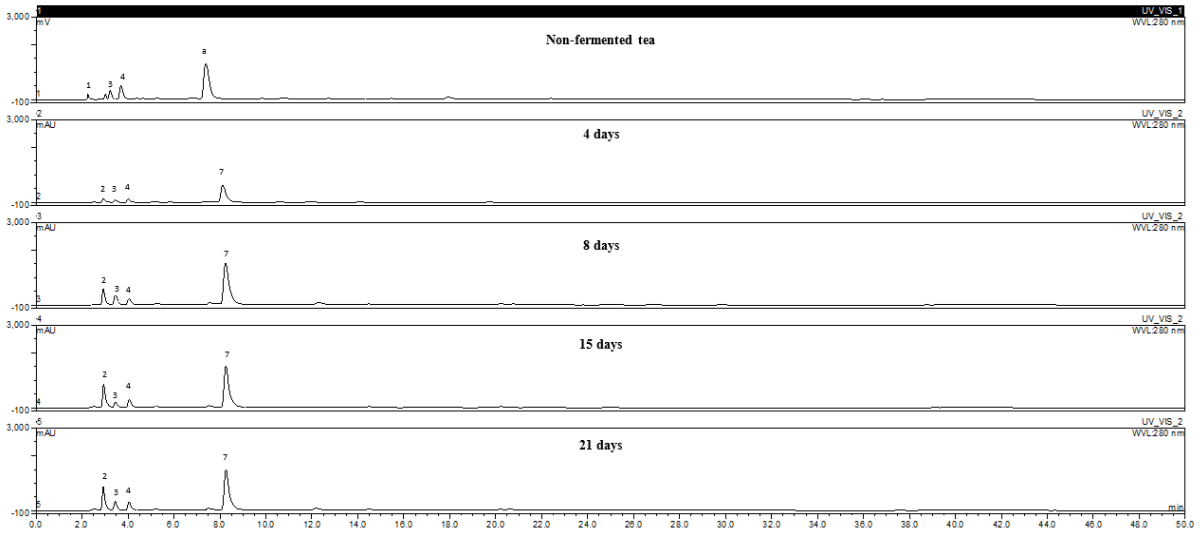

Figure 45. HPLC chromatograms of butanol kombucha extracts during 21 days of fermentation.

6.3.4 GC-MS Analysis of volatile compounds

Gas chromatography-mass spectrometry allowed the identification of several compounds in Kombucha tea extracts (**Table 18**). 2,2'-Methylenebis,4-methyl-6-tert-butylphenol was detected in both fermented and non-fermented samples, and remained quite stable through the 21 days of fermentation. The derivatization of thermally stable metabolites can be obtained by the silylation reaction, which in this study allowed the identification of thirty-eight compounds. Except from sugars, mainly all of the detected metabolites were produced after fermentation with the consortium. 2,3 Butanediol, propanoic acid and glycerol were present in all the samples. The highest presence of some organic acids (succinic, isovaleric, and gallic) was found between 8 and 15 days, which was also the period where an increase in bioactivity was observed. A similar behavior of this flavonoid was observed by Jayabalan *et al*., (2007) who found an increase of its concentration in the 12^{th} day and observed the same tendency of degradation with longer fermentation times. Succinic acid, glycerol, malic acid, catechin, gluconic acid, 2,3 butanediol, gallic and propanoic acids have been already detected in a Kombucha tea obtained from oak leaves (*Quercus resinosa*) (Vázquez-Cabral *et al*., 2014), in fermented garlic with the Kombucha consortium (Ebrahimi *et al*., 2017) and in Kombucha tea from black tea (Villarreal-soto *et al*., 2019). This similarity in the chemical composition despite the different substrates, suggests that its microbiota mainly determine the final composition of Kombucha teas and not the fermented plants. This could also be observed by comparing the chemical composition obtained in this study with one from a previous research (Villarreal-soto *et al*., 2019), where a different consortium was used. Differences can be observed regarding the

detected compounds, e.g., malic acid, succinic acid and glycerol highly increased its value, while gluconic acid decreased its presence by 50%. However, besides the microbial consortium, the geometry of the vessel and the fermentation times also differed between both studies. Isovaleric acid increased its value after 8 days and then decreased and remained similar in both consortia. Gallic acid increased 10-fold its value after 15 days of fermentation before decreasing and catechin production increased with fermentation time, reaching its highest value after 8 days. Chen & Liu (2000) observed a high increase in gluconic acid production after 20 days of fermentation, reaching its highest concentration of 3.9 g 100 mL^{-1} after 60 days. Gluconic acid is a valuable compound as it has applications in the food and pharmaceutical industries (Ramachandran *et al*., 2006), and its production tends to increase with longer fermentation times. However, according to the FDA Kombucha tea fermentations longer than 10 days are not suitable for human consumption (Nummer, 2013). Therefore, according to the industrial interests, the Kombucha could be considered as a tea or as an alternative source for the production of metabolites of interest.

Table 18. GC-MS analysis (area x10^6) of black tea (NF) and Kombucha tea samples without and with derivatization.

Without derivatization																
Chemical group	**Compound**	**NF**			**4 Days**			**8 Days**			**15 Days**			**21 Days**		
		EtOAc	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**	**EtOAc**	**BuOH**	**H_2O**
Phenolic	**2,2'-Methylenebis(4-methyl-6-tert-butylphenol**	550			831	13	256	955	26	300	531	19	218	370	21	450
With derivatization																
Organic acids	**Propanoic acid**				76			68			66			48		
	Isovaleric acid				3			23			10			4		
	Succinic acid				257			432	761		844	888		358	2120	
	Malic acid							22			16	182		23	213	
	2-(Hydroxymethyl)Benzoic Acid				21			9			5					
	2-Isopropylmalic acid							22						1		
	Threonic acid														19	
	Pentanedioic acid														20	
	Citric acid													7	292	
	L-Gluconic acid 1,4-lactone														975	
	D-Gluconic acid									387		38			297	
	Gluconic acid, 1,4-lactone														1328	
	Ribonic acid											815.0				
Sugars	**D-(-)-Ribofuranose**			1854					120			179				
	L-(-)-Arabitol														142	
	D-Arabino-1,4-lactone				251			157			83			54		
	D-(-)-Tagatofuranose		200	377					690	639	2	546			226	
	D-(-)-Fructofuranose		410						1390			2155			922	

	D-(+)-Talofuranose		36	716					1362	1327		212				
	D-(-)-Fructopyranose														78	
	α-D-Mannopyranose			8940					3696	1135						
	D-glucose			3752					27	335		4235			3313	
	D-Psicofuranose								26			132			78	
	L-(-)-Sorbose											300				
	Lactulose							210			10					
	D-(+)-Turanose		135	1790					1321	96		1737			70	
	D-(+)-Trehalose		22149													
Alcohols	**2,3 Butanediol**				21			26			36			25		
	2-Phenylethanol				2.6			11.9			11.5					
	Glycerol				58			141	3918	872	153	4301		83	6775	
	Mannitol														238	
Phenolics	**Ethyl cinnamate**		25	385												
	2-Tert-Butyl-6-Methylphenol	226			543			199			65			77		
	Catechin		17		173			128			64				18	

6.3.5 Biological activities

As far as we know this is the first study that compares the biological profile of the fermented beverage against the one of the solvent extracts. We decided to give this approach to our study in order to obtain a better understanding of its functionality, not only as a potential healthy beverage but also as a source of pharmacologically bioactive molecules that could be used for the development of new products in the cosmetic or food industries. The measured activities are described next.

6.3.5.1 Antioxidant activity

The antioxidant activity of solvent extracts and the dried Kombucha sample was tested against the DPPH radical. High inhibition percentages were obtained in the case of ethyl acetate extracts tested at 50 µg/mL and maintained through all the fermentation process (**Figure 46)**. It can be observed that in the case of the dried sample the scavenging activity increased progressively all along the fermentation process, going from 15.7 until 75.7% after 21 days; where the most significant increase was observed between 15 and 21 days of fermentation. This behavior could be due to the increase in the yield of ethyl acetate extract, which was the most bioactive extract. The transformation of sugars into several non-polar compounds by the microorganisms could have concentrated the antioxidant compounds that were present in the Kombucha tea. Similar results were observed by Chu & Chen (2006), who obtained a maximal inhibition value of 69.2% after 15 days using a sample collected from a household in Taiwan.

