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Etude de la dynamique microbienne pour la maîtrise de la fabrication de kombucha

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Titre : Etude de la dynamique microbienne pour la maîtrise de la fabrication de kombucha

Mots clés : Kombucha ; Interactions microbiennes ; Fermentation ; Levures ; Bactéries acétiques.

Résumé : La kombucha est une boisson fermentée issues de la transformation d'une infusion de thé sucrée par l'activité d'un consortium microbien de levures et de bactéries donnant lieu à la production d'un biofilm cellulosique. L'étude des interactions microbiennes au sein de cette matrice a pour but d'améliorer la maîtrise du procédé de fabrication à échelle industrielle. La méthodologie employée dans ces travaux s'appuie principalement sur l'isolement et la sélection de levures et de bactéries acétiques d'un consortium donné, puis de leur mise en œuvre en monocultures et cocultures dans du thé noir sucré. Différents paramètres ont été suivi aux niveaux microbiologique (populations, composition en genres et espèces), chimique (composition en sucres, acides organiques, acides aminés, protéines, composés volatils, oxygène) et sensoriel (descriptions gustative et olfactive). Les résultats montrent le caractère essentiel de l'association levure-bactérie acétique dans le processus de fabrication de la kombucha au niveau des composés fixes et volatiles. Ils soulignent en particulier l'importance du métabolisme levurien dans ces transformations. La matrice joue également un rôle déterminant dans la composition chimique et le profil organoleptique de la kombucha de part son abondance en susbstrats carbonés et sa pauvreté en substrats azotés. Bien qu'étroitement lié aux interactions microbiennes tant au niveau de sa formation que de sa fonction, le biofilm de kombucha ne conditionne pas les activités microbiennes essentielles à la production de la boisson. L'ensemble des interactions mises en évidence, incluant commensalismes et compétitions, forment un système d'interactions globalement mutualiste entre les levures et les bactéries acétiques de la kombucha.

Title: Study of microbial dynamics for the control of Kombucha production

Keywords: Kombucha; Microbial interactions; Fermentation; Yeasts; Acetic acid bacteria.

Abstract: Kombucha is a fermented beverage obtained from the transformation of sugared tea infusion through the activity of a consortium of yeasts and bacteria, during which a cellulosic biofilm is formed. The study of microbial interaction that occur in this matrix aims at improving the control over the production process at industrial scale. The methodology used in this work is mainly based on the isolation and selection of yeasts and acetic acid bacteria from a determined kombucha consortium. The selected microorganisms are then used to inoculate monocultures and cocultures in sugared black tea. Different parameters were followed at the microbiological level (population counts, composition in genera and species), at the chemical level (composition in sugars, organic acids, amino acids, proteins, volatile compounds, oxygen) and at the sensory level (gustative and olfactive descriptions). Results point towards the essential role of yeast-acetic acid bacteria association during the production process of kombucha, regarding both volatile and non-volatile compositions. They underline specifically the importance of yeast metabolism in those transformations. The matrix also plays a determining role in the chemical composition and organoleptic profile of kombucha because of its abundance in carbon substrates and its poorness in nitrogenous substrates. The kombucha biofilm possesses a strong relationship with microbial interactions, both regarding its formation and its function. However, it is not mandatory for the essential microbial activities involved in the beverage's production to occur. Highlighted interactions, including commensalisms and competitions, form as a whole a mutualistic system of interactions between kombucha yeasts and acetic acid bacteria.

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Cette thèse est dédiée à la mémoire d'Alan Lucid (1989-2017), mon mentor et ami. Alan, it won't bring you back, but this PhD is also yours. I'll always be your kombucha happy maker.

Sommaire

Liste des communications scientifiques

Articles scientifiques acceptés pour publication

Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2020. Microbiological and technological parameters impacting the chemical composition and sensory quality of kombucha. Comprehensive Reviews in Food Science and Food Safety 19, 2050–2070. https://doi.org/10.1111/1541-4337.12574

Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2020. Microbial Dynamics between Yeasts and Acetic Acid Bacteria in Kombucha: Impacts on the Chemical Composition of the Beverage. Foods 9, 963. https://doi.org/10.3390/foods9070963

Tran, T., Grandvalet, C., Winckler, P., Verdier, F., Martin, A., Alexandre, H., *et al.* (2021). Shedding Light on the Formation and Structure of Kombucha Biofilm Using Two-Photon Fluorescence Microscopy. Frontiers in Microbiology 12, 13. doi:10.3389/fmicb.2021.725379.

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Tran, T., Billet K., Torres-Cobos, B., Vichi, S., Verdier, F., Martin, A., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R.. Origins of kombucha flavor: role of microbial interactions, process, and tea type. Food Chemistry.

Tran, T., Verdier, F., Martin, A., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R.. Oxygen management during kombucha production: roles of the matrix, microbial activity, and process parameters. Food Microbiology.

Mas, P., Tran, T., Verdier, F., Martin, A., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R.. Evolution in composition of kombucha consortia over three consecutive years in production context. LWT.

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Rodriguez-Rey, J., Tran, T., Loupiac, C., Verdier, F., Martin, A., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R.. Production and consumption kinetics of proteins and amino acids during kombucha production. Food Chemistry: Molecular Sciences.

Tran, T., Romanet, R., Roullier-Gall, C., Verdier, F., Martin, A., Schmitt-Kopplin, P., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R.. Metabolomic analysis of kombucha's production process. Food Chemistry: Molecular Sciences.

Tran, T., Roullier-Gall, C., Verdier, F., Martin, A., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R.. Metabolomic analysis of kombucha's production process. Food Chemistry: Molecular Sciences.

Articles de vulgarisation scientifique

Tran, T., Grandvalet, C., Winckler, P., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2020. If It Tastes Great, Blame Science. Part 1 of 3 series: The Chemistry of Fermentation. Symbiosis (Official Journal of Kombucha Brewers International) 38–40.

Communications orales

Tran, T., Grandvalet, C., Winckler, P., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2020. Sensory quality of kombucha: the role of chemical composition and ways to control it. Présenté au Kombucha Summit 2020 (octobre), Online - Berlin.

Tran, T., Grandvalet, C., Winckler, P., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2021. Impact of yeasts-acetic acid bacteria interactions on the composition of kombucha beverage. Présenté au 2nd VLB Symposium on Acidic Fermented non-alcoholic Beverages (SAFB), Versuchs- und Lehranstalt für Brauerei in Berlin (VLB) (avril), Online - Berlin.

Tran, T., Grandvalet, C., Winckler, P., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2021. Microbial Interactions in Kombucha: Impacts on the Beverage's Chemical Composition. Présenté à la 6ème ISEKI-Food Conference 2021 (juin), ISEKI Food Association, Online.

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Communications affichées

Joran, A., Bordet, F., Tran, T., 2019. Fermented beverages: a social network for microorganisms. Présenté à la Journée des Doctorants de l'UMR PAM, Dijon.

Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2021. Polyphenols in kombucha: impact of infusion time on extraction and investigation of their behavior during "fermentation." Présenté au Forum Jeunes Chercheurs, Ecole Doctorale E2S, Dijon (online).

Liste des abréviations

AAB	*Acetic Acid Bacteria*
ADN / DNA	Acide désoxyribonucléique / Desoxyribonucleic acid
AFLP	*Amplified Fragment Length Polymorphism*
AI	*Acetobacter indonesiensis*
ANOVA	*Analysis Of Variance*
AP	*Acetobacter papayae*
ATP	Adénosine triphosphate
BB	*Brettanomyces bruxellensis*
BLAST	*Basic Local Alignment Search Tool*
BTK	*Black Tea Kombucha*
CFU	*Colony Forming Unit*
CoA	Coenzyme A
DM	*Dietary Microorganisms*
ERIC	*Enterobacterial Repetitive Intergenic Consensus*
FAN	*Free Amino Nitrogen*
FEL	*Faster-Evolving Lineage*
FT-ICR-MS	*Fourier Transform – Ion Cyclotron Resonance – Mass Spectrometry*
GAAC	*General AminoAcid Control*
GE	*Glycine Equivalent*
GTK	*Green Tea Kombucha*
HCA	*Hierarchical Clustering Analysis*
HPLC	*High Performance Liquid Chromatography*
HS-SPME-GC-MS	*HeadSpace - Solid Phase Micro Extraction -Gaz Chromatography – Mass Spectrometry*
HV	*Hanseniaspora valbyensis*
ITS	*Internal Transcribed Spacer*

KBC	Kombucha
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
KS	*Komagataeibacter saccharivorans*
LC-MS	*Liquid Chromatography – Mass Spectrometry*
MRS	Man, Rogosa, Sharpe
NAD	Nicotinamide adénine dinucléotide
NCBI	*National Center for Biotechnology Information*
NCR	*Nitrogen Catabolite Repression*
OTU	*Operational Taxonomic Unit*
P1	Phase 1
P2	Phase 2
PAGE	*PolyAcrylamide Gel Electrophoresis*
PCA	*Principal Component Analysis*
PCR	*Polymerase Chain Reaction*
PI	*Propidium Iodide*
RAPD	*Random Amplified Polymorphic DNA*
REP	*Repetitive Extragenic Palindromics*
RFLP	*Restriction Fragment Length Polymorphism*
SBT	*Sugared Black Tea*
SC	*Saccharomyces cerevisiae*
SCOBY	*Symbiotic Culture Of Bacteria and Yeast*
SDS	*Sodium Dodecyl Sulfate*
SEL	*Slower-Evolving Lineage*
SGT	*Sugared Green Tea*
SIS	*Specific Interfacial Surface*
SW	*Sugared Water*
T	Trio

YPD	*Yeast Peptone Dextrose*
W	*Water*
WL	Wallerstein Lab

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Les titres de figures sont présentés dans leur ordre d'apparition. Les chapitres de cette thèse sont rédigés en anglais sous forme d'article ou revue scientifique. Par souci de référencement des tableaux, les légendes des tableaux dans les parties rédigées en anglais apparaîtront avec la désignation « Tableau » et non pas « Table ».

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A Avant-Propos

Avant de plonger dans l'introduction bibliographique, un avant-propos détaille le contexte dans lequel ces travaux de thèse ont été entrepris sous un angle sociétal et scientifique. Cette entrée en matière très générale m'a semblé être nécessaire afin que les lecteurs et lectrices puissent mieux appréhender la pertinence des problématiques abordées. De plus, ce manuscrit de thèse sur articles a été rédigé avec l'ambition d'être accessible autant que cela soit possible aux acteurs et actrices de la branche kombucha, qu'ils ou elles produisent cette boisson, en fassent commerce, l'étudient ou la dégustent tout simplement. Cette démarche découle d'une volonté d'ouverture et de diffusion du savoir scientifique dont le monde académique est le garant et que je souhaite promouvoir.

A.1 Le rôle des aliments fermentés dans les sociétés humaines

Les aliments fermentés, dont les premières traces ont été datées du Néolithique (-14 400 ans), font partie intégrante de l'alimentation humaine (Liu *et al.*, 2018). La fermentation représente un moyen de conservation des aliments qui a été peu à peu délaissé au XXème avec le développement de l'industrie agroalimentaire et d'autres alternatives telles que la réfrigération, surgélation, congélation des matières premières. Les aliments fermentés connaissent actuellement un regain porté par un mouvement général de retour vers des valeurs sociétales liées au bien-être, à la santé et au respect de l'environnement (Arranz-Otaegui *et al.*, 2018 *;* Paul Ross *et al.*, 2002). Il est attendu que le marché mondial des aliments fermentés croisse de 4,3 % (taux de croissance annuel composé) entre 2019 et 2024 (Debailly *et al.*, 2018 *;* Ilango and Antony, 2021).

Sur le plan scientifique, des questions ont été soulevées concernant le lien entre l'appauvrissement de l'alimentation en micro-organismes des sociétés industrialisées et l'émergence de pathologies, notamment liées à l'alimentation (allergies, intolérances). De là, a émergé l'idée que les micro-organismes, dont ceux du microbiote intestinal, sont de « vieux-amis » (*old friends*) jouant un rôle essentiel pour la santé humaine (Guarner *et al.*, 2006). Ainsi, la stimulation du système immunitaire passerait également par l'alimentation *via* l'exposition à une diversité de micro-organismes qu'ils soient pathogènes ou non. Au-delà des hypothèses, des études cliniques ont conclu sur l'existence d'effets bénéfiques de la consommation de produits fermentés sur la santé humaine (Marco *et al.*, 2017). Les mécanismes à l'origine de

ces effets impliqueraient l'interaction entre les micro-organismes et leurs métabolites présents dans l'aliment, et le microbiote intestinal et les cellules du système immunitaire (Figure 1).

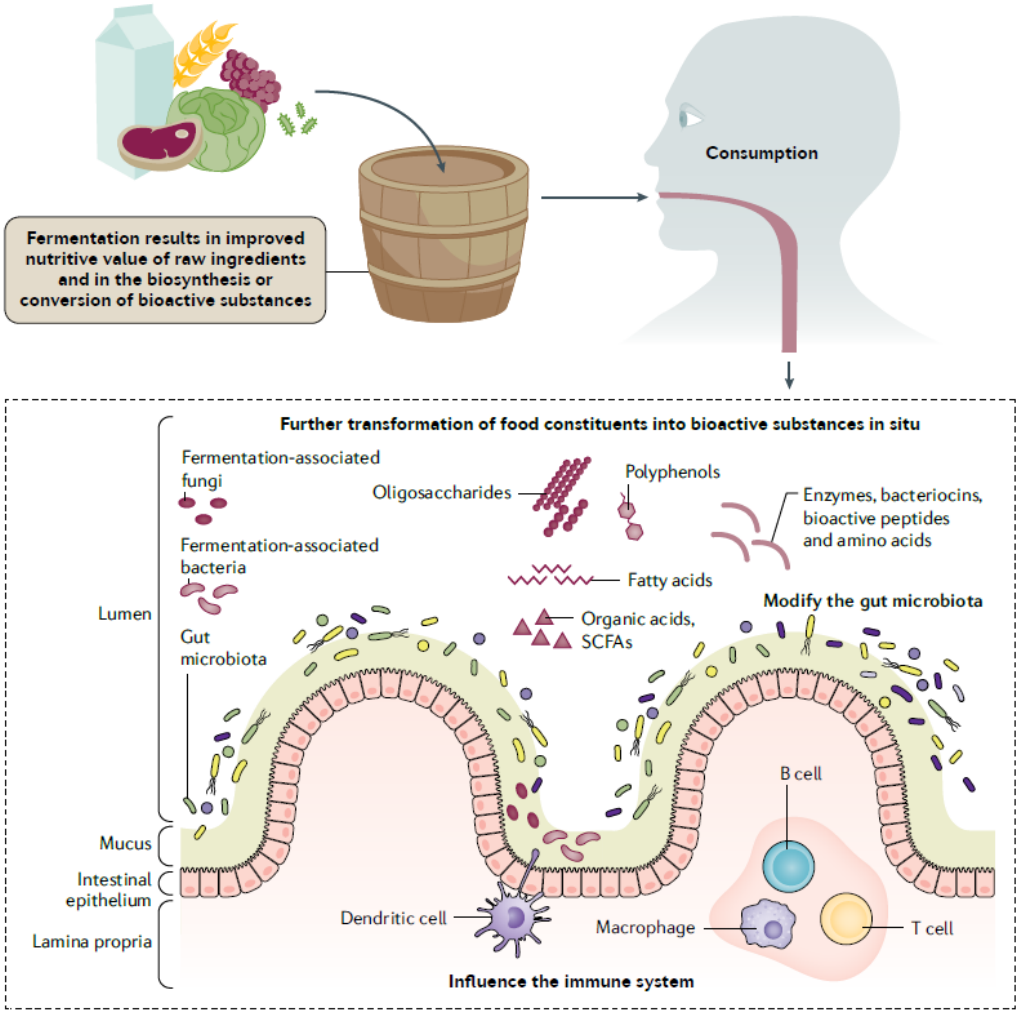

Figure 1 : Bases mécanistiques des effets bénéfiques pour la santé des aliments fermentés (Marco *et al.*, 2021).

Certains gouvernements, dont celui de l'Inde, recommandent la consommation de produits fermentés. Ce n'est pas le cas dans les sociétés occidentales, notamment au sein de l'Union Européenne. Percevant un enjeu sociétal important, un groupe multidisciplinaire de scientifiques (nutritionnistes, médecins, microbiologistes, immunologistes, biochimistes) s'est réuni à l'initiative de l'ISAPP (*International Scientific Association for Probiotics and Prebiotics*) et s'est récemment exprimé afin d'interpeler gouvernements et institutions sur le potentiel que représente la consommation d'aliments fermentés pour la santé publique (Marco *et al.*, 2021).

A.2 La consommation d'aliments fermentés, probiotiques, prébiotiques et « *dietary microorganisms* »

Dans la publication résultante de cette rencontre scientifique de l'ISAPP, une nouvelle définition de la notion d'aliment fermenté a été proposée, s'éloignant de la définition biochimique et restrictive de la fermentation comme « un processus générant de l'ATP dans lequel des composés organiques jouent les rôles de donneurs et accepteurs d'électrons à la fois » (Tortora *et al.*, 2010). Englobant une plus grande diversité de métabolismes et transformations microbiologiques, l'aliment fermenté serait « un aliment résultant d'une croissance microbienne et de transformations enzymatiques de composés désirés dans une matrice alimentaire ». A noter que l'absence de micro-organismes viables dans le produit fini (comme le pain qui est cuit), ne porte pas préjudice à ce statut. A l'inverse, l'ajout artificiel de micro-organismes viables dans un produit fini sans lien avec sa transformation ne satisfait pas à cette définition (Marco *et al.*, 2021). C'est en effet souvent le cas pour les produits probiotiques.

Les probiotiques sont, selon la définition de l'Organisation des Nations unies pour l'alimentation et l'agriculture et l'Organisation Mondiale de la Santé et revisitée par l'ISAPP, « des micro-organismes vivants qui, lorsqu'administrés en quantité adéquate, confèrent un bénéfice pour la santé de l'hôte. » (Hill *et al.*, 2014). La consommation de ces souches sélectionnées a montré des effets sur la santé (Govender *et al.*, 2014). D'un point de vue réglementaire, la mention d'allégation santé en lien avec la présence de probiotiques a engendré une réglementation stricte dans l'Union Européenne (*Règlement (CE) n º 1924/2006*, 2007). Parallèlement, l'engouement du grand public pour les probiotiques a donné lieu à d'importants enjeux économiques, malgré la difficulté de satisfaire cette réglementation (Ilango and Antony, 2021). En effet, le produit doit contenir des micro-organismes issus d'une liste positive de souches dont les propriétés sur la santé sont prouvées, avec une teneur minimum en micro-organismes viables, parfois incompatible avec les formulations et procédés alimentaires (Bertazzoni *et al.*, 2013). Par conséquent, ces allégations concernent principalement des compléments alimentaires (Govender *et al.*, 2014 *;* Marco *et al.*, 2021).

L'utilisation d'allégations santé liées aux « prébiotiques » offre une plus grande flexibilité. Il s'agit d'un « substrat utilisé sélectivement pour les micro-organismes d'un hôte et conférant un bénéfice pour la santé » (Gibson *et al.*, 2017). Dans cette idée, l'Union Européenne reconnaissait la présence des « fibres alimentaires » (par exemple l'inuline) comme allégation santé, 2007). En effet, la consommation de ces fibres stimule la production d'acides gras à

courte chaîne qui jouent un rôle de régulateur dans l'absorption des nutriments dans l'intestin (Baxter *et al.*, 2019).

Enfin, dans le cas où l'aliment fermenté n'est pas stabilisé microbiologiquement, la présence de micro-organismes actifs pourrait également jouer un rôle positif pour le microbiote intestinal. D'où la notion émergente de « *dietary microorganisms* » (DM) ou « micro-organismes alimentaires », désignant la flore présente dans les produits fermentés. Ingérés avec la matrice alimentaire, l'augmentation générale de leur consommation serait bénéfique à la santé humaine sans cibler spécifiquement des genres, espèces ou souches comme c'est le cas pour les micro-organismes probiotiques (Pot, 2021). Cependant, beaucoup de recherches doivent être menées avant de rassembler les éléments nécessaires permettant d'apporter la preuve d'effets bénéfiques pour la santé humaine, et éventuellement mener à la conception d'une réglementation allant dans ce sens.

A.3 L'étude des interactions microbiennes comme clé de la maîtrise de la production d'aliments fermentés traditionnels

La consommation d'aliments fermentés et des DM renvoie principalement à la consommation d'aliments fermentés traditionnels catégorisés comme « *novel food* » (nouveaux aliments). Malgré l'existence d'éléments et d'hypothèses scientifiques, cette mouvance actuelle est davantage basée sur des croyances, dont on leur reproche un manque de rigueur concernant les effets supposés sur la santé (Marco *et al.*, 2021). Or, la production de ses propres aliments fermentés à l'échelle du foyer s'appuie sur une connaissance profane et expose à des risques de dérives microbiologiques (production de toxines, développement de flores pathogènes), mais elle expose un nombre limité d'individus (Debailly *et al.*, 2018). Cela n'est pas envisageable pour une production d'aliments fermentés à échelle industrielle, dont l'envergure serait à la hauteur des enjeux sociétaux énoncés plus tôt. Dans ce contexte, la sécurité alimentaire reste bien sûr primordiale et nécessite de s'appuyer sur un savoir expert et une connaissance poussée des matrices alimentaires, du procédé de fabrication et donc du matériel biologique utilisé pour la fermentation. Cela impose notamment le contrôle de la composition microbienne, la compréhension des processus biologiques en œuvre et de l'influence des paramètres de production sur ces derniers.

Si cette connaissance est bien établie concernant les produits comme le yaourt, le pain, la bière ou le vin, cela n'est pas le cas pour les produits traditionnels qui émergent sur le marché des

nouveaux aliments fermentés comme le kéfir ou la kombucha. Dans leur version traditionnelle, tous les aliments fermentés sont issus de l'activité microbienne de communautés complexes (consortia) involontairement sélectionnées par l'homme pour leurs aptitudes technologiques et composées de micro-organismes peu étudiés. La réduction de ces communautés à une ou deux souches domestiquées ou non (comme ce fût le cas pour la levure *Saccharomyces cerevisiae*), a été la solution apportée par l'industrie agroalimentaires (Aldrete-Tapia *et al.,* 2018 *;* Kuthan *et al.*, 2003). Dans le cas où l'usage d'un consortium microbien doit être maintenu, la transition vers une production industrielle impose de passer par la caractérisation de sa composition, des micro-organismes qui la composent et de leurs interactions entre eux et avec la matrice alimentaire. Bien que la maîtrise, voire la domestication de micro-organismes en communauté soit plus délicate que pour une seule souche de levure ou de bactérie, l'utilisation de consortia microbiens représente des opportunités d'innovation en procédé alimentaire. En effet, sur le modèle de procédés biotechnologiques non-alimentaires utilisant des consortia microbiens (souvent synthétiques, c'est-à-dire assemblés artificiellement), il peut être envisagé de réaliser des conversions chimiques qui ne peuvent être réalisées (ou moins efficacement) par un seul micro-organisme dans le cadre de production alimentaire (Lawson *et al.*, 2019). Cette approche est d'ores et déjà étudiée pour la production de vins moins alcoolisés, en réalisant des inoculations séquentielles de levures, dont la première possède un rendement alcoolique inférieur à *Saccharomyces cerevisiae*, cette dernière inoculée en second a pour fonction d'achever la fermentation (Contreras *et al.*, 2015 *;* Quirós *et al.*, 2014).

Ainsi, au-delà de la nécessité de caractériser les souches technologiques en elles-mêmes, tout l'enjeu de l'utilisation de consortia réside dans l'étude des interactions microbiennes. Or, ces interactions sont d'autant plus nombreuses que la diversité des populations microbiennes en présence est élevée. Ces interactions interviennent à de multiples niveaux : nutritionnel, métabolique, génomique voire spatial (Ivey *et al.*, 2013).

B Introduction bibliographique

Cet état de l'art débute sur des rappels concernant la classification des interactions microbiennes et leurs mécanismes. Il se poursuit sur des connaissances générales relatives à la kombucha et aux autres boissons fermentées. Enfin, elle conclut avec la description des micro-organismes appartenant aux consortia de kombucha et de leurs métabolismes. Cette dernière partie demeure relativement générale afin d'épargner aux lecteurs et lectrices de nombreuses redondances d'éléments bibliographiques plus spécifiques introduits et développés dans les chapitres/articles

de la thèse. Le format de thèse sur articles implique également que les éléments relatifs aux méthodes et techniques mises en œuvre soient également distribués au sein des chapitres/articles concernés.

B.1 Les interactions microbiennes dans les aliments fermentés

B.1.1 Les types d'interactions microbiennes

Les interactions entre deux micro-organismes A et B sont catégorisées en fonction de l'effet favorable ou néfaste de la présence ou de l'activité d'un micro-organisme sur l'autre. Ces différents types d'interactions ont été observés au cours de la fabrication d'aliments fermentés qui représentent par ailleurs des modèles intéressants pour l'étude des interactions microbiennes (Ivey *et al.*, 2013).

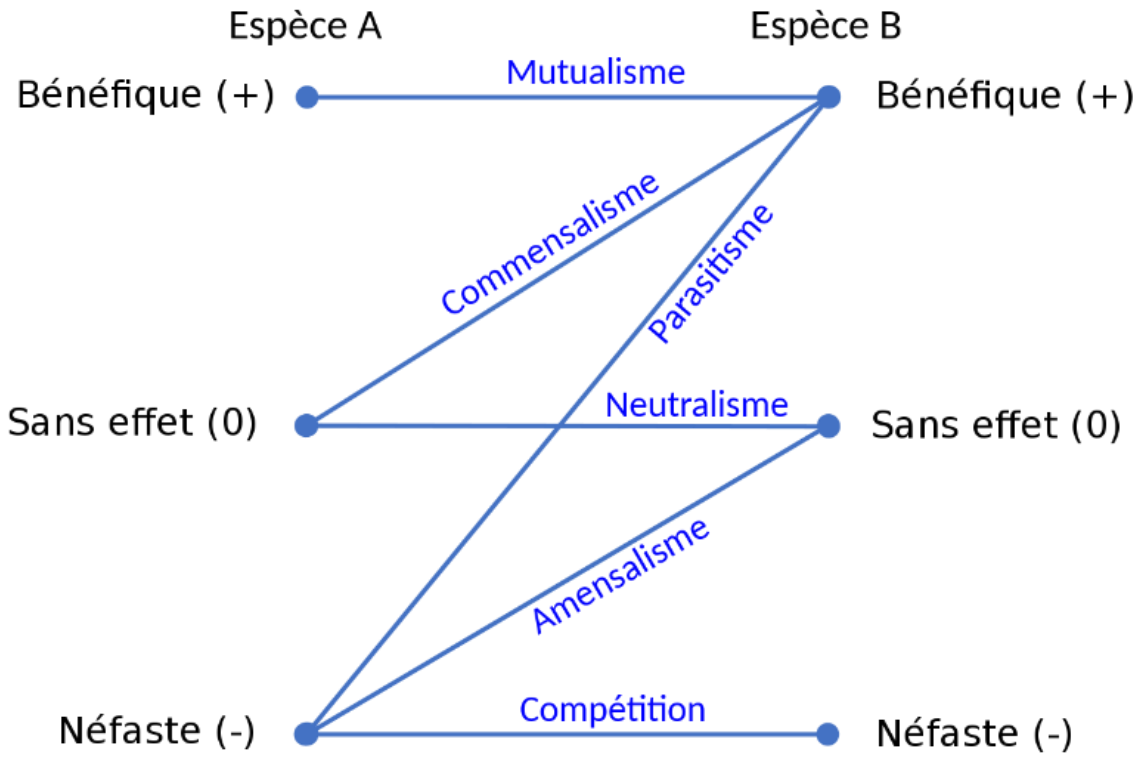

Figure 2 : Diagramme simplifié des six principales interactions biologiques (Alexander, 2018).

La compétition désigne une interaction où les populations des micro-organismes A et B sont en compétition pour des ressources communes, et par conséquent ont un effet néfaste l'un sur l'autre. Il est possible de citer la compétition pour les acides aminés assimilables par les levures

dans le contexte œnologique. Au début d'une fermentation spontanée du moût de raisin, la biodiversité levurienne est élevée et les levures entrent alors en compétition pour les ressources en azote. L'existence de profils de consommation spécifiques chez les levures qu'elles appartiennent au genre *Saccharomyces* ou non (on parle alors de levures non-*Saccharomyces*) implique des besoins et préférences différentes qui va donc induire une compétition plus ou moins intense selon les genres et espèces en présence (Gobert *et al.*, 2017).

L'amensalisme désigne une interaction où la population du micro-organisme B a un effet néfaste sur la population A, sans que la population B n'en reçoive de bénéfice, ni ne soit affectée. Ce cas s'applique classiquement dans la production du yaourt où la production d'acide lactique à partir du lactose entraîne une diminution de la valeur du pH qui inhibe le développement de micro-organismes non adaptés à ces conditions physico-chimiques, tels que certains contaminants. L'augmentation de la teneur en éthanol par fermentation alcoolique levurienne au cours de la vinification est un autre exemple d'amensalisme inhibant le développement des micro-organismes non adaptés (Grangeteau *et al.*, 2015 *;* Sieuwerts *et al.*, 2008). La sécrétion de toxines, comme les bactériocines fait également partie de ce type d'interaction, au même titre que certains peptides antimicrobiens d'origine levurienne identifiés dans le contexte œnologique (Albergaria and Arneborg, 2016 *;* Nehme *et al.*, 2010 *;* O'Connor *et al.*, 2020).

Le commensalisme désigne une interaction où la population microbienne A a un effet favorable pour la population B sans que la population A n'en reçoive de bénéfice, ni ne soit affectée. Ce type d'interaction a été identifié dans le cadre de la production de Gouda entre deux souches de *Lactococcus lactis*, dont l'une possédait une protéase extracellulaire et l'autre non permettant l'accès aux acides aminés (Juillard *et al.*, 1996).

Le mutualisme désigne une interaction où la population A et la population B ont un effet bénéfique l'une pour l'autre. Ce type d'interaction a été étudié en détail dans la production de yaourt entre *Streptococcus thermophilus* et *Lactobacillus delbrueckii* mettant en jeu la mise à disposition de nutriments azotés *via* une activité protéase de *L. delbrueckii* bénéfique pour *S. thermophilus*, et en retour, une libération de facteurs de croissance tels que les acides formique, pyruvique et folique (Sieuwerts *et al.*, 2008). Dans des systèmes avec des compositions microbiennes moins maîtrisées, tels que les levains de panification, des interactions mutualistes entre levures et bactéries ont été rapportées. Celles-ci impliquent l'hydrolyse de macromolécules telles que l'amidon ou les protéines rendant disponibles maltose et acides

aminés respectivement pour des bactéries lactiques, qui en retour convertissent le maltose en glucose. Une partie de la ressource en nutriment carboné est, de ce fait, rendue disponible pour les micro-organismes qui sont incapables d'utiliser le maltose (Gobbetti, 1998).

Enfin, le parasitisme désigne le cas où la population microbienne A a un effet bénéfique sur la population B à son propre détriment. Si l'on inclut les virus dans le monde microbien, l'exemple classique dans la production d'aliments fermentés porte sur les contaminations de bactériophages impactant négativement les bactéries utilisées comme ferments (Sieuwerts *et al.*, 2008). Détectés dans les aliments à base de lait, de viande ou dans des boissons (vins et cidres), les phages pourraient également être des moteurs de l'évolution des consortia microbien dans les aliments en appliquant une pression de sélection (Ledormand *et al.*, 2020).

Il est nécessaire de souligner que cette catégorisation permettant de caractériser les interactions microbiennes n'intègre que peu de nuances, d'autant plus que différents types d'interactions peuvent coexister au sein d'un aliment. Pour deux micro-organismes donnés, plusieurs types d'interaction ont pu être observés avec des comportements opposés en lien avec des caractères souche-dépendants. C'est par exemple le cas pour *Saccharomyces cerevisiae* et *Oenococcus oeni* dans le contexte de vinification, (Alexandre, 2004 *;* Balmaseda *et al.*, 2021)

B.1.2 Les mécanismes impliqués dans les interactions microbiennes

En termes de mécanismes, une distinction est faite entre les interactions directes, impliquant un contact physique entre les cellules, et les interactions indirectes qui sont opérées par la production de substances qui diffusent dans le milieu pour agir plus ou moins spécifiquement sur d'autres populations (Sieuwerts *et al.*, 2008). Parmi les interactions basées sur des contacts entre cellules, il est possible de citer l'induction de la synthèse d'exopolymères par le contact entre *Lactobacillus kefiranofaciens* et *S. cerevisiae* au cours de la production de kéfir (Cheirsilp *et al.*, 2003). La floculation est également impliquée dans les interactions levure-levure au cours de la production de bière et de vin (Soares, 2011). Concernant les interactions indirectes, outre les flux de métabolites, de nutriments ou l'action des toxines, le phénomène de « *Quorum Sensing* » désigne spécifiquement la sécrétion d'une substance proportionnellement à la taille de la population émettrice, permettant la communication avec une seconde population microbienne intraspécifique. Toutefois, des cas de communication interspécifique par *Quorum Sensing* ont été identifiés chez *Lactiplantibacillus plantarum* (ex-*Lactobacillus plantarum*) par l'intermédiaire de peptides (Maldonado *et al.*, 2004 *;* Zheng *et al.*, 2020). Chez les bactéries, ce

phénomène, impacte la motilité, la sécrétion de toxines ou la formation de biofilm (González and Keshavan, 2006). Il a également été mis en évidence chez les levures, dont *S. cerevisiae*, avec l'utilisation d'alcools comme le phenyléthanol comme molécule signal pour initier le passage vers la forme filamenteuse (Albuquerque and Casadevall, 2012).

Enfin, il est important de mentionner d'autres mécanismes encore peu étudiés qui peuvent impliquer des transferts horizontaux de gènes (par exemple chez *L. delbrueckii* subsp. *bulgaricus* (van de Guchte *et al.*, 2006)), ou bien la sécrétion de prion, comme avec le prion [*GAR*+] produit par des bactéries acétiques et lactiques du vin ayant pour effet l'inhibition de l'activité fermentaire de *S. cerevisiae* (Ramakrishnan *et al.*, 2016).

On remarque que les interactions microbiennes impliquent indifféremment des individus de règnes différents (par exemple levures et bactéries), d'espèces différentes au sein d'un même règne (entre levures *Saccharomyces* et non-*Saccharomyces* par exemple) ou même entre deux souches de la même espèce (par exemple *L. lactis* protéase $^{+/-}$ (Juillard *et al.*, 1996)).

Bien que la plupart des interactions microbiennes aient été étudiées avec des micro-organismes en conditions planctoniques, le mode de vie sessile est également observé dans le contexte alimentaire (Alvarez-Ordóñez *et al.*, 2019). Un biofilm peut être défini par des agrégats de micro-organismes dans lesquels les cellules sont encapsulées dans une matrice extracellulaire auto-produite composée de substances polymériques, les rendant adhérentes entre elles ou à une surface. Ces structures supra-cellulaires, observées chez les levures et chez les bactéries, sont des formes de résistance et possèdent de multiples fonctions, notamment pour les interactions microbiennes en abritant différentes espèces (Flemming *et al.*, 2016 *;* Zara *et al.*, 2020). Outre la protection physique et chimique offerte par la production de polymères extracellulaires, la formation de biofilm permet le piégeage des nutriments et de l'eau, qui sont autant d'avantages pour résister au stress comparé à un mode de vie planctonique. De plus, l'organisation spatiale des micro-organismes s'effectue selon différents gradients chimiques (comme l'oxygène ou les nutriments) au sein du biofilm. De plus, la formation de micro-colonies peut permettre un meilleur contrôle des conditions physico-chimiques (par exemple la valeur de pH) (Figure 3). Par ailleurs, les biofilms ont été également identifiés comme le support des interactions microbiennes par diffusion de molécules signal (*Quorum Sensing*) ou des transferts de gènes horizontaux. Par conséquent, ce mode de vie peut amener des changements dans les types d'interactions microbiennes mis en jeu comparé aux conditions planctoniques en favorisant coopération ou compétition entre populations (Flemming *et al.*, 2016). Chez les

levures, dont *S. cerevisiae*, la formation de biofilm peut s'accompagner d'un passage vers la forme filamenteuse (pseudo-hyphe) induit par une carence nutritive (Sanna *et al.*, 2012 *;* Zara *et al.*, 2020, 2005).

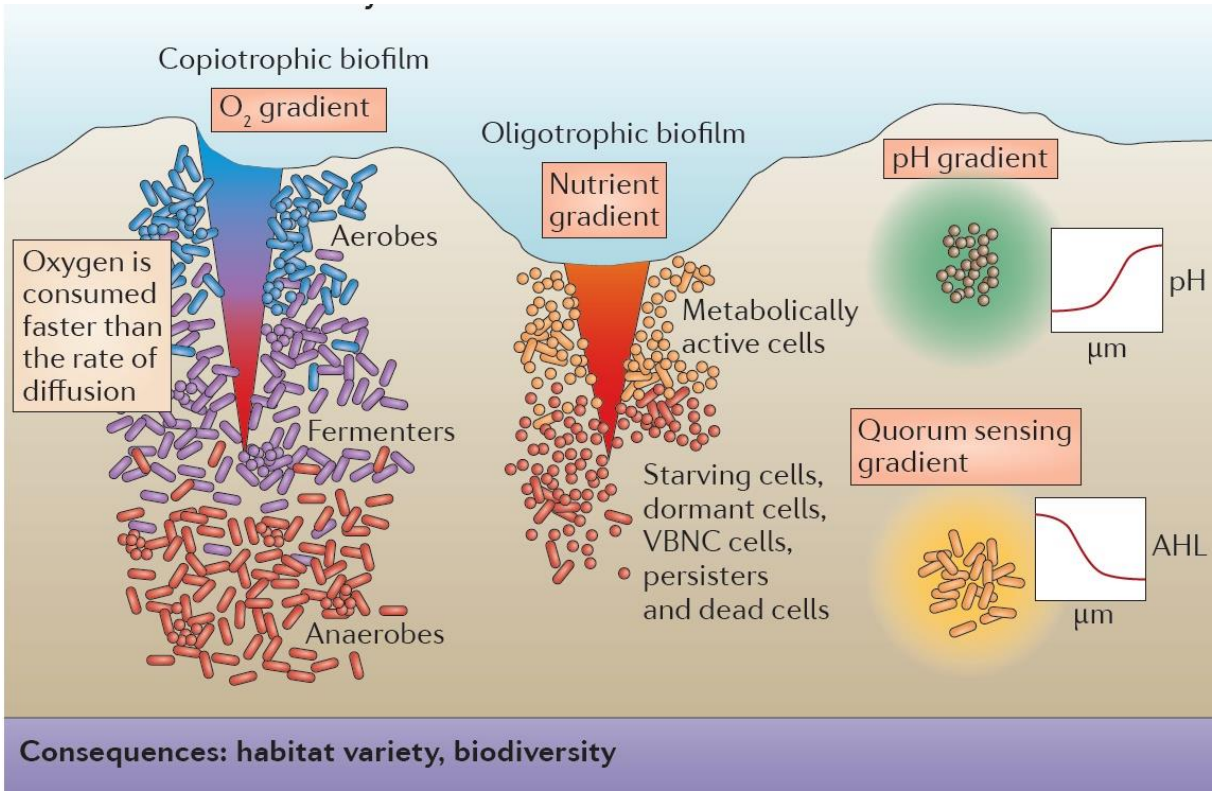

Figure 3 : Caractéristiques remarquables des biofilms microbiens : Les biofilms sont caractérisés par leur hétérogénéité et les interactions sociales. Les gradients sont stabilisés par l'immobilité des cellules du biofilm dans la matrice (Flemming *et al.*, 2016).

Les conditions environnementales, les modes de vie et les paramètres abiotiques peuvent ainsi induire des interactions différentes. L'étude des interactions microbiennes dans le contexte alimentaire est donc dépendante d'une autre interaction, celle entre les micro-organismes et la matrice alimentaire.

B.2 La kombucha

B.2.1 Bref historique de la kombucha

La matrice alimentaire dont cette thèse fait spécifiquement l'objet est la kombucha. La kombucha est une boisson fermentée acide non alcoolisée préparée à partir d'une infusion de thé sucré. Cette base est ensuite inoculée avec un consortium de levures et de bactéries acétiques, avec parfois la présence facultative de bactéries lactiques. La transformation majeure

réalisée par cette association de micro-organismes est la conversion du sucre, le saccharose, en acides organiques (Blanc, 1996 *;* Jayabalan *et al.*, 2014 *;* Sievers *et al.*, 1995). Durant la première phase de production, le système est laissé ouvert afin de permettre les échanges gazeux. Les monosaccharides (glucose et fructose) rendus disponibles par l'activité invertase des levures sont utilisés par ces dernières pour la fermentation alcoolique avec production d'éthanol et de dioxyde de carbone (Jayabalan *et al.*, 2014 *;* Villarreal-Soto *et al.*, 2018). Les bactéries acétiques convertissent le glucose et l'éthanol produits en acides gluconique et acétique principalement et acidifient la matrice (Lynch *et al.*, 2019). Ce métabolisme oxydatif caractéristique des bactéries acétiques nécessite un accès à l'oxygène, d'où l'utilisation d'un récipient ouvert en condition statique. Parallèlement, elles produisent un biofilm cellulosique flottant à la surface du liquide (Goh *et al.*, 2012a). La seconde phase de production intervient à la suite de la mise en bouteille, un système fermé imperméable aux gaz. L'épuisement de la ressource en oxygène inhibe le métabolisme oxydatif des bactéries acétiques et donc l'acidification. D'autre part, la fermentation alcoolique est favorisée chez les levures, entrainant l'accumulation du gaz carbonique et donc une prise de mousse (Kim *et* Adhikari, 2020). Cependant l'éthanol qui n'est plus converti en acide acétique s'accumule également et expose les producteurs au dépassement des teneurs maximales autorisées pour les boissons non-alcoolisées (1,2 %(v/v) dans l'Union Européenne) (*Règlement (CE) n º 1924/2006*, 2007 *;* Talebi *et al.*, 2017). Le procédé de production ainsi que la microbiologie de la kombucha sont détaillées dans la partie B.2.3.

La kombucha est considérée comme une boisson fermentée traditionnelle millénaire ayant pour origine la Chine. Cette idée s'est propagée sur la base d'interprétations erronées de légendes chinoises ou japonaises et largement disséminée sans vérification des sources bibliographiques (Zagrabinski, 2020a, 2020b). La présence d'un biofilm cellulosique flottant à la surface du liquide, la mère de kombucha, a été source de confusion car longtemps pris pour un champignon macroscopique, d'où le qualificatif de « champignon de thé » (Jayabalan *et al.*, 2014). Récemment, le gérant du « Café Bärbucha » à Berlin a entrepris un travail rigoureux de recherches concernant l'origine de la kombucha. Sur la base de recherches documentaires, les mentions les plus anciennes de la kombucha remontent au début du XX^{ème} siècle en Russie et en Allemagne dans des publications scientifiques. L'auteur de ce travail fait l'hypothèse que la kombucha a été importée de Russie vers l'Occident pour la première fois durant cette période marquée par les deux Guerres Mondiales impliquant la Russie et l'Europe de l'Ouest. La fabrication de kombucha « maison » a connu des hauts et des bas dans l'après-guerre en Europe

(Zagrabinski, 2021a). Ce n'est qu'au début des années 90 que la commercialisation de la kombucha débuta en Allemagne, mais c'est à partir de 1995 en Californie que les « *kombucha breweries* », entreprises productrices de kombucha, ont réellement pris leur essor pour continuer à se développer dans le monde entier jusqu'à aujourd'hui (Zagrabinski, 2021b).

Entre temps, la réputation de la kombucha concernant ses effets bénéfiques sur la santé s'est construite sur la base d'écrits prétendument scientifiques entre les années 60 et 90, mettant en avant des propriétés curatives non prouvées contre le cancer ou le SIDA (Frank, 1990 *;* Tietze, 1996). En revanche, d'autres travaux s'intéressant plus spécifiquement à la microbiologie de la boisson ont été menés à partir des années 90 (Blanc, 1996 *;* Greenwalt *et al.*, 2000 *;* Reiss, 1994 *;* Sievers *et al.*, 1995). Du fait d'un marché global de plus en plus dynamique, la kombucha est devenue le sujet de nombreux travaux de recherche sur le plan de ses propriétés pour la santé, mais également concernant sa qualité en tant que boisson produite industriellement (Kim and Adhikari, 2020). Ainsi, malgré l'accumulation significative de connaissances, la kombucha reste une boisson fermentée dont la production non standardisée rend la maîtrise de sa qualité difficile. De ce fait, les connaissances concernant la microbiologie, l'écologie, la physico-chimie et la sensorialité de cette boisson restent à ce jour encore lacunaires, ce qui impose de replacer cette boisson dans un contexte plus large d'aliments fermentés afin d'identifier des repères pouvant guider son étude.

B.2.2 La kombucha et les autres boissons fermentées

La kombucha en sa qualité de boisson fermentée peut être rapprochée de produits très étudiés tels que les vins, les cidres ou les bières. En effet, ces boissons résultent de la transformation, principalement par fermentation alcoolique, d'une base liquide sucrée d'origine végétale contenant les nutriments nécessaires au développement des populations et activités microbiennes (Torres-Guardado *et al.*, 2021). Toutefois, ces boissons sont principalement fermentées par des levures avec majoritairement des souches sélectionnées appartenant à l'espèce *Saccharomyces cerevisiae*. Si l'activité de bactéries est souhaitée, il s'agit en général de bactéries lactiques. On peut citer la fermentation malolactique pour le vin et les bières sûres (*sour beers*) faisant intervenir la fermentation lactique (De Roos and De Vuyst, 2019 *;* Ribéreau-Gayon *et al.*, 2004). Dans la plupart des cas, l'activité de bactéries acétiques, dont la présence est indispensable pour la kombucha, représente un risque de déviation pour ces boissons alcoolisée du fait de la conversion de l'éthanol en acide acétique (De Roos and De Vuyst, 2018). En ce sens, il est possible de rapprocher également la kombucha du vinaigre

produit par la transformation d'une base de vin, bière ou cidre grâce à l'activité des bactéries acétiques sans être associée à une activité levurienne. Par opposition, les bières gueuzes et lambics produites dans la région de Bruxelles (Belgique) font intervenir levures (*Saccharomyces* et non-*Saccharomyces*), bactéries lactiques et bactéries acétiques. Ces fermentations spontanées sont menées par une flore technologique indigène issues du lieu de production (De Roos and De Vuyst, 2019a *;* Spitaels *et al.*, 2014). De manière générale, en ce qui concerne le vin et la bière, le développement de co-fermentations simultanées ou séquentielles impliquant levures *Saccharomyces* et non-*Saccharomyces* (incluant les genres *Lachancea, Metschnikowia, Torulaspora* ou *Hanseniaspora* également présents dans consortia de kombucha) est en plein essor (Bellut *et al.*, 2018 *;* Bordet *et al.*, 2020 *;* Morata, 2019 *;* Vejarano, 2020). Ces nouveaux procédés visent différentes problématiques comme la réduction de la teneur en alcool, la bioprotection ou bien la diversification des profils sensoriels (Contreras *et al.*, 2015 *;* Gobert *et al.*, 2017 *;* Morata, 2019 *;* Morata *et al.*, 2019 *;* Sadoudi *et al.*, 2012 *;* Simonin *et al.*, 2020, 2018).

Bien que présentant des similitudes en termes de compositions microbiologiques, les matrices fermentescibles que sont les moûts de raisin, de pomme ou d'orge se distinguent de la kombucha par le substrat carboné de base. Le saccharose n'est en effet pas un substrat carboné assimilable directement comme le glucose ou le fructose qui sont en revanche directement disponibles dans les moûts de raisin, de malt d'orge (grâce à l'action des amylases) ou de pomme (Ribéreau-Gayon *et al.*, 2004). On peut donc faire l'hypothèse que l'accès primordial au substrat carboné conditionne l'écologie des communautés microbiennes peuplant ces milieux, qui dans le cas de la kombucha nécessite une hydrolyse du saccharose (Manoochehri *et al.*, 2020). Une récente étude a démontré que c'était effectivement le cas en comparant les compositions en genres et espèces de produits fermentés non alcoolisés basés sur des substrats carbonés différents (Leech *et al.*, 2020). Dans le groupe ayant pour base le saccharose, divers produits fermentés ont été analysés : kombucha, kéfir d'eau, kvass. Cette étude amis en évidence la dominance des bactéries acétiques (*Acetobacteraceae*) dans les matrices à base de saccharose. Ces bactéries acétiques sont trouvées en association avec des bactéries lactiques (notamment *Oenococcus oeni*) et des levures (comme *Brettanomyces bruxellensis*) et relèvent la forte représentation de l'espèce *Hanseniaspora valbyensis*.

Tableau 1 : Répartition des espèces microbiennes communes dans différentes boissons fermentées

Genre	Glucose/Fructose		Glucose seul	Saccharose		Espèces communes	Références
	Moût et baies de raisin	Moût de pomme	Moût de malt d'orge	Kéfir d'eau	Kombucha		
Levures		x					
Saccharomyces sp.	x		x	x	x	*S. cerevisiae*	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017 *;* Gulitz *et al.*, 2011 *;* Valles *et al.*, 2007)
Zygosaccharomyces sp.	x				x	*Z. bailii*	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017)
Schizosaccharomyces sp.	x				x	*Sc. pombe*	(Barata *et al.*, 2012 *;* Villarreal-Soto *et al.*, 2018)
Lachancea sp.	x			x	x	*L. thermotolerans*	(Barata *et al.*, 2012 *;* Bellut *et al.*, 2018 *;* Gulitz *et al.*, 2011)
Metschnikowia sp.	x	x				*M. pulcherrima*	(Barata *et al.*, 2012 *;* Valles *et al.*, 2007)
Torulaspora sp.	x				x	*T. delbrueckii*	(Barata *et al.*, 2012 *;* Teoh *et al.*, 2004)
Hanseniaspora sp.	x	x	x	x	x	-	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017 *;* De Roos and De Vuyst, 2019 *;* Gulitz *et al.*, 2011 *;* Valles *et al.*, 2007)
Brettanomyces sp.	x	x	x		x	*B. bruxellensis*	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017*;* De Roos and De Vuyst, 2019 *;* Morrissey *et al.*, 2004)
Pichia sp.	x	x	x		x	-	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017 *;* Freek Spitaels *et al.*, 2014 *;* Valles *et al.*, 2007)
Candida sp.	x				x	*C. stellata*	(Barata *et al.*, 2012 *;* Teoh *et al.*, 2004)
Debaryomyces sp.	x	x	x		x	-	(Ahmed *et al.*, 2020 *;* Barata *et al.*, 2012 *;* Morrissey *et al.*, 2004 *;* Freek Spitaels *et al.*, 2014)
Bactéries lactiques							
Lactobacillus sp.	x	x					(Barata *et al.*, 2012 ; Morrisey *et al.*, 2004)

Liquorilactobacillus sp.				x	x	*L. nagelii*	(Gulitz *et al.*, 2011 *;* Savary *et al.*, 2021)
Oenococcus sp.	x	x			x	*O. oeni*	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017 *;* Sánchez *et al.*, 2012)
Pediococcus sp.	x	x	x		x	-	(Barata *et al.*, 2012 *;* Sánchez *et al.*, 2012 *;* Freek Spitaels *et al.*, 2014 *;* Villarreal-Soto *et al.*, 2018)
Levilactobacillus sp.	x	x	x			*Lb. brevis*	(Barata *et al.*, 2012 *;* Sánchez *et al.*, 2012 *;* Freek Spitaels *et al.*, 2014)
Secundolactobacillus sp.		x	x			-	(De Roos and De Vuyst, 2019 *;* Sánchez *et al.*, 2010)
Lentilactobacillus sp.		x		x		-	(De Roos and De Vuyst, 2019 *;* Sánchez *et al.*, 2010)
Leuconostoc sp.	x		x	x		*L. mesenteroides*	(Barata *et al.*, 2012 *;* Gulitz *et al.*, 2011 *;* Freek Spitaels *et al.*, 2014)
Bactéries acétiques							
Acetobacter sp.	x		x	x	x	-	(Barata *et al.*, 2012 *;* De Roos and De Vuyst, 2018 *;* Freek Spitaels *et al.*, 2014 ; Marsh *et al.*, 2014)
Komagataeibacter sp.	x				x	*K. saccharivorans*	(Barata *et al.*, 2012 *;* De Filippis *et al.*, 2018)
Gluconobacter sp.	x		x		x	*G. oxydans*	(Barata *et al.*, 2012 *;* Coton *et al.*, 2017 *;* De Roos and De Vuyst, 2018)
Gluconacetobacter sp.	x				x	-	(Barata *et al.*, 2012 *;* Marsh *et al.*, 2014)

B.2.3 Paramètres microbiologiques et technologiques impactant la composition chimique et la qualité sensorielle de la kombucha (review)

La revue "*Microbiological and technological parameters impacting the chemical composition and sensory quality of kombucha*" publiée dans le journal *Comprehensive Reviews in Food Science and Food Safety* décrit le procédé de production de la kombucha et les composantes de sa qualité organoleptique. Elle détaille notamment sa physico-chimie en lien avec les transformations microbiennes et fait le lien avec les changements au niveau sensoriel survenant entre le thé et la kombucha.

Résumé

La kombucha est une boisson produite à partir de thé sucré transformé par des levures et des bactéries acétiques. Fait-maison à l'origine, elle est devenue une boisson non-alcoolisée produite à échelle industrielle dont les standards de qualité sont encore mal définis et dont le procédé de production est encore mal maîtrisé. En se basant sur les connaissances actuelles relatives aux boissons, des liens ont été établis entre la composition chimique et les composantes sensorielles de la kombucha. Les macromolécules créent de la turbidité et les dérivés de pigments issus du thé, encore mal caractérisés, participent à la couleur. Les sucres résiduels amènent de la sucrosité et les acides organiques produits principalement par les bactéries acétiques donnent à la kombucha son acidité caractéristique. L'acide acétique fait partie du profil aromatique, bien que peu de données soient disponibles concernant l'odeur de la kombucha. Le dioxyde de carbone, potentiellement les polyphénols et l'éthanol résiduel sont impliqués dans la texture en bouche.

Après avoir défini les composantes clé délimitant les propriétés sensorielles de la kombucha, cette revue discute l'impact des différents paramètres de production. La composition de l'eau est déterminante pour l'extraction des composés du thé, de même que le type de thé utilisé, la durée d'infusion et la température. Le type et la quantité de sucre jouent un rôle dans la sucrosité et influencent la cinétique de production. De manière similaire, la quantité d'inoculum et sa composition microbienne ont un effet sur la production. De plus, le rôle de la géométrie du contenant utilisé en production et la température sont également des paramètres essentiels pouvant être maîtrisés pour ajuster le temps de la phase d'acidification lors de la production. Malgré toutes les recherches déjà menées, de nouvelles investigations doivent être conduites concernant les caractéristiques sensorielles de la kombucha. De tels travaux permettraient une meilleure définition de la qualité de la kombucha et ainsi d'améliorer la maîtrise du procédé de production.

Microbiological and technological parameters impacting the chemical composition and sensory quality of kombucha

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Abstract

Kombucha is a beverage made from sugared tea transformed by yeasts and acetic acid bacteria. Being originally homemade, it has become an industrially produced soft drink whose quality standards are poorly defined and whose production process is still not fully controlled. Based on current knowledge in beverages, links between kombucha's chemical composition and sensorial compounds are drawn. Macromolecules create turbidity, whereas uncharacterized tea pigments derivatives participate in the color. Residual sugars bring sweetness and organic acids produced by acetic acid bacteria form its characteristic sour taste. Acetic acid is also part of its aroma profile, although little data are available on the smell of kombucha. Carbon dioxide, potentially polyphenols, and residual ethanol are involved in the mouthfeel. In this review, after defining the key compounds that shape the characteristic sensory properties of kombucha, the impact of different production parameters is discussed. Water composition is determinant in the extraction of tea compounds along with the tea type and infusion duration and temperature. The type and amount of sweeteners play a role in the sweetness and influences the production kinetics. Similarly, the amount of inoculum and its microbial composition have an effect on the production, but the role of the vessels' geometry and temperature are also essential parameters that can be used to adjust the acidification phase's duration. Despite the amount of research carried out, further investigations of

kombucha's sensory characteristics are needed. Such research could lead to a better definition of kombucha's quality and to an improved control over its production process.

Keywords

fermentation, Kombucha, process, quality, sensory.

1 Introduction

Kombucha, also named "kombucha tea," is a fermented beverage resulting from the activity of a microbial consortium including yeasts, acetic acid bacteria, and often (but not always) lactic acid bacteria in sugared tea liquor as liquid medium (Dufresne and Farnworth, 2000; Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). It is believed to originate from Asia but all the elements appearing in the scientific literature, especially about the Chinese origins of kombucha, are supported by unverified sources (Dufresne and Farnworth, 2000). Nevertheless, these stories continue to feed the mythology of kombucha and could be used to support the marketing communication of industrialized products. Indeed, the main marketing arguments of kombucha are its putative benefits for human health. Numerous in vivo and in vitro studies were carried out to establish the existence of antioxidant (Bhattacharya *et al.*, 2013; Gamboa-Gómez *et al.*, 2016; Jayabalan *et al.*, 2008), antimicrobial (Battikh *et al.*, 2012; Sreeramulu *et al.*, 2000; Steinkraus *et al.*, 1996) and hepato-protective effects (Murugesan, 2009; Wang *et al.*, 2014). Nevertheless, the existence of beneficial effects of kombucha for human health remains controversial because of the insufficient amount of decisive scientific data (Ernst, 2003; Jayabalan *et al.*, 2014; Martínez Leal *et al.*, 2018) and because of multiple cases of diseases or unexplained death following the overconsumption of kombucha (Holbourn and Hurdman, 2017; Phan *et al.*, 1998; SungHee Kole *et al.*, 2009). The active compounds of kombucha with potential benefits for human health originate from tea polyphenols, in particular epigallocatechin gallate (Jayabalan *et al.*, 2007; Khan and Mukhtar, 2007), hydrolytic enzymes, vitamins (B1, B2, B6, B12, and C) (Bauer-Petrovska and Petrushevska-Tozi, 2000; Kumar and Joshi, 2016), and organic acids such as gluconic acid, glucuronic acid (Nguyen *et al.*, 2015, 2014) or D-saccharic-1,4lactone acid (Wang *et al.*, 2010) produced by the microorganisms. To preserve these potential benefits and/or promote a clean label, some kombucha producers do not pasteurize nor filter their products.

During the last decades, kombucha transitioned from a homemade fermented beverage to a commercialized soft drink produced industrially. A striking result is that the market of kombucha is expected to exhibit a strong growth rate of 17.5% in the United States between 2019 and 2024 (Mordor Intelligence, 2019). Despite a strong development of kombucha-producing companies, the producers of this beverage suffer from a lack of technical knowledge similar to what exists for the production of other beverages, such as wines or beers. This review aims at giving the kombucha brewers, R&D staff, and researchers an innovative approach of kombucha as a commercialized fermented beverage. It will revolve around the identification and control of the different dimensions, or components, of kombucha's quality through the lens of the current available scientific literature. The concept of quality developed on wine was studied by Charters and Pettigrew (2007) and distinguished between extrinsic and intrinsic qualities. This review will focus on the intrinsic quality of tea-based kombucha that is bound to its structural features, in other words its sensory properties. This then excludes all the aspects related to price, packaging, or marketing. Also, fermented products based on infusions of plants that are not tea (*Camellia sinensis*) or on other food matrices will not be discussed, nor will flavored kombuchas. Indeed, the use of aromatization ingredients such as fruits extracts, herbs, or spices implies the addition of a variety of compounds. Among them, nitrogen sources, sugars, or antimicrobial compounds can be the origin of significant modification in microbial dynamics and elaboration kinetics before and after bottling. The complexity of the effects that could be induced by the addition of such ingredients makes inclusion of this topic injudicious and thus it is not addressed in the present review. For the review on kombucha made with plants other than tea, the reader can be directed to the review of Emiljanowicz and Malinowska-Pańczyk (2019).

After defining kombucha and the way it is generally produced, due to the lack of published sensory data on kombucha beverage, the components of kombucha's flavor will be discussed by breaking down its chemical composition and discussing the known relationship of these parameters with sensory characteristic. Then, with all these elements in mind, different aspects of the production process will be presented in order to give the reader all the known levers that can be used to shape the product's flavor and quality.

2 General knowledge about kombucha and its process

The traditional way of making kombucha consists in the brewing of black tea liquor to which sucrose is added. Most of the scientific literature reports the use of infusion (initial temperature between 70 and 95 °C) rather than decoction in order to perform tea extraction (Ali and Shivanna, 2017; Dufresne and Farnworth, 2000; Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). Thus, infusion will be used as the initial process in the context of the present review. After the liquid reaches room temperature, the infusion is inoculated with a kombucha culture in the form of a pellicle fragment or as whole (traditionally referred to as "tea fungus") and/or broth (Dufresne and Farnworth, 2000; Greenwalt *et al.*, 2000; Jayabalan *et al.*, 2008). In the course of kombucha production a new cellulosic pellicle forms itself at the surface of the liquid phase (Chen and Liu, 2000). Despite the lack of consensual definition of a biofilm, the pellicle satisfies the definition of a biofilm with aggregated and sessile cells without a solid surface (Alhede *et al.*, 2011). In the case of kombucha, the cellulosic pellicle is an air–liquid interface biofilm. Such structure has already been reported by two studies focusing on the wine matrix (David-Vaizant and Alexandre, 2018; Zara *et al.*, 2005). Kombucha pellicle will therefore be referred to as "biofilm" in the context of the present review.

The different steps of kombucha elaboration are not standardized. According to previous studies, sucrose concentration and tea amount can range from 50 to 100 g/L and from 1.5 to 10 g/L, respectively, with steeping time between 5 and 15 min (Blanc, 1996; Chen and Liu, 2000; Chu and Chen, 2006; De Filippis et al., 2018; Goh et al., 2012b; Jayabalan et al., 2014; Kallel et al., 2012; Lončar et al., 2006; Malbaša et al., 2006, 2008; Neffe-Skocińska et al., 2017; Reiss, 1994; Sievers et al., 1995). Namely, the order and length of each step and the various amounts of tea, sugar, and inoculum can vary and be adapted depending on personal and empirical appreciations.

To trigger the transformation of sugared tea infusion in kombucha, a microbial culture must be added to the sweetened tea medium. Jayabalan *et al.* (2014) mention, while describing the inoculation process, that kombucha culture as tea fungus has to be placed in the sugared tea broth. Nevertheless, whether the process is home-made, produced at industrial scale, or in research labs, several methods are reported. For example, the inoculation step has been reported to be achieved by the addition of the broth (Blanc, 1996; Jayabalan *et al.*, 2007; Loncar *et al.*, 2014; Malbaša *et al.*, 2008), the addition of

only the biofilm or biofilm fragments (Jayabalan *et al.*, 2010; Reiss, 1994; Sievers *et al.*, 1995), or the addition of both (Chen and Liu, 2000; Goh *et al.*, 2012b; Kallel *et al.*, 2012).

There is no single "culture" or microbial consortium for developing kombucha but instead a multitude of matrix dependent consortia whose origins are unknown. It appears that the only constant element that defines a kombucha culture is the simultaneous presence of yeasts and acetic acid bacteria, lactic acid bacteria not being always present.

The acetic acid bacteria community is mostly represented by the genera: *Acetobacter* (sp. *okinawensis* and *tropicalis*), *Gluconobacter* (sp. *oxydans*), *Gluconacetobacter* (sp. *europaeus* and *saccharivorans*), and *Komagataeibacter* (sp. *kombucha* and *xylinus*) (Chakravorty *et al.*, 2016; De Filippis *et al.*, 2018; Marsh *et al.*, 2014; Reva *et al.*, 2015).

The yeast community is more variable and includes genera such as *Zygosaccharomyces* (sp. *lentus*, *bisporus* and *bailii*), *Candida* (sp. *stellimalicola* and *tropicalis*), *Lachancea* (sp. *thermotolerans* and *fermentati*), *Kloeckera*/*Hanseniaspora* (sp. *valbyensis*), *Torulaspora* (sp. *delbrueckii*), *Rhodotorulaspora* (sp. *mucilagenosa*), *Pichia* (sp. *mexicana* and *occidentalis*), *Brettanomyces*/*Dekkera* (sp. *bruxellensis* and *anomala*), *Saccharomyces* (sp. *cerevisiae*), *Schizosaccharomyces* (sp. *pombe*) and *Saccharomycoides* (Chakravorty *et al.*, 2016; Coton *et al.*, 2017; De Filippis *et al.*, 2018; Markov *et al.*, 2005; Marsh *et al.*, 2014; Reva *et al.*, 2015; Teoh *et al.*, 2004).

Other microbial families have been identified and involve lactic acid bacteria with namely the genera *Lactobacillus*, *Bifidobacterium*, and *Oenococcus* (Chakravorty *et al.*, 2016; Coton *et al.*, 2017; Marsh *et al.*, 2014).

The biological transformation driving the elaboration of kombucha is a combination of microbial metabolic interrelationships, which is not limited to a plain fermentation. Consequently, this microbial process will be referred to as "elaboration" in the context of the present review. Kombucha elaboration occurs generally following the process shown in Figure A1. At room temperature, the sucrose added in the tea liquor is hydrolyzed by the yeasts into fructose and glucose, which are subsequently converted into ethanol through alcoholic fermentation. This step is the basis of the symbiosis that occurs in kombucha because acetic acid bacteria are unable to metabolize sucrose. But yeasts provide them carbonated substrates that they can use. Since the system is in contact with air, the obligate aerobe acetic acid bacteria use the available glucose and ethanol to

produce organic acids (Chen and Liu, 2000; Sievers *et al.*, 1995), mainly acetic acid from the oxidation of ethanol and gluconic acid obtained from glucose as part of their oxidative metabolism (De Ley, 1961; Lynch *et al.*, 2019). Although glucose is used by acetic acid bacteria, it is thought that a part can remain available for yeasts as well as fructose as substrates for alcoholic fermentation (Sievers *et al.*, 1995). The acidification of the medium leads to the decrease of pH, which prevents the development of pathogenic microorganisms under the value of 3 (Leistner, 2000). It is worth noting that the initial pH after inoculation is dependent on the total acidity of the inoculum. In parallel, the formation of a gelatinous mass is observed. This biofilm composed of cellulose is produced by some acetic acid bacteria, more specifically *Komagataeibacter xylinus* (Wen Zhang *et al.*, 2018) (formerly named *Acetobacter xylinum* and *Gluconacetobacter xylinus* [Yamada *et al.*, 2012]). In a context of industrial production, the biofilm might be removed before bottling and smaller pieces can be eliminated by filtration. After bottling, further cellulose synthesis should be stopped because of oxygen deprivation after consumption of residual oxygen by microorganisms, namely acetic acid bacteria or yeast with respiratory metabolism. Similarly, the acetic conversion can be stopped at any time according to the desired taste of the beverage by putting the system in anaerobic conditions, which inhibits acetic acid bacteria and favors alcoholic fermentation of yeasts, turning the residual sugars into ethanol and carbon dioxide. A sour and sparkling beverage is obtained. To our knowledge, little investigation has been carried out on this secondary fermentation. Since different elaboration times are used, chemical compositions reported in the literature are very diverse (Tableau A1 [(Villarreal-Soto *et al.*, 2018)]). In order to provide a good acceptability of the product, the brewer will have to make sure that the acidity is not too high and the pH not too low in the case of long-lasting elaboration, else the taste will not remain pleasant. The pH value of 3 appears to be a minimum threshold for kombucha acceptability; a lower pH would be too acidic (Lončar *et al.*, 2006).

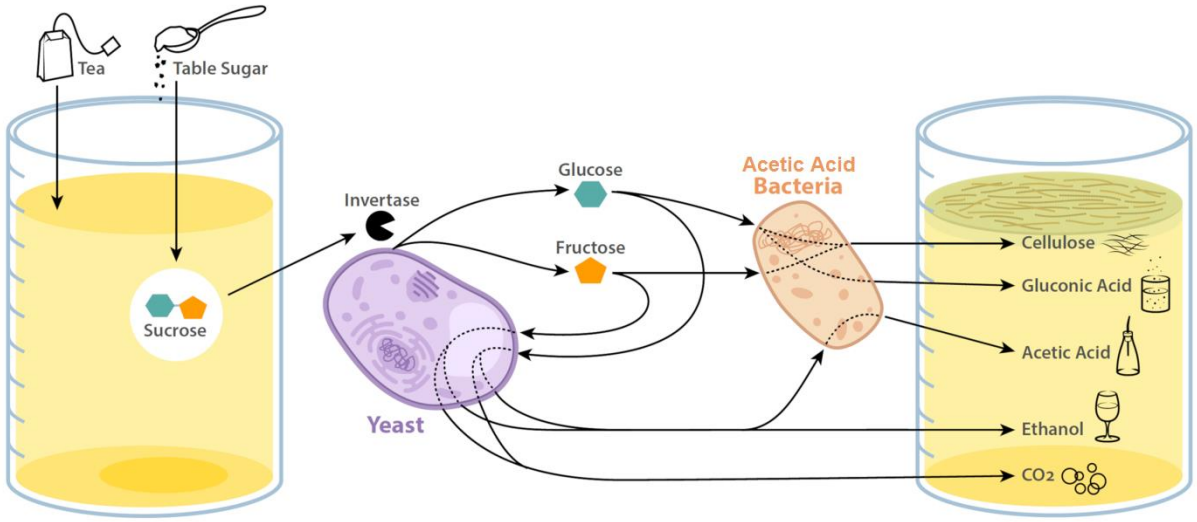

Figure A1 : Kombucha metabolism and microbial interactions. (a) Kombucha is brewed by adding tea and table sugar to a small amount of kombucha starter that contains yeast and acetic acid bacteria. These microbes begin to break down the sugar, leading to a metabolic cascade that ends with a bubbly, acidic, and slightly alcoholic beverage. (b) During the process of elaboration, cooperative and competitive interactions occur among microbes. The production of the public good invertase by yeast, the removal of waste products through metabolization of alcohol, and the generation of the cellulose pellicle by bacteria are potentially cooperative functions. Antimicrobial metabolites, low pH, and the generation of a physical barrier inhibit the growth of competitors (adapted from [May *et al.*, 2019])

Tableau A1 : General chemical composition of Kombucha (Villarreal-Soto *et al.*, 2018)

	Compound	Average composition	Initial sucrose	Fermentation time (days)	References
Organic acids	Acetic acid	5.6 g L^{-1}	70 g L^{-1}	15	(Blanc, 1996)
	Acetic acid	8.36 g L^{-1}	100 g L^{-1}	18	(Jayabalan *et al.*, 2007)
	Acetic acid	11 g L^{-1}	100 g L^{-1}	30	(Chen and Liu, 2000)
	Gluconic acid	39 g L^{-1}	100 g L^{-1}	60	(Chen and Liu, 2000)
	Glucuronic acid	0.0160 g L^{-1}	70 g L^{-1}	21	(Lončar *et al.*, 2006)
	Lactic acid	0.18 g L^{-1}	100 g L^{-1}	18	(Jayabalan *et al.*, 2007)
Vitamins	Vitamin B_1	0.74 mg L^{-1}	70 g L^{-1}	15	(Bauer-Petrovska and Petrushevska-Tozi, 2000) (Malbaša *et al.*, 2011)
	Vitamin B_2	8 mg 100 mL^{-1}	70 g L^{-1}	10	(Bauer-Petrovska and Petrushevska-Tozi, 2000)
	Vitamin B_6	0.52 mg L^{-1}	70 g L^{-1}	15	(Bauer-Petrovska and Petrushevska-Tozi, 2000) (Malbaša *et al.*, 2011)
	Vitamin B_{12}	0.84 mg L^{-1}	70 g L^{-1}	15	
	Vitamin C	25 mg L^{-1}	70 g L^{-1}	10	
General composites	Ethanol	5.5 g L^{-1}	100 g L^{-1}	20	(Chen and Liu, 2000)
	Proteins	3 mg mL^{-1}	100 g L^{-1}	12	(Jayabalan *et al.*, 2007)
	Tea polyphenols	7.8 mM (gallic acid equivalent)	100 g L^{-1}	15	(Chu and Chen, 2006)
Minerals	Cu, Fe, Mn, Ni, Zn	0.1 to 0.4 µg mL^{-1}	70 g L^{-1}	15	(Bauer-Petrovska and Petrushevska-Tozi, 2000)
Anions	F^-, Cl^-, Br^-, I^-, NO_3^-, HPO_4^-, SO_4^-	0.04 to 3.20 mg g^{-1}	100 g L^{-1}	7	(Kumar and Joshi, 2016)

The microbiological studies of kombucha elaboration show an increase in yeast and bacteria population during the two first days following the inoculation of the sugared tea liquor (Coton *et al.*, 2017; De Filippis *et al.*, 2018; Teoh *et al.*, 2004). Next, different variations in populations were reported according to the consortium used (Chen and Liu, 2000), the type of tea (Coton *et al.*, 2017), or the temperature (De Filippis *et al.*, 2018). The impact of these parameters on the process and the final product will be developed in Section 4.

The microbial dynamics occurring in a given kombucha consortium during the elaboration highlighted the domination of one to three genera for the yeasts (Chakravorty *et al.*, 2016; Reva *et al.*, 2015; Teoh *et al.*, 2004) and for bacteria (Chakravorty *et al.*, 2016; Coton *et al.*, 2017; Reva *et al.*, 2015) over the important diversity of the other detected genera (with an abundance inferior to 1% per genus). One predominant genus is often found per kingdom, for example: *Candida stellimalicola*, (Chakravorty *et al.*, 2016) or *Dekkera anomala* (Reva *et al.*, 2015) for the yeasts and *K. xylinus* (Reva *et al.*, 2015) for the bacteria. Some studies have reported important variations of the proportion of the dominant species over time, suggesting the occurrence of interaction between yeast species (Chakravorty *et al.*, 2016; Teoh *et al.*, 2004) and between bacteria species (Coton *et al.*, 2017).

Currently, the control of kombucha production is mostly empiric despite the increasing amount of knowledge and understanding provided by the scientific community. Yet, professional producers of kombucha are confronted with several difficulties for the control of its elaboration. First, there exists a wide variability of elaboration kinetics due to the complex and hard-to-control microbial consortium, as opposed to a single culture fermentation (Villarreal-Soto *et al.*, 2018). Moreover, the production of batches by successive inoculations (also called propagation) could lead to a modification or evolution of the consortium in terms of composition, microbial dynamics, or both. Finally, in the current context, refrigeration is not always sufficient to completely prevent microbial activity after commercialization of unstabilized bottled kombucha (using thermal or filtration processes). Because of the possibility of yeast refermentation, the production of carbon dioxide resulting in bottle explosions is a real risk for kombucha producers. Moreover, the possible increase in alcohol content (Talebi *et al.*, 2017) could have consequences on regulatory levels. In fact, kombucha is classified as nonalcoholic beverage only as long as its alcohol content does not exceed a threshold value. In United States, it is set at 0.5%

(Office of the Federal Register, 1993), whereas in the European Union, this limit is set at 1.2% (Official Journal of the European Union, 2011).

3 Dissection of the chemical composition of kombucha in relationship to its potential sensorial impact

The perception of all food and beverages is conveyed by the consumer's five senses: sight, hearing, touch, smell, and taste. When drinking kombucha, the consumer experiences a mix of visual (aspect of the product), olfactive (aroma profile before and after ingestion), taste (sweetness, sourness), and touch sensations (for example, via chemosensation of the tingling of carbon dioxide bubbles on the tongue) (Redondo *et al.*, 2014).

There are very few data available of descriptive sensory analysis on tea kombucha beverages. The study of Neffe-Skocińska *et al.* (2017) includes a sensory analysis of kombucha beverages elaborated during 10 days, made of a mix of black and green tea (2 and 4 g/L, respectively) and 100 g/L sucrose at 20, 25, and 30 °C. The sensory analysis was performed by a panel of 16 untrained people and no statistical analysis was performed on the results obtained (Figure A2 [Neffe-Skocińska *et al.*, 2017]). Therefore, the interpretation of these results should be handled with care. Nevertheless, the descriptors used are representative of the sensory characteristics of kombucha and help to define its main features. The descriptors with highest scores are: "color intensity" and "clarity" for the visual attributes; "tea" and "citric" for the olfactory attributes; and "tea," "citric," and "acid" for the flavor attributes. The descriptor "sweetness" was not assessed despite residual sugars ranging from 27 to 70 g/L. The descriptors "yeast flavor," "acetic acid flavor," "bitter flavor," "stinging flavor" have low scores. The low "stinging flavor" score could be explained by a lack of secondary fermentation in closed vessels. Recent studies incorporated sensory analysis but did not bring further detailed descriptive elements (Ivanišová *et al.*, 2019; Shahbazi *et al.*, 2018).