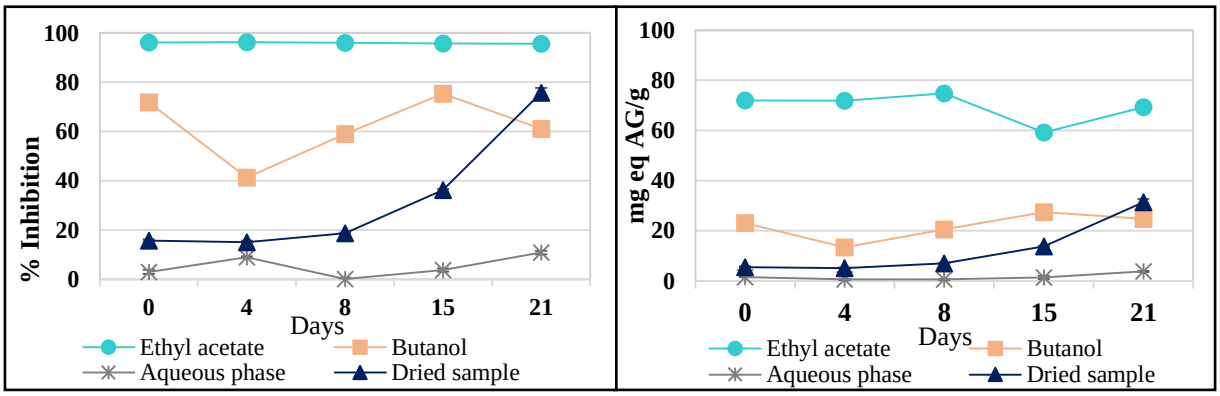

Figure 46. Changes in DPPH scavenging activity tested at 50 µg/mL and evolution of total phenolic composition during Kombucha fermentation. Data are the means of three independent experiments ± standard deviations (n=3).

In the case of Kombucha extracts and taking into account that the molecules were extracted according to their polarity, we can assume that the compounds with higher antioxidant properties are non-polar. Ethyl acetate is regularly used to extract lipophilic antioxidants, for example the flavonoids, which are commonly found in tea. According to Chakravorty *et al*., (2016), the concentration of this compounds was increased during the fermentation, meaning that the high inhibition percentage of this solvent extracts may be due to the combination of the flavonoids already present in the tea and those obtained by the hydrolysis of microorganisms. Regarding the variation of the inhibition obtained with the butanol and water extracts, this may be due to the synergy effect between the bioactive extracted compounds. IC_{50} value was calculated for the inhibitions percentages higher than 60% and the results are showed in **Table 19**. Lower IC_{50} values show more antioxidant potential, being the ethyl acetate extracts, after four and eight days of fermentation, the lowest ones obtaining a half-maximal inhibitory concentration of 7 µg/mL compared to 28.0 µg/mL in the case of the butanol extract of the non-fermented tea.

Table 19. Half-maximal inhibitory concentration of the antioxidant activity of Kombucha samples, black tea (NF=0 days) and dried sample (total dry extract at day 21).

Solvents	**Samples IC_{50} (µg/mL)**				
	0 days	**4 days**	**8 days**	**15 days**	**21 days**
EtOAc	6.6±0.2	7.0±0.8	7.1±0.3	12.4±0.1	11.3±0.1
BuOH	28.0±0.7	>50	>50	18.7±0.6	>50
H_2O	>50	>50	>50	>50	>50
Dried total extract	>50	>50	>50	>50	25.4±1.0

IC_{50} values were calculated for inhibition percentages higher than 70% at 25 and 5 µg/mL ± standard deviations (n=3)

6.3.5.2 Determination of Phenolic composition

Phenolic compounds are well known for their reducing properties as hydrogen or electron donating agents which gives them their antioxidant potential. **Figure 46** shows a similar tendency as the one obtained for the scavenging activity of the DPPH radical. In order to confirm the relationship between the antioxidant potential and the obtained phenolic composition of the different samples, the correlation coefficient was determined, obtaining

values of 0.571, 0.921, 0.616 and 0.999 for the ethyl acetate, butanol, aqueous phase and dried sample respectively. This allowed to observe that in all the cases the phenolic profile exhibited a strong relationship with the scavenging activity of Kombucha extracts.

6.3.5.3 Anti-inflammatory activity

The 15-LOX inhibition of solvent extracts and dried Kombucha sample is presented in **Figure 47**, where it can be observed that interesting inhibition values where obtained in the case of the ethyl acetate extract. This non-polar extract showed a strong inhibition percentage of 81.6 % even in the non-fermented tea and obtained the lowest IC_{50} value of 25±1.3 after 15 days of fermentation. In the case of butanol and aqueous phase, a progressive increase was observed, going from 4.7% until 24.2%. Conversely, the dried Kombucha sample did not show any interesting activity. All the extracts showed the same behavior, obtaining its higher inhibition on the 15th day followed by a fast decrease. The action of several enzymes, which are mainly produced during the growth of microorganisms result in the hydrolysis and release of bioactive compounds (Huynh *et al.*, 2014), and according to Chen & Liu (2000) the growth of yeasts and bacteria reached its maximum values after 14 days and then decreased in the latter period of fermentation. Thus, the above results suggest that several molecules of interest, including the LOX inhibitors may be produced at the idiophase, which in this case is normally reached around 15 days.

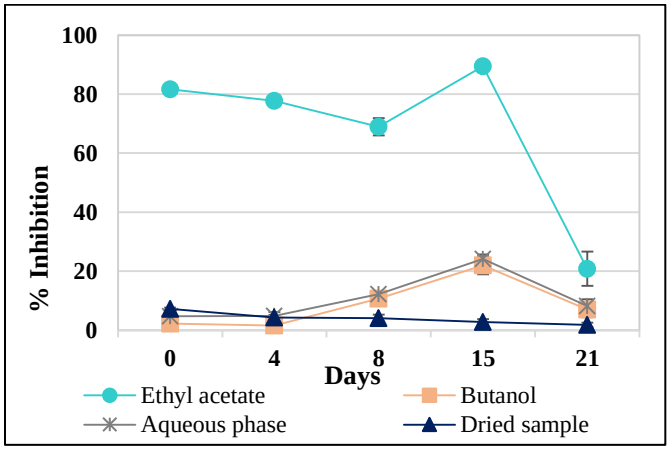

Figure 47. Changes in 15-LOX inhibition during Kombucha fermentation.

6.3.5.4 Antiproliferation evaluation on human cancer cells

Cytotoxic activity of Kombucha extracts and dried sample was assessed against human breast cancer cell (MCF-7) and human colon cancer cell (HCT-116) at a concentration of 50 µg/mL using the MTT assay. It can be observed (**Figure 48**) that in the case of MCF-7 the cell metabolic activity was not diminished after fermentation by the tested extracts; however, the dried sample increased its inhibition percentage going from 15.6 in the non-fermented tea until 32.0% after 21 days of fermentation. Regarding the HCT-116 cells no cytotoxic activity was detected in the dried sample, and in the case of the extracts samples they showed a tendency to decrease, nevertheless the ethyl acetate extract maintained a not negligible inhibition percentage of 46.1% after 8 days of fermentation.