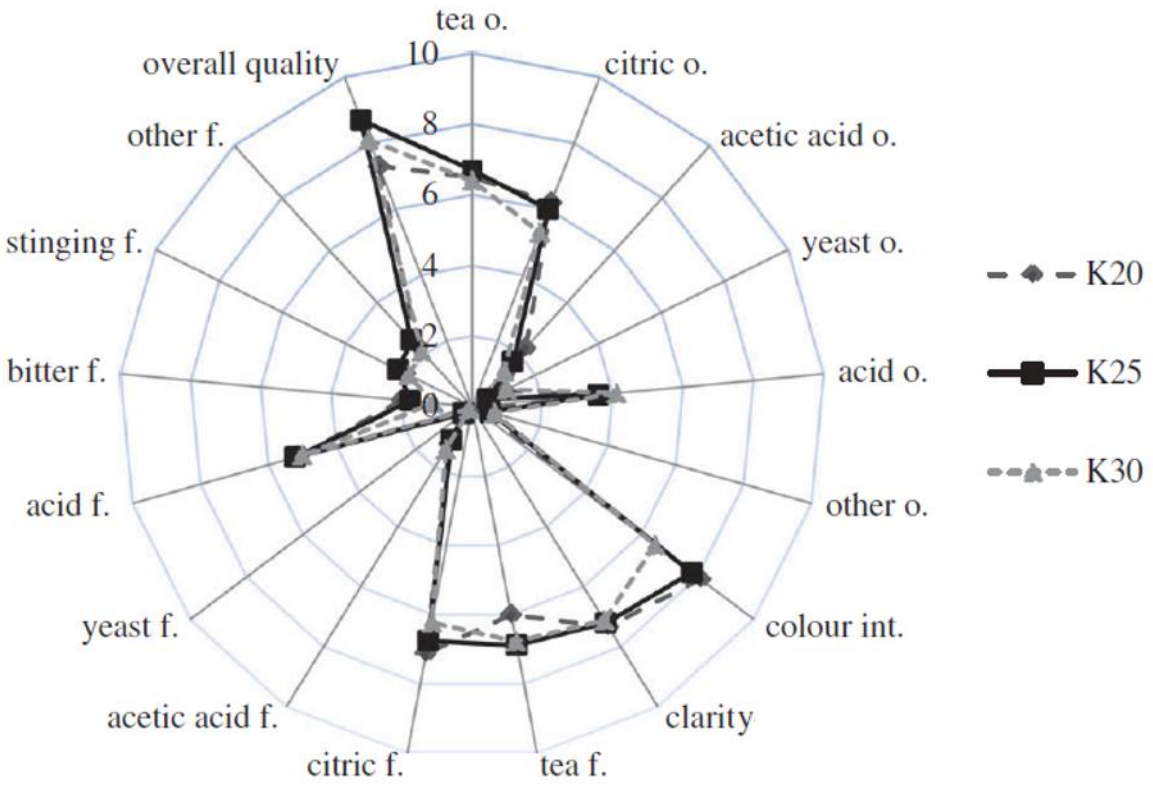

Figure A2 : Sensory profiles of the kombucha beverages after 10 days of elaboration process at 20, 25, and 30 °C (K20, K25, and K30, respectively). “o.” stands for “olfactive,” “int.” stands for “intensity,” and “f.” for “flavor” (Neffe-Skocińska *et al.*, 2017).

The use of a more precise and standardized set of descriptors could allow deeper investigations of the olfactive and gustative dimensions of kombucha. So, a more indirect approach is necessary to explore the sensory characteristics of kombucha by relating the chemical constituents of kombucha to the known sensory properties of those compounds.

3.1 The sight: the aspect of kombucha

Kombucha beverages can be either clear filtered or turbid (as nonfiltered version). The turbidity of the latter is mainly due to the colloidal state of the aqueous beverage, defined as a suspension of particles. These particles are composed of microorganisms and large molecules or aggregates ranging from 1 to 1000 nm. The presence of such bodies induces the scattering of the light. This phenomenon is known as the Tyndall Effect (Petrucci *et al.*, 2011). Although little investigation was carried on the colloids of kombucha, it can be speculated that they can result from the aggregation of proteins (Jayabalan *et al.*, 2007; Petrović *et al.*, 1999), polyphenols, and cellulose fibrils produced by acetic acid bacteria (Goh *et al.*, 2012a; Lin *et al.*, 2013; Wen Zhang *et al.*, 2018). Colloids play a huge part of

soft drinks' intrinsic quality (Kappes and Schmidt, 2007), but to our knowledge there are no data available mentioning the mouthfeel of kombucha.

The color hue of kombucha is mainly due to the presence of the pigment polyphenols extracted from the tea. The characteristic color of black tea results from polyphenols oxidase type enzymes, or the so-called "fermentation" of fresh tea leaves (Harris and Ellis, 1981). This process allows the oxidation and polymerization of native polyphenols composed mainly of catechins (epicatechin, epigallocatechin, and their gallic acid ester derivatives) into different classes of polymers (Balentine *et al.*, 1997; Harbowy *et al.*, 1997). Two of those classes are pigments: one of them is theaflavin, a red-orange pigment dimer that gives black tea its characteristic color (Harbowy *et al.*, 1997). Nonetheless, theaflavin is not the main contributor of black tea's color. The second family named thearubigins, results from a higher degree of polymerization and acts as the main pigment. The molecular structure of those polymeric molecules is still not fully elucidated (Haslam, 2003). Chemical structures of some of those compounds are displayed in Figure A3.

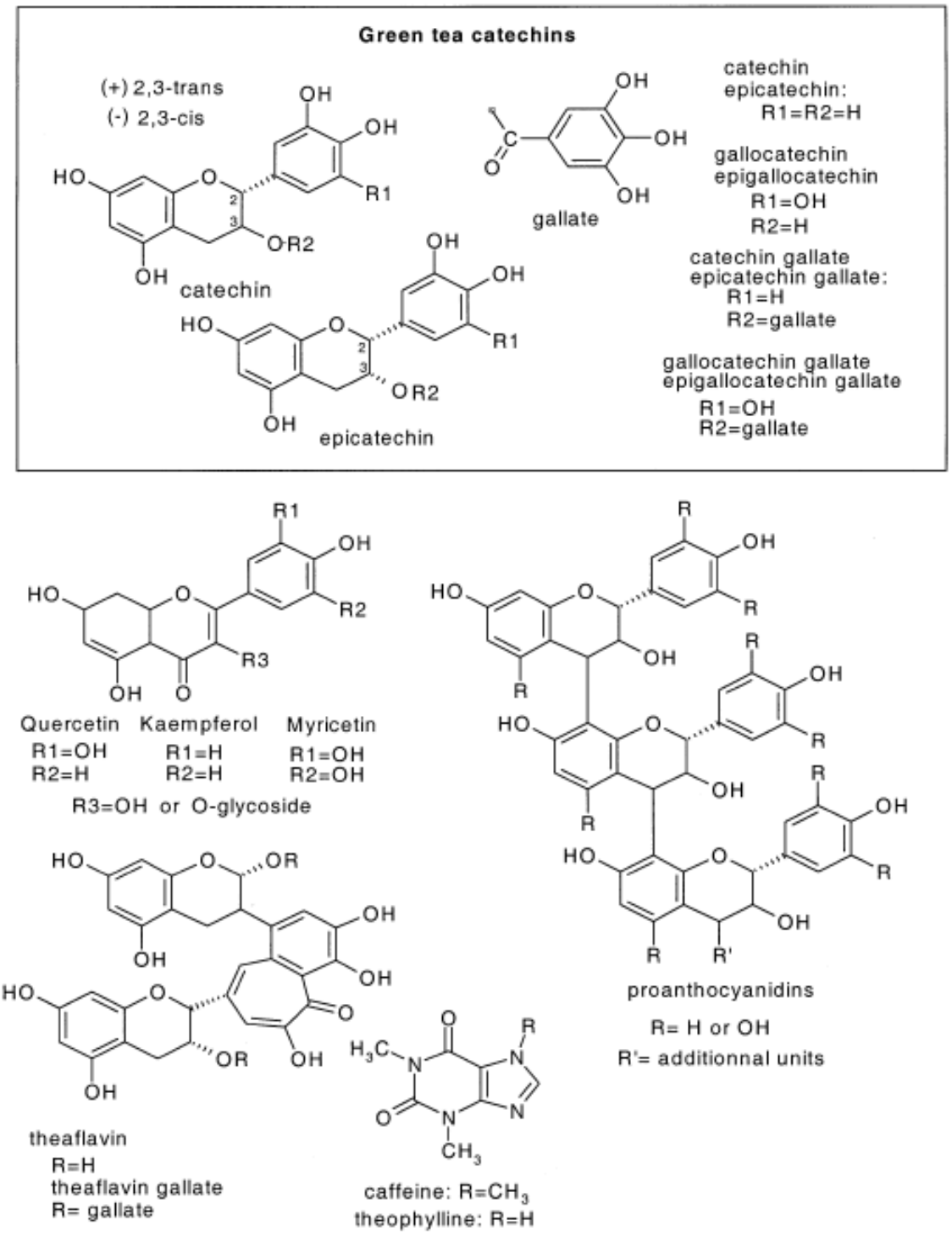

Figure A3 : Chemical structure of some tea constituents (adapted from [Dufresne and Farnworth, 2000])

Although little attention was given to the color and polyphenols of kombucha, two studies reported a significant decrease in color intensity and increase in total phenolic content during kombucha elaboration (Chakravorty et al., 2016; Chu and Chen, 2006). The decrease of pH could be the cause of the change of color (Tse, 1985). It has also been hypothesized that the biological activity of the kombucha consortium may alter or even depolymerize the pigments extracted from the tea (Haslam, 2003). Thus, a large part of kombucha pigments may be derivatives from the tea polyphenols.

Another component of kombucha's visual identity as a carbonated beverage is the aspect and abundance of the bubbles that are dependent on the carbonation process that can be obtained naturally after fermentation in closed vessel or by injecting carbon dioxide artificially (forced carbonation) (Barker *et al.*, 2002; Descoins *et al.*, 2006). Bubbles number is dependent on nucleation sites, which mainly consist in particles (belonging to the product itself or its vessel) (Lubetkin and Blackwell, 1988; Wilt, 1986). The formation kinetics and size of bubbles is dependent on the interfacial tension between the gas and liquid phase (Jones *et al.*, 1999), which means that macromolecules such as polysaccharides and proteins play a role in the visual aspect of a carbonated drink (Barker *et al.*, 2002).

3.2 The smell: a vaporous idea of kombucha's aroma profile

Little information is available about the volatile compounds of kombucha, their origins, and their relationship to olfactory experience (as for most food and beverages) (Acree and van Ruth, 2003), which is why this section relies mainly on speculations. Kombucha's smell has been widely described as "cidery" (Dufresne and Farnworth, 2000; Greenwalt *et al.*, 2000; Jayabalan *et al.*, 2014). As many fermented beverages, the odorant compounds originate from both the raw material (the tea) and volatile metabolites produced by the microorganisms. Although black tea hosts numerous volatile molecules such as 3-hexenol (greenish), linalool (floral), geraniol (sweet, honey-like), 2-phenylethanol (honey-like), damascenone (rose-like), or 2,5-dimethyl-4-hydroxy-3(2H)furanone (DMHF) (caramel-like) (Ho *et al.*, 2015; Robinson and Owuor, 2013; Teranishi *et al.*, 1999), typical tea aroma never seems to be part of the characteristic aroma profile of kombucha. Instead, the elaboration-related aromas dominate: the vinegary odor associated with acetic acid produced by acetic acid bacteria and the cidery odor associated with the activity of yeasts (Rosend *et al.*, 2019; Wei *et al.*, 2019). In cider, higher alcohols are largely produced: amyl

alcohols (banana, pear), butanol (balsamic), propanol (fermented, fruity), ethyl acetate (solvent, fruity like), and ethyl lactate (creamy, fruity). 2-phenylethanol, hexanol (green), octanol (citrus), and butanoic acid (cheesy) were also identified in a significant amount (Mangas *et al.*, 1996; Rosend *et al.*, 2019; Valles *et al.*, 2007; Williams and Rosser, 1981).

It is noteworthy that many yeast genera are common to both kombucha and cider, namely *Candida*, *Hanseniaspora*, *Pichia*, *Dekkera*, and *Saccharomyces* (Morrissey *et al.*, 2004; Valles *et al.*, 2007; Wei *et al.*, 2019). Although it can be suggested that the production of volatile metabolites follows similar pathways, there are currently too few elements to make any further statement on this topic. Thus, many questions remain open: what are the volatile compounds essential to kombucha aroma and what are their origins? If a comparison with wine is conducted, the deglycosylation of some aroma precursors by yeast β-glucosidase during fermentation might have very little impact on kombucha (Fia *et al.*, 2005; Hernandez *et al.*, 2003). The enzymatic oxidation of polyphenols (see Section 3.1.) can lead to aroma release in black tea before kombucha elaboration (Ho *et al.*, 2015; Zhou *et al.*, 2017). On the contrary, the precursors are present in green tea in their glycosylated form, which means that the use of this raw material for kombucha elaboration could unlock aroma potential through the enzymatic activity of yeasts.

3.3 The taste: a complex combination of sapid substances

3.3.1 Sweetness

The traditional sweetener used for kombucha, which also acts as carbonated substrate for the microorganisms, is sucrose (Blanc, 1996; Dufresne and Farnworth, 2000; Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). The use of sucrose constitutes the basis of the symbiosis between yeast and bacteria, the former breaking down sucrose via the activity of invertase into glucose and fructose that are usable by the latter (May *et al.*, 2019). It is possible to bypass this necessary step for sucrose hydrolysis by introducing sugars in the form of purified or mixed ingredients such as glucose syrup, agave syrup, or molasses (Malbaša *et al.*, 2008; Reiss, 1994). These ingredients differ on two major aspects. The first is the difference in relative sweetness: glucose and fructose possess a sweetness intensity of 65 to 75% and 120% (w/w), respectively, compared to sucrose (although those values are modulated by their concentrations [Stone and Oliver, 1969]). The second is other compounds contained in those ingredients, such as those produced by the Maillard reactions in agave syrup or molasses (Willems and Low, 2012). These compounds can be

volatile impacting the aroma profile or nonvolatile such as minerals or pigments that can impact the visual aspect of the beverage.

The sweetness of the final product is thus dependent on the residual amount of sugars, which is conditioned by the initial amount of sweetener added to the tea and the consumption of this substrate by microorganisms during the elaboration. More details about the influence of the substrate and its initial quantity on elaboration are developed in Section 4.3.

3.3.2 Sourness

Organic acids of kombucha are mainly produced by acetic acid bacteria, although the contribution of yeasts and lactic acid bacteria should not be neglected. The major organic acids contributing to kombucha's taste are acetic acid, gluconic acid, and glucuronic acid, whereas the minor ones are lactic acid, malic acid, and succinic acid (Blanc, 1996; Chakravorty *et al.*, 2016; De Filippis *et al.*, 2018; Jayabalan *et al.*, 2007; Malbaša *et al.*, 2008; Neffe-Skocińska *et al.*, 2017). The sensory properties of these metabolites are detailed in Tableau A2.

Tableau A2 : Chemical and sensorial properties of organic acids of kombucha (Da Conceicao Neta *et al.*, 2007; Li and Liu, 2015; Ramachandran *et al.*, 2006).

Acid	Molecular weight (g mol^{-1})	pKa	Number of carboxylic functions	Taste perception threshold (mg L^{-1} water)	Sensory quality
Acetic	60	4.75	1	52.6	Tart and sour
Lactic	90	3.86	1	80.1	Acrid
Gluconic	196	3.86	2	Not determined	Mild, soft, refreshing taste
Malic	134	3.40 and 5.11	2	7.3	Smooth tartness
Succinic	118	4.19 and 5.50	2	22	Tart, slightly bitter in aqueous solutions
Citric	192	3.14, 4.77 and 6.39	3	4.3	Tart; delivers a "burst" of tartness

It should be noted that organic acids also generate, on a lower level, a bitter and astringent taste (Rubico and McDaniel, 1992; Siebert, 1999). Yet, those chemical species are not the main origin of the bitter perception of kombucha.

3.3.3 Bitterness

Bitterness in kombucha, if not masked by the sweetness, can take origin from tea caffeine and polyphenols (Balentine *et al.*, 1997; Harbowy *et al.*, 1997). The average perception taste threshold for caffeine has been reported of 0.2 g/L (Paulus and Reisch, 1980) and Chakravorty *et al.* (2016) reported a caffeine content ranging above this value from 0.6 to 1 g/L in the course of black tea kombucha elaboration.

Polyphenols are bitter and astringent secondary metabolites produced by plants, including tea (*C. sinensis*) (Harbowy *et al.*, 1997; Lesschaeve and Noble, 2005). Before any treatment, tea leaves are composed of 30 to 40% (w/w dry weight) of polyphenols. After infusion in hot water, the proportion in dry matter extracted is about the same (Balentine *et al.*, 1997; Harbowy *et al.*, 1997). The phenolic composition of a green tea infusion can be summed up (in weight percentage of solid extract) as a majority of catechins (30 to 42%): epicatechin, epigallocatechin, and their gallic acid esters (epicatechin gallate and epigallocatechin gallate). Minor compounds (2%) are represented by flavonols (kaempferol, quercetin, and myricetin) as aglycones and glycosides. Phenolic acids such as gallic acid and theogallin are also found (2% in aqueous solutions total). Tea tannins are frequently mentioned, but tannins amounts are in very low concentrations (catechins being wrongly included under the term “tannin”). As a matter of fact, proanthocyanidins (or condensed tannins) are catechins polymers and are present in very low amounts in green tea and are mainly dimers (trimers are even rarer) (Fraser *et al.*, 2012). Chemical structures of these compounds are detailed in Figure A3.

Flavan-3-ol, including catechins and proanthocyanidins, possess bitter and astringent sensory properties (Fontoin *et al.*, 2008; Kielhorn and Thorngate III, 1999; Lesschaeve and Noble, 2005; Peleg *et al.*, 1999) that may contribute to the mouthfeel of kombucha. The fate of polyphenols during kombucha elaboration still remains enigmatic. Jayabalan *et al.* (2007) reported a general decrease of epicatechin, epigallocatechin, and their gallate derivatives during the first 9 days of elaboration and then an increase of the non-gallated species until the 12th day, suggesting the hydrolysis of the ester bound. Based on the work of (Zhu *et al.*, 1997), an acidic hydrolysis is rather unlikely, which is why an enzymatic

origin is speculated. However, the consequences of this phenomenon on the taste and mouthfeel of kombucha remain unknown. A new study (Cardoso *et al.*, 2020) using UPLC-MS (ultra-performance liquid chromatography – mass spectrometry) reported a significant change of phenolic profiles between black tea infusion and kombucha resulting from it. The diversity of compounds increased (27 new compounds), whereas the abundance of compounds decreased. Nevertheless, the global profile did not change drastically with flavonoids remaining the main phenolic compounds followed by phenolic acids. New compounds produced during elaboration mainly belonged to the class of flavonoids. No significant change was observed for green tea and the kombucha made from it. This suggests that kombucha elaboration could affect the phenolic profile, especially for black tea, but would not change it drastically. The initial phenolic profile obtained from infusion is therefore a defining step for the final product regarding this class of compounds.

3.4 The touch: the booze and the fizz

To our knowledge, despite the presence of astringent polyphenols, kombucha is never described as astringent. This is probably due to the presence of sugars that inhibit the perception of astringency, as reported by (Lyman and Green, 1990).

The perception of ethanol and its influence on other perceptions have been intensively studied to elucidate its impact on the quality of alcoholic beverages. The perception of ethanol occurs across gustatory, olfactory, and trigeminal (or irritation) systems (Cometto-Muñiz and Cain, 1990; Greenwalt *et al.*, 2000; Laska *et al.*, 1997). Several studies have reported that the olfaction and nasal irritation thresholds of ethanol ranged around 0.01% (v/v) in water or below, with the irritation (also referred to as “trigeminal”) threshold being always higher (Cometto-Muñiz and Cain, 1990; Martin and Pangborn, 1970; Mattes and DiMeglio, 2001). The taste threshold of ethanol ranges around 1 to 2%. This means that ethanol, even if not identifiable on the olfactory level, can impact the aromatic profile of kombucha. On the other hand, the taste of a regular kombucha, with alcohol content below 1%, should not induce a perception of alcohol taste. At near threshold concentration of ethanol, Mattes and DiMeglio (2001) obtained a predominant description of ethanol taste as bitter. Therefore, the crossing of this threshold could impact the perception of kombucha for some consumers and potentially decrease the global appeal of the product, as it can be that case after commercialization on the shelves of retailer (Talebi *et al.*, 2017). Carbonation affects visual, taste, and trigeminal components. The characteristic oral

perception of carbonated drinks is the irritation or trigeminal sensation of tingling (Dessirier *et al.*, 2000). Although few investigations have been carried out, the results indicate an enhancement of sourness by carbonation (Cometto-Muniz *et al.*, 1987; Yau and McDaniel, 1992). In addition, carbonation has been shown to enhance the perception of cold and vice versa (Green, 1992). However, no interaction seems to occur between sweetness and carbonation (Odake, 2001).

3.5 Perceptual interactions

As seen for ethanol and carbon dioxide, chemosensory stimuli do not work independently on perceptions but are involved in interactions even below their own thresholds (Dalton *et al.*, 2000). It has been shown that the visual aspect of beverages influenced significantly both olfactory and taste perception in noncarbonated aqueous solutions (DuBose and Cardello, 1980; Stillman, 1993). The amplification of fruitiness by sourness (and to a lesser extent sweetness) has been reported as a taste–smell interaction. On the contrary, suppressive effects of sourness on sweetness have also been determined as taste–taste interactions (Bonnans and Noble, 1993; Nahon *et al.*, 1996, 1996).

Finally, temperature is a key parameter that impacts the physical chemistry of food and beverages and in particular the volatility of molecules. Kombucha is usually consumed at cold temperature (around 4 °C), as it is commonly marketed and perceived by the consumer as a soft drink. Consequently, sensory evaluation of kombucha should then be assessed at the appropriate temperature.

3.6 What makes kombucha "refreshing"?

Even though the quality components of kombucha have not been defined, one of the most frequently used descriptors for this beverage is the adjective "refreshing" (Dufresne and Farnworth, 2000; Jayabalan *et al.*, 2014; Reiss, 1994; Villarreal-Soto *et al.*, 2018). The real temperature of the food product plays indeed a major role in the perception of "freshness" (Guinard *et al.*, 1998; Labbe *et al.*, 2009; Zellner and Durlach, 2002) but it is not the only one. Several studies attempted to define the different components of "freshness." Among them, low viscosity or thickness was judged more "refreshing" in liquids than gels (Labbe *et al.*, 2009; McEwan and Colwill, 1996; Zellner and Durlach, 2002). We can cite also some aroma such as mint, citrus, or peach (Labbe *et al.*, 2009), acidity and low sweetness (Labbe *et al.*, 2009; McEwan and Colwill, 1996). These elements echo the

sweetness/sourness balance that has been described as being “basic typical taste profile of all flavored soft drinks.” “Without this sweetener and acid balance, the beverage would taste totally wishy-washy and unexciting” (Shachman, 2005).

Kombucha possesses all the characteristics of a refreshing beverage: it is served cold, it is carbonated, sour with low sweetness and viscosity, and exhibits fruity aroma. The identity of a defined product can be modulated by working on different parameters of kombucha’s production process ranging from:

- the initial formulation of the sweetened tea;
- the elaboration conditions;
- to bottling and stabilization steps if applicable.

These aspects are discussed in details in the next section of this review.

4 Process parameters impacting the composition and quality of kombucha

The production of kombucha has been mainly studied by focusing on the elaboration kinetics and the influence of different conditions ranging from the choice of the substrate, the temperature, the inoculum, and even the vessel geometry (Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). This section proposes to expand this approach by adding elements relative to the production of the tea liquor.

4.1 Parameters impacting the quality of the tea liquor

The initial composition of water plays a role in the final composition of the tea liquor: the medium in which microorganisms of kombucha grow and exert their activities. Indeed, the mineral and organic composition of water is variable from a region to the other and associated with its hardness, dependent on the calcium and magnesium contents. It was demonstrated that the calcium concentration in water is an important parameter of mineral and organic compounds of tea leaves (Mossion *et al.*, 2008; Spiro *et al.*, 1987; Spiro and Price, 1987) along with the time/temperature conditions of the infusion (Price and Spitzer, 1993, 1994; Spiro *et al.*, 1992).

The minerals had different behaviors during the extraction depending on the ions when using distilled water (Matsuura *et al.*, 2001; Ødegård and Lund, 1997):

- Sodium, potassium, and nickel are strongly extracted (>55%);

- Magnesium, aluminum, manganese, and zinc are moderately extracted (between 20 and 55%);

- Calcium, iron, and copper are poorly extracted (<20%).

When mineral water is used, the ions with the lowest concentrations show variation of their extraction yield of 10% maximum according to Mossion (2007) (approximately several mg/L). The nature of the tea induces a strong variability in extraction behaviors.

Calcium possesses a particular behavior, especially if the infusion water possesses a significant amount of it as it is the case for mineral water: an inversed flux was observed from water to the tea leaves (Anderson *et al.*, 1971; Mossion, 2007; Mossion *et al.*, 2008). It is speculated that pectins present in the cell walls of the tea cells bind to Ca^{2+} ions (Capel *et al.*, 2006; Spiro *et al.*, 1987), inducing structure modifications that inhibit the extraction of compounds (Figure A4 [Mossion *et al.*, 2008]).

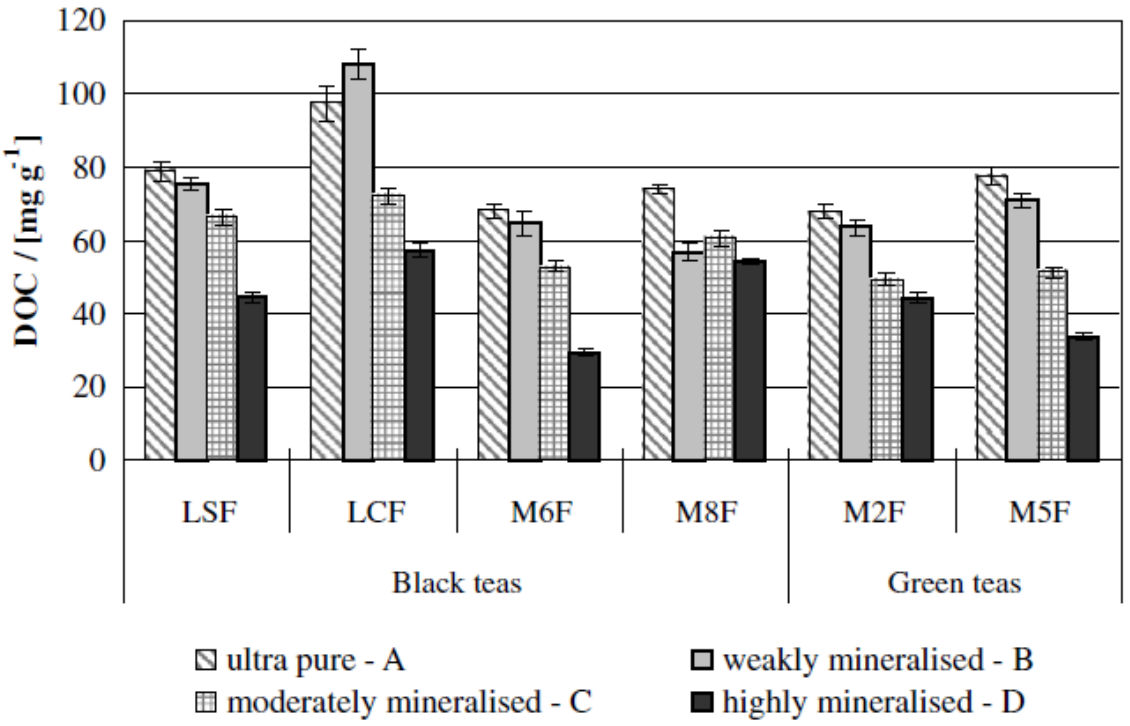

Figure A4 : Effect of water composition on dissolved organic carbon content extracted from tea leaves per gram of brewed leaves (mg C/g) (Mossion *et al.*, 2008)

It has also been shown that the extraction of organic matter was enhanced by:

- the increase in temperature (ranging above 70 °C) and infusion duration (between 1 min and 1 hr);
- a weaker mineral content;
- more specifically, a lower calcium content (corresponding to lower water hardness).

In conclusion, temperature, calcium content of water, and the nature of the tea are the main parameters involved in the making of the tea liquor. This matrix will affect the growth and activities of the kombucha cultures' microorganisms.

4.2 Impact of the nature of tea on microbial dynamics

According to Kallel *et al.* (2012), a consumption of sucrose, glucose, fructose, and the production of organic acids and cellulose are faster and more intense in black tea than in green tea in identical elaboration conditions. This was not observed in other studies, in which no impact on the physical chemistry could be observed (Jayabalan *et al.*, 2007) or gave the opposite effect (Coton *et al.*, 2017). On the contrary, at the microbiological level, it was reported that the nature of tea did not impact the dynamics of yeasts but the use of green tea allowed the development of *Oenococcus oeni* that was absent in the black tea modality (Figure A5 [(Coton *et al.*, 2017)]). In the same study, a higher bacterial biodiversity could be observed in green tea than in black tea, in which domination phenomenon is more present in the liquid phase. In the biofilm, the domination of *Gluconobacter* is effective in all modalities after 2 days of elaboration to the detriment of *O. oeni*.

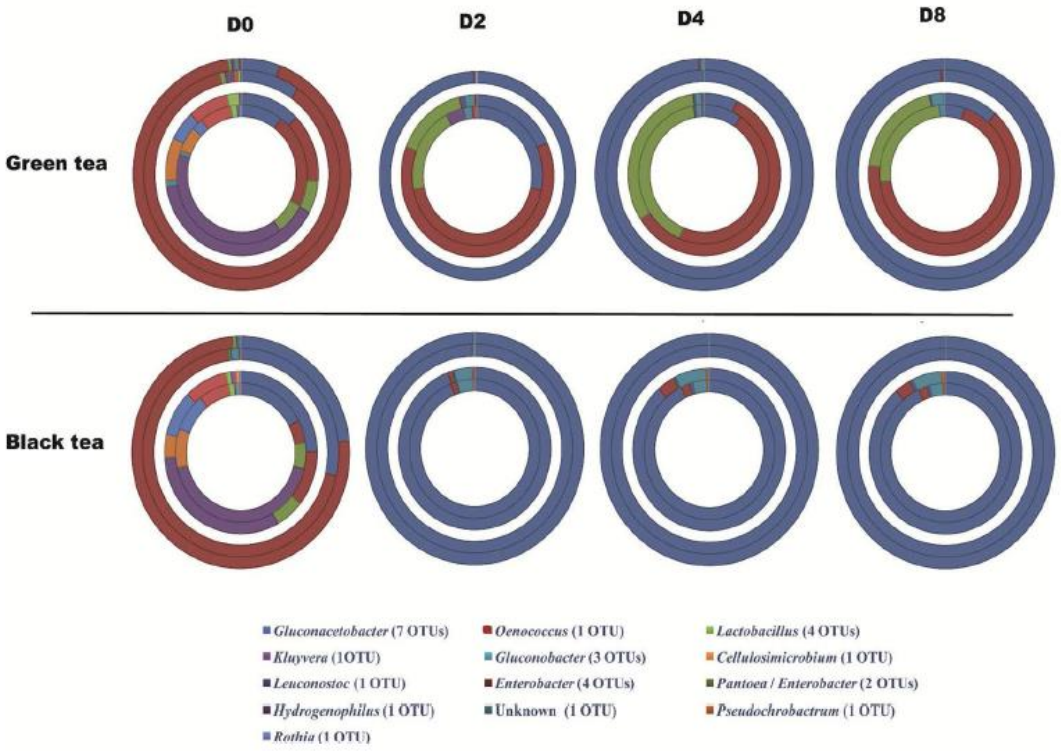

Figure A5 : Relative abundance of bacterial species in green and black tea Kombucha elaboration based on 16S rDNA metabarcoding. The 28 most abundant OTUs (Operational Taxonomic Units) out of total 354 OTUs are presented on this figure (>1% relative abundance). Sampling was performed at days 0, 2, 4, and 8 on both tea (two inner circles) and biofilm (two outer circles) samples. The two circles represent replicate samples for each sample type (Coton *et al.*, 2017).

4.3 Impact of the carbohydrate substrate and of its initial amount

The use of different carbohydrates as substrate has been investigated by (Reiss, 1994). Sucrose is hydrolyzed by yeasts into glucose and fructose. In this study, glucose favors the production of lactic acid and fructose the production of ethanol. Moreover, maltose has been poorly consumed and lactose did not affect the yield of ethanol nor stimulated the production of lactic acid. Acetic acid bacteria transform glucose in gluconic acid and fructose in acetic acid. Beside carbohydrates, it was also reported that lactic acid enhanced the production of biomass.

The utilization of molasses with 50% sucrose content (wet weight) was studied at different rates (35, 50, and 70% of total batch volume) (Malbaša *et al.*, 2008). With 35 and 50 g/L of molasses, kinetics of sucrose consumption, pH variation, and production of organic acids were similar; whereas with 70 g/L of molasses, the consumption of sucrose was

much faster and the production of lactic acid was enhanced to the detriment of acetic acid but with identical total acidity values. Moreover, the decrease in pH was less intense, probably due to the buffering capacity of molasses.

The increase of the initial content in sucrose led to the increase of the production of cellulose until a limit concentration (90 g/L) after which a decrease of cellulose production could be observed. The increase of initial sucrose concentration between 70 and 110 g/L accelerates the decrease of pH following a dose effect. The growth of yeast and bacterial populations was also stimulated by the increase of initial sucrose concentration (Goh *et al.*, 2012b). A similar trend was observed in the work of Blanc (1996) but to a lesser extent.

4.4 The inoculum

As stated in Section 2, several methods are reported: using a previous batch of kombucha, only the broth can be added (Blanc, 1996; Jayabalan *et al.*, 2007; Loncar *et al.*, 2014; Malbaša *et al.*, 2008), only the biofilm or fragments (Jayabalan *et al.*, 2010; Reiss, 1994; Sievers *et al.*, 1995), or both (Chen and Liu, 2000; Goh *et al.*, 2012b; Kallel *et al.*, 2012).

4.4.1 Nature of the inoculum

Several studies have compared elaboration kinetics with different kombucha inocula. (Chu and Chen, 2006) observed different kinetics of antioxidant capacities and total phenolic content increases over eight differently sourced kombucha cultures used as inocula.

The elaboration kinetics of a kombucha inoculum and two controlled inocula prepared from kombucha isolates (a mix of acetic acid bacteria and one yeast: *Zygosaccharomyces* sp. and *Saccharomyces* cerevisiae, respectively) were compared (Malbaša *et al.*, 2011). Similar pH variations in black tea and green tea matrices were observed but the production of C and B vitamins showed significant differences with the original consortium producing always the highest amount of vitamin B. The highest amount of vitamin C was produced by the original consortium and the controlled consortium including *S. cerevisiae*. Another study using cocultures from isolated microorganisms (*Gluconobacter intermedius* coinoculated with *Dekkera bruxellensis*) was carried out in order to optimize the production of health beneficial glucuronic acid by playing with the relative proportion of each strain with significant results (Nguyen *et al.*, 2014). Currently, no link has been established between the presence of particular genera or species and general chemical profiles of kombucha.

4.4.2 Amount of inoculum used

The addition of 15% inoculum (unknown chemical and microbiological composition "from previous batch") compared to 10% demonstrated a consumption of sucrose, glucose, fructose and acidification faster at a given temperature (22 °C or 30 °C) on the first 10 days of elaboration (Figure A6 [Lončar *et al.*, 2006a]). This suggests that increasing the quantity of inoculum accelerates the elaboration kinetics (Lončar *et al.*, 2006).

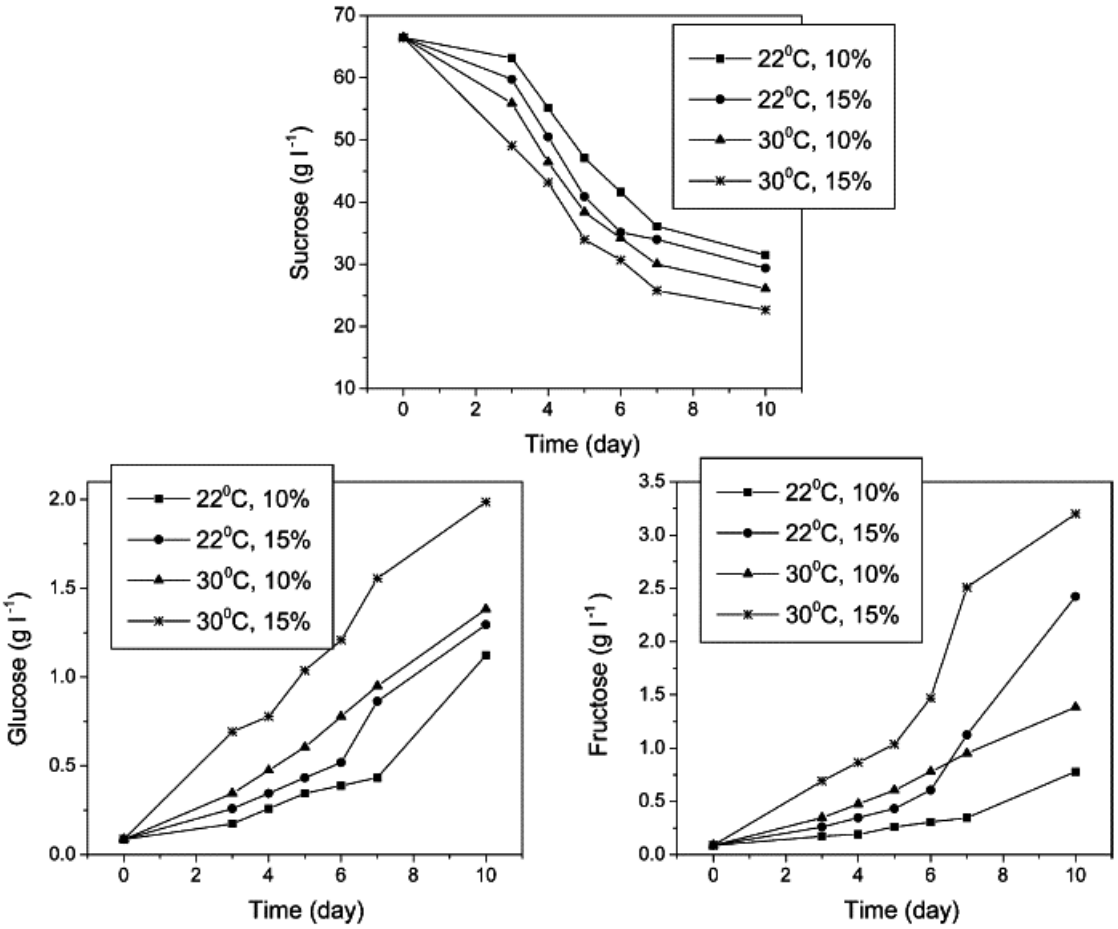

Figure A6 : Sugar quantities as functions of time, temperature, and inoculum concentration (Lončar *et al.*, 2006)

4.5 The temperature

In the study of Lončar *et al.* (2006), temperature was determined to be a parameter more impactful on elaboration kinetics compared to the amount of added inoculum. When 22 °C and 30 °C elaboration temperatures were compared, the consumption of sugars and the acidification appeared to be faster at 30 °C for a given quantity of added inoculum (10% and 15%) on the first 10 days. Conclusion is that higher temperature also accelerates the elaboration kinetics. This is contradictory with the results of Neffe-Skocińska *et al.* (2017)

who evaluated an optimal temperature of 25 °C because it could favor the yeasts' activity whose metabolites (monosaccharides and ethanol) are the only available substrates for acetic acid bacteria at the beginning of the process (Tableau A3). Thus, the growth and metabolic activity of yeasts in sucrose-based kombucha is the limiting step of the process. Yet, no significant difference in sensory profile could be determined between the kombucha produced at 20, 25, and 30°C.

Tableau A3 : Changes in total count of acetic acid bacteria, yeast, lactic acid bacteria, and pH values in Kombucha beverages during 10 days of elaboration at 20, 25, and 30 °C (adapted from Neffe-Skocińska *et al.* [2017])

Microbial species	Temperature of elaboration (°C)	Days of elaboration			
(log CFU mL^{-1}) / pH		0	3	7	10
AAB	20	3.57 ± 0.2^{aA}	4.60 ± 0.3^{aB}	6.72 ± 0.1^{aC}	7.39 ± 0.1^{bD}
	25	3.93 ± 0.1^{aA}	4.90 ± 0.2^{aB}	6.90 ± 0.2^{aC}	7.61 ± 0.2^{bD}
	30	3.65 ± 0.2^{aA}	5.15 ± 0.1^{aB}	7.10 ± 0.2^{aC}	6.77 ± 0.2^{aC}
Yeast	20	4.02 ± 0.1^{aA}	5.86 ± 0.2^{bB}	7.00 ± 0.1^{bC}	7.83 ± 0.2^{cD}
	25	4.24 ± 0.1^{aA}	7.00 ± 0.2^{cB}	7.22 ± 0.1^{bB}	7.43 ± 0.1^{bB}
	30	4.01 ± 0.2^{aA}	4.73 ± 0.1^{aB}	5.48 ± 0.1^{aC}	36.40 ± 0.2^{aD}
pH	20	3.08 ± 0.1^{aA}	2.85 ± 0.1^{aA}	2.88 ± 0.1^{aA}	2.67 ± 0.1^{aA}
	25	3.07 ± 0.1^{aB}	2.80 ± 0.1^{aA}	2.79 ± 0.1^{aA}	2.77 ± 0.2^{aA}
	30	3.04 ± 0.1^{aB}	2.81 ± 0.1^{aA}	2.71 ± 0.1^{aA}	2.63 ± 0.2^{aA}

Data are expressed as mean ± standard deviation of n = 3 samples.

Means in the same column followed by different lowercase letters represent significant differences ($p < 0.05$).

Means in the same row followed by different uppercase letters represent significant differences ($p < 0.05$).

Another study (De Filippis *et al.*, 2018) reported that a temperature of 30°C compared to 20°C increased the population of acetic acid bacteria. Each modality operated a selection of bacteria species: *Gluconobacter saccharivorans* was in majority at 30°C to the detriment of *Gluconobacter xylinus*, which was dominant at 20°C. At 30°C, the enhancement of *Gluconobacter saccharivorans* population also enhanced the production of gluconic and glucuronic acids.

4.6 The geometry of the vessel

According to Malbaša *et al.* (2006), the geometry of the elaboration tank or vessel was a parameter more impactful than the amount of added inoculum. In the same study, the parameter of geometric similarity for a cylindrical vessel was highlighted and defined as:

$$\frac{D_{T2}}{D_{T1}} = (\frac{V_{T2}}{V_{T1}})^{1/3}$$

D_{Tx} is defined as the diameter of the cylinder x and V_{Tx} as the volume of the cylinder x. The elaboration kinetics seems to be characterized by a relationship between the vessel's diameter and volume.

Another parameter of the same type was investigated: the specific interfacial surface (SIS) defined as:

$$Specific\ Interfacial\ Surface\ (cm^{-1}) = \frac{Surface\ of\ liquid\ (cm^2)}{Volume\ of\ liquid\ (cm^3)}$$

The accuracy of this model was tested with different geometries on 56 vessels from 90 L reactors to 330 mL flasks and could be validated (Cvetković *et al.*, 2008). This model was judged to be more efficient than the geometric similarity because the study showed a higher fidelity of elaboration kinetics and durations to the SIS mathematic model (Figure A7) (Cvetković *et al.*, 2008; Malbaša *et al.*, 2006). The study shows that regardless of the shape of the vessel (flask, cylinder, or reactor) and the size, a larger SIS induced faster acidification kinetics. The increase of SIS can be achieved by using a larger air/liquid interface and/or by reducing the volume of liquid. Consequence results in better conditions for oxygen access for acetic acid bacteria located in the biofilm or the liquid phase. A smart use of the SIS parameter can help controlling the speed of the acidification phase of kombucha elaboration.

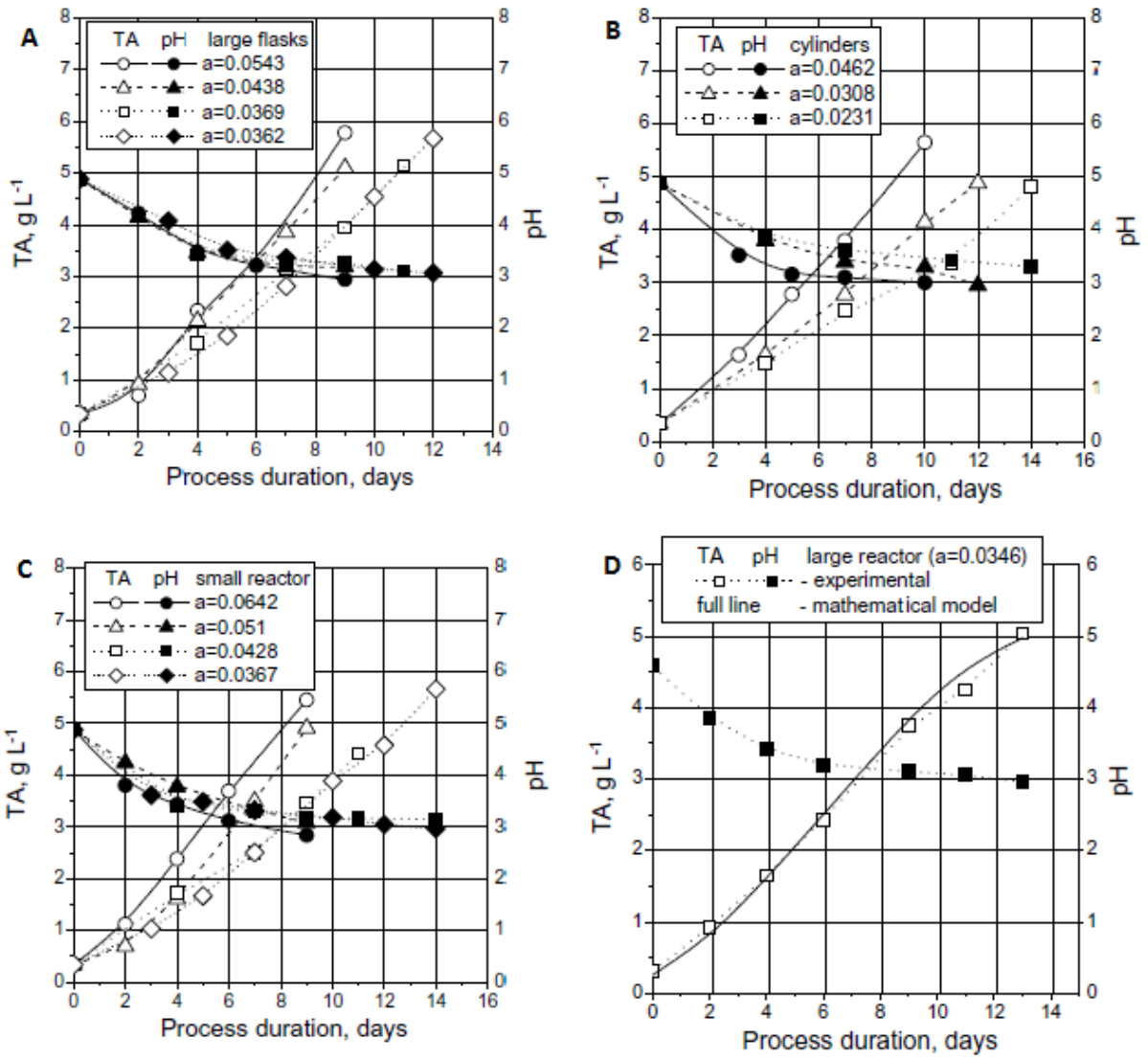

Figure A7 : Changes in pH value and total acidity of kombucha produced in flasks (a), cylinders (b), small reactors (c), and large reactors (d) (adapted from Cvetković *et al.*, 2008a]).

As a conclusion, ways to increase the speed of the acidification phase of kombucha elaboration is to increase the amount of inoculum (ranging between 10% and 15%), increase the temperature up to around 30 °C, and to maximize the SIS. The water composition, tea type, and the choice of substrates interdependently influence composition of the initial matrix. Thus, these parameters need to be assessed on a case-by-case basis depending on the targeted result. Eventually, the influence of sugared tea liquor and microbial composition and the interaction of these both elements on the final product remain shrouded in mystery and there is a need for research to be carried out on these problematics.

5 Conclusion and perspectives

Kombucha can be approached the same way as a carbonated soft drink: emphasis is put on its visual aspect, the aroma profile, and the taste, in particular the sweetness/sourness balance. Yet, due to the infinity of combination of microbial compositions, the multiple processes used at home and in the industry and the lack of specific quality standards in regulation, the characterization of kombucha's quality dimension remains a challenge (Watson, 2019). If the authenticity of kombucha had to be defined by the expectations of the consumer (Monaco, 2019), it would need to take the following elements into account:

- The rawness of the product, namely the preservation of potential beneficial properties;
- The stability of the product over time;
- The accessibility of the product by offering a pleasant sensory profile from appearance to taste.

Therefore, decisions need to be taken about the microbial and chemical stabilization of the product and the production process needs to be adapted accordingly. Consequently, interdependent parameters during the production process will impact the consumer perception of the final product. The knowledge of such complex systems is still lacking as producers and scientists face many grey areas.

The process of kombucha production was mainly studied by the determination of the microbial composition and the understanding of the main technological roles attributed to yeasts and bacteria through the consumption of carbohydrates and the production of organic acids. The impact of environmental factors, such as temperature, the substrate

content, and the vessel geometry, was investigated. A characterization of kombucha consortia composition involving a large number of samples at worldwide scale would help define kombucha better and highlight signature genera, species, and cooccurrences between microorganisms. The existence of an effect of the geographical sourcing on the microbial composition could even lead to the concept of kombucha typicity. As a matter of fact, few studies have investigated the microbial dynamic and tried to explain the reasons why certain genera or species were dominant in given conditions during elaboration (Coton *et al.*, 2017; De Filippis *et al.*, 2018; Teoh *et al.*, 2004). Moreover, the intraspecific diversity was not investigated to our knowledge. Indeed, the questions of the origin of kombucha consortia and their stability or evolution over time, namely in relationship to the matrix composition (tea, substrate) and the process conditions, still remain unanswered. Nevertheless, the available knowledge can already allow the orientations of the microbial activity and the kinetics of elaboration. Further control or prediction on kombucha elaboration remains difficult when it comes to the organoleptic profile.

Surprisingly, little data are available about the sensory of kombucha per se and namely the compounds involved in its aroma profile. To our knowledge, no research was carried out to draw lines between the odorant volatile molecules and the microbial composition. As a matter of fact, beside the main metabolites such as organic acids, ethanol and the substances of interest such as vitamins, gluconic, glucuronic, or D-saccharic-1,4-lactone acids, little is known of the metabolic activity of the consortium. Without a doubt, microbial interactions are occurring during the elaboration (Teoh *et al.*, 2004), first through the symbiosis between yeasts and acetic acid bacteria and possibly through nutrient competition or/and targeted chemical signals such as peptides or messenger RNAs (Ivey *et al.*, 2013; Leroi and Pidoux, 1993; Sieuwerts *et al.*, 2008; Wang *et al.*, 2016). The utilization of “omics” techniques such as metabolomics or transcriptomics, which are nontargeted analyses of metabolites and gene expression, respectively, could open doors in the study of microbial interactions in products as it is the case for wine (Liu *et al.*, 2016). Kombucha is seen as a promising model system for the study of microbial interaction in symbiotic systems (May *et al.*, 2019). The elucidation of such interactions could give way to better control of elaboration processes in terms of timing and repeatability, and thus spark significant interest in the dynamic kombucha community of producers and consumers.

Les interactions microbiennes au sein d'un consortium de kombucha nécessitent d'être distinguées des interactions entre le consortium et les micro-organismes contaminants et/ou pathogènes. Ce second sujet a en effet été plus intensément traité dans la littérature, avec l'objectif de démontrer des effets antimicrobiens de cette boisson permettant d'éviter la contamination du produit (Bhattacharya *et al.,* 2013 ; Cardoso *et al.,* 2020 ; Hou *et al.,* 2021 ; Kaewkod *et al.,* 2019 ; Sreeramulu *et al.,* 2000). Trois principales familles de molécules ont été étudiées pour leur capacité antimicrobienne dans la kombucha : les acides organiques d'origine microbiennes, les polyphénols issus du thé et les bactériocines produites par les bactéries lactiques. L'effet de ces substances a été testé sur des bactéries et levures pathogènes, principalement *Escherichia coli* et *Staphylococcus aureus* et le genre *Candida* (Hou *et al.*, 2021). Les résultats rassemblés sont contradictoires et par conséquent, les activités antimicrobiennes semblent avoir des causes multifactorielles. En effet, la diminution de la valeur de pH n'est pas toujours suffisante pour expliquer l'inhibition des pathogènes. L'acide acétique possède en effet une activité antimicrobienne propre et distinct de celle du pH. Sous sa forme protonée non chargée il peut diffuser à travers la membrane plasmique des cellules. Une fois dans le cytosol, il relargue alors un proton, forçant la cellule à dépenser de l'énergie pour évacuer les protons afin de prévenir la chute de la valeur de pH intra-cellulaire (Nakano and Ebisuya, 2016). Cet effet antimicrobien a été étudié chez *Escherichia coli* et *Staphylococcus aureus* (Entani *et al.,* 1998). Une activité antimicrobienne a également été démontrée pour les polyphénols (Cardoso *et al.*, 2020). Enfin, la présence de bactériocines produites par les bactéries lactiques nécessite la présence de ces dernières dans les consortia de kombucha, ce qui n'est pas toujours le cas puis que leur présence est matrice dépendante (Tran *et al.,* 2020a). Ces études montrent bien que les consortia de kombucha résultent d'une adaptation des micro-organismes qui les composent et cela suppose le développement de résistance contre ces effets inhibiteurs. Un état de l'art sur les micro-organismes caractéristiques des consortia de kombucha et leurs activités a donc été réalisé car c'est bien sur l'interaction entre ces micro-organismes que les travaux de thèse se concentrent.

B.3 Etat de l'art des micro-organismes de la kombucha et leur métabolisme

B.3.1 Levures Saccharomyces et non-Saccharomyces

La levure est typiquement définie comme un champignon (règne *Fungi*) microscopique unicellulaire. D'un point de vue taxonomique, cette définition ne correspond pas à un groupe uniforme. Toutefois, les organismes dont il est question dans le cadre de la thèse appartiennent tous au groupe *Saccharomycetes* du phylum *Ascomycota* (Bisson *et al.,* 2017). Dans le cadre de la production d'aliments, le mot levure désigne couramment l'espèce *Saccharomyces cerevisiae*

qui est l'agent principal de la fermentation alcoolique intervenant dans la fabrication du pain, de la bière, du cidre et du vin. L'inoculation de *S. cerevisiae* sous forme de LSA (« Levure Sèche Active ») ou de culture liquide commercialisée est courante et permet des fermentations en culture dite pure (Kurtzman *et al.*, 2011). Des souches de cette espèce levurienne ont fait l'objet d'une domestication par l'être humain et ont donc évolué spécifiquement pour subvenir à des besoins technologiques (Fay and Benavides, 2005 ; Kuthan *et al.*, 2003). Cependant l'intérêt technologique croissant pour les levures non domestiquées qui n'appartiennent pas au genre *Saccharomyces* (levures « non-*Saccharomyces* ») se développe. En effet, la grande majorité des travaux de recherche sur les levures fermentaires se sont concentrées sur *S. cerevisiae* et très peu concernent d'autres espèces et d'autres genres. De plus, les souches levuriennes de la kombucha sont *a priori* des souches « indigènes » qui n'ont pas été isolées en laboratoire auparavant. Ainsi les souches de *S. cerevisiae* utilisées dans la littérature ne constituent pas forcément une référence absolue pour l'étude des activités levuriennes dans cette matrice (Kuthan *et al.*, 2003). En effet, *B. bruxellensis* est la levure la plus représentée dans les consortia de kombucha (Harrison and Curtin, 2021). L'étude bibliographique veillera donc à aborder les spécificités des genres et espèces levuriennes principales de la kombucha tout en prenant dans un premier temps appui sur les connaissances accumulées chez la levure modèle *S. cerevisiae* concernant le détail des voies métaboliques.

Dans le contexte des boissons fermentées, la levure a pour fonction principale la production d'éthanol par fermentation alcoolique, définie précisément par la transformation de sucres, principalement glucose et fructose, en éthanol et dioxyde de carbone selon l'équation suivante :

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

Cette voie représente un métabolisme alternatif à la respiration pour la production d'énergie sous forme d'ATP en l'absence d'oxygène, avec un rendement énergétique plus faible : 2 molécules d'ATP par mole de glucose consommée contre environ 36 pour la respiration (Zamora, 2009). Les deux voies métaboliques sont décrites dans la Figure 4. Toutes deux reposent dans un premier temps sur la glycolyse qui convertit un hexose en deux pyruvates. C'est à partir de cette molécule que les deux voies divergent, avec la décarboxylation du pyruvate en éthanol suivi d'une réduction aboutissant à l'éthanol (et régénérant les co-facteurs réduits lors de la glycolyse) dans le cadre de la fermentation alcoolique. Ainsi, selon cette voie les chaînes respiratoires dépendantes de l'oxygène ne sont pas sollicitées pour réoxyder les coenzymes. Toutefois, l'absence d'oxygène n'est généralement pas l'inducteur de la fermentation alcoolique chez *S. cerevisiae* qui est une levure dite Crabtree-positive. L'effet Crabtree désigne l'inhibition de la voie respiratoire lorsque la teneur en glucose dans

l'environnement est supérieure à un certain seuil malgré la présence d'oxygène (Crabtree, 1928 ; De Deken, 1966), ce qui est le cas dans les moûts de raisin. Toutes les espèces de levures ne partagent pas ce trait, induisant donc une régulation différente des métabolismes (Wardrop *et al.*, 2004).

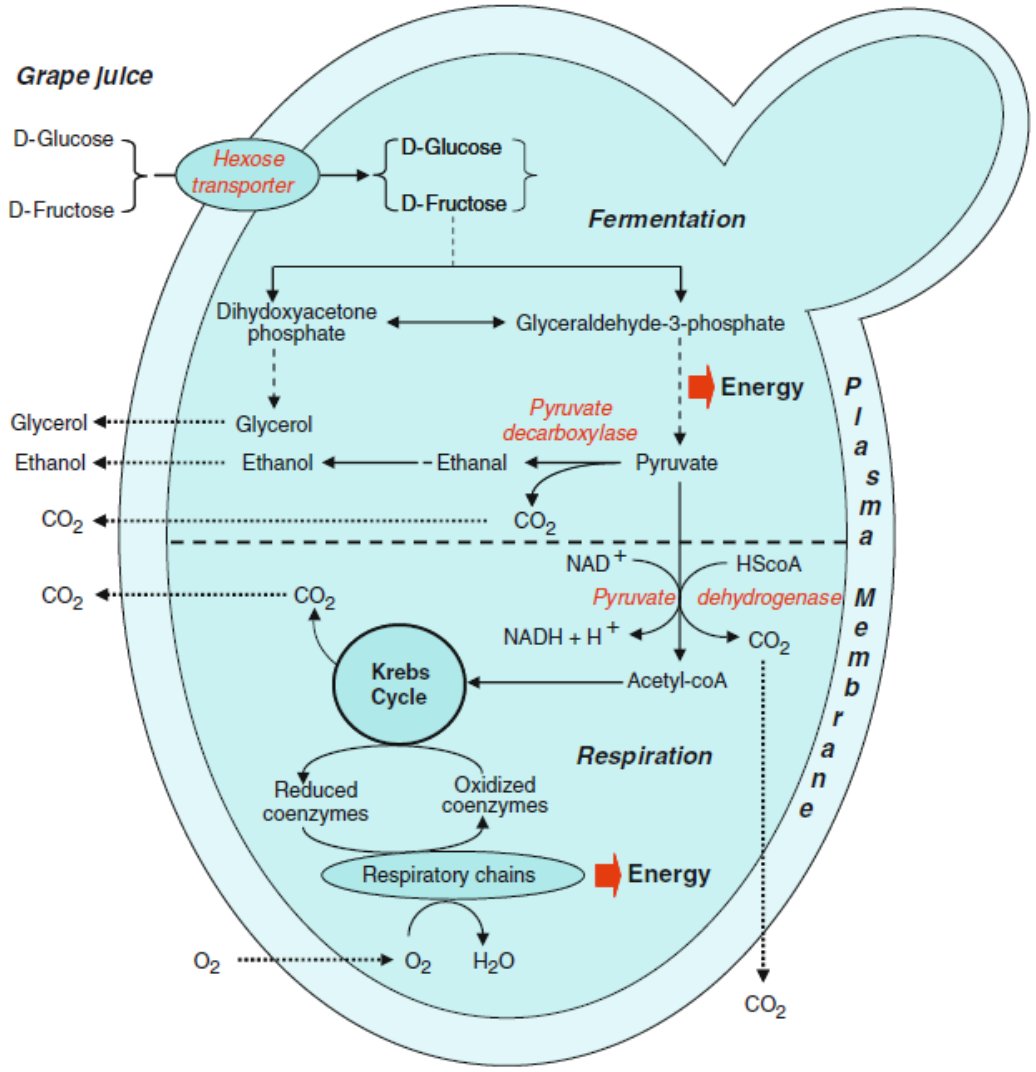

Figure 4 : Schéma métabolique simplifié de la fermentation alcoolique et de la respiration chez S. cerevisiae (Zamora, 2009).

L'activité levurienne dans les boissons fermentées a également pour conséquence la production de composés organoleptiques fixes ou volatils. Les composés fixes concernent notamment des acides organiques issus du métabolisme du carbone. Par exemple, l'acide succinique s'accumule et est rejeté dans le milieu par altération du fonctionnement du cycle de Krebs en conditions fermentaires. En effet, l'inhibition de la succinate déshydrogénase en condition anaérobie entraîne la formation d'une branche oxydante débouchant sur le 2-oxoglutarate et d'une branche réductrice débouchant sur le fumarate (Camarasa *et al.*, 2003). La production de composés volatils de type esters ou alcools supérieurs est liée surtout au métabolisme de l'azote dans le contexte œnologique. L'azote assimilable (l'ion ammonium NH_4^+ et les acides aminés à l'exception de la proline) entre dans la cellule grâce à différents transporteurs (Marini *et al.*, 1997 ; Zamora, 2009). La proline ne peut être transportée qu'en condition aérobie (Salmon *et al.*, 1998 ; Zamora, 2009). Les acides aminés peuvent être utilisés pour la production de peptides, protéines, acides nucléiques ou vitamines nécessaires à leur fonctionnement. La production de composés volatils serait un moyen de réguler le potentiel oxydo-réducteur intracellulaire (Belda *et al.*, 2017). Les acides aminés sont convertis en alcools supérieurs *via* la voie de Ehrlich, composée de trois réactions enzymatiques successives : une transamination donnant un céto-acide, une décarboxylation donnant un aldéhyde et une réduction donnant un alcool (Ehrlich, 1907 ; Hazelwood *et al.*, 2008) (Figure 5).

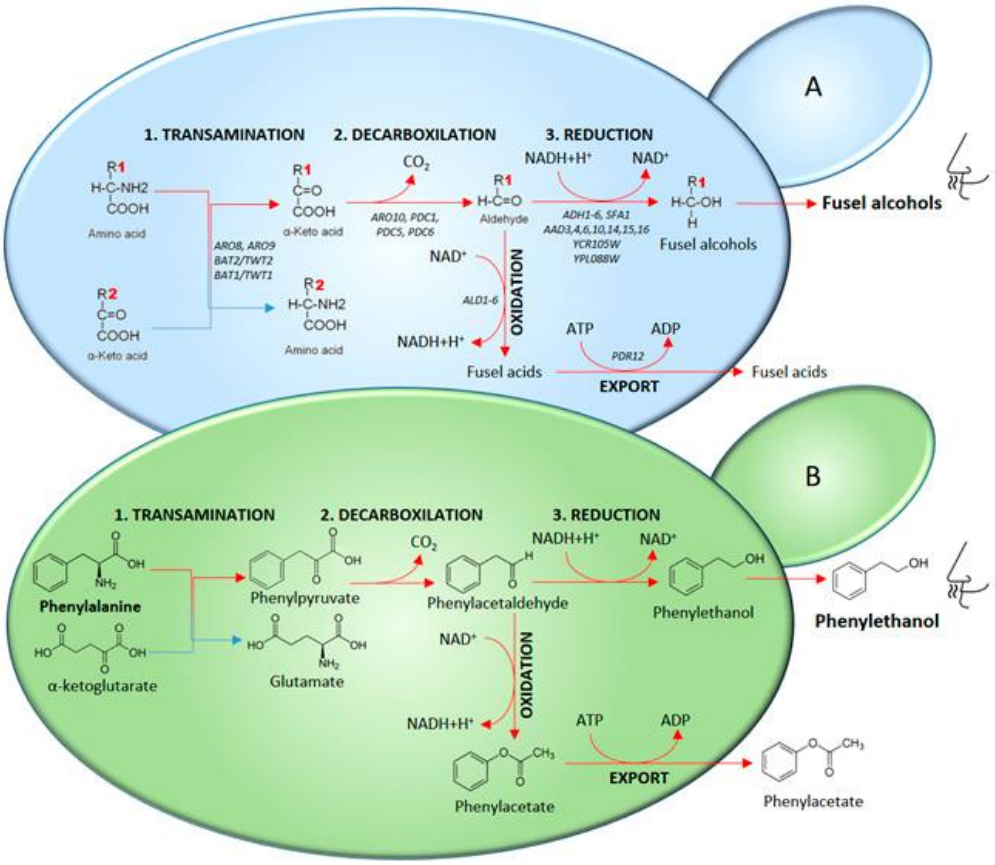

Figure 5 : Schéma métabolique de la voie de Ehrlich chez *S. cerevisiae* (Belda *et al.*, 2017).

L'ajout d'une réaction d'estérification faisant intervenir l'acétyl-Coenzyme A (acétyl-CoA) aboutit à la formation d'esters d'acétate. Une autre voie de formation d'esters indépendante du métabolisme de l'azote existe par estérification de l'éthanol avec un acide gras-CoA (Belda *et al.*, 2017) (Figure 6).

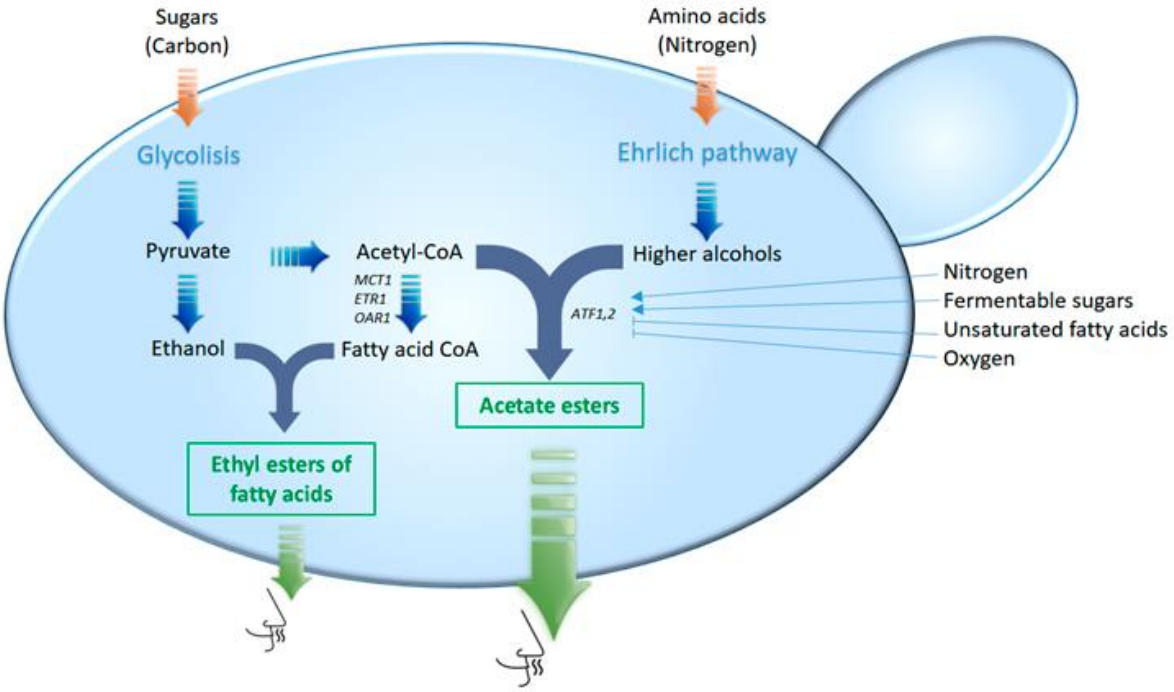

Figure 6 : Schéma métabolique de la formation d'esters chez *S. cerevisiae* (Belda *et al.*, 2017).

Il est important de mentionner la possibilité d'engendrer des composés volatils organoleptiques par activités β-glucosidase ou cystéine-β-lyase hydrolysant la liaison reliant un groupement glucoside ou cystéine à une molécule d'arôme, alors présent sous forme de précurseur dans la matrice (par exemple le moût de raisin) et permettant la volatilisation de la molécule aromatique. Ces activités sont variables en fonction des genres, espèces et souches de levures (Belda *et al.*, 2017 ; Fia *et al.*, 2005 ; Holt *et al.*, 2012).

La capacité d'hydrolyser les protéines afin d'accéder aux acides aminés liés non-disponibles est également recherchée. Bien que les protéinases A et B intracellulaires présentes dans la vacuole aient été caractérisées en détail (Parr *et al.*, 2007), un flou persiste sur le mécanisme à l'origine des activités extracellulaires observées chez *S. cerevisiae* de manière souche-dépendante (Ogrydziak, 1993 ; Sturley and Young, 1988). Celles-ci pourraient être liées à l'autolyse des levures ou bien à un mécanisme de sécrétion (Alexandre *et al.*, 2001 ; Mette'Wolff *et al.*, 1996). L'existence d'une activité protéase extracellulaire peut jouer un rôle dans les interactions microbiennes commensales ou mutualistes par la mise à disposition de substrat azoté au reste

du consortium (Sieuwerts *et al.*, 2008). Au-delà de l'aspect nutritionnel, une telle activité peut potentiellement diminuer la turbidité dans les boissons en cas de formation de colloïdes protéiques (Bilinski *et al.*, 1987).

Après avoir énoncé les fonctions principales des levures dans la fabrication de boissons fermentées, les genres et espèces principaux de levures vont être présentés en fonction de critères propres à la fabrication de kombucha. Les caractéristiques importantes à considérer sont :

- Leurs besoins en nutriments carbonés et azotés (sucres, acides aminés, vitamine, *etc*.) ;
- Le comportement vis-à-vis de l'oxygène (métabolisme respiratoire et métabolisme oxydatif) ;
- L'impact des conditions de culture : teneurs en oxygène, température, pH, tolérance aux pressions osmotiques (osmotolérance) et aux teneurs en acides organiques élevées (acidotolérance) ;
- Leur capacité à hydrolyser le saccharose (activité invertase) pouvant impacter le métabolisme des micro-organismes invertase-négatifs ou à faible activité invertase comme les bactéries acétiques.
- La production d'éthanol qui sera ensuite oxydé en acide acétique par les bactéries acétiques ;
- Le transport et la consommation des sucres (glucose, fructose) ;
- L'activité β-glucosidase pouvant impacter le profil aromatique au niveau des arômes variétaux ;
- La production de composés aromatiques fermentaires ;
- La production d'acides organiques voire d'autres substances ayant un impact sur les propriétés organoleptiques ;
- Les mécanismes d'interactions avec les autres micro-organismes (facteurs biotiques et abiotiques).

Et plus secondairement :

- L'activité protéase (en conditions aérobies, la biosynthèse des acides aminés est possible) ;
- La production de biofilm (potentiellement associé à la mère cellulosique).

B.3.1.1 Le genre *Saccharomyces*

Les levures du genre *Saccharomyces* sont fortement associées aux produits fermentés et à un rendement fermentaire élevé. Ces levures sont bourgeonnantes, de forme globulaire à ellipsoïde et peuvent former des pseudo-hyphes (Rainieri *et al.*, 2003 ; Vaughan-Martini and Martini, 2011). L'espèce la plus souvent isolée dans la kombucha est *S. cerevisiae* (Jayabalan *et al.*, 2010 ; Villarreal-Soto *et al.*, 2018).

S. cerevisiae est une levure Crabtree positive acidotolérante (pH = 3,0 - 3,5) et osmotolérante (croissance en présence de 140 à 260 g/L de sucre) (Albergaria and Arneborg, 2016) avec des besoins en oxygène faibles voire nuls (Holm Hansen *et al.*, 2001 ; Visser *et al.*, 1990) et de faibles besoins en azote (Albergaria and Arneborg, 2016 ; Bely *et al.*, 1990). Cette levure est capable de métaboliser le saccharose mais ne possède pas de transporteur dédié. Sept copies de gènes *SUC* ont été identifiés dans le génome de *S. cerevisiae*, *SUC1* à *SUC5*, *SUC7* et *SUC8*, codant pour des invertases fonctionnelles (Carlson and Botstein, 1982 ; Marques *et al.*, 2016 ; Naumov and Naumova, 2010). Le gène *SUC2* code deux formes de l'enzyme : une invertase intracellulaire non glycosylée (Beteta and Gascon, 1971 ; Oliver, 1968) et une forme sécrétée glycosylée (Andjelković *et al.*, 2015 ; Carlson and Botstein, 1982 ; Margetić and Vujčić, 2017 ; Neumann and Lampen, 1969). L'invertase intracellulaire est exprimée constitutivement, tandis que l'expression de la forme glycosylée est régulée par la teneur en glucose (répression catabolique) (Özcan *et al.*, 1997). Lorsque la concentration en glucose est très faible, comme cela peut être le cas dans la kombucha, la répression catabolique par le glucose n'est pas effective, l'expression des gènes *SUC* n'est donc pas réprimée. L'invertase produite hydrolyse le saccharose libérant du glucose. Si le glucose s'accumule et que sa concentration vient à trop augmenter, la mise en place de la repression catabolique permet de moduler l'expression des gènes *SUC* diminuant la synhèse d'invertase. Cette régulation assure une hydrolyse progressive du saccharose au fur et à mesure de la consommation du glucose. L'entrée des hexoses dans le cytoplasme se fait par diffusion facilitée *via* des transporteurs notés HTX (Johnston, 1999 ; Kruckeberg, 1996). La levure *S. cerevisiae* possède des rendements fermentaires élevés et est résistante à des teneurs en éthanol allant jusqu'à 18 % (v/v) (Fleet, 2003). Outre l'éthanol, son métabolisme conduit également à la production d'acides organiques, de composés aromatiques dont des alcools supérieurs et des esters (Styger *et al.*, 2013, 2011 ; Walker and Stewart, 2016). Certaines souches disposent d'une β-glucosidase leur permettant d'accéder à une réserve de glucose supplémentaire liée aux précurseurs d'arômes. En conséquence, la libération des molécules aromatiques peut jouer un rôle dans le profil olfactif du produit (Hernandez *et al.*, 2003).

L'interaction de *S. cerevisiae* avec d'autres levures « non-*Saccharomyces* » dans la fermentation du vin a été très étudiée. Outre la modification des conditions environnementales telle que l'élévation de la température, la consommation de l'oxygène, l'élévation de la teneur en éthanol au-delà de 5% et de l'épuisement des nutriments carbonés et azotés, d'autres mécanismes ont pu être mis en évidence (Albergaria and Arneborg, 2016) :

- Inhibition des « non-*Saccharomyces* » par contact cellule-cellule (Renault *et al.*, 2013) ;
- Production de substances toxiques vis-à-vis d'autres levures (Albergaria *et al.*, 2010 ; Branco *et al.*, 2015 ; Pereznevado *et al.*, 2006 ; Wang *et al.*, 2016) et/ou bactéries (Nehme *et al.*, 2010) ;
- Capacité à floculer (Soares, 2011) ;

Dans le contexte de la production de kombucha, et sous réserve de la présence de *S. cerevisiae*, il est attendu que le métabolisme soit respiro-fermentaire du fait de l'accès à l'oxygène et de l'absence de glucose initialement (par absence d'effet Crabtree). De plus, l'accumulation d'éthanol n'a pas lieu grâce à l'activité oxydative des bactéries acétiques. *A priori*, la compétition pour les nutriments azotés ainsi que les mécanismes de contact cellule-cellule, la production de toxines et la floculation sont des interactions envisageables dans la kombucha vis-à-vis des bactéries et des autres levures « non-*Saccharomyces* ».