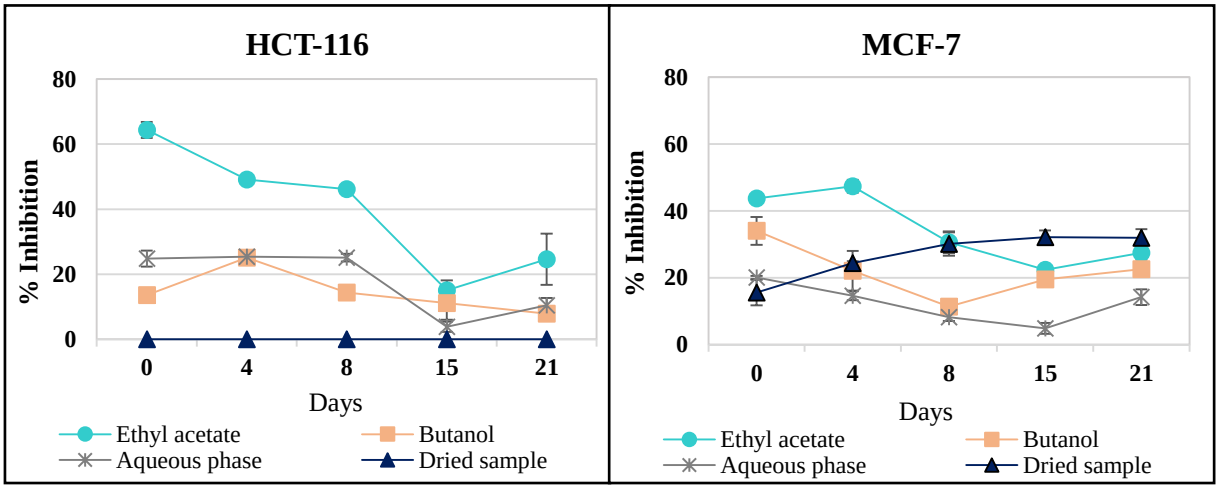

Figure 48. Effect of fermentation time and Kombucha tea samples on cell viability of HCT-116 and MCF-7 cells. . Tested at 50 µg/mL. Data are the means of three independent experiments ± standard deviations (n=3).

6.4 Conclusion

A significant effect of the fermentation time was observed in all the bioactivity evaluations and produced metabolites. We can conclude that Kombucha tea presents interesting biological properties after 8 to 15 days and that no longer fermentation is required, especially if it's intended for direct consumption as the concentration of organic acids might reach an unsafe level. The results also showed that the solvents polarities highly influenced the extraction of bioactive molecules. Being the non-polar one the most effective regarding biological activity but not regarding extraction yields. Nevertheless, it is also important to consider the industrial concern towards a specific bioactivity potential, which may allow choosing the optimal fermentation time in order to produce the molecules of interest. Our work highlighted the

therapeutic and nutraceutical potential of Kombucha extracts. Further work is needed in order to confirm these beneficial effects with *in-vivo* studies.

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6.6 Analyse en composantes principales

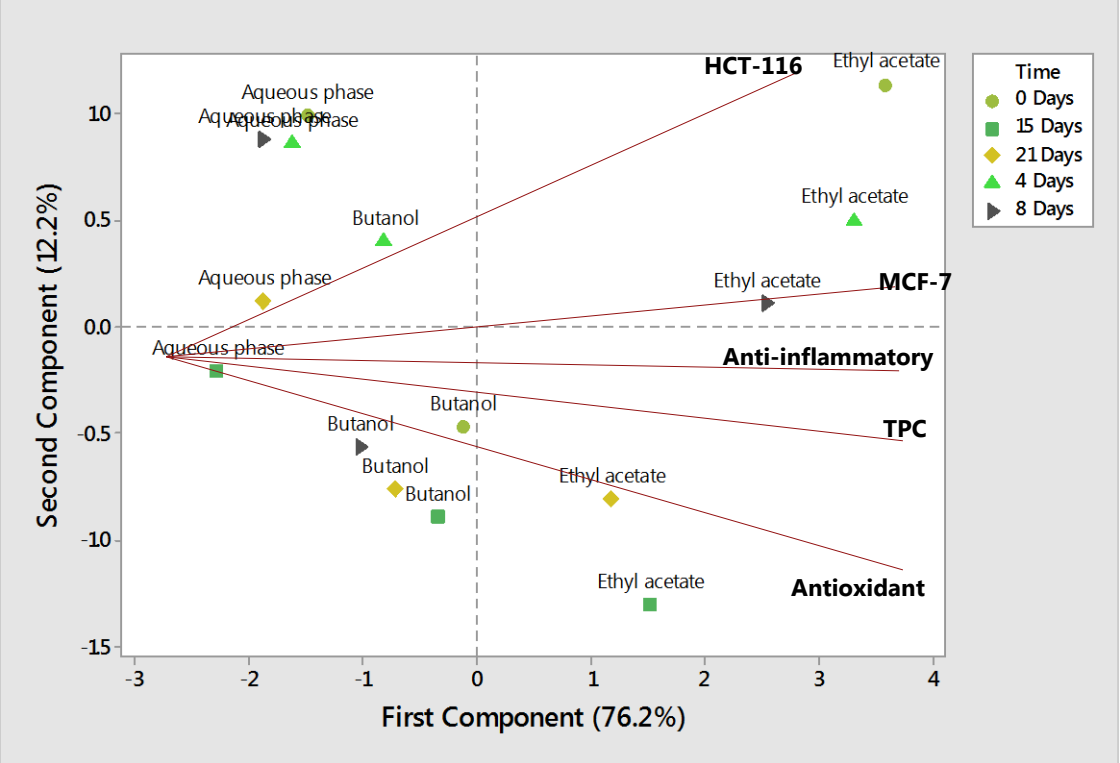

Figure 49. Analyse en composantes principales de l'évolution du profil biologique en fonction du temps de fermentation.

L'analyse de composantes a permis de représenter visuellement toute différence entre les activités biologiques et la durée de fermentation, ainsi comme le solvant d'extraction avec le meilleur profil biologique. Dans cette ACP, les deux premiers composants représentaient 88.4% de la variance expliquée. Les échantillons représentés dans le quadrant du premier composant sont ceux qui ont montré les profils cytotoxiques et anti-inflammatoires les plus élevés et correspondent aux échantillons avec le temps de fermentation le plus court (0, 4 et 8 jours). Concernant le profil antioxydante, les extraits d'éthyle acétate et butanol ont montré une bonne corrélation y compris des échantillons de 15 et 21 jours de fermentation. Ce résultat est cohérent avec ce qui a été observé précédemment concernant la stabilité de l'activité antioxydante pendant tout le processus de fermentation. Ainsi que sa forte corrélation avec la teneur en polyphénols totaux. Cette analyse nous a permis de conclure qu'une fermentation à court terme permet d'obtenir un bon profil biologique tout en réduisant les coûts qu'une fermentation à long terme pourrait générer.

6.7 Conclusions du chapitre

Conformément à l'agence américaine des produits alimentaires et médicamenteux (FDA) le thé de Kombucha doit être fermenté entre 7 et 10 jours pour pouvoir être consommé. Cependant, des études ont montré aussi que le Kombucha est une source potentielle de composés bioactifs avec des applications intéressantes.

Dans les expériences précédentes ont avait toujours caractérisé le thé non fermenté et ensuite le thé après fermentation (15-21 jours). En conséquence, pour ce chapitre on a trouvé pertinent d'étudier l'évolution des métabolites secondaires produits au cours de la fermentation ainsi que leurs respectives activités biologiques.

Un temps de fermentation entre 8 à 15 jours a été déterminé comment optimal en ce qui concerne la production maximale de métabolites et les meilleures inhibitions des enzymes pour le cas des extraits organiques. Le the fermenté non fragmenté a montré une augmentation progressive de son profil biologique en obtenant ses inhibitions maximales après 21 jours de fermentation.

Ces résultats ont montré qu'une durée de fermentation de 8 à 10 jours peut produire un thé de Kombucha approprié pour la consommation humaine et en ayant un profil biologique élevé.