B.3.1.2 Les levures « non-*Saccharomyces* »

Bien que très diverses, il est couramment admis que les levures « non-*Saccharomyces* » possèdent des besoins en oxygène plus importants que *S. cerevisiae* et présentent des rendements fermentaires plus faibles. Selon Jolly *et al.* (2014) on peut distinguer trois profils : les levures largement aérobies (notamment le genre *Pichia*), les levures apiculées avec une faible activité fermentaire (notamment le genre *Hanseniaspora*) et les levures avec un métabolisme fermentaire (notamment *Zygosaccharomyces bailii*). Les valeurs de températures de l'ordre de 15°C leur sont aussi favorables vis-à-vis de *S. cerevisiae* dans le contexte œnologique (Ciani *et al.*, 2006). En cas d'inoculation séquentielle, une levure « non-*Saccharomyces* » inoculée avant *S. cerevisiae* peut inhiber le développement de cette dernière à cause de l'épuisement des nutriments azotés. Dès lors, l'activité protéasique de *S. cerevisiae* est un facteur clé pour surmonter la carence en acides aminés (Charoenchai *et al.*, 1997). Les genres de levures non-*Saccharomyces* détaillées par la suite ont été sélectionné vis-à-vis de leur occurrence dans la kombucha (Tableau 1).

B.3.1.2.1 Les levures du genre *Brettanomyces/Dekkera*

Les levures du genre *Brettanomyces/Dekkera* sont à l'origine fortement associées à la production de bière avec le premier dépôt de brevet de l'histoire (UK patent GB190328184) portant sur un micro-organisme (Steensels *et al.*, 2015). Cependant, la présence de ces levures s'est avérée être plus étendue au sein des aliments fermentés (boissons non alcoolisées, produits laitiers, levains). Le nom *Brettanomyces* est associée à la forme anamorphique ou reproduction asexuée, tandis que *Dekkera* est associée à la forme téléomorphique ou reproduction sexuée. La présence de *Brettanomyces* dans ces matrices alimentaires fermentées permettent de caractériser ce genre levurien comme étant extrêmement résistant aux stress tels que les teneurs élevées en éthanol, les basses valeurs de pH, les faibles ressources en oxygène et en substrats carbonés et azotés disponibles (Steensels *et al.*, 2015). Au laboratoire, le genre *Brettanomyces* pose des difficultés lorsqu'on cherche à mettre ces levures en culture puisqu'elles croissent lentement sur milieu nutritif et entrent en état viable non cultivable (VBNC) (du Toit *et al.*, 2005 ; Longin *et al.*, 2016 ; Serpaggi *et al.*, 2012 ; Smith, 2011a). Le genre *Brettanomyces* a surtout été étudié car il est à l'origine d'altérations organoleptiques dans le vin rouge dues à sa production de phénols volatils à partir des acides hydroxycinnamiques présents dans la pellicule des baies de raisin donnant lieu à des odeurs de cuir, écurie, sueur, connues sous le nom de « caractère Brett », (Romano *et al.*, 2008 ; Suárez *et al.*, 2007). Il est intéressant de noter que contrairement aux baies de raisin, les précurseurs de ces phénols volatils ne sont pas présents de manière significative dans les infusions de thé, limitant le risque de développement de tels arômes dans la kombucha (Horžić *et al.*, 2009 ; Pagliosa *et al.*, 2010).

B. bruxellensis est l'espèce levurienne la plus représentée dans les consortia de kombucha (76,9% d'occurrence parmi 103 échantillons analysés) (Harrison and Curtin, 2021). Elle a été également isolée dans le vin (Agnolucci *et al.*, 2009 ; Conterno *et al.*, 2006 ; Curtin *et al.*, 2007), le cidre (Morrissey *et al.*, 2004 ; Valles *et al.*, 2007), la bière (De Roos and De Vuyst, 2019 ; Schifferdecker *et al.*, 2014) et le kéfir (Laureys and De Vuyst, 2014). Ses cellules sont en forme d'ogives, mais elles montrent un important polymorphisme avec des formes en lien avec son groupe génétique (Lebleux *et al.*, 2021). *B. bruxellensis* est une levure tolérante à l'éthanol (12-15% (v/v)), anaérobie facultative, Crabtree positive comme *S. cerevisiae* avec qui elle partage une stratégie de « faire-accumuler-consommer » (*make-accumulate-consume*) reposant sur une rapide consommation de sucre et accumulation d'éthanol (Cibrario *et al.*, 2020 ; Oswald and Edwards, 2017 ; Rozpędowska *et al.*, 2011). Elle est caractérisée également par une inhibition de la fermentation alcoolique en anaérobiose, c'est l'effet « Custers » appelé aussi effet Pasteur négatif (Custer, 1940). Cette levure a en effet une forte tendance à produire de l'acétate à partir du glucose réduisant de manière concomitante le NAD^+. En condition aérobie, les coenzymes

sont réoxydés par la chaîne respiratoire. Tandis qu'en anaérobiose, en absence d'accepteur de protons, la moindre production d'acétate entraîne un déséquilibre redox (diminution du ratio NAD^+/NADH) et une stagnation du flux glycolytique au niveau de la glycérate-3- phosphate déshydrogénase. (Aguilar Uscanga *et al.*, 2003 ; Carrascosa *et al.*, 1981). Tolérante à l'éthanol et à l'acétate, *B. bruxellensis* accumule ainsi dans son environnement ces deux métabolites, qu'elle peut ensuite utiliser comme substrat lorsque le glucose est épuisé. Ceci la distingue de *S. cerevisiae* et joue un rôle dans son interaction avec d'autres levures (Rozpędowska *et al.*, 2011). Elle est également tolérante aux variations d'acidité et de température (Conterno *et al.*, 2006 ; Rozpędowska *et al.*, 2011). *B. bruxellensis* est connue pour ses besoins nutritionnels très faibles et sa capacité à utiliser une large gamme de sources de carbone (glucose, galactose, mannose, saccharose, maltose, xylose, cellulose et amidon) et d'azote (Crauwels *et al.*, 2015 ; Galafassi *et al.*, 2011 ; Godoy *et al.*, 2017). Chez certaines souches, en plus de posséder une invertase intracellulaire (Blondin and Ratomahenina, 1983), on a pu également identifier des invertases extracellulaires (Fia *et al.*, 2005). Il est intéressant de mentionner que l'étude du génome de *B. bruxellensis* a permis de soulever l'hypothèse de l'acquisition d'un gène codant pour une invertase d'origine bactérienne par transfert de gène horizontal. La séquence du gène montre une similarité avec celle de bactérie acétique appartenant au genre *Asaia* (Roach and Borneman, 2020). *B. bruxellensis* et *B. anomalus* sont les deux seules espèces du genre à posséder ce gène d'origine bactérienne et de ce fait à pouvoir métaboliser le saccharose. La Figure 7 résume les principales voies métaboliques identifiées chez *B. bruxellensis*.

Enfin, *B. bruxellensis* a été isolée sur diverses surfaces en cuverie et en brasserie (Suiker *et al.*, 2021) et sa capacité à produire des biofilms et à former des pseudo-hyphes a pu être mise en évidence (Dimopoulou *et al.*, 2019 ; Joseph *et al.*, 2007 ; Lebleux *et al.*, 2020 ; Tristezza *et al.*, 2010). Il est suggéré que la grande adaptabilité de *B. bruxellensis* soit liée à divers états de polyploïdie. Les souches caractérisées sont diploïdes ou triploïdes et aucune souche haploïde n'a été identifiée à l'état sauvage, en lien avec l'absence de sporulation (Avramova *et al.*, 2018). Elle présente également une grande variabilité du nombre de chromosomes entre les souches (de 4 à 9) (Hellborg and Piškur, 2009).

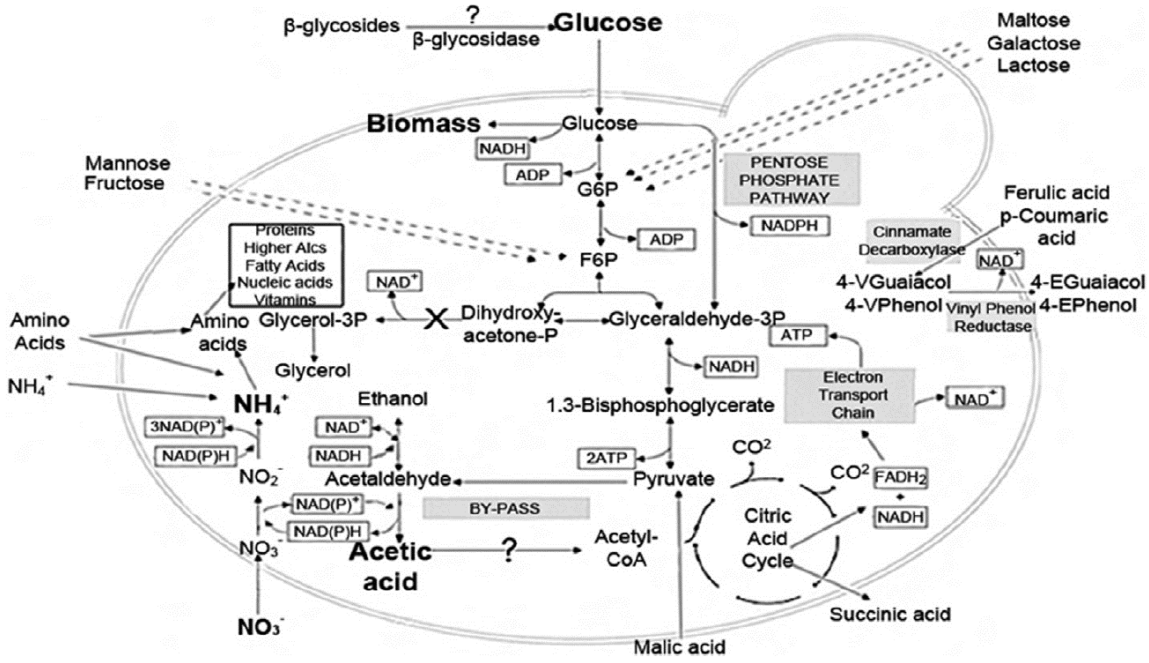

Figure 7 : Représentation du métabolisme des sucres et de l'azote chez *Brettanomyces bruxellensis* en conditions aérobie et anaérobie, adaptée de Smith and Divol (2016). Le « ? » et le « X » expriment un effet souche-dépendant rendant la réaction réalisable ou non.

B.3.1.2.2 Les levures du genre *Hanseniaspora/Kloeckera*

Les levures du genre *Hanseniaspora/Kloeckera* possèdent une forme apiculée caractéristique avec une bourgeonnement polaire (Kurtzman *et al.*, 2011). Le nom *Kloeckera* est associée à la forme anamorphe, tandis que *Hanseniaspora* est associée à la forme téléomorphe. Les levures appartenant à ce genre consomment le glucose et la majorité des espèces n'assimile pas le saccharose (Cadez and Smith, 2011). Dans le contexte œnologique, les levures *Hanseniaspora* sont associées aux débuts de fermentations de par leur faible rendement fermentaire et leur faible tolérance à l'éthanol, entrainant leur mortalité au cours de la vinification. De plus, le caractère Crabtree est variable en fonction des espèces (Díaz-Montaño and de Jesús Ramírez Córdova, 2009). Comparées à *S. cerevisiae*, les levures du genre *Hanseniaspora* sont intéressantes technologiquement puisqu'elles produisent davantage de monoterpènes, acétoïne et esters d'acétate (surtout acétate d'éthyle) dans le vin (Díaz-Montaño and de Jesús Ramírez Córdova, 2009 ; Martin et al., 2018). La production de ces composés organoleptiques est décrite dans la Figure 8.

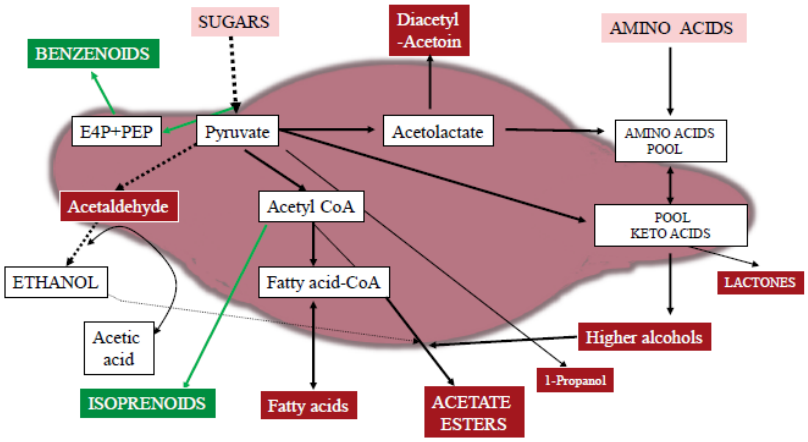

Figure 8 : Composés organoleptiques fermentaires produits par les levures *Hanseniaspora/Kloeckera* au cours de la vinification (boîtes vertes et rouges). Les métabolites spécifiques de certaines espèces de ce genre sont présentés en vert (boîtes et flèches). Les boîtes rouges représentent les nutriments du milieu de culture et les flèches en pointillées symbolisent la voix métabolique de la glycolyse et de la fermentation alcoolique (Martin *et al.*, 2018).

Les levures du genre *Hanseniaspora* sont réparties sur deux lignées évolutives caractérisées toutes deux par des pertes de gènes comparativement à *S. cerevisiae* et qui sont impliqués dans la régulation du cycle cellulaire. La première est une lignée à évolution plus lente ou « *Slower-Evolving Lineage* » (SEL) dont l'analyse des enzymes glycolytiques et de la fermentation alcoolique révèle une plus grande proximité avec celles de *S. cerevisiae* et associées à des rendements fermentaires plus élevés (Valera *et al.*, 2020). Les espèces appartenant à cette lignée, *H. vinae* et *H. osmophila*, sont fortement associées à la fermentation du moût de raisin (Martin *et al.*, 2018). La seconde lignée est à évolution plus rapide ou « *Faster-Evolving Lineage* » (FEL) et est caractérisée par une perte de gènes encore plus importante qui sont liés principalement à la réparation de l'ADN et aux polymérases. En plus d'avoir subi davantage d'altérations sur les gènes codant les enzymes glycolytiques et liées à la fermentation alcoolique, les espèces appartenant à cette branche ont perdu le gène codant l'invertase, les rendant incapables d'hydrolyser et exploiter le saccharose (Steenwyk *et al.*, 2019). Par ailleurs, l'analyse des caryotypes a révélé des contextes de ploïdie différents : allodiploïdie, allotriploïdie et allotétraploidie, soulignant le dynamisme important du génome des levures *Hanseniaspora* (Saubin *et al.*, 2020). De plus, une étude rapporte que les levures appartenant à la branche SEL possèdent 5 chromosomes, alors que celles appartenant à la branche FEL possèdent entre 8 et 9 chromosomes (Esteve-Zarzoso *et al.*, 2001).

H. valbyensis est une espèce récurrente de la kombucha et plus largement des aliments fermentés dont le saccharose est le substrat principal (Coton *et al.*, 2017 ; Leech *et al.*, 2020). Paradoxalement, cette espèce appartient à la branche FEL incapable d'utiliser le saccharose. De plus, elle fait partie d'une branche à part au sein de la branche FEL mais les implications de ce placement n'ont pas encore été précisément déterminées (Esteve-Zarzoso *et al.*, 2001 ; Saubin *et al.*, 2020). Cette espèce a également été isolée dans des sols, sur des arbres et dans des boissons fermentées, notamment le cidre (Cadez, 2003 ; Valles *et al.*, 2007). Une activité β-glucosidase a été mise en évidence chez cette espèce (Fia *et al.*, 2005 ; Pando Bedriñana *et al.*, 2012). Dans la fermentation du cidre, *H. valbyensis* produit davantage d'acétate d'éthyle et d'acétate de phenyléthyl comparé à *S. cerevisiae* (à l'origine respectivement d'arômes fruités et de rose) (Xu *et al.*, 2006).

B.3.1.2.3 Les levures du genre *Pichia*

Les levures du genre *Pichia* sont caractérisées par un bourgeonnement multilatéral. Les cellules sont sphériques, ovoïdes ou légèrement allongées et la formation de pseudo-hyphes a été observée. De manière générale, peu de sucres sont utilisables mis à part le glucose pour les levures appartenant à ce genre (Kurtzman, 2011).

P. occidentalis (ex-*Issatchenkia occidentalis*) a été isolée dans la kombucha (Mayser *et al.*, 1995 ; Reva *et al.*, 2015), mais également sur fruits (Trindade *et al.*, 2002), en cuverie (vin) (Sabate *et al.*, 2002), dans le fromage (Biagiotti *et al.*, 2018) et le kéfir (Diosma *et al.*, 2014). *P. occidentalis* désigne la forme téléomorphe, alors que la forme anamorphe est connue sous le nom de *Candida sorbosa* (Kurtzman, 2011). Peu de données sont disponibles concernant cette espèce. Elle croît en conditions aérobie et ses colonies sont caractérisées par la présence importante de pseudo-hyphes. En milieu liquide, cette espèce peut, en plus du glucose, utiliser l'éthanol, le glycérol, le lactate, le succinate et le citrate. Des activités de dégradation des phytates et de l'aflatoxine B_1 ont été identifiées chez cette espèce (Ben Taheur *et al.*, 2020 ; Nuobariene *et al.*, 2012). *P. occidentalis* est également capable de former des biofilms (Arroyo López *et al.*, 2007 ; Giobbe *et al.*, 2007 ; Lebleux *et al.*, 2020).

B.3.1.2.3 Les levures du genre *Candida*

Ce genre de levures est très diversifié et compte de nombreuses espèces mais il existe des caractères communs : ce sont des levures aérobies avec une température de croissance optimum située entre 25 et 30°C et une valeur optimale de pH située entre 4,0 et 6,0 (Kieliszek *et al.*, 2017 ; Kurtzman *et al.*, 2011). Elles sont aussi capables de produire des exopolysaccharides (Kieliszek *et al.*, 2017).

L'espèce *C. californica* a été isolée dans la kombucha et également sur raisin (Agustini *et al.*, 2018 ; Chakravorty *et al.*, 2016 ; Puig-Pujol *et al.*, 2016). Elle possède des caractéristiques intéressantes pour la fermentation en co-inoculation avec *S. cerevisiae* de par sa consommation efficace des sucres associée à un rendement fermentaire inférieur à *S. cerevisiae* (Aplin *et al.*, 2019). Elle peut consommer le glucose, l'éthanol, le glycérol, le lactate, le succinate et le citrate. La production d'éthanol, d'acétoïne, d'acide acétique, d'alcools supérieurs (dont le 2-phényl éthanol) et d'esters volatils (dont le 2-phényléthyl acétate) a été rapportée pour cette espèce en lien avec leur pouvoir attractif vis-à-vis de son hôte *Drosophila melanogaster*, conjointement avec *Pichia kluyveri* (Becher *et al.*, 2018). Il est intéressant de mentionner que l'analyse phylogénétique montre une proximité avec des souches du genre *Pichia* (Meyer *et al.*, 1998).

B.3.2 Les bactéries

Bien que les bactéries acétiques soient moins étudiées que les bactéries lactiques, une attention particulière sera apportée à la description des bactéries acétiques dont la présence est indispensable dans les consortia de kombucha.

B.3.2.1 Les bactéries acétiques

Elles correspondent à la famille des *Acetobacteraceae* qui constitue une classe des *Alphaproteobacteria* (Yamada, 2016). Ce sont des bactéries à Gram négatif, aérobies stricts, non sporulantes, se présentant sous forme ellipsoïde, en bâtonnets individuels ou organisés en courtes chaînes. Certaines espèces sont motiles et peuvent être pourvues de flagelles. Leur température optimale de croissante est de 30 °C, pour une valeur optimale de pH située entre 5,0 et 6,5 mais elles peuvent se développer à des pH plus bas. Les bactéries acétiques possèdent un métabolisme oxydatif caractéristique qui implique une association de déshydrogénases membranaires (comme l'alcool déshydrogénase et l'aldéhyde déshydrogénase) et une chaîne respiratoire située dans la membrane cytoplasmique de la bactérie (Figure 9) (Matsushita *et al.*, 1994 ; Matsushita and Matsutani, 2016). Les pyrroloquinoline quinone (PQQ) et les déshydrogénases flavine-dépendantes sont liées à la membrane dont les sites actifs stéréo- et régio-sélectifs sont placés vers l'extérieur de la cellule. Du côté intracellulaire, on trouve d'autres déshydrogénases également liées ou non à la membrane (Prust *et al.*, 2005). La localisation de ces enzymes est décrite plus précisément dans la Figure 10. La transformation principale, l'acétification, correspond à l'oxydation de l'éthanol en acide acétique (De Roos and De Vuyst, 2018). La production de cellulose bactérienne, qui est à l'origine des mères de kombucha et de vinaigre est un caractère souche-dépendant (Esa *et al.*, 2014 ; Goh *et al.*, 2012b, 2012a ; Ross *et al.*, 1991). L'espèce modèle utilisée pour étudier la production de cellulose est *Komagataeibacter xylinus* (Cook and Colvin, 1980 ; Esa *et al.*, 2014 ; Lee *et al.*, 2015 ; Volova *et al.*, 2018), dont le mécanisme de production sera développé dans la partie J.

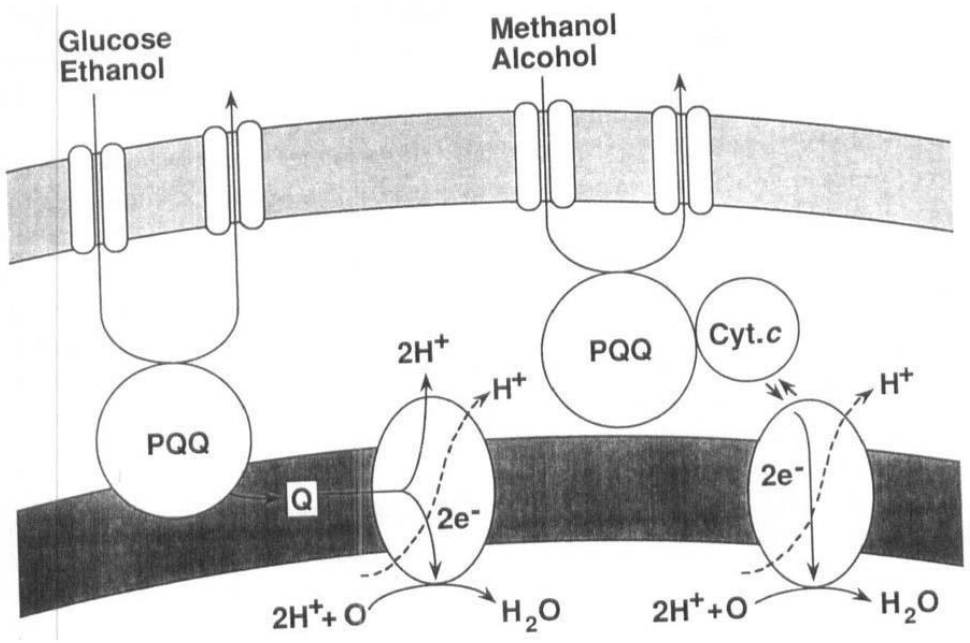

Figure 9 : Réactions réalisées par le complexe d'oxydases périplasmiques comprenant l'oxydation les alcools et les sucres par les bactéries acétiques. Le glucose et l'éthanol peuvent être oxydés par la quinoprotéine déshydrogénase (PQQ) associée à la surface de la membrane cytoplasmique. La déshydrogénase cède les électrons à l'ubiquinone (Q), cédant elle-même les électrons à l'ubiquinol oxydase terminale. La voie impliquant le méthanol et les alcools comprend l'éthanol, le butanol et l'isopropanol. Ces derniers peuvent être oxydés par une quinoprotéine déshydrogénase (PQQ) qui est présente sous forme soluble dans l'espace périplasmique. Cette déshydrogénase cède des électrons à la cytochrome c oxydase terminale *via* le cytochrome c (Cyt. c) également sous forme soluble. Ceci génère un gradient protonique par dissociation de charge et/ou pompe à protons (Matsushita *et al.*, 1994).

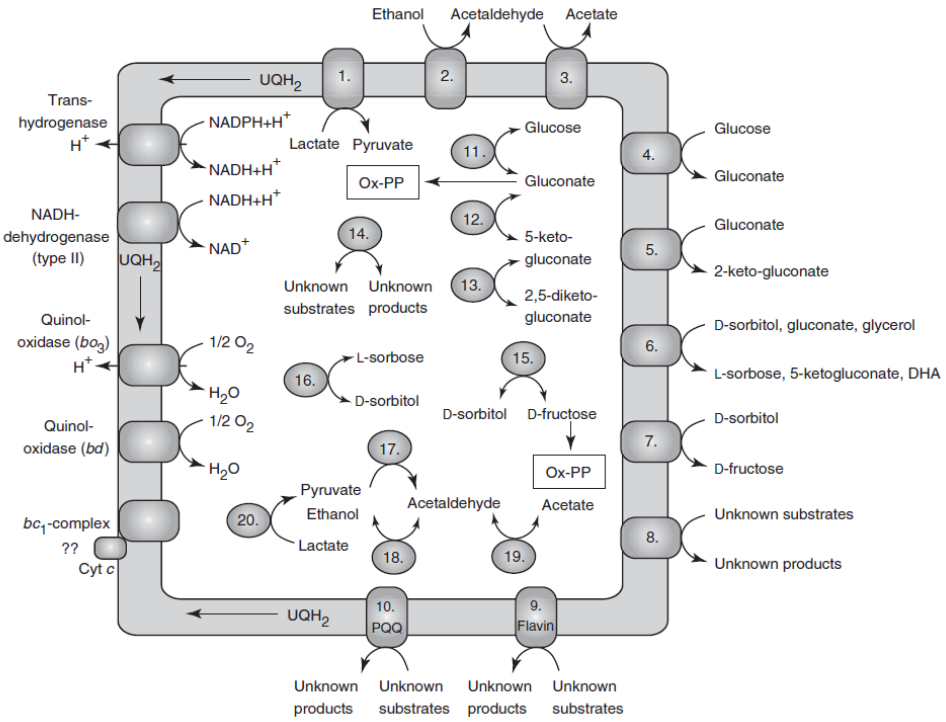

Figure 10 : Chaîne respiratoire et métabolisme des sucres et des alcools chez *Gluconobacter oxydans* (Prust *et al.*, 2005).

Dans le domaine de la production d'aliments, les bactéries acétiques sont les agents clés de la production de vinaigre à partir de substrats fermentés comme le cidre, la bière ou le vin (Ho *et al.*, 2017). Elles interviennent aussi dans la fabrication de produits fermentés tels que les bières lambic (gueuzes), la kombucha, le cacao et parfois le kéfir (De Roos and De Vuyst, 2019, 2018 ; Jayabalan *et al.*, 2014 ; F. Spitaels *et al.*, 2014). Ce même phénomène est aussi perçu sous l'angle de l'altération de ces mêmes produits puisque les métabolites produits sont à l'origine de défauts olfactifs et gustatifs (De Roos and De Vuyst, 2018 ; Gomes *et al.*, 2018).

Les genres représentés parmi les bactéries acétiques isolées sont *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, et *Komagataeibacter*. Les cellules peuvent se présenter sous forme de batônnets, voire ellipsoïdes chez *Acetobacter* et *Gluconobacter*. A l'exception du genre *Gluconobacter*, les cellules peuvent être organisées sous forme de chaînettes, voire par paires chez *Gluconacterobacter* et *Komagataeibacter*. La flagellation est généralement péritriche chez *Acetobacter* et *Gluconacetobacter*, polaire pour *Gluconobacter* et absente chez *Komagataeibacter* (Yamada, 2016). Toutefois, la motilité qui conditionnée par la présence de flagelles péritriches est décrite comme largement souche-dépendante chez le genre *Acetobacter*, alors qu'elle est majoritaire pour le genre *Gluconacetobacter* (Lisdiyanti *et al.*, 2000 ; Sievers and Swings, 2015). Toutes les bactéries appartenant à ces quatre genres sont catalase positives et oxydase négatives à l'exception de *Acetobacter peroxydans* qui est catalase négative. Le

genre *Gluconobacter* se distingue des autres par son incapacité à oxyder complétement l'acétate et le lactate en eau et dioxyde de carbone. Cela est dû à l'absence d'un cycle de Krebs complet (absence de succinate thiokinase et de succinate déshydrogénase) et à l'absence du cycle de l'acide glyoxylique et de voie de la gluconéogenèse (Deppenmeier and Ehrenreich, 2009 ; Ehrenreich and Liebl, 2017). Aucun gène codant la 6-phosphofructokinase n'a été identifié chez *G. oxydans*, *A. pasteurianus* et *K. xylinus*, suggérant un dysfonctionnement dans la voie de la glycolyse chez ces bactéries acétiques (Azuma *et al.*, 2009 ; Deppenmeier and Ehrenreich, 2009 ; Rezazadeh *et al.*, 2020). Les bactéries appartenant à ces quatre genres sont capables d'utiliser le glucose pour former du gluconate, du 2-ceto-D-gluconate 5-ceto-D-gluconate (Yamada, 2016). En revanche seules les bactéries du genre *Gluconacetobacter* produisent du 2,5-diceto-D-gluconate. La distribution des caractéristiques suivant les genres de bactéries acétiques est résumée dans le Tableau 2.

Tableau 2 : Caractéristiques phénotypiques différenciant les genres de bactéries acétiques (d'après Yamada (2016)).

	Acetobacter	***Gluconobacter***	***Gluconacterobacter***	***Komagataeibacter***
Forme cellulaire	Ellipsoïde ou en bâtonnet	Ellipsoïde ou en bâtonnet	En bâtonnet	En bâtonnet
Présence de chainettes	oui	non	oui	oui
Flagelles	péritriches	polaires	péritriches	absents
Catalase	+ (sauf *A. peroxydans*)	+	+	+
Oxydase	-	-	-	-
Oxydation complète de l'acétate et du lactate	+	-	+	+
Production de 2- et 5-céto-D-gluconate	+	+	+	+
Production de 2,5-céto-D-gluconate	-	-	+	-

Un grand nombre de bactéries acétiques a évidemment été isolé à partir du vinaigre (Milanović *et al.*, 2018). D'autres espèces l'ont été à partir de fruits comme *A. papayae* issu de la papaye, *A. tropicalis* de la fève de cacao et de la noix de coco, *A. senegalensis* de la mangue ou *A. indonesiensis* du fruit zirzak (lino *et al.*, 2012 ; Lisdiyanti *et al.*, 2001, 2000 ; Ndoye *et al.*, 2007). Les bactéries du genre *Gluconacetobacter* ont, en plus des sources citées précédemment, été isolées dans des boissons fermentées telles que la kombucha (Sievers and Swings, 2015).

Selon les souches, les sources de carbone utilisables sont diverses : fructose, glycérol et sorbitol (De Ley, 1961). La croissance peut avoir lieu en présence de saccharose pour les bactéries *Gluconobacter*, *Gluconacteobacter* et *Komagataeibacter* (Yamada, 2016). Toutefois, le mécanisme d'utilisation du saccharose a peu été décrit. Une étude a mesuré une faible activité invertase par une sucrose phosphorylase chez *K. xylinus* (Balasubramaniam and Kannangara, 1982)

De manière concomitante avec le genre levurien *Brettanomyces,* une étude portant sur 103 échantillons (Harrison and Curtin, 2021) fait apparaître le genre bactérien *Komagataeibacter* a la plus haute occurrence dans les consortia de kombucha., A l'inverse des levures où une espèce dominante a été identifiée (*B. bruxellensis*), ce n'est pas le cas pour les bactéries acétiques. L'espèce *K. xylinus* possède une occurrence légèrement supérieure aux autres (8%) et est une des espèces la plus étudiée pour sa production de cellulose et, en revanche, peu étudiée pour son métabolisme oxydatif (Volova *et al.,* 2018). L'analyse du génome de la souche de *K. xylinus* K1G4 a mis en évidence un lien étroit entre la production de cellulose la voie des pentoses phosphate et le cycle de Krebs (Rezazadeh *et al.,* 2020). Par ailleurs, l'analyse du génome de la souche *A. pasteurianus* 1023 montre qu'une modification du cycle de Krebs par la substitution de la malate déshydrogénase et de la succinyl CoA synthase par la malate : quinone oxydoréductase et la succinyl -CoA : acétate CoA transférase, permettant la consommation de l'acétate intracellulaire (Matsutani *et al.*, 2011 ; Mullins *et al.,* 2008). Cette modification est complémentée par d'autres mécanismes intervenant dans la tolérance à l'acide acétique : la production de cellulose inhibe l'entrée de l'acétate dans la cellule, les transporteurs couplés ou non à des pompes à protons, l'induction de chaperons pour la stabilisation des protéines intracellulaires (Nakano and Ebisuya, 2016).

Il est toutefois important de souligner que ces études de physiologie ont été menées sur des espèces qui n'ont pas été isolées de la kombucha. En effet, selon Barja *et al.* (2016), les souches isolées de matrices avec un taux en acide acétique élevé (environ 8%) comme le vinaigre produit par méthode de submersion (aération) sont plus difficiles à cultiver sur milieu gélosé, potentiellement à cause d'exigence en termes d'apport en oxygène très important. L'adaptation des souches impacte donc potentiellement fortement leur physiologie. De plus, une étude génomique suggère que les bactéries acétiques pourraient survivre et fonctionner en conditions micro-aérobies car certaines enzymes clés ne sont pas dépendante de l'oxygène. Cela leur permettrait d'assurer leur rôle de symbiotes dans le système digestif de leurs hôtes, les drosophiles (Crotti *et al.,* 2010).

Les caractéristiques importantes à considérer chez les bactéries acétiques en ce qui concerne la fabrication de la kombucha sont :

- Leur capacité à produire des acides organiques, l'acidification de la matrice étant le paramètre principal de la fermentation de la kombucha (Chakravorty *et al.*, 2016 ; De Roos and De Vuyst, 2018) ;
- Leur capacité à produire de la cellulose qui formera un biofilm ou mère (Goh *et al.*, 2012a ; Matsushita and Matsutani, 2016 ; Ross *et al.*, 1991) ;
- Leur capacité à produire de l'acide gluconique. Outre la consommation des monosaccharides issus de l'hydrolyse du saccharose par les levures et la production d'acides organiques, entraînant respectivement une diminution de la sucrosité et une augmentation de l'acidité en termes organoleptiques, l'impact et l'intérêt de l'activité des bactéries acétiques sur le produit est mal connu. Notamment, et à l'instar des nuances d'acidité du vin, l'influence des rapports de teneurs en acides organiques tels que l'acide acétique, l'acide gluconique et l'acide lactique sur la sensation en bouche n'a, à notre connaissance, pas été étudiée (Chidi *et al.*, 2018). La tendance veut cependant que la production d'acide gluconique soit favorisée par rapport à celle d'acide acétique afin d'atténuer le caractère acescent de la boisson.
- Les besoins en azote des bactéries acétiques ont peu été étudiés. Toutefois, des études menées dans le cadre de la production de vinaigre montrent une utilisation préférentielle de certains acides aminés (proline ou arginine) selon le procédé utilisé et selon la matrice (Callejón *et al.*, 2008 ; Valero *et al.*, 2005). En milieux synthétiques, il a été montré que les besoins en azote pour une croissance optimale étaient souches-dépendants (Nakano and Ebisuya, 2016). Cependant, il est évident que la matrice de base utilisée pour la production de vinaigre (vin, bière ou cidre dilués) possède peu de nutriments puisque consommés en grande partie lors de la fermentation alcoolique. On peut donc supposer que les exigences en nutriments azotés sont faibles.

B.3.2.2 Les bactéries lactiques

Les bactéries lactiques correspondent à l'ordre des *Lactobacillales* appartenant aux *Firmicutes* (Mozzi *et al.*, 2016). Ce sont des bactéries à Gram positif, aérobie facultatives, catalase négatives. L'acide lactique est le composé principal résultant du métabolisme des sucres. Comme c'est le cas dans les produits laitiers, carnés, végétaux ou le kéfir, l'activité de ces bactéries peut impacter positivement ou négativement le profil organoleptique de la kombucha (production d'acide lactique, acide acétique, acide succinique, acide formique, diacétyle,

éthanol, gaz carbonique et exopolysaccharides notamment) (Gänzle, 2015 ; Lynch *et al.*, 2018 ; Stiles, 1996).

Les fermentations lactiques réalisées par les bactéries lactiques peuvent être dites « homofermentaire » ou « hétérofermentaire ». La fermentation lactique homofermentaire aboutit à la formation de deux moles de lactate à partir d'une mole de glucose et la fermentation lactique hétérofermentaire induit la production de produits autres que le lactate comme l'acétate et l'éthanol (Gänzle, 2015). Les bactéries homofermentaires obligatoires utilisent la voie de la glycolyse/Embden-Meyerhof car elles possèdent une aldolase mais pas de phosphocétolase (Figure 11a). C'est le cas pour toutes les espèces du genre *Pediococcus*. Les bactéries hétérofermentaires obligatoires utilisent la voie de la phosphocétolase car elles possèdent à l'inverse une phosphocétolase mais pas d'aldolase (Figures 11b et 11d). Les bactéries possédant cette particularité sont les espèces *Lentilactobacillus brevis*, *Lentilactobacillus hilgardii*, *O. oeni* et celles appartenant au genre *Leuconostoc*. Enfin, certaines bactéries telles que *Lacticaseibactillus casei* et *Lactiplantibacillus plantarum* sont hétérofermentaires facultatives utilisent à la fois les voies de Emden-Meyerhof Parnas et de la phosphocétolase pour le métabolisme des hexoses, et les voies cataboliques des pentoses, car elles ne possèdent que l'aldolase (Figure 11c) (Saguir *et al.*, 2019). Il également possible de citer la voie des acides mixtes identifiée chez *Lactococcus lactis* pouvant mener à la formation des acides formique, lactique et acétique à partir de glucose, lactose ou galactose (Garrigues *et al.*, 1997). Si elle est possible, l'utilisation du saccharose par les bactéries lactiques se fait par l'intermédiaire d'une sucrose-6-P hydrolase qui intervient dans le cytosol après internalisation par un transporteur spécifique PTS (*Phosphoenolpyruvate-Dependent phosphotransferase System*) (Reid and Abratt, 2005).

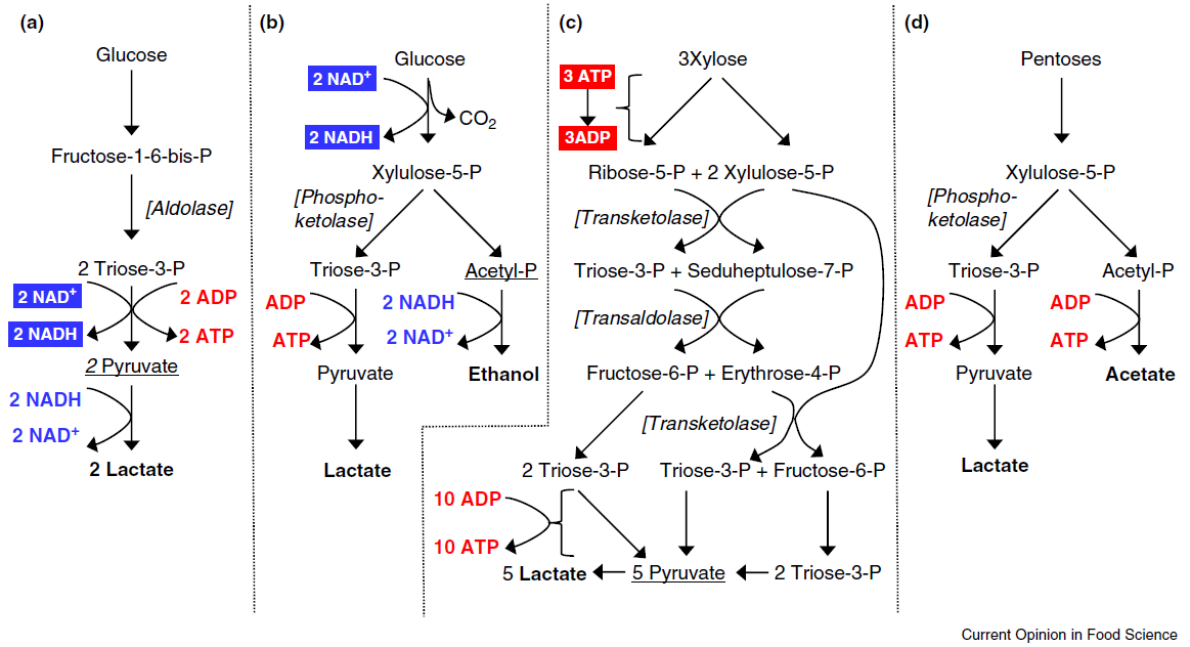

Figure 11 : Schéma métabolique des fermentations lactiques chez les bactéries lactiques : (a) Métabolisme homofermentaire des hexoses par la voie de Emden-Meyerhof Parnas, (b) métabolisme hétérofermentaire des hexoses par la voie de la phosphocétolase, (c) métabolisme homofermentaire des pentoses par la voie des pentoses phosphate et (d) métabolisme hétérofermentaire des pentoses par la voie de la phosphocétolase (Gänzle, 2015).

Le genre *Lactobacillus* a été redécoupé en 25 nouveaux genres sur la base du degrés de similitude génétique et de critères physiologiques et écologiques (Zheng *et al.*, 2020). Les espèces appartenant à cet ancien groupe sont étroitement liées à la nutrition humaine puisque présent dans de nombreux aliments et certaines souches sont catégorisées comme probiotiques. Ces bactéries sont généralement aérotolérantes ou anaérobies ainsi qu'acidophiles. Leur température de croissance optimale est située entre 30 et 40°C (Hammes and Vogel, 1995). L'étude des bactéries lactiques, notamment celles appartenant à l'ancien genre *Lactobacillus*, devrait bénéficier de la nouvelle taxonomie en faisant apparaitre des spécificités liées aux genres nouvellement formés (Zheng *et al.*, 2020). Cette nouvelle nomenclature n'affecte pas l'espèce *Oenococcus oeni*, qui est une bactérie lactique isolée dans diverses boissons fermentées dont le vin, le cidre et la kombucha. Les études génomiques informent sur la plasticité de son génome, son évolution et sa domestication en lien avec la production de boissons fermentées (Lorentzen *et al.*, 2019 ; Lorentzen and Lucas, 2019).

Etant donnée la diversité des micro-organismes et des métabolismes, on peut faire l'hypothèse de nombreuses et complexes interconnections entre métabolismes levuriens et bactériens pouvant intervenir dans les interactions microbiennes lors de l'élaboration d'une kombucha.

B.5 Contexte de la thèse et problématiques

Le projet de thèse est le fruit de ma rencontre avec les deux fondateurs de l'entreprise Biomère, Antoine Martin et François Verdier une année seulement avant la fondation de leur entreprise en 2017 en Île-de-France. Notre intérêt commun pour la kombucha, sur laquelle je travaillais dans le cadre d'un projet de recherche industriel à l'Université de Cork (Irlande), nous a amené à envisager une collaboration par l'intermédiaire d'un travail de thèse. La thématique ciblée des interactions microbiennes dans une boisson fermentée m'a naturellement poussé à reprendre contact avec mes anciennes enseignantes Raphaëlle Tourdot-Maréchal et Cosette Grandvalet rattachées à l'équipe VAlMiS (Vin Aliment Microbiologie Stress) de l'UMR PAM (Procédés Alimentaires et Microbiologiques). Elles ont accepté de m'aider à consolider le sujet de thèse en vue de la demande de financement. Ainsi, Biomère a financé avec l'aide du dispositif CIFRE, ma thèse intitulée « Etude de la dynamique microbienne pour la maîtrise de la fabrication de la kombucha » entre 2018 et 2021.

L'objectif général de la thèse est d'apporter des éléments de compréhension concernant les interactions microbiennes dans la kombucha, afin de mieux maîtriser le procédé de fabrication. Il apparait en effet clairement que cette boisson fermentée est dépendante de la symbiose et des interconnexions métaboliques entre levures et bactéries. L'occurrence d'interactions microbiennes semble donc indispensable et impose par conséquent leur étude. Ces travaux visent donc à apporter de nouvelles connaissances à portées applicatives pour la maîtrise du procédé fermentaire, mais également à un niveau plus fondamental pour la compréhension du fonctionnement des communautés microbiennes alimentaires.

Ces travaux de thèse s'intéressent à la caractérisation des interactions levures-levures et levures-bactéries au cours de la production de kombucha. La méthodologie employée se base principalement sur la comparaison d'un consortium de kombucha original et des cultures sur infusion de thé sucré de micro-organismes issus de ce consortium en faisant varier la composition microbienne. Les interactions sont étudiées sur plusieurs plans :

- Sur le plan microbiologique en suivant les variations des populations de levures et de bactéries et en étudiant la structure et la formation du biofilm cellulosique,
- Sur le plan chimique en caractérisant les métabolismes levuriens et bactériens lors de mono- et co-cultures et leurs effets sur la composition du milieu. Le focus est particulièrement porté sur la gestion des substrats carbonés, azotés, de l'oxygène à l'échelle des espèces et de la communauté entière,

- En lien avec la composition chimique, sur le plan sensoriel, afin de déterminer le rôle des micro-organismes et de la matrice sur le profil organoleptique de cette boisson dont les standards de qualité n'ont pas été encore clairement définis aujourd'hui.

Au-delà de la caractérisation des interactions trophiques, spatiales ou métaboliques ayant lieu dans les *consortia* de kombucha, ces travaux visent également à mieux définir la boisson vis-à-vis de ses composantes essentielles, qu'il s'agisse de micro-organismes, de composition chimique ou de caractéristiques sensorielles. Or, définir la kombucha passe aussi par la compréhension des rôles et mécanismes en jeu lors de sa production. Ainsi, les travaux présentés ont pour objectif de répondre aux questions suivantes : quels rôles remplissent la composition en genres et espèces de levures et de bactéries ainsi que leurs interactions dans la composition chimique et le profil sensoriel ? Quels rôles jouent la matrice thé sucré et les substrats qu'elle contient pour les consortia et le produit final ? Quelles sont les fonctions du biofilm ? Obtenir des éléments de réponse à ces questions permet également d'envisager la mise en place de *consortia* simplifiés, maîtrisés, et donc mieux adaptés à la production industrielle de kombucha.

Techniquement, les travaux prennent appui sur des plans expérimentaux d'étude des interactions et des outils analytiques classiques de microbiologie, de chimie et d'évaluation sensorielle, couplés à certaines techniques innovantes et de pointe. Ces dernières comprennent la microscopie biphotonique et la spectrométrie de masse permettant une analyse métabolomique.

Ce manuscrit se découpe en huit chapitres abordant une ou plusieurs des problématiques énoncées plus haut. Dans un premier chapitre, les interactions microbiennes dans la kombucha ont été étudiées sur le lieu de production à travers l'étude de l'évolution de la composition microbienne sur trois années consécutives. Dans le second chapitre, l'étude des interactions levures-bactérie acétique porte sur les métabolismes principaux permettant la fabrication de kombucha, en particulier par la consommation des sucres. Cette étude a permis la sélection de deux levures et d'une bactérie acétique dont les interactions seront étudiées plus en détails dans les chapitres suivants. Le troisième chapitre bascule sur une approche métabolomique non ciblée afin d'appréhender les transformations des composés non-volatils ayant lieu au cours de la production de kombucha en fonction des deux phases de production et du type de thé utilisé. Cette approche est également utilisée dans le quatrième chapitre pour réaliser une étude fine des interactions levure-levure et levure-bactérie acétique. Après les composés non-volatils, ce sont principalement de la formation de composés volatils dont il est question dans le cinquième chapitre. La confrontation des analyses de composition chimique avec une analyse sensorielle permet de mieux comprendre l'origine du profil sensoriel de la kombucha. Jusque-là réalisés en point final, les travaux des deux chapitres suivants s'appuient sur le suivi de cinétiques. Le

sixième chapitre analyse la cinétique de l'utilisation des substrats azotés et des fractions protéiques produites. Le septième chapitre se concentre sur la cinétique de consommation de l'oxygène au cours de la production de kombucha. Enfin, le huitième et dernier chapitre propose une incursion dans le biofilm de la kombucha et apporte de nouveaux éléments concernant sa formation et sa structure à l'aide de la microscopie biphotonique.

Les résultats obtenus sont mis en perspective dans la discussion générale, à l'issue de laquelle les principales conclusions sont énoncées dans la dernière partie de ce manuscrit.

C Chapitre 1 : Evolution de la composition de consortia de kombucha sur trois années dans un contexte de production.

La présentation des résultats de cette thèse s'ouvre dans ce premier chapitre avec une étude descriptive menée sur le terrain. Ces travaux ont été réalisés dans le cadre de stages de Master 1 avec l'implication de Fatou Sahono (2020) et Perrine Mas (2021). Ce chapitre a été soumis sous forme de publication au journal *Lebensmittel-Wissenschaft und Technologie* (*LWT)*. L'étude a pour but de poser un constat sur les problématiques rencontrées aux cours de la production de kombucha à l'échelle industrielle. En effet, depuis que la production de kombucha s'est industrialisée, l'étude de l'évolution des communautés microbiennes est devenue nécessaire afin de contrôler le procédé. La composition microbienne des kombuchas de thés noir et vert produits sur le site de l'entreprise Biomère a été analysée sur trois années consécutives (2019, 2020 et 2021). Afin de déterminer la composition en genres et espèces, une approche culture-dépendante a été mise en œuvre. Les micro-organismes ont été isolés du liquide et du biofilm à l'aide de différents milieux nutritifs gélosés puis mis en culture liquide pour être conservés. Dans le cas des levures, un travail de caractérisation des morphotypes des colonies sur milieu différentiel WallersteinLab et des cellules par observations microscopiques a permis de distinguer les populations levuriennes à l'échelle de l'espèce. Un dénombrement différencié des populations levuriennes a ainsi pu être mis en œuvre. L'ADN des isolats a été extrait individuellement, amplifié par PCR (*Polymerase Chain Reaction*) 26S et 16S pour les levures et les bactéries respectivement, puis séquencés selon la méthode Sanger (Genewiz®). Les identités ont été obtenues par comparaison des séquences avec la base de données NCBI (*National Center for Biotechnology Information*). Les espèces levuriennes principalement identifiées sont *Brettanomyces bruxellensis*, *Hanseniaspora valbyensis*, *Saccharomyces cerevisiae* et les espèces bactériennes appartiennent au genre *Liquorilactobacillus* et *Acetobacter*.

Les résultats soulignent la persistance d'espèces levuriennes telle que *B. bruxellensis* et suggère une sensibilité des levures face à des évènements stressants telles que la fluctuation de la température au cours de la production. Par conséquent, des espèces levuriennes initialement identifiées n'ont plus été détectées l'année suivante et seules certaines d'entre elles ont pu être de nouveau isolées par la suite, comme *H. valbyensis*. La dominance de *B. bruxellensis* est démontrée dans les kombuchas thé noir et thé vert, mais la proportion en levures varie selon le type de thé et la phase (liquide ou biofilm). La composition en bactéries acétiques et lactiques a montré une plus grande variabilité comparée aux levures avec de nombreux changements d'espèces au cours du temps.

L’étude révèle donc une variabilité de la composition microbiologique des consortia de kombuchas utilisés en production sur plusieurs années. Elle montre également que ces variations peuvent avoir pour cause des évènements de production stressants, impactant particulièrement les espèces levuriennes. La réapparition d’espèces non détectées l’année précédente suggère également une possibilité de rémanence.

Les modifications au sein des consortia peuvent potentiellement avoir un impact sur leur fonctionnalité et donc sur la production-elle-même, ce qui représente une difficulté pour les producteurs souhaitant contrôler le procédé de production et garantir les qualités organoleptiques du produit. Cette première étude souligne ainsi l’importance de gagner en compréhension sur les micro-organismes de la kombucha, leur physiologie et leurs interactions. Les isolats obtenus en 2019 ont servi de base pour la première étude d’interactions mettant en jeu quatre espèces de levures et trois espèces de bactéries acétiques différentes, décrite dans le chapitre suivant. Une analyse métagénomique complémentaire a été réalisée grâce à une prestation de service réalisée par l’entreprise Genoscreen®. Cependant, les résultats ont montré des incohérences pouvant être imputées à l’extraction de l’ADN total, et ne sont donc pas présentés.

Evolution in composition of kombucha consortia over three consecutive years in production context.

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Abstract

Kombucha is a traditional drink obtained from sugared tea transformed by a community of yeasts and bacteria. Its production has become industrialized, and the study of microbial community's evolution is needed to improve control over the process. This study followed the microbial composition of black and green tea kombucha over three consecutive years in a production facility using a culture-dependant method. Microorganisms were isolated and cultivated using selective agar media. DNA of isolates was extracted, amplified using 26S and 16S PCR, and sequenced. Identities were obtained after comparison to NCBI database. *Brettanomyces bruxellensis*, *Hanseniaspora valbyensis*, *Saccharomyces cerevisiae* were the major yeast species, and major bacterial genera were *Liquorilactobacillus* and *Acetobacter*. Results highlight the persistence of yeast species like *B. bruxellensis* detected in 2019 and suggest a sensitivity of yeast towards stressful events such as a hot period in 2020. Some yeasts species were resilient and isolated again in 2021, for example *H. valbyensis*. Dominance of *B. bruxellensis* was clear in green and black tea kombucha, but proportions in yeasts varied depending on tea type and phase (liquid or biofilm). Composition in acetic acid and lactic acid bacteria showed a higher variability than yeasts with many changes in species over time.

Keywords

kombucha, evolution, microbial composition, yeast, acetic acid bacteria.

1 Introduction

Kombucha is a drink obtained from sugared tea infusion through the activity of a symbiotic microbial community composed of yeasts (including *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis*) and bacteria, including acetic (mainly *Komagataeibacter*, *Gluconobacter* and *Acetobacter* genera) and sometimes lactic acid bacteria (from the genera *Lactobacillus* and *Liquorilactobacillus*) (Marsh *et al.*, 2014; Villarreal-Soto *et al.*, 2018; Zheng *et al.*, 2020). The production process occurs in two phases: one in open condition for acidification and another in closed condition (after bottling for example) for natural carbonation (Tran *et al.*, 2020a). During the first phase, kombucha is a combination of several microbial activities, including alcoholic fermentation by yeasts, and the oxidative metabolism by acetic bacteria (Jayabalan *et al.*, 2014). Tea infusion brings the nitrogenous substrates. The sucrose added to the tea is hydrolysed into glucose and fructose by the invertase activity of yeasts, which also produce ethanol through alcoholic fermentation. Acetic bacteria oxidize ethanol into acetic acid and glucose into gluconic acid (May *et al.*, 2019). The use of the yeast metabolites by bacteria raises the question of interactions between yeast and bacteria (Tran *et al.*, 2020b). During that phase, a cellulosic biofilm develops thanks to acetic acid bacteria and includes two environments for the development of microorganisms: a liquid phase, where the microorganisms have a planktonic lifestyle, and a solid phase, a cellulosic biofilm also called kombucha pellicle or SCOBY (Symbiotic Culture Of Bacteria and Yeasts) produced by acetic acid bacteria, where sessile cells remain (May *et al.*, 2019; Tran *et al.*, 2021b).

As a result of these transformations, kombucha contains residual sugars, polyphenols, vitamins, organic acids (glucuronic, acetic, gluconic acids) resulting in a low pH value and low alcohol content (generally below 1% (v / v)) working as a barrier effect against microbial contamination (Coton *et al.*, 2017; May *et al.*, 2019). To meet the increasing consumers demand, kombucha production has gradually become industrialized, leading to the development of many kombucha breweries of small to intermediate sizes. Therefore, this branch is relatively young in the process of business and technical structuring (Kim and Adhikari, 2020). Moreover, kombucha microorganisms are poorly studied with complex interactions, whose study is relatively recent. Kombucha production meets difficulties related to reproducibility, the control of fermentations and therefore the quality of the product, because of the complex nature of the consortia. Finally, the microbial community is likely to evolve as kombucha production progresses, with changes in

presence and proportions of species and field data about such evolution are lacking. Therefore, the long-term study of these microbial community's evolution appears crucial. Indeed, changes in the population could impact the functionality of the inoculum, which would lead to consequences in an industrial production process.

To determine and identify the genera and species composition of the kombucha microbial community used in a production unit, a culture-dependent approach has been chosen. Microorganisms were isolated using different selective or differential agar media, followed by the individual extraction, amplification, and sequencing of DNA through 26S and 16S, for yeasts and bacteria respectively. Finally, the results obtained over three consecutive years (2019, 2020 and 2021) have been summarized and discussed.

2 Materials and Methods

2.1 Origin and composition of kombucha samples

Black tea and green tea kombucha samples were obtained from the kombucha brewery Biomère (Paris, France) each year in January over three years in 2019, 2020 and 2021. Those samples were kombucha liquid phase and biofilm with liquid sampled separately at the end of the acidification phase, before aromatization and bottling. Inoculation of new batches traditionally occurs by propagation (Tran *et al.*, 2020a).

Kombucha of batch n-1 at the end of the acidification phase (approximately 7 days) was used to inoculate batch n at the rate of 12% (v/v). Production was carried out without temperature control, as no temperature regulation system was set up before summer 2020. Afterwards, temperature was maintained at around 25 ± 2 °C. It should be noted that a heat wave occurred during summer of year 2019, which occasionally implied production temperatures above 35°C (Figure C1).

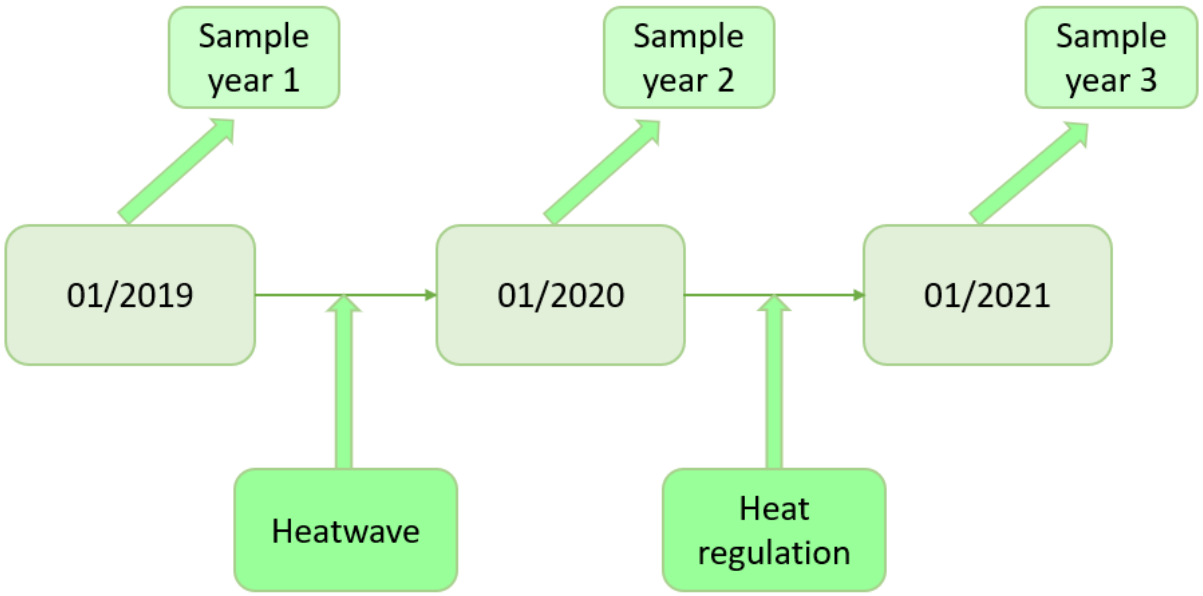

Figure C1 : Timeline of experimentations and events at the production facility.

2.2 Isolation and identification of yeasts and bacteria using culture-depending methods

2.2.1 Sampling and extraction of microorganisms from the biofilm

Liquid samples were directly used and diluted for plating whereas microorganisms needed to be extracted from the biofilm samples. The biofilm was gently removed and put in a sterile Petri dish. Then, a punch sample of biofilm was cut with the help of a sterile beaker (3 cm diameter) and a sterile scalpel. The sample obtained was then rinsed twice with 10 mL of sterile physiological water. In parallel, an empty Petri dish filled with 10 mL of physiological water was weighted with and without the punch sample to calculate the fresh pellicle weight. Finally, the sample was placed in a sterile stomacher bag and homogenized for 30 minutes.

2.2.2 Determination of biofilm dry weight

Determination of biofilm dry weight was performed on separate pieces of biofilm than the ones described in part 2.2.1. Biofilm punching and washing procedure was carried out in triplicates for each condition. Then, an aluminum cup was weighted with and without the punch to obtain its fresh weight. Finally, the sample was dried in an oven at 102 °C for 24 hours, then weighted a second time to obtain the dry weight after calculation. Populations levels in the biofilm were expressed in CFU g^{-1} dry biofilm.

2.2.3 Isolation of microorganisms and population determination

Bacteria and yeasts were isolated using different agar media. The Wallerstein Laboratory medium of Thermo Fischer Scientific (Waltham, USA) has been used to isolate yeasts (Green and Gray, 1951; Hall, 1971; Pallmann *et al.*, 2001). Bromocresol Green allowed macroscopic discrimination of colonies based on their appearance and color. Different media were used to isolate bacteria with different growth requirements. De Man Rogosa and Sharpe (MRS) medium (pH 6.2) from Condalab (Madrid, Spain) in aerobic incubation was used as unspecific agar medium to isolate bacteria. Mannitol agar medium with aerobic incubation was used to isolate acetic acid bacteria specifically (Ndoye *et al.*, 2006). LAC (pH 5.1) (Salma et al., 2013) and M17 (pH 6.9; only used in 2020 and 2021) agar media with anaerobic incubation were used to isolate anaerobe lactic acid bacteria specifically with different nutritional requirements (Terzaghi and Sandine, 1975). All reagents used in culture media come from Merck (Darmstadt, Germany) if no details are given. The compositions of the agar media are provided in Annexe SC1.

For each agar medium, 100 µL of each decimal dilution was spread over the entire surface of the plate. Each plate counting was performed in triplicates. For liquid phase samples, dilutions ranged from 10^{-2} to 10^{-4} and for biofilm samples from 10^{-2} to 10^{-5}. Detection limit was 1.10^{3} CFU mL^{-1}. WL agar media were incubated for 72 hours at 28 °C. The other agar media were incubated for 48 hours at 28 °C.

2.2.4 Macroscopic and microscopic examinations

Colony and cell morphotyping of yeasts and bacteria were carried out by macroscopic observation on agar plate and using an EVOS® microscope from Thermo Fisher (Carlsbad, USA) with 400 to 1000 magnification. However, because of the more similar aspects among bacteria colonies and cells, the same way of characterization could not be achieved.

2.2.5 Preparation of isolates for identification

Five yeast colonies per colony morphotype were picked from WL agar media, inoculated each in 0.5 ml of Yeast Peptone Dextrose medium (YPD), then incubated 48 hours at 28 °C in closed tube. Then, the sample was centrifuged to 14,500 g three minutes, at 15 ° C and the yeast pellets were re-suspended in 50% (v/v) YPD, 20%(v/v) glycerol.

The process was similar for lactic acid bacteria. However, fifteen colonies were picked without morphological distinctions on LAC as well as five on M17 (only in 2020 and 2021). Each colony was inoculated in 1.5 ml of LAC medium. Then, liquid cultures were incubated 48 hours at 28 °C without agitation. Finally, after centrifugation (14,500 g, three minutes at 15°C), the bacteria were resuspended in 50% (v/v) LAC medium containing 20% (v/v) glycerol.

For acetic acid bacteria, the fifteen colonies on MRS and five on Mannitol agar media were picked to inoculate each 0.5 ml of Mannitol medium. Incubation was the same as described before, but the tubes were open, protected by a clean piece of cloth. After incubation and centrifugation (14,500 g, three minutes at 15 °C), the cells were re-suspended in 50% (v/v) Mannitol medium,20% (v/v) glycerol. All isolates were stored at -20°C.

2.2.6 DNA extraction

DNA extraction was carried out on each isolate using an InstaGeneTM Matrix kit (BioRad, Hercules, CA USA). The process was the same for all samples. First, 40 µL of InstaGeneTM Matrix reagent was mixed with 5 µL of thawed cell suspension. This suspension was incubated at 56 °C for fifty minutes, then at 99 °C for eight minutes before cooling down to room temperature. After, 30 µL of milliQ water were added. Finally, the sample was vortexed for thirty seconds, then centrifuged at 2,500 g 10 minutes, at 10 ° C, to separate extracted DNA from cellular debris.

2.2.7 Amplification

To achieve interspecific discrimination for yeasts, an amplification of the 26S region of ribosomal DNA (D1/D2 domain) by PCR was performed. NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GTCCGTGTTTCAAGACGG-3') primers were used (Maoura *et al.*, 2005). Two microliters of DNA previously extracted with the InstaGeneTM Matrix kit were added to 25µL of PCR mix (1.8 mM $MgCl_2$, 0.25 mM dNTPs, 1.25 µM of each primer, and 0.025U Taq polymerase (Promega Corp., Madison, WI, USA), then amplification was performed using a BioRad thermocycler (Hercules, CA, USA) as described in Esteve-Zarzoso *et al.* (1999).

Amplified DNA samples were analyzed by capillary electrophoresis with a MultiNa MCE 202 (Shimadzu, France), at 37°C for 75 seconds using the DNA-1000 kit (Shimadzu,

France), and an internal size calibrator. The peaks were identified after excitation of a LED (470 nm) and then detection of fluorescence. The size of the amplified DNA fragments was calculated by the software using the gene ruler 50 pb DNA Ladder molecular weight marker (Thermoscientific, USA) as a reference.

To achieve species discrimination for bacteria, RAPD (Random Amplified Polymorphic DNA) PCR amplification was performed using the primer M13, (5'-GAGGGTGGCGGTTCT-3') (Huey and Hall, 1989). Two microliters of DNA previously extracted with the InstaGeneTM Matrix kit were added to 25µL of PCR mix (4 mM $MgCl_2$, 0.20 mM dNTPs, 4 µM of each primer, and 0.05U Taq polymerase). The amplification was performed using a thermocycler (Qbiogen, Illkirch Graffenstaden, France), as described in Reguant & Bordons (2003).

After selecting samples representative of the diversity of acetic and lactic bacteria through capillary electrophoresis analysis, 16S PCR amplification was performed. Ribosomal DNA extracted from the selected group was amplified using the following primers: E517F (5'-GCCAGCAGCCGCGGTAA-3') and E106R (5'CTCACGRCACGAGCTGACG-3') (Wang and Qian, 2009). The amplification was carried out under the same conditions as for yeast samples.

2.2.8 Identification

Amplified DNA samples obtained through 26S and 16S PCR were sequenced according to the Sanger method by Genewiz® (Leipzig, Germany). Sequencing was performed on both strands, using primers E517F and E106R for bacteria, and NL1 and NL4 for yeasts. Finally, the sequences obtained were analyzed using the Geneious R7 software (version 7.1.5), and the BLAST tool (basic alignment search), which, after comparison with the NCBI databases, returned the names of genera and species associated to a E-value (number of expected hits of similar quality) and percentage of pairwise identity. Validation of genus and species per sample was made based on the lowest E-value.

2.3 Statistical analyses

The confidence intervals have been calculated with Excel (Microsoft 365), from technical repetitions.

3 Results

3.1 Populations of yeasts and bacteria

The yeasts total population was determined using the Wallerstein Laboratory (WL) medium. Microbial populations were determined in green tea kombucha and black tea kombucha (Tableaux C1 and C2). Comparison of bacteria counts between the different agar media showed significant differences, highlighting selective growths. When comparing total yeasts and total bacteria in the liquid or in the biofilm, yeasts population were systemically inferior or equal to the highest bacterial population count regardless of the medium, except for the black and green tea biofilms in 2019. It is remarkable that bacteria counts on LAC agar medium in liquid and biofilm was always higher in black tea than green tea kombucha for each year except in the biofilm in 2021. Additionally, total yeasts count was equivalent between green and black tea kombucha for every year. In contrast, total yeast count in the biofilm was higher in green tea kombucha, except in 2019. It is worth noting that it was not possible to rigorously compare the population between the solid and liquid phases. Indeed, living conditions were not comparable and the accuracy of the count for the biofilm samples depended on the quality of the extraction. It was observed that yeast populations decreased in 2020, while the differences between 2019 and 2021 were more tenuous, and yeast and bacteria populations increased again in 2021, for the liquid phase (Tableaux C1 and C2). For biofilms, the bacterial population did not decrease in 2020, while the yeast population decreased for green and black tea kombuchas (Tableaux C1 and C2).

Tableau C1 : Populations in yeasts and bacteria in green tea kombucha liquid and biofilm samples over three years. nd = not determined.

Green tea kombucha	January 2019		January 2020		January 2021	
	Liquid (CFU mL^{-1})	Biofilm (CFU g^{-1} dry mass)	Liquid (CFU mL^{-1})	Biofilm (CFU g^{-1} dry mass)	Liquid (CFU mL^{-1})	Biofilm (CFU g^{-1} dry mass)
Bacteria						
LAC	$7.13.10^5$ $\pm 3.28.10^5$	$2.34.10^6$ $\pm 3.60.10^6$	$3.18.10^5$ $\pm 3.44.10^4$	$8{,}98.10^6$ $\pm 3.10.10^3$	$8.33.10^6$ $\pm 1.27.10^7$	$5.18.10^8$ $\pm 1.59.10^7$
Mannitol	$8.47.10^6$ $\pm 6.23.10^5$	$9.29.10^7$ $\pm 1.16.10^6$	$1.79.10^6$ $\pm 4.33.10^5$	$5.51.10^7$ $\pm 3.90.10^4$	$9.45.10^6$ $\pm 1.95.10^6$	$7.79.10^7$ $\pm 4.22.10^6$
MRS	$8.67.10^6$ $\pm 5.09.10^6$	$1.15.10^6$ $\pm 3.64.10^5$	$2.23.10^5$ $\pm 1.51.10^4$	$8.98.10^6$ $\pm 1.36.10^3$	$1.19.10^6$ $\pm 1.57.10^6$	$8.57.10^7$ $\pm 2.91.10^6$
M17	nd	nd	$1.73.10^5$ $\pm 4.09.10^4$	nd	$1.74.10^6$ $\pm 6.23.10^5$	$1.66.10^8$ $\pm 5.88.10^6$
Yeasts						
Wallerstein Lab nutrient (WL)	$1.66.10^6$ $\pm 3.39.10^5$	$1.03.10^8$ $\pm 2.43.10^5$	$3.40.10^4$ $\pm 1.72.10^4$	$1.60.10^6$ $\pm 1.55.10^3$	$9.83.10^6$ $\pm 4.27.10^6$	$5.70.10^7$ $\pm 4.53.10^6$
B. bruxellensis	$6.03.10^5$ $\pm 1.64.10^5$	$1.02.10^8$ $\pm 3.16.10^6$	$3.10.10^4$ $\pm 2.63.10^4$	$1{,}60.10^6$ $\pm 2.92.10^5$	$7.05.10^6$ $\pm 1.30.10^5$	$1.95.10^7$ $\pm 6.66.10^5$
Hanseniaspora sp.	$1.05.10^6$ $\pm 7.42.10^4$	$1.09.10^6$ $\pm 4.46.10^5$	$<1.10^3$	$<1.10^3$	$7.80.10^6$ $\pm 2.74.10^4$	$1.95.10^6$ $\pm 6.86.10^3$
S. cerevisiae	$<1.10^3$	$<1.10^3$	$<1.10^3$	$<1.10^3$	$1.86.10^7$ $\pm 2.55.10^4$	$1.47.10^7$ $\pm 8.04.10^4$
Z. florentinus	$<1.10^3$	$<1.10^3$	$<1.10^3$	$<1.10^3$	$5.70.10^6$ $\pm 3.14.10^4$	$1.05.10^6$ $\pm 2.94.10^3$
Candida sp	$<1.10^3$	$3.68.10^4$ $\pm 6.53.10^2$	$3.00.10^3$ $\pm 7.84.10^2$	$<1.10^3$	$3.00.10^5$ $\pm 1.96.10^3$	$1.20.10^6$ $\pm 1.18.10^4$

Tableau C2 : Populations in yeasts and bacteria in black tea kombucha liquid and biofilm samples over three years. nd = not determined.

Black tea kombucha	January 2019		January 2020		January 2021	
	Liquid (CFU mL^{-1})	Biofilm (CFU g^{-1} dry mass)	Liquid (CFU mL^{-1})	Biofilm (CFU g^{-1} dry mass)	Liquid (CFU mL^{-1})	Biofilm (CFU g^{-1} dry mass)
LAC	$5.43.10^7$ $\pm 2.83.10^7$	$3.47.10^7$ $\pm 6.63.10^5$	$1.92.10^6$ $\pm 5.37.10^4$	$3.22.10^7$ $\pm 4.83.10^3$	$6.31.10^7$ $\pm 1.46.10^6$	$1.48.10^8$ $\pm 7.37.10^6$
MAN	$5.73.10^6$ $\pm 1.31.10^5$	$>1.76.10^8$	$1.97.10^5$ $\pm 2.94.10^3$	$2.13.10^8$ $\pm 2.65.10^2$	$8.47.10^6$ $\pm 6.23.10^5$	$4.75.10^7$ $\pm 2.72.10^6$
MRS	$2.93.10^7$ $\pm 4.71.10^6$	$3.00.10^7$ $\pm 1.11.10^6$	$8.50.10^5$ $\pm 1.35.10^4$	$1.45.10^8$ $\pm 1.21.10^3$	$8.67.10^6$ $\pm 5.09.10^6$	$8.25.10^6$ $\pm 1.77.10^6$
M17	nd	nd	$2.62.10^5$ $\pm 4.12.10^4$	$2.22.10^6$ $\pm 3.70.10^3$	$4.28.10^6$ $\pm 4.02.10^5$	$5.99.10^7$ $\pm 5.05.10^6$
Wallerstein Lab nutrient (WL)	$1.26.10^6$ $\pm 1.59.10^5$	$>6.29.10^8$	$4.51.10^4$ $\pm 1.20.10^4$	$4.70.10^5$ $\pm 1.08.10^3$	$9.50.10^6$ $\pm 2.45.10^6$	$2.78.10^7$ $\pm 1.14.10^6$
B. bruxellensis	$4.50.10^5$ $\pm 2.35.10^5$	$>1.76.10^8$	$4.51.10^4$ $\pm 1.47.10^3$	$4.70.10^5$ $\pm 1.63.10^4$	$1.74.10^7$ $\pm 2.20.10^5$	$9.75.10^6$ $\pm 2.89.10^5$
Hanseniaspora sp.	$8.07.10^5$ $\pm 1.63.10^5$	$2.63.10^8$ $\pm 1.16.10^6$	$<1.10^3$	$<1.10^3$	$4.05.10^6$ $\pm 4.21.10^4$	$6.15.10^6$ $\pm 2.06.10^4$
S. cerevisiae	$6.67.10^1$ $\pm 9.80.10^1$	$5.52.10^4$ $\pm 2.26.10^3$	$<1.10^3$	$<1.10^3$	$1.56.10^7$ $\pm 2.55.10^4$	$2.28.10^7$ $\pm 5.68.10^4$
P. fermentens	$<1.10^3$	$1.82.10^8$ $\pm 2.79.10^5$	$<1.10^3$	$<1.10^3$	$<1.10^3$	$<1.10^3$
Z. florentinus	$<1.10^3$	$<1.10^3$	$<1.10^3$	$<1.10^3$	$1.05.10^6$ $\pm 1.27.10^4$	$3.30.10^6$ $\pm 2.74.10^4$
G. geotrichum	$<1.10^3$	$8.81.10^6$ $\pm 1.13.10^5$	$<1.10^3$	$<1.10^3$	$<1.10^3$	$<1.10^3$
Candida sp	$<1.10^3$	$<1.10^3$	$<1.10^3$	$<1.10^3$	$1.05.10^6$ $\pm 2.94.10^3$	$2.40.10^6$ $\pm 3.92.10^3$

3.2 Identification of microorganisms

3.2.1 PCR and electrophoresis

In 2019, 120 yeast clones and 200 bacteria clones were isolated. In 2020, 55 yeasts clones and 195 bacteria clones were isolated. In 2021, 88 yeasts clones and 168 bacteria clones were isolated. For yeasts, 26S PCR was performed using NL1 and NL4 primers, and amplified DNA samples were sequenced. The capillary electrophoresis profiles of amplified DNA could be observed for each sample. For 2019, 2020 and 2021, 24, 11 and 6 isolates respectively were selected for DNA sequencing. For bacteria, RAPD PCR was carried out using M13 primer. Each isolate possessed several bands, and their position formed a profile that was dependent on the strain. The comparison of electrophoretic bands profiles allowed the formation of groups with common profiles, and a representative isolate was selected from each of these groups. Then for 2019, 2020 and 2021, 22,19 and 14 representative isolates respectively were amplified by PCR 16S and sequenced. Exemples of electropheris profiles for 26S, RAPD and 16S PCRs are available in Annexes SC2, SC3, SC4 and SC5. Comparison of sequences to NCBI database returned identities with e-values equal to zero or very close ($\leq 1.10^{-173}$). For some acetic acid bacteria, two species were proposed, because the targeted sequence did not allow their discrimination. The identifications results are presented in Tableau C3.

Tableau C3 : Identification of yeasts and bacteria in black and green tea kombucha liquid and biofilm samples over 3 years.