Chapitre VII: Caractérisation physico-chimique de la cellulose bactérienne

Sommaire

Problématique de recherche et résumé du septième chapitre

La cellulose bactérienne possède deux fonctions dans la fermentation du thé de Kombucha, au début elle sert de source de nutriments porteuse de la culture de départ, néanmoins, elle continue à être produit au cours de la fermentation en augmentant son poids à 80% de la valeur initiale, devenant ainsi un sous-produit important. Plusieurs auteurs ont étudié les caractéristiques physiques, thermiques et structurelles, ainsi que certains facteurs pouvant affecter la production de cellulose (Huang *et al*., 2010; Gea *et al*., 2011; Mohite & Patil, 2014). Cependant, la plupart des études ont été réalisées à partir de cellulose produite par un seul type de bactérie, d'où l'intérêt d'étudier la cellulose obtenue auprès d'un consortium afin de voir si elle présente des caractéristiques similaires lui permettant d'être valorisée comme sous-produit du Kombucha.

Dans le dernier chapitre de cette thèse nous nous sommes intéressés à caractériser la cellulose produite à partir de trois fermentations différentes :

- Statique (témoin)

- Avec 2 g/L d'extrait de levure

- Avec agitation magnétique à 100 rpm

Les images en haute résolution obtenues à partir des structures par microscopie électronique à balayage ont révélé la présence des cristaux de calcium dans les trois conditions, ainsi comment les cellules microbiennes pièges entre les microfibrilles. Le carbone et l'oxygène ont été les deux atomes majeurs constituant la structure. Quelques traces d'azote ont été détectées, pouvant provenir des résidus de thé, d'acides aminés, de protéines, etc. Concernant l'analyse thermogravimétrique aucune différence significative n'a été observée entre les échantillons malgré les conditions différentes. En revanche, les résultats de la calorimétrie différentielle à balayage ont montré des différences en ce qui concerne la température de cristallisation de la condition agité. Cette caractérisation a montré que la cellulose bactérienne produit par le consortium de Kombucha a des propriétés intéressantes qui pourraient le conférer une utilisation pour certaines applications potentielles (Czaja *et al*., 2006).

Physicochemical properties of bacterial cellulose obtained from different Kombucha fermentation conditions

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Abstract

The production of bacterial cellulose has been limited due to its high cost and low productivity. Alternative low-cost sources of this biopolymer of high purity and biocompatibility are needed in order to benefit from its enormous potential. Kombucha tea is a trend functional beverage whose production is growing exponentially worldwide, and the bacteria present in this fermented beverage belonging to the genus *Komagataeibacter* are capable of producing a crystalline biofilm with interesting properties. Obtaining bacterial cellulose from Kombucha tea has already been studied, however several fermentation conditions are being optimized in order to scale-up its production. In this study, we characterized the bacterial cellulose produced from three different Kombucha fermentation conditions. SEM images revealed the crystalline structure of the biofilms. EDX analysis exhibited the chemical composition of the crystals. TGA analysis showed a rate of degradation between 490 °C to 560 °C and the DSC confirmed the presence of crystalline and amorphous regions in the bacterial cellulose samples. The results suggested that crystalline cellulose could be obtained by varying the fermentation conditions of Kombucha tea.

Keywords: Bacterial cellulose, Kombucha, SEM, TGA, Fermentation conditions

7.1 Introduction

Nowadays, bacterial cellulose (BC) is a promissory material for the development of biotechnological devices in many different fields, especially in the medical one, where it have been already used as wound dressing to heal lower extremity ulcers and it was observed that it shortened the healing time as compared to standard care (Lustri *et al.*, 2015). Its production from different microorganisms is being optimized with the aim of reducing the production costs, and scale up optimization is a technological prerequisite to be adopted at large scale (Cacicedo *et al.*, 2015). During the formation process of the cellulose pellicle various carbon compounds of the nutrition medium are utilized by the *Gluconacetobacter xylinum*, formerly polymerized into single, linear ß-1,4-glucan chains and finally secreted outside the cells through a linear of pores located on their outer membrane (Czaja *et al.*, 2006). These chains with a ribbon-like structure will self-assembly into fibrils creating a macrofibril network that may vary depending on the used strains, culture time and chemical additives present in the culture media (Lee *et al.*, 2015). Bacterial cellulose has distinctive advantages over traditional sources, no delignification is required following harvest (De Oliveira Barud *et al*., 2016), its physical properties such as crystallinity, hydrophilicity and degree of polymerization are superior than those from the plant-derived cellulose (George & Sabapathi, 2015), and it can be produced from a wide variety of substrates (Mohammadkazemi *et al*., 2015). One particular source for bacterial cellulose production is Kombucha tea, where it is produced as a floating biofilm associated with a consortium of bacteria and yeasts, and according to Nguyen *et al*., (2008) it may also have different characteristics than those from typical sources. However, Kombucha tea elaboration process is facing a standardization phase in order to do its scaling-up for industrial production (Coton *et al*., 2017) and several parameters which could modify the bacterial cellulose yield have already being studied. Such as, temperature (El-Salam, 2012), fermentation time (Kalifawi, 2014), depth and surface area (Phunsri *et al*., 2003), between others. Moreover, although yield is important, further research is needed in order to study the impact of the processing over the physicochemical properties of the bacterial cellulose. The aim of this study was to compare different fermentation parameters of Kombucha tea and their influence over the characteristics of the final biofilm.

7.2 Materials and methods

7.2.1 Culture conditions and inoculum preparation

Starter inoculum

The tea fungus was purchased from the website www.je-mange-vivant.com and maintained in sugared black tea according to an optimized protocol in the laboratory.

Preparation of Kombucha teas

10 g of Ceylon black tea and 70 g of sucrose were added to 1 L of boiling water and allowed to infuse for 15 min, the tea was then removed and the infusion was left to cool. Once the temperature was around 25°C the tea was inoculated with 20 g of the starter inoculum and 20 mL of previously fermented medium. Finally, the beakers were covered with cheesecloth for allowing a proper aeration, and were then incubated at 25 °C for 15 days.

Three different cultivation methods were tested:

- Tea broth in a static fermentation (Control)
- Tea broth with 2 g/L of yeast extract
- Tea broth with magnetic stirring at 100 rpm

7.2.2 Bacterial cellulose samples preparation

The BC biofilms were removed from Kombucha fermented tea and directly dried at 60 °C during 24-36 hours without any specific cleaning procedures. Once dried they were cut in small pieces according to each analytical technology.

7.2.3 Scanning electron microscopy (SEM) images

1cm² of the sample was plunged into a 4% glutaraldehyde solution during 2 h in order to preserve the biological material. Then it was washed during 5 min into different mixes of Acetone/water (70-30%) and Acetone/HMDS (hexamethyldisilazane) (50-50%) to be dehydrated. Finally, a metallic coating was performed under vacuum conditions to provide the

sample with a conductive layer during analysis. Images were performed with a JEOL-JSM-6700F instrument. Magnification range used on SEM images was X100 – X25000.

7.2.4 Thermogravimetric (TG) and Derivative Thermogravimetric (DTG) analysis

Both scans were performed under air condition by a TGA Q600 analyzer from TA Instruments. About 8 mg were introduced inside an alumina crucible for each test. Temperatures were tested in the range of 25 to 1000 °C. TG and DTG analysis were used to approach the thermal properties of the Kombucha membranes as onset temperature degradation (T_{on}) or temperature of maximum rate of degradation (T_d).

7.2.5 Differential Scanning Calorimetry (DSC) scans

The analysis were performed with a Q2000 instrument from TA Instruments. The Q2000 DSC is a heat-flux DSC with Modulated DSC capability. 5mg of sample were placed in an aluminum DSC support with a standard aluminum lid. Samples were tested under an air flow of 50 mL/min. The rank of temperature used was 10°C/min from -50 °C to 400 °C. DSC analysis was used to study the thermal properties of the Kombucha biofilms as glass transition temperature (T_g), crystalline phase transition temperature (T_c) and denaturation temperatures.

7.3 Results and discussion

7.3.1 SEM analysis

The cellulose matrix acts as a nutrient source by containing several proteins, extra cellular enzymes, nucleic acids, lysed cells, etc. (Flemming & Wingender, 2010). These components represent an important energy source during fermentation; however, they are considered organic impurities when studying the biofilm structure, as they remained attached to the surface of the gel as seen in **Table 21**. Scanning Electron Microscopy offered important visual information on the morphology of the biofilm samples. One-micron resolution scans showed entanglements of microfibrils with a diameter lower than 100 nm. Ovoid and stick forms were microorganisms from 1 to 3 microns, some of them were present on the surface of the sample and others were caught into by the fibrils and forced to remain into the matrix. It can be seen

that the higher microbial population was found in the magnetic stirring condition, probably due to the agitation, which deliver most of the cells into suspension. A higher presence of microbial cells can prevent the contact between the fibrils within the network, reducing the number of hydrogen bonds and thus the strength of the biofilm (Gea *et al*., 2011). This theory was observed in the studied samples, as the microfibrils seemed to constitute a more organized network in the case of the static condition. Crystalline forms of 0,5-1 µm were present on the SEM images of all the different cellulose biofilms and bacterial cellulose is known for having a high crystallinity of around 60-80% (Czaja *et al*., 2006). Our obtained biofilms present a denser matrix than those observed by Lee et al., (2015) where their biofilm was characterized after 7 days.

7.3.2 EDX analysis

Energy-Dispersive X-ray (EDX) analysis applied on the matrix showed two major atoms represented in the samples (**Figure 50**): Carbon (50 to 62%) and Oxygen (38 to 50%). These two major atoms constituted the structure of the fibrils. These results were consistent with the cellulose structure because cellulose formula $(C_6H_{10}O_5)_n$ presents 55% of C and 45% of O if H atom are neglected in the calculation. H atoms are not considered because these are not measurable in EDX analysis because of its extremely small photoelectron cross-section. However, traces of Nitrogen were observed in some spectra, which could probably come from contaminants of the Kombucha biofilms as amino-acids or/and proteins from entangled microorganisms. Jayabalan *et al.*, (2010) performed a proximate analysis of the biochemical constituents of a Kombucha biofilm and found Oxygen and Carbon as the major compounds. Nevertheless, they detected also other compounds such as, Na, K, Mg, Zn and Ca, whose concentrations increased progressively during fermentation.

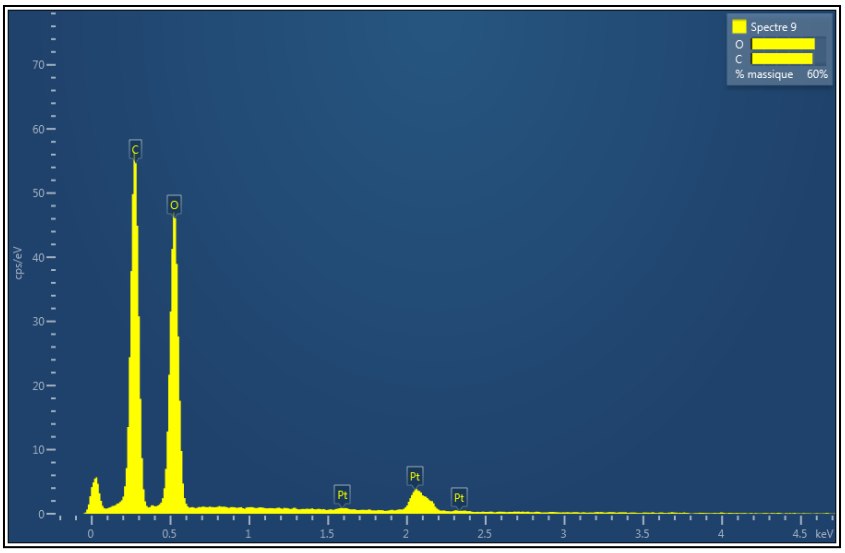


Figure 50. EDX spectrum of the bacterial cellulose matrix

Figure 51 shows the comparison between the EDX analysis of a crystal (spectrum 5) and of the matrix (spectrum 6). Spectrum 5 showed a Calcium peak which was not present on spectrum 6 and showed also a higher peak of Oxygen. The detected elements in the spectrum 5 suggest that it could be formed of $Ca(OH)_2$ crystals. Podolich *et al*., (2017) carried out similar analysis and detected Calcium and Potassium in their Kombucha biofilms. However, these elements were not detected in the characterization done by Mohite & Patil (2014), who just detected carbon and oxygen in 54.06 and 45.94% respectively in a biofilm produced by *Gluconacetobacter hansenii*. The differences observed in the chemical composition of the bacterial biofilms may be due to the initial microbial population used for its production and could lead to different final properties.

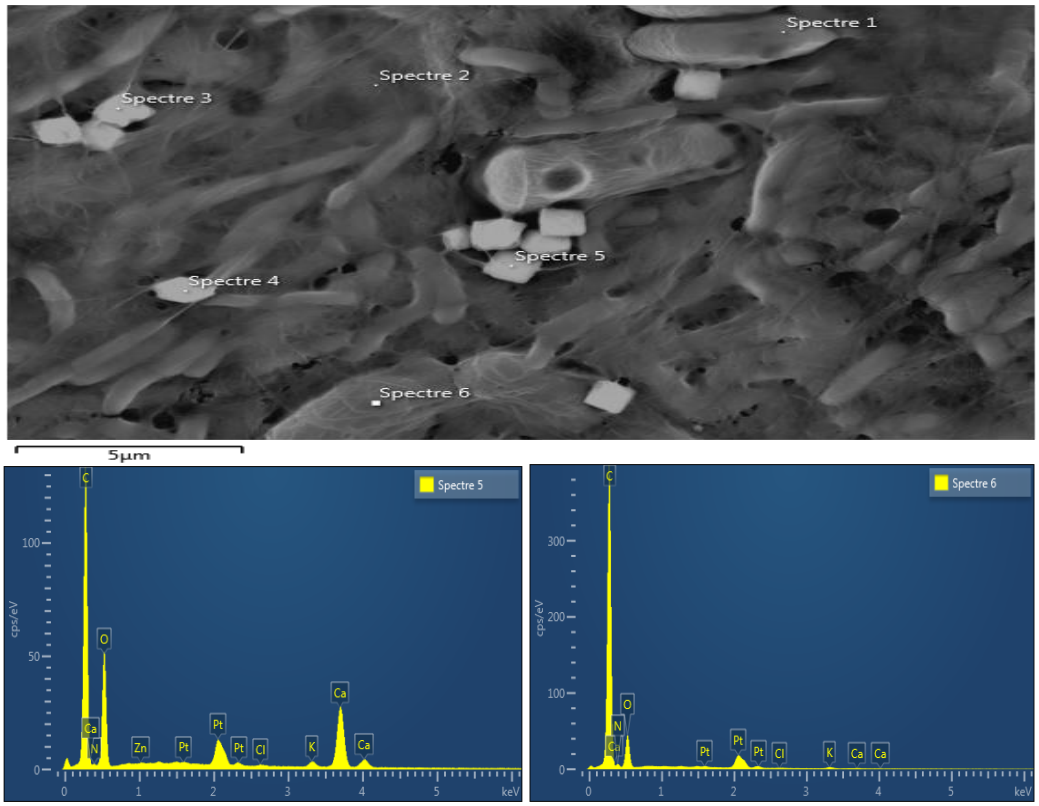


Figure 51. Corresponding EDX spectra of the bacterial cellulose matrix and crystals.