Species	Green tea 2019	Green tea 2020	Green tea 2021	Black tea 2019	Black tea 2020	Black tea 2021
Yeasts						
Brettanomyces bruxellensis	L+B	L+B	L+B	L+B	L+B	L+B
Hanseniaspora valbyensis	L+B	nd	nd	L+B	nd	L+B
Hanseniaspora opuntiae	nd	nd		B	nd	nd
Saccharomyces cerevisiae	nd	nd	L+B	L+B	nd	L+B
Pichia aff. fermentans	nd	nd	nd	B	nd	nd
Galactomyces geotrichum	nd	nd	nd	B	nd	nd
Zygosaccharomyces florentinus	nd	nd	L+B	nd	nd	L+B
Candida boidinii	B	nd	nd	nd	nd	
Candida californica	B	L	L+B	nd	nd	L+B
Acetic bacteria						
Acetobacter indonesiensis	nd	nd	nd	L+B	nd	nd
Acetobacter tropicalis or *senegalis*	L+B	B	nd	nd	L	nd
Acetobacter pasteurianus or *cibinongensis*	nd	nd	L	nd	nd	nd
Acetobacter okinawensis	nd	nd	B	nd	nd	nd
Acetobacter aceti	nd	nd	B	nd	nd	nd
Acetobacter peroxydans or *papayae*	nd	nd	nd	L+B	B	nd
Gluconobacter sp	nd	nd	nd	nd	nd	L
Gluconacetobacter liquefaciens	L+B	nd	nd	nd	L	nd
Gluconacetobacter takamatsuzukensis	L+B	nd	nd	nd	nd	nd
Komagataeibacter rhaeticus	nd	nd	B	nd	nd	nd
Komagataeibacter saccharivorans	nd	nd	B	L+B	nd	nd
Lactic bacteria						
Liquorilactobacillus ghanensis	nd	nd	nd	nd	L	L+B

Liquorilactobacillus hordei	nd	B	nd	nd	nd	nd
Liquorilactobacillus satsumensis ou *oeni*	nd	nd	nd	nd	nd	L+B
Liquorilactobacillus mali	nd	L	L	nd	L	nd
Liquorilactobacillus nagelii	L+B	nd	nd	nd	B	B

L = detected in the liquid, B = detected in the biofilm, L+B = detected in both phases, nd = not detected in any phase.

For the yeasts, *Brettanomyces bruxellensis* was found in green and black tea kombucha in both liquid and biofilm every year. It was the only yeast species identified in 2020 in black tea kombucha, and it was identified along with *Candida californica* in green tea kombucha. *Hanseniaspora valbyensis* was also widely detected for both tea types in 2019 and 2021. *Saccharomyces cerevisiae* was detected in 2019 in black tea kombucha only, and in 2021 for both tea types. Species such as *Hanseniaspora opuntiae*, *Galactomyces geotrichum* and *Pichia aff. fermentans* were only found in the biofilm of black tea kombucha, in 2019. The same year, *C. californica* and *Candida boidinii* were isolated from green tea kombucha biofilm. Finally, the species *Zygosaccharomyces florentinus* was detected for the first time in 2021.

Regarding bacteria, the genera *Acetobacter* has been widely identified in black and green tea kombuchas over the years, except in 2021 black tea kombucha. However, the presence of species was not consistent between the years or tea types, but *A. indonesiensis tropicalis* or *senegalensis* and *A. peroxydans* or *papayae* were identified more often than the other species. Other genera included *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter* were not specific to a year or a tea type. About lactic acid bacteria, *Liquorilactobacillus* (formerly belonging to the genus *Lactobacillus* (Zheng *et al.*, 2020)) was the only lactic acid bacteria genus identified and it was present in all samples except in 2020 black tea kombucha. Continuity in species could be observed between 2020 and 2021 for *L. mali* for green tea kombucha and for *L. ghanensis* and *L. nagelii* in black tea.

It appears that the yeast species *H. opuntiae*, *G. geotrichum* and *P. aff. fermentans* were specific to the biofilm along with many acetic acid bacteria species (*A. okinawensis*, *A. aceti* and *K. rhaeticus*), whereas it was not the case for lactic acid bacteria.

Overall, the loss of species diversity of yeasts in 2020 was not as marked in bacteria, where several different species were still found, under all conditions. Moreover, it seems that the diversity of the bacterial population was less impacted than that of the yeast population in the biofilm.

3.3 Macroscopic and microscopic examinations

To get more detail on populations according to genera and species, images were acquired using light microscopy to link the cellular aspect and colony morphotype on agar media to an identity. This was not possible with bacteria because of overall very similar colony aspect. For yeasts, morphological differences could be easily observed unlike bacteria. Since the morphotypes stayed consistent on all the samples regardless of the year, location (liquid of biofilm) or tea type. Only an image of each species detected in 2021 is presented (Figure C2).

There was a visible diversity of morphology, with very round and opaque cells for *S. cerevisiae* (Figure C2A), apiculate yeasts in the case of *H. valbyensis* (Figure C2B), and very granular cells for the species *Z. florentinus* (Figure C2C). On agar medium, *B. bruxellensis* (Figure C2D) was associated with small white (1-2 mm diameter, sometimes light green), bulging, opaque colonies, with a diameter of less than 2 mm, which took 48 to 72 hours to appear. Colonies of *H. valbyensis* (Figure C2C) were dark green, bulging, opaque, with a darker center. *S. cerevisiae* yeasts (Figure C2A) gave large white colonies (5-6 mm diameter, sometimes with a light green border), bulging and opaque. *Z. florentinus* (Figure C3D) was associated with very pale blue colonies, bulging, opaque, measuring between 1 and 2 mm. Finally, the colonies of *C. californica* (Figure C2E) were large (4-5 mm diameter), whitish, dull, opaque, and flat.

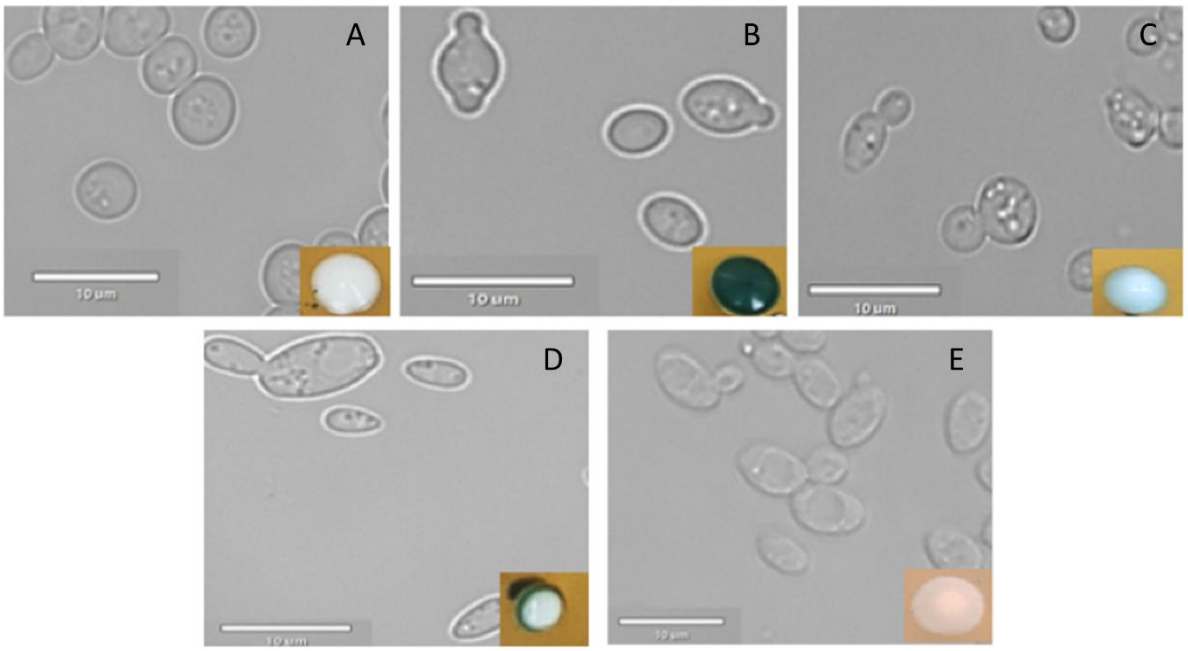

Figure C2 : Cell and colony morphologies on WL agar medium of yeast identified in green and black tea kombuchas: (A) *Saccharomyces cerevisiae*, (B) *Hanseniaspora. valbyensis*, (C) *Zygosaccharomyces florentinus*, (D) *Brettanomyces bruxellensis*, (E) *Candida californica*.

3.4 Proportions in yeast species

As yeasts have identifiable morphotypes, a morphotype count could be performed on WL medium to determine proportions. Figure C4 represents a translation of the "absolute" yeast populations shown in Tableaux C1 and C2. The diversity within green tea kombucha has fluctuated over the three years. Indeed, it was observed that in 2019, the two genera *Hanseniaspora* and especially *Brettanomyces* were found in a major proportion in both phases, the genus *Candida* being in minority in SCOBY only (< 1%) (Tableau C1). In 2020, the diversity decreased especially for the biofilm where only *B. bruxellensis* was detected, the liquid phase having preserved *C. californica* in smaller proportion (9%). On the other hand, the genus *Hanseniaspora* was not detected. Finally, in 2021, diversity has been restored and was even higher than in 2019. The genera *Saccharomyces* and *Zygosaccharomyces* were detected for the first time in the green tea matrix, in important proportion for *Saccharomyces cerevisiae* (47% in liquid phase) and the specie *H. valbyensis* was again present (Tableau C2). It was observed that the species *Brettanomyces* was still represented, especially in the biofilm, where it represented 51%

of the colonies (Figure C3). This species has been detected in an important proportion (even mostly) every year.

In black tea kombucha, the representation of different genera also fluctuated during the three years. In 2019, the genus *Hanseniaspora* and *Brettanomyces bruxellensis* were together in majority, respectively 64% and 36% for the liquid phase. In the biofilm, *Pichia aff. fermentans* was also well represented (29%), whereas *G. geotrichum* was a minority (< 1%). Other genera were detected but their presence was very low (Tableau C2). In 2020 as in green tea, a massive drop in diversity was observed to the point that only *B. bruxellensis* was detected for each of the phases. In January 2021, the diversity partly recovered as well in black tea kombucha: the species *H. valbyensis* was detected again and represented 10% and 14% of the colonies (Figure C3), but the other two genera (*Pichia* and *Galactomyces*) were not detected. Two new species were detected for black tea kombucha, which were *Zygosaccharomyces florentinus* and *Candida californica*, in minor proportions.

Comparison of tea types showed that five yeast species were detected for the black tea matrix, three for the green tea matrix in 2019. Oppositely, in 2020 only the species *B. bruxellensis* was detected in both matrices, as well as *Candida* for green tea. Nevertheless, the diversity increased again in 2021, where five species were detected for each matrix, some of which were already present in 2019 and 2020, such as *B. bruxellensis* for example, or *Candida*. Black tea kombucha had a greater diversity than green tea, especially in the biofilm. Even if the proportions varied, the species found in 2021 were the same for both tea matrices. Finally, the species *Brettanomyces bruxellensis* was the most represented yeast species for the three years studied.

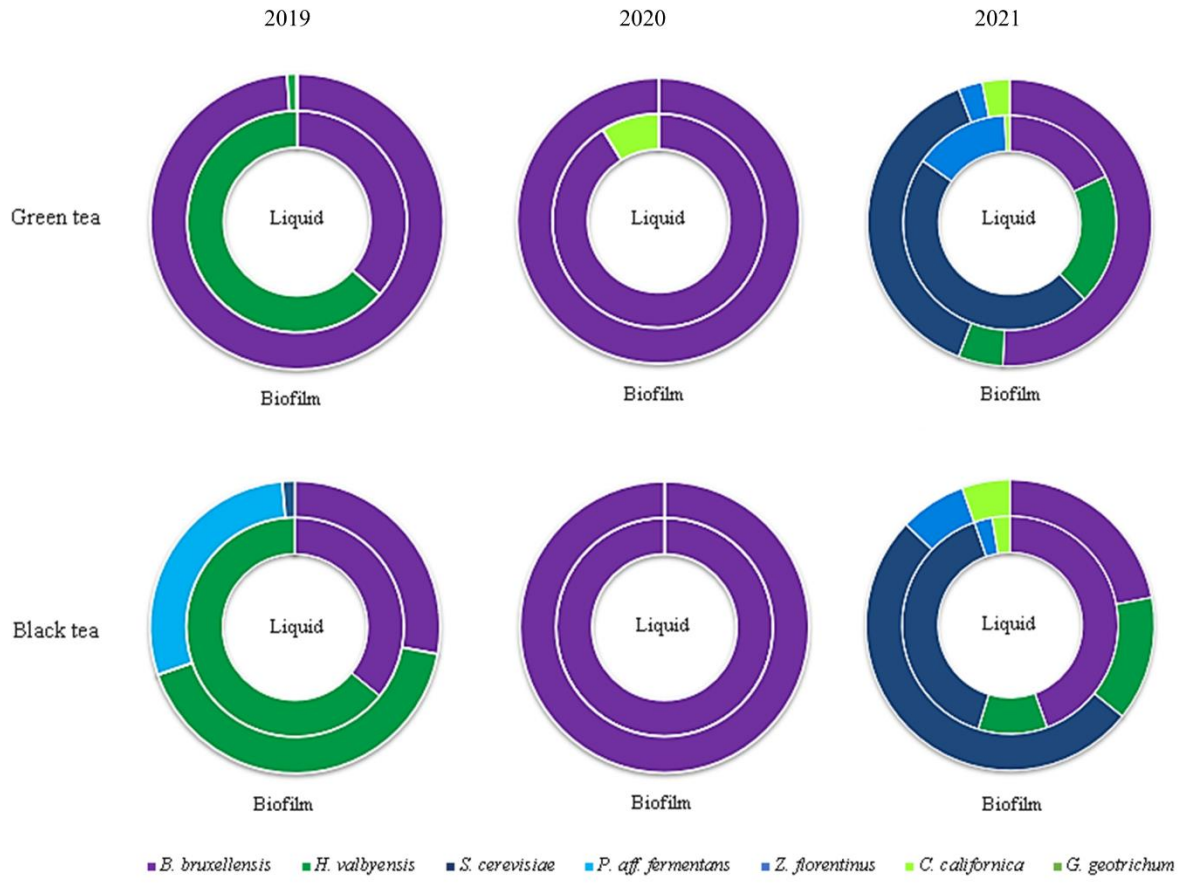

Figure C3 : Proportions in yeasts species in green and black tea kombuchas liquid and biofilms samples over 3 years. Internal circles refer to the liquid phase and external circles to biofilm.

4 Discussion

Analysis of microbial populations and the identification of isolates in kombucha liquid and biofilm samples gave insights into proportion of yeasts and bacteria species. Several species of yeasts have been identified for several years and in variable proportions (Figure C4): *B. bruxellensis, H. valbyensis, C. californica* and *S. cerevisiae*, which have already been identified in kombucha (Villarreal-Soto *et al.*, 2018). When comparing the 3 years, *B. bruxellensis* was found in all conditions (biofilm and liquid of the two teas) every year, which suggests high adaptation to the sugared tea matrix. Moreover, the species *H. valbyensis* was found in every kombucha sample of 2019 and 2021. Thus, *B. bruxellensis* and *H. valbyensis* were predominant yeast species in kombuchas according to recent studies (Harrison and Curtin, 2021; Leech *et al.*, 2020). Additionally, the proportions also varied from one year to the other. For example, *H. valbyensis* was more present in the two kombucha matrices in 2019 than in 2021(Figure C5). Dominance of the genus *Brettanomyces*, specifically *B. bruxellensis* has been reported along with the presence of

the genera *Saccharomyces* and *Zygosaccharomyces* (Coton *et al.*, 2017). Additionally, the genus *Hanseniaspora* was sometimes found in greater or lesser proportions according to studies (Coton *et al.*, 2017; Harrison and Curtin, 2021). Other works reported the presence of the genera *Torulaspora, Starmerella* and *Zygotorulaspora,* which were not found in our samples (Coton *et al.*, 2017; Harrison and Curtin, 2021; Marsh *et al.*, 2014; Reva *et al.*, 2015). The differences observed may be due to the specificities intrinsic to each kombucha consortia, but also to the fact that the studies cited have used a metagenomic approach, which allowed the detection of viable but nonculturable or dead microorganisms.

The loss of yeasts diversity could be explained by the sampling itself, because the entire kombucha production or the entire mother could not be analyzed, but the most probable hypothesis is a problem of thermal regulation within the place of production (a heat wave that took place during the summer of 2019; Figure C1), which would have negatively impacted some yeast species more sensitive to heat. The implementation of a temperature regulation of the premises between 2020 and 2021 would have made it possible to find a diversity for the samples of January 2021, with nevertheless a modification of the species present and the representativeness. In all cases, the resilience of *B. bruxellensis* against the perturbation that caused the loss of diversity observed suggests high adaptability in this species, as it has been reported in wine (Smith and Divol, 2016). The detection of species that were detected in 2019 and not in 2020, shows that they were not destroyed by the stressful event, but suggests remanence instead.

Different species of bacteria could be identified, with a predominance of genera such as *Liquorilactobacillus* and *Acetobacter* (Tableau C3). The comparison of the three years showed different dynamics in comparison with the yeasts. Indeed, diversity was greater in bacteria, especially for the year 2020. But even though all genera were present in January 2021, differences between species were noticeable between the three years. These genera were also reported in the literature, along species like *O. oeni* and *A. okinawensis* in the green tea matrix, but also like *L. nagelii* and *L. satsumensis* for the black tea matrix. The genera *Gluconobacter* and *Komagataeibacter* were also present, but with different species than those described by (Coton *et al.*, 2017) More broadly, the genera *Komagataeibacter*, *Liquorilactobacillus* and *Acetobacter* were found in significant proportions in many kombucha biofilms (Harrison and Curtin, 2021)

Many lactic acid bacteria species were identified with eight different species in 2021, while the previous two years have combined six (Tableau C3). This could be explained by a change in their representation in the community or by the introduction of the M17 medium in 2020, which allowed the growth of these bacteria. It is worth noting that all identified lactic acid bacteria species were ex-*Lactobacillus* species that were re-classified as *Liquorilactobacillus* (Zheng *et al.*, 2020). Therefore, the change in nomenclature was beneficial in this study since it allowed to highlight a relationship between this new genus and kombucha and calls further investigation. The problem of thermal regulation mentioned upstream did not have a great impact on the bacterial population. The decrease in total bacterial population and the loss of diversity were less intense. Results regarding bacteria highlights higher resilience of the overall bacteria population associated to higher variability in genera and species in the consortia over time compared to yeasts. It is possible to hypothesize that the under-representation of the genera *Acetobacter* and *Gluconobacter* was due to sampling or to the diversity specific to each kombucha consortia.

Finally, the culture-dependent approach did not allow the detection of viable but in nonculturable microorganisms, in opposition to metagenomics. Nevertheless, it reflected more accurately on active populations involved in the transformation of the matrix, by putting aside dead and viable nonculturable microorganisms. Moreover, the use of colony morphotyping allowed a satisfactory degree of detail for the investigation yeast population at the genus and species level.

5 Conclusion

The microbial communities of kombucha have a complex nature because of their diversity, which could easily be observed under a microscope, more specifically for yeasts. Yet, this diversity could be investigated in detail thanks to the identification of different isolates through culture-dependent method. The use of differential and selective agar media, morphotyping and sequencing provided an accessible insight in the microbial composition of the kombucha cultures in an industrial context.

Changes in representations of population could be observed qualitatively a quantitatively. *B. bruxellensis* was constant in presence and dominance in all samples while other yeasts presence and proportion varied. Differences in representations between the different tea types (black or green tea) and the different phases (biofilm or liquid) allowed to conclude

on a certain preference of environment depending on the microbial species (Coton *et al.*, 2017; Teoh *et al.*, 2004; Tran *et al.*, 2021b). Stressful events such as heat waves in absence of temperature control during production can lead to changes in proportions and diversity within the consortium, especially in the case of the yeasts' community, which appears to be more sensitive to thermal stress than bacteria. However, remanence of certain species such as *Hanseniaspora valbyensis* could be observed. Composition in bacterial species was very variable but *Acetobacter* ad *Liquorilactobacillus* were predominant genera identified over the three years. If yeasts are more impacted by events and stresses related to production conditions, the functionality of the consortium for kombucha production will be strongly impacted, since yeasts' metabolism impacts more the fermentation kinetics (Tran *et al.*, 2020b). Therefore, it would be interesting to study the stress response of these microbial communities, and the effects on manufacturing processes, to increase the control over kombucha production.

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Author contributions

Perrine Mas and Thierry Tran took the lead of the writing of this article, but all other authors provided critical and complementary elements to the manuscript. Perrine Mas and Thierry Tran carried out the experiments. François Verdier and Antoine Martin provided the kombucha cultures used in the experiments.

Competing interest

Authors declare no conflict of interest.

D Chapitre 2 : Dynamique microbienne entre levures et bactéries acétiques dans la kombucha : impacts sur la composition chimique de la boisson.

Ce deuxième chapitre correspond à l'article "*Microbial dynamics between yeasts and acetic acid bacteria in kombucha: impacts on the chemical composition of the beverage*" publié dans le journal *Foods*. Il s'agit de la première analyse des interactions entre levures et bactéries acétiques isolées d'une kombucha thé noir (voir Chapitre 1).

Afin d'étudier les interactions microbiennes et leur impact sur la composition chimique de la boisson, un plan expérimental avec neuf couples associant une levure à une bactérie acétique a été mis en œuvre. Les espèces levuriennes sélectionnées étaient *Brettanomyces bruxellensis*, *Hanseniaspora valbyensis* et *Saccharomyces cerevisiae*, et les espèces bactériennes étaient *Acetobacter indonesiensis*, *Acetobacter papayae* et *Komagataeibacter saccharivorans*. Les monocultures de chaque micro-organisme réalisées dans du thé noir sucré (saccharose) ont été analysées afin de déterminer leur comportement individuel en conditions d'incubation ouverte ou fermée (14 jours). Les niveaux de population ont été déterminés par dénombrement sur milieux nutritifs gélosés sélectifs. Divers paramètres chimiques ont été analysés : sucres (glucose, fructose, saccharose), éthanol, acidité totale, acides organiques et azote aminé libre par analyse colorimétrique, kits enzymatiques et HPLC (chromatographie liquide haute performance). L'effet Custer a notamment été observé chez *B. bruxellensis*. Par la suite, une culture de kombucha originale (d'où les micro-organismes ont été isolés) a été comparée aux cocultures levure-bactérie acétique en thé sucré afin de mettre en évidence des interactions microbiennes au cours des deux phases de production (incubation ouverte 14 jours puis fermée 10 jours). Les résultats ont montré que le métabolisme levurien impactait principalement la composition chimique du produit et que l'influence secondaire des bactéries acétiques portait sur la composition en acides organiques. La mise à disposition des substrats carbonés que sont les monosaccharides et l'éthanol par certaines levures conditionne la cinétique d'acidification opérée par les bactéries à travers la production des acides acétique et gluconique. Les interactions ainsi mises en évidence peuvent être expliquées par différentes stratégies d'utilisation du saccharose. Les bactéries acétiques et la levure *H. valbyensis* incapables d'hydrolyser efficacement le saccharose tirent profit des autres levures présentes dont l'activité invertase est plus intense, afin d'accéder aux monosaccharides ainsi libérés. De plus, la présence des bactéries acétiques stimule à leur avantage l'activité invertase et le métabolisme fermentaire de *S. cerevisiae* (cas de « dilemme du prisonnier »).

Cette étude nous informe sur le rôle clé du métabolisme des levures comme facteur limitant pour l'acidification, paramètre principal de la transformation du thé sucré en kombucha. Ainsi, il peut être établi qu'un consortium de kombucha « minimal », doit associer une levure à hautes activités invertase et fermentaire en association avec une bactérie acétique. L'étude a permis également d'établir une sélection de micro-organismes pour l'étude fine des interactions, en respectant le principe énoncé précédemment. L'espèce *B. bruxellensis* a été sélectionnée pour remplir le rôle de levure à hautes activités invertase et fermentaire en plus d'être l'espèce avec la population la plus élevée dans le consortium original. *A. indonesiensis* est l'espèce de bactérie acétique sélectionnées pour réaliser l'acidification. Ce choix a été fait car *K. saccharivorans* a montré une capacité à maintenir sa production d'acide acétique durant la seconde phase de production, rendant plus difficile la maîtrise du niveau d'acidité dans le produit final. Le choix entre *A. indonesiensis* et *A. papayae* a été fait arbitrairement faute d'élément décisif les distinguant. *H. valbyensis* a également été sélectionnée pour investiguer les interactions levure-levure, du fait de sa représentation importante au sein du consortium contrastant avec une absence de rôle technologique apparent (faibles activités invertase et fermentaire) par opposition à *B. bruxellensis*. A défaut de rôle technologique, cette espèce de levure pourrait revêtir une fonction pour l'écologie du consortium.

Microbial Dynamics between Yeasts and Acetic Acid Bacteria in Kombucha: Impacts on the Chemical Composition of the Beverage

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Abstract

Kombucha is a traditional low-alcoholic beverage made from sugared tea and transformed by a complex microbial consortium including yeasts and acetic acid bacteria (AAB). To study the microbial interactions and their impact on the chemical composition of the beverage, an experimental design with nine couples associating one yeast strain and one AAB strain isolated from original black tea kombucha was set up. Three yeast strains belonging to the genera *Brettanomyces*, *Hanseniaspora*, and *Saccharomyces* and three strains of *Acetobacter* and *Komagataeibacter* species were chosen. Monocultures in sugared tea were analyzed to determine their individual microbial behaviors. Then, cultivation of the original kombucha consortium and cocultures in sugared tea were compared to determine the interactive microbial effects during successive phases in open and closed incubation conditions. The results highlight the main impact of yeast metabolism on the product's chemical composition

and the secondary impact of bacterial species on the composition in organic acids. The uncovered microbial interactions can be explained by different strategies for the utilization of sucrose. Yeasts and AAB unable to perform efficient sucrose hydrolysis rely on yeasts with high invertase activity to access released monosaccharides. Moreover, the presence of AAB rerouted the metabolism of *Saccharomyces cerevisiae* towards higher invertase and fermentative activities.

Keywords

kombucha; yeasts; acetic acid bacteria; interactions; symbiosis; sucrose hydrolysis; pellicle.

1 Introduction

Fermented food is often designated as a convenient model for the study of microbial communities and interactions (Ivey *et al.*, 2013; Sieuwerts *et al.*, 2008; Wolfe and Dutton, 2015). Kombucha is an increasingly exploited example of a fermented beverage obtained by microbial communities. Kombucha, also named “kombucha tea”, results from the metabolic interplay of a microbial consortium including acetic acid bacteria (AAB), yeasts, and often (but not always) lactic acid bacteria in sugared tea liquor (Dufresne and Farnworth, 2000; Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). Infusion provides nitrogenous substances extracted from tea necessary for the growth of microorganisms. Sucrose is converted into glucose and fructose by periplasmic yeast invertase and ethanol is produced as a result of alcoholic fermentation. AAB oxidize glucose into gluconic acid and ethanol into acetic acid through oxidative metabolism. This metabolic scheme raises the question of a possible trophic dependency of AAB towards yeasts (Lynch *et al.*, 2019). Glucose and fructose are also used for bacterial cellulose production, leading to the formation of the pellicle, also known as “mother”, “tea fungus”, or Symbiotic Culture of Yeast and Bacteria (“SCOBY”), since it can be used as inoculum with or instead of liquid culture (Jayabalan *et al.*, 2014; La China *et al.*, 2018; Villarreal-Soto *et al.*, 2018). As a result, the beverage has the profile of a soda with a sweet/sour balance and can also be carbonated naturally if the vessel is left closed after a first phase of biological acidification at ambient temperature (Tran *et al.*, 2020b). There is no single “culture” or microbial consortium for the production of

kombucha, but a multitude of matrix-adapted consortia whose origins are unknown. This matrix also offers two distinct environments: A liquid phase, where microorganisms are in a planktonic state, and a pellicle, where they are entrapped. Biofilms are known to host numerous interaction mechanisms among microbial communities and the cellulosic pellicle produced by the AAB of kombucha is no exception (Carlier *et al.*, 2015; Flemming *et al.*, 2016; Gullo *et al.*, 2018). Beyond this trophic interaction, little is known about other types of interactions that might occur during kombucha fermentation at genus or species levels. The formation of the pellicle, as well as the drop of pH, can work as protection against contamination by exogenous spoilage agents such as molds (May *et al.*, 2019). Inside the consortium, inter-kingdom and intra-kingdom interaction mechanisms remain poorly documented.

Some studies have provided clues on the existence of interactions through the lens of microbial dynamics. The results have shown that some bacterial species are dominant, but the dynamics are dependent on the type of tea used (black or green) (Coton *et al.*, 2017). It has also been shown that the population of some yeast species decreases during kombucha fermentation, while others remain stable, as was the case for *Torulaspora delbrueckii* or *Schizosaccharomyces pombe* in the presence of *Zygosaccharomyces bailii*. In this case, the impacted yeast species were dependent on the consortium and this phenomenon did not occur in pellicles, with all populations being maintained (Teoh *et al.*, 2004). Microbial interaction data based on indigenous complex consortia are difficult to summarize because of the complexity of the original system. Focus has been placed on microbial dynamics in terms of populations, without investigating the impact on the chemical composition of the beverage. To the best of our knowledge, only one study has investigated the interactions between individual isolates from kombucha by cultivating AAB (*Acetobacter* sp.) in sugared black tea with the addition of autoclaved sugared black tea fermented by a single strain of yeast (*Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Zygosaccharomyces bailii*) (Liu *et al.*, 1996). This study concluded on the positive impact of the addition of fermented medium on AAB growth and also suggested that acetic acid produced by AAB could stimulate the production of ethanol by yeast. A recent study compared an original kombucha consortium with an unknown microbial composition, and a synthetic consortium including purchased isolates of species commonly found in kombucha

(Wang *et al.*, 2020). Two AAB species, consisting of *Acetobacter pasteurianus* and *Komagataeibacter xylinus*, were used with the yeast species *Z. bailii*. The study confirmed the technical suitability of using a synthetic consortium of yeasts and AAB for the fermentation of kombucha beverages and highlighted the metabolic interplay between yeasts and AAB.

The present study goes further in the study of the yeast–bacterium interaction that occurs during kombucha fermentation in terms of both microbiological levels and the composition of the main metabolites produced. Yeasts and bacterial strains were isolated from an original black tea kombucha culture, identified, and selected to develop an experimental design with three yeast strains and three AAB strains. Each yeast and AAB was characterized individually, co-inoculated (one yeast strain x one AAB strain, with "x" indicating a coculture) in sugared black tea, and compared to the original consortium. This methodology was inspired by the integrated design for the study of microbial communities described by Lawson *et al.* (2020), consisting of joint studies of the original consortium, with simplified and deconstructed consortia reassembled using isolates (Lawson *et al.*, 2019). Moreover, on top of the aerobic acidification phase, the anaerobic carbonation phase in a closed bottle was investigated for the first time.

2 Materials and Methods

A diagram summing up the experiment is available as supplementary data (Annexe SD1).

2.1 Isolation and Identification of Microbial Species

Yeast and bacterial strains were isolated from liquid and pellicle samples of black tea kombucha from the company Biomère (Paris, France). WallersteinLab (WL) agar medium from Thermo Fisher Scientific (Waltham, MA, USA) was used to isolate yeasts (Green and Gray, 1951). Bromocresol Green allowed a macroscopic discrimination of colonies based on their aspect and color for the set of yeasts isolated in the present study (Hall, 1971; Pallmann *et al.*, 2001). De Man Rogosa and Sharpe (MRS) (pH 6.2) from Condalab (Madrid, Spain), LAC (pH 5.1), and Mannitol agar media (Simonin *et al.*, 2018) were used for the isolation of bacteria that may have different nutritional

requirements in both aerobic and anaerobic conditions of incubation (Salma *et al.*, 2013). All reagents used in agar media were purchased from Merck (Darmstadt, Germany), if not otherwise specified.

Five yeast colonies per colony morphotype were separately picked-up for inoculation in Yeast Peptone Dextrose (YPD) liquid medium (48 h at 28 °C). Twenty-five bacterial colonies per agar medium were separately picked-up for inoculation in LAC medium (48 h at 28 °C in the aerobic condition of incubation), since all species identified on Mannitol agar medium were also identified on LAC agar medium. After incubation, culture media were eliminated by centrifugation (14.500 *g*, 3 min at 15 °C). A half volume of appropriate liquid medium with a half volume of 40% (v/v) glycerol was then added to the samples to make a stock kept at −20 °C.

DNA extraction from each isolated strain was performed using an Instagen Matrix kit (Bio-Rad, Hercules, CA USA). For yeasts, the 26S rDNA region ribosomal non transcribed spacer 2 (NTS 2) was amplified using the following primers: NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and *NL4* (5′-GTCCGTGTTTCAAGACGG-3′) (Maoura *et al.*, 2005). Two microliters of extracted DNA were added to 25 µL PCR mix (1.8 mM $MgCl_2$, 0.25 mM dNTPs, 1.25 µM of each primer, and 0.025 U Taq polymerase) (Promega Corp., Madison, WI, USA). A Biorad (Hercules, CA, USA) thermocycler was used as described previously (Esteve-Zarzoso, 2019).

For bacteria, 16S ribosomal DNA was amplified using the following primers: E517F (5′-GCCAGCAGCCGCGGTAA-3′) and E106R (5′-CTCACGRCACGAGCTGACG-3′). Amplification was performed in the same conditions as for the yeasts, except for the annealing temperature, which was changed to 58 °C (Wang and Qian, 2009). Both strands Sanger sequencing was performed on amplified DNA by Genewiz® (Leipzig, Allemagne) using NL1 and NL4 primers for yeasts and E515F and E106R primers for bacteria. The sequences obtained were then analyzed using the software Geneious R7 (version 7.1.5) and the Basic Local Alignment Search Tool (BLAST) available on NCBI's website (https://blast.ncbi.nlm.nih.gov/Blast.cgi), thus returning genus and species names.

2.2 Growth Conditions

In order to generate pure precultures, yeasts were restreaked on WL agar and bacteria were restreaked on MRS agar from stocks kept at −20 °C and incubated at 28 °C. MRS agar was chosen because it is a commonly used growth medium for exigent bacteria, and the growth of each isolated AAB species on this medium was tested prior to the experiment.

Cultivation of the original kombucha consortium and cocultures was initiated in sugared tea. To produce 1 L of sugared black tea, tap water was filtered through a Brita (Taunusstein, Allemagne) charcoal cartridge and sterile filtered by a Steritop® cartridge by Merck (Darmstadt, Allemagne). Then, 200 mL of sterile tap water was boiled and directly taken off the heating source. One gram of black tea (Pu'er Grade 1 TN4107 from "Les jardins de Gaïa") (Wittisheim, France) was immediately added and left for infusion for one hour at room temperature. Tea was then removed using a sieve and transferred to a sterile vessel. A total of 800 mL of sterile tap water at 26 °C was added. Then, 60 g sucrose was added and dissolved completely. The mix was kept at 26 °C in a closed Schott® flask for one hour, prior to inoculation. Yeast and AAB strains were cultivated in YPD and MRS liquid medium, respectively. The tubes were not fully closed, in order to allow gaseous exchange during the incubation at 28 °C in static conditions for 3 days. Before inoculation in sugared tea, cells from pure cultures were washed with sugared black tea and then centrifugated (3.000 *g*; 10 min at 4 °C). The populations of pure cultures were determined using a BD Accuri C6 (Franklin Lakes, NJ, USA) flow cytometer coupled with 0.1 µg mL^{-1} propidium iodide marking for cell mortality (Stiefel *et al.*, 2015).

The choice of the target inoculation rate for individual species was based on the population levels determined for original kombucha at the inoculation time. Consequently, sugared black tea was then inoculated using precultures with an initial population of yeast or AAB of 1.10^5 cells mL^{-1}. For cocultures, the same process as that employed for pure cultures was used, with initial populations of 1.10^5 cells mL^{-1} for both yeast and bacterium strains.

The production of kombucha was carried out using a kombucha mother culture maintained in the lab from the original black tea kombucha sample from Biomère (Paris, France) by adding a half volume of sugared black tea each month. Kombucha mother culture was added to sugared black tea at the rate of 12% (v/v) and kept at 26 °C for 14 days in a 500 mL Schott® bottle, in order to produce a fresh kombucha inoculum. 12% of this inoculum was added to fresh sugared black tea to produce the traditional kombucha used in this study.

Monocultures, cocultures, and original kombucha incubations were performed in 125 mL Boston flasks with a Specific Interfacial Surface (SIS) of 0.01 cm^{-1} in a final volume of 123 mL (Cvetković *et al.*, 2008). For monocultures in an open incubation condition (IC), bottlenecks were loosely covered with tin foil to allow gas exchange, whereas for closed IC, flasks were closed using a cap to reflect bottling. Monocultures were characterized in both open and closed IC at 26 °C after 7 and 14 days for microbiological analysis and after 14 days for chemical analyses. Cocultures and kombucha incubations were carried out in two phases. The first phase (P1) of fermentation was performed for 14 days at 26 °C in the same open IC as monocultures. Then, half of the volume was withdrawn for analysis ("P1" samples). The bottles were then sealed, in order to trigger natural carbonation. This phase (P2) was performed at 26 °C for 10 days. At the end, the samples were analyzed ("P2" samples). Open and closed IC do not strictly reflect aerobic or anaerobic systems, but rather the production process of kombucha making in industrial conditions. Dissolved oxygen concentrations were not measured in samples. A description of the research samples and abbreviations is detailed as supplementary data (Annexe SD2).

The populations of yeasts and/or bacteria during fermentation were determined by plating successive decimal dilutions of samples on WL agar for yeasts and MRS agar for bacteria, with technical triplicates for each biological triplicate.

2.3 Chemical Analyses

For chemical analyses, samples were kept frozen at −20 °C and centrifuged prior to chemical analyses (3.000 *g*; 15 min, 10 °C). pH values were measured with a Mettler Toledo Five Easy pH meter coupled with an LE498 probe. The total acidity was

determined by titration with 0.1 N NaOH and 0.2% phenolphthalein as a color indicator (OIV, 2009).

Acetic, lactic, malic, and succinic acid concentrations were determined by HPLC with a VWR Hitachi (Tokyo, Japan) control unit, L-2350 oven, L-2200 autosampler injection device, and L-2130 pump. The column used was a Raptor ARC-18 2.7 µm 150 × 2.1 mm from RESTEK (Lisses, France) and the detector was a UV Diode Array Detector VWR Hitachi L-2455 (Tokyo, Japan) at 210 nm. The mobile phase was 97% 20 mM KH_2PO_4 (pH 2.4) and 3% methanol. A flow gradient was applied at 35 °C, with the following sequence for a total duration time of 10 min: From 0.1 mL min^{-1} to 1.0 mL min^{-1} for 5 min, 1.0 mL min^{-1} for 3 min, and 0.1 mL min^{-1} for 2 min.

Sucrose, glucose, and fructose concentration determinations by HPLC involved the same equipment, except for the column. A HyperREZ XP Carbohydrate Ca++ 8% column from Thermofisher (Waltham, Etats-Unis) was used with a Spectrasystem RI-150 refractometer from de JMBS (Mandelieu-Napoule, France). The flow rate was 0.6 mL min^{-1} with ultrapure water at 80 °C.

Gluconic acid and ethanol concentrations were determined using enzymatic kits from Biosentec (Auzeville-Tolosane, France).

The free amino nitrogen (FAN) concentration was determined according to the protocol from MEBAK® (2013), with the following adaptation. In total, 0.4 mL 20-fold diluted sample was added to 0.2 mL reactive mix (0.71 M Na_2HPO_4, 0.44 M KH_2PO_4, 28 mM ninhydrin, and 17 mM fructose). The mix was heated for 16 min at 100 °C and left to cool down for 20 min at room temperature. Then, 0.8 mL dilution solution (40% (v/v) ethanol, 12 mM potassium iodide) was added before the absorbance reading at 570 nm using a UV-1800 spectrophotometer from Shimadzu (Kyoto, Japan). Distilled water was used as the blank. A calibration curve was made using a glycine solution.

Analytical chemistry results were expressed as the balance, according to the following formula (1):

$$\Delta\text{Concentration} = \Delta\text{Endpoint concentration} - \Delta\text{Initial concentration.} \quad (1)$$

Initial corresponds to day 0 and the "endpoint" corresponds to day 14 for monocultures and "P1" or "P2" for cocultures.

2.4 Statistical Analyses

All samples were made in triplicate. Values were treated with ANOVA and in the case of significant differences ($p < 0.05$), the Newman–Keuls pair test was applied. Principal Component Analysis (PCA) coupled with Hierarchic Ascending Classification (HCA) was also performed for P1 and P2 values separately. All statistical analyses were performed with R software (version 3.5.2.).

3 Results and Discussion

3.1 Isolation and Identification of Yeast and Bacterial Strains

Based on the colony morphology on WL agar, yeast species in the original black tea kombucha sample were isolated and then identified by DNA sequencing of the 26S ribosomal region (Maoura *et al.*, 2005). In the liquid phase, *Brettanomyces* (*Dekkera*) *bruxellensis* (small white colonies), *Hanseniaspora valbyensis* (dark green colonies), and *Saccharomyces cerevisiae* (large white colonies) were identified. In the biofilm, the same species were isolated, in addition to *Hanseniaspora opuntiae* (bright green colonies), *Pichia aff. fermentans* (white star-shaped colonies), and *Galactomyces geotrichum* (white filaments). Yeast colony morphologies were all distinct for each species. On agar plates, all AAB colonies had a sticky and translucid aspect. DNA sequencing of the 16S ribosomal region (Wang and Qian, 2009) allowed the identification of the species *Acetobacter indonesiensis*, *Acetobacter papayae*, and *Komagataeibacter saccharivorans*, which were all isolated in both liquid and biofilm phases. All species names were associated with E-values equal to 0. No lactic acid bacteria were identified, despite the use of LAC medium in both aerobic and anaerobic conditions of incubation. Except for *Galactomyces geotrichum*, the genera of isolated yeasts and bacteria are commonly found in kombucha (Villarreal-Soto *et al.*, 2018). For this study, species found in the liquid phase were chosen because they were present in

both liquid and biofilm. Each of the selected strains was characterized in sugared black tea.

3.2 Characterization of Pure Cultures in Sugared Black Tea

Figure D1 presents the population levels of each selected strain inoculated in sugared black tea after inoculation on day 0 and after incubation for 7 days and 14 days at 26 °C in open and closed incubation conditions (IC).

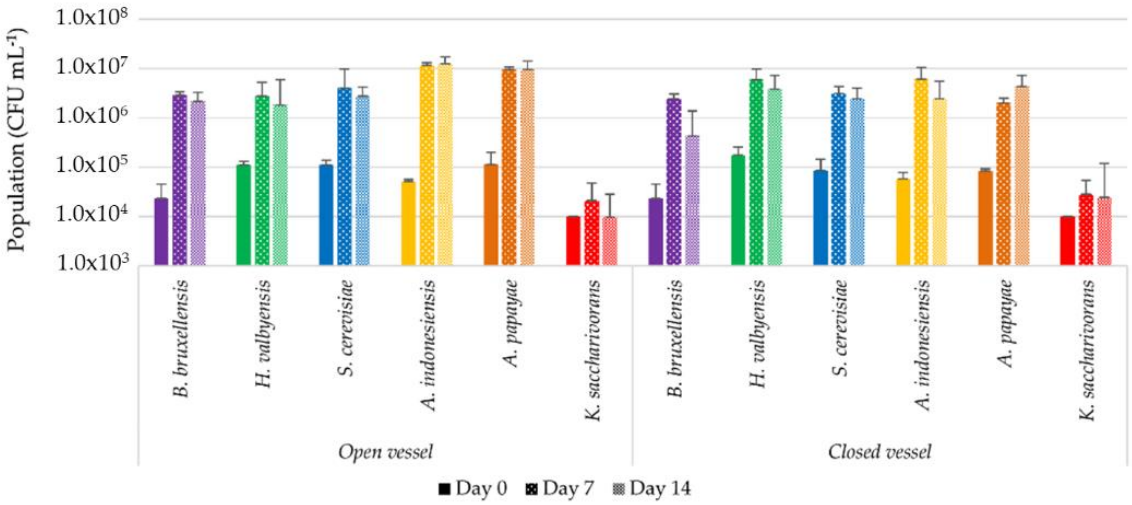

Figure D1 : Microbial populations during cultivation in sugared black tea of a monoculture of yeast and bacterial strains isolated from black tea kombucha determined by plate counting (CFU mL^{-1}). Error bars correspond to the confidence interval with α = 0.05 (*n* = 3). Cultures were conducted in open vessel (left) or closed (right) conditions of incubation for 14 days.

Yeast population levels increased from $1.0.10^5 \pm 5.10^4$ CFU mL^{-1} to values between $1.0.10^6$ CFU mL^{-1} and $6.0.10^6$ CFU mL^{-1}, regardless of the open or closed IC after 7 days. These levels of population were maintained 7 days later.

Except for *K. saccharivorans*, AAB's population levels increased from $1.0.10^5 \pm 5.10^4$ CFU mL^{-1} to values between $1.0.10^6$ CFU mL^{-1} and $1.0.10^7$ CFU mL^{-1}, regardless of the open or closed IC after 7 days. It can be supposed that, on top of assimilable nutrients, the medium initially possessed a sufficient dissolved oxygen level to allow the

growth of AAB in closed IC. These levels of population were maintained 7 days later. *K. saccharivorans* possessed a lower population at day 0 and the population only increased by less than 1 log. Discrepancies between the target inoculation rate and plate counting results at day 0 could be due to the viable but not culturable (VBNC) state, as reported for *Komagataeibacter xylinus* (Millet and Lonvaud-Funel, 2000; Zou *et al.*, 2020).

The average initial composition of sugared black tea was 66.6 ± 3.3 g L^{-1} total sugars (66.4 g L^{-1} sucrose, 0.2 g L^{-1} fructose, and no glucose detected). The FAN concentration was 63 ± 4 µg L^{-1}, which is very low compared to grape must, for example (between 50 and 150 mg L^{-1}) (Lee and Schreiner, 2010). The initial average pH value was 6.64 ± 0.47 units and average total acidity was lower than 1 meq L^{-1}. No organic acids nor ethanol were detected. The chemical composition variation of sugared black tea after the incubation of each culture (day 14) is detailed in Tableau D1.

For yeasts, the decrease of total sugars indicated a maximal sugar variation of −10.7 g L^{-1}. No groups could be formed among the different values, despite significant differences ($p < 0.05$). Sucrose underwent variations from null to total disappearance (−68.3 g L^{-1}), with significant differences between the values ($p < 0.05$). The monosaccharide content varied significantly from 0.0 to 28.5 g L^{-1} for glucose ($p < 0.05$) and from 0.0 to 31.8 g L^{-1} for fructose ($p < 0.05$). Their production succeeded sucrose hydrolysis. A significant production of ethanol was also observed, with variations between 0.1 and 3.2 from 0.0 to 28.5 g L^{-1} ($p < 0.05$), which is representative of the fermentation activity. The results show very different sugar consumption behaviors across the three yeasts species and highlight the link between invertase activity, respiration, and fermentation. *B. bruxellensis* is characterized by high invertase activity and fermentative metabolism in both open and closed IC. For this strain, ethanol production was significantly higher in open IC than closed IC ($p < 0.05$). The inhibition of alcoholic fermentation under anaerobic conditions is called the Custer effect and was reported for *B. bruxellensis* (Aguilar Uscanga *et al.*, 2003; Custer, 1940). *H. valbyensis* is characterized by poor sucrose hydrolysis and fermentative capacities, particularly in open IC. The very low consumption of sucrose was reported in the context of cider production (Nogueira *et al.*, 2008). The same applied to *S. cerevisiae* in open IC, but

the presence of invertase activity is more evident and points to the induction of *SUC* genes in the presence of a low glucose concentration (Meijer *et al.*, 1998; Özcan *et al.*, 1997). This mechanism allows *S. cerevisiae* to progressively hydrolyze sucrose and consume monosaccharides, without inducing catabolic repression by glucose. However, oxygen limitation in closed IC induced fermentation and increased sugar consumption (Pasteur effect), as reported by the data summarized in Marques *et al.* (2016). According to this review, the increase in sugar consumption may be a consequence of the lower energetic yield of fermentative metabolism compared to respiration. Then, it can be supposed that the low sucrose consumption observed in *S. cerevisiae* monoculture in open IC is associated with respiratory metabolism, which could also be the case for *H. valbyensis*. In addition, slightly lower glucose concentrations could be observed for modalities associated with fermentative metabolism. Studies have reported preferential glucose consumption during the alcoholic fermentation of grape must (Berthels *et al.*, 2004, 2008). The free amino nitrogen (FAN) concentration increased in all modalities between 3 and 54 µg L^{-1} and it also seems to be enhanced by fermentative metabolism induced by oxygen limitations, although a significant increase between open and closed IC was only observed for *S. cerevisiae* ($p < 0.05$)

Tableau D1 : Change in the chemical composition of sugared black tea by pure cultures of yeast and bacterial strains isolated from black tea kombucha after 14 days in open and closed conditions of incubation.

Microorganism	*B. bruxellensis*		*H. valbyensis*		*S. cerevisiae*		*A. indonesiensis*		*A. papayae*		*K. saccharivorans*	
Incubation Condition	Open Vessel	Closed Vessel	Open Vessel	Closed Vessel	Open Vessel	Closed Vessel	Open Vessel	Closed Vessel	Open Vessel	Closed Vessel	Open Vessel	Closed Vessel
ΔTotal sugars (g L^{-1}) *	−10.7 ^{a}	−10.6 ^{a}	−2.2 ^{a}	−9.4 ^{a}	0.0 ^{a}	−7.9 ^{a}	−3.6 ^{a}	−0.9 ^{a}	−0.7 ^{a}	0.0 ^{a}	−6.2 ^{a}	−10.0 ^{a}
ΔSucrose (g L^{-1})	−68.3 ^{d}	−68.3 ^{d}	−2.2 ^{a}	−15.4 ^{ab}	−36.1 ^{c}	−68.3 ^{d}	−6.9 ^{ab}	−0.9 ^{a}	−1.0 ^{a}	0.0 ^{a}	−12.3 ^{ab}	−19.6 ^{b}
ΔGlucose (g L^{-1})	27.0 ^{a}	26.9 ^{a}	0.0 ^{d}	0.9 ^{d}	18.9 ^{b}	28.5 ^{a}	2.1 ^{d}	nd	nd	nd	4.2 ^{d}	9.0 ^{c}
ΔFructose (g L^{-1})	29.8 ^{a}	30.8 ^{a}	0.0 ^{d}	5.0 ^{c}	17.2 ^{b}	31.8 ^{a}	1.1 ^{cd}	nd	0.2 ^{d}	0.4 ^{d}	1.9 ^{cd}	0.6 ^{d}
ΔEthanol (g L^{-1})	3.2 ^{a}	2.5 ^{b}	0.1 ^{d}	1.5 ^{c}	0.4 ^{d}	2.4 ^{b}	nd	<0.1	nd	nd	nd	<0.1
ΔFree amino nitrogen (µg L^{-1})	37 ^{a}	52 ^{a}	7 ^{b}	31 ^{ab}	3 ^{b}	54 ^{a}	3 ^{b}	30 ^{ab}	8 ^{b}	29 ^{ab}	8 ^{b}	28 ^{ab}
ΔpH	−2.13 ^{cd}	−1.86 ^{cd}	−1.07 ^{b}	−2.26 ^{cd}	−0.38 ^{a}	−1.67 ^{c}	−2.64 ^{d}	−2.25 ^{cd}	−2.05 ^{cd}	−2.04 ^{cd}	−2.61 ^{d}	−2.50 ^{d}
ΔTotal acidity (meq L^{-1})	8.0 ^{abc}	6.3 ^{abc}	2.0 ^{c}	6.7 ^{abc}	1.0 ^{c}	4.7 ^{bc}	11.0 ^{abc}	19.3 ^{a}	7.0 ^{abc}	5.7 ^{bc}	19.3 ^{a}	16.7 ^{ab}
ΔAcetic acid (g L^{-1})	0.38 ^{a}	0.25 ^{a}	nd	<0.1	<0.1	nd	0.25 ^{a}	0.49 ^{a}	0.28 ^{a}	0.38 ^{a}	0.40 ^{a}	0.56 ^{a}
ΔGluconic acid (g L^{-1})	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.91 ^{a}	0.11 ^{b}	0.56 ^{ab}	0.18 ^{b}	1.94 ^{a}	0.12 ^{b}
ΔLactic acid (g L^{-1})	nd	nd	0.46 ^{a}	0.20 ^{abc}	0.32 ^{ab}	nd	nd	nd	nd	nd	<0.1	nd
ΔSuccinic acid (g L^{-1})	1.27 ^{a}	1.19 ^{a}	0.11 ^{c}	0.76 ^{b}	0.34 ^{c}	1.27 ^{a}	<0.1	<0.1	nd	nd	0.14 ^{c}	0.16 ^{c}
ΔMalic acid (g L^{-1})	0.92 ^{a}	1.4 ^{a}	nd	1.00 ^{a}	nd	1.24 ^{a}	nd	0.22 ^{b}	<0.1	0.18 ^{b}	nd	<0.1

*: ΔConcentration = ΔEndpoint concentration − ΔInitial concentration; nd = not detected; common letters imply non-significant differences between average values (ANOVA test with $\alpha = 0.05$ and $n = 3$).

The increase in total acidity (between +1 and +8 meq L^{-1}) led to a decrease of the pH value, ranging from −1.7 to −2.7 units. This increase in total acidity was significantly more intense for *B. bruxellensis* compared to *S. cerevisiae* and the increase for *S. cerevisiae* was also more intense than for *H. valbyensis* in open IC ($p < 0.05$). *B. bruxellensis* is characterized by the production of acetic acid. Indeed, this species is strongly associated with the production of this organic acid (Aguilar Uscanga *et al.*, 2003). For the whole set of yeasts, the accumulation of succinic and malic acid and the absence of lactic acid production seem to be linked to fermentative metabolism in these conditions.

Despite the initial absence of monosaccharides added, AAB were able to consume between 0.7 and 10 g L^{-1} total sugars. The presence of monosaccharides after 14 days points to the hydrolysis of sucrose in the external medium. This phenomenon has been reported in several studies (De Ley, 1961; Sharma and Bhardwaj, 2019; Velasco-Bedrán and López-Isunza, 2007; Wang *et al.*, 2020). The total acidity increased between +5.7 and +19.3 meq L^{-1} and induced a drop of pH between −2.05 and −2.64 units, with significant differences between the values ($p < 0.05$). The production of gluconic acid from the oxidation of released glucose between +0.11 and +1.94 g L^{-1} was significantly lower with closed IC for all AAB species except *A. papayae* ($p < 0.05$) and could be explained by the limitation of oxygen (Lynch *et al.*, 2019). The production of acetic acid between +0.25 and +0.56 g L^{-1} without the initial presence of ethanol can be explained through the glycolysis and pyruvate metabolism of AAB (De Ley, 1961; De Ley and Schell, 1962; Prust *et al.*, 2005) and has been reported in the study of Wang *et al.* (2020) (Wang *et al.*, 2020). Succinic acid production occurred between +0.14 and +0.16 g L^{-1} for *K. saccharivorans* monocultures and malic acid production only occurred for *Acetobacter* sp. cultures in closed IC. It is worth noting that no consistent biofilm was produced after 14 days in all conditions; only floating cellulose fragments were visible. Overall, AAB could hydrolyze sucrose and consume monosaccharides. Oxygen limitations in closed IC impacted oxidative metabolism converting glucose into gluconic acid, and thus a limited acidification of the medium.

The interpretation of coculture results in comparison with original kombucha fermentation on the basis of monoculture characterization will help clarify the impact of each metabolic profile on the yeast–AAB interactions.

3.3 Comparison of Yeast-Acetic Acid Bacteria Cocultures with Original Kombucha Fermentation

3.3.1 Microbial Dynamics

The experimental plan for yeast-AAB cocultures and their abbreviations are presented in Tableau D2. These pairings can be seen as minimal kombucha consortia that allow the metabolic interplay necessary for the fermentation of kombucha. This process occurred at 26 °C in two phases: An initial 14-day phase in open IC and a second phase of 10 days in closed IC.

Tableau D2 : "Yeast x Acetic acid bacteria" couples used for cocultures.

Cocultures	*Acetobacter indonesiensis*	*Acetobacter papayae*	*Komagataeibacter saccharivorans*
Brettanomyces bruxellensis	BB x AI	BB x AP	BB x KS
Hanseniaspora valbyensis	HV x AI	HV x AP	HV x KS
Saccharomyces cerevisiae	SC x AI	SC x AP	SC x KS

All yeast populations in coculture (Figure D2a,c,e) at day 14 remained significantly lower (1 to 0.5 log) than those in yeast monocultures at day 14 in open IC (Figure D1). This could mean that the presence of AAB lowered the population of yeasts, regardless of the species. Possible explanations could be nutritional competition (Ivey *et al.*, 2013) or inhibition through the production of acetic acid (see Tableau D3) (Dong *et al.*, 2017; Drysdale and Fleet, 1989). AAB populations increased significantly above 1.10^5 CFU mL^{-1} at day 7. Only cocultures BB x AP, BB x KS, and HV x AI (Figure D2b,d,f) reached bacterial populations beyond 1.10^6 CFU mL^{-1} on day 7. Between day 14 and day 24, populations remained stable in the range between 1.10^5 and 1.10^6 CFU mL^{-1}. The strongest variations could be seen with the modalities SC x AP and SC x KS, with populations below 1.10^5

CFU mL^{-1}. Populations of *Acetobacter* sp. in coculture at day 14 were lower than those in monocultures in open IC at day 14 by around 1 log. On the contrary, populations of *K. saccharivorans* were higher in cocultures by around 1.5 log.

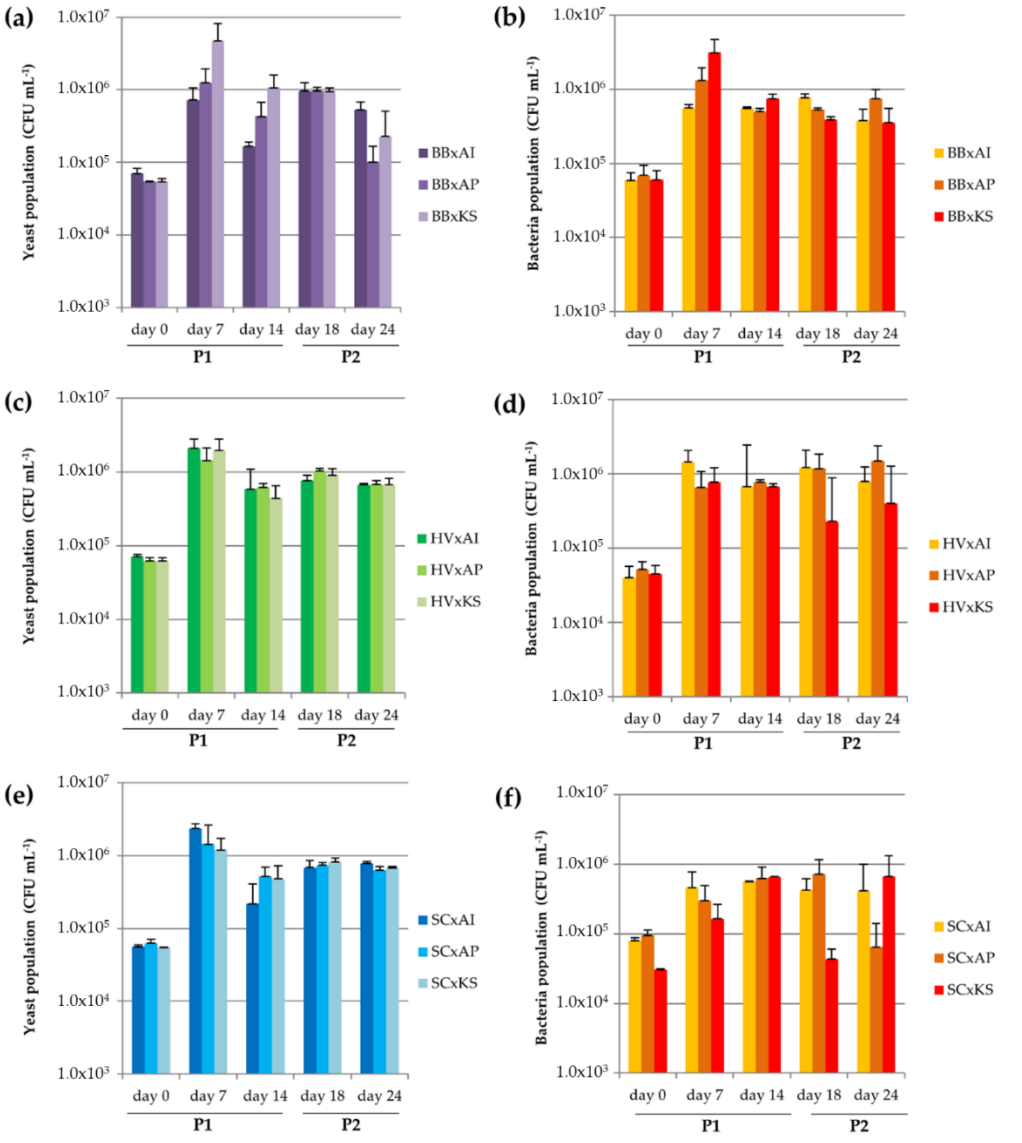

Figure D2 : Microbial populations of yeast and bacterial cocultures in sugared black tea determined by plate counting (CFU mL^{-1}). Error bars correspond to the confidence interval with α = 0.05 (*n* = 3). (a) and (b) Yeast and bacterial populations, respectively, in cocultures involving *Brettanomyces bruxellensis* (BB). (c) and (d) Yeast and bacterial populations, respectively, in cocultures involving *Hanseniaspora valbyensis* (HV). (e) and (f) Yeast and bacterial populations, respectively, in cocultures involving *Saccharomyces cerevisiae* (SC). AI = *Acetobacter indonesiensis*, AP = *Acetobacter papayae*, and KS = *Komagataeibacter saccharivorans*.

During original kombucha fermentation, total yeasts and total bacterial populations at inoculation were $3.5.10^5 \pm 4.6.10^4$ CFU mL^{-1} and $8.5.10^4 \pm 1.3.10^4$ CFU mL^{-1}, respectively (Figure D3). Yeast and bacterial populations increased to $3.2.10^6 \pm 1.8.10^6$ CFU mL^{-1} and $1.8.10^6 \pm 3.2.10^5$ CFU mL^{-1}, respectively, and stayed stable until day 24. The total yeasts population thus remained slightly higher than the total bacteria population throughout the fermentation. This was not observed for cocultures. Both yeast and AAB populations were lower in cocultures than in original kombucha fermentation (Figure D2). The general higher population in original kombucha could indicate different microbial dynamics of the consortium compared to cocultures. The differences in the yeast/AAB balance could be explained by the absence of consistent pellicle formation in cocultures, with a fragile veil appearing instead. The AAB population present in the biofilm in original kombucha could then be found in the liquid phase of cocultures instead (Chen and Liu, 2000; Goh *et al.*, 2012b). The different yeast colony morphotypes allowed a discrimination of populations by species. At the inoculation time, *H. valbyensis* was the major yeast species ($6.6.10^4 \pm 5.1.10^3$ CFU mL^{-1}), followed by *B. bruxellensis*. ($2.8.10^5 \pm 4.3.10^4$ CFU mL^{-1}). The *S. cerevisiae* population was clearly lower, with $1.3.10^3 \pm 1.7.10^3$ CFU mL^{-1} (Figure D3). After 7 days, all populations increased beyond 5 log and *B. bruxellensis* became the major species, followed by *H. valbyensis* and then *S. cerevisiae*. The same levels of population were found at day 14. It is worth noting that these proportions differ in comparison to those of the inoculum (fermentation of 14 days) and consequently the sample at day 0 (inoculation). This could be explained by differences in oxygen access due to the greater SIS of the preculture compared to the SIS used during the experiment. Change occurred at day 18 and day 24 after closing the bottle, with a parallel decrease of the *H. valbyensis* population ($-2.7.10^5$ CFU mL^{-1}) at day 24 and an increase of *S. cerevisiae* ($+1.8.10^5$ CFU mL^{-1}). The *B. bruxellensis* population remained stable between day 7 and day 24, which highlights a predominant role during the whole process. The population dynamics induced by vessel closing were not observable in cocultures (Figure D2).

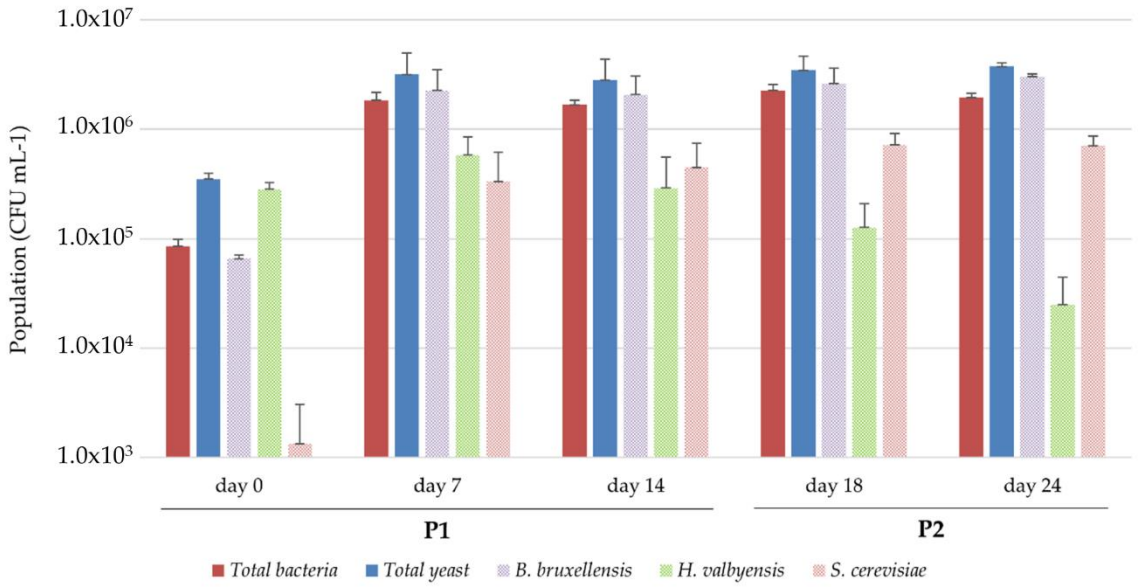

Figure D3 : Microbial populations during cultivation in sugared black tea of the black tea kombucha consortium determined by plate counting (CFU mL^{-1}). Error bars correspond to the confidence interval with α = 0.05 (*n* = 3).

3.3.2 Utilization of Carbohydrates

No groups could be determined among the total sugar variation values, ranging between −5 and −27 g L^{-1}, despite the presence of significant differences (p = 0.008) (Figure D4a). However, the average values of total sugar variations are all lower between the aerobic acidification phase (14 days after inoculation, P1) and anaerobic acidification phase (10 days in closed IC, P2). This means that most of the sugars were consumed during P1. The sucrose hydrolysis of cocultures, reflected by the disappearance of sucrose, was similar to the capacities observed by the corresponding yeast monoculture, except for *S. cerevisiae.* This species behaved closer to monocultures in the closed IC, with the complete degradation of sucrose during P1 (Figure D4a and Tableau D1). All cocultures except those including *H. valbyensis* achieved significantly stronger sucrose hydrolysis at P1 compared to kombucha (p < 0.05), but this gap was filled at P2 (Figure D3a). This highlights a strong impact of yeasts on the release of monosaccharides that are used by AAB. The results displayed in Figure D4-b show a significantly greater release of monosaccharides of *B. bruxellensis* and *S. cerevisiae* compared to *H. valbyensis*, thus confirming the release of glucose and fructose for cocultures including yeasts with strong known invertase activity (p < 0.05 for glucose and p < 0.05 for fructose). It is noteworthy

that in all cases at P1, the fructose content was higher than glucose at P1 and P2, which was characteristic of yeast metabolism in monocultures (Tableau D1), but preference for glucose could also occur for AAB.

Original kombucha underwent an incomplete hydrolysis of sucrose at P1 (−52.0 g L^{-1} sucrose), but the process was completed at P2 (−63.4 g L^{-1} sucrose), although the total sugar consumption did not evolve significantly (−15.9 and −18.2 g L^{-1} total sugars at P1 and P2, respectively; the initial amount of total sugar was 72.5 g L^{-1} with 71 g L^{-1} sucrose and 1.5 g L^{-1} fructose, and glucose was not detected). It can be supposed that the conditions in original kombucha led to a different rate of substrate consumption.

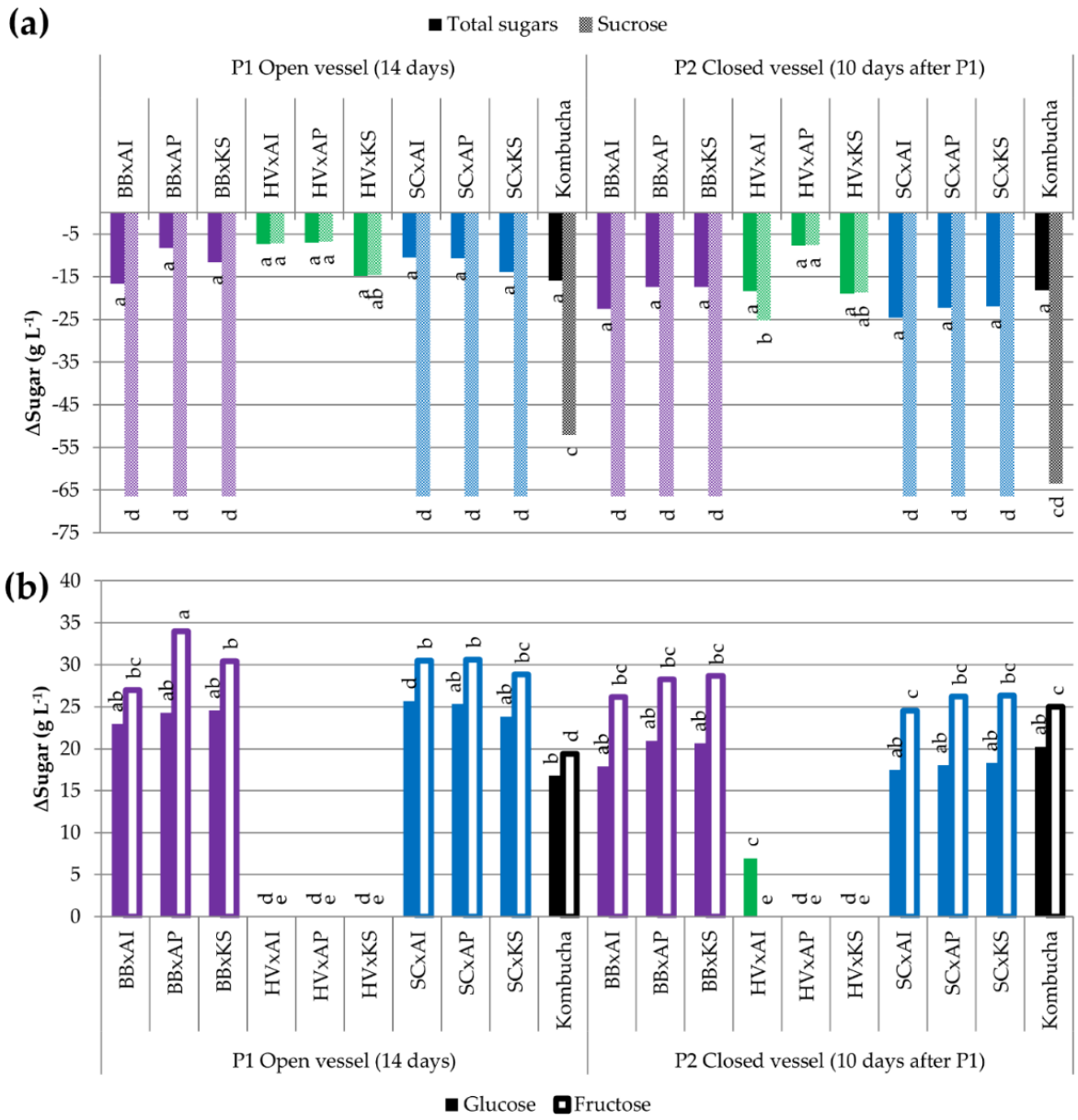

Figure D4 : Difference in total sugars and sucrose (a) and in glucose and fructose (b) (g L^{-1}) of samples between day 0 (after inoculation) and the end point (14 days for pure cultures, 14 days in an open vessel for P1, and 10 days in a closed vessel following P1 for P2). ANOVA was performed with $\alpha = 0.05$ and $n = 3$. Common letters imply non-significant differences between means. Colors reflect the yeast species in the coculture. BB = *Brettanomyces bruxellensis* (purple), HV = *Hanseniaspora valbyensis* (green), SC = *Saccharomyces cerevisiae* (blue), AI = *Acetobacter indonesiensis*, AP = *Acetobacter papayae*, KS = *Komagataeibacter saccharivorans*, and "x" = a coculture.

3.3.3 Variations in Ethanol Content

The ethanol increase was lower at P1 (from +0.3 to +1.2 g L^{-1}) than P2 because of the inhibition of oxidative metabolism with oxygen deprivation (from +0.5 to +4.3 g L^{-1}) (Figure D5). The production of ethanol by cocultures involving *S. cerevisiae* at P1 was higher than those occurring in monocultures in open IC (+0.1 g L^{-1}) (Tableau D3). This shows that this species in cocultures switched to fermentative metabolism, even in open IC. This switch also involved an increase in invertase activity due to the lower energetic yield of glucose consumed through fermentation compared to respiration (Figure D3). Between P1 and P2, the ethanol content increased significantly for BB x AI, BB x AP, HV x AP, and all cocultures involving *S. cerevisiae* ($p < 0.05$). The ethanol production of original kombucha was +1.0 g L^{-1} at P1, with no significant variation at P2. Most ethanol production was significantly higher in cocultures, except BB x KS, HV x AI, and HV x KS. This could suggest that biological ethanol oxidation is influenced by microbial dynamics. The presence of the pellicle in original kombucha could play a specific role in the enhancement of ethanol oxidation in particular.

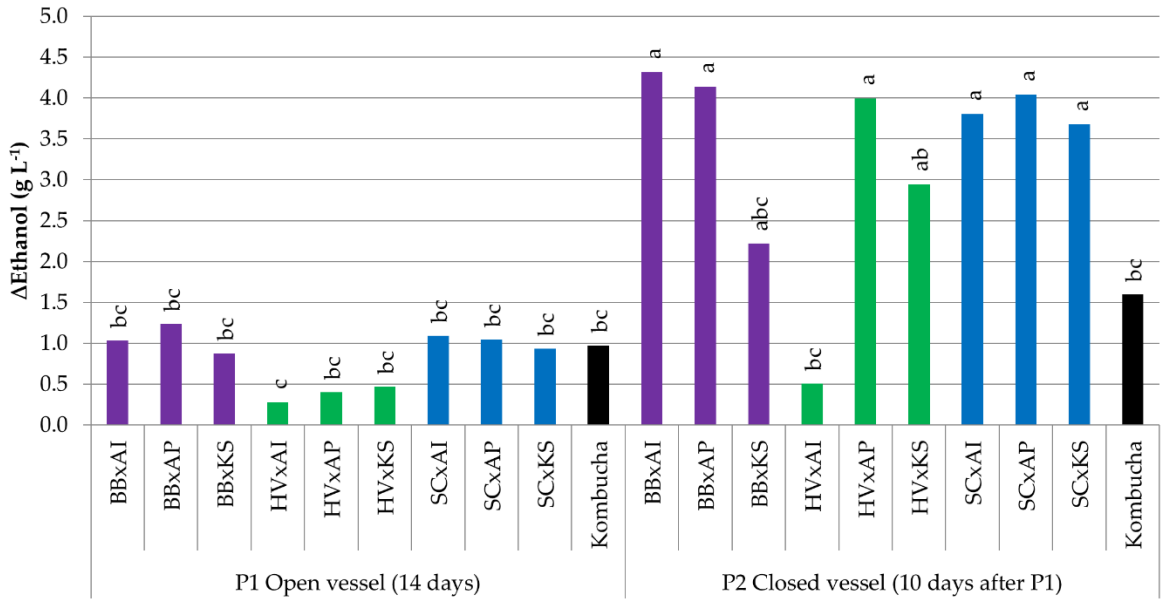

Figure D5 : Difference in ethanol (g L^{-1}) of samples between day 0 (after inoculation) and the end point (14 days for pure cultures, 14 days in an open vessel for P1, and 10 days in a closed vessel for P2 following P1). ANOVA was performed with α = 0.05 and *n* = 3. Common letters imply non-significant differences between means. Colors reflect the yeast species in the coculture. BB = *Brettanomyces bruxellensis* (purple), HV = *Hanseniaspora valbyensis* (green), SC = *Saccharomyces cerevisiae* (blue), AI = *Acetobacter indonesiensis*, AP = *Acetobacter papayae*, KS = *Komagataeibacter saccharivorans,* and "x" = a coculture.