7.3.3 TG and DTG analysis

Thermogravimetric (TG) and differential thermogravimetric (DTG) analysis were performed under air conditions. All TG analysis showed three distinguished weight losses and DTG analysis presented a two step-process to degrade Kombucha biofilm elements (**Table 20**). Kombucha biofilm samples from the magnetic stirring condition showed the highest first weight loss of 32,67% instead of 25,79 or 12,05% for the other conditions. This difference may be explained by a higher presence of impurities, which could be normal either because of the higher yeast concentration or by the fact that magnetic stirring promoted a greater homogeneity of the bacteria and yeasts in the medium that could have ended trapped in the biofilm matrix. This non-cellulosic molecules inside the cellulosic matrix could also disturb the crystalline zones of the biofilm structure by releasing free hydroxyl functional groups on glycosidic units. These free hydroxyl groups could bind water molecules with hydrogen bonds and retain them into the matrix increasing its water content. This first weight loss was generally higher in comparison with values found by Machado *et al*., (2016) who obtained a weight loss from water vaporization of ~5%. However, the samples showed a similar profile to the one observed by Mohammadkazemi *et al*., (2015), were the loss was observed at 100 °C and it was due to the

loss of water. They worked with five different carbon sources and observed that the moisture content was the same for all the bacterial cellulose samples. Onset degradation temperature (T_{on}) and first maximum rate of degradation temperature (1^{st} T_d) were lower in the yeast extract sample (T_{on}: 220 °C/1^{st} T_d: 320 °C) than in the control and stirred samples (T_{on}: 240-260 °C/1^{st} T_d: 320-340 °C). These degradation temperatures concerned first non-cellulosic compounds degradation, then cellulose depolymerisation and the decomposition of glycosidic units into gases. And according to the literature, the decrease in the thermal stability can be related to a higher crystallinity index (Dima *et al*., 2017). The second maximum rate of degradation temperature occurred between 490 °C to 560 °C, which was higher than the one obtained by Gea *et al*., (2011) of around 360 °C from cellulose produced by *A. xylinum*. This could suggest that bacterial cellulose obtained from the Kombucha consortium seems to have more thermal stability than the one produced from only one bacterial species. The broad range of temperature values observed in the thermogravimetric analysis were probably linked to the different natures, sizes and concentrations of the char residues. In order to confirm the presence of these carbonaceous materials TG scans under nitrogen atmosphere should be performed.

Table 20. Thermogravimetric results from the different biofilm samples.

Samples	1^{st} Weight loss (% of total mass)	2^{nd} Weight loss (% of total mass)	3^{rd} Weight loss (% of total mass)	T_{on} (°C)	1^{st} T_d (°C)	2^{nd} T_d (°C)
Control	25,79	27,97	43,50	240	320	560
Yeast extract	12,05	44,80	37,73	220	320	490
Magnetic stirring	32,67	28,23	36,14	260	340	515

7.3.4 DSC analysis

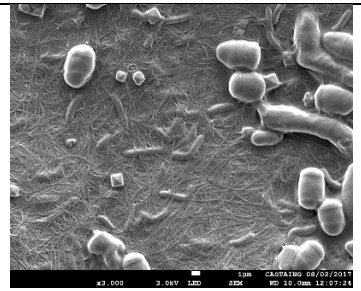
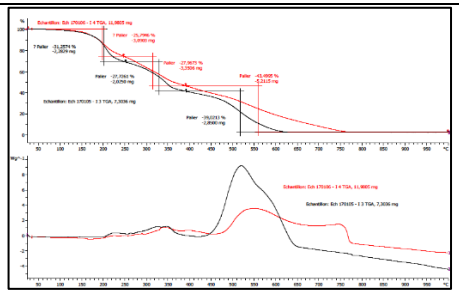
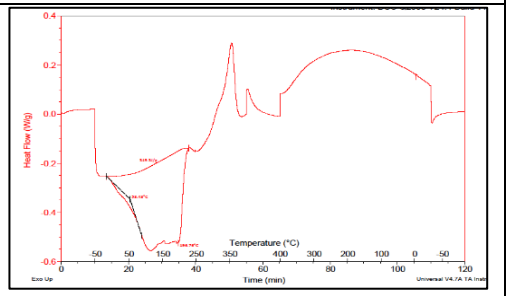
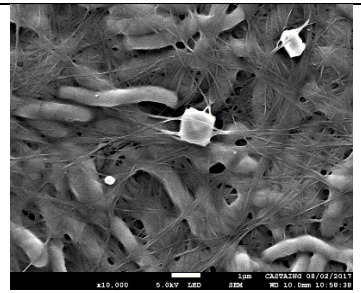
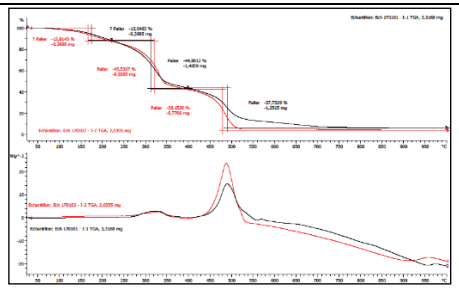
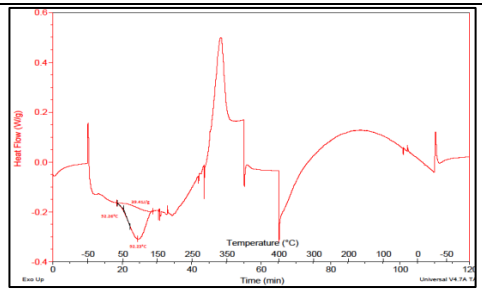
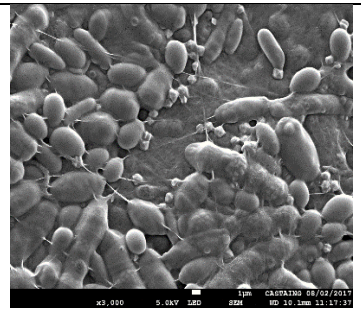
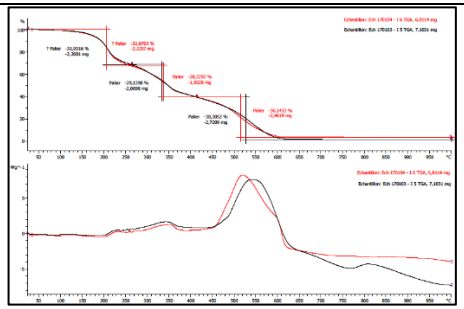
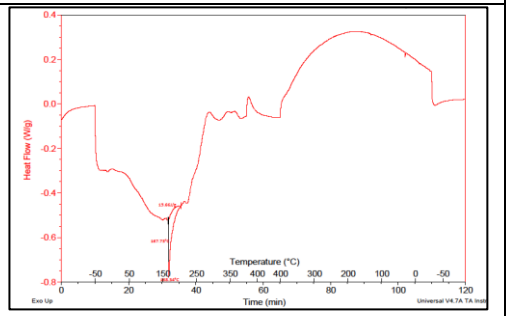
DSC curves indicated the energy consumed and released by Kombucha biofilms under temperature variations (**Table 21**). The DSC thermograms exhibited rough results because of the high contamination of the samples. However, the yeast extract sample presented a more accurate curve with isolated peaks. This confirmed the higher purity of the sample compared to the control and the stirred one as shown before. A first endothermic peak around 100 °C was

observed. The endothermic reactions occurred at this temperature were mainly attributed to the removal of the water and other volatile non-cellulosic compounds absorbed inside the cellulose matrix (Ashok *et al*., 2015). In this first peak, glass transition probably occurred but glass transition temperature (Tg) was difficult to be determined. A temperature decrease after water removal might be a solution to approach Tg. The melting of the crystalline phase of cellulose is obtained at a temperature of 80-140°C (Mohite & Patil, 2014), which corresponds to the endothermic peaks observed in the case of the control and yeast extract samples (**Table 21**). Nevertheless, nearly no phase transition was observed in the case of the magnetic stirring condition, probably meaning that this polymer showed an amorphous structure. A second endothermic peak in the range of 200-250 °C corresponded to the non-cellulosic contaminants and cellulose degradation. A first exothermic peak around 350 °C indicated exothermic chemical reactions in the oxidization of cellulose. The second exothermic peak around 375 °C indicated exothermic chemical reactions in the oxidization of char residues. The last part of the curves from 400 °C to -50 °C did not show interesting elements because all volatile compounds were already removed, and samples were completely degraded. These results completed TG and DTG results and highlight the high thermal properties of Kombucha cellulosic biofilms.