3.3.4 Variations in the Free Amino Nitrogen Content

An increase of the FAN concentration between +19 and +80 µg L^{-1} was also observed in cocultures compared to sugared tea (initial average concentration of 63 µg L^{-1}) (Figure D6). The FAN increase was significantly lower for HV x KS compared to BB x KS and SC x KS at P1 ($p < 0.05$). The FAN increase in original kombucha was 12 µg L^{-1} at P1 and was not significantly different compared to P2. The fact that FAN increases were similar between P1 and P2 suggests that oxygen deprivation could occur at P1 in cocultures because of the oxidative metabolism of AAB, as this behavior contrasts with that of yeast monocultures (Tableau D1).

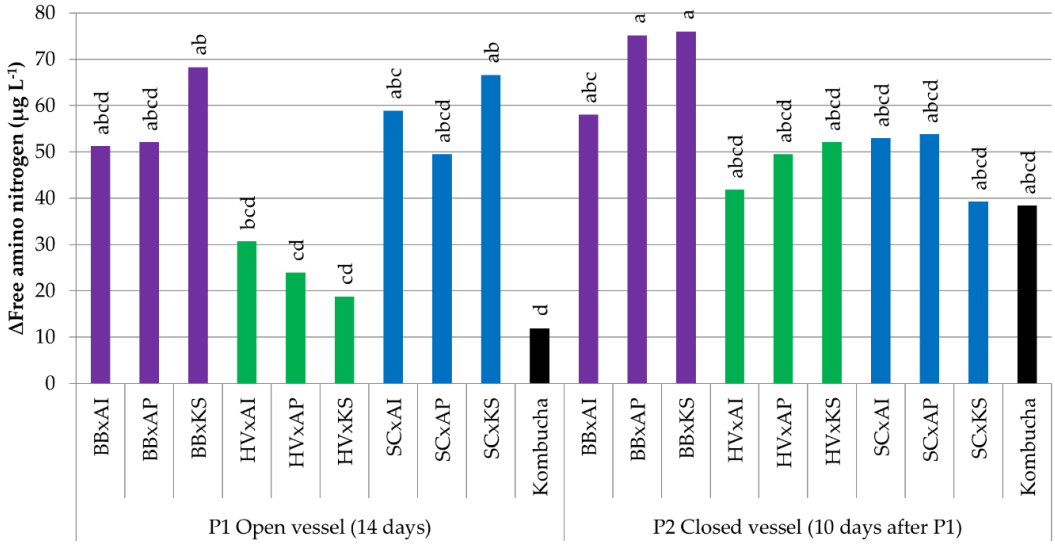

Figure D6 : Difference in free amino nitrogen (µg L^{-1}) of samples between day 0 (after inoculation) and the end point (14 days for pure cultures, 14 days in an open vessel for P1, and 10 days in a closed vessel for P2 following P1). ANOVA was performed with α = 0.05 and *n* = 3. Common letters imply non-significant differences between means. Colors reflect the yeast species in the coculture. BB = *Brettanomyces bruxellensis* (purple), HV = *Hanseniaspora valbyensis* (green), SC = *Saccharomyces cerevisiae* (blue), AI = *Acetobacter indonesiensis*, AP = *Acetobacter papayae*, KS = *Komagataeibacter saccharivorans*, and "x" = a coculture.

3.3.5 Acidification by the Production of Organic Acids

The pH of all cocultures dropped between −2 and −4 units (initial average of 6.64) and the total acidity increased from +4 to +70 meq L^{-1} (initial value < 1 meq L^{-1}) (Figure D7). After 14 days, cocultures involving *H. valbyensis* displayed a drop of pH that was significantly weaker than the others ($p < 0.05$) and their average total acidity values were the lowest ($p < 0.05$). Between P1 and P2, a significative drop of pH could be observed for HV x AP and HV x KS only ($p < 0.05$). Moreover, the total acidity increased significantly for BB x KS, HV x AP, HV x KS, and all cocultures involving *S. cerevisiae*, which suggests that the oxidative metabolism of AAB was maintained after the closing of the vessel. This could have been allowed by the presence of residual oxygen after the closing of bottles.

The drop of pH of original kombucha remained the same at P1 and P2, with −1.7 units (average initial pH of 6.04). The increase of total acidity was +47 meq L^{-1} at P1 and +63.7 meq L^{-1} at P2, but these average values were not significantly different (average initial total acidity of 3 ± 1 meq L^{-1}). It is noticeable that despite having one of the highest values for the total acidity, the pH decrease of original kombucha was less intense than all cocultures and supposes a stronger buffering capacity. While a consistent cellulosic biofilm was present in original kombucha, no consistent pellicle was visible for cocultures. Instead, cellulosic fragments were floating at the surface. Therefore, in the absence of a consistent pellicle, cocultures were able to produce organic acids in similar quantities to original kombucha, which implies that the biofilm is not necessary to complete the P1 phase.

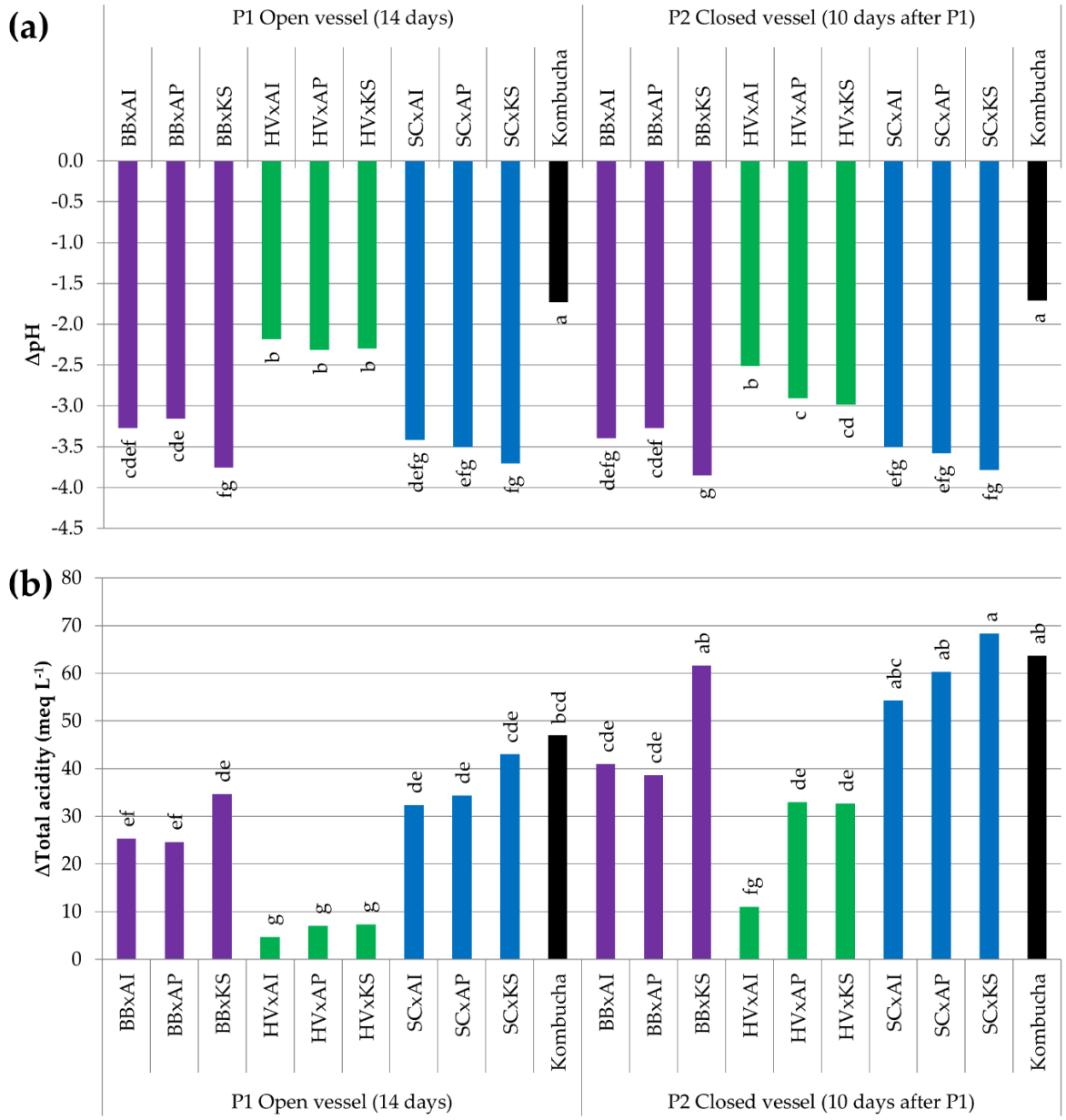

Figure D7: Difference in the (a) pH value (arbitrary unit) and (b) total acidity (meq L^{-1}) of samples between day 0 (after inoculation) and the end point (14 days for pure cultures, 14 days in an open vessel for P1, and 10 days in a closed vessel following P1 for P2). ANOVA was performed with $\alpha = 0.05$ and $n = 3$. Common letters imply non-significant differences between means. Colors reflect the yeast species in the coculture. BB = *Brettanomyces bruxellensis* (purple), HV = *Hanseniaspora valbyensis* (green), SC = *Saccharomyces cerevisiae* (blue), AI = *Acetobacter indonesiensis*, AP = *Acetobacter papayae*, KS = *Komagataeibacter saccharivorans*, and "x" = a coculture.

The production of organic acids in cocultures and original kombucha is detailed in Tableau D3. No target organic acid could be detected in sugared black tea and among them, no citric acid was detected in any sample.

Tableau D3 : Difference in the organic acid content of samples between day 0 (after inoculation) and the end point (14 days for pure cultures, 14 days in an open vessel for P1, and 10 days in a closed vessel following P1 for P2).

Fermentation Phase	Coculture	ΔAcetic Acid (g L^{-1})	ΔGluconic Acid (g L^{-1})	ΔLactic Acid (g L^{-1})	ΔSuccinic Acid (g L^{-1})	ΔMalic Acid (g L^{-1})
P1 Open vessel (14 days)	BB x AI	0.7 ^{bcd}	1.6 ^{abc}	nd	0.3 ^{abc}	nd
	BB x AP	0.6 ^{bcd}	1.4 ^{abcd}	nd	0.3 ^{abc}	nd
	BB x KS	2.7 ^{bc}	1.7 ^{ab}	nd	nd	nd
	HV x AI	nd	0.1 ^{ef}	0.3 ^{a}	0.2 ^{bc}	nd
	HV x AP	0.2 ^{cd}	0.1 ^{f}	0.4 ^{a}	0.2 ^{abc}	nd
	HV x KS	0.4 ^{c}	<0.05	0.2 ^{bc}	0.2 ^{abc}	0.3 ^{a}
	SC x AI	1.0 ^{bcd}	1.2 ^{abcde}	nd	0.3 ^{abc}	nd
	SC x AP	0.8 ^{c}	2.2 ^{a}	nd	0.3 ^{abc}	nd
	SC x KS	2.7 ^{bcd}	1.3 ^{abcde}	nd	0.4 ^{abc}	nd
	Kombucha	1.6 ^{bcd}	1.7 ^{ab}	0.1 ^{c}	0.2 ^{abc}	nd
P2 Closed vessel (10 days after P1)	BB x AI	2.0 ^{bcd}	1.6 ^{abc}	nd	0.6 ^{abc}	nd
	BB x AP	1.6 ^{bcd}	1.2 ^{abcde}	nd	0.7 ^{ab}	nd
	BB x KS	12.7 ^{a}	2.0 ^{a}	nd	nd	nd
	HV x AI	0.7 ^{cd}	0.3 ^{def}	0.2 ^{bc}	0.7 ^{ab}	1.0 ^{a}
	HV x AP	2.4 ^{bcd}	0.5 ^{cdef}	0.1 ^{cd}	0.7 ^{ab}	nd
	HV x KS	2.9 ^{bcd}	0.4 ^{def}	0.3 ^{ab}	0.5 ^{abc}	0.2 ^{a}
	SC x AI	2.2 ^{bcd}	2.3 ^{a}	nd	0.8 ^{a}	nd
	SC x AP	2.2 ^{bcd}	2.4 ^{a}	nd	0.8 ^{a}	nd
	SC x KS	11.3 ^{a}	2.2 ^{a}	nd	nd	nd
	Kombucha	2.1 ^{bcd}	0.8 ^{bcdef}	0.1 ^{cd}	0.3 ^{abc}	nd

ANOVA was performed with α = 0.05 and *n* = 3, and common letters imply non-significant differences between means. BB = *Brettanomyces bruxellensis*, HV = *Hanseniaspora valbyensis*, HO = *Hanseniaspora opuntiae*, SC = *Saccharomyces cerevisiae*, AI = *Acetobacter indonesiensis*, AP = *Acetobacter papayae*, KS = *Komagataeibacter saccharivorans*, and "x" = a coculture.

The main organic acids produced during P1 were acetic and gluconic acids, with the concentration increase ranging below +2.7 g L^{-1} and below +2.2 g L^{-1}, respectively. The lowest increases were associated with cocultures involving *H. valbyensis*. These organic acids are mainly produced by AAB as a result of oxidative metabolism from ethanol and glucose. At P1, the gluconic acid concentrations of cocultures involving *H. valbyensis* were significantly lower than those of cocultures involving *B. bruxellensis* ($p < 0.05$). The results suggest that the production of organic acids reflects the capacity of the paired yeast to make these substrates available, as it has been established that *H. valbyensis* possesses poor fermentative and invertase activities. Between P1 and P2, no significant change in the concentration of acetic and gluconic acids occurred for most cocultures, except BB x KS and SC x KS, whose acetic acid concentrations increased from +7.3 to +12.7 g L^{-1} ($p < 0.05$). This indicates a capacity of *K. saccharivorans* to maintain the production of acetic acid in conditions of oxygen limitations compared to *Acetobacter* sp., possibly through a pathway other than oxidative metabolism (De Ley, 1961; De Ley and Schell, 1962). Other organic acids, such as succinic acid, were detected in all cocultures except for BB x KS at P1 and BB x KS and SC x KS at P2, which suggests a link with the presence of *K. saccharivorans,* especially in oxygen limitation conditions. Malic acid was only detected in HV x KS at P1 and HV x AI and HV x KS at P2. Lactic acid was only detected in all cocultures involving *H. valbyensis*, which seems to characterize this species (Tableau D1). The links established between the production of malic, succinic, and lactic acids and the type of metabolism do not appear clearly for cocultures, probably due to the complexification of metabolic pathways in cocultures.

In original kombucha, an increase of acetic acid and gluconic acid production of +1.6 and +1.7 g L^{-1}, respectively (initial average concentrations of acetic acid of 0.4 g L^{-1} and gluconic acid of 0.3 g L^{-1}) and low production of lactic and succinic acids inferior to +0.2 g L^{-1} at P1 (lactic and succinic acids not detected at day 0) (Tableau D3) were observed. Qualitatively, the organic acid composition of kombucha gathers all organic acids detected in cocultures, with the exception of malic acid. On the contrary, lactic acid was detected and was characteristic of cocultures with *H. valbyensis*. Quantitatively, the original kombucha composition in acetic and gluconic matched those of all cocultures involving fermentative yeasts (excluding *H. valbyensis*) at P1, but at P2, this only applied

to cocultures involving *Acetobacter* sp., since the presence of *K. saccharivorans* induced an intense production of acetic acid that was probably due to the intensification of ethanol through alcoholic fermentation induced by oxygen deprivation. Indeed, this phenomenon did not occur in cocultures including *H. valbyensis*.

The coculture results underline the impact of yeasts and AAB metabolism on the matrix and variation occurring between yeast and AAB species. The use of statistical treatment can help in visualizing the phenomenon occurring in such complex systems and determine the similarities with original kombucha.

3.3.6 Principal Component Analysis as a Visual Tool for Understanding Complex Microbial Interactions

Principal Component Analysis (PCA) was performed on cocultures and original kombucha for P1 and P2 separately, in order to characterize the two different phases in terms of the impact on the chemical composition of the sugared tea matrix (microbial population data were excluded). Both PCA possessed a satisfactory sum of proper values with Dim1 and Dim2 axes (77.40% for P1 and 67.91% for P2).

The parameter plot of P1 (Figure D8a) was primarily structured by the Dim1 axis positively characterized by the glucose, fructose, acetic acid, gluconic acid, ethanol, FAN concentrations, and total acidity ($\cos^2 \geq 0.7$), and negatively by the pH, and sucrose and lactic acid concentrations ($\cos^2 \leq -0.7$). Dim1 translated the biological acidification process targeted during P1 in an open vessel. This acidification (increase of total acidity and decrease of pH) was mainly caused by the production of acetic and gluconic acids by AAB. Their production was dependent on the release of monosaccharides (glucose and fructose) from sucrose that can efficiently be hydrolyzed by yeast invertase activity. Lactic acid production was associated with low invertase and fermentative activities, as observed in *H. valbyensis* or *S. cerevisiae* monocultures in open IC and in cocultures with *H. valbyensis* (Tableaux D1 and D3). Dim 2 was positively characterized by total sugars concentrations and negatively characterized by malic acid, and translated differences in the profiles of organic acids from yeasts and the sugar consumption of cocultures. As a result, the sample plot (Figure D8b) coupled with hierarchical clustering discriminated cocultures with different levels. The predominant difference separated cocultures

including *H. valbyensis* from other yeasts. This was caused by the low sucrose hydrolysis and fermentative capacity of *H. valbyensis* that prevented the access of AAB to monosaccharides and ethanol and thus efficient acidification of the medium. Secondarily, the clustering discriminated the cocultures, including *K. saccharivorans* and *Acetobacter* sp., for all coupled yeasts except *S. cerevisiae*. Original kombucha belonged to the cluster including BB x KS, highlighting the closeness in chemical compositions.

The parameter plot of P2 (Figure D8c) was mainly structured by Dim1, which was positively characterized by glucose, fructose, and gluconic acid concentrations ($cos^2 \geq 0.7$), and negatively characterized by the pH, and lactic acid and sucrose concentrations. Dim2 was positively characterized by the ethanol concentration and negatively characterized by the total acidity ($cos^2 \leq -0.7$). Specifically, Dim1 could be representative of the invertase activity and Dim2 of AAB activity (oxidation of ethanol and increase of the total acidity). It can be noticed that total sugar and ethanol are not anticorrelated, since, during P1 and even P2, the balance between total sugars and ethanol is biased by its conversion in acetic acid by AAB. The clustering (Figure D8d) again discriminated cocultures including *H. valbyensis* from the others and cocultures including *K. saccharivorans* from those with *Acetobacter* sp. (except for cocultures including *H. valbyensis*). Moreover, original kombucha again belonged to the cluster including BB x KS and SC x KS.

The comparison of parameter plots of P1 and P2 reflects the shift from the combined yeasts and AAB metabolisms, with a positive correlation of invertase activity, fermentation, and acidification during the acidification phase (all strongly contributing to Dim1) (Figure D8a), towards the prevalence of yeast metabolism (strong contribution to Dim1), with a lower correlation with AAB oxidative metabolism during the carbonation phase (ethanol and total acidity strongly contributing to Dim 2) (Figure D8c).

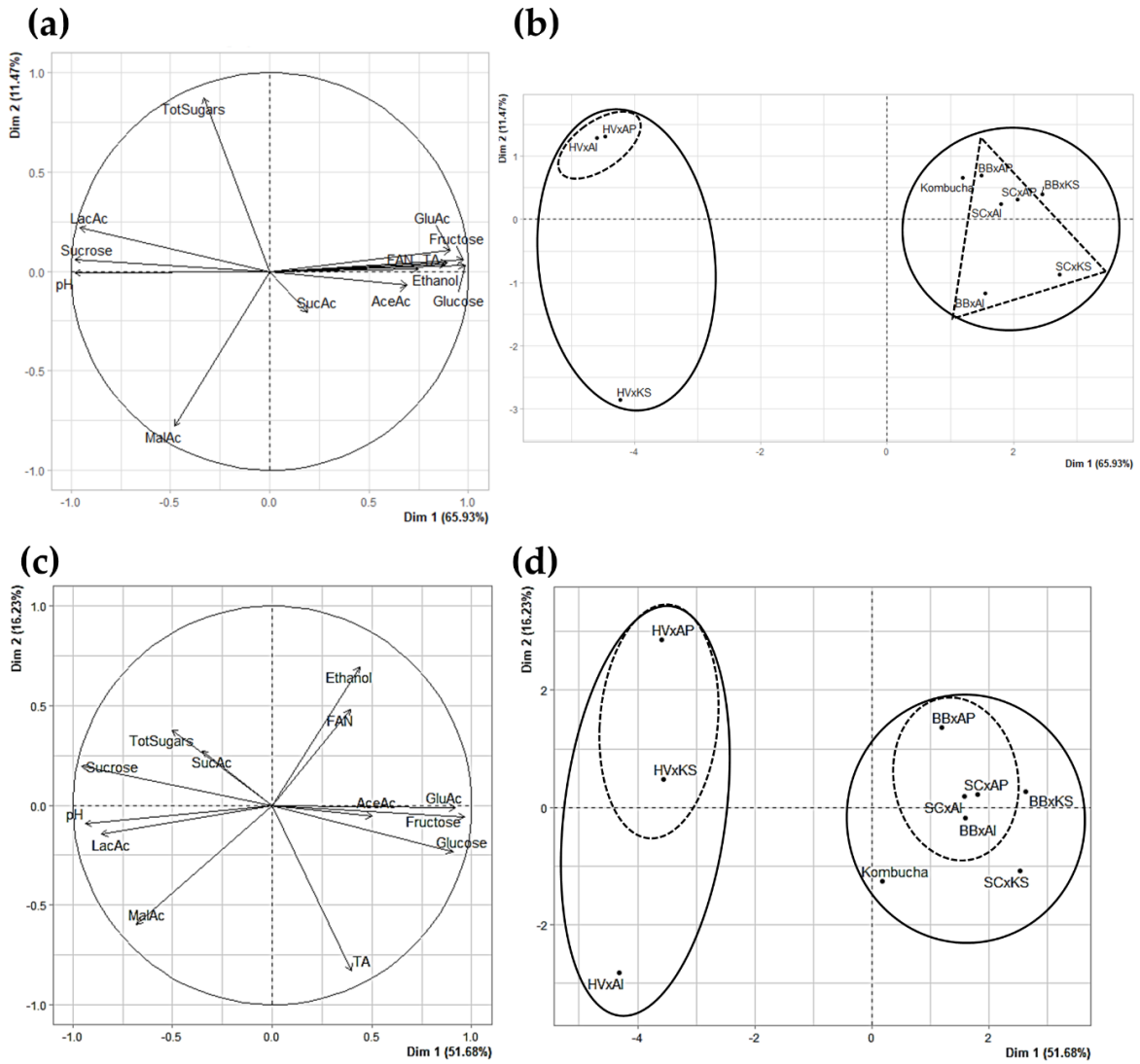

Figure D8 : Principal Component Analysis of coculture and original kombucha samples using parameters of the chemical composition. (a) Parameter or vector plot for P1, (b) sample plot for P1, (c) parameter or vector plot for P2, and (d) sample plot for P2. Continuous circles gather samples of the same primary clusters and dashed line circles gather samples of the same sub-clusters according to hierarchical ascendant clustering analysis.

These results show that yeast metabolism was key throughout the process of fermentation: P1 required yeasts to hydrolyze sucrose and produce ethanol for their conversion into acetic and gluconic acids by AAB and P2 relied on fermentation to complete natural carbonation. Nevertheless, *K. saccharivorans* still strongly impacted the chemical composition during P2 because of its ability to maintain oxidative metabolism, especially when ethanol production is abundant, which was not as visible for *Acetobacter* sp. More generally, yeast metabolism is the main factor influencing the kombucha composition and the influence of AAB is secondary and mostly impacts the organic acid profile.

3.4 Sucrose Utilization Strategies as a Basis of Microbial Interactions in Kombucha

The experimental results obtained in this study confirm the metabolic interplay between yeasts and acetic acid bacteria that has been theorized for kombucha made from sugared black tea (Blanc, 1996; Chen and Liu, 2000; Sievers *et al.*, 1995; Wang *et al.*, 2020). They highlight, for the first time, the key role of yeasts and their metabolisms in terms of invertase activity and the fermentation capacity, which can differ, depending on yeast strains (Tableau D1). Bibliographic resources and the NCBI database have reported the existence of genes encoding invertase in *S. cerevisiae* (internal and extracellular enzyme) (Andjelković *et al.*, 2015; Beteta and Gascon, 1971; Margetić and Vujčić, 2017; Neuman Jr *et al.*, 1973) and *B. bruxellensis* (internal and extracellular enzyme) (Blondin and Ratomahenina, 1983; Conterno *et al.*, 2006; Fia *et al.*, 2005; Roach and Borneman, 2020), but few data is available for *H. valbyensis*. However, genomic studies on the *Hanseniaspora* genus reported the loss of the *SUC* gene of the branch to which *H. valbyensis* belongs, thus supporting the low sucrose hydrolysis observed (Tableau D1) (Steenwyk *et al.*, 2019). On top of glucose, fructose, and ethanol, yeasts may also release nitrogenous substrates through the conversion of bound amino-acids, such as proteins and peptides, into free amino acids, since the results suggest a correlation between FAN and fermentation (Figure D8a). Yeast can release free amino acid through extracellular proteolytic activity and/or autolysis (Alexandre et al., 2001; Charoenchai et al., 1997; Guilloux-Benatier et al., 2001; Younes et al., 2011), which could benefit AAB as well (Guilloux-Benatier and Chassagne, 2003). As a result, the metabolism of yeasts directly impacts the flux of available substrates for AAB. Nevertheless, AAB studied in this work

were able to grow in sugared black tea without the presence of yeasts to hydrolyze sucrose, similar to the AAB strains used in Wang *et al.* (2020) (Wang *et al.*, 2020). From the perspective of the present results and the experimental conditions of the work cited before, the hypothesis of a snowball effect is supported: Initial spontaneous hydrolysis of sucrose in acidic conditions would be amplified by a pH drop resulting from organic acid production by AAB (Figure SD3) (Torres *et al.*, 1994). It could even be hypothesized that such a phenomenon also applied for cultures involving *H. valbyensis* (Tableau D1, Figures D4 and D7). However, the existence of enzymatic invertase activity in AAB is not to be excluded and more research needs to be carried out in terms of the metabolic pathways of AAB (Jakob *et al.*, 2019). However, Balasubramaniam and Kannangara (1982) reported low invertase activity of *K. xylinus* and determined that sucrose consumption was achieved through the activity of sucrose phosphorylase (Balasubramaniam and Kannangara, 1982). In all cases, sucrose hydrolysis performed by yeasts except *H. valbyensis* was more efficient than that induced by AAB alone (Tableau D1). It should be underlined that besides oxidative metabolism, AAB can use monosaccharides through glycolysis, tricarboxylic acids (TCA), and pentose phosphate pathways (Prust *et al.*, 2005). The presence of acetic, succinic, and malic acids in AAB monocultures are markers of these metabolisms (Tableau D1), in agreement with coculture results. However, the metabolic activity of AAB mostly depends on the substrates released by yeast and the composition in AAB can impact the profile of produced organic acids (Tableau D3 and Figure D8).

The microbial dynamics of original kombucha after bottling (P2) were investigated for the first time and highlighted the microbial interaction resulting in the decrease in the population of *H. valbyensis* triggered by oxygen limitations (Figure D3). As *S. cerevisiae* exhibited more metabolic activity in monocultures and cocultures in oxygen-limited conditions, it can be hypothesized that a negative interaction with *H. valbyensis* could occur in original kombucha. *Saccharomyces*–non-*Saccharomyces* interactions have been studied and are not fully understood, but possible mechanisms include the production of toxic compounds (Branco *et al.*, 2015; Pereznevado *et al.*, 2006; Wang *et al.*, 2016), competition with other nutrients such as sugars or amino acids (Gobert *et al.*, 2017), cell–cell contact mechanisms (Renault *et al.*, 2013), and flocculation (Soares,

2011). It was observed that *S. cerevisiae*'s metabolism in cocultures switched to high invertase activity and fermentation during P1 (Figures D4 and D5). This difference of behavior with the monocultures (Tableau D1) could be explained by an evolutionary mechanism in *S. cerevisiae*, often compared to "The Prisoner's Dilemma", which modulates its invertase activity according to the presence of "cheaters" without such enzymatic activity (Celiker and Gore, 2012; Greig and Travisano, 2004; May *et al.*, 2019). Therefore, the *SUC* gene coding for the invertase is kept at a minimum expression in monocultures, so that *S. cerevisiae* can hydrolyze sucrose progressively. A shift to a higher expression level can occur in the presence of other "cheater" species, leading *S. cerevisiae* to rapidly consume sugars to outgrow other populations and inhibit them through the release of toxic metabolites, such as ethanol (Marques *et al.*, 2016). However, in the case of kombucha, this benefits AAB because of the increase of oxidizable substrates and possibly through the increased availability of amino acids. Analytical data regarding oxygen consumption during kombucha fermentation is needed to assess its role in microbial interactions, namely taking into account the presence of the pellicle, which is thought to reduce the access to oxygen in the liquid phase (Goh *et al.*, 2012b; Jayabalan *et al.*, 2007). It is worth noting that the pellicle was not necessary for AAB in cocultures to perform efficient organic acid production, thus raising questions about additional functions other than facilitating oxygen access to AAB during kombucha fermentation (Goh *et al.*, 2012b).

Differences between cocultures and kombucha also appeared in the chemical composition of the liquid phase. Some kinetics in original kombucha seemed to be intermediate compared to cocultures, especially with sucrose hydrolysis at P1 (Figure D3), the ethanol concentration at P2 (Figure D5), and the FAN concentration (Figure D6). This could be explained by the balance in the yeast population, namely between *B. bruxellensis* and *H. valbyensis*, which could lead to a leveling of global yeast metabolism during original kombucha fermentation (Figure D3). The role of *H. valbyensis* remains enigmatic regarding the technological aspect of kombucha fermentation. As the present study did not explore volatile compounds, it cannot be excluded that this yeast strain could contribute to the olfactive profile of kombucha beverages. More subtle microbial interactions could also take place in terms of amino acid consumption or chemical

signaling between *H. valbyensis* and other yeasts and bacteria (Gobert *et al.*, 2017). Overall, the results point to an eco-evolution in kombucha between species that possess and do not possess *SUC* genes necessary for the use of sucrose as a substrate. *H. valbyensis* and AAB would then be labeled as "cheaters" by taking benefits from the invertase activity of other yeasts, such as *B. bruxellensis* and *S. cerevisiae*, without contributing themselves to the production of public goods (here, monosaccharides) (Harrington and Sanchez, 2014; Sanchez and Gore, 2013). However, it has been reported in *S. cerevisiae* x *Escherichia coli* cocultures that, by lowering the population of public good producers (*S. cerevisiae*) at high population levels, cheaters (*E. coli*) could consequently stabilize the sucrose hydrolysis rate, thus maintaining both population levels (Celiker and Gore, 2012). A similar phenomenon could also have taken place in the present study, since lower yeast populations in cocultures with AAB compared to monocultures were also observed (Figures D1 and D2). *H. valbyensis* and AAB could therefore contribute to the stability of the community, as it was reported that *Hanseniaspora* sp. could achieve fast growth due to the evolutionary loss of cell-cycle components, thus limiting nutrient access (Langenberg *et al.*, 2017), and acidification of the medium by AAB induced the selection of acidophilic species (Leistner, 2000).

4 Conclusions

Microbial interactions occurring in kombucha have often been described as "symbiotic", which does not imply mutual benefits, as described by the word "mutualistic", but rather that all species manage a stable coexistence, possibly through interactions that are yet to be defined (Ivey *et al.*, 2013; May *et al.*, 2019). Although the present study lacks an exhaustive insight into the volatile and non-volatile metabolites, it was possible to characterize nutritional interactions. As AAB are not dependent on yeasts to access substrates, it appears that yeast–AAB interactions display a non-strict parasitism relationship (as sucrose hydrolysis occurred in AAB monocultures and was sufficient to increase their population at the same level as in original kombucha). No evidence of a beneficial nutritional interaction for yeast could be determined. There is also evidence that AAB induce the production of substrates by *S. cerevisiae* by indirectly rerouting its metabolism, thus enhancing the benefits of this parasitic relationship. Moreover, sucrose appears to play a central role in kombucha symbiosis not only in yeast–bacteria

interactions, but also potentially between yeast species. While the AAB composition secondarily impacts the profile in organic acids, the yeast composition in kombucha consortia is crucial for the global microbial dynamics, the chemical composition of the beverage, and the sensory characteristics of the product (Tran *et al.*, 2020a).

This study only includes one kombucha consortium, but many different microbial compositions exist. Nevertheless, many research works have focused on the identification of yeast and bacteria genera and it could be interesting to determine if the yeast profiles include genera or species with variable invertase activities and fermentative capacities. In silico studies relying on genome and protein databases combined with the results of past identification works of kombucha consortia could highlight the existence of patterns in yeast compositions in terms of links between species and activities. Yeast–yeast interactions in the context of kombucha fermentation should also be addressed in future research works, now that the importance of their role has been uncovered.

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E Chapitre 3 : Analyse métabolomique du procédé de production de la kombucha.

Le troisième chapitre a pour but d'investiguer les transformations de la matrice thé sucré au cours des deux phases de production de kombucha et l'impact du type de thé sur la composition chimique fixe du produit pour un consortium donné. Il doit être soumis sous forme d'article au journal *Food Chemistry: Molecular Sciences*. Pour se faire, une analyse métabolomique a été menée à l'aide de l'outil FT-ICR-MS (*Fourier Transform - Ion Cyclotron Resonance – Mass Spectrometry*) dont l'accès nous a été donné par le Centre Helmholtz de Munich. Cet outil permet une analyse semi-quantitative non-ciblée des composés fixes détectables à de très faibles concentrations. Après extraction sur cartouche SPE (*Solid Phase Extraction*) des échantillons, permettant l'élimination partielle des sucres résiduels, les extraits ont été analysés dans leur composition fixe. Les masses, détectées de manière très précise, ont pu être annotées avec une formule élémentaire. Ainsi, il a été possible de connaître l'impact des étapes de production (première phase d'acidification de 7 jours puis seconde phase de prise de mousse de 5 jours) sur les métabolites du thé sucré, ainsi que l'impact des transformations selon l'utilisation de thé noir ou de thé vert. Les modifications intervenant sur les métabolites ont été évaluées au niveau des proportions en compositions élémentaires (CHO, CHOS, CHON, CHONS) et des familles moléculaires (acides gras, acides organiques, acides aminés, peptides, polyphénols et glucides). L'annotation des formules avec des identités putatives a permis de soulever de nouvelles hypothèses en mettant en évidence de potentiels métabolites clés.

Indépendamment du type de thé utilisé, la première phase d'acidification a mis en évidence la production de composés marqueurs : l'acide gluconique issu du métabolisme oxydatif des bactéries acétiques, ainsi qu'une libération d'acide gallique probablement issus de polyphénols polymériques gallatés. La seconde phase de prise de mousse en condition fermée est caractérisée par une diminution de l'acide oléique, dont la consommation potentielle par les levures pourrait être induite par la limitation en oxygène. Toutefois, c'est la première phase qui impacte le plus la composition fixe du produit, la désignant comme étape déterminante dans le procédé de production. Des polysaccharides semblent également avoir été produits durant cette phase. Malgré une diversité moins importante en métabolites du thé noir sucré comparé au thé vert sucré avant inoculation, la tendance s'inverse à la suite de l'activité microbienne du consortium. En effet, les polyphénols subissent davantage de transformations dans la kombucha thé noir que dans la kombucha thé vert, dont la composition reste la plus proche des thés sucrés.

Il apparait que le type de thé est le paramètre le plus important agissant sur la composition fixe globale du produit au travers des modifications plus ou moins importantes induites par l'activité microbienne. Cette influence est par ailleurs supérieure à celle amenée par la première phase de production.

L'analyse métabolomique de la kombucha nous procure des informations précieuses sur l'impact des paramètres de production sur la composition fixe globale du produit. Elle permet de caractériser les transformations ayant lieu au cours phases de production et a mis en évidence l'impact du type de thé utilisé en particulier concernant les modifications subies par les polyphénols. Cette étude réalisée avec la kombucha originale possède néanmoins le désavantage d'apparaitre comme une boîte noire puisque l'impact spécifique des différents micro-organismes ne peut être distingué. Cette lacune est comblée dans le chapitre suivant.

Non-Targeted metabolomic analysis of kombucha's production process

Running Title: Metabolomic analysis of kombucha processing

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Abstract

Kombucha is a traditional fermented beverage obtained from the transformation of sugared black tea by a community of yeasts and bacteria. Kombucha production recently became industrialized, but its quality standards remain poorly defined. Metabolomic analyses were applied using FT-ICR-MS to characterize the impacts of production phases and the type of tea on the non-volatile chemical composition of kombucha. Independently from tea type, the first phase of acidification in open vessel was characterized by the release of gluconate and gallate from acetic acid bacteria metabolism and probably from polymeric polyphenols, respectively. The second phase of carbonation in closed vessel

induced a consumption of oleic acid that could be consecutive of oxygen limitation. The first phase was the most impactful on molecular diversity, but tea type mainly influenced the global composition in polyphenol profile. Black tea polyphenols were more impacted by microbial activity compared to green tea polyphenols.

Keywords

kombucha, metabolomics, process, fermentation, tea, polyphenols.

1 Introduction

Metabolomics has known an important development in the field of food and nutrition sciences (Mashego *et al.*, 2007). It is defined as the high-throughput identification, and quantification of small molecules (metabolites) from different molecular families and constitutive of a metabolome. The study of metabolomes in food aims at determining their composition by analyzing the highest number of compounds possible, which is why this approach is often coupled with non-targeted methods. Deep and comprehensive understanding of food composition allows the identification of parameters, markers, signatures linked to the identity or authenticity of a given food, thus helping defining it and distinguishing from others, or adulterated versions (Wishart, 2008). Indeed, the identity of food product are closely tied to their production process. In the context of fermented beverages, metabolomics was successfully applied to wine or beer, and allowed to determine the impacts of vintage and terroir on the composition of Burgundy red wines, or highlight the role of starch source on the metabolomes of German beers (Roullier-Gall, Boutegrabet, *et al.*, 2014; Pieczonka *et al.*, 2021).

Therefore, such approach is of great use when it comes to foods or beverages that are poorly caracterised and raise many problematics regarding their production and quality management. This relevantly applies to kombucha, a traditional fermented beverage from sugared tea infusion formerly produced at home and now experiencing high market development as commercialized product (Kim and Adhikari, 2020). Its putative health benefits are promoted but conclusive clinical trials remain to be conducted to confirm them (Ernst, 2003; Vargas *et al.*, 2021). Kombucha is produced by inoculating a sugared tea infusion with a microbial consortium composed of yeasts and bacteria, mainly acetic

acid bacteria, which can occur as multiple compositions in species (Harrison and Curtin, 2021). The production process of kombucha occurs mainly as following: during a first phase in open vessel, yeasts with invertase and fermentative activity convert sucrose in glucose and fructose, which are in turn converted in ethanol and carbon dioxide through alcoholic fermentation. Acetic acid bacteria use those yeast metabolites to produce acetic and gluconic acids, thus acidifying the matrix from the consumption of sugars. When satisfying acidity is reached, bottling or tight sealing of the vessel induces oxygen deprivation and retention of carbon dioxide and natural carbonation is thus achieved (Tran *et al.*, 2020b). While this main metabolic scheme was significantly investigated, many grey area remain associated with this complex microbiological process, involving interkingdom and interspecies interactions in an very unusual matrix. Metabolomics can help raise new hypotheses and uncover metabolites linked to biological activities or the transformations occurring during the process (Tran *et al.*, 2020a).

Metabolomics has already been used to study kombucha. In the study of Villarreal-Soto *et al.* (2020), three different kombucha consortia were analyzed by HPLC-DAD and GC/MS in addition to metagenomics. The different microbial compositions led to different microbial activities and chemical compositions. Phenolic compounds such as phenolic acids or catechin were reported to be impacted by microbial activity. Moreover, some concentration in metabolites varied significantly across the consortia, such as propanoic acid, 2-phenylethanol, as well as carbohydrates. The establishment of links between chemical composition and antioxidant, anti-inflammatory and antiproliferative activities was also attempted. A similar approach was employed in the study of Savary *et al.* (2021), with the coupling of LC-Q-TOF/MS, enzymatic kits and GC/MS with metagenomics to follow changes in metabolites throughout a 27 days kombucha production process in open vessel. Microbial dynamics could then be linked to microbial activities in terms of kinetics of production of organic acids and volatile compounds. Several phases could be characterized based on microbial dynamics and specific metabolite compositions using clustering and multiple factor analyses. While these two studies relied on targeted chemical analyzed, the study of Cardoso *et al.* (2020) used LC-Q-TOF/MS as non-targeted analytical method with a focus on phenolic compounds. In this study, black and green tea kombucha phenolic profiles were compared to corresponding unfermented

teas. Results showed high diversity of polyphenols in black tea kombucha than in green tea kombucha, which corresponded also to a higher antioxidant activity in black tea kombucha, highlighting the significant impact of the type of tea used on the kombucha's phenolic profile. So, the utilization of untargeted techniques shows to greatly expand the knowledge on the metabolic landscape of kombucha. One of those techniques, Fourier-Transform-Ion Cyclotron Resonance/Mass Spectrometry (FT-ICR/MS) has been successfully used for the analysis of tea metabolites for the unravelling of thearubigins formation (Kuhnert *et al.*, 2010).

This study aims at analyzing kombucha samples FT-ICR/MS for the first-time as non-targeted analytic tool, to determine the impact of production phases (open and closed vessel) from sugared tea to the finished product, as well as the effect of the tea type on the kombucha non-volatile metabolome. Fixed parameters are the kombucha microbial consortium and the preparation of sugared tea infusions (same amount of sugar and tea, whether green or black). The evaluation of the effects focused on the comparison non-volatile molecular diversity (number of detected features), variations in signal intensity and determination of markers or pathways in association to the conditions studied for their characterization.

2 Materials and Methods

2.1 Generation of biological samples

Samples analyzed in this study came from the same experiment conducted in another study (Tran *et al.* 2021a - Under Review ; Chapitre 5). Briefly, sugared black and green tea infusion were produced by steeping 1 g L^{-1} of black tea (Pu'er Grade 1 TN4107) or green tea (Sencha Zhejiang TV4217) from Les Jardins de Gaia© (Wittisheim, France) in boiling water for one hour. After cooling down at room temperature, 50 g L^{-1} blond cane sugar from Ethiquable© (Fleurance, France) was dissolved. Then 12% (v/v) of 7 days kombucha was added. The mother culture used to produce this 7 days kombucha was obtained from Biomère (Paris, France). Black tea kombucha (BTK) was produced from sugared black tea (SBT) and green tea kombucha (GTK) from sugared green tea (SGT). After inoculation, incubation occurred at 26°C in static conditions during 7 days for the first phase of production in open vessel (P1), that corresponded to a biological

acidification (Tran *et al.*, 2020a). Aluminum foil was loosely applied on the bottle neck to prevent contact with particles and insects. Samples obtained at this stage were labelled D7. Then, bottles were tightly capped for 5 days to initiate the second phase of production (P2), corresponding to a natural carbonation. Samples obtained at the end of the process were labeled D12. Each culture was performed in triplicates in 123 mL Boston flasks with a Specific Interfacial Surface (SIS) of 0.01 cm^{-1}(Cvetković *et al.*, 2008). Sugared tea samples possessed a total sugar content of 59.0 ± 1.0 g L^{-1}, a pH value of 6.90 ± 0.01 and a total acidity inferior to 1 meq L^{-1}. Kombucha samples at D7 possessed a total sugar content of 51.0 ± 10.0 g L^{-1}, a pH value of 4.2 ± 0.1 and a total acidity of 20 ± 1 meq L^{-1}. Kombucha samples at D12 possessed a total sugar content of 46.4 ± 4.0 g L^{-1}, a pH value of 3.84 ± 0.5 and a total acidity between 28 ± 2 and 41 ± 3 meq L^{-1}. Important discrepancies in sugar contents, pH and total acidity values of inoculated samples can be explained by biological variability of microbial activities. Sugar content was measured using Sucrose Glucose Fructose enzymatic kit from Biosentec (Portet-sur-Garonne, France). pH values were measured with a Mettler Toledo Five Easy pH meter coupled with a LE498 probe and total acidity was determined by titration with 0.1 N NaOH and 0.2% phenolphthalein as color indicator, with reagents purchased from Merck (Darmstadt, Germany).

2.2 Sample preparation

Samples were centrifuged at 3000 x g for 10 minutes at 4°C to remove cells and particles before freezing at -18°C. Once all samples were available, solid phase extraction (SPE) was performed using Bond Elut C18 cartridges from Agilent (Santa Clara, USA). The aim of this step was to reduce the amount of sugar in the sample. Sugars are highly ionizable compounds that can suppress the signal of other ion when present in large quantity (Roullier-Gall *et al.*, 2020). Samples were acidified to reach a pH value between 1.5 and 2.0 using 50% (v/v) formic acid. Then, the column was conditioned using 2 mL of methanol and 1 mL of 2% (v/v) formic acid. One mL of sample was added, followed by 1 mL of formic acid for washing. Extract was harvested by adding 1 mL of methanol. This extract was then diluted in methanol at the rate of 1/40 (v/v). Diluted samples were kept at -18°C before analysis. All reagents were purchased from Fischer Scientifics (Hampton USA).

2.3 Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry (FT-ICR-MS)

Ultrahigh-resolution FT-ICR-MS was performed with a 12 T Bruker Solarix mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an APOLLO II electrospray source in negative ionization mode (Roullier-Gall *et al.*, 2014b). The diluted samples were infused into the electrospray ion source at a flow rate of 120 $\mu L.h^{-1}$. Settings for the ion source were the following: drying gas temperature 180 °C, drying gas flow 4.0 $L.min^{-1}$, capillary voltage 3,600 V. Spectra were externally calibrated by ion clusters of arginine (10 mg L^{-1} in methanol). Internal calibration of each spectrum was carried out using a reference list including selected markers and ubiquitous fatty acids at 0.1 ppm. The spectra were acquired with a time-domain of 4 megawords and 400 scans were accumulated within a mass range of m/z 92 to 1000. A routine resolving power of 400,000 at m/z 300 was achieved (Petitgonnet *et al.*, 2019; Roullier-Gall *et al.*, 2015).

2.4 Processing of FT-ICR-MS data

Raw spectra were post-processed using the software Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany). Peaks processing was very conservative with a signal-to-noise ratio (S/N) of at least 4 that were exported to mass lists (Roullier-Gall *et al.*, 2015). For all samples, exported m/z features were aligned into a matrix containing averaged m/z values (peak alignment window width: ±1 ppm) and corresponding peak intensities. Molecular formulae were assigned to the exact m/z values by mass difference network analysis using an in-house developed software tool NetCalc (Tziotis *et al.*, 2011). In total, the matrix containing the entire sample set presented 506 detected features that could be assigned to distinct and unique molecular formulae. More than 90% of all assignments were found within an error range lower than 0.2 ppm. All further calculations and filtering were done in Perseus 1.5.1.6 (Max Planck Institute of Biochemistry, Germany) and R Statistical Language (version 3.1.1). To validate the detection of a mass for a given condition (for example SBT or BTKD7), a given mass had to be detected in at least 2 of the 3 replicates which in consequence left only 471 masses in total. Annotation of formulae was made using the METLIN database and assignation to metabolic pathways was performed using MASSTRIX database coupled with KEGG Color Mapper tool.

2.5 Repeatability of FT-ICR-MS measurements

Quality control (QC) samples were produced by mixing all extract samples in equal amounts. To monitor the reproducibility of the measurements overtime, QC samples were injected at the beginning and after every 10 samples (Annexe SE1). The coefficient of variation was then calculated from the peak intensities of all elemental compositions detected in the QC samples (Annexe SE1D). More than 90% of all elemental compositions showed a CV-value lower than 20%.

2.6 Statistical analysis

Treatment of mass lists and analysis of variance (ANOVA) with $\alpha = 0.05$ were performed using Perseus 1.5.1.6 (Max Planck Institute of Biochemistry, Germany). Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA) were performed using R software (version 4.0.1). Van Krevelen diagrams (O/C versus H/C elemental ratios) and multidimensional stoichiometric compounds classification (MSCC) have been used to elucidate main compound categories commonly defined as lipids, peptides, amino sugars, carbohydrates, nucleotides and polyphenols compounds (Rivas-Ubach *et al.*, 2018; Roullier-Gall *et al.*, 2020). Venn diagrams were generated using Molbiotools' Multiple List Comparator (https://www.molbiotools.com/listcompare.php).

3 Results and discussion

3.1 Comparison of general chemical compositions and data visualization

Peak intensities of sugared tea and kombucha samples were analyzed using HCA to display similarity in compositions (Figure E1). Clusters separated mainly BTK samples from the others and secondarily GTK samples from sugared teas (SBT and SGT). Microbial activity significantly impacted the chemical composition of the matrix. The type of tea also had an effect, since the composition of GTK samples appeared to be closer to sugared teas and thus less impacted. The production phases Showed minor impacts on the chemical composition, with separation of D7 and D12 samples inside BTK and GTK clusters, especially for GTK. P1 et P2 phases were clearly separated for GTK, whereas only one BTKD7 replicate grouped BTKD12 cluster. The next step of our approach attempted to explain the formation of those clusters.

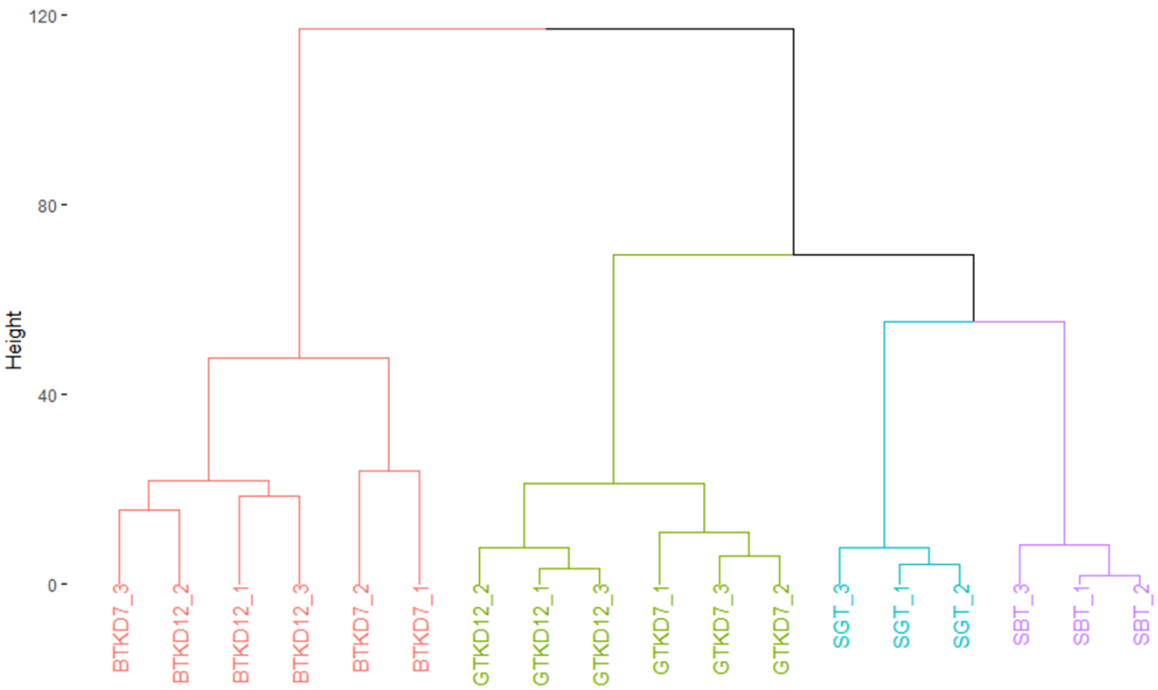


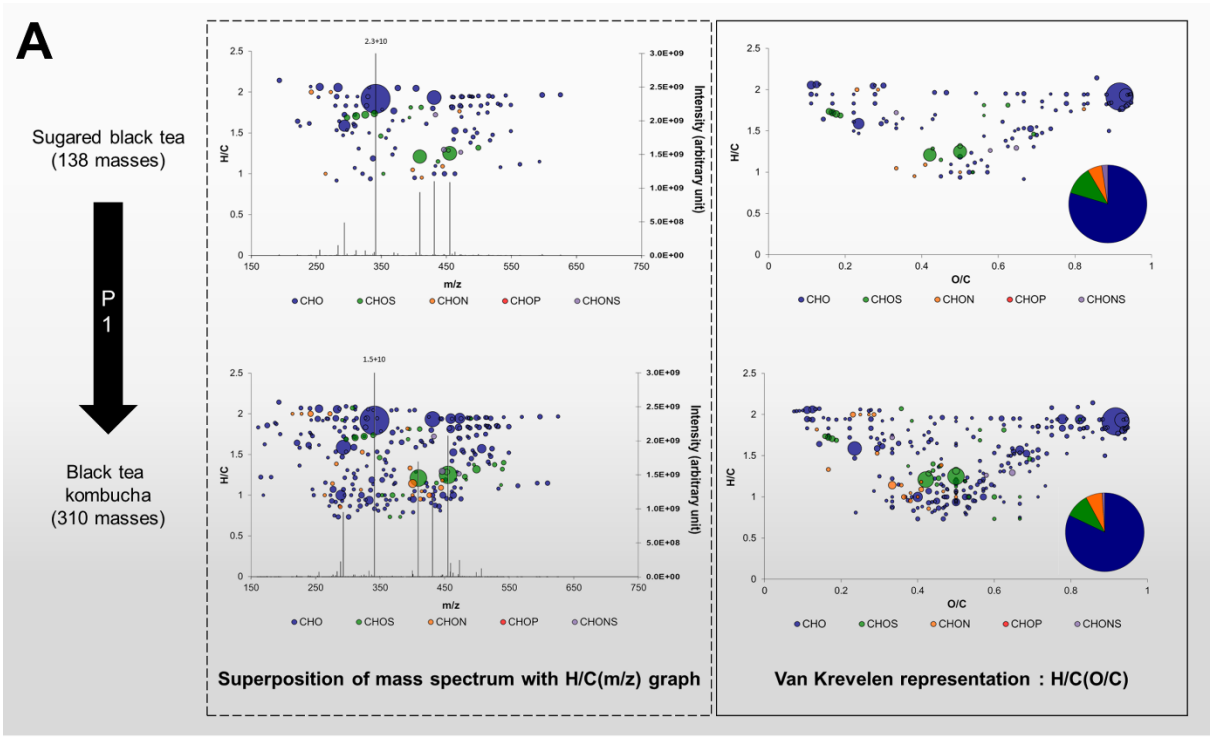
Figure E1 : Dendrogram showing composition similarities between the three replicates of sugared black tea (SBT), sugared green tea (SGT) and corresponding kombucha samples (BTK and GTK) at day 7 (D7) and day 12 (D12) samples, structured after Hierarchical Clustering Analysis.

To achieve that, it is necessary first to define terms and the way data is represented. The approach is detailed in Figure E2.

Direct infusion FT-ICR-MS was used for metabolite profiling, enabling a wide dynamic range in intensity (10^6) and we focused on the most abundant compounds (S/N>4). A peak obtained on mass spectra after FT-ICR-MS data processing associates an ion mass (m/z) to an intensity, thus providing semi-quantitative data (Figure E2A). For example, more masses were detected in BTK at D7 (310) compared to SBT (138), which corresponded to an enrichment in molecular diversity induced by P1. Based on highly precise measured m/z values, each value was annotated with a molecular or elemental formula, which distinguishes FT-ICR-MS from other tools for metabolomics. The formulae obtained in the CHONS-space can then be visually represented with different colors based on their elemental compositions and spatially using elemental ratios, for example H/C. Moreover, relative intensities can be expressed through the surface of the bubbles associated to each formula. The utilization of H/C and O/C ratios corresponds to the Van

Krevelen diagram, and it can be complemented with a diagram of the proportion in elemental compositions. Figure E2B represents the 99 formulae common to all samples and this group was labeled as “core metabolites” in a Van Krevelen diagram. This representation gives more information on the formulae, since it allowed the determination of zones corresponding to chemical families such as lipids, amino acids, carbohydrates and polyphenols (Rivas-Ubach *et al.*, 2018). The core metabolites group included mainly CHO formulae and CHOS, CHON and CHONS in lesser proportion. Also, different chemical families were represented, such as lipids, amino acids, carbohydrates, and polyphenols. By comparing the masses with databased such as METLIN used in the present study, it was possible to annotate them with putative identities (level 3 annotation level; Annexe SE2) (Roullier-Gall *et al.*, 2015, 2014b). In the case of the core metabolites group, putative identities from the sugared tea matrix could be obtained, like sucrose, glucose, fructose, palmitic, stearic, and oleic acids along with various polyphenols and phytochemicals (plant compounds).

Additionally, coupled utilization of MASSTRIX application (Wägele *et al.*, 2012) for annotation with KEGG Mapper Color application allowed to associate putative identities with molecular pathways. Annotation was performed using specific databases for *Saccharomyces cerevisiae* (model yeast) and *Acetobacter pasteurianus* (acetic acid bacterium). Distribution of putative identities according to metabolic pathways is available in Annexe SE3. Main metabolic pathways were specific to plants and involved the synthesis of polyphenols and phytochemical. Other common pathways were also represented, such as sugar metabolism or the biosynthesis of cofactors. Pathways exclusive to yeast involved starch and sucrose metabolism, and alpha linoleic acid metabolism, whereas only aromatic compounds degradation pathways were specific to the acetic acid bacterium.



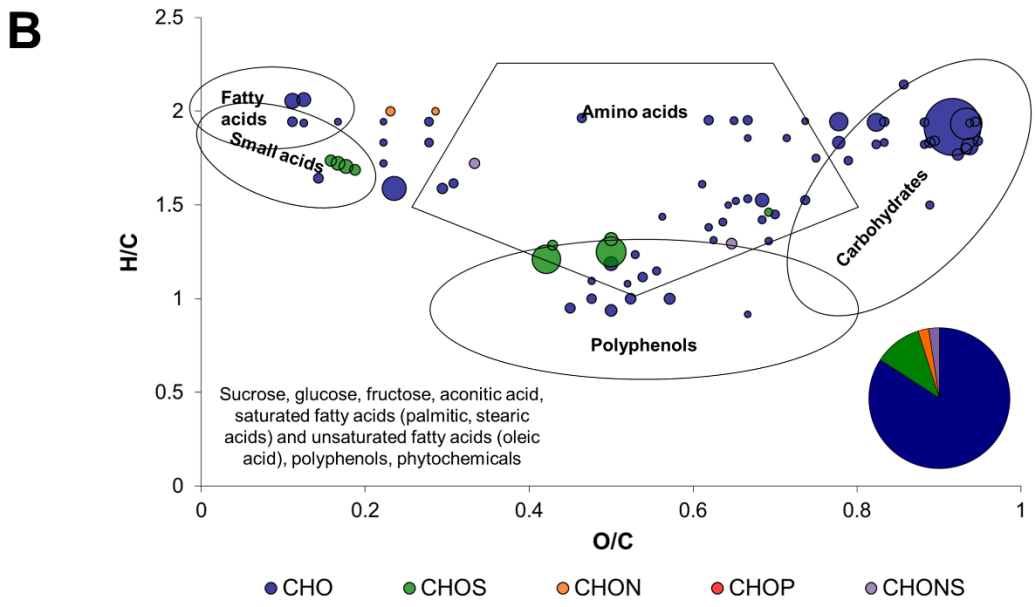


Figure E2 : Construction of graphic representations of FT-ICR-MS data. (A) From mass spectra, annotation of elemental formula allows to arrange each formula according to elemental ratios (for example H/C) and to distinguish different elemental composition using colors. By arranging formulae using H/C and O/C ratios, Van Krevelen diagrams are obtained and can be complemented with the proportions in different elemental compositions. Enrichment in composition between Sugared Black Tea and corresponding kombucha during the first phase of production (P1) can be observed. (B) Van Krevelen diagram of common formula across Sugared Teas and kombuchas at day 7 and day 12,

complemented with the proportions in different elemental compositions. The utilization of Van Krevelen diagrams allows the determination of regions corresponding to chemical families such as lipids, small acids, amino acids, polyphenols or carbohydrates, based on the analysis of a large number of compounds (Rivas-Ubach *et al.*, 2018). The surface of bubbles expresses relative formulae's intensity. With a comparison of mass lists with databased such as METLIN, putative compound identities can be obtained. Most probable candidates were added in the bottled left-hand corner of the diagram.

3.2 Impact of production phases on the molecular composition of kombucha

Firstly, the impact of production phases P1 and P2 was investigated without taking account of the tea type. Therefore, in this part the ST (sugared tea) group corresponded to common formulae of SBT and SGT. D7 group corresponded to common formulae of BTKD7 and GTKD7 and D12 group corresponded to common formulae of BTKD12 and GTKD12.

Figure E3 shows the modifications induced by P1 by comparing the variations in intensity of formulae between ST and D7. A Venn diagram indicates formulae common or exclusive to ST, D7 and/or D12 (Figure E3A). The core metabolites group of 99 formulae is represented at the intersection of the 3 ensemble circles. It is clearly visible that the main changes in molecular diversity occurred during P1 with the detection of 81 new formulae in D7 compared to ST. In contrast, only 41 new formulae were detected in D12 exclusively, while 69 were common with D7.

Between ST and D7, formulae that did or did not undergo variations were mainly CHO and a minority were CHON, CHOS or CHONS, similarly to the core metabolites group (Figure E2B). 86 formulae, including CHONS remained stable in intensity (no significant difference) and expectedly belonged to the core metabolites group: sucrose, monosaccharides, fatty acids and aconitic acid, the intermediate between citrate and isocitrate in the TCA cycle (Figure E3B). Only 26 formulae decreased in intensity belonging to small acids, polyphenols, and carbohydrates (Figure E3C). No meaningful putative identity could be included. Oppositely, 89 formulae increased in intensity, which belonged mainly to the polyphenol family, namely epigallocatechin (Figure E3D). Other compounds belonged to the carbohydrate family, lipids family (namely oleic acid) but also

organic acids: citric, gluconic and gallic acids. Citric acid is involved in the TCA cycle, whereas gluconic acid is a biomarker for the oxidative metabolism of acetic acid bacteria and highlights their activity during P1, the acidification phase of kombucha production (Lynch *et al.*, 2019; Tran *et al.*, 2020b). Finally, increase of gallic acid during kombucha production has been reported in other studies(Ivanišová *et al.*, 2019; Jayabalan *et al.*, 2007; Villarreal-Soto *et al.*, 2019). Along with the increase in epigallocatechin, this suggests the release of gallic acid through hydrolysis of gallate groups attached to polyphenols *via* ester bonds (for example epigallocatechin gallate). More evidences regarding this phenomenon were investigated in part 3.4.

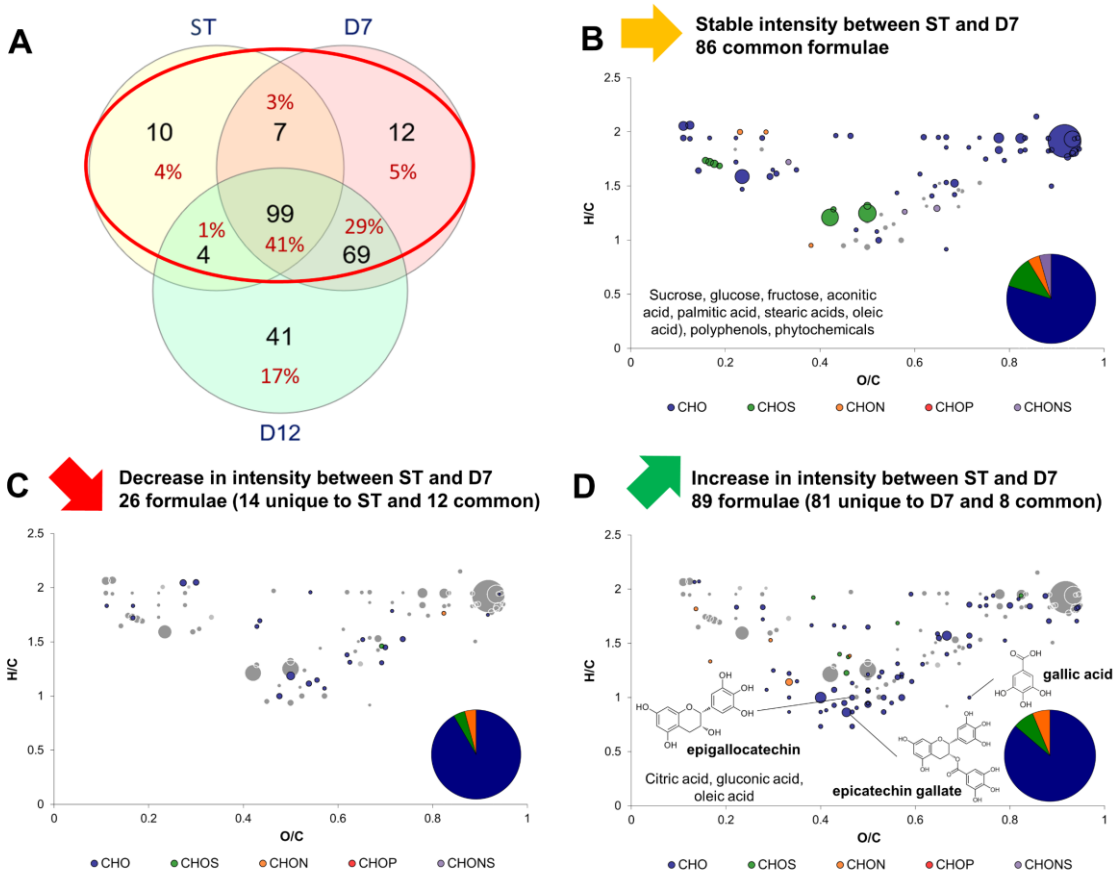


Figure E3 : Change in composition between sugared teas (common formula between sugared black and green teas) and day 7 (D7) kombuchas (common formula between black and green tea kombuchas). (A) Venn diagram of formulae identified at each process steps: Sugared tea (ST), day 7 (D7) and day 12 (D12). Van Krevelen diagrams and putative identities of (B) stable, (C) decreasing and (D) increasing formula between ST and D7. The surface of bubbles expresses relative formulae's intensity. Core metabolites formulae (Figure E2B) are represented in the background in grey.

Figure E4 shows the modifications induced by P2 by comparing the variations in intensity of formulae between D7 and D12. As stated previously, less new formulae were detected between D7 and D12 compared to between ST and D7 (Figure E4A). Both stable and varying formulae were mainly CHO. However, it is remarkable to note that the proportion in CHON, CHOS and CHONS of decreasing formulae was larger than for stable and increasing formulae during P2. Stable formulae counted 133 and included those of the core metabolites group, but also formulae that increased during P1: citric acid and gallic acid, as well as gallated and non gallated flavan-3-ols (epiafzelechin and epicatechin

gallate). 1,4-beta-glucan can be interpreted as being cellulose since they share the same molecular formula (Figure E4B). Cellulose is a biomarker of acetic acid bacteria metabolism, which produce it from monosaccharides (Lynch *et al.*, 2019). Decreasing formulae were only 30 and included fatty acids, namely oleic acid an unsaturated fatty acid, which are constituents of yeasts and acetic acid bacteria plasmic membranes (van Roermund *et al.*, 2003; Yamada *et al.*, 1981; Figure E4C). Therefore, it can be hypothesized that uptake of fatty acids from the medium was stimulated during P2, since oxygen is necessary for the synthesis of unsaturated fatty acids and sterols (Mbuyane *et al.*, 2021). It is worth noting that during P1, where microorganism have access to oxygen, oleic acid was produced (Figure E3). Additionally, the production of oleic acid during P1 could be stimulated by the acidification occurring through the oxidative metabolism of acetic acid bacteria. In *Saccharomyces cerevisiae*, oleic acid production was reported to act as a resistance mechanism against acetic acid (Guo *et al.*, 2018). Increasing formulae intensities were associated with polyphenols, phytochemicals but also carbohydrates, potentially polysaccharides (Figure E4D).

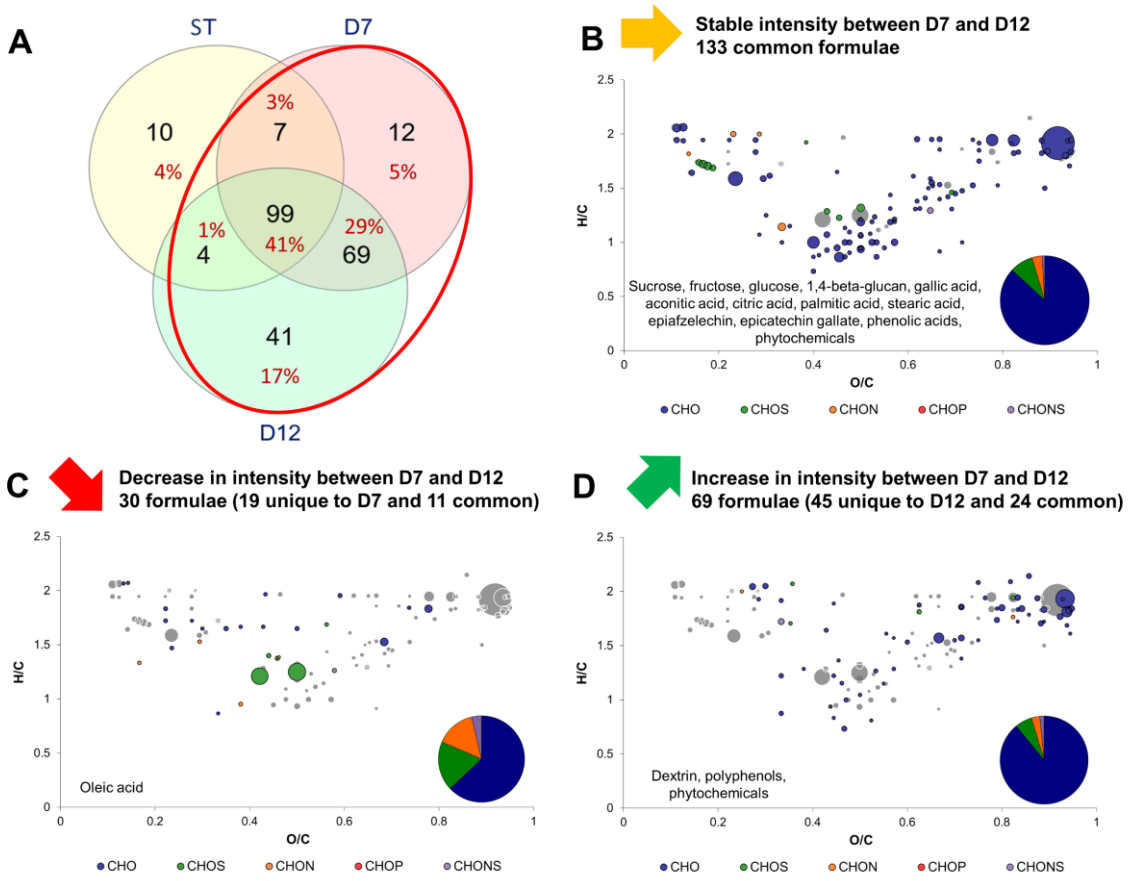


Figure E4 : Change in composition between day 7 (D7) and day 12 (D12) kombuchas (common formula between black and green tea kombuchas). (A) Venn diagram of formulae identified at each process steps: Sugared tea (ST), day 7 (D7) and day 12 (D12). Van Krevelen diagrams and putative identities of (B) stable, (C) decreasing and (D) increasing formula between D7 and D12. The surface of bubbles expresses relative formulae's intensity. Core metabolites formulae (Figure E2B) are represented in the background in grey.

To sum up, during kombucha production, sucrose, glucose fructose and fatty acids such as palmitic and stearic acids remained stable in intensity throughout the two phases. In contrast, polyphenols were widely impacted during the whole process and this suggests a strong impact of microbial activity on their structures as reported in a recent study using UPLC-QTOF-MS (Cardoso *et al.*, 2020). P1 was characterized by the production of gluconic acid through the oxidative metabolism of acetic acid bacteria, but also by the release of gallic acid. The production of those markers did not carry on during P2 and their intensity remained stable. Instead, signs of fatty acids consumption were observed,

probably due to the limitation in oxygen and diverse carbohydrates appeared to be released.

3.3 Impact of tea type on the molecular composition of kombucha

Echoing the approach used in part 3.2., this part investigates the effect of tea type on the molecular composition of kombucha. Firstly, sugared teas SBT and SGT were compared to assess the intrinsic differences of both studied teas (Figure E5). The Venn diagram (Figure E5A) highlights the striking differences regarding unique formulae, with only 18 formulae unique to SBT and 121 to SGT, while 120 were common. Therefore, the green tea used in this study was much richer in terms of molecular diversity than the black tea. Formulae equivalent in intensity belonged to the core metabolites group (Figure E5B). Formulae associated with higher intensities in SBT are CHO in majority, but proportions in CHOS and CHON represented more than 40%. They included fatty acids, polyphenols and phytochemicals (Figure E5C). Formulae that had higher intensities in SGT were mainly CHO with lower proportion in CHOS and CHON. Similarly with SBT, SGT distinguished itself through its composition in fatty acids and polyphenols that included epicatechin, epigallocatechin and epigallocatechin gallate, as well as phytochemicals and peptides (Figure E5D).

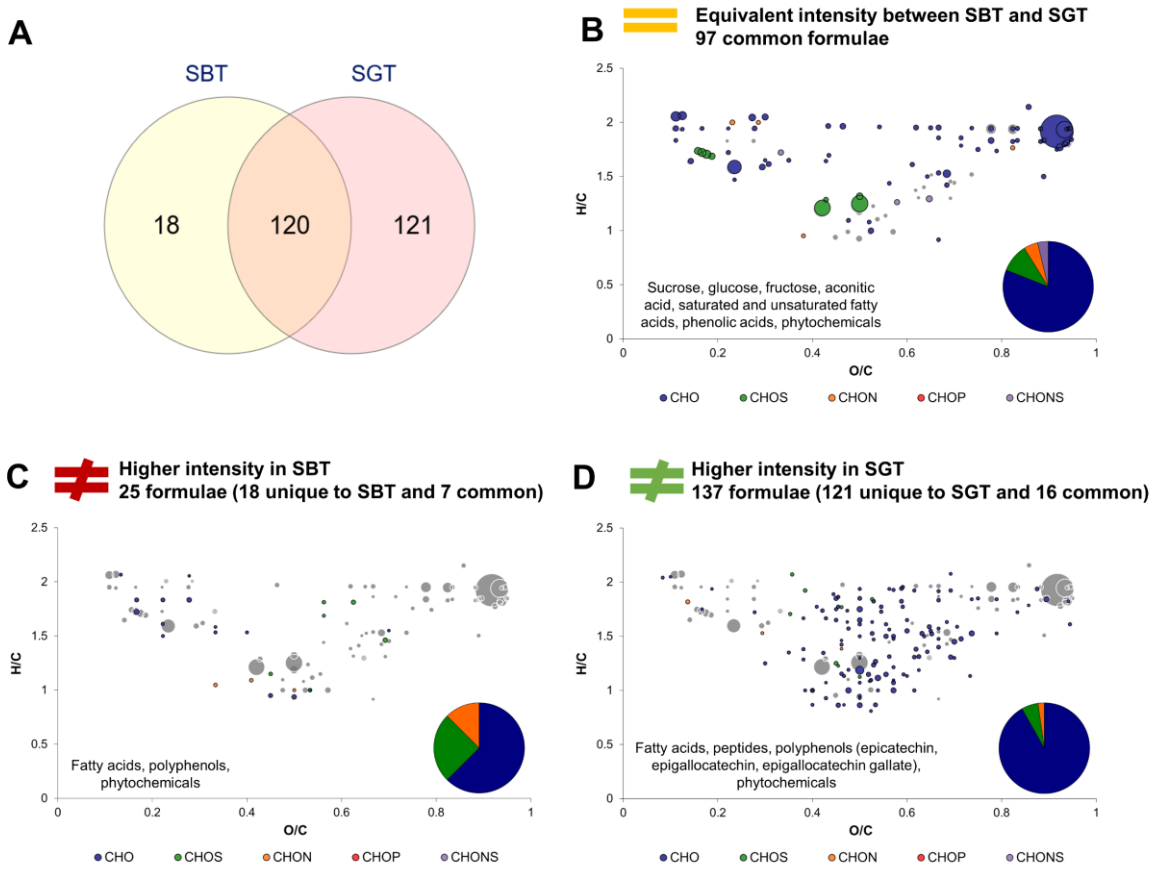


Figure E5 : Comparison of composition between Sugared Black Tea (SBT) and Sugared Green Tea (SGT). (A) Venn diagram of formulae identified in SBT and SGT. Van Krevelen diagrams and putative identities of formula (B) equivalent in intensity in both conditions, (C) higher in SBT and (D) higher in SGT. The surface of bubbles expresses relative formulae's intensity. Core metabolites formulae (Figure E2B) are represented in the background in grey.

With the differences between sugared teas in mind, corresponding kombuchas can be compared (Figure E6). In this part, BTK and GTK regrouped for each tea type the common formulae of D7 and D12, so that the impact of the process steps was left aside. For example, BTK corresponded to the common formulae of BTKD7 and BTKD12. The Venn diagram display in Figure E6A strongly contrasts with the one comparing the sugared teas (Figure E5A). While SGT showed higher molecular diversity than SBT, GTK showed less molecular diversity than BTK with respectively 48 and 87 unique formulae. Stable formulae in intensity were mainly CHO with small proportions in CHOS, CHON and CHONS (Figure E6B). They include core metabolites formulae as well as carbohydrates such as 1,4-beta-

glucan (potentially cellulose), gluconic acid (marker of acetic acid bacteria activity), palmitoleic acid, phenolic acids, phytochemicals and polyphenols (including flavan-3-ols epiafzelechin and epicatechin gallate). BTK distinguished itself by higher proportions in CHOS and CHON formulae, with specific fatty acids, peptides, polyphenols, and phytochemicals (Figure E6C), while GTK did with phenolic acids, phytochemicals, and polyphenols (Figure E6D). Interestingly, the flavan-3-ols specific to GTK were the same than for SGT (epicatechin, epigallocatechin and epicatechin gallate). Also, sucrose's signal was more intense in GTK and CHOP formulae were detected.

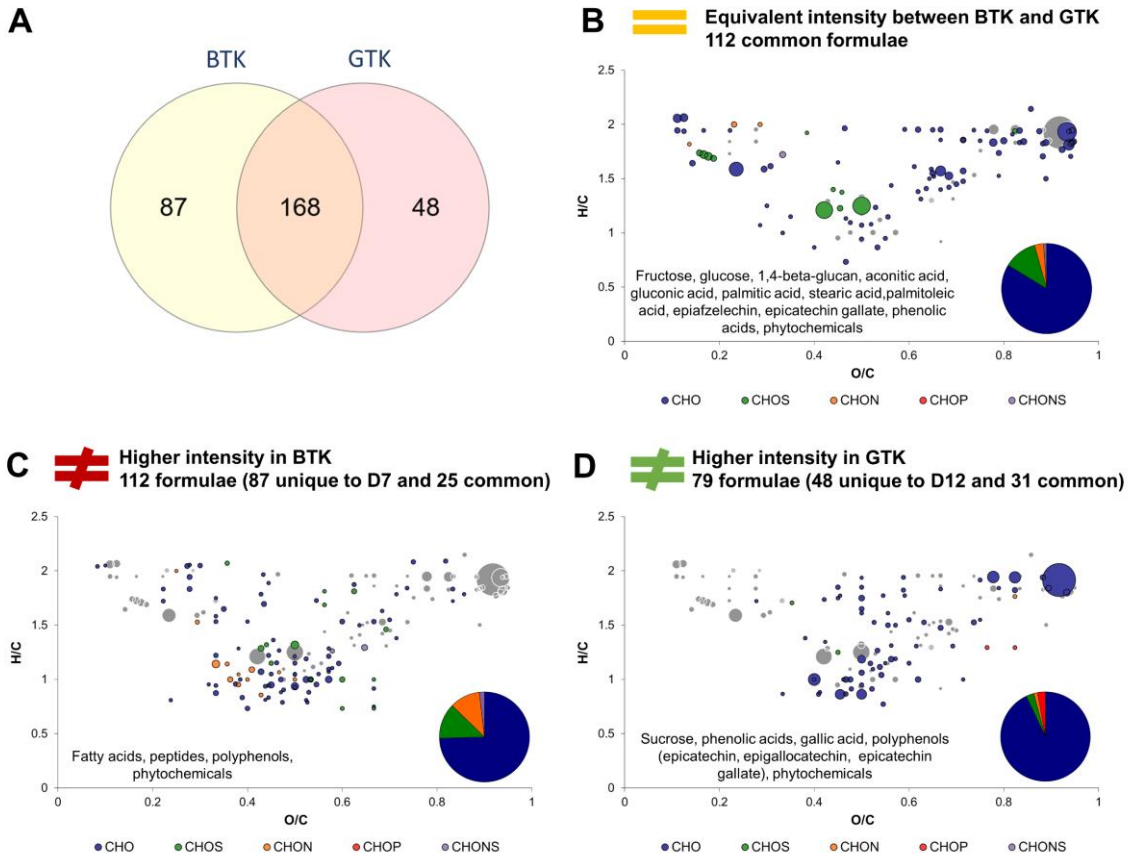


Figure E6 : Comparison of composition between Black tea kombucha (BTK) and Green Tea Kombucha (GTK) (common formulae between day 7 and day 12). (A) Venn diagram of formulae identified in BTK and GTK. Van Krevelen diagrams and putative identities of formula (B) equivalent in intensity in both conditions, (C) higher in BTK and (D) higher in GTK. The surface of bubbles expresses relative formulae's intensity. Core metabolites formulae (Figure E2B) are represented in the background in grey.

To sum up on the effect of tea type on the molecular diversity of kombucha, it appeared that BTK achieved higher molecular diversity than GTK despite SBT having initially lower diversity than SGT. While having common formulae in equivalent amount such as fatty acids and flavan-3-ols, BTK and GTK differed significantly on their fatty acids, polyphenols and phytochemical compositions. This highlights the fact that tea types did not only affect microbial metabolism but mainly the interaction between, microbial activity and tea compounds, with the generation of different polyphenols and phytochemical derivates. The same phenomenon was also observed in a recent study that compared the polyphenol composition of black and green teas and corresponding kombuchas (Cardoso *et al.*, 2020). This study also reported higher molecular diversity in polyphenols in black tea kombucha compared to green tea kombucha.

3.4 Further investigations on the release of gallic acid during P1

Release of gallic acid was highlighted during P1 along with potential presence of gallated flavan-3-ol, particularly in SGT and GTK throughout the process. Further investigation regarding the origin of this gallic acid was carried out. The hypothesis was that gallic acid was released from a degallation reaction, meaning the hydrolysis of an ester bound between a gallate group and another molecule, for example a flavan-3-ol such as epicatechin gallate. To achieve this, a R script was conceived to screen formulae with a difference in elemental composition of 7C, 4H and 3O, corresponding to the elemental composition of gallic acid $C_7H_6O_5$ minus H_2O consumed by hydrolysis reaction. This returned 63 candidates that were either precursors or products of the degallation reaction. After verification, only 3 couples could possibly be part of such reaction: epicatechin/epicatechin gallate, epigallocatechin/epigallocatechin gallate and epiafzelechin/epiafzelechin gallate. Positions of gallic acid, epigallocatechin, epicatechin gallate on theVan Krevelen diagram are indicated on Figure E3D. Although none of those putative identities were detected in SBT, all of them were detected in BTK except epicatechin gallate and epiafzelechin gallate (Annexe SE3). Epigallocatechin and epiafzelechin gallate putative identities were not detected in SGT but all putative identities were detected in GTK with significantly higher intensity if detected in SGT. This means that, when detected, all putative formulae underwent an increase during P1 along with gallic acid. Since monogallated forms did not undergo a decrease or stagnation in

intensity, it implies that degallation might occur in digallated forms, or polymeric polyphenols with multiple gallate groups that could not be annotated. Such compounds' masses might be higher than the range of analysis or they did not belong in the databases. It has been hypothesized that polyphenol transformation was induced by microbial activity, either spontaneously due to physical chemical changes, such as the decrease in pH value, or biologically through microbial enzymatic activity, that has yet to be determined (Chen and Liu, 2000; Jayabalan *et al.*, 2007). Echoing this observations, a two-way interaction between wine polyphenol and gut microbiota has been identified, leading to a regulation of the microbiota and an alteration of polyphenols structures through microbial activities (Dueñas *et al.*, 2015). Although this non-targeted investigation could not conclude on the origin of the gallic acid release during P1, it provided strong basis for experimentation based on targeted methods. Indeed, the increase of gallic acid content in kombucha is valuable because the lowering of polyphenols' molecular mass increases their bioavailability as antioxidants in the context of human nutrition. Namely, gallic acid and epicatechin showed better absorption than epicatechin gallate (Scalbert *et al.*, 2002). Thus, further investigation is needed to determine the benefits of kombucha microbial activity for the bioavailability of tea polyphenols.

5 Conclusions

The non-targeted analysis of black and green sugared tea and kombuchas samples allowed to characterize the impact of production phases and the type of tea used on the molecular changes occurring during the process. The clustering based on global metabolome shown in Figure E1 showed that microbial activity had a major impact on the non-volatile composition of the sugared tea matrix. The biological acidification phase P1 in open vessel was the production step that transformed the most the matrix with the production of news compounds including gluconic acid, marker of acetic acid bacteria activity. This phase was also characterized by the transformation of polyphenols and the release of gallic acid from bound forms. The natural carbonation phase P2 in closed vessel was characterized by a less changes and the consumption of fatty acids in a context of oxygen deprivation. Clustering also showed an effect of the type of tea on the compositions of kombuchas. Black tea and green tea induced the production of different metabolites, but black tea kombucha underwent a much stronger modification of

polyphenols than green tea kombucha, which explains why green tea kombucha was closer to the sugared tea according to clustering analysis (Figure E1).

This study gave extensive insight into the nature of the transformations happening to sugared tea during kombucha production, and how the type of tea influenced it. Those new elements are useful to further define kombucha and guide the conception of its regulation worldwide. New hypotheses were raised and can be used as the basis of further investigation using targeted methods. For example, the mechanisms of polyphenol transformation and its role in potential health benefits of kombucha consumption is a potential topic to cover. However, similar non-targeted approach could be used to investigate microbial interaction in kombucha with experimental designs involving controlled microbial compositions.

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Declaration of interest

Authors declare no conflict of interest.

Author contributions

Thierry Tran took the lead of the writing of this article, but all other authors provided critical and complementary elements to the manuscript. Thierry Tran performed the generation of samples and the data treatment. Rémy Romanet provided the R script for the screening of formulae. Chloé Roullier-Gall provided extensive help on data treatment and writing of the manuscript. Philippe Schmitt-Kopplin performed FT-ICR-MS analysis and provided the raw data. François Verdier and Antoine Martin provided the kombucha cultures used in the experiments.

F Chapitre 4 : Interactions levure-levure et levure-bactérie acétique de kombucha à travers le prisme de la métabolomique.

Reposant exactement sur la même méthode analytique de métabolomique précédente (Chapitre 3), l'étude décrite dans ce quatrième chapitre se concentre sur les interactions entre micro-organismes mis en culture dans du thé noir sucré au cours de la première phase d'acidification en condition ouverte de 7 jours. Il doit être soumis sous forme d'article au journal *Food Chemistry: Molecular Sciences*. Les interactions levure-levure ont été investiguées avec les espèces *B. bruxellensis* et *H. valbyensis*, et les interactions entre levures et bactérie acétique avec ces deux espèces levuriennes en coculture avec *A. indonesiensis* séparément en duo ou en trio.

L'interaction entre *B. bruxellensis* et *H. valbyensis* induit la consommation de peptides et la transformation de composés phénoliques ou d'origine végétale plus largement. La production de composés appartenant aux familles moléculaires des acides gras et des peptides a également été observée. Les interactions entre levures et bactérie acétique ont montré des comportements très différents selon l'espèce de levure en présence. En présence de *A. indonesiensis*, *B. bruxellensis* a principalement induit la production d'acides gras, de peptides et la transformation de composés phénoliques. A l'inverse, *H. valbyensis* a principalement induit la disparition de composés impliqués dans le métabolisme des acides aminés, dont des peptides, ainsi que des acides gras avec une très faible production de nouveaux composés. Ce type de comportement a également été observé pour l'interaction entre le couple de levure et la bactérie acétique (trio). Il faut cependant noter que la production des métabolites issus de l'interaction levure-levure est, pour la plupart d'entre eux, diminuée en présence de *A. indonesiensis*. Ceci a pour conséquence de rapprocher cette coculture en trio des monocultures et de la matrice thé sucré, en termes de composition globale. La consommation de l'acide hydroxy stéarique d'origine levurienne semble être un trait constant dans les interactions levure-bactérie. Elle pourrait signaler une consommation par *A. indonesiensis* qui pourrait mener à la production de γ-dodecalactone. L'impact de la levure sur *A. indonesiensis* est similaire quelle que soit l'espèce levurienne. En outre, les composés propres à *A. indonesiensis* diminuant en présence de levure, indifféremment de l'espèce, sont des polyphénols, des acides gras et l'acide déhydroquinique. Ce métabolite universel faisant la jonction entre la glycolyse et la biosynthèse des acides aminés pourrait être mis à disposition pour les levures en coculture avec *A. indonesiensis*. Cette mise à

disposition réciproque de métabolites entre micro-organismes caractérise une interaction mutualiste.

L'analyse métabolomique permet de soulever de nouvelles hypothèses concernant les interactions microbiennes ayant lieu au cours de la production de kombucha. La mise en évidence des métabolites clés putatifs appelle à l'utilisation de méthodes analytiques quantitatives comme la LC-MS afin de confirmer ces hypothèses.

Microbial interactions in kombucha through the lens of metabolomics

Running Title: Kombucha microbial interactions using metabolomics

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Abstract

Exo-metabolomes of kombucha microorganisms were analyzed using FT-ICR-MS to investigate their interactions. Kombucha is a fermented beverage obtained through the activity of a complex microbial community of yeasts and bacteria. A simplified set of micro-organisms including two yeasts (*Brettanomyces bruxellensis* and *Hanseniaspora valbyensis*) and one acetic acid bacterium (*Acetobacter indonesiensis*)

was used to investigate yeast-yeast and yeast-acetic acid bacterium interactions. Yeast-yeast interaction was characterized by the consumption of peptides and the production of fatty acids and peptides. Yeast-acetic acid bacterium interaction was different depending on yeast species. With *B. bruxellensis*, fatty acids and peptides were mainly produced, whereas with *H. valbyensis*, peptides and fatty acids were mainly consumed. With all three microorganisms, the formulae produced from yeast-yeast interaction were consumed or not produced in presence of *A. indonesiensis*. Hydroxystearate from yeasts and dehydroquinate from *A. indonesiensis* were consumed in all cases of yeast(s)-acetic acid bacterium pairing, highlighting mutualistic behavior.

Keywords

kombucha, interaction, metabolomics, yeast, acetic acid bacteria, fermentation.

1 Introduction

As the knowledge linking human microbiota (especially in the gut) to human health deepens, the interest regarding the consumption of fermented food with or without live microorganisms (dietary microorganisms) is increasing (Bell *et al.*, 2018; Marco *et al.*, 2017). Evidence pointing the benefits of dietary microorganisms and their activity on the nutritional quality of food accumulates as new strong hypotheses emerged on the interactions of live food-borne microorganisms and their metabolites with the gut microbiota (Marco *et al.*, 2021). Therefore, the study of microbial interactions is relevant to understand phenomena and mechanisms that occur in the context of food fermentations, whether those interactions involve food borne microorganisms and the host, or the fermenting microorganisms themselves in relationship to the control of fermentations involving complex microbial communities (Aldrete-Tapia *et al.*, 2018).

Kombucha is a traditional beverage of interest regarding the consumption of dietary microorganisms. It is produced through the transformation of sugared tea infusion by a consortium of yeasts and acetic acid bacteria, with or without the presence of lactic acid bacteria (Tran *et al.*, 2020a). It has been determined that those consortia (occurring with many different compositions in genera and species) used as starter cultures induced different microbial interactions. On the metabolic level, acetic acid bacteria are dependent

on yeasts for the release of assimilable nutrients that are glucose, fructose and ethanol released through invertase activity then alcoholic fermentation (Tran *et al.*, 2020b). Microbial interactions in foods were traditionally studied through the use of culture-dependent microbiological analysis and targeted chemical analyses as it was the case in sourdough (De Vuyst and Neysens, 2005). However, the growing accessibility of "omics" approaches involving non culture-dependent microbiological analyses (metagenomics) and nontargeted chemical analysis (metabolomics, proteomics) opened new perspectives for the study of food matrices and fermentations (Leech *et al.*, 2020). Therefore, the use of other approaches and analytic tools, namely non-targeted ones, could help to uncover other types of interactions in kombucha (May *et al.*, 2019; Tran *et al.*, 2020a).

Microbial interactions can be categorized according to positive or negative impact of one microorganism population on another (Ivey *et al.*, 2013). Competition for resources can occur between population sharing similar needs in nutrients. Amensalism refers to a negative impact of one population on another, for example through the secretion of toxins. Commensalism corresponds to the production of one metabolite by one population that can be exploited by another. In the case that one population benefits from another while impacting the later negatively, the interaction is characterized by parasitism. Finally, if both population benefits from each other's presence, it is characterized by mutualism.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS) is a non-targeted analysis of nonvolatile compounds that has already been used to study microbial interaction using metabolomics in fermented beverages, particularly grape must in the context of wine fermentation. Metabolites present in the food matrix, including those released by microorganisms, are named as a whole "exo-metabolome" and the qualitative study of exo-metabolomes is called "footprinting" (Mashego *et al.*, 2007). Interspecies yeast-yeast interactions between *Saccharomyces* and non-*Saccharomyces* species in grape must have been investigated by comparing exo-metabolomes obtained by FT-ICR-MS (Petitgonnet *et al.*, 2019; Roullier-Gall *et al.*, 2020). These studies confirmed the impact of such interaction on the composition in metabolites and namely the effect of cell-cell contact on yeast metabolism which involved compounds from different molecular families (carbohydrates, amino acids, nucleic acids, and polyphenols). Interkingdom yeasts-bacteria interactions have also been investigated between

Saccharomyces cerevisiae and *Oenococcus oeni* following a similar approach (Liu *et al.*, 2016). Detailed interpretation of data highlighted individual markers such as gluconic acid, citric acid, trehalose and palmitic acid, which were reported to be metabolized by both microorganisms and be the support of metabolic interplay.

The present study aims at analyzing and comparing exo-metabolomes of monocultures and cocultures in sugared tea of microorganisms isolated from the same kombucha consortium using FT-ICR-MS tool. The study involved two yeasts (*Brettanomyces bruxellensis* and *Hanseniaspora valbyensis*) and one acetic acid bacterium (*Acetobacter indonesiensis*) that are representative of the microbial composition of the original kombucha consortium in terms of population levels. Additionally, those genera and species have been reported to be widely represented in kombucha consortia in general and sucrose-based fermented food (Harrison and Curtin, 2021; Leech *et al.*, 2020). The qualitative and quantitative comparison of exo-metabolomes is expected to highlight the stimulation or inhibition of non-volatile metabolites release or pathways associated with microbial interactions between yeasts and the acetic acid bacterium.

2 Materials and Methods

2.1 Generation of biological samples

Samples analyzed in this study came from the same experiment conducted in another study (Tran *et al.* 2021a - Under Review ; Chapitre 5). Briefly, sugared black tea infusion was produced by steeping 1 g L^{-1} of black tea (Pu'er Grade 1 TN4107) from Les Jardins de Gaia© (Wittisheim, France) in boiling water for one hour. After cooling down at room temperature, 50 g L^{-1} blond cane sugar from Ethiquable© (Fleurance, France) was dissolved. Then 12% (v/v) of 7 days kombucha was added. The mother culture used to produce this 7 days kombucha was obtained from Biomère (Paris, France). After inoculation, incubation occurred at 26°C in static conditions during 7 days for the first phase of production in open vessel, that corresponds to a biological acidification (Tran *et al.*, 2020a). Aluminum foil was loosely applied on the bottle neck to prevent contact with particles and insects. Samples obtained from original kombucha culture, labelled BTK, were used to compare global metabolic signature with cultures from isolated

microorganisms. Those cultures were carried out by inoculating sugared black tea with microorganisms isolated from the same kombucha consortium than BTK samples. Isolation and selection process was described in detail in a previous study (Tran *et al.*, 2020b). Yeasts and bacteria were isolated from the broth and the pellicle using differential agar media. Identification of microorganisms was performed using biomolecular methods (26S and 16S PCR for yeasts and bacteria, respectively). Selection of yeasts and acetic acid bacteria for further investigation was made based on their representativity and functionality in the context of kombucha production (Tran *et al.*, 2020b). As a result, two yeasts, *Brettanomyces bruxellensis* and *Hanseniaspora valbyensis*, and one acetic acid bacteria *Acetobacter indonesiensis* were selected to investigate yeast interspecies interactions and yeasts-acetic acid bacterium interkingdom interactions. Consequently, monocultures of *B. bruxellensis* (BB), *H. valbyensis* (HV), *A. indonesiensis* (AI), yeast-yeast coculture (BBHV), yeasts-acetic acid bacterium (BBAI, HVAI) and the trio made of two yeasts and one acetic acid bacterium (T) were produced. Cells washed with sugared black tea were inoculated at the rate of $1.0.10^5$ cells mL^{-1} using the same procedure as in our previous study (Tran *et al.*, 2020b). Each culture was performed in triplicates in 123 mL Boston flasks with a Specific Interfacial Surface (SIS) (Cvetković *et al.*, 2008) of 0.01 cm^{-1}. Sugared tea samples possessed a total sugar content of 58.9 ± 1.0 g L^{-1}, a pH value of 6.90 ± 0.01 and a total acidity inferior to 1 meq L^{-1}. Samples possessed a total sugar content ranging from 39.1 to 56.1 g L^{-1}, pH values between 4.09 and 4.75 and a total acidity between 19 and 21 meq L^{-1}. Important discrepancies in sugar contents, pH and total acidity values can be explained by very different activities between yeast and acetic acid bacteria monocultures compared to yeast-acetic acid bacteria cocultures, in which metabolic interplay is known to stimulate sugar metabolism and organic acid production (Tran *et al.*, 2020b). Sugar content was measured using Sucrose Glucose Fructose enzymatic kit from Biosentec (Portet-sur-Garonne, France). pH values were measured with a Mettler Toledo Five Easy pH meter coupled with a LE498 probe and total acidity was determined by titration with 0.1 N NaOH and 0.2% phenolphthalein as color indicator, with reagents purchased from Merck (Darmstadt, Germany).

2.2 Sample preparation

Samples were centrifuged at 3,000 x g for 10 minutes at 4°C to remove cells and particles before freezing at -18°C. Once all samples were available, solid phase extraction (SPE) was performed using Bond Elut C18 cartridges from Agilent (Santa Clara, USA). The aim of this step was to reduce the amount of sugar in the sample. Sugars are highly ionizable compounds that can then suppress the signal of other ion when present in large quantity (Roullier-Gall *et al.*, 2020). Samples were acidified to reach a pH value between 1.5 and 2.0 using 50% (v/v) formic acid. The column was conditioned using 2 mL of methanol and 1 mL of 2% (v/v) formic acid. One mL of sample was added, followed by 1 mL of formic acid for washing. Extract was harvested by adding 1 mL of methanol. This extract was then diluted in methanol at the rate of 1/40 (v/v). Diluted samples were kept at -18°C before analysis. All reagents were purchased from Fischer Scientifics (Hampton USA). Formic acid (CAS 64-18-6) mother solution (50% (v/v) possessed puriss. p.a. grade and pure methanol (CAS 67-56-1) was UHPLC-MS grade with purity ≥ 99.9%.

2.3 Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry (FT-ICR-MS)

Ultrahigh-resolution FT-ICR-MS was performed with a 12 T Bruker Solarix mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an APOLLO II electrospray source in negative ionization mode (Roullier-Gall *et al.*, 2014b). The diluted samples were infused into the electrospray ion source at a flow rate of 120 µL h^{-1}. Settings for the ion source were: drying gas temperature 180 °C, drying gas flow 4.0 L min^{-1}, capillary voltage 3,600 V. Spectra were externally calibrated by ion clusters of arginine (10 mg L^{-1} in methanol). Internal calibration of each spectrum was carried out using a reference list including selected markers and ubiquitous fatty acids at 0.1 ppm. The spectra were acquired with a time-domain of 4 megawords and 400 scans were accumulated within a mass range of m/z 92 to 1000. A routine resolving power of 400,000 at m/z 300 was achieved (Petitgonnet *et al.*, 2019; Roullier-Gall *et al.*, 2015).

2.4 Processing of FT-ICR-MS data

Raw spectra were post-processed using the software Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany). Peaks processing was very conservative with a signal-to-

noise ratio (S/N) of at least 4 that were exported to mass lists (Roullier-Gall *et al.*, 2015). For all samples, exported m/z features were aligned into a matrix containing averaged m/z values (peak alignment window width: ±1 ppm) and corresponding peak intensities. Molecular formulae were assigned to the exact m/z values by mass difference network analysis using an in-house developed software tool NetCalc (Tziotis *et al.*, 2011). In total, the matrix containing the entire sample set presented 506 detected features that could be assigned to distinct and unique molecular formulae. More than 90% of all assignments were found within an error range lower than 0.2 ppm. All further calculations and filtering were done in Perseus 1.5.1.6 (Max Planck Institute of Biochemistry, Germany) and R Statistical Language (version 3.1.1). To validate the detection of a mass for a given condition (for example SBT or BBAI), a given mass had to be detected in at least 2 of the 3 replicates which in consequence left only 307 masses in total. Annotation of formulae was made using the METLIN database and assignation to metabolic pathways was performed using MASSTRIX database coupled with KEGG Color Mapper tool.

2.5 Repeatability of FT-ICR-MS measurements

Quality control (QC) samples were produced by mixing all extract samples in equal amounts. To monitor the reproducibility of the measurements overtime, QC samples were injected at the beginning and after every 10 samples (Annexe SF1). The coefficient of variation was calculated from the peak intensities of all elemental compositions detected in the QC samples (Annexe SF1D). More than 90% of all elemental compositions showed a CV-value lower than 20%.

2.6 Statistical analysis

Treatment of mass lists and analysis of variance (ANOVA) with $\alpha = 0.05$ were performed using Perseus 1.5.1.6 (Max Planck Institute of Biochemistry, Germany). Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA) were performed using R software (version 4.0.1). Van Krevelen diagrams (O/C versus H/C elemental ratios) and multidimensional stoichiometric compounds classification (MSCC) have been used to elucidate main compound categories commonly defined as lipids, peptides, amino acids, carbohydrates, nucleotides and polyphenols compounds (Rivas-Ubach *et al.*,

2018; Roullier-Gall *et al.*, 2020). Venn diagrams were generated using Molbiotools' Multiple List Comparator (https://www.molbiotools.com/listcompare.php).

3 Results and discussion

3.1 Comparison of general chemical compositions and data visualization

Peak intensities of sugared tea and cultures samples were analyzed using Hierarchical Cluster Analysis (HCA) to display similarity in non-volatile chemical compositions (Figure F1A). BTK (Black Tea Kombucha) has been included to assess the closeness in composition of cultures from isolated kombucha microorganisms with original kombucha. Clusters separated mainly BTK samples from those cultures and SBT (Sugared Black Tea), highlighting a strong distance between original kombucha and the cultures in terms of nonvolatile chemical composition. This can be explained by the difference in microbial compositions with BTK possessing much higher microbial diversity than the other cultures (Tran *et al.*, 2020b). Moreover, the inoculation methods were different, with the inclusion of metabolites from the previous batch in the case of BTK corresponding to traditional kombucha production method. This did not happen for the other cultures which occurred through the addition of washed cells grown on culture medium. Inside the cluster excluding BTK, the main subclusters discriminated cocultures involving *B. bruxellensis* except the coculture T with the simultaneous presence of the three microorganisms. The yeast *B. bruxellensis* appeared to strongly impact the composition of the medium in association with *H. valbyensis* only or with *A. indonesiensis* only. In the remaining branch, AI and HVAI were opposed to SBT, BB, HV and T. It appears that the presence of *H. valbyensis* in HVAI coculture had little impact when compared to the AI monoculture, suggesting an important impact of *A. indonesiensis*. Also, it seems that yeasts (BB and HV) induced the least changes of the matrix SBT in comparison with the acetic acid bacterium in monoculture since they belonged to the same cluster. The presence of the coculture of the two yeasts and one acetic acid bacterium T in this cluster was surprising, because of its apparent dissimilarity with other cocultures (BBAI, HVAI, BBHV) that are closer to this condition on the microbiological level. Additionally, with a higher biodiversity, T could be expected to not share the same cluster than the unfermented matrix SBT. It is worth noting that yeasts monocultures were closer than the acetic acid bacterium

monoculture, highlighting proximity between organisms of the same kingdom. Detailed study of metabolite transformations will help understanding the reason of such clustering. However, several elements of data visualization need to be explained beforehand.

Direct infusion FT-ICR-MS was used for metabolite profiling, enabling a wide dynamic range in intensity (10^6) and we focused on the most abundant compounds (S/N>4). After data processing, peaks appearing on FT-ICR-MS mass spectra associate a set of ion masses (m/z) with intensity, thus providing semi-quantitative data (Figure F1B). Based on highly precise measured m/z values, each value was annotated with a molecular or elemental formula, which distinguishes FT-ICR-MS from other tools for metabolomics. The formulae obtained in the CHONS-space can be visually represented with different colors based on their elemental compositions and spatially using elemental ratios, for example H/C. The utilization of H/C and O/C ratios corresponds to the Van Krevelen diagram and it can be complemented with a diagram of the proportion in elemental compositions. Figure F1C represents the 136 formulae common to all samples. This group was labeled as “core metabolites” in a Van Krevelen diagram. This representation gives more information on the formulae, since it allowed the determination of zones corresponding to chemical families such as lipids, amino acids, carbohydrates and polyphenols (Rivas-Ubach *et al.*, 2018). Moreover, relative intensities can be expressed through the surface of the bubbles associated to each formula. The core metabolites group included mainly CHO formulae and CHOS and CHON in lesser proportion. Different chemical families were represented (lipids, amino acids, carbohydrates, and polyphenols). By comparing the masses with databases such as METLIN used in the present study, it is possible to annotate them with putative identities (level 3 annotation; Annexe SF2) (Roullier-Gall *et al.*, 2015, 2014b). In the case of the core metabolites group, putative identities from the sugared tea matrix could be obtained, like sucrose, glucose, fructose, dextrin, isopropyl malate (intermediate of leucine biosynthesis), palmitic, stearic and oleic acids, along with various polyphenols and phytochemicals (plant compounds).

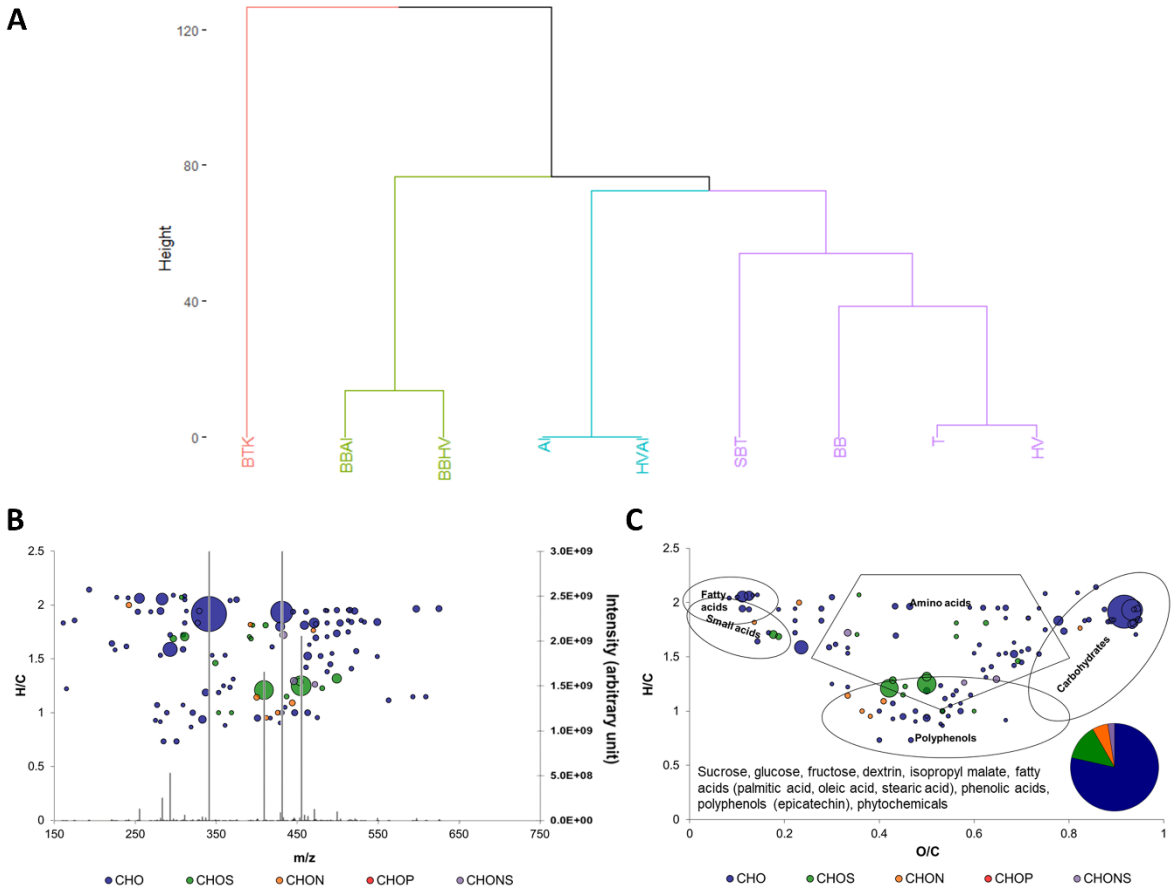

Figure F1 : Visualization of composition differences between the samples analyzed by FT-ICR-MS (A) HCA dendrogram showing composition similarities between sugared black tea (SBT), cultures at day 7 including *Brettanomyces bruxellensis* (BB), *Hanseniaspora valbyensis* (HV) and *Acetobacter indonesiensis* (AI) isolated from black tea kombucha (BTK), structured after Hierarchical Clustering. (B) Superposition of mass spectrum and distribution of annotated element formula according to H/C ratio and m/z. Colors distinguish different elemental compositions. (C) Van Krevelen diagram of common formulae across Sugared Black Tea and all cultures (“core metabolites” group), complemented with the proportions in different elemental compositions. The utilization of Van Krevelen diagrams allows the determination of regions corresponding to chemical families such as lipids, small acids, amino acids, polyphenols or carbohydrates, based on the analysis of a large number of compounds (Rivas-Ubach *et al.*, 2018). The surface of bubbles expresses relative formulae’s intensity. Comparison of mass lists with databased such as METLIN, putative compound identities can be obtained. Most probable candidates were added in the bottled left-hand corner of the diagram.

Additionally, coupled utilization of MASSTRIX application (Wägele *et al.*, 2012) for annotation with KEGG Mapper Color application allowed to associate putative identities with molecular pathways. Annotation was performed using specific databases for *Saccharomyces cerevisiae* (model yeast) and *Acetobacter pasteurianus* (acetic acid bacterium). Distribution of putative identities according to metabolic pathways is available in Annexe SF3. Common pathways, such as sugar metabolism or the biosynthesis of cofactors were mainly represented. Pathways exclusive to yeasts involve starch and sucrose metabolism, and alpha linoleic acid metabolism, whereas only aromatic compounds degradation pathways were specific to the acetic acid bacterium.

3.2 Metabolic signature of individual microorganisms

Before comparing monocultures and cocultures exo-metabolomes, metabolic signatures of each microorganism studied were represented in Van Krevelen diagrams (Figure F2). Those formulae were associated with one of the microorganisms if they were detected significantly in higher intensity than one of the two other microbes. *H. valbyensis* and *A. indonesiensis* possessed a similar number of signature formulae (61 and 64 respectively), whereas *B. bruxellensis* had less (38). The signature formulae of all three microorganisms were composed mostly of CHO. For yeasts, they were composed to a lesser extent of CHOS and CHON, in opposition with the signature formulae of *A. indonesiensis* that possessed no CHOS. Signature formulae also belonged to different molecular families: mainly fatty acids, carbohydrates, and polyphenols. Plant metabolites, polyphenols and phytochemicals, were molecular families that widely discriminated the microorganisms. It is worth noting that epicatechin gallate was potentially associated to *A. indonesiensis* and was reported in kombucha by several studies (Jayabalan *et al.*, 2007; Villarreal-Soto *et al.*, 2019) (Figure F2C). Additionally, *B. bruxellensis* and *H. valbyensis* distinguished themselves from the acetic acid bacterium with fatty acids (potentially linoleic and hydroxy stearic acids; Figures F2A and F2B). *H. valbyensis* and *A. indonesiensis* shared azelaic acid and aconitic acid (intermediate between citrate and isocitrate in the TCA cycle) as putative signature compounds (Figures F2B and F2C). However, the putative compound phenylpyruvate was specific to *H. valbyensis*. This metabolite is a precursor of the volatile organoleptic compounds phenylethanol and phenylethyl acetate, which have been reported to be produced in higher amounts by this

yeast species in both kombucha and cider (Nogueira *et al.*, 2008). The fact that peptides were also specific of this yeast species could be linked to the fact that it experienced more mortality inducing the release of intracellular peptides. Indeed, the genome of this species informs that it is part of a fast-evolving lineage that lost genes related to DNA repair and cell cycle that could induce higher mortality (Steenwyk *et al.*, 2019). Regarding *A. indonesiensis*, gluconic acid appeared to be a signature compound. This metabolite results from the oxidation of glucose (Lynch *et al.*, 2019). Another signature formula was associated to 1,4-β-D-glucan putative identity, probably cellulose which shares the same formula. Cellulose is also a typical product of acetic acid bacteria leading to the formation kombucha pellicle through the utilization of monosaccharides (glucose and fructose) (Tran *et al.*, 2021b; Villarreal-Soto *et al.*, 2018). Globally, metabolic signatures differentiated microorganisms, in relation with their kingdom. The next step examined the changes brought by the interaction of those microorganisms when cultured together in sugared black tea.

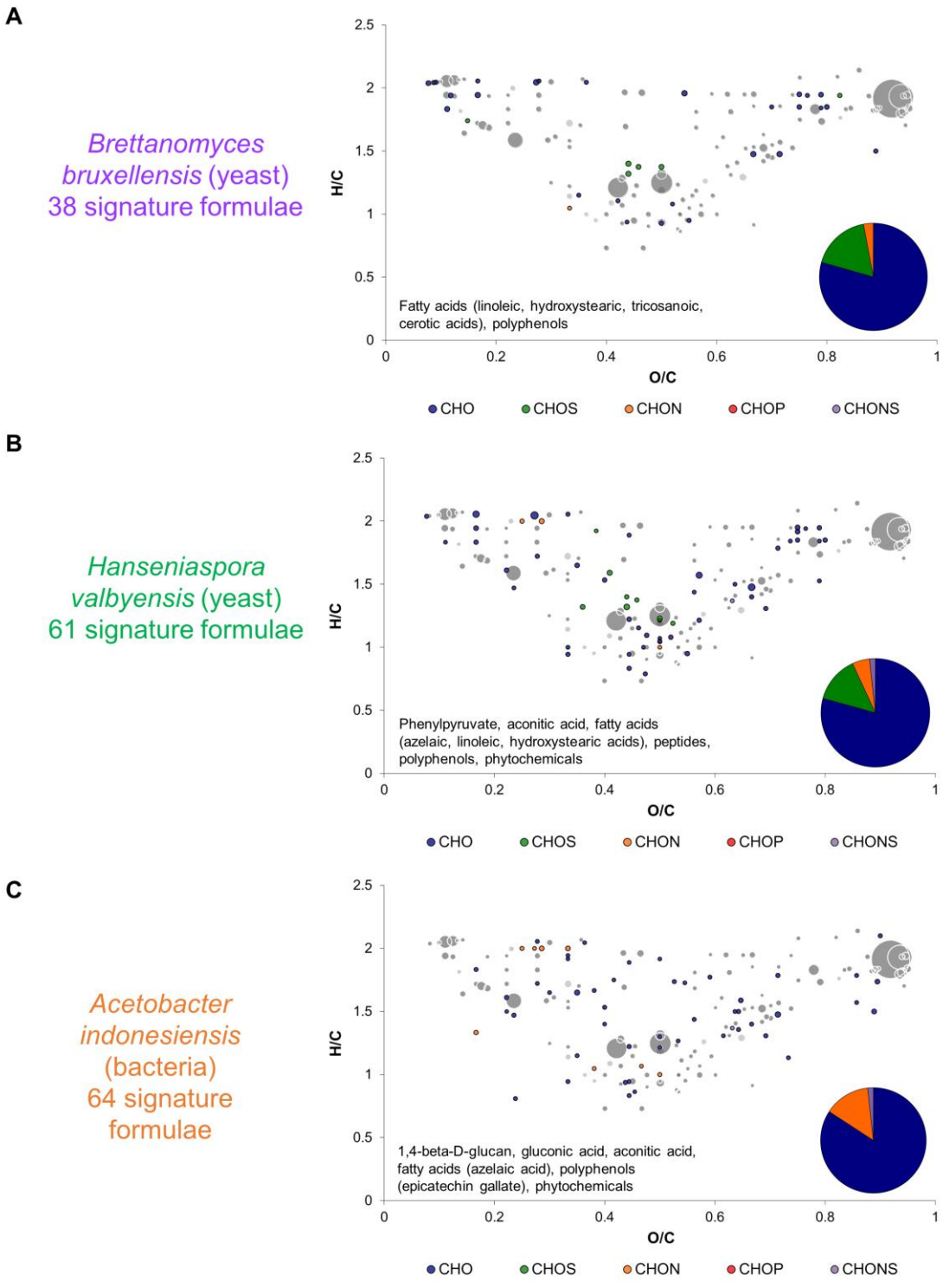

Figure F2 : Van Krevelen diagrams of formula that are not common to all three microorganisms (signature formulae): (A) *Brettanomyces bruxellensis*, (B) *Hanseniaspora valbyensis* and (C) *Acetobacter indonesiensis*, with proportions in elemental compositions and putative identities. Core metabolites formulae (Figure F1C) are represented in the background in grey.

3.3 Yeast-yeast interspecies interactions

Exo-metabolomes of *B. bruxellensis* and *H. valbyensis* in separate monocultures in sugared tea (BB and HV) were compared to the exo-metabolome of both yeasts in coculture (BBHV). Figure F3A shows that most of metabolites were detected in all conditions (162 over 265) and that 39 new formulae were detected exclusively in the coculture. In contrast, fewer formulae present in monoculture were not detected in the coculture (20 in total). Only 11 formulae underwent a significant decrease in intensity for BB and were CHO in majority, along with CHOS and CHONS. The same observation occurred for HV with 9 formulae. According to putative compound annotations, phytochemicals and polyphenols were involved. The decrease in *H. valbyensis*'s peptides intensities could be due to their consumption by *B. bruxellensis* in the coculture, which would characterize a case of commensalism. In turn, 59 formulae significantly increased in intensity because of the coculture, with a majority of CHO, CHOS and CHON. Annotation with putative identities implied the production of fatty acids, one peptide, polyphenols and phytochemicals. Overall, yeast-yeast interaction between *B. bruxellensis* and *H. valbyensis* influenced microbial metabolism by inducing the release or consumption of fatty acids and peptides, possibly in relationship to commensalism. Moreover, it impacted the structure of tea compounds.

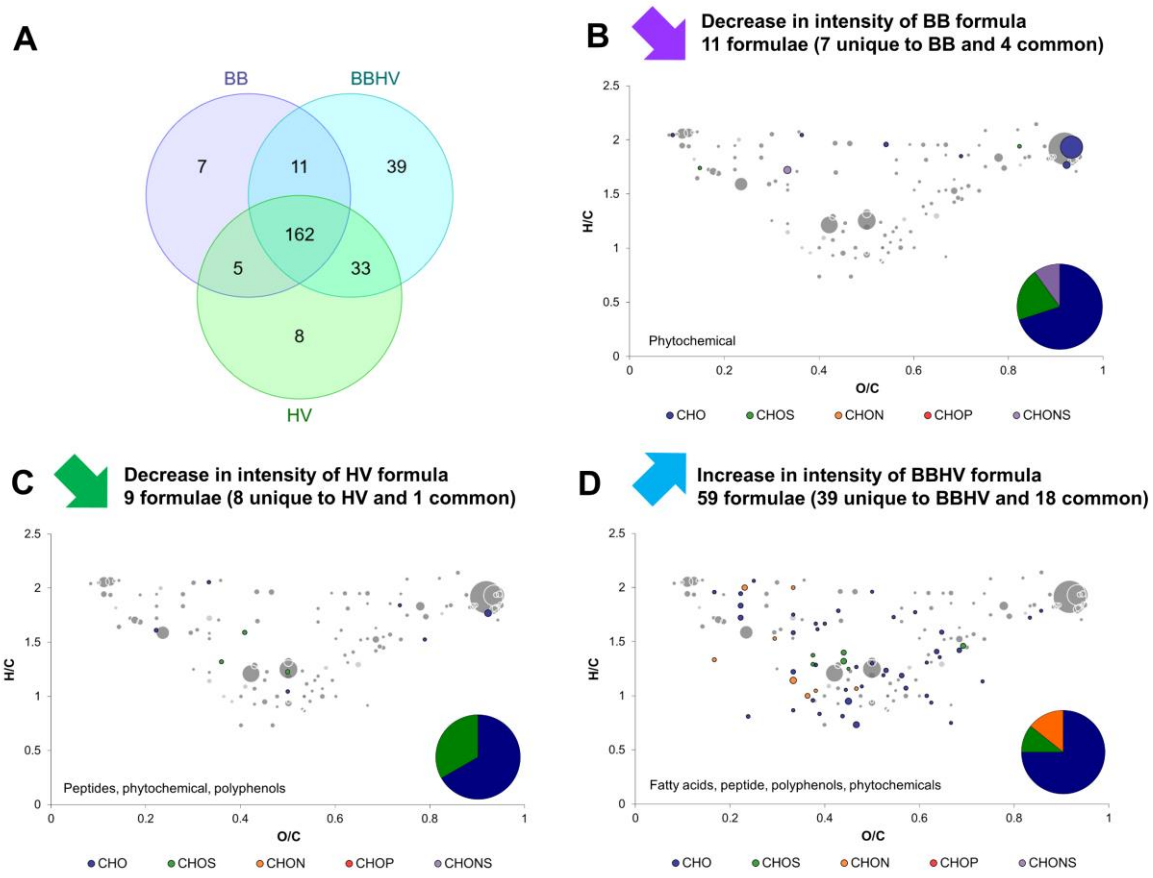

Figure F3 : Change in composition induced by the yeast-yeast interaction of *B. bruxellensis* (BB) and *H. valbyensis* (HV). (A) Venn diagram of formulae identified in BB and HV monocultures and the BBHV coculture. Van Krevelen diagrams and putative identities of formula (B) decreasing in BB monoculture, (C) decreasing in HV monoculture and (D) increasing in BBHV coculture. Core metabolites formulae (Figure F1C) are represented in the background in grey.

3.4 Yeast-acetic acid bacterium interactions

Exo-metabolomes of *B. bruxellensis* and *A. indonesiensis* in separate monocultures (BB and AI) were compared to the exo-metabolome of both microorganisms in coculture (BBAI). The same was applied with the yeast *H. valbyensis* in association with the cocultures (HVAI). In both cases, most of formulae were detected in all conditions (140 over 280 and 149 over 255 for the comparisons involving *B. bruxellensis* and *H. valbyensis* respectively; Figures F4A and F5A). Based on the Venn diagrams, two very distinct behaviors could be observed. BBAI coculture induced the production of 37

formulae and the disappearance of 28 formulae unique to AI monoculture, and 9 formulae unique to BB (Figure F4A). In contrast, the HVAI coculture only produced 5 new and unique formulae. The number of formulae unique to AI monoculture was 31 in similar amount as those affected by the BBAI coculture. However, 25 formulae were unique to HV, which is more than those of BB (Figure F5A).

CHO represented the majority of yeast formulae that underwent a decrease in intensity, along with CHOS and CHONS. CHON were only detected for HV monoculture (Figures F4B and F5B). Among formulae annotated with putative identities, only hydroxy stearic acid underwent a decrease in intensity in both cases. Sucrose was significantly more consumed in BBAI than BB along with other fatty acids and polysaccharides. Greater sucrose consumption could be explained by the adequation of *B. bruxellensis* and *A. indonesiensis* cocultures, leading to efficient sugar consumption and production of organic acids in the context of kombucha production (Tran *et al.*, 2020b). *B. bruxellensis* was reported to have higher invertase and fermentative activities allowing better accessibility of substrate for acetic acid bacteria (Tran *et al.*, 2020b). In contrast, HV formulae that underwent a decrease in intensity due to HVAI coculture were polyphenols and metabolites that could be involved in commensalism interactions: peptides, fatty acids, phenolic acids, isopropyl malate and phenylpyruvate, that are involved respectively in leucine and phenylalanine metabolism and widely useable by other organisms (Braus, 1991).

When *A. indonesiensis* was paired with a yeast, a majority of CHO compounds and some CHON formulae (no CHOS or CHONS) underwent a decrease in intensity (Figures F4C and 5C). Putative identities involved phytochemicals and dehydroquinic acid (an intermediate in the shikimate pathway) regardless of the yeast in coculture. In coculture with *B. bruxellensis*, a decrease in intensity of putative aconitic acid and 1,4-β-D-glucan could be observed, whereas phenolic acids and polyphenols including epicatechin gallate appeared to be impacted in presence of *H. valbyensis*. In the first case, carbon metabolism of *A. indonesiensis* seemed to be influenced, whereas secondary plant metabolisms were transformed on the second case.

The formulae significantly produced in coculture were mainly CHO and CHON or CHOS to a lesser extent (Figures F4D and F5D). They included fatty acids in both cases, highlighting an effect on microbial metabolism (van Roermund *et al.*, 2003; Yamada *et al.*, 1981). In the case of BBAI, stearic acid was specifically released as well as peptides, possibly due to yeast autolysis. Additionally, phenolic acids could have been converted and polyphenols were modified. In the case of HVAI, less sucrose was consumed and polysaccharides were produced, whereas phytochemicals were affected.

Overall, yeast-acetic acid bacterium interactions were substantially different depending on the yeast species, with apparent effects on compounds from different metabolic pathways. The interaction of *A. indonesiensis* with *B. bruxellensis* seemed to affect the exo-metabolome towards higher molecular diversity through the stimulation of carbon metabolism, while the interaction with *H. valbyensis* led to a lower molecular diversity through the consumption of *H. valbyensis* metabolites. In all cases however, tea compounds were affected by microbial interactions.

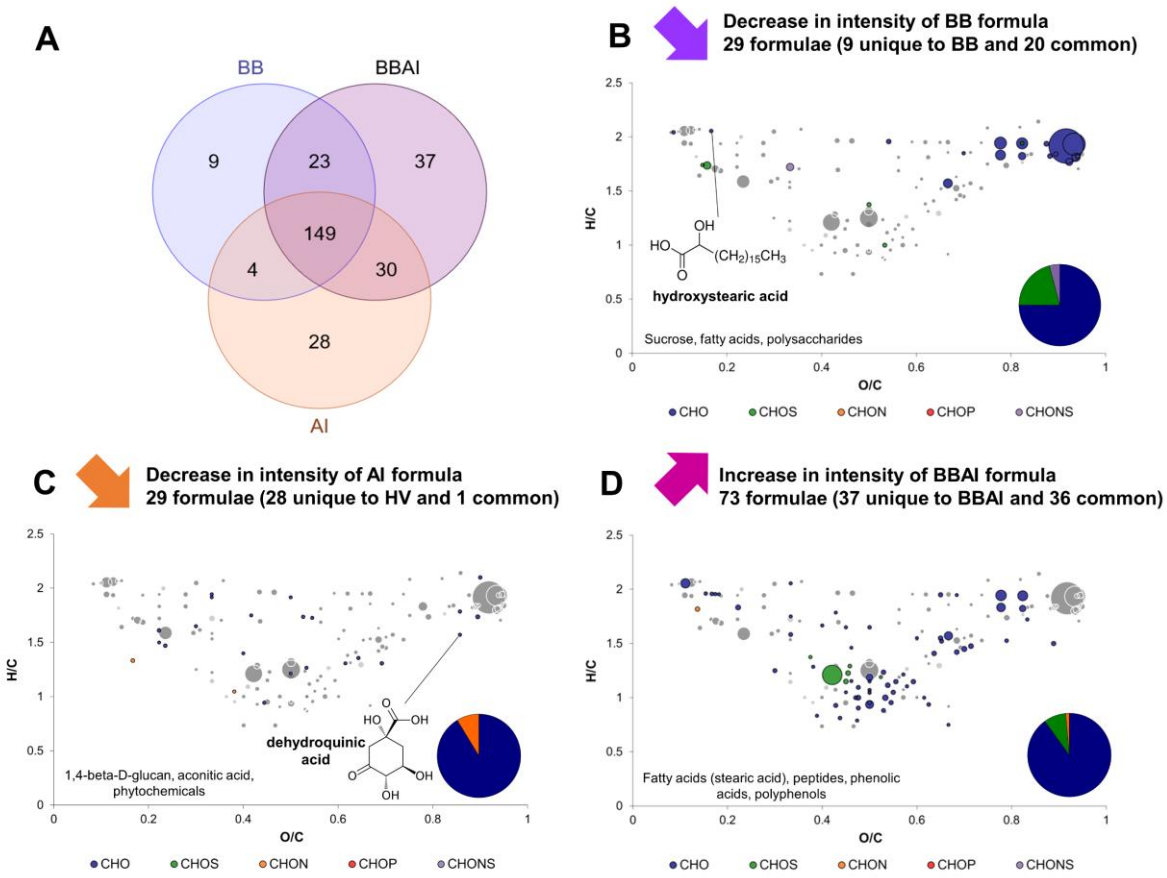

Figure F4 : Change in composition induced by the yeast-acetic acid bacteria interaction of *B. bruxellensis* (BB) and *A. indonesiensis* (AI). (A) Venn diagram of formulae identified in BB and AI monocultures and the BBAI coculture. Van Krevelen diagrams and putative identities of formulae (B) decreasing in BB monoculture, (C) decreasing in AI monoculture and (D) increasing in BBAI coculture. Core metabolites formulae (Figure F1C) are represented in the background in grey.

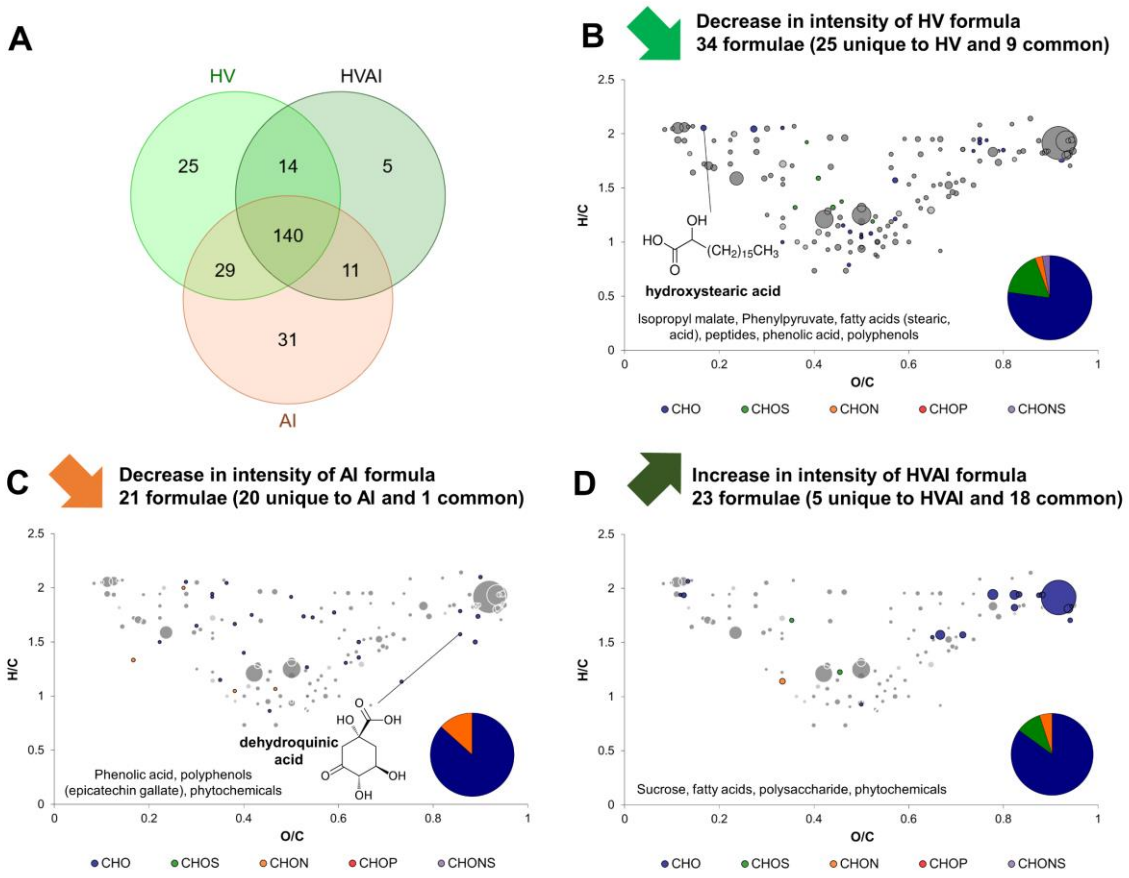

Figure F5 : Change in composition induced by the yeast-acetic acid bacteria interaction of *H. valbyensis* (HV) and *A. indonesiensis* (AI). (A) Venn diagram of formulae identified in HV and AI monocultures and the HVAI coculture. Van Krevelen diagrams and putative identities of formulae (B) decreasing in HV monoculture, (C) decreasing in AI monoculture and (D) increasing in HVAI coculture. Core metabolites formulae (Figure F1C) are represented in the background in grey.

3.5 Complex interactions involving yeast-yeast and yeast-acetic acid bacterium interaction

Exo-metabolomes of *B. bruxellensis* and *H. valbyensis* yeast-yeast coculture (BBHV) and *A. indonesiensis* in monoculture (AI) were compared to the exo-metabolome of the coculture gathering the three microorganisms (T). Similarly with previous comparisons, a big part of formulae was detected in all conditions (154 over 281 in total; Figure F6A). The Venn diagram shows that only 9 formulae were unique to T, while 35 and 24 unique formulae from BBHV and AI respectively (plus 30 common formulae) were not detected

in T. This case was similar to the interaction between *H. valbyensis* and *A. indonesiensis* with a loss in molecular diversity due to the interaction between the yeast and the bacterium. 44 formulae from BBHV underwent a decrease in intensity and included mainly CHO, CHON and CHOS in lesser amounts (Figure F6B). Very diverse molecular families were impacted. According to putative identity annotations, these families included fatty acids, hydroxy stearic acid, a peptide, a phenolic acid, polyphenols and phytochemicals. Twenty-five formulae from AI underwent a decrease in intensity, with similar amounts compared to the BBAI and HVAI interactions (28 and 31 respectively). These formulae were mainly represented by CHO, with a smaller portion of CHON. No CHOS was detected. Annotations with putative identities suggested a decrease in intensity of the same compounds as for BBAI: 1,4-βD-glucan (or cellulose), dehydroquinic acid, gluconic acid and epicatechin gallate, along with gluconolactone, other polyphenols and phytochemicals. Finally, 17 formulae underwent an increase in intensity in T composed of CHO, CHOS and CHON, including potentially a peptide, a polysaccharide and a phytochemical. The result of this complex interaction of two yeasts and one acetic acid bacterium is surprising because of the loss of molecular diversity, when microbial interactions rather stimulate the production of compounds in reaction to the presence of other microorganisms (Roullier-Gall *et al.*, 2020). This was namely the case for the yeast-yeast interaction between *B. bruxellensis* and *H. valbyensis*.

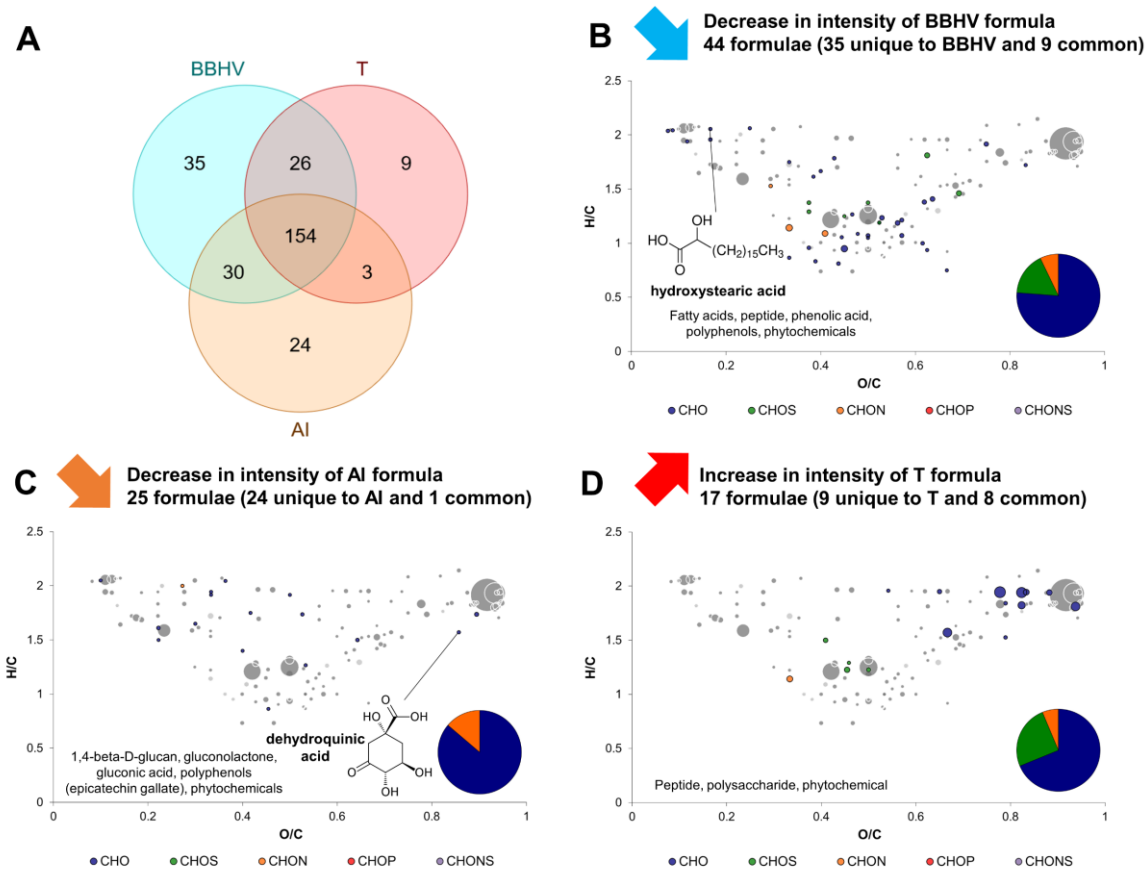

Figure F6 : Change in composition induced by the complex yeast-acetic acid bacteria interaction of *B. bruxellensis* and *H. valbyensis* coculture (BBHV) and *A. indonesiensis* (AI). (A) Venn diagram of formulas identified in BBHV and the T coculture including the two yeasts and the acetic acid bacteria. Van Krevelen diagrams and putative identities of formulae (B) decreasing in BBHV coculture, (C) decreasing in AI monoculture and (D) increasing in T coculture. Core metabolites formulae (Figure F1C) are represented in the background in grey.

The formulae unique to BBHV produced by the interaction of the two yeasts were compared with the formulae unique to BBHV that where not detected in presence of *A. indonesiensis* in T (Annexe SF4). It appears that 30 formulae out of 57 that were produced from the yeast-yeast interaction were inhibited by the presence of *A. indonesiensis*. It may be assumed that the yeast-acetic acid bacterium interaction had either consumed or prevented the production of the metabolites produced by the yeast-yeast interaction. Potential metabolite identities include few fatty acids, phenolic acids, polyphenols and phytochemicals. Therefore, it is not clear if metabolites, potentially

signaling molecules could be consumed by *A. indonesiensis* as part of commensalism. However, the interaction of microbial activity with polyphenols was clearly inhibited in presence of the bacterium. The mechanism behind this phenomenon remains unclear, but it can be stated that the transformation underwent by tea compounds were dependent on microbial activity with variations according to the species. Modification of phenolic compounds during kombucha production have been reported in a recent study using metabolomics (Cardoso *et al.*, 2020).

Regarding the effects of yeast-acetic acid bacterium interaction, formula associated to putative hydroxystearic acid from yeast cultures (BB, HV and BBHV) decreased systematically in intensity in presence of *A. indonesiensis.* The position of hydroxy stearic acid is indicated on Van Krevelen diagrams on Figures F3B, F4B, F5B and F6B. This mono-unsaturated fatty acid was reported as the intermediate between stearic acid and oleic acid. This reaction was thought to be performed by *Saccharomyces cerevisiae* for a long time. A recent study suggested that it was instead caused by bacterial contaminants, in contradiction with our results with an apparent hydroxy stearic production by *B. bruxellensis* and *H. valbyensis* (Serra and De Simeis, 2018). However, the decrease in intensity observed in presence of *A. indonesiensis* could be explained by the conversion of hydroxy stearic acid into volatile organoleptic γ-dodecalactone, which has been reported for bacteria, and namely lactic acid bacteria in the context of whisky production (Gocho *et al.*, 1995; Wanikawa *et al.*, 2000). As supporting elements, acetic acid bacteria have been reported to convert 1,4-nonandiol into γ-nonanoic lactone (Romano *et al.*, 2002) and gluconolactone was detected as putative compound in our study. This metabolite acts as an intermediate in the oxidation of glucose into gluconic acid by *Acetobacter senegalensis* according to KEGG database (Kanehisa, 2000). Consequently, the decrease in hydroxystearic acid, which is strongly associated to yeast-bacteria interactions in the literature, could be explained by a conversion performed by the acetic acid bacterium present, as part of a commensalism.

Moreover, a pattern regarding the formulae associated to AI negatively affected by yeast-acetic acid bacterium interaction could be observed. The number of those compounds was consistent in each case (between 24 and 31) and consisted in CHO and CHON exclusively, with no CHOS detected. The lists of AI formulae that were negatively impacted

by the presence of yeast across the three cases of yeast-acetic acid bacterium interaction were compared using a Venn diagram (BBAI, HVAI and T; Annexe SF5). Fourteen formulae out of 37 systematically underwent a decrease in intensity in presence of yeasts in all three cases, and other 14 formulae did in 2 cases out of 3. This suggests that the impact of the yeasts' presence on *A. indonesiensis* was consistent regardless of the yeast species. In opposition, the impact of the acetic acid bacterium on the yeast formulae differed significantly according to the yeast species. Putative compound identities involve polyphenols, phytochemicals, fatty acids and dehydroquinic acid. This last metabolite is a key intermediate of the shikimate pathway that connects glycolysis to the biosynthesis of aromatic amino acids. The decrease in presence of yeasts could suggest a case of commensalism if it is internalized by yeast to supply their own production of aromatic amino acids (Braus, 1991). Moreover, the presence of dehydroquinic acid in the medium can be explained by the presence of quinate dehydrogenase enzyme (converting quinic acid into dehydroquinic acid) on the membrane of acetic acid bacteria (Adachi *et al.*, 2003). The position of dehydroquinic acid is indicated on Van Krevelen diagrams on Figures F3C, F4C, F5C and F6C. By providing such a key metabolic intermediate directly in the medium, acetic acid bacteria clearly favor commensalism regarding this compound. When put in perspective with their non-strict parasitic interaction towards yeast reported in the context of kombucha production (Tran *et al.*, 2020b), this element could advocate for a mutualistic interaction between yeasts and acetic acid bacteria.

5 Conclusions

Exo-metabolomes of kombucha microorganisms grown in sugared black tea in monocultures and cocultures allowed to highlight new hypotheses regarding microbial interactions that could occur during kombucha production. Interaction between the two major yeast species *B. bruxellensis* and *H. valbyensis* of the original kombucha culture induced an increase in molecular diversity and so did the yeast-acetic acid bacterium pairing *B. bruxellensis* and *A. indonesiensis*. Oppositely, the interactions that occurred in cocultures involving *H. valbyensis* and *A. indonesiensis*, with or without the presence of *B. bruxellensis* (HVAI and T), induced lower molecular diversity. Those phenomena explain the dendrogram shown in Figure F1A, because except when all three microorganisms where together, *B. bruxellensis* influenced greatly the metaboloms in

BBAI and BBHV (but not T). To a lesser extent, the presence of *H. valbyensis* with *A. indonesiensis* did not bring as many changes compared to the acetic acid bacterium monoculture. Finally, the interaction of all three microorganisms appeared to cancel the effects of the other interactions (in particular those from the yeast-yeast interaction), thus bringing the exo-metabolome closer to sugared tea and the monocultures. Polyphenols and phytochemicals were widely affected by microbial interactions, although metabolic pathways including polyphenols have not been characterized in kombucha species. Such enzymatic activity of polyphenol conversion have been identified in the context of the gut microbiota (Dueñas *et al.*, 2015). Nevertheless, it can also be supposed that microbial interactions generated different compounds and physical chemical conditions that could interact and modify plant secondary metabolites in different ways. Moreover, it has been demonstrated that polyphenols could be absorbed by yeast cell wall (Mazauric and Salmon, 2005). However, detailed interpretation of the most abundant metabolites analyzed with FT-ICR/MS raised new hypotheses regarding microbial activities and metabolites from different molecular families: fatty acids, peptides, and carbohydrates. It highlighted potential recurring commensalism between yeasts and the acetic acid bacterium through hydroxy stearic acid (released from yeasts and consumed by the acetic acid bacterium) or dehydroquinic acid (released from the acetic acid bacterium and consumed by the yeasts). Such exchanges of metabolites point toward mutualistic interactions that characterize stable communities of organisms. By taking support from these results, further investigation on less abundant metabolites and using targeted analyses could confirm the existence of such interactions with a focus on specific novel compounds and pathways. Moreover, acetic acid bacteria remain unproperly studied. Similar metabolomic approaches using non-targeted analytic tools could bring significant advances in the understanding of interspecies interactions for this group of bacteria, in the context of kombucha for example.

Acknowledgments

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Declaration of interest

Authors declare no conflict of interest.

Author contributions

Thierry Tran took the lead of the writing of this article, but all other authors provided critical and complementary elements to the manuscript. Thierry Tran performed the generation of samples and the data treatment. Chloé Roullier-Gall provided extensive help on data treatment and writing of the manuscript. Philippe Schmitt-Kopplin performed FT-ICR-MS analysis and provided the raw data. François Verdier and Antoine Martin provided the kombucha cultures used in the experiments.

G Chapitre 5 : Les origines de la flaveur de la kombucha : rôle des interactions microbiennes, du process et du type de thé.

Après les composés fixes, les composés volatils ont été étudiés par analyse métabolomique dans ce cinquième chapitre. Ce travail a été soumis sous forme d'article au journal *Food Chemistry*. Des analyses microbiologiques, chimiques et sensorielles ont été menées afin de comprendre l'origine des composés organoleptiques de la kombucha ainsi que leurs implications dans la perception du produit. Les effets de l'interaction levure-levure et levure-bactérie acétique ont été investigués pour les deux phases de production (ouverte durant 7 jours puis fermée pendant 5 jours) en comparant une kombucha originale avec des monocultures et des cocultures des micro-organismes sélectionnés (*B. bruxellensis*, *H. valbyensis* et *A. indonesiensis*) et isolés de cette même kombucha. L'influence du type de thé, noir ou vert, a également été évaluée.

La composition en micro-organismes, déterminée par dénombrement sur milieux gélosés, influence les dynamiques de populations. *B. bruxellensis* est la levure majoritaire dans les kombuchas originales alors qu'il s'agit de *H. valbyensis* pour la culture en trio (dont la composition microbienne est la plus proche), bien que dans les deux cas on puisse parler de codominance entre les deux espèces. De plus, la croissance de *A. indonesiensis* est stimulée en présence de *H. valbyensis* et inhibée en présence de *B. bruxellensis* avec ou sans *H. valbyensis* à la fin de la première phase. L'activité microbienne est également impactée, en particulier l'acidification, comme observé dans le Chapitre 2. Trente-deux composés volatils ont été identifiés et quantifiés par HS-SPME-GC-MS (*Head Space – Solid Phase Micro-Extraction – Gas Chromatography – Mass Spectrometry*), puis classifiés selon leur origine : du thé et/ou des micro-organismes. La production d'éthanol et des alcools supérieurs (comme l'isobutanol) est associée aux deux levures. Cependant, *H. valbyensis* se caractérise par la production de nombreux esters (comme l'acétate d'éthyle ou l'acétate d'isoamyle). La production d'acide acétique caractérise *A. indonesiensis.* Tandis que la production des acides gras saturés (acides octanoïque, nonanoïque et décanoïque) est commune à tous les micro-organismes. Certains aldéhydes (comme l'hexanal) et cétones (comme le 2-pentanone) sont associés au thé. La concentration de ces composés est soumise à des effets d'interactions, principalement en lien avec l'activité microbienne, la composition en levures et la phase de production. Le type de thé n'influence pas fortement le profil en composés volatils. Toutefois, l'analyse sensorielle descriptive réalisée par un panel entrainé a révélé que le type de thé influence la perception olfactive des produits, bien que la composition microbienne demeure le facteur principal.

L'association de *B. bruxellensis* et *A. indonesiensis* permet d'obtenir le produit le plus proche de la kombucha originale en termes de composition chimique et de profil sensoriel. Enfin, l'arôme de jus de pomme caractéristique de la kombucha apparait être le produit d'une interaction sensorielle entre les composés volatils d'origines levuriennes et bactériennes, appuyant la nécessité de l'association levure-bactérie acétique dans la production de kombucha précédemment évoquée dans le chapitre 2 (partie D).

Dans cette étude, des liens entre la composition chimique et le profil sensoriel de la kombucha dont il est fait l'hypothèse dans la *review* présentée en partie B, ont pu être établis sur la base de résultats expérimentaux. La sucrosité et l'acidité représentent les descripteurs gustatifs principaux et semblent éclipser les sensations d'amertume et d'astringence amenées par les polyphénols. Au niveau olfactif, les arômes fruités sont associés principalement aux esters et peuvent également être conjointement associés à des alcools et acides gras (comme c'est le cas pour le descripteur « jus de pomme »). La composition des composés volatils semble par ailleurs être fortement influencée par la matrice thé sucré, étant donnée la représentation prédominante de composés associés à des voies de synthèse dérivées du métabolisme du carbone (à partir du glucose) et indépendantes de celui de l'azote (voie de Ehrlich notamment). Cela est en accord avec le fait que la ressource en sucre est abondante dans la matrice thé sucré, en comparaison avec la ressource en composés azotés très limitée. Cette observation pointe donc vers un rôle déterminant de la matrice dans la composition chimique (en particulier volatile) et le profil sensoriel de la kombucha.

Origins of kombucha flavor: role of microbial interactions, process, and tea type

Running Title: Origins of kombucha flavor

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Abstract

Microbiological, chemical, and sensory analyses were coupled to understand the origins of kombucha organoleptic compounds and their implication in the flavor of the beverage. By isolating microorganisms from an original kombucha and comparing it to monocultures and cocultures of two yeasts (*Brettanomyces bruxellensis* and *Hanseniaspora valbyensis*) and an acetic acid bacterium (*Acetobacter indonesiensis*), interaction effects were investigated during the two phases of production. 32 volatile compounds identified and quantified by HS-SPME-GC/MS were classified according to their origin from tea or microorganisms. Many esters were associated to *H. valbyensis*, while alcohols were associated to both yeasts, acetic acid to *A. indonesiensis* and saturated fatty acids to all microorganisms. Concentration of metabolites were dependent on microbial activity, yeast composition, and phase of production. Sensory analysis showed that tea type

influenced the olfactive perception, although microbial composition remained the strongest factor. Association of *B. bruxellensis* and *A. indonesiensis* induced characteristic apple juice aroma.

Keywords: kombucha, interaction, metabolites, sensory, volatile compounds, flavor, yeasts, acetic acid bacteria.

1 Introduction

When tasting a beverage, olfaction precedes the gustation itself, which establishes the odor as a key organoleptic component during the consumption of a product. Kombucha, a sour nonalcoholic fermented beverage obtained from sugared tea infusion is no exception. However, kombucha producers face difficulties with the control of kombucha's stability of organoleptic quality including the olfactive profile, amid a very dynamic global market development (Kim and Adhikari, 2020). This is due to several factors among which: the absence of standard production procedure, highly biodiverse composition in yeasts and bacteria consortia in kombucha cultures and the lack of knowledge regarding the product itself and the process parameters impacting its composition and organoleptic quality (Harrison and Curtin, 2021; Tran *et al.*, 2020a). This also applies to the organoleptic stability of those products when they are put on the market without microbiological stabilization.

Indeed, the main established knowledge regarding kombucha is the symbiotic biological acidification process that constitutes the first phase of the production process in open vessel. Yeasts provide available carbon substrate to acetic acid bacteria by hydrolyzing sucrose into monosaccharides and producing ethanol through alcoholic fermentation. In turn, acetic acid bacteria convert glucose into gluconic acid and ethanol into acetic acid through oxidative metabolism (Tran *et al.*, 2020b). Parallelly, acetic acid bacteria generate a cellulosic biofilm (pellicle) at the air / broth interface. Once satisfactory degree of acidity is achieved, it is possible to perform natural carbonation as a second production phase by bottling the product. As a consequence, this stimulates yeast alcoholic fermentation and allows accumulation of carbon dioxide and unfortunately also ethanol (Talebi *et al.*, 2017). However, little is known about the contribution of the metabolism of kombucha

microorganisms in volatile compounds production, besides the evident vinegary note brought by acetic acid (Tran *et al.*, 2020a).

Although some studies helped understanding the management of the main non-volatile parameters related to taste (meaning residual sugar and acidity) (Chakravorty *et al.*, 2016; Chen and Liu, 2000; Tran *et al.*, 2020b), few studies have attempted to analyze kombuchas volatile composition (Savary *et al.*, 2021; Zhang *et al.*, 2021), by providing first insights into the type and quantity of volatile compounds (aldehyde, ketones, alcohols, fatty acids and esters mainly). In the study of (Zhang *et al.*, 2021), GC/MS was performed on 6 days kombuchas and led to the detection of 22 volatile compounds common to all samples (including ethyl octanoate, ethyl guaiacol and phenylethyl ethanol). Variations in composition between kombuchas made from different tea types appeared to involve only tea compounds and no metabolites produced by microorganisms. In the study of Savary *et al.* (2021), evolution of volatile composition of black tea kombucha was followed during 27 days of production. Clustering analysis showed an evolution of volatile profile with an increase in diversity from day 7 and peaking between day 11 and day 14. Produced compounds are fatty acids (acetic acid, short and medium chain fatty acids), alcohols (butanol, methylbutanol, methylpropanol, hexanol), esters (ethanol, ethyl acetate, ethyl esters of short and medium chain fatty acids). Aldehydes (methylbutanal, heptanal, nonanal) were rather represented during the first days and ketones (heptanone, hexanone) during the last days of the process. Despite the several studies that performed descriptive sensory analysis (Ivanišová *et al.*, 2019; Neffe-Skocińska *et al.*, 2017; Zhang *et al.*, 2021), no traditional descriptive sensory analysis using trained panel has been used. Untrained panel size varied between 16 and 60 individuals. Besides hedonic evaluations, descriptive analyses involved descriptors related to the sight (“color intensity”, “clarity”, “turbidity”, “darkness”), to the smell (“tea”, “lemon”, “acetic acid”, “yeast”, “fruity”, “floral”, “herbal”, “medicinal”) and to the taste (“acid/acidity”, “sweetness”, “bitter”). The sense of touch was once investigated with the descriptor “stinging”. Sourness or acidity appeared indeed to be a fundamental descriptor of kombucha regarding the taste, whereas, the acetic acid, fruity, herbal and tea notes were characteristic of the smell. Coupled GC-MS and sensory analysis reported adequation of sweetness with residual sugar content and hypothesized on the link between isovaleric acid concentration and “unpleasant acidity”.

The present study aimed at investigating the topic of kombucha flavor by coupling microbiology, analytical chemistry, and sensory analyses. The objectives were to verify several hypotheses regarding the origins of kombucha flavor and how it is impacted by process parameters. Is kombucha olfactive profile affected by its microbial composition? If yes, is kombucha sensory components the result of microbial interactions? Do the secondary natural carbonation production phase or tea type play a role on sensory components? To achieve that, a similar approach as used in our previous study was conducted (Tran *et al.*, 2020b). It consisted in comparing an original kombucha consortium with monocultures and cocultures of yeasts and acetic acid bacteria previously isolated from the original consortium. Thus, yeasts – acetic acid bacteria interactions as well as yeast – yeast interactions were investigated and included a condition gathering two yeasts and one acetic acid bacteria. Additionally, both production phases were investigated, and the matrix effect was evaluated by using both black and green teas in association to the original kombucha culture used in the study.

2 Materials and Methods

2.1 Generation of kombucha, monocultures and cocultures of yeasts and acetic acid bacteria isolated from kombucha

Sugared teas and traditional kombuchas were produced according to our previous work (Tran *et al.*, 2020b) with some modifications. First, 1% (m/v) of tea was steeped for one hour, then 50 g of sucrose was added. Black tea (Pu'er Grade 1 TN4107) and green tea (Sencha Zhejiang TV4217) were used for kombucha production to investigate the matrix effect. Both teas were sourced at Les Jardins de Gaïa (Wittisheim, France). Sugared black tea (SBT) and sugared green tea (SGT) were obtained and used to produce black tea kombucha (BTK) and green tea kombucha (GTK) from the same kombucha culture. After cooldown to room temperature, 12% (v/v) of 7 days black tea kombucha broth was added. A primary inoculum was produced using the same procedure by using a mother culture obtained from the company Biomère (Paris, France) to ensure a physiological state of microorganism comparable to regular industrial scale production. The mother culture is a kombucha culture that was refreshed monthly with sugared black tea.

Yeasts and acetic acid bacteria strains were isolated from the broth of the black tea kombucha previously mentioned. Those microorganisms were selected for the present study according to their representation in terms of population during the elaboration of kombucha and their functionality (Tran *et al.*, 2020b). The selection included *B. bruxellensis*, *H. valbyensis* and *A. indonesiensis*. The different modalities of monocultures and cocultures are detailed in Tableau G1.

Tableau G1 : Description of the different cultures.

	Monocultures in sugared black tea	Yeast-Yeast coculture in sugared black tea	Yeast(s)-acetic acid bacteria cocultures (minimal consortia) in sugared black tea	Black or green tea kombucha (detected microorganisms)
Microbial composition (Code)	*B. bruxellensis* (BB) *H. valbyensis* (HV) *A. indonesiensis* (AI)	*B. bruxellensis* and *H. valbyensis* (BBHV)	*B. bruxellensis* and *A. indonesiensis* (BBAI) *H. valbyensis* and *A. indonesiensis* (HVAI) *B. bruxellensis*, *H. valbyensis* and *A. indonesiensis* (T)	*B. bruxellensis, H. valbyensis, Saccharomyces cerevisiae,* and bacteria including *A. indonesiensis* (BTK, GTK)

The association of at least one yeast and one acetic acid bacteria (BBAI, HVAI and T) can be seen as simplified kombucha consortium and will be referred to “minimal consortium”.

To generate precultures, yeasts and the acetic acid bacterium were restreaked on Wallerstein Lab (WL) agar (Hall, 1971) and De Man Rogosa and Sharpe (MRS) agar respectively from stocks kept at -20°C and incubated at 28°C. Yeasts and acetic acid bacteria isolates were inoculated in YPD liquid medium and MRS medium for yeasts and the bacterium, respectively. Those precultures were incubated at 28°C in static conditions for 2 days in sterile vessels with untight caps to allow gas exchanges. After incubation, cells were washed with sugared black tea then centrifugated (3,000 g, 10 minutes at 4°C). Each population was inoculated at the rate of 1.10^5 cell mL^{-1} in sugared black tea only. Inoculation loading was determined through flow cytometry using a BD Accuri C6 (Franklin Lakes, USA) with 0.1 µg mL^{-1} propidium iodide in ultrapure water from

ThermoFischer Scientific (Waltham, USA) to evaluate non-viable cells proportion (Stiefel *et al.*, 2015).

Cultures occurred in triplicates in 123 mL Boston flasks with a Specific Interfacial Surface (SIS) (Cvetković *et al.*, 2008) of 0.01 cm^{-1} with bottlenecks loosely covered with tin fold to allow gas exchanges during 7 days first phase (P1) of production. Then, flasks were fully closed for 5 more days of natural carbonation phase (P2).

2.2 Microbiological analysis

Plate counting was performed to determine the populations of yeasts and/or acetic acid bacteria after inoculation (d0), at the end of P1 after 7 days (d7) and at the end of P2 after 12 days (d12). Despite the apparent production cellulose, due to the absence of consistent cellulosic pellicle in cultures other than kombucha, microbiology data regarding the kombucha pellicles were not included. Successive decimal dilutions of samples on WL agar for yeasts and MRS agar for bacteria respectively, were performed in triplicates. Characterization of the yeasts present in the cultures on WL agar plates allowed the quantification of yeasts subpopulation according to the species based on morphological aspect, as described previously (Tran *et al.*, 2020b).

2.3 Analysis non-volatile compounds

Endpoint chemical analyses at d0, d7 and d12 were performed on supernatants obtained by centrifugation (3,000g; 15 minutes, 4°C) and kept frozen at -20°C. Total acidity was determined by titration with 0.1 N NaOH and 0.2% phenolphthalein as color indicator. pH values were measured with a Mettler Toledo Five Easy pH meter coupled with a LE498 probe. Acetic, lactic, malic, succinic acids concentrations were determined by HPLC as previously described (Tran *et al.*, 2020b). Sucrose, glucose and fructose concentrations were determined using enzymatic kits from Biosentec (Auzeville-Tolosane, France).

2.4 Analysis of volatile compounds

Volatile compounds were analyzed using Headspace-Solid Phase-MicroExtraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS). Volatile compounds were extracted by Solid Phase Microextraction using a Combi-pal autosampler (CTC Analytics,

Zwingen, Switzerland). An aliquot of 2 mL of sample spiked with 4-methyl-2-pentanol (Internal standard, 400 µg/L) was placed into a 10 mL vial kept at 40 °C under constant agitation. After 10 min of sample conditioning, a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (2 cm length, 50/30 µm film thickness) provided by Supelco (Bellefonte, PA) was exposed to the sample headspace for 30 min. Then, it was desorbed in the gas chromatograph (GC) injection port at 260 ˚C for 10 min.

Volatile compounds were analyzed by gas chromatography coupled to quadrupolar mass selective spectrometry using an Agilent 6890N Network GC system coupled to a quadrupolar mass selective analyzer Agilent 5975C Inert MSD (Agilent Technologies, Santa Clara, California, USA) using helium as carrier gas, at a flow of 1.5 mL/min. Analytes were separated on a Supelcowax-10 capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness) (Supelco, Bellefonte, PA). The GC oven temperature was held at 40 °C for the first 10 min, then increased to 150 °C at 3 °C/min and finally to 200 °C at 15 °C/min, holding 5 min at that ending temperature. The temperature of the ion source and the transfer line were 200 °C and 275 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionization energy in full scan mode (m/z range from 35 to 300), 5.1 scans/s.

Identification of compounds was carried out by comparison of their mass spectra and linear retention indices with those of standard compounds or with those available in mass spectrum library Wiley 6 and in the literature, respectively. Response factors of volatile compounds were calculated using calibration curve obtained by analyzing different concentrations of reference compounds in the ranges 0.2-10000 µg/L (3-methylbutanal, ethyl acetate, butane-2,3-dione, 2-methylpropan-1-ol, 3-methylbutan-1-ol, 6-methyl-5-hepten-2-one, hexan-1-ol, 1-octen-3-ol, phenyl ethanol), 0.25-2.5 mg/L (3-methylbutanoic, nonanoic and decanoic acids) and 12.5-2500 mg/L (acetic acid). The quantitative assessment of organic acids was carried out in Extracted Ion Chromatogram (EIC) by selecting the m/z 60. The rest of compounds were quantified in the Total Ion Chromatogram (TIC). Reagents were purchased by Sigma-Aldrich (St Louis, MO, USA). Analytical repeatability, expressed as relative standard deviation (%), was assessed by

analyzing 5 replicates of a pooled sample obtained by mixing different kombucha samples (Annexe SG1).

2.5 Sensory analysis

Descriptive sensory profiles were performed using a trained panel. Training occurred in 2 sessions. The first session included descriptor generation, qualitative and quantitative tasting and smelling of standards. The second session included an evaluation of qualitative and quantitative perception of the standards followed by validation and selection of 12 trained panelists. Selected descriptors and corresponding training standards with concentrations are described in Annexe SG2.

Sensory samples were produced using the same procedure as in part 2.1. but were produced later separately and kept unfiltered, decarbonated and frozen (-20°C). The absence of filtration reflects the fact that kombucha is often sold unfiltered and the decarbonation eliminates the effect of carbon dioxide on the perception of the product both at visual and olfactive levels (Tran *et al.*, 2020a). Thirty mL of thawed sample at room temperature was presented simultaneously to panelist in 50 cL white plastic beakers labelled with a 3-digits code. A latin square was used to eliminate bias of the order of tasting. A first evaluation session included the kombucha samples: BTKd7, GTKd7, BTKd12, GTKd12. A second evaluation session included the cultures in sugared tea: BBd7, HVd7, AId7, BBHVd7, BBAId7, HVAId7, Td7, BBAId12, HVAId12 and Td12. Each descriptor was evaluated using a horizontal intensity scale with 11 levels, going from 0 to 10, with 0 corresponding to “absence” and 10 to “very intense”. Samples were firstly evaluated on the olfactive descriptors, then on the gustative descriptors except the conditions BBd7, HVd7, AId7 and BBHVd7, which were only characterized on the olfactive level, since they did not correspond to minimal consortia and therefore, they could not be comparable to kombuchas (original or issued from minimal consortium).

2.6 Statistical analyses

All samples were made in triplicates. Microbiological, non-volatile chemical composition and sensory data sets were treated using univariate analysis. Average values were compared using ANOVA and Newman-Keuls pair test was applied in the case of

significant difference ($p < 0.05$). In the case of sensory data, two-way ANOVA was performed. Statistics test as well as multivariate analysis (MVA) were performed using R Statistical Computing software (v4.0.3), with the R studio interface (v 1.4.1106). Prior to MVA, variables were mean-centered and unit-variance scaled. To visualize similarities and differences between samples, the FactoMineR package (v2.4) was employed to performed Principal Component Analysis. Hierarchical Cluster analyses (HCA) using the Ward's minimum variance method were used to identify sub-groups among samples. The Fowlkes-Mallows (B_K) similarity index was computed with the "dendextend" package (v1.14.0) and enabled the comparison between the sensory clustering and those obtained from GC-MS data set (Fowlkes and Mallows, 1983). Pair-wise Spearman correlations between metabolites abundancy and the sensory score were calculated with the "cor_test" function of statix package (v0.7.0). Correlation results and the significance of the interaction ($p<0.05$) were showed as a heatmap using the pheatmap package (v1.0.12).

3 Results and discussion

3.1 Microbiology and non-volatile composition

Population levels of yeasts and bacteria at day 7 (d7) and day 12 (d12) in the different cultures were determined using plate counting on selective and differential agar plates. The results are shown in Annexe SG3.

All cultures were conducted by inoculation of sugared tea at the rate of 1.10^5 cells mL^{-1}, excepted kombuchas which underwent traditional inoculation with 12 % (v/v) kombucha from previous batch. Initial populations for kombuchas (BTK and GTK) were $5.4.10^5$ and $9.0.10^4$ CFU mL^{-1} for total yeasts and total bacteria, respectively. The effect of microbial interactions has been assessed by comparing populations in cocultures compared to those in monocultures. At day 7 (end of P1), no significant differences among yeasts population could be observed among the cocultures except for *H. valbyensis* with significantly higher populations in cocultures when including *B. bruxellensis* (BBHVd7 and Td7) compared to kombuchas (BTKd7 and GTKd7; $p < 0.05$). However, the population of *A. indonesiensis* was significantly inferior in all cultures compared to the monoculture (AId7), except when associated with *H. valbyensis* only (HVAId7; $p < 0.05$). Between day

7 and day 12 (end of phase P2) the population of *B. bruxellensis* increased significantly in kombuchas (BTK and GTK, respectively +6.7.10^{6} and +7.1.10^{6} CFU mL^{-1}). The same increase was observed for *H. valbyensis* when in coculture with *B. bruxellensis* only (BBHV). Oppositely, a decrease of *A. indonesiensis* population occurred in the monoculture (AI) (-2.5.10^{6} mL-1) and in the coculture with *H. valbyensis* (HVAI) (-5.0.10^{6} CFU mL^{-1}), thus erasing any significant difference with other culture including the bacterium. In kombuchas (BTK and GTK), the population of *S. cerevisiae* increased between 9.0.10^{3} and 1.0.10^{4} CFU ml^{-1}, but it was not detected (inferior to 1.10^{3} CFU mL^{-1}) at day 7. It is worth noting that the dominant yeast species was different between the kombucha issued from the minimal consortium T (for the "Trio" composed of two yeast and one acetic acid bacteria) and the original kombuchas: *H. valbyensis* was the dominant species in the kombucha issued from the minimal consortium T whereas, *B. bruxellensis* was predominant in original kombuchas.

Microbial dynamics were influenced by the nature of the consortium, whether a minimal one or original kombucha culture, and impacted the identity of the dominant yeast species. The first phase of production P1 favored *A. indonesiensis* growth in monoculture or when associated with *H. valbyensis* only. The presence of *B. bruxellensis* appeared to cancel this effect, possibly due to substantial differences in metabolism between the two yeast species (Tran *et al.*, 2020b). This effect disappeared at the end of the natural carbonation phase P2 that influenced positively the dominant yeast species in black and green tea kombuchas as well as the yeasts cocultures (BBHV). This could also be due to the inhibition of acetic acid bacteria induced by the limitation of oxygen access, if they were present.

Endpoint chemical analyses were performed at the end of each production phase, respectively at day 7 and day 12. Initial sucrose, glucose, and fructose average concentrations in sugared teas (SBT and SGT) were respectively 58.3 ± 0.9 g L^{-1}, 0.3 ± 0.4 g L^{-1} and 0.4 ± 0.4g L^{-1}. The results are presented in Annexe SG4. No significant difference of sucrose concentrations at d7 or d12 were obtained with values between 39.1 and 56.1 g L^{-1} (Annexe SG4), except between BBAId7 and BBAId12(respectively 55.3 and 39.1 g L^{-1}; $p < 0.05$). For all cultures between d7 and d12, sucrose has never been fully hydrolyzed and the monosaccharides concentrations did not exceed 5 g L^{-1}. This

suggests a progressive consumption of monosaccharides obtained from sucrose hydrolysis. Moreover, the concentration of glucose was significantly higher for the *A. indonesiensis* monoculture (AI) at d12 (4.9 g L^{-1}) compared to other cultures where the bacterium was present ($p < 0.05$), which suggests a different kinetics of glucose consumption due to coculture with yeasts. Little effect of microbial composition, phase or tea type could be seen on the consumption of sugars, which was not the case in our previous studies (Tran *et al.*, 2020b). However, process duration and initial sugar concentration were different (24 days instead of 12 days for the total process duration and 66.6 g L^{-1} of initial total sugars).

The acidity parameters (pH and total acidity) were measured (Annexe SG4) and showed significant differences between the cultures ($p < 0.05$). Initial pH average value of sugared teas was 6.90 ± 0.10 with an average total acidity below 1 meq L^{-1}. All cultures underwent a decrease in pH value ($p < 0.05$). At day 7, the coculture of BBAI had a significantly lower pH than *B. bruxellensis* monoculture (BB) and was equivalent to the *A. indonesiensis* monoculture (AI) (respectively 4.43, 4.75 and 4.36), although no difference in total acidity was noted. No other effect of interaction or matrix was observed at day 7. Oppositely, kombucha issued from the minimal consortium T possessed a significantly higher total acidity compared to BBHV ($p < 0.05$), which highlights the contribution of *A. indonesiensis* in the production of organic acids (+ 5.7 meq L^{-1}), without significant effect on the pH value. Kombuchas (BTK and GTK) had significantly higher total acidity values (respectively 20.7, 19.0 meq L^{-1}) than minimal consortia at day 7 (ranging between 7.0 and 12.0 meq L^{-1}). Between d7 and d12 an increase in total acidity occurred for all cultures except for the *H. valbyensis* monoculture (HV) (+9.3, +8.7, +10.3, +8.7, +7.7, +8.0, +20.0, +9.3 meq L^{-1}, for BB, AI, BBAI, HVAI, BBHV, T, BTK and GTK respectively). This increase was not systematically associated to a decrease of pH value (not the case for kombucha issued from minimal consortium T and GTK). This suggests that the production of organic acids continued even with reduced oxygen access during P2. This phenomenon has already been observed in our previous study (Tran *et al.*, 2020b). At d12, comparison of cultures of BB, HV, AI and BBHV with BBAI, HVAI and kombucha issued from minimal consortium T respectively, showed that yeasts-acetic acid bacterium association induced a significant increase of total acidity. It is consistent with the acetic acid bacteria's

expected role in a minimal consortium. However, there was no significant difference between cultures of BBAI, HVAI and T. Minimal consortia BBAI, HVAI, T and kombuchas (BTK and GTK) possessed total acidity average values at day 12 ranging between 15.7 and 40.7 meq L^{-1} with a more efficient acidification for original kombuchas. Differences in inoculation process must be here considered. Original kombuchas were traditionally inoculated with 12% (v/v) of a previous acidic broth. As a result, initial average total acidity of kombuchas were 5.0 ± 0.1 meq L^{-1} and appeared to favorize the process, whereas initial total acidity of minimal consortia was inferior to 1 meq L^{-1}. Moreover, BTK pH value was significantly lower than GTK's (3.69 and 4.00, respectively), in association with a significantly higher total acidity at day 12 (40.7 and 28.3 meq L^{-1}, respectively), which indicates an effect of the matrix on the microbial activity without significant difference on the microbial populations (Annexe SG3).

Behavior in terms of sugar consumption and acidification were globally consistent with our previous study (Tran *et al.*, 2020b). Minimal consortia, as well as kombuchas consumed sugars to produce organic acids more efficiently than monocultures, but the performance of minimal consortia was not as good as the kombuchas. Additionally, no meaningful difference in sugars consumption could be observed. Thus, the microbial composition appeared to play a significant role on the production process, with a more intense acidification of yeast(s)-acetic acid bacteria cultures, despite the decrease of *A. indonesiensis* population induced by the presence of *B. bruxellensis*. The transition from P1 to P2 impacted the microbial dynamics by increasing yeasts and decreasing *A. indonesiensis* populations due to limitation of oxygen access. Finally, the tea type did not influence the microbial dynamics but had an impact on the level of acidification at the end of P2. Those three factors: microbial composition, phase and matrix clearly have an impact on microorganisms' dynamics and on the kombucha non-volatile composition. In contrast, the next part assesses the microbial role on the volatile composition.

3.2 Volatile composition

The analysis of the samples by HS-SPME-GC-/MS allowed the identification and quantification of 32 volatile metabolites belonging to different molecular families: alcohols, aldehydes, ketones, esters, phenol and saturated fatty acids (including acetic acid;

Tableau G2). Many metabolites such as phenylethyl ethanol, acetic acid, valeric and isovaleric acids were previously quantified in kombucha (Savary *et al.*, 2021; Zhang *et al.*, 2021). ANOVA was performed for the monocultures (BBd7, HVd7, AId7, BBd12, HVd12 and AId12) and SBT to determine if some metabolites were significantly more concentrated in one of those conditions (Annexe SG5). If a metabolite was detected in higher concentration in SBT, it was classified as “varietal”. If a metabolite was present in SBT and its concentration increased positively in one of the cultures, it was classified as “varietal and fermentative”. If a metabolite was not detected in SBT and produced in significant quantity in the monocultures, it was classified as “fermentative”. Signature metabolites were assigned to SBT of one microorganism if metabolites levels were significantly higher in one specific condition.

Tableau G2 : Detected and quantified volatile metabolite with chemical, sensory and origin features (Lambrechts and Pretorius, 2000; Luebke, 1980).

Code	IUPAC name	Common name	Chemical Family	Origin	Microorganism and phase where metabolite acts as signature	Smell
m01	ethanal	acetaldehyde	aldehyde	fermentative	none	sour, green apple (Lambrechts and Pretorius, 2000)
m02	methyl acetate	methyl acetate	ester	varietal and fermentative	none	solvant (Luebke, 1980)
m03	ethyl acetate	ethyl acetate	ester	varietal and fermentative	HVd7, HVd12	varnish, nail polish, fruity (Lambrechts and Pretorius, 2000)
m04	2-methylbutanal	2-methylbutanal	aldehyde	varietal and fermentative	none	malt (Luebke, 1980)
m05	3-methylbutanal	isovaleraldehyde	aldehyde	varietal	none	warm, herbaceous, slightly fruity (Lambrechts and Pretorius, 2000)
m06	ethanol	ethanol	alcohol	fermentative	BBd7, BBd12, HVd7, HVd12	alcohol (Lambrechts and Pretorius, 2000)
m07	ethyl propanoate	ethyl propanoate	ester	fermentative	HVd7, HVd12	weet fruity rum juicy fruit grape pineapple (Luebke, 1980)
m08	ethyl 2-methylpropanoate	ethyl isobutyrate	ester	fermentative	BBd12	sweet, rubber (Luebke, 1980)
m09	propyl acetate	propyl acetate	ester	fermentative	HVd7, HVd12	solvent-like pungency, lifting, fusel, amyl alcohol, sweet and fruity (Luebke, 1980)
m10	pentan-2-one	2-pentanone	ketone	varietal	none	etherial, diffusive and sweet banana-like with fermented woody nuance (Luebke, 1980)

m11	butane-2,3-dione	diacetyl	ketone	fermentative	none	buttery (Lambrechts and Pretorius, 2000)
m12	hexanal	hexanal	aldehyde	varietal	none	grass, tallow, fat (Luebke, 1980)
m13	2-methylpropan-1-ol	isobutanol	primary alcohol	fermentative	none	wine, solvent, bitter (Luebke, 1980)
m14	3-methylbutan-1-ol	isoamylacetate	ester	fermentative	none	banana, pear (Lambrechts and Pretorius, 2000)
m15	2-methyl-2-propanol	tert-butanol	primary alcohol	varietal and fermentative	none	camphor (Luebke, 1980)
m16	3-methylbutan-1-ol	isoamyl alcohol	primary alcohol	varietal and fermentative	HVd7, HVd12	marzipan (Lambrechts and Pretorius, 2000)
m17	3-hydroxybutan-2-one	acetoin	ketone	fermentative	none	butter, cream (Luebke, 1980)
m18	6-methyl-5-hepten-2-one	6-methyl-5-hepten-2-one	ketone	varietal	none	fruity, apple, musty, ketonic and creamy with slight cheesy and banana nuances (Luebke, 1980)
m19	hexan-1-ol	hexanol	primary alcohol	varietal and fermentative	HVd7, HVd12	resin, flower, green (Luebke, 1980)
m20	1-octen-3-ol	1-octen-3-ol	secondary alcohol	varietal and fermentative	BBd7, HVd7	earthy, green, oily, vegetative and fungal (Luebke, 1980)
m21	heptanol	heptanol	alcohol	varietal and fermentative	HVd7, HVd12	mushroom, green (Luebke, 1980)
m22	benzaldehyde	benzaldehyde	aldehyde	varietal and fermentative	none	bitter almond (Lambrechts and Pretorius, 2000)
m23	nonan-1-ol	nonanol	alcohol	varietal and fermentative	HVd7, HVd12	fresh clean fatty floral rose orange dusty wet oily (Luebke, 1980)
m24	2-phenylethyl acetate	2-phenylethyl acetate	ester	varietal and fermentative	HVd7	rose, honey, fruity, flowery (Lambrechts and Pretorius, 2000)

m25	2-phenylethan-1-ol	phenylethanol	alcohol	varietal and fermentative	HVd12	floral, rose (Lambrechts and Pretorius, 2000)
m26	phenol	phenol	phenol	varietal and fermentative	none	phenol (Luebke, 1980)
m27	acetic acid	acetic acid	saturated fatty acid	fermentative	Ald12	vinegar, pungent (Lambrechts and Pretorius, 2000)
m28	3-methylbutanoic acid	isovaleric acid	saturated fatty acid	fermentative	none	rancid, cheese, sweaty, rancid, fatty, pungent (Lambrechts and Pretorius, 2000)
m29	pentanoic acid	valeric acid	saturated fatty acid	fermentative	HVd12	unpleasant (Lambrechts and Pretorius, 2000)
m30	octanoic acid	caprylic acid	saturated fatty acid	fermentative	none	oily, fatty rancid, soapy, sweet, faint fruity, butter (Lambrechts and Pretorius, 2000)
m31	nonanoic acid	nonanoic acid	saturated fatty acid	fermentative	none	waxy, dirty and cheesy with cultured dairy nuance (Luebke, 1980)
m32	decanoic acid	capric acid	saturated fatty acid	fermentative	none	fatty, unpleasant, rancid, citrus, phenolic (Lambrechts and Pretorius, 2000)

3

Each class gathered different chemical families (Tableau G2). However, we can underline that saturated fatty acids were all classified as fermentative and that the yeasts signature metabolites were mainly marked by alcohols and esters, as it can be expected based on other matrices such as wine and cider (Hirst and Richter, 2016; Mo *et al.*, 2003). Ethanol and 1-octen-3-ol appeared to be signature metabolites of both *B. bruxellensis* and *H. valbyensis*. Most metabolites were associated with *H. valbyensis* only (ethyl acetate, isoamyl alcohol, hexanol, heptanol, nonanol, 2-phenyl acetate, phenyl ethanol, ethyl propanoate, ethyl isobutyrate, propyl acetate). It has been reported in cider that *H. valbyensis* in coculture with *S. cerevisiae* enhanced the production of esters (ethyl acetate, phenylethyl acetate). Those compounds in pure solution are associated with solvent, floral and fruity aroma (Lambrechts and Pretorius, 2000). Identified saturated fatty acids were small and medium-chain fatty acids produced during alcoholic fermentation and were implied in the biosynthesis of long chain fatty acids (Lambrechts and Pretorius, 2000; Stevens and Hofmeyr, 1993; van Roermund *et al.*, 2003; Viegas *et al.*, 1989). Medium-chain fatty acids were reported to have an inhibitory effect on the growth and fermentative activity of *S. cerevisiae* in conjunction with ethanol concentration and low pH value. Inhibition of lactic acid bacteria *Oenococcus oeni* by caprylic and lauric acids have also been reported in the context of interaction with *S. cerevisiae* during wine fermentation (Guilloux-Benatier *et al.*, 1998). Little data is available for acetic acid bacteria (Yamada *et al.*, 1981). However, release of medium-chain fatty acids was reported for lactic acid bacteria (Nakae and Elliott, 1965). According to the study of (Chen *et al.*, 1980), those compounds could be released during the autolysis of yeasts and could be involved in the "yeasty" aroma of lees. Indeed, acetic acid was the signature metabolite of *A. indonesiensis* (Tran *et al.*, 2020b). Metabolites classified as varietal such as isovaleraldehyde, hexanal, benzaldehyde, heptanol, nonanol and phenyl ethanol are typically found in tea (Ho *et al.*, 2015). Aldehydes and ketones were mainly classified as purely varietal.

Each metabolite was labelled with a code and was used as parameter to visualize similarities between samples through PCA and HCA (Figure G1). The sum of eigenvalues of the dimension PCA1 (26.0%) and PCA2 (22.8%) was equal to 48.8%. The interpretation of those two dimensions was judged satisfactory to describe the

phenomenon. The loading plot (Figure G1A) showed that metabolites vectors of the same family projected in similar PCA area, except aldehydes and ketones. Alcohols projected positively on the PC1 axis, esters positively on PC2, saturated fatty acid and phenol positively on PC1 and negatively on PC2. This result showed that the production of volatile metabolites occurred by chemical families for the three main ones, which were all fermentative metabolites (Tableau G2), involved possibly common metabolic pathways.

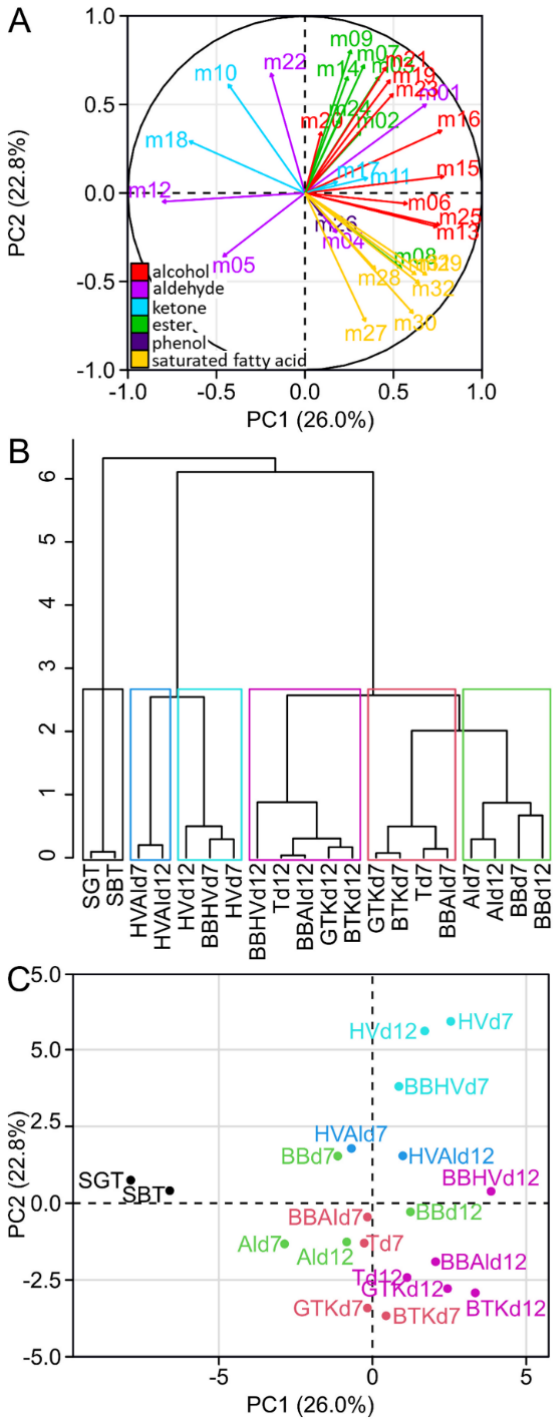

Figure G1 : Unsupervised classification using principal component analysis (PCA) and hierarchical clustering (HCA) on the relative concentration of the 32 metabolites from the 18 cultures (n = 3). Loading plot (A) shows the projection of each metabolite numbered consistently with Tableau 2. The metabolite coloration corresponds to its chemical family: alcohol (red), aldehyde (magenta), ketone (blue), ester (green), phenol (purple) and saturated fatty acid (yellow). HCA (B) with cut-off enabling a separation in 6 clusters depicted in the PCA score plot (C) with each cluster associated to one color.

The HCA allowed the discrimination of samples into clusters of similar volatile compositions (Figure G1B). The first cluster gathered the sugared teas (SBT and SGT). The second cluster gathered coculture of *H. valbyensis* and *A. indonesiensis* (HVAId7 and HVAId12). The third cluster gathered *H. valbyensis* monocultures (HVd7 and HVd12) and BBHVd7. The fourth cluster gathered *B. bruxellensis* and *A. indonesiensis* monocultures (BBd7, AId7, BBd12 and AId12). The fifth cluster gathered minimal consortia including *B. bruxellensis* and the original kombuchas at day 7 (BBAId7, Td7, BTKd7 and GTKd7). The sixth cluster gathered the same cultures at day 12 (BBAId12, Td12, BTKd12, GTKd12) and the coculture BBHVd12. The organization of the dendrogram's branches enables to hierarchize the factors that affect the samples volatile composition. The main factor was the microbial activity (sugared teas against cultures) and separated the samples according to their volatile composition. Within the cultures, the effect of yeast composition separated secondarily the samples including *H. valbyensis* with and without the presence of *B. bruxellensis*, apart from BBHVd7 that seemed to be mainly influenced by the presence of *H. valbyensis*, in opposition to BBHVd12 that seemed influenced by *B. bruxellensis*. In the case of BBHV, the phase of production appeared to modify the microbial activities without effect on yeast species dominance (Annexe SG3). Therefore, associations can be made between *H. valbyensis* and P1, and between *B. bruxellensis* and P2. Indeed, the third effect of the production phase could be clearly observed for the minimal consortia including *B. bruxellensis* and the kombuchas, which were dominated by this yeast species (Annexe GS3). Finally, monocultures of *B. bruxellensis* and *A. indonesiensis* were closer to the minimal consortia including *B. bruxellensis* (BBAI and T), which highlighted a fourth effect of yeast – acetic acid bacteria interaction. It is noteworthy that the difference in tea types induced no matrix effect on the clustering, both before and after microbial activity. So, regardless of the tea type, kombuchas were in the same clusters. The cultures that showed the closest similarity to the kombuchas were the cocultures including at least both *B. bruxellensis* and *A. indonesiensis* both at day 7 and day 12, with or without the presence of *H. valbyensis* (BBAI and T). Consequently, the minimal presence of *B. bruxellensis* and *A. indonesiensis* defined a functional consortium on the level of volatile composition. Interestingly, this echoes the results of our previous study (Tran *et al.*, 2020b), where this couple of microorganism was also a suitable combination for a functional consortium regarding acidification. The major volatile

metabolites found in those clusters besides ethanol and acetic acid were ethyl acetate and isovaleric acid with concentrations ranging from 600 to 7000 µg L^{-1}.

Figure G1C presents the PCA's score plot and demonstrates the association of clusters to chemical families in relationship to the PCA loading plot (Figure G1A). Sugared teas, located on the left part of the plot, were associated with ketones and aldehydes that were mainly signature metabolites of SBT. Clusters with minimal consortia including *B. bruxellensis* and kombuchas were clearly associated with the production of alcohols and saturated fatty acids, while cultures involving *H. valbyensis* without *B. bruxellensis* (HV, HVAI) and BBHVd7 were associated to the production of alcohols and esters.

More details can be obtained from the ANOVA of minimal consortia (BBAI, HVAI and T) and BTK (Annexe SG6). *H. valbyensis* significantly contributed on signature metabolites in Td7 (ethyl acetate, ethyl isobutyrate and isoamyl alcohol) compared to BBAId7 ($p < 0.001$), but this contribution sustained only with ethyl isobutyrate and isoamyl alcohol at day 12. Therefore, *H. valbyensis* could possess a functionality in the minimal consortium T by enrichment of ester and alcohols of the volatile profile and potentially the olfactive profile of the product. Moreover, for all cocultures, the minimal consortia and SBT, P2 significantly increased the concentrations of isobutanol and isoamyl alcohol ($p < 0.001$), which became major volatile compounds (between 700 and 2000 µg L^{-1}). This could be the consequence of redox potential regulation due to restricted oxygen access (Dijken and Scheffers, 1986; Hazelwood *et al.*, 2008; Quain, 1988).

By comparing monocultures and cocultures at day 7 using ANOVA (Annexe SG7), it is possible to observe that the presence of *B. bruxellensis* and/or *A. indonesiensis* decreased the production of ethyl propanoate, isoamyl alcohol, hexanol, heptanol and nonanol by *H. valbyensis*. Two hypotheses can be made: either the microorganisms competed for the substrate or precursor needed to produce those metabolites, or the metabolites were used by the other microorganisms (commensalism) (Ivey *et al.*, 2013). Namely, the presence of *A. indonesiensis* significantly decreased the concentration of alcohols (ethanol, isobutanol, hexanol, nonanol, $p < 0.01$). For example, nonanol could be oxidized into nonanoic acid in the same manner that ethanol is oxidized in acetic acid or glycerol in dihydroxyacetone (Matsushita *et al.*, 1994), although no specific enzyme for

the conversion of nonanol has been yet reported. Consequently, the functionality of *H. valbyensis* was limited by the presence of other microorganisms due to microbial interactions. Additionally, it is worth noting that pure varietal metabolites either showed no significant variation linked to microbial activity (2-pentanone, 6-methyl-5-hepten-2-one) or showed significant decrease regardless of microbial composition (isovaleraldehyde, hexanal; $p < 0.001$).

A putative metabolic scheme based on metabolic pathways of *S. cerevisiae* was described in the literature and KEGG database (Kanehisa, 2000) and gathered most of fermentative metabolites identified in the present study (Figure G2). It highlights the metabolic pathways that are dependent on sugar (glucose and fructose) and those dependent on nitrogenous substrates (ammonium NH_4^+ and amino acids). Production of volatile compounds in wine for example are mainly attributed to the Ehrlich pathways, that relies on the transamination of an amino acid, the decarboxylation of the keto acid (phenylpyruvate in the case of phenylalanine metabolism) then the reduction of the acid into volatile alcohol (Ehrlich, 1907). The alcohols can then be esterified to produce volatile esters (Belda *et al.*, 2017). However, keto acids and phenylpyruvate can be produced from pyruvate using amino acids biosynthesis pathways (Kanehisa, 2000; Yu *et al.*, 2016). Consequently, volatile alcohols such as isoamyl alcohol, isobutanol and phenylethanol and their corresponding esters can be produced independently from the corresponding amino acids (leucine, valine, phenylalanine). Other metabolites unrelated from the Ehrlich pathway can be produced solely from glucose (ethyl esters, ketones, saturated fatty acids). Although amino acids can be synthetized *de novo* from ammonium (Ljungdahl and Daignan-Fornier, 2012), the initial amount of free amino nitrogen in tea infusion (63 µg L^{-1} in our previous study: Tran *et al.*, 2020b) is insufficient to produce the amount of volatile metabolites quantified in the present study. Moreover, no volatile metabolite absolutely dependent on the Ehrlich pathways were detected in the present study, nor in recent studies (Savary *et al.*, 2021; Zhang *et al.*, 2021). In these studies, the detection of ethyl esters of caprylic and caproic acids tends to corroborate this hypothesis. Therefore, the volatile profile of kombucha appears to be determined by the composition of the sugared tea infusion matrix, that consists in a low N/C ratio. For example, N/C ratio of sugared teas

in the present study was around $1.3.10^{-6}$, which is 577 times lower than the N/C ratio of a grape must of 200 g L^{-1} sugars and 150 mg L^{-1} of assimilable nitrogen ($7.5.10^{-4}$).

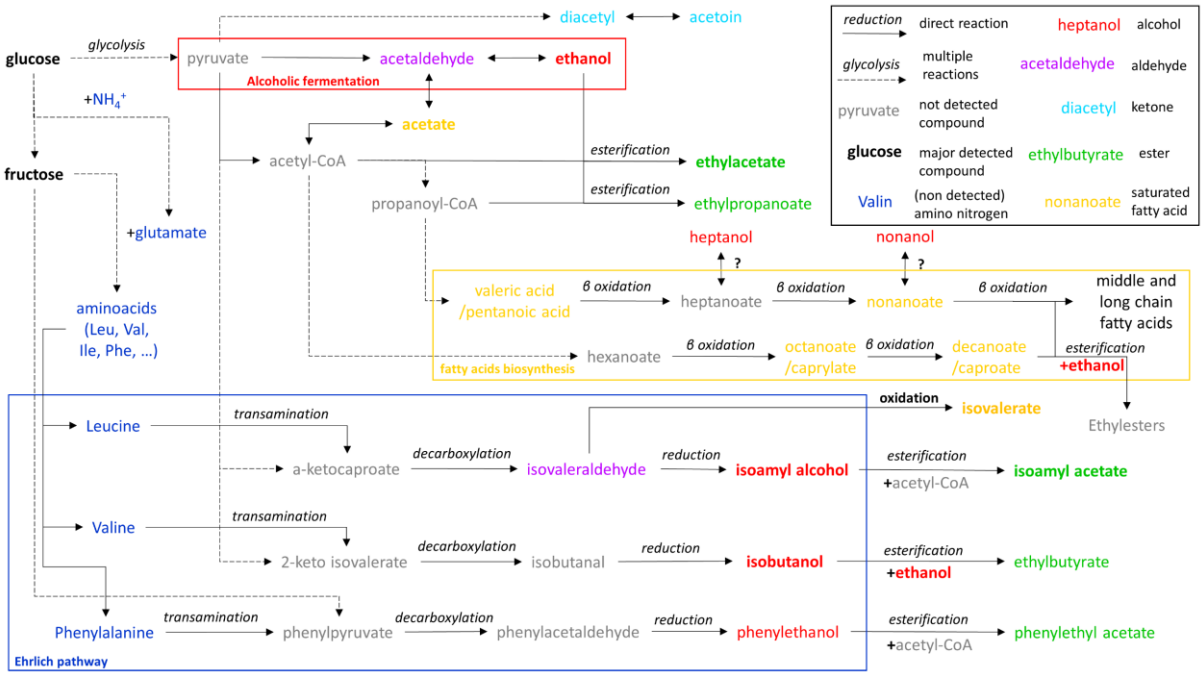

Figure G2 : Putative metabolic pathways of fermentative volatile metabolites detected based on *S. cerevisiae* metabolism (Ardö, 2006; Belda *et al.*, 2017; Ljungdahl and Daignan-Fornier, 2012; Yoshizawa, 1964; Yu *et al.*, 2016).

The volatile profile of kombucha appeared to be mainly impacted by the microbial composition and interactions. The production phase induced changes in the volatile profile. Tea type had little effect on it. Also, the volatile compounds of kombucha are suspected to be determined by the particular composition of the sugared tea matrix in terms of carbon and nitrogen substrates availability. However, the real implications of those effects on the product can only be evaluated through sensory analysis.

3.3 Linking volatile composition to sensory profiles

Among the 20 conditions analyzed by GC/MS, 14 underwent descriptive sensory analysis through the evaluation of newly produced samples by 12 trained panelists. The samples gathered minimal consortia and kombuchas at day 7 and day 12, and monocultures at day 7.

ANOVA revealed no significant difference in sweetness, bitterness, and astringency between the samples (Annexe SG8). In contrast, sourness was significantly higher for BBAI and HVAI samples at day 12 than at day 7 ($p < 0.001$). Regarding kombuchas only, sweetness and sourness reached highest scores (respectively 5.5 and 3.7), whereas bitterness and astringency showed lower scores (1.9 and 2.1), which clearly established the sweetness and sourness balance as main gustative parameters of kombucha and probably mask bitterness and astringency of tea as previously hypothesized (Tran *et al.*, 2020a).

Volatile compositions were confronted to olfactive sensory scores per descriptor using pair-wise Spearman correlations (Figure G3). The results showed significant correlations of esters and ketones with tea (ethyl acetate, isoamyl acetate, 6-methyl-5-hepten-2-one, ethyl propanoate, propyl acetate; $R > 0.54$, $p < 0.048$) and together with white fruits (methyl acetate, 2-pheynlethyl acetate; $R > 0.55$, $p < 0.042$). Consistently, those compounds are generally associated to fruity aroma (Lambrechts and Pretorius, 2000). All those metabolites are varietal and most of them participate to the fermentative signature of *H. valbyensis*. This signifies that those metabolites enhanced tea and white fruits aroma, which could be a positive contribution to the olfactive profile. In contrast, saturated fatty acids and alcohols are involved in the expression of vinegar, apple juice and exotic fruits aroma. Together with decanoic and octanoic acid, acetic acid expectedly contributed to the vinegar aroma ($R > 0.54$, $p < 0.045$) whereas the pure varietal 2-pentanone was negatively correlated ($R = -0.58$; $p = 0.029$). Acetic acid also played a significant role in exotic fruits aroma with isovaleric acid and isobutanol ($R > 0.56$; $p < 0.040$). Additionally, isovaleric acid is significantly correlated to the apple juice aroma ($R = 0.56$, $p = 0.036$). Besides acetic acid, the odor of those compounds in pure solutions are generally associated with oily, fatty and rancid notes and isobutanol with solvent (Lambrechts and Pretorius, 2000). The correlation with fruity odors must then result of sensory interaction between the compounds involved on global perception (Chambers and Koppel, 2013). Finally, the cheesy aroma was significantly correlated only negatively with methyl acetate and 2-phenylethyl acetate ($R < -0.56$, $p < 0.042$). This might be related to a masking effect on fruity aroma.

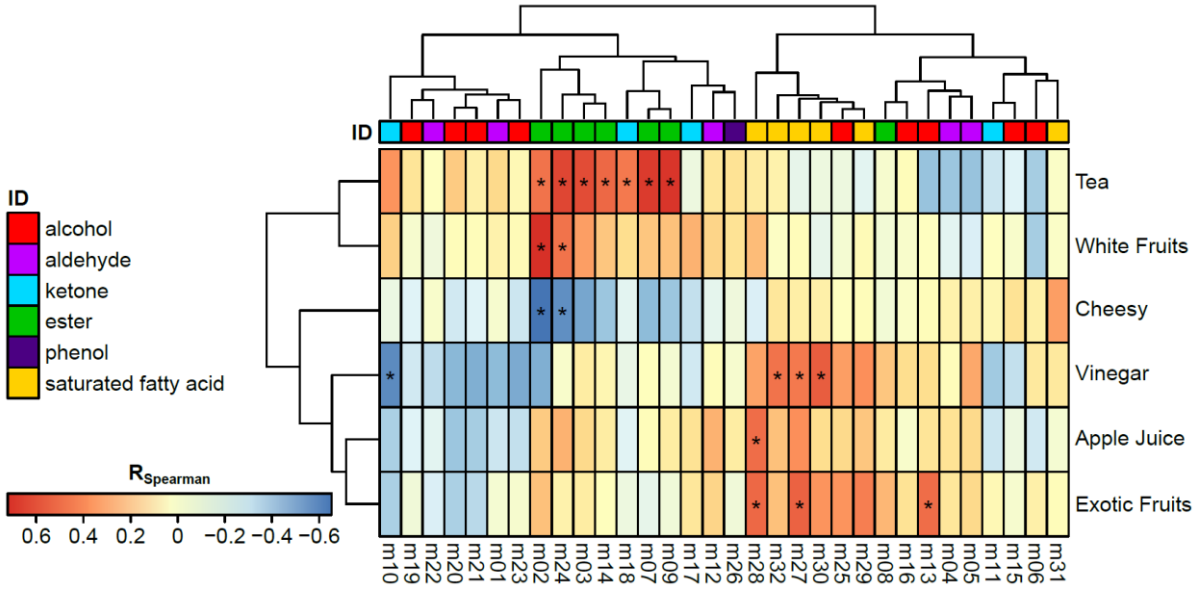

Figure G3 : Heatmap depicting pair-wise Spearman correlations (R; p<0.05: *) between a sensory descriptor score and a single metabolite numbered as in the Tableau G2 and colored consistently as its chemical family.

Figure G4 displays a dendrogram comparison of the 14 conditions treated by GC-MS and by sensory analyses. To go further on the similarity between the GC-MS and sensory dendrograms, the Fowlkes-Mallows index (B_K) was used. The B_K index is linked to the *k* cut-off threshold used for both dendrograms and to the number of matching entries in the resulting *k* clusters of each tree. The resulting B_K plot (Annexe SG9) shows increased similarity for *k* clusters between 4 and 5 and thus indicates relationships between volatile composition and sensory perception. Differences in clustering were visible but the general organisation remained similar with separation of *B. bruxellensis* and *A. indonesiensis* monocultures from cocultures and kombucha. Also, HVAId12 and BBAId7 samples remained in the same clusters. The conservation of the clustering of Td12 with BBAId12 and BTKd12 samples was striking and showed the closeness of functional consortia and kombucha in volatile composition carried on to the olfactive profile. Considering the sensory dendrogram alone, monocultures were separated from cocultures except for *H. valbyensis* monoculture that was related to HVAId7 sample. Within the coculture and kombucha branch, cultures dominated by *H. valbyensis* (HVAId7, HV, Td7 and BBHVd7) shared closeness (Annexe SG3). A striking difference involved GTKd7 sample that was separated to BTKd7 sample. This suggests a matrix effect that was not expressed through

GC-MS volatile compounds analysis. It is likely that some organoleptic varietal compounds specific to green tea were not detected. GTKd12 sample was related to *B. bruxellensis* and *A. indonesiensis* monocultures (BBd7 and AId7) due to unexpected intense cheesy off-flavor. Those three samples showed significant higher cheesy notes ($p < 0.001$) (Annexe SG8). In contrast, *H. valbyensis* (HV) monoculture and HVAId7 sample showed significantly higher tea aroma compared to BBd7, AId7 and GTKd12 samples ($p < 0.001$), confirming the masking effect of the cheesy note. Interestingly, BBAId7 showed higher white fruits and apple juice notes than BBd7 and AId7 samples. This worked similarly with HVAId7, HVd7 and AId7 samples for the apple juice aroma only. Therefore, the characteristic apple juice aroma of kombucha appears to result from the yeast-acetic acid bacteria interaction (Tran *et al.*, 2020a). Moreover, this interaction relied on sensory interaction of compounds produced by each microorganism and not from compounds issued from microbial interaction.

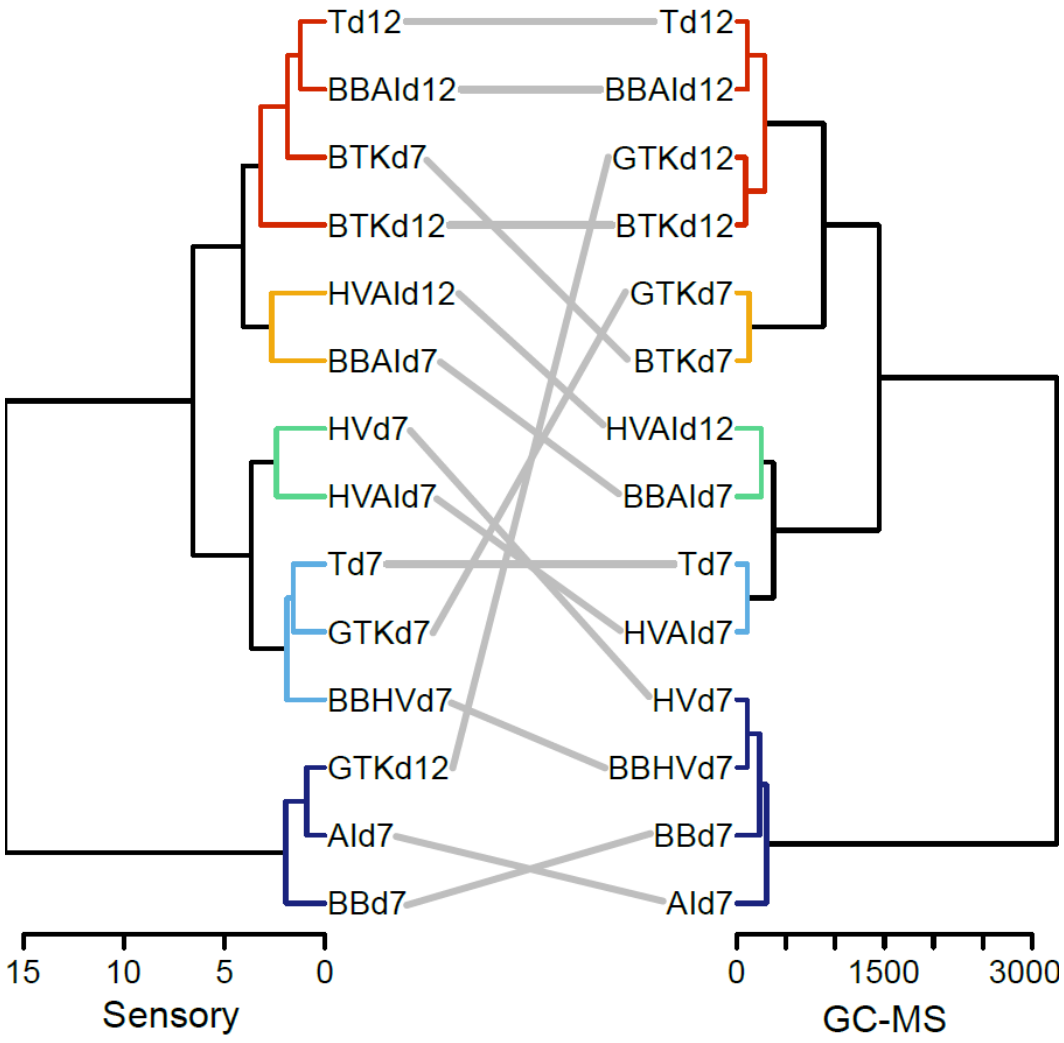

Figure G4 : Dendrogram comparison based on Ward's clustering performed on sensory scores (left) and volatile metabolite concentrations (right) in the 32 detected metabolites among the 14 cultures evaluated by sensory analysis.

In contrast with the volatile composition, the production phase had less effect on the sensory profile of the samples, while the tea type appeared to induce a matrix effect. The effect of microbial composition remained preponderant, and the simultaneous presence of *B. bruxellensis* and *A. indonesiensis* was confirmed to be key for the typicity of kombucha's olfactive profile that includes the apple juice note.

5 Conclusions

Through this study coupling microbiology, analytical chemistry and sensory analysis, many questions regarding the origins of kombucha's organoleptic components could be answered(Tran *et al.*, 2020a). Olfactive profiles of cultures and kombuchas were clearly associated to volatile profiles and identified the association of at least one yeast with the tested acetic acid bacterium, such as *B. bruxellensis* and *A. indonesiensis* as minimal functional consortium to produce the closest beverage to kombucha. This statement was previously made for the main non-volatile compounds of kombucha (organic acids, sugars, and ethanol; Tran *et al.*, 2020d). The typical apple juice aroma of kombucha emerged from yeast-acetic acid bacteria association as well. *H. valbyensis*' contribution in the olfactive and volatile profiles was beneficial in terms of quality, with a potential enhancement of fruity aroma through the production of esters but it was limited because of interactions with other microorganisms and the secondary natural carbonation phase. This production phase also stimulated the production of alcohols.

This study contributed to better understand the kombucha's volatile composition in terms of origins (varietal and/or fermentative). It also rose a new hypothesis about kombucha's composition determinism and its relationship with the sugared tea matrix composition. Ultimately, a matrix effect could be observed on the sensory level although it had the least impact on the composition in detected volatile compounds. Those results should enable further investigation to understand, improve and control the organoleptic quality of kombucha. Namely, the influence of acetic acid bacteria or lactic acid bacteria species and their interactions would constitute research topics of high interest.

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Declaration of interest

Authors declare no conflict of interest.

Author contributions

Thierry Tran took the lead of the writing of this article, but all other authors provided critical and complementary elements to the manuscript. Thierry Tran performed microbiological and non-volatile chemical analyses, as well as ANOVA of volatile compounds and sensory analyses. Kevin Billet performed statistical treatment and figures conception of PCA, HCA, heatmap, dendrogram comparison analysis and Bk-plot. Berta Torres-Cobos and Stefania Vichi performed HS-SPME-GC-MS analysis of volatile compounds. François Verdier and Antoine Martin provided the kombucha cultures used in the experiments.

H Chapitre 6 : Cinétiques de production et consommation des protéines et des acides aminés au cours de la production de kombucha.

Ce sixième chapitre et le suivant délaissent les analyses en point final pour adopter des suivis en cinétiques. Les travaux ont été réalisés en très grande partie par Jacqueline Rodriguez-Rey dans le cadre de son stage de Master 2 en 2021. Après la réalisation d'analyses complémentaires, ce chapitre constituera la base pour la rédaction d'un article qui sera soumis au journal *International Journal of Food Microbiology*. Cette étude se concentre sur la gestion des substrats azotés (ammonium, acides aminés et protéines) par les micro-organismes. Comme évoqué dans le chapitre précédant, la ressource en azote est en effet très limitée dans la matrice thé sucré. La quantité des protéines solubles et d'azote aminé libre (ou FAN pour *Free Amino Nitrogen*) a été analysée par méthodes colorimétriques pendant les deux phases de production de la kombucha originale, des monocultures et des cocultures de *B. bruxellensis*, *H. valbyensis* et *A. indonesiensis*. De plus, les fractions protéiques ont été analysées qualitativement en point final avant inoculation et à la fin des deux phases par électrophorèse en conditions native et dénaturante.

Les cinétiques de consommation de FAN et de production de protéines diffèrent selon les cultures. Les monocultures de *B. bruxellensis* et *A. indonesiensis* montrent une corrélation entre les teneurs en FAN et en protéines, suggérant que la faiblesse des ressources en azote limite la production de protéines. Pour les autres cultures, l'absence de corrélation suggère des phénomènes plus complexes, qui dans le cas des cocultures, peut être expliquée par la multiplicité des flux de consommation de FAN par la présence de plusieurs micro-organismes. Il est intéressant de relever que les teneurs en FAN et en protéines dans la kombucha originale n'ont montré que peu de variations, ce qui suggère une meilleure gestion des flux de nutriments. Enfin, l'analyse par électrophorèse des protéines a montré la présence d'une fraction de masse apparente d'environ 15 kDa absente du thé sucré, commune à toutes les cultures et donc tous les micro-organismes. En revanche, une fraction de masse apparente d'environ 21 kDa est détectée uniquement dans les cultures comprenant *A. indonesiensis*. Selon la littérature, cette protéine pourrait correspondre à une protéine de stress identifiée chez cette espèce bactérienne et impliquée dans la tolérance de la bactérie à l'acide acétique.

La nutrition azotée dans la kombucha est un intéressant objet d'études, étant donnée la limitation importante des ressources en nutriments azotés dans cette matrice. Le « modèle » kombucha offre donc un cadre intéressant pour des études de stress microbien vis-à-vis des ressources en nutriments azotés. Cette première étude ouvre un champ futur d'investigations afin d'élucider les conséquences de cette limitation sur les micro-organismes et leurs activités. L'obtention d'une telle compréhension pourrait à terme mener à une optimisation accrue du pilotage du process.

Production and consumption kinetics of proteins and amino acids during kombucha production

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Abstract

Kombucha is a beverage obtained from the transformation of sugared black tea infusion by a community of microorganisms composed mainly of yeasts and acetic acid bacteria. In tea infusion, nitrogen is mainly represented by proteins and amino acids. but to the best of our knowledge there is no data on the consumption of these compounds during the production of kombucha.

This study aimed at studying quantitatively proteins and amino nitrogen and qualitatively the soluble proteins profile during a 12-day process of kombucha production. The growth in sugared black tea of major microorganisms previously isolated from the kombucha consortia was analyzed (monocultures and cocultures of *Brettanomyces bruxellensis*, *Hanseniaspora valbyensis* and *Acetobacter indonesiensis*). A quantitative and qualitative analysis was carried out by comparing cultures with a control sample inoculated by the whole set of microorganisms.

Protein and Free Amino Nitrogen (FAN) kinetics were different depending on microbial compositions of the cultures, thus highlighting microbial interactions. Monocultures of *B. bruxellensis* and *A. indonesiensis* showed a correlation between FAN and protein contents, suggesting that FAN deprivation through consumption limited the synthesis of protein. Such correlation was not observed for the other cultures, thus pointing to a more complex management of nitrogen, that is present in limiting amounts. Less variations in FAN and protein contents were observed in the original kombucha compared to the cultures with isolated microorganisms, suggesting the existence of interactions allowing a better management of the available nutrients. Protein analysis by electrophoresis showed the presence of a microbial 15 kDa fraction common to all cultures. However, a 21 kDa signature protein fraction related with *Acetobacter indonesiensis* has been detected and could be related to stress proteins.

Keywords

Interactions, kombucha, proteins; free amino nitrogen; amino acids

1 Introduction

Kombucha is a beverage commonly obtained from the fermentation of sugared black or green tea infusion. The transformation is carried out by a consortium of microbes added in the sugared tea infusion under the form of liquid suspension or using a biofilm carrying those microbes and involving interplays between the microorganisms (Jayabalan *et al.*, 2014; May *et al.*, 2019; Villarreal-Soto *et al.*, 2018). Several yeasts have been isolated from the liquid phase of the kombucha, belonging to the genera *Saccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Brettanomyces* or *Hanseniaspora* (Villarreal-Soto *et al.*, 2018). Recently, *Brettanomyces* has been found to be the predominant yeast genus between 103 samples issued from breweries in North America (Harrison and Curtin, 2021). Besides yeasts, acetic acid bacteria (AAB) have been predominantly isolated from kombucha, with different genera encountered, such as *Acetobacter*, *Gluconobacter* and *Komagataeibacter.* These bacteria can produce a cellulose network where yeasts and other bacteria can adhere or be embed, producing a biofilm called “kombucha mother”, “pellicle” or “SCOBY” (Symbiotic Culture of Bacteria and Yeast) (Jayabalan *et al.*, 2010). The process of kombucha production starts with the hydrolysis of sucrose by yeasts and

bacteria, to produce fructose and glucose that are converted to ethanol by the yeasts through alcoholic fermentation. During the first phase of acidification in open vessel, AAB oxidise glucose and ethanol into organic acids (mainly acetic and gluconic acid). The second phase of natural carbonation in closed vessel favours the activity of yeasts that convert residual sugars in ethanol and carbon dioxide (Tran *et al.*, 2020a, 2020b). It has been established that many process parameters and non-standardized procedure induced great variability in kombucha composition, namely in organic acids, vitamins, minerals, and tea polyphenols content (Villarreal-Soto *et al.*, 2018).

Mutualistic interactions are crucial for microorganism's survival and have been described in multiple fermented foods (Sieuwerts *et al.*, 2008; Smid and Lacroix, 2013). Better understanding of the process of kombucha production necessitates to gain insight into the microorganisms' metabolism and interactions with the nutrients present in the medium. In the case of the present work, the focus was made on the nitrogen compounds present in the liquid matrix. Indeed, this topic has been poorly investigated previously in contrast to the utilization of the carbon substrate (Chen and Liu, 2000; Jayabalan *et al.*, 2010; Loncar *et al.*, 2014; Malbaša *et al.*, 2008; Reiss, 1994; Tran *et al.*, 2020b).

The sugared tea matrix is characterized by an initial low concentration of nitrogen compounds (Hormann and Engelhardt, 2013; Jayabalan *et al.*, 2007; Kallel *et al.*, 2012; Sreeramulu *et al.*, 2000). Composition is variable quantitatively and qualitatively depending on the type and amount of tea leaves used to prepare the infusion. Protein levels are between 0.1-0.2 mg mL^{-1}, comparable to the content in white grape must, that vary around 0.27 mg mL^{-1} (Vincenzi *et al.*, 2005). In the case of Free Amino Nitrogen (FAN), the content in black tea infusion can reach 10 mg L^{-1} (Kocadağlı *et al.*, 2013), much lower than in grape must that can vary around 120 mg mL^{-1} (Bely *et al.*, 1990). The composition in amino acid in tea is particular with high abundance of tea-specific theanine. Other amino acids reported are glutamic acid, asparagine serine, tryptophan (Alcázar *et al.*, 2007; Horanni and Engelhardt, 2013; Syu *et al.*, 2008).

Regarding the relationship of yeasts to nitrogen compounds, the highly studied species *Saccharomyces cerevisiae* can be used as a reference. This yeast can assimilate both ammonia and amino acids to produce proteins and purines. Amino acids can be directly

used or be degraded in two key compounds: ammonium and glutamate, which can be used for the biosynthesis of all other amino acids through the activity of transaminases (Ljungdahl and Daignan-Fornier, 2012). Ammonia and amino acids are internalized in yeast cells thanks to permeases, respectively active uniports and symports (Casal *et al.*, 2008; Marini *et al.*, 1997). Regulation in amino acid incorporation involves several transcriptional and post-transcriptional mechanisms. For example, Nitrogen Catabolic Repression (NCR) is active when preferential nitrogen sources are available (glutamine, glutamate or ammonium for *S. cerevisiae*) and is lifted otherwise, thus increasing up-take in other nitrogen sources (Bianchi *et al.*, 2019). In the oenological context, preference in amino acid did not highlight general trends, but strain specific profiles, however proline and hydroxyproline could be discriminated as secondary amino acids that are significantly less assimilated than the other primary amino acids (Bell and Henschke, 2005; Gobert *et al.*, 2019). In a context of deprivation of one or more amino acids, General Amino Acids Control (GAAC) inhibits translation initiation and impacts the regulation of at least 539 genes (Bianchi *et al.*, 2019). Down-regulation of genes related to protein synthesis and nucleic acid metabolism occurs (Weiping Zhang *et al.*, 2018). Oppositely, there is an up-regulation of the genes involved in energy generation, carbohydrate metabolism, oxidoreductase activity, respiratory chain phosphorylation, transporter activity, respiration, response to oxidative stress, oxygen, and reactive oxygen species metabolism (Mendes-Ferreira *et al.*, 2007). Consequently, nitrogen deficiency in grape must during stationary phase can lead to a decrease of sugar uptake (Bisson, 1999).

Brettanomyces (*Dekkera) bruxellensis* nitrogen requirements have been well-studied due to its ability to grow in deprived environment such as wine, thus leading to spoilage (Steensels *et al.*, 2015). Indeed, *B. bruxellensis* has been characterised by the fact that it does not need high amounts of nitrogen to grow, and it adapts to whatever is available. In synthetic wine medium, *B. bruxellensis* was able to grow using ammonium, proline, arginine, or leucine as a sole source of nitrogen (Crauwels *et al.*, 2015; Smith and Divol, 2016). Moreover, it has been determined that *B. bruxellensis* nitrogen up-take was regulated by NCR (de Barros Pita *et al.*, 2013). However, unlike *S. cerevisiae*, glutamine appears to be a preferred amino acid for *B. bruxellensis* in both aerobic or anaerobic conditions, but glutamate and aspartate are preferred in anaerobiosis specifically (Parente

et al., 2018). Nitrogen depletion induced a moderate decrease of glucose consumption and ethanol production in the studied strain (*B. bruxellensis* GDB 248) (de Barros Pita *et al.*, 2011). Ethanol yield was lower in these conditions, indicating that some carbon has been diverted from fermentation to other metabolic processes, most probably respiration.

A different yeast majorly found in kombucha is *Hanseniaspora valbyensis* (Leech *et al.*, 2020; Villarreal-Soto *et al.*, 2018). Aside from its low fermentative capacity observed in kombucha, particularly under aerobic conditions, *Hanseniaspora* species are not able to grow under non-carbohydrate sources of energy, such as pyruvate, amino acids, or glycerol, because of the lack of gluconeogenic enzymes genes (Valera *et al.*, 2020). Some *Hanseniaspora* species have been characterized by a similar profile of amino acid consumption than *S. cerevisiae* in synthetic must. Lysine, glutamic acid, cysteine, isoleucine, leucine, and phenylalanine were first consumed during alcoholic fermentation (Lleixà *et al.*, 2019). In a comparative genomic study, it was found that *Hanseniaspora* species isolated from different wineries possessed general amino acid permeases with a broad range of substrates instead of specific ones, as a mean of adaptation (Seixas *et al.*, 2019).

The other major players in kombucha are AAB and their nitrogen requirements are dependent on the strain, as for yeasts. Acetic acid bacteria, including *Acetobacter malorum* DSM 14337 and CECT 7742 strains have been found to prefer proline as nitrogen source, but they could also grow using alanine, glutamic acid, glutamine, serine, leucine, methionine, and ornithine (Álvarez-Cáliz *et al.*, 2012; Maestre *et al.*, 2008; Sainz *et al.*, 2017). Ammonium has not been found to stimulate growth as a sole source of nitrogen, but it was consumed in high proportions during vinegar production together with other amino acids (Maestre *et al.*, 2008; Sainz *et al.*, 2017). However, in this matrix, in response to an abrupt change in the environmental conditions, the cells tend to use free amino acids preferentially over ammonium ion. Under mild conditions, AAB might use ammonium ion to produce amino acids that were partly released to the medium as a reserve for more adverse future conditions (Álvarez-Cáliz *et al.*, 2012).

The aim of our study is to investigate the management of different nitrogen compounds by microorganisms during the production of kombucha. Based on the protocol previously

described (Tran *et al.*, 2020b), monocultures of *Brettanomyces bruxellensis, Hanseniaspora valbyensis* and *Acetobacter indonesiensis* have been carried out, as well as cocultures of yeast and the bacterium representing a minimal microorganism's combination able to produce kombucha. The original isolated consortium was tested as control. The potential impact of these different microbial composition under two different production phases in open and closed vessel on proteins and Free amino nitrogen (FAN) was analysed. Quantitatively, the kinetics have been followed during the process and qualitatively protein fractions have been analysed using electrophoresis.

2 Materials and methods

2.1 Microorganisms and cultures

Strains of *Brettanomyces bruxellensis, Hanseniaspora valbyensis* and *Acetobacter indonesiensis* were previously isolated from black tea kombucha liquid and pellicle samples from the company Biomère (Paris, France), as described by Tran et al, (2020d). Sugared black tea was prepared as previously described by Tran et al, (2020d). Briefly, to produce 1 L of sugared tea, 200 mL of charcoal filtered tap water was boiled and, after removed from the heater, 1 g of black tea Pu'er Grade 1 TN4107 from "Les Jardins de Gaïa" (Wittisheim, France) was added and left at room temperature for one hour. Then, the tea was removed using a sieve, transferred to a sterile vessel, and mixed with 800 mL of distilled water. Finally, 50 g of sucrose were dissolved and left at 26 °C for one hour.

Precultures of microorganisms were produced from stocks kept at -20 °C. Yeasts were restreaked on WallersteinLab (WL) agar medium from Thermo Fisher Scientific (Waltham, MA, USA) and the acetic acid bacterium on De Man Rogosa and Sharpe (MRS) (pH 6.2) agar medium from Condalab (Madrid, Spain). After growth at 28°C, YPD and MRS liquid medium were inoculated with yeasts and acetic bacterium isolates respectively. Incubation occurred at 28°C for 2 days in static conditions and sterile vessels with untight caps to allow gas exchanges. Cells were washed twice with sugared black tea, before centrifugation (3000 x g for 10 minutes at 4 °C). Finally, cultures in sugared black tea were inoculated at the rate of 1.10^5 cell mL^{-1} per population. The loading was determined using flow cytometer (BD Accuri C6, Franklin Lakes, USA) coupled with 0.1 µg mL^{-1} propidium iodide for marking non-viable cells.

One culture was used as a control and inoculated with the original set of microorganisms previously identified byTran et al, (2020d), named as kombucha (KBC) culture. In that case, inoculation was performed by adding 12% (v/v) of a 7 days kombucha in sugared black tea. The 7 days kombucha was produced from a mother culture refreshed once per month and aimed at simulating real kombucha production. Tableau H1 shows the letter codes used to identify each culture. All cultures were realised in triplicate.

Incubation of cultures were carried out in sterile 125 mL Boston flasks, at 26 °C, under open and closed conditions, to simulate the kombucha production process, including the natural carbonation after bottling. For the first phase, the flask necks were loosely covered with aluminium foil, allowing gas exchange for 7 days (these cultures were referred as D7). For the second phase, the bottles were closed with a cap, and incubated until completing 12 days (cultures referred as D12).

Tableau H1 : Letter codes indicating monocultures and cocultures.

Microoganisms	*Brettanomyces bruxellensis*	*Hanseniaspora valbyensis*	*Acetobacter indonesiensis*	Trio	KBC
Brettanomyces bruxellensis	BB	BBxHV	BBxAI	BBxHVxAI	BBxHVxAI
Hanseniaspora valbyensis	-	HV	HVxAI		+
Acetobacter indonesiensis	-	-	AI		Other microorganisms*

*including *Saccharomyces cerevisiae, Acetobacter papayae, Komagataeibacter saccharivorans* (Tran *et al.*, 2020b)

The production of samples was performed twice. The first batch was used for the quantification of proteins and amino nitrogen at days 0, 1, 3, 7, 9 and 12. The second batch was used for the qualitative analysis of proteins at endpoints of both incubation conditions (D7 and D12).

All the values were relative with the microbial population of yeasts and bacteria, determined at each point. The enumeration was done by plating serial dilutions of the samples on WL agar for the yeasts and MRS agar for the bacterium. Three technical

repetitions were performed for each biological triplicate. For chemical analyses described in part 2.2. and 2.3., cells were removed by centrifugation (3000 x g for 10 minutes at 4 °C).

2.2 Quantitative study of proteins and free amino nitrogen

2.2.1 Free amino nitrogen (FAN) determination

Free amino acids and ammonia were determined in samples collected at days 0, 1, 3, 7, 9 and 12 of fermentation. The procedure was based on the protocol published by MEBAK (2013) with some modifications. 500 µL of a 1:5 (v/v) diluted sample was mixed with 250 µL of Ninhydrin Colour Reagent (0.7 M Na_2HPO_4, 0.44 M KH_2PO_4, 28 mM ninhydrin and 17 mM fructose). The mixed was placed in a water bath at 100°C for 16 minutes. After cooling for 20 minutes at room temperature, 1.25 mL of the Dilution Solution was added (12 mM KI, 38.4% v/v ethanol). Absorbance was read at 570 nm in a spectrophotometer UV-1800 from Shimadzu (Kyoto, Japan). Calibration curves were made using glycine solutions as standards, at appropriate concentrations. The results were expressed as mg L^{-1} of glycine equivalents (GE). By dividing FAN values by total microbial population in $\log_{10}$ (CFU mL^{-1}), the unit mg mL^{-1} GE / $\log_{10}$ (CFU mL^{-1}) was used and called "relative mg mL^{-1} GE".

2.2.2 Protein determination

The protein content was determined in the same samples as before (corresponding to days 0, 1, 3, 7, 9, and 12 of fermentation). Pierce™ Coomassie (Bradford) Protein Assay Kit of Thermo Fisher Scientific™ (Rockford, USA) was used, following the Micro Test Tube Protocol. Samples were diluted to reach the described working range: 100 µL of sample were mixed with 900 µL of distilled water. Then, 1 mL of Bradford reagent from Thermo Fisher Scientific (Waltham, USA) was added, and the absorbance was read in a spectrophotometer UV-1800 from Shimadzu (Kyoto, Japan), at 595 nm in the following 5 to 10 minutes. Calibration curves were constructed using BSA standard from Thermo Fisher Scientific (Waltham, USA) in the appropriate concentrations. By dividing protein values by total microbial population in $\log_{10}$ (CFU mL^{-1}), the unit µg mL^{-1} / $\log_{10}$ (CFU mL^{-1}) was used and called "relative µg mL^{-1}".