7.4 Conclusions

EDX scans from the practical work revealed cellulose as the main constituent of Kombucha biofilms. SEM images revealed an organized and tight entanglement of 80 nm macrofibrils that constitute a homogeneous and dense cellulose network. This cellulose network confers high mechanical and thermal properties to the biofilm as shown on the different thermogravimetric scans. The different tested conditions did not change the composition of the cellulose network, which could allow obtaining different Kombucha teas according to the industrial needs and producing a crystalline cellulose biofilm at the same time. Bacterial cellulose has proven to be a remarkably versatile biomaterial and can be used in a wide variety of specific applications. However, the availability and production costs of this biofilm still need to be improved. Kombucha tea fermentation proved to be a promising alternative for the production of this renewable natural resource.

Table 21. Comparison of the physicochemical properties of the biofilms obtained with each fermentation condition.

Sample	SEM	TGA/DTG	DSC
Control			
Yeast extract			
Magnetic stirring			

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7.6 Conclusion du chapitre

La valorisation de la cellulose bactérienne ne répond pas seulement à l'utilisation potentielle d'un sous-produit de fermentation mais aussi à un besoin actuel pour une production moins chère de ce polymère. Le biofilm obtenu à partir de la production du thé de Kombucha semble avoir un profil chimique et thermique similaire à celui produit déjà à partir de souches pures de bactéries.

Cependant l'industrie de ce thé fermenté est aujourd'hui en plein développement et diverses études sont en cours afin d'optimiser son processus de fabrication. L'intérêt de cette étude a été d'étudier l'impact de diffèrent procédés sur les caractéristiques de la cellulose produite.

Les résultats ont montré que la structure du polymère semble être assez stable et similaire entre les échantillons. Par contre le biofilm de la condition agité n'a pas montré une phase de transition pour la température de cristallisation, ce qui suggère que ce polymère a présenté une structure plus amorphe probablement due à l'agitation.

Les biofilms de la Kombucha semblent avoir une composition chimique plus riche et une stabilité thermique plus grande que celle obtenue à partir d'une seule espèce de bactérie. De ce fait, le thé de Kombucha pourrait être considéré comme une source alternative d'obtention de la cellulose bactérienne.

Chapitre VIII : Conclusion et perspectives

Ce parcours de recherche doctorale nous a permis d'approfondir les connaissances dans l'extraordinaire monde de la fermentation par un consortium mixte qu'est le Kombucha. Ce thé fermenté de façon originale est une boisson qui, malgré son ancienneté, représente toujours un défi en termes de production industrielle et de valorisation en tant que produit bénéfique pour la santé. L'originalité de l'approche scientifique utilisée réside dans l'approche multidisciplinaire et complémentaire de cette fermentation. Un des objectifs principaux était l'étude des paramètres clés permettant d'impacter le processus d'élaboration du Kombucha, afin de maintenir une production élevée en métabolites bioactifs. Tout en s'appuyant sur plusieurs domaines et outils de recherche nous permettant d'atteindre les objectifs proposés, ce travail nous a conduits aux points principaux discutés ci-dessous :

Facteurs qui influencent le transfert d'oxygène lors de la fermentation

Les analyses de séquençage ont montré que les écosystèmes microbiens utilisés étaient constitués majoritairement par des bactéries acétiques (Section 4.3.2), qui sont des microorganismes strictement aérobies ; de ce fait, il est important d'assurer un bon apport en oxygène lors de la fermentation. Étant donné que la fermentation du Kombucha est traditionnellement réalisée en mode statique, nous avons proposé l'utilisation de géométries différentes afin d'augmenter le contact avec l'oxygène (Chapitre III). A partir de cette expérience, nous avons constaté qu'un ratio surface/hauteur plus important semblait améliorer le profil biologique des extraits obtenus ainsi que la production de certains composés phénoliques tel que la catéchine, connue pour ses effets antioxydants et anti-inflammatoires (Jhang *et al*., 2015). Subséquemment, avec l'intérêt d'optimiser le processus de fermentation du Kombucha en s'appuyant sur le transfert d'oxygène, il a été décidé d'ajouter deux sources d'aération externes (Chapitre IV) en utilisant respectivement l'agitation orbitale et un bullage d'air continu dans le milieu. Notre hypothèse partait du principe qu'augmenter le coefficient de transfert d'oxygène (kLa) résulterait dans une amélioration du bioprocédé, que ce soit en termes de temps de fermentation, comme pour la production de métabolites d'intérêt ayant des rôles dans des activités biologiques potentiellement bénéfiques pour la santé. Plusieurs valeurs du kLa ont été obtenues (0,042-7,44 h^{-1}) à partir des expériences menées dans les deux chapitres, et les caractérisations chimiques et biologiques ainsi que la cinétique de fermentation ont été évaluées pour chaque condition. D'après l'évaluation chimique des échantillons obtenus il a été observé que la condition « aération » a augmenté significativement la production de l'acide gallique, obtenant 22 mg/kg d'extrait sec avec la condition aérée (E) contre 13 mg/kg d'extrait

sec avec la condition statique (H), le transfert d'oxygène étant le seul paramètre variable (même volume, même géométrie de réacteur, même consortium..). Ceci pourrait indiquer que des concentrations plus élevées d'oxygène dans le milieu de fermentation renforcent l'activité de plusieurs enzymes responsables de la production de cet acide. Par contre, pour la production de catéchine, la concentration plus élevée a été obtenue avec le ratio s/h plus grand (B) en conditions statiques. Même si dans ce cas la valeur de *k*La de 0.042 h^{-1}, était la plus faible, la géométrie présentait la surface exposée la plus grande en conditions statiques. Cela a sans doute permis d'avoir un bon flux d'oxygène entre l'atmosphère et les bactéries en surface. Cela pourrait expliquer le fait que dans cette condition on ait obtenu le profil anti-inflammatoire le plus intéressant de toutes les conditions étudiées, et une production élevée de certains composés d'intérêt, notamment la catéchine, connue pour des fonctions anti-inflammatoires (Nakanishi *et al*., 2010; Jhang *et al*., 2015).

Dans le cadre de cette thèse plusieurs paramètres ont été étudiés afin de faciliter le transfert d'oxygène dans la fermentation du thé de Kombucha à l'échelle laboratoire (1-6 litres), soit par la géométrie du fermenteur dans des conditions statiques, soit en utilisant des sources externes telles que l'agitation ou l'aération. Les résultats obtenus nous ont permis d'observer que les deux facteurs peuvent avoir des impacts sur la production des métabolites primaires et secondaires ainsi que sur le profil biologique final. Parmi les différents échantillons, la condition statique avec le ratio s/h plus grand (B) a montré un bon équilibre entre la présence de molécules et ses propriétés anti-inflammatoires et antioxydantes. Ces résultats ont montré que la production de métabolites bioactifs peut être optimisée en conditions statiques, à condition d'utiliser un fermenteur ayant une grande interface gaz-liquide, permettant d'augmenter l'accès à l'oxygène pour les micro-organismes à la surface. Néanmoins, pour les volumes de fermentation au-delà de 200 litres l'apport d'oxygène par l'interface sera limitée (Fuchs & Ryu, 1971)

Au-delà de ce volume ou plus précisément pour faire la mise en échelle industrielle, le maintien d'une valeur de *k*La à l'aide de sources externes d'aération est conseillé afin d'assurer une oxygénation correcte du milieu. L'apport d'oxygène permet aussi un brassage du milieu liquide, et permet une meilleure homogénéité.