2.3 Qualitative study of protein fractions

2.3.1 Protein purification

Protein purification was carried out by precipitation using a mixture of trichloroacetic acid (TCA) and acetone. Samples corresponding to the end of the two phases of incubation, at day 7 and day 12 of fermentation, were treated by triplicates. The protocol outlined by Niu *et al.* (2018) was followed with some minor modifications. 5 mL of sample were mixed with an ice-cold 20% TCA / acetone (w/v) solution. This mixture was placed on ice for 5 minutes. It was then centrifuged at 15000 x g for 3 minutes at 4 °C and the supernatant was discarded. The pellet was washed twice with an ice-cold 80% acetone solution, left to air-dry for 10 minutes, and resuspended in 1 mL of a solution of 10 mM NaCl. Therefore, the samples were concentrated five times. All reagents were purchased from Merck (Darmstadt, Germany).

2.3.2 Polyacrylamide Gel Electrophoresis (PAGE)

Pre-casted polyacrylamide and Tris-Glycine gels with a gradient concentration from 8 to 16% of Thermo Fisher Scientific™ (Waltham, USA) were used for electrophoresis. Protein determination was carried out on the samples according to part 2.2.2. to determine necessary dilutions for normalization of the level of protein loaded in each well of the gel. Final amount in each well ranged from 0.2-0.3µg according to recommendations (Kumar, 2018). 20 µL of each sample was mixed 1:1 (v/v) with loading buffer (6.25 mM Tris-HCl pH8.9, glycerol 1% (m/v), bromophenol blue, +/- SDS 0.2% (m/v)). This protocol was performed for native PAGE (without SDS) as well as SDS-PAGE (with of SDS in loading and migration buffers). The electrophoresis was carried out in migration buffer (25 mM Tris (hydroxymethyl)-aminomethane, 192 mM glycine, +/- 0.2% (m/v) SDS) at 35 mV for 2 hours.

2.3.3 Silver Staining

The electrophoresis gel was stained using the Pierce™ Color Silver Stain Kit of Thermo Fisher Scientific™ (Waltham, USA). Incubation procedure was applied according to manufacturer protocol for 1 mm thick gels.

2.4 Extraction of intracellular proteins of Acetobacter indonesiensis and gel electrophoresis

To have a clearer view of the origin of the proteins derived from *Acetobacter indonesiensis*, i.e., if there are intracellular or extracellular, monoculture of acetic acid bacterium in sugared black tea was carried out according to part 2.1. with some modifications. Cultures were made in triplicate and incubated for 7 days at 26°C. Bacterial density was determined by measuring absorbance at 600 nm (DO_{600nm}). The equivalent of 20 UDO of a culture were centrifugated at 10000 x g for 10 minutes at 4°C. After washing with NaCl 10 mM, cells were resuspended in 800 µL of a phosphate buffer ((NaH_2PO_4, 100 mM; NaCl, 300 mM, pH 8.0 from Merck (Darmstadt, Germany). This suspension was mixed with 0.2 g of glass beads of 70-100 µm diameter in a 2 mL tube and treated in a tissue homogenizer Precellys® Evolution from Bertin Technologies (Paris, France). A total of 4 cycles of 60 seconds at 6500 rpm were applied with a pause of 1 minute between each other where the cells were placed on ice. Finally, the cell lysate was centrifuged at 11000 x g for 10 minutes and the supernatant was kept. For gel electrophoresis, 20 µL of sample were mixed with 20 µL of loading buffer, corresponding to a quantity of 0.28-0.29 µg of proteins for the three repetitions loaded.

2.5 Statistical analysis

Statistical analyses were performed using the software R (version 4.0.5). Protein and FAN determination was performed on biological triplicates and average values were compared with one-way ANOVA. Newman–Keuls pair test was used when significant differences were detected by ANOVA ($p \leq 0.05$).

3 Results and discussion

3.1 Kinetics of FAN and proteins contents during kombucha production

3.1.1 Free amino nitrogen (FAN) variations

FAN content, represented by amino acids and ammonia, was determined for samples collected from day 0 to day 12. The average value obtained in sugared black tea before inoculation was 5.88 ± 0.33 mg L^{-1} GE, slightly lower than values previously reported

(Kocadağlı *et al.*, 2013). The microbial populations in each condition were determined and the quotient between FAN concentration and population (in logarithmic scale) was calculated. Generally, microbial population increased during exponential phase and reached stationary phase after 1 to 3 days for yeasts and after 5 to 7 days for bacteria. Population then generally maintained for the rest of the process. Figure H1 shows FAN concentrations obtained at days 0, 1, 3, 7, 9 and 12 of process.

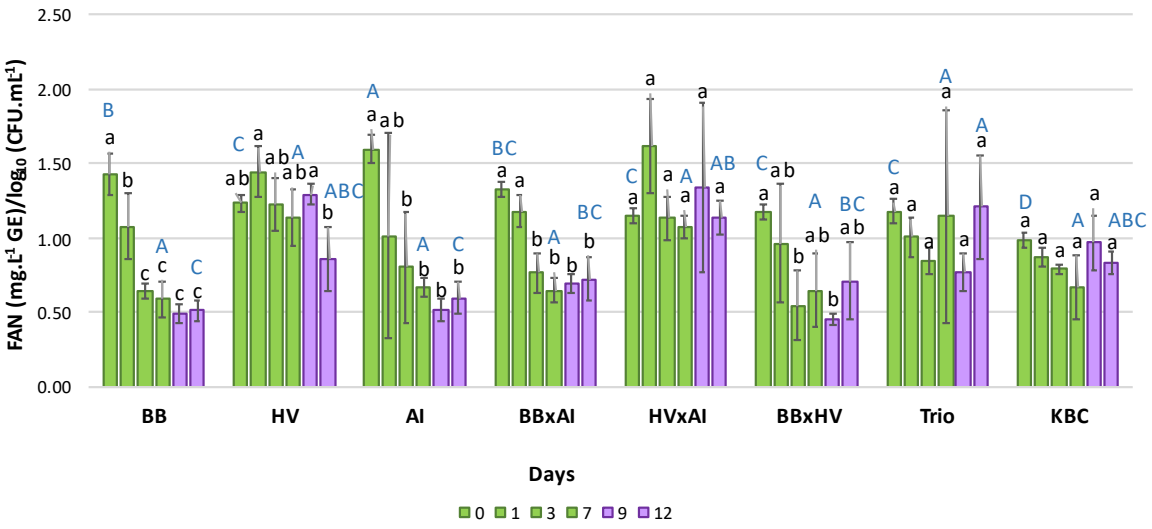

Figure H1 : Free Amino Nitrogen evolution relative to microbial population in mono and cocultures. Green and pink colours represent phase 1 (open vessel) and phase 2 (closed vessel), respectively (average values, n = 3). Error bars represent standard deviations. Black small letters indicate significant differences between the days for a given culture. Blue capital letters represent significant differences between cultures for the days 0, 7 or 12 according to ANOVA ($p < 0.05$). BB = *B. bruxellensis*; HV = *H. valbyensis*, AI = *A. indonesiensis*, Trio = coculture of all three microorganisms, KBC = kombucha.

Comparisons between the cultures at days 0, 7 and 12 are presented in Figure H1, with significant differences indicated with capital letters. At day 0, immediately after inoculation, values separated the samples into different groups. Considering that the initial level of free amino nitrogen in the tea infusion was the same for all conditions, the differences were due to the different population levels at the inoculation instance (the more species present, the more total population). However, no difference could be observed at day 7 between the cultures. Independently of the initial population, at the end of the first phase (open

vessel) the microorganisms adapted their consumption in FAN. However, at the end of the second phase (closed vessel) differences were obtained. The presence of *A. indonesiensis* in the Trio coculture induced a higher value at D12 compared to BBxHV despite the presence of more population, thus pointing at interactive effects.

Except for the samples with no significant difference between the values (HVxAI, KBC and Trio), a general decrease in FAN concentration was observed during the process. This decrease was more pronounced for the monocultures of *B. bruxellensis* and *A. indonesiensis* (BB and AI), while variations were the smallest for the *H. valbyensis* monoculture (HV). For *B. bruxellensis* and *A. indonesiensis* monocultures, the concentration decreased from day 0 to day 3 and showed no significant variation afterwards. Values varied between 1.4 and 0.5, and 1.6 and 0.5 relative mg mL^{-1} GE for *B. bruxellensis* and *A. indonesiensis,* respectively. In the case of *H. valbyensis* monoculture, a maximum was reached at day 1 that was significantly different from the value at day 12, varying between 1.5 and 0.9 relative mg L^{-1} GE. This suggests slower kinetics of FAN consumption for this yeast. A fast initial decrease in the FAN concentration could be observed for the *B. bruxellensis* monoculture only, with a significant difference between day 0 and day 1 (respectively 1.4 and 1.1 relative mg.L^{-1} GE).

A global FAN consumption kinetics was also observed for the cocultures BBxAI and BBxHV, with values ranging between 1.3 and 0.7, and between 1.2 and 0.7 relative mg L^{-1} GE, respectively. In contrast, the cultures HVxAI, KBC and Trio possessed stable values along the process with no significant difference. These values varied between 1.62 and 1.07, 0.99 and 0.67, 1.2 and 0.8 relative mg L^{-1} GE, respectively. For the coculture HVxAI, it can be hypothesised that the slow consumption observed for *H.valbyensis* resulted in more available FAN. The fact that KBC and Trio cultures also presented stable values suggests that the microbial consortia could induce an optimized nutrients management through microbial interactions. These interactions could include the release and the consumption of amino acids by different microorganisms resulting in a beneficial exchange. Mutualistic interactions between yeasts and bacteria have been described in previous studies. *S. cerevisiae* and *L. plantarum* were forced to co-evolve in a medium that lacked determined amino acids, such as isoleucine, alanine, valine, methionine, and leucine. After several generations, both microorganisms were able to provide the other

with the amino acids they needed, and to develop new adaptations to support each other growth (du Toit *et al.*, 2020). Through nitrogen catabolite repression - sensitive pathways, yeasts can excrete amino acids, although in small quantities, when there is no excess of nitrogen in the medium (Ponomarova *et al.*, 2017). Regarding the two phases of the process, most of the samples did not present significant changes during the second phase (closed vessel). Only the *H. valbyensis* monoculture (HV) underwent a drastic decrease in FAN concentrations, suggesting a late activation of the nitrogen metabolism. During previous kombucha elaborations, it has been observed that this yeast presented low sucrose hydrolysis and fermentative capacities, more particularly under aerobic conditions (Tran *et al.*, 2020b). Therefore, it could be possible that anaerobic conditions stimulated its activity.

3.1.2 Proteins content variations

The protein content in the culture supernatants were analysed along 12 days of process using Bradford assay (Figure H2). The values were relative according to the population in the same way as FAN analysis. The average value of proteins measured in the sugared tea before inoculation was 27.9 ± 7.2 µg mL^{-1}. Considering that the amount of tea leaves used for the preparation could be proportionally related with the level of proteins in the infusion, this value is consistent with other reports (between 100 and 470 µg mL^{-1}) (Jayabalan *et al.*, 2007; Kallel *et al.*, 2012; Sreeramulu *et al.*, 2000).

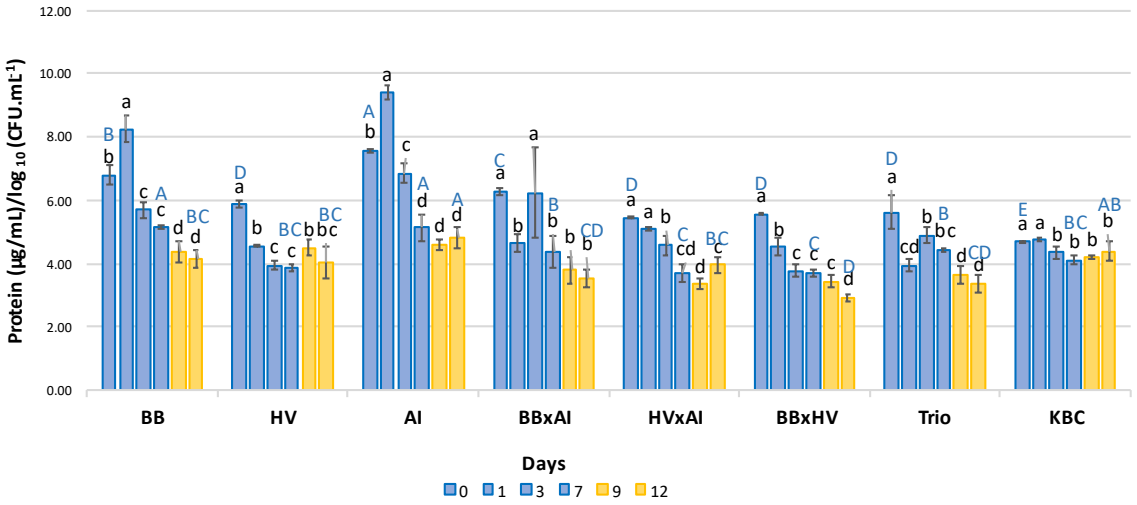

Figure H2 : Kinetic of protein content (µg mL^{-1}) evolution relative to population during the process. Monocultures (BB, HV, AI), and the cocultures (BBxAI, Trio and KBC) were followed during 7 days of open vessel fermentation (blue) and subsequent 5 days of closed vessel (yellow). Error bars represent standard deviations (average values, n = 3). Black small letters indicate significant differences inside each modality separately. Blue capital letters represent significant differences between cultures for the days 0, 7 or 12 according to ANOVA ($p < 0.05$). BB= *B. bruxellensis*; HV= *H. valbyensis*, AI = *A. indonesiensis*, Trio = coculture of all three microorganisms, KBC= kombucha.

Regarding the comparisons between the values at day 0 (indicated with capital letters in Figure H2), all of them were significantly different, which could be explained by the different population levels of each culture. At day 7, the value obtained for KBC (4.12 relative µg mL^{-1}) was not statistically different from the cocultures or the *H. valbyensis* monoculture (HV), but it was different from the *B. bruxellensis* and *A. indonesiensis* monocultures (BB and AI). At day 12, the association of *B. bruxellensis* and *H. valbyensis* induced lower protein content in coculture (BBxHV) than in monocultures (BB and HV). The same phenomenon occurred with *A. indonesiensis* regardless of the paired yeast with lower protein content in BBxAI and HVxAI than AI. Moreover, despite having a higher microbial diversity, protein content of KBC was higher those of Trio, suggesting different dynamics.

For most cultures, the proteins content was stable during the second phase after an initial decrease during the first phase. This was the case of all monocultures (BB, HV, AI), and the cocultures BBxAI, Trio and KBC. The other two cocultures (HVxAI and BBxHV) showed a different trend, with significant differences between values at day 9 and 12. For monocultures of *B. bruxellensis* and *A. indonesiensis* (BB and AI), a peak in protein content was observed at day 1, followed by a decrease. Values varied between 8.3 and 4.1, and 9.4 and 4.6 relative µg mL^{-1}, respectively. The same behavior was observed in the BBxAI coculture, with a peak at day 3 (6.2 relative µg mL^{-1}) followed by a decrease (3.6 relative µg mL^{-1}). Oppositely, the *H. valbyensis* monoculture (HV) presented a decrease in protein content during the first phase, from 5.9 to 3.9 relative µg mL^{-1}, with a subsequent increase at the beginning of the second phase (4.5 relative µg mL^{-1}) and a decrease towards the end of the process. For the coculture HVxAI, the protein content decreased from 5.5 to 3.4 relative µg mL^{-1} between days 0 and 9. The last day, a slight significant increase was observed. The coculture BBxHV and Trio showed only a decrease of protein content from 5.6 to 2.9, and from 5.6 to 3.4 relative µg mL^{-1}, respectively. It is worth noting that a different trend was observed for KBC, where the protein content slightly decreased between day 1 and 3 from 4.8 to 4.4 relative µg mL^{-1} with no further variation afterwards.

Overall, after an initial increase in the protein content for the cultures BB, AI, and BBxAI, no protein release was observed along the process. The only exception involved the *H. valbyensis* monoculture, with a slight increase between days 7 and 9, and the coculture HVxAI, that showed a final increase at day 12. The values were stable for a longer time in the kombucha (KBC), with no variations from day 3 to day 12. This could indicate that the microbial management of the proteins, regarding consumption or release, was better balanced. No absolute release was observed during kombucha fermentation, in opposition with other previous studies (Jayabalan *et al.*, 2007; Kallel *et al.*, 2012).

Considering both FAN and protein kinetics, different situations could be encountered. For the monocultures of *B. bruxellensis* and *A. indonesiensis*, a decrease in FAN concentration occurred at the beginning of the process together with a peak in the protein values. This could indicate that free amino nitrogen was used by the cells for protein synthesis. A plot of FAN against protein concentrations revealed a positive correlation (R^2

= 0.96) for *B. bruxellensis* and *A. indonesiensis* monocultures between the two parameters from day 1 to 12 (Figure H3). This was not the case for the *H. valbyensis* monoculture and all cocultures with weak R^2 values (≤ 0.66) possibly due to the complexity of cocultures (Tableau H2).

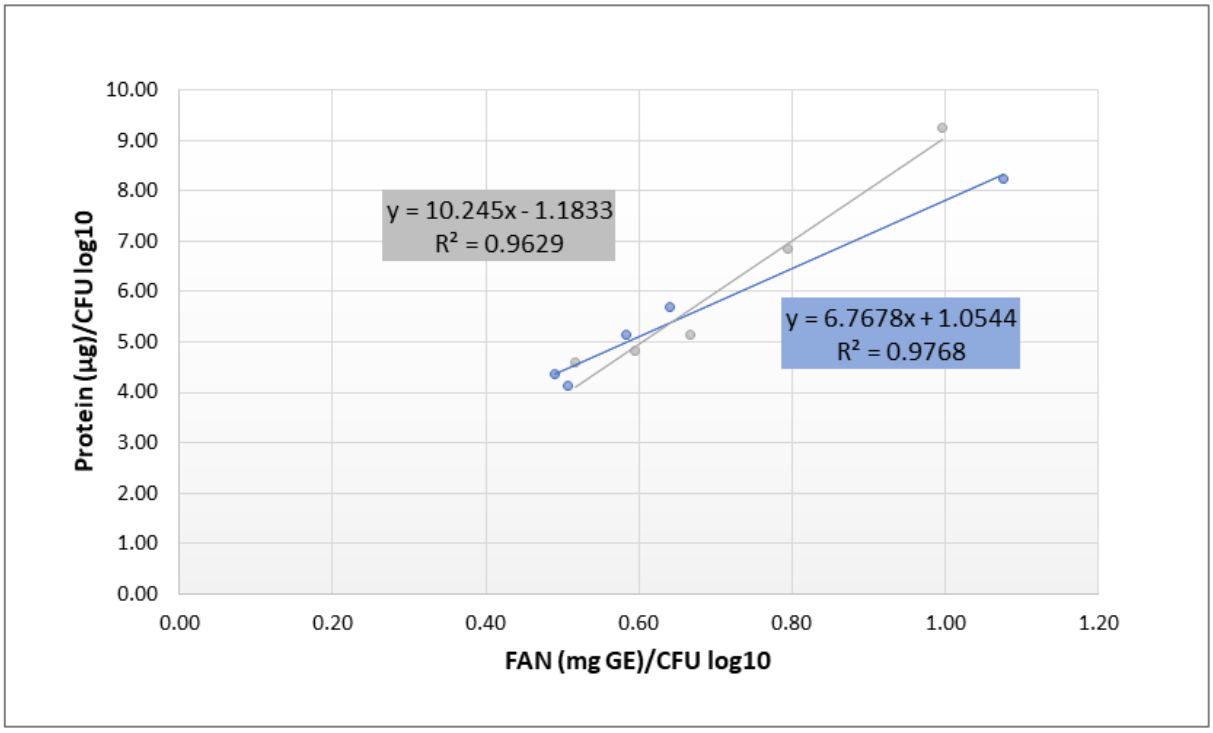

Figure H3 : Correlation between Free Amino Nitrogen (relative mg of GE) and protein (relative µg). In blue: *B. bruxellensis*; in grey: *A. indonesiensis*. Correlation coefficients (R^2 values) are indicated for each tendency line.

Tableau H2 : Coefficient of determination of plots between FAN and protein concentration. BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*, Trio = coculture of all three microorganisms, KBC = Kombucha.

Sample	HV	BBxAI	HVxAI	BBxHV	Trio	KBC
R^2	0.66	0.03	0.27	0.30	0.06	0.20

Figure H3 shows that the less FAN content, the less protein content in *B. bruxellensis* and *A. indonesiensis* monocultures. This could be explained by the fact that, as the cells have less amino acids available, they are not able to synthesize new proteins. It has been observed that when cells of *S. cerevisiae* grow in a medium rich in amino acids, they can reallocate their proteome from amino acid biosynthesis to translation of other proteins (Björkeroth *et al.*, 2020). The opposite could be occurring when there is lack of amino acids. In the case of our cultures, the lack of nutrients in the medium might not support the synthesis and excretion of protein from the cells.

In the HVxAI and Trio cocultures, proteins values decreased, and FAN values were maintained. This could indicate that proteolysis with consumption of free amino acids could be occurring. Oppositely, the coculture BBxHV was not able to increase the protein content at any time of the process, with only a consumption of free amino nitrogen. Kinetics differences in FAN and protein contents underline microbial interactions impacting the management of nitrogenous substrates and subsequent synthesis of proteins.

3.2 Qualitative study of proteins released during kombucha production

3.2.1 Analysis of proteins by (SDS)-PAGE Electrophoresis.

Gel electrophoresis under denaturing and native conditions were performed on supernatant protein extracts after TCA/acetone precipitation to eliminate possible interferences by tea polyphenols (Figure H4). Failing precipitation failing were observed for some samples. Therefore, the samples corresponding to *H. valbyensis* monoculture at D7 and D12, KBC at D7 and BBxAI and BBxHV cocultures at D12 were loaded in the gel without precipitation treatment with equivalent protein content as other samples. The darker colour of the lane (as observed in Figure H4) probably indicates the presence of polyphenols that interact with the staining, less intense for the other lanes, where they were eliminated during the precipitation.

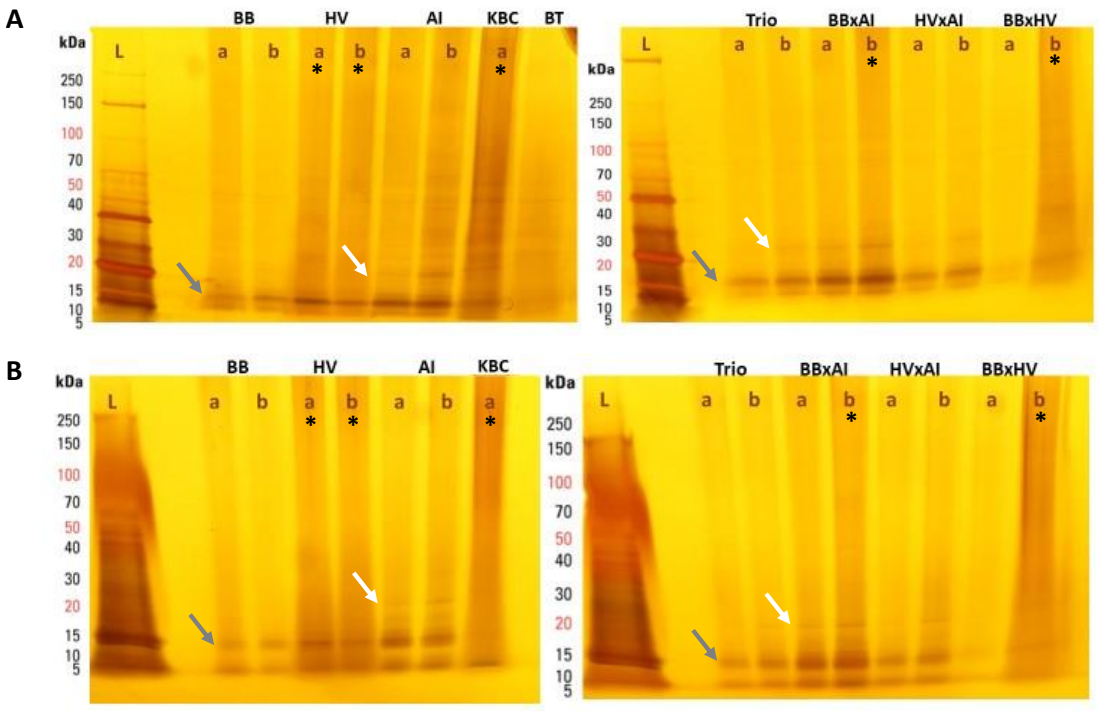

Figure H4 : Electrophoresis analysis of proteins during kombucha process in mono and cocultures. Culture supernatant harvested at day 7 (a) or day 12 (b) were precipitated and concentrated 5 times (except for the ones marked with * which are raw samples). (A) SDS-PAGE. (B) Native PAGE. L= Ladder. BB = *B. bruxellensis;* HV = *H. valbyensis;* AI = *A. indonesiensis;* KBC = Kombucha; SBT = Sugared black tea. Protein of interest of around 15 and 21 kDa are marked by grey and white arrows, respectively.

For most samples, two bands of interest were observed: one dark-coloured with an apparent molecular weight close to 15 kDa and another lighter one closer to 21 kDa. None of these bands was present in the lane corresponding to sugared black tea before inoculation. Therefore, these proteins were related to the microorganisms. The darker band (signalled by a grey arrow in Figure H4) was present in all the samples, suggesting a common protein fraction to all studied microorganisms. The other one (signalled by a white arrow in Figure H4) appeared in the of *A. indonesiensis* monoculture and was absent in the samples where this microorganism was not present, suggesting a bacterial origin of this protein. Moreover, this protein did not appear to be related to a specific phase of the process, since it was detected at the end of the first phase (D7) and at the end of the second phase (D12).

The fact that the same profiles were observed in native condition, and SDS-PAGE (denaturating condition) indicates that the proteins probably included only one subunit, and both proteins fractions (15 and 21 kDa) are likely the same in the samples where they were detected (Figure H4B).

The only proteins previously found in kombucha were characterized to have a molecular weight of around 60 kDa (Kallel *et al.*, 2012), which appeared during the elaboration process of green and black tea kombucha,. The protein fraction's molecular weight did not match the ones found in our study, but this can be explained by the use of a kombucha culture with a different microbial composition. However, it is noteworthy that in the gel electrophoresis image of Kallel *et al.* (2012), a protein around 15 kDa was observed, which could match our findings, but this data was not commented by the authors.

3.2.2 Further investigation on the origins of the 21 kDa fraction

An extra experiment was carried out to observe if there is any protein inside the cells that could match with the molecular weight of 21 kDa. *Acetobacter indonesiensis* was grown and the supernatant and cytosolic fractions were compared using SDS-PAGE. As shown in Figure H5, the profile of the proteins presented in the liquid matrix (lanes 1 and 2) and the ones corresponding to the cellular components (lanes 3 to 5) appears to be similar. Black arrows indicate a protein of around 21 kDa, and white arrows indicate a protein with a molecular weight around 15 kDa, both present in extra and intracellular fractions. This could suggest that the two proteins observed in the previous gels are part of the cytosol. In a previous study focused on the proteome of *Acetobacter pasteurianus* during oxidative fermentation, Andrés-Barrao *et al.* (2012) identified a Heat Shock Protein (GrpE) with a molecular weight of 19.6 kDa, according to its amino acid sequence. The authors found that the production of this protein, together with other chaperones, were induced by acetic acid and growth. Those two factors are encountered during kombucha production. Therefore, the same protein could potentially have been detected in this present study.

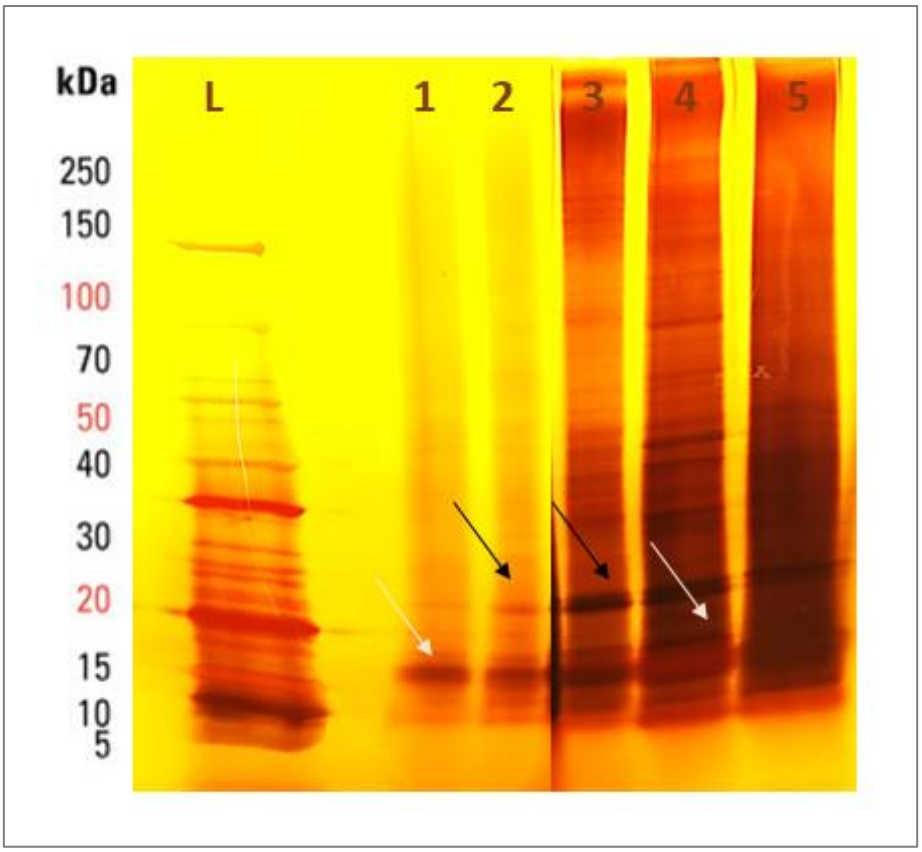

Figure H5 : SDS-PAGE protein profile of *A. indonesiensis*. Supernatant samples and crude cell extract were analysed by SDS-PAGE. Supernatant samples of *A. indonesiensis* (AI) grown in sugar tea were harvested at D7 (lane 1) or D12 (lane 2) and compared to crude cell extracts from AI grown in MRS medium (lanes 3, 4, 5 are triplicates). Black and white arrows signal a protein of around 21 kDa, and between 15kDa, respectively. L = ladder. Picture of the gel was edited to show relevant lanes only. The original image can be consulted in Annexe SH1.

4 Conclusion and prospects

Based on current scientific literature, this work presents the first investigation about the variations in FAN and proteins contents during kombucha production. The impact of microbial interactions on the consumption and release of FAN and proteins was assessed by controlling the microbial composition in different cultures.

FAN and proteins contents kinetics indicated a correlation between both parameters for *B. bruxellensis* and *A. indonesiensis*. That suggests an initial use of amino acids to produce proteins, followed by a decrease of both components in the medium. It can be

hypothesized that the microorganisms stopped releasing proteins due to deprivation of FAN, or that there was an imbalance between synthesis and degradation of proteins. The *H. valbyensis* and *A. indonesiensis* coculture was characterized by a decrease in protein concentration, as FAN (represented by free amino acids and ammonia) remained stable. These data could indicate a proteolysis linked to free amino acids consumption. The presence of *B. bruxellensis* in cocultures also underlined more competition for nutrients.

Original kombucha consortia was analysed together with the other cultures of isolated microorganisms and showed less variations in the parameters studied. If compared with the coculture Trio, closest in microbiological composition, a similar behaviour in the use of FAN could be observed. It was not the case for the proteins. So, the results obtained for original kombucha suggest a better management of the nitrogen nutrients. Indeed, harsh conditions such as low pH values due to the activity of AAB and a low amount of nutrients due to tea infusion, could force microorganisms to efficiently share the available resources. Further work could help determine if the nitrogen deprivation observed is the cause of the slow sugar consumption occurring during kombucha production (Chen and Liu, 2000; Loncar *et al.*, 2014; Sievers *et al.*, 1995).

Moreover, a protein fraction has been detected related to the cultures involving the acetic acid bacterium *Acetobacter indonesiensis*. A 15 kDa protein fraction was found in the culture supernatant which apparent size could correspond to a protein detected in the crude cell extract. According to the literature it could be a chaperon that has been detected in *A. pasteurianus* (Andrés-Barrao *et al.*, 2012). Further analyses are needed to confirm this hypothesis.

This study gives an insight in the management of nitrogenous nutrients during kombucha production by microbial communities. Deeper knowledge of microbial metabolic exchanges could help to better understand the process and achieve higher control over an up-scaled kombucha production.

I Chapitre 7 : Gestion de l'oxygène durant la production de kombucha : rôle de la matrice, de l'activité microbienne et des paramètres de production.

La gestion de l'oxygène un autre paramètre-clé pour l'optimisation de la production de kombucha car l'acidification, principalement réalisée par les bactéries acétiques, est dépendante de leur métabolisme oxydatif. Ce septième chapitre porte sur le suivi la consommation d'oxygène au cours de la fabrication de kombucha originale et dans les monocultures et cocultures de *B. bruxellensis*, *H. valbyensis* et *A. indonesiensis* à l'aide du dispositif non intrusif Nomasense®. Les mesures ont été effectuée à deux niveaux : la phase liquide et l'espace de tête. Cette étude et les résultats qui en découlent ont été soumis sous forme d'article au journal *Food Microbiology*. En ajoutant des modalités non inoculées, il a été montré que les composés extraits du thé étaient principalement responsables de la consommation de l'oxygène durant les premières 24 heures dans le liquide, avec un impact très limité de l'activité microbienne sur ce paramètre. Ainsi, durant la première phase de production de 7 jours, la phase liquide représente un environnement globalement anaérobie dont seule la surface en contact avec l'air permet l'accès à l'oxygène. Après mise en bouteille pour initier la seconde phase de prise de mousse, les cinétiques de consommation de l'oxygène diffèrent selon la composition microbiologique des cultures. Seules les cultures incluant une ou plusieurs bactéries acétiques (respectivement les cultures avec *A. indonesiensis* et la kombucha originale) consomment efficacement l'oxygène de l'espace de tête dans un intervalle de 3 à 6 heures après obturation. L'accès en oxygène limité au cours de la seconde phase stimule la production d'éthanol dans les cultures associant *B. bruxellensis* et *H. valbyensis* avec ou sans *A. indonesiensis*. Dans le cas où cette dernière est présente, la teneur en alcool est limitée.

A l'instar du chapitre précédant, cette étude met en avant le rôle déterminant de la matrice et de ses composés endogènes sur l'accès à l'oxygène dans le système, forçant potentiellement la spatialisation des micro-organismes dépendants de cette ressource. En corollaire, ceci suggère que la position du biofilm à l'interface air / liquide est une conséquence de cette spatialisation induite par l'accès à l'oxygène et non l'inverse (elle n'induit pas l'anaérobiose du liquide), comme cela a pu être avancé. Ces résultats ouvrent des perspectives concernant la gestion de l'oxygène et les technologies associées au cours du procédé de fabrication.

Oxygen management during kombucha production: roles of the matrix, microbial activity, and process parameters.

Running Title: Oxygen management in kombucha

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Abstract

Oxygen plays a key role in kombucha production, since the production of main organic acids, acetic and gluconic acids, is performed through acetic acid bacteria's oxidative metabolism. Oxygen consumption during traditional kombucha production was investigated by comparing kombucha to mono and cocultures in sugared tea of microorganisms isolated from kombucha. Two yeasts, *Brettanomyces bruxellensis* and *Hanseniaspora valbyensis* and one acetic acid bacterium *Acetobacter indonesiensis* were used. Results showed that tea compounds alone were mainly responsible for oxygen depletion during the first 24 hours following inoculation. During the first 7 days phase of production in open vessel, the liquid surface was therefore the only access to oxygen for microorganisms, as anaerobic conditions were sustained below this area. During the 5 days second phase of production after bottling, comparison of cultures with different microbial compositions showed that oxygen was efficiently depleted in the head space of

the bottles in 3 to 6 hours if the acetic acid bacterium was present. Lower access to oxygen after bottling stimulated ethanol production in *B. bruxellensis* and *H. valbyensis* cocultures with or without *A. indonesiensis*. This study provides insights into the management of oxygen and the roles of the tea and the biofilm during kombucha production.

Keywords

kombucha, oxygen, microbial dynamics, yeasts, acetic acid bacteria.

1 Introduction

During the production of fermented beverages, the management of oxygen is an important parameter and can be either needed, monitored, or must be avoided. In wine production for example, contact with oxygen must be managed to avoid oxidation of polyphenols before and after alcoholic or malolactic fermentation, since soluble oxygen is being consumed by active metabolism (Ribéreau-Gayon *et al.*, 2004). Microoxygenation can be used to bring a determined quantity of oxygen improve yeast growth, but uncontrolled aeration can lead to the development of aerobic acetic acid bacteria, which are spoilage agent in this matrix (Gomes *et al.*, 2018). Oppositely, aeration is a process used for vinegar production from wine, beer or cider by culturing acetic acid bacteria in aerated tanks (Lynch *et al.*, 2019). Traditional kombucha production can be seen as the incomplete acetification of a sugared tea infusion in static conditions (Villarreal-Soto *et al.*, 2018). A metabolic interplay between symbiotic yeasts and acetic acid bacteria occurs in kombucha cultures. Starting from a sugared tea infusion, a kombucha culture is introduced under the form of liquid suspension or a cellulosic pellicle hosting live microorganisms as well (Tran *et al.*, 2020a). Kombucha cultures do no occur spontaneously but have to be obtained from a preexisting culture (Villarreal-Soto *et al.*, 2018). During the first phase of production in open vessel, acidification of the matrix is performed by acetic acid bacteria that oxidize ethanol and glucose into acetic acid and gluconic acid respectively using oxidative metabolism. Those substrates are made available by yeasts that break down sucrose into glucose and fructose through invertase activity and release ethanol through fermentation pathway. Parallelly, a biofilm is formed at the surface of the liquid, mainly from acetic acid bacteria (Teoh *et al.*, 2004; Tran *et al.*,

2021b). A second phase occurs if natural carbonation is performed, by bottling the product in the presence of active microorganisms. At this stage, the limitation in oxygen is expected to inhibit oxidative metabolism of acetic acid bacteria and allows accumulation of ethanol and carbon dioxide since yeast alcoholic fermentation is still able to occur (Tran *et al.*, 2020b). So, as long as oxygen is accessible, ethanol content of kombucha remains low, but this is not the case after bottling if yeasts are still active. Since kombucha is typically sold as alcohol-free beverage, the control of the legal threshold of alcohol content (0.5 % (v/v) and 1.2 % (v/v) in the USA and European Union, respectively) is a major problem for the kombucha brewers (Office of the Federal Register, 1993; Official Journal of the European Union, 2011; Talebi *et al.*, 2017). The result of a controlled production is a beverage with a organoleptic profile comparable to a soda: sour, sweet and sparling (Tran *et al.*, 2020a). In case of "overfermentation", a tea vinegar is obtained (Chen and Liu, 2000).

So, oxygen appears to be a major process parameter to produce kombucha. Yet, it remains to be investigated, since, to the best of our knowledge, there are no studies on the consumption of oxygen during the production of kombucha. In our previous study, characterization of kombucha yeasts and acetic acid bacteria monocultures and cocultures in sugared tea medium was assessed and showed differences of behavior whether oxygen was available or not (Tran *et al.*, 2020b). The most striking effect could be observed for yeasts with different impacts on invertase and fermentative activities, depending on the species. For exemple, *B. bruxellensis* fermentative activity was efficient although oxygen was accessible due to Custer effect (Custer, 1940). Oxygen limitation impacted mainly the production of gluconic acid by acetic acid bacteria, but no acetic acid production. It was hypothesized that the biofilm plays a role in the access to oxygen by allowing acetic acid bacteria to maintain themselves at the surface to perform oxidative metabolism in optimal conditions (May *et al.*, 2019; Schramm and Hestrin, 1954). This sessile state in aerobic conditions was also supposed to have an impact on the yeasts dynamics by favoring species over others or allowing better conditions for higher biodiversity compared to the planktonic state in the liquid (Coton *et al.*, 2017; Teoh *et al.*, 2004). However, another function of the biofilm could be that it acts as a lid, preventing

dissolution of oxygen in the liquid phase, which would orientate yeasts metabolism towards fermentation (May *et al.*, 2019).

The present study aims at investigating how and where oxygen is available during the production of kombucha. During the first phase, is it available in the liquid or only at the surface in the biofilm? How fast is it consumed during the second phase? How does oxygen availability impact microbial dynamics and metabolism? What is the role of the biofilm regarding the microorganisms' access to oxygen? To achieve it, experimental design was based on the comparison of monocultures and cocultures in sugared tea of microorganisms isolated from one control kombucha. Both production phases were investigated by analysis of microbial dynamics using culture dependent methods and chemical analyses. Oxygen content measurement was performed in the liquid and in the headspace using a non-intrusive method.

2 Materials and Methods

2.1 Generation of kombucha, monocultures and cocultures of yeasts and acetic acid bacteria isolated from kombucha.

Sugared black tea, kombucha and cultures from microorganisms isolated from the same kombucha culture were produced according to a previous work (Tran *et al.*, 2020b) with some modifications. To produce sugared black tea, 1% (m/v) of tea was infused for one hour, then 50 g L^{-1} of sucrose was added. Black tea (Pu'er Grade 1 TN4107) from Les Jardins de Gaïa (Wittisheim, France) was used. The sugared black tea (SBT) obtained was used to produce kombucha and the cultures from isolated kombucha microorganisms. For the kombucha, 12% (v/v) of a 7 days black tea kombucha broth was added after SBT cooling. A primary inoculum was produced using the same procedure by using a mother culture obtained from the kombucha company Biomère (Paris, France) to ensure a physiological state of microorganism comparable to regular industrial scale production. The mother culture is a kombucha culture refreshed monthly with sugared black tea.

Yeasts and acetic acid bacteria strains were isolated from the broth of the black tea kombucha previously mentioned, as described in our previous study (Tran *et al.*, 2020b).

Those microorganisms were previously selected according to their representation in terms of population during the elaboration of kombucha and their functionality (Tran *et al.*, 2020b). The selection includes: *Brettanomyces bruxellensis*, *Hanseniaspora valbyensis* and *Acetobacter indonesiensis*. The different modalities of monocultures and cocultures are detailed in Tableau I1.

Tableau I1 : Description of the different cultures.

	Monocultures in sugared black tea	Yeast-Yeast coculture in sugared black tea	Yeast(s)-acetic acid bacteria cocultures (minimal consortia) in sugared black tea	Black tea kombucha (detected microorganisms in the liquid)
Microbial composition (Code)	*B. bruxellensis* (BB) *H. valbyensis* (HV) *A. indonesiensis* (AI)	*B. bruxellensis* and *H. valbyensis* (BBHV)	*B. bruxellensis* and *A. indonesiensis* (BBAI) *H. valbyensis* and *A. indonesiensis* (HVAI) *B. bruxellensis*, *H. valbyensis* and *A. indonesiensis* (T)	*B. bruxellensis, H. valbyensis, Saccharomyces cerevisiae,* and *bacteria including A. indonesiensis, Acetobacter papayae and Komagataeibacter saccharivorans* (Kombucha)

Precultures of yeasts and the acetic acid bacterium were produced by restreaking material from stocks kept at -20°C on Wallerstein Lab (WL) agar (Hall, 1971) and De Man Rogosa and Sharpe (MRS) agar respectively, with an incubation at 28°C. Yeasts and acetic acid bacterium isolates were respectively inoculated in YPD liquid medium and MRS medium. Incubation occurred at 28°C in static conditions for 2 days in sterile vessels with untight caps to allow gas exchanges. After incubation, cells were washed with sugared black tea then centrifugated (3,000 g; 10 minutes at 4°C). Each population was inoculated at the rate of 1.10^5 cell mL^{-1} in sugared black tea. The level of microorganisms was determined by flow cytometry using a BD Accuri C6 (Franklin Lakes, USA) with 0.1 µg mL^{-1} propidium iodide to evaluate non-viable cells proportion (Stiefel *et al.*, 2015).

Cultures of 123 mL occurred in triplicates in 125 mL Boston flasks with a Specific Interfacial Surface (SIS) (Cvetković *et al.*, 2008) of 0.01 cm^{-1} with bottlenecks loosely covered with tin fold to allow gas exchanges during 7 days (first phase P1) of production. Then, flasks were tightly capped for 5 more days of natural carbonation (second phase P2).

2.2 Microbiological analysis of liquid cultures and kombucha pellicles

Plate counting was performed to determine the populations of yeasts and/or acetic acid bacteria from the broth after inoculation (day 0), during P1 (days 1, 3, 5 and 7) and during P2 (days 10 and 12). Due to the absence of consistent cellulosic pellicle in cultures except for original kombucha (control), microbiology data regarding the kombucha pellicle only was included. To extract microorganisms from the pellicle, the whole 10 mm diameter biofilm was washed twice with 10 mL of sterile physiological water. Then, 10 mL sterile physiological water was added, and the pellicle was cut in bits using a sterile scalpel blade. Next, it was vortexed 3 minutes at full speed. This suspension was used as sample similarly to liquid culture samples. Successive decimal dilutions of samples were plated in triplicates on WL agar for yeasts and MRS agar for bacteria. Characterization of the yeasts on WL agar plates allowed the quantification of yeasts subpopulation according to the species based on morphological aspect, as described in our previous study (Tran *et al.*, 2020b). Identification of unknown or unclear yeast morphotype was performed as described in (Tran *et al.*, 2020b). After DNA extraction, 26S PCR was carried out using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GTCCGTGTTTCAAGACGG-3') primers (Maoura *et al.*, 2005). Amplified DNA was sequenced by Genewiz (Leipzig, Germany) according to Sanger method and identity was obtained by comparing the sequence to NBCI database. Results were expressed in CFU mL^{-1} for liquid samples and in CFU g^{-1} dry biofilm for biofilm extracts. Dry weight of biofilm was calculated at day 2, 3, 5 and 7 using gravimetric method before et after drying at 102°C for 24 hours. The pellicles were discarded in the flasks just before P2.

2.3 Chemical analyses

Chemical analyses were performed at days 0, 1, 2, 3, 5, 7, 10 and 12. Cell-less samples were obtained by centrifugation (3,000g; 15 minutes, 4°C) and kept frozen at -20°C. Total acidity was determined by titration with 0.1 N NaOH and 0.2% phenolphthalein as color indicator. Acetic and succinic acids concentrations were determined at days 7 (end of P1) and 12 (end of P2) by HPLC as described previously (Tran *et al.*, 2020b). Sucrose, glucose and fructose, gluconic acid and ethanol concentrations were determined using enzymatic kits from Biosentec (Auzeville-Tolosane, France).

Results were expressed, according to the following formula (1):

$$\Delta Concentration = \Delta Endpoint\ concentration - \Delta Initial\ concentration \quad (1)$$

“Initial” corresponds to day 0 and the “endpoint” corresponds to the day of analysis.

Measurement of oxygen in the liquid and in the headspace of the culture flasks was performed using a Nomasense O2 P300 device and compatible Pst3 captors from Vinventions (Thimister-Clermont, Belgium). This device allows the quantification of headspace or soluble oxygen in liquids. Two captors per flask were placed on the inner part of the flask: one in the middle of the main body for measurement of soluble oxygen in the liquid and one in the bottleneck above the surface of the liquid to measure the oxygen in the headspace. Signals of captors could be read using the devices optical fiber from the outside of the flask, which make this analysis method non-intrusive. The device was calibrated according to user’s manual. Pst3 captors can accurately measure oxygen content up to 22 mg L^{-1} and has a lower detection limit of 15 µg L^{-1}. Oxygen consumption was followed during the first eight hours after inoculation, then after 1, 2, 3, 5 and 7 days during P1. At P2, oxygen consumption in the headspace was followed during the first six hours following the capping of flasks, then at day 8, 10 and 12. Oxygen partial pressure in the headspace was expressed in kPa. Those measurements were performed on separate samples than the ones described in part 2.1. and included initial saturation in oxygen using preliminary air injection for 10 minutes, but they were rigorously generated in the same way. A preliminary study determined the effect of sugar and tea in the medium without inoculation during the first eight hours following saturation.

2.6 Statistical analyses

All samples were made in triplicates. Confidence intervals were calculated using Microsoft Excel for Microsoft 365. Values were treated with ANOVA and Newman-Keuls pair test was applied in the case of significant difference ($p < 0.05$) with R software (version 3.5.2.).

3 Results and discussion

3.1 Microbial dynamics

Plating allowed the quantification of yeast and bacteria population. Colony morphotyping using WL agar medium and complementary identification using 26S PCR allowed the discrimination of yeast populations according to genera and species. The microbial dynamics occurring in kombucha is described in Figure I1. In the liquid phase, total bacteria population increased from an average initial population of $3.6.10^4 \pm 1.8.10^3$ CFU mL^{-1} to a maximum population of $9.2.10^5 \pm 4.3.10^5$ CFU mL^{-1} reached on day 7 (Figure I1A). A similar behavior was observed for the yeast *B. bruxellensis* with an increase from initial population of $5.2.10^5 \pm 1.8.10^4$ CFU mL^{-1} to a maximum of $1.1.10^7 \pm 3.4.10^5$ CFU mL^{-1}. *H. valbyensis* population reached its maximum in a shorter time from initial population of $3.5.10^5 \pm 8.8.10^3$ CFU mL^{-1} to maximum of $7.9.10^6 \pm 1.2.10^6$ CFU mL^{-1} at day 3. No significant difference was detected before day 12 with a significant decrease to $3.2.10^6 \pm 1.4.10^6$ CFU mL^{-1}. A similar behavior was observed in our previous study (Tran *et al.*, 2020b). It is noteworthy that in the liquid phase, yeasts population dominated the bacterial one with *B. bruxellensis* as dominant species. In contrast, bacteria dominated yeasts in terms of population in the biofilm from day 3. Starting from above $8.6.10^6$ CFU g^{-1} dry biofilm at day 2 at the time of biofilm apparition, the population in yeasts and bacteria followed similar variations with a significant increase at day 3 ($1.1.10^9 \pm 2.4.10^8$ and $4.3.10^9 \pm 3.2.10^8$ CFU g^{-1} respectively), followed by significant decrease to $2.1.10^8 \pm 7.4.10^7$ and $4.3.10^8 \pm 3.2.10^8$ CFU g^{-1}, respectively (Figure I1B). No further variation was observed at day 7 before the biofilm was discarded with the end of P1. The growth of the biofilm in terms of dry weights showed a significant increase only between day 3 and day 5 from 1.71 ± 0.4 mg to 11.8 ± 5.4 mg. Consequently, the decrease in total microbial population per dry weight of biofilm between day 3 and day 5 can be explained by the production of exopolymers, mainly bacterial cellulose (Betlej *et al.*, 2020), and not by a microbial mortality. Yeasts dynamics in the biofilm involved the two species identified in the liquid phase (*B. bruxellensis* and *H. valbyensis*) and *Pichia occidentalis* (Figure I1C). This species was characterized by filamentous colonies and pear-shapes cells when not filamentous (pseudo-hyphae; data not shown). This yeast was the main species in terms of yeasts population in the biofilm, with a maximum absolute population at day 3 when

total microbial population peaked. No other significant variation in yeasts populations was detected in the biofilm. In conclusion, microbial dynamics between the liquid and the biofilm were distinct for kombucha, both between yeasts and bacteria and between yeast species in terms of dominance.

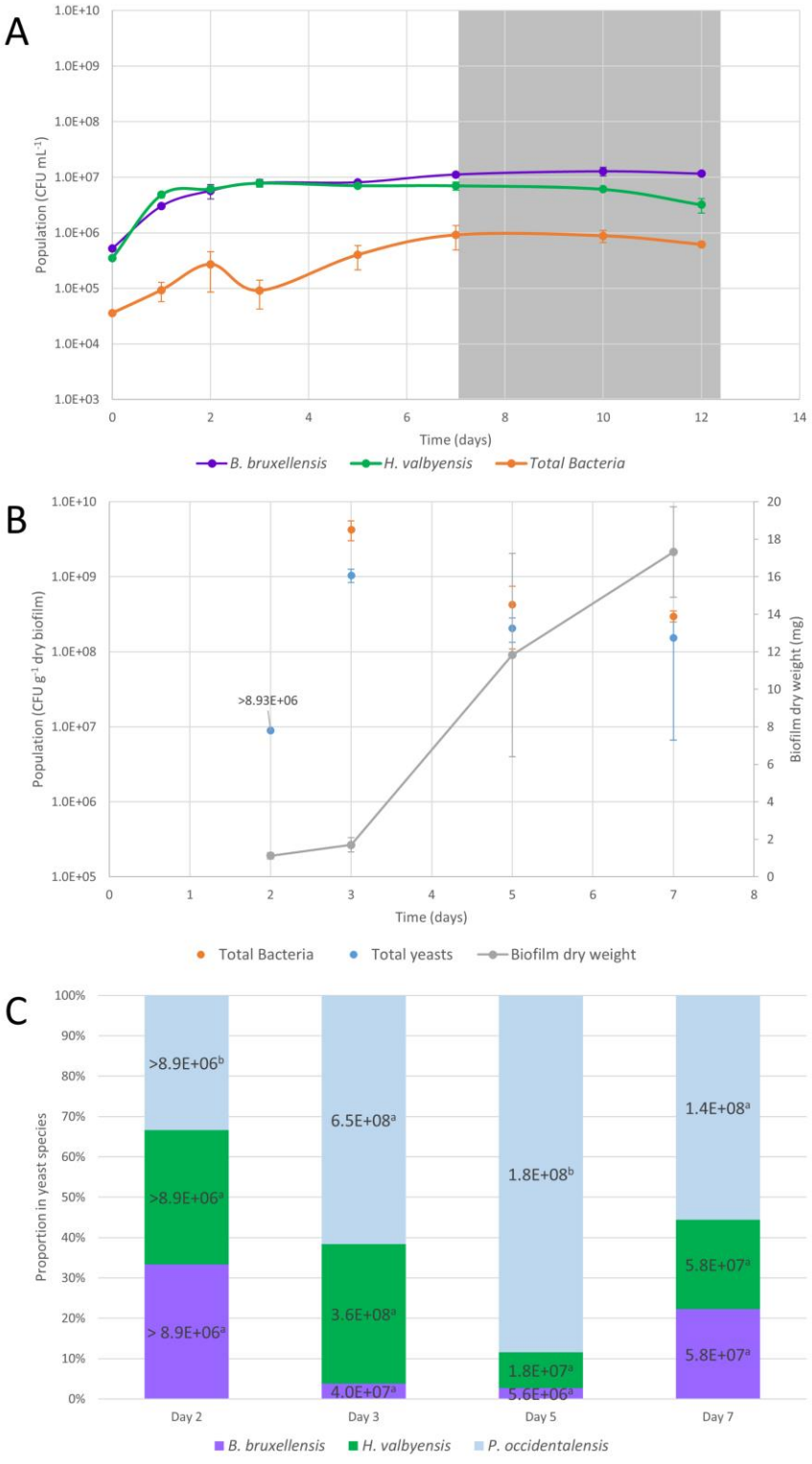

Figure I1 : Microbial dynamics of kombucha production (A) in the broth, (B) in the biofilm with (C) focus on the yeast species (average values, n = 3). Curves are meant to ease the reading. The dark background corresponds to closed vessel phase. Error bars correspond to confidence intervals (α = 0.05). Common letters correspond to no significant differences between values according to ANOVA (α = 0.05).

Figure I2 shows the population dynamics of monocultures and cocultures of microorganisms isolated from the kombucha culture mentioned before. Growth is shown per species in the different conditions and the effect of interaction are assessed by using the monoculture as a control. The kinetics of *B. bruxellensis* were similar in the monoculture and in the cocultures including *A. indonesiensis* (BBAI and T; Figure I2A). Maximum population was reached between day 4 and day 6 for the cocultures ($5.3.10^6 \pm 5.1.10^5$ and $4.2.10^6 \pm 9.3.10^5$ CFU mL^{-1}, for BBAI and T respectively), and at day 7 for the monoculture ($2.3.10^6 \pm 4.5.10^5$ CFU mL^{-1}). However, the cocultures were equivalent or significantly higher in population at all points except at day 7 and day 12, which highlights a slightly better growth of *B. bruxellensis* when in presence of the acetic acid bacterium. In contrast, *B. bruxellensis* in yeasts coculture BBHV reached the stationary phase at day 1 and a maximum population at day 12 ($2.8.10^6 \pm 3.5.10^5$ CFU mL^{-1}). The presence of *H. valbyensis* without *A. indonesiensis* (BBHV) stimulated initial growth, but lowered population levels between day 3 and day 10 ($1.3.10^6 \pm 5.0.10^5$ CFU mL^{-1}). A different behavior could be observed for *H. valbyensis* (Figure I2B). Cultures including *A. indonesiensis* (HVAI and T) showed identical growth between day 0 and day 3. Then, the presence of *A. indonesiensis* alone (HVAI) induced lower population levels, except at day 12 where maximum population was achieved for any condition ($1.5.10^7 \pm 5.4.10^6$ CFU mL^{-1}). On the contrary, the simultaneous presence of *A. indonesiensis* and *B. bruxellensis* (T) induced higher or equivalent population levels. This observation is also true for the yeasts coculture (BBHV), but with an additional boost of growth between day 0 and day 2, similarly to *B. bruxellensis*. In this culture, *H. valbyensis* dominated *B. bruxellensis* from day 2 to day 12. This dominance was also observable for T from day 0 to day 3, but after codominance was observed. Regarding *A. indonesiensis*, data showed that the presence of *H. valbyensis* stimulated the growth with the maximum population of $6.4.10^7 \pm 1.3.10^7$ CFU mL^{-1} (HVAI), whereas the presence of *B. bruxellensis* (BBAI and T) induced equivalent or lower population levels (Figure I2C). In detail, *A. indonesiensis* population was equivalent in monoculture and in BBAI from day 1 to day 3, then it showed similar behavior with T from day 7 to day 10. The population in T was always lower than in monoculture. To sum up, the presence of yeasts impacted the maximum population of *A. indonesiensis*, positively or negatively depending on the yeast species. However, *B. bruxellensis* had a dominant inhibiting influence and cancelled the effect induced by

H. valbyensis. Yet, it is noticeable that T was the only culture in which no decrease in *A. indonesiensis* population was observed between day 10 and day 12. This decrease in population could be induced by the closing of the vessel during P2 and seemed to be prevented by the simultaneous presence of the two yeasts species. Difference in initial populations at day 0 might be due to known discrepancies between plate counting and flow cytometry, possibly because of viable non cultivable state (Millet and Lonvaud-Funel, 2000; Zou *et al.*, 2020). In all cases, the inoculation process was based on flow cytometry was rigorously the same for all conditions.

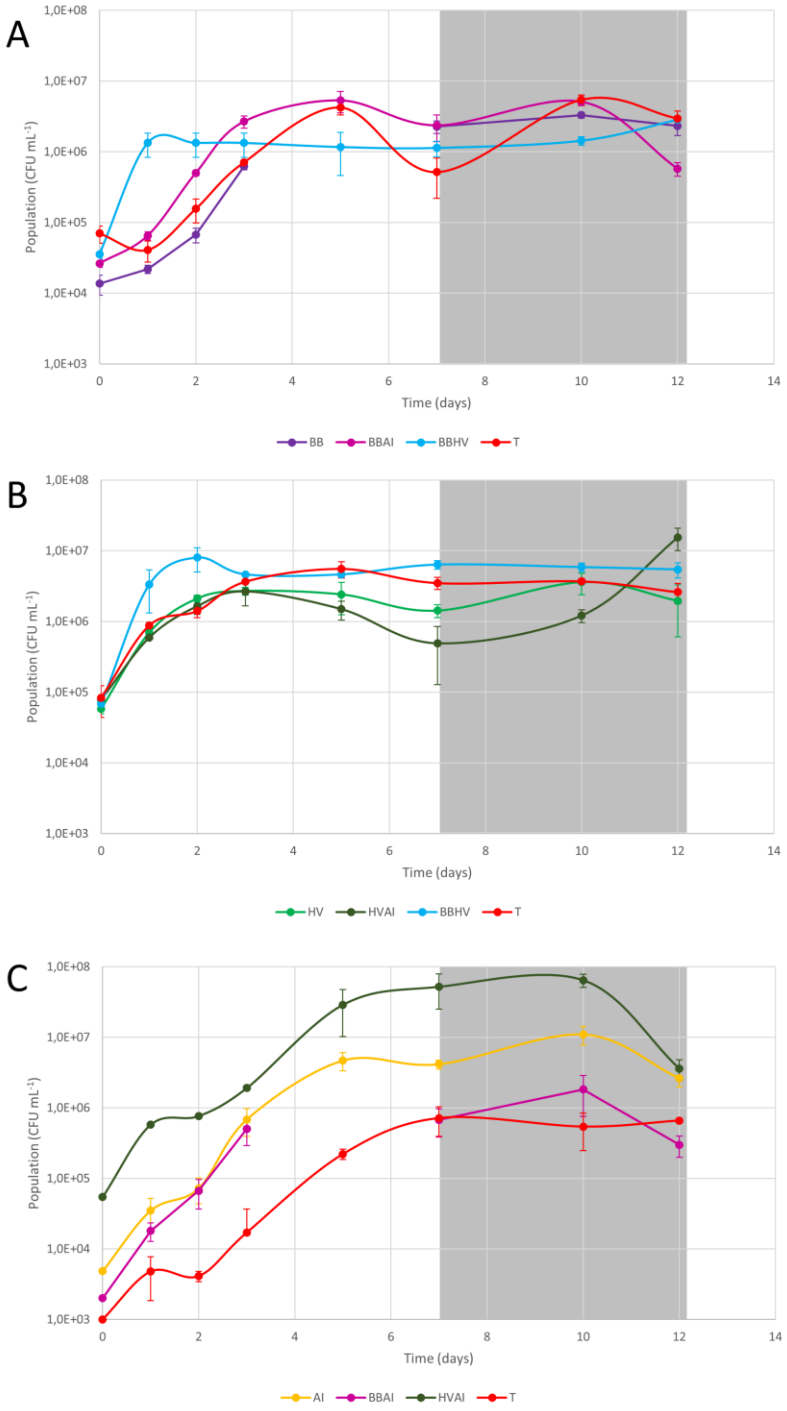

Figure I2 : Comparison of microbial dynamics per microorganism during open and closed vessel phases (average values, n = 3) for (A) *B. bruxellensis*, (B) *H. valbyensis* and (C) *A. indonesiensis* (AI) in the different cultures: BB, HV, AI, BBAI, HVAI, BBHV and T. BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis* and T corresponds to the coculture of all three microorganisms. Curves are meant to ease the reading. The dark background corresponds to the closed vessel phase. Error bars correspond to confidence intervals (α = 0.05).

In conclusion, the comparison of cultures informed on the microbial dynamics of kombucha (Figures I1 and I2). The closest microbial composition with kombucha is the T culture that exhibited similar microbial dynamics with slight differences. The main common aspect is the yeasts dominance in the liquid phase and with the codominance of the two yeasts *B. bruxellensis* and *H. valbyensis* during most of P1. At P2, the decrease of *H. valbyensis* population was observed only in kombucha. Through the lens of the present results, the yeast dominance is due to *B. bruxellensis*. Additionally, it appears that the difference of initial growth between the two yeasts species (slower for *B. bruxellensis* and faster for *H. valbyensis*) was due to the presence of *A. indonesiensis* that cancelled the boost of growth provided by a yeast-yeast interaction, bringing initial dynamics back to those of yeast monocultures. The decrease in *H. valbyensis* and *A. indonesiensis* between day 10 and day 12 seemed to be induced by the closed vessel condition, but it might also involve other microbial interaction that could not be clearly determined experimentally. Namely, a possible influence of *P. occidentalis* from the biofilm to the liquid could be supposed. The study of this yeast species was no carried out due to its non-cultivability in our conditions of analysis.

3.2 Oxygen consumption after inoculation (open vessel phase)

The concentration in soluble oxygen was followed in the cultures and kombucha's liquid during the first phase of production P1 in open vessel. Focus was made on the first eight hours following the inoculation. One measurement per day was performed at day 1, 2, 3, 5 and 7 (Tableau I2). As preliminary study, the effect of sugar and tea on oxygen concentration in the liquid was assessed. Results show that no significant oxygen consumption occurred in water and sugared water, whereas important oxygen consumption occurred in sugared black tea infusion (SBT). After an initial brutal decrease from saturation at 9.6 to 5.5 mg L^{-1} in one hour, a second more gentle consumption occurred afterward from 4.9 to 3.371 mg L^{-1} after 2 and 8 hours, respectively. After 24 hours, the oxygen concentration was below 0.1 mg L^{-1}. This first result demonstrated that tea compounds alone induced a chemical consumption of oxygen in the liquid, leading to near anaerobic conditions after 24 hours. Spontaneous oxidation of tea polyphenols was likely the main phenomenon occurring (Cardoso *et al.*, 2020). Inoculated cultures except kombucha followed a similar behavior, but significant differences could be observed.

Cocultures of *H. valbyensis* without *B. bruxellensis* (HV and HVAI) showed significantly higher oxygen concentrations than SBT, between one and five hours after inoculation. Oppositely, cocultures including both *B. bruxellensis* and *H. valbyensis* (BBHV and T) possessed lower oxygen concentrations than SBT. This highlights an effect of yeasts coculture with an increase in oxygen consumption regardless of the presence of *A. indonesiensis* (no significant differences between the two cultures). The behavior of kombucha was different from SBT and the other cultures with higher oxygen levels. This is probably due to the traditional method of inoculation involving kombucha from previous batch. The introduction of kombucha compounds might have changed oxygen solubility in the medium. Linear regression was applied to oxygen concentration curves between 2 and 8 hours after inoculation (Figure I3 and Tableau I2). Correlation coefficients (R^2) for SBT was the weakest with 0.87, whereas it was above 0.90 for all cultures. All inoculated cultures possessed greater slopes in absolute value than SBT except kombucha, with HV reaching the greatest value (-0.37). This slope was close to the one of BBHV (-0.35), which suggests the influence of *H. valbyensis*. The presence of *A. indonesiensis* implied greater slopes in absolute value for HVAI and T, compared to HV and BBHV respectively with values below -4.0. In conclusion, microbial composition and activity appeared to affect oxygen consumption kinetics during the first hours after inoculation but it followed in any case a linear model after the initial brutal drop occurring in the two first hours.

Tableau I2 : Oxygen concentration of the liquid in non-inoculated and inoculated media for 24 hours (average values, n = 3) and linear regression parameters between 2 and 8 hours. Common letters correspond to no significant differences between values of the same time of analysis according to ANOVA (α = 0.05). Bold values highlight significant difference with sugared black tea control (SBT). BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the coculture of all three microorganisms. nd = not determined.

	Time (hours)											Linear regression parameters (y = ax + b)		
in mg L^{-1}	0	1	2	3	4	5	6	7	8	24	48 to 168	a	B	R^2
Water	9.2a	8.5a	8.5a	8.5a	8.4a	8.4a	8.4a	8.4a	8.4a	nd	nd	nd	nd	nd
Sugared Water	9.3a	8.5a	8.5a	8.4a	8.4a	8.3a	8.3a	8.3a	8.3a	nd	nd	nd	nd	nd
SBT	9.6a	5.5c	4.9d	4.4d	3.8d	3.6d	3.5cd	3.4c	3.3c	0.1b	nd	-0.25	5.09	0.87
BB	9.6a	5.4c	4.6d	4.1e	3.6de	3.3de	3.0de	2.9cd	2.7cd	1.6a		-0.31	5.01	0.95
HV	9.6a	6.5b	5.7c	4.9c	4.4c	4.0c	3.7c	3.5c	3.5c	0.1b		-0.37	6.08	0.92
AI	9.6a	5.3c	4.6d	4.1e	3.6de	3.4de	3.1de	2.9cd	2.8cd	0.2b		-0.30	4.98	0.95
BBAI	9.6a	5.3c	4.6d	4.1e	3.7de	3.4de	3.1de	2.9cd	2.8cd	0.9ab		-0.30	4.98	0.95
HVAI	9.6a	6.5b	5.6c	4.9c	4.4c	3.9c	3.6cd	3.3c	3.1cd	0.1b	< 0.1	-0.42	6.19	0.96
BBHV	9.6a	5.4c	4.5d	3.8e	3.5ef	3.0ef	2.8ef	2.5de	2.4de	0.1b		-0.35	4.98	0.95
T	9.6a	5.1c	4.4d	3.9e	3.5f	2.9f	2.5f	2.1e	1.8e	0.1b		-0.44	5.20	0.99
Kombucha	9.6a	6.2b	6.1b	6.0e	6.0b	5.7b	5.6b	5.3b	5.2b	0.1b		-0.17	6.53	0.95

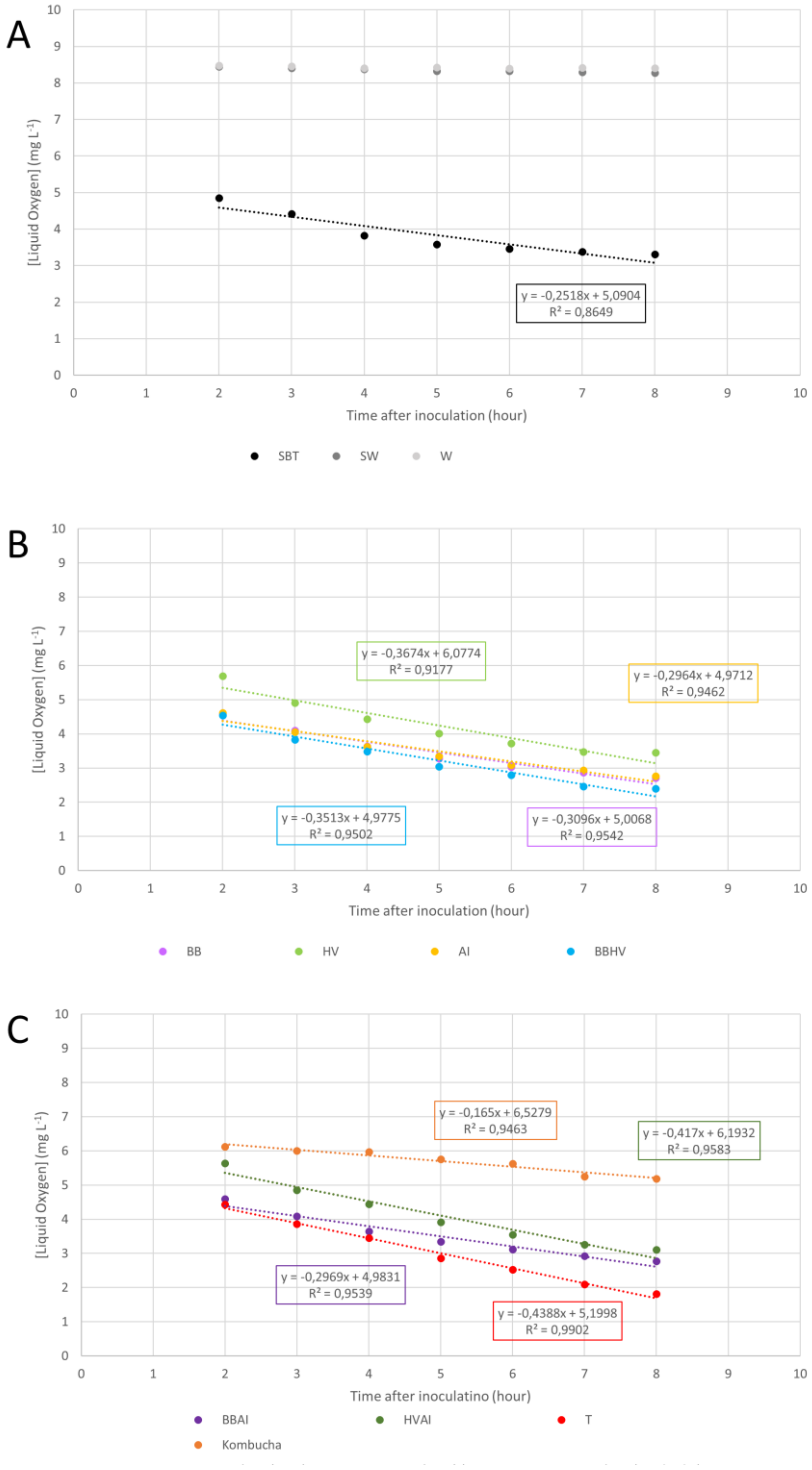

Figure I3 : Soluble oxygen consumption in the liquid for (A) non inoculated media, (B) monocultures and yeast coculture and (C) minimal consortia and kombucha cultures. Pointed lines show linear regression of the oxygen consumption kinetics with corresponding equation (y = ax + b) and correlation coefficient (R^2). SBT = sugared black tea, SW= sugared water, W = water, BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the coculture of all three microorganisms.

In general, highlighted differences had a small impact on the entire process, since after 24 hours, all culture were near anaerobiosis except *B. bruxellensis* monoculture (BB). However, from 48 hours until the end of P1, all cultures including kombucha reached anaerobiosis with oxygen concentrations below 0.1 mg L^{-1}. In conclusion, presence of microorganisms had a very limited impact on oxygen concentration of sugared black tea in the first 24 hours following inoculation. Antioxidative compounds extracted from tea appeared to induce anaerobiosis in kombucha and suggests a matrix determinism, imposing oxygen access only at the surface of the liquid. It is noteworthy that no biofilm was present in any culture at that point. Anaerobiosis induction is therefore not attributable to the biofilm, but rather to the tea compounds of the matrix.

3.3 Oxygen consumption after bottling (closed vessel phase)

All cultures achieved P1 in anaerobic conditions in the liquid phase, with oxygen access only at the surface of the liquid. Kombucha produced a consistent biofilm, whereas other cultures showed only inconsistent ones. After discarding the biofilm when present, all cultures were capped for the second phase that aims at natural carbonation in kombucha production (Tran *et al.*, 2020a). Oxygen consumption was followed in the headspace, during the six first hours following the capping of the flasks and during P2 (Tableau I3).

Tableau I3: Oxygen concentration of the headspace of cultures during the next 48 hours and after 120 hours after bottling (average values, n = 3). Common letters correspond to no significant differences between values of the same time of analysis according to ANOVA (α = 0.05). BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the coculture of all three microorganisms.

	Oxygen Partial Pressure (kPa)							
Time after capping (hours)	0	0.5	1	3	6	24	72	120
BB	21.4ab	21.5a	21.8a	20.5a	19.6a	14.3a	7.5a	1.5a
HV	21.2ab	21.8a	21.7a	20.7a	20.3a	13.1a	5.3a	1.1a
AI	21.6a	17.9b	15.1b	5.8b	0.5b	0.1b	0.1a	0.1a
BBAI	18.4c	14.9b	8.4cd	4.9b	1.3b	0.1b	0.8a	0.3a
HVAI	17.9c	15.5b	10.7c	6.2b	0.7b	0.1b	0.3a	0.3a
BBHV	20.5	22.1a	20.2a	19.0a	18.2a	14.0a	7.9a	1.4a
T	17.7c	10.5c	6.0d	1.4b	1.0b	0.1b	0.1a	0.1a
Kombucha	20.5ab	18.1b	10.3c	0.9b	0.1b	0.1b	0.2a	0.1a

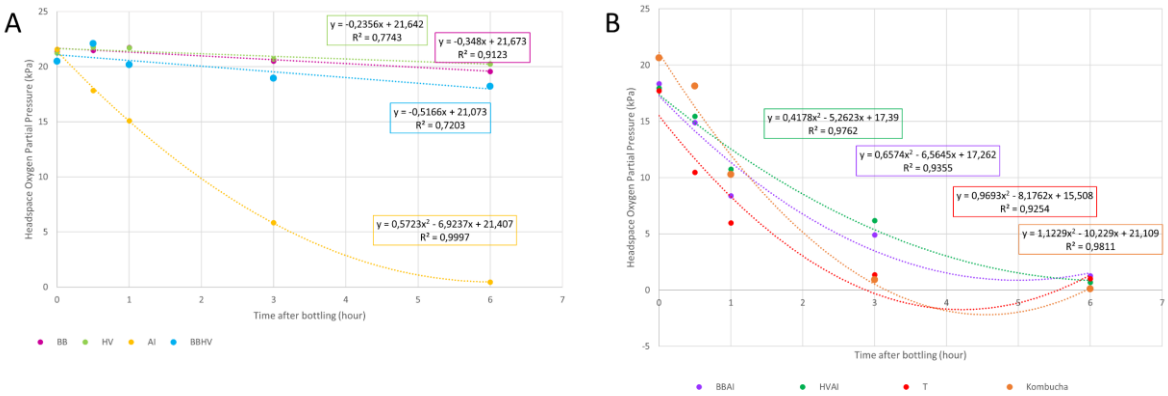

Figure I4: Oxygen consumption in the headspace for (A) monocultures and yeast coculture and (B) minimal consortia and kombucha cultures. Pointed lines and curves show regression of the oxygen consumption kinetics with corresponding equation (y = ax + b for linear regression and y = ax² + bx +c for polynomial