Impact de l'origine du consortium microbien sur les profils chimique et biologique

Dans le chapitre V, nous nous sommes intéressés à l'étude de trois consortia de Kombucha différents, nous permettant d'étudier la diversité biologique de ce type de produit. Nous avons pu identifier les genres et espèces majoritaires de bactéries et levures, et comparer ces compositions selon l'origine du consortium. L'analyse metagénomique a révélé *Komagataeibacter rhaeticus, Gluconacetobacter sp, Brettanomyces bruxellensis* et *Schizosaccharomyces pombe* comme les groupes dominants de bactéries et levures. Cependant, leur abondance relative a montré des différences distinctives entre chaque consortium, à la fois dans la phase solide (biofilm) ainsi comme dans la phase liquide (Figure 23). Pour cette expérience les mêmes conditions de fermentation ont été utilisées afin de montrer comment la population microbienne peut impacter les cinétiques, la production de métabolites et la composition chimique des thés de Kombucha, et ainsi les propriétés finales étudiées. En ce qui concerne le profil biologique, le consortium (G) a obtenu les meilleurs pourcentages d'inhibition contre l'enzyme 15-LOX (activité anti-inflammatoire), montrant une inhibition de 44% par rapport à 28 ou 7% obtenu avec (H) et (F), respectivement. De plus, il a été le seul échantillon montrant une inhibition de la croissance des cellules du cancer des ovaires de 29,1% du même ordre que celui obtenu avec le thé non fermenté, utilisé comme témoin. Par contre, la production d'acide gallique et d'autres molécules organiques comme l'acide propanoïque et l'acide malique a été augmentée avec le consortium (H). L'abondance relative des espèces (Table 13) a montré que les deux consortia ayant les profils biologiques les plus intéressants comportent *K. rhaeticus* et *K. xylinus* comme bactéries dominantes suivi de *B. bruxellensis* et *S. pombe* dans le cas de levures. A l'inverse, l'échantillon (F) a été dominé par les levures, et présente une diversité chimique et biologique moindre, ce qui suggère que les bactéries du genre *Komagataeibacter* pourraient être l'un des principaux responsables de la production de métabolites secondaires bioactifs.

La provenance du consortium s'est avéré être un facteur d'impact, cependant l'étude réalisée dans le chapitre IV a montré que les paramètres de fermentation tels que l'aération du milieu, peuvent impacter significativement la croissance des populations microbiennes ainsi que leurs respectives voies métaboliques et par conséquent les métabolites produits. Ceci a été remarqué grâce à l'étude de trois conditions de fermentation avec le même consortium. Des nettes différences ont été observées dès le premier jour de fermentation. La condition (E) ayant la valeur de kLa plus élevé de 7.4 h^{-1} a obtenu la production maximale d'acide gallique, qui est un métabolite d'intérêt avec des applications intéressantes (Badhani *et al*., 2015). La condition

statique (C) avec un *k*La de 0.12 h^{-1} a obtenu environ 50% d'inhibition de plus contre les cellules de cancer du côlon que les autres deux conditions. Néanmoins c'est la condition (D) avec un *k*La de 1.5 h^{-1}, qui a montré le meilleur équilibre entre la production de métabolites secondaires et ses activités biologiques.

Les résultats obtenus dans ces deux chapitres (IV et V) ont révélé que l'impact du procédé semble être plus grand que celui de l'origine du consortium microbien, ce qui pourrait être avantageux pour sa commercialisation au niveau industriel.

Biotransformation des composés organiques et leurs activités biologiques au cours du temps

La fermentation du thé noir a montré une amélioration du profil fonctionnel dans la plupart des cas étudiés. Le suivi chromatographique réalisé dans le chapitre VI au cours du temps a révélé l'évolution des principaux métabolites pendant la durée du procédé (21 jours). L'ensemble des résultats obtenus a montré que les quantités maximales des métabolites produits ont été obtenues à des temps différents (4, 8 et 15 jours) variable selon le composé. On pourrait attribuer cette augmentation ou production à l'effet des activités des différents microorganismes, qui fonctionnent en symbiose, mais pas de façon synchronisée (en même temps), se traduisant par une cinétique fermentation particulière. Ceci menant aussi à l'hydrolyse des composés phénoliques déjà présents dans le thé. A l'inverse, une forte diminution de composés a été observée après 15 jours, pouvant être dû à la dégradation des composés par des enzymes (Pham *et al*., 2000).

Toutes les caractérisations chimiques et biologiques dans ce travail de thèse ont été faites sur des extraits par solvants organiques, ce qui nous a permis d'identifier et de quantifier certains des principaux métabolites produits par le consortium au cours de la fermentation ainsi que d'évaluer son potentiel biologique. Cependant le thé de Kombucha étant un produit destiné principalement à la consommation comme une boisson bioactive, il a été pertinent d'évaluer le thé fermenté complet (sans fractionnement) afin d'observer si celui avait le même profil que les extraits. L'analyse des résultats a montré qu'à l'exception de la rutine, l'acide chlorogénique et la théobromine, aucun autre composé phénolique n'a été détecté par HPLC dans le thé non fractionnement. En ce qui concerne les activités biologiques des extraits, elles sont pour la plupart supérieures à celles du thé fermenté complet, et apparaissent dès le début de la fermentation. Dans l'ensemble, les échantillons ont montré une augmentation majoritairement

entre 4 et 8 jours. Cette expérience nous a permis d'établir un temps de fermentation convenable pour avoir un produit final avec un profil biologique et possiblement sensorielle acceptable.

Valorisation d'un sous-produit avec des propriétés intéressantes : la cellulose bactérienne

Le biofilm de cellulose est un sous-produit d'intérêt issu de la fermentation du thé de Kombucha. Environ 80 g/L ont été produits en poids humide par échantillon dans toutes les conditions. La caractérisation réalisée a montré que le procédé n'a aucun impact dans la composition chimique du biofilm. La caractérisation effectuée a montré que le biofilm produit a des propriétés thermiques intéressantes et couplé à sa grande capacité de rétention d'eau, il pourrait avoir des applications dans le domaine de la santé et produits de soin, notamment pour soigner les brulures de la peau. Néanmoins, une purification du polymère est fortement recommandée afin d'enlever les impuretés et faciliter le contact entre les microfibrilles et augmenter ainsi la force du biofilm.

Afin de pouvoir continuer à optimiser la production et mise en échelle du procédé, nous proposons quelques perspectives d'étude qui serviront pour compléter et continuer nos travaux :

- Quelques paramètres clés pour l'optimisation du procédé ont été identifiés, cependant son évaluation avec des volumes à l'échelle pilote (à partir de 250 litres) pourrait contribuer à l'établissement des conditions optimales pour la production du thé de Kombucha.

- La consommation de boissons fermentées est en pleine croissance au niveau mondial. Ainsi, fermenter d'autres plantes avec le consortium de la Kombucha pourrait être une alternative prometteuse pour la conception de nouvelles boissons fonctionnelles.

- Une identification et purification de molécules formées après fermentation est fortement recommandée afin de permettre d'augmenter son utilisation potentielle dans les industries pharmaceutiques, alimentaires et cosmétiques.

- L'évaluation anti-inflammatoire *in-vivo* ainsi qu'une analyse de composition nutritionnelle et des effets probiotiques contribuerait à l'évidence des bienfaits pour la santé qui peut apporter la consommation de ce thé.

- Les extraits de Kombucha ont montré une activité d'anti prolifération cellulaire contre les lignées tumorales du sein, ovaires et côlon. Réaliser une évaluation contre les lignées de cellules saines complèterait bien les études déjà menées et pourrait fournir des informations importantes sur le profil cytotoxique du thé de Kombucha.

Toutes les expériences réalisées dans le cadre de cette thèse ont mis en évidence l'immense complexité qui implique l'étude de cette boisson ancienne et unique qui est en train de transformer le marché international. Une maitrise de plusieurs aspects scientifiques a été nécessaire afin de pouvoir établir quelques bases primordiales pour une production durable de la Kombucha en gardant un profil bioactif. Ce challenge de recherche nous a permis de comprendre un peu plus sur la vie en symbiose et le potentiel que cette collaboration mutualiste peut avoir sur le développement de boissons fermentés, notamment sur le thé de Kombucha. Nous espérons que nos résultats puissent contribuer à l'avancer du développement de nouveaux bioprocédés et produits fermentés dans le passionnant monde de systèmes microbiens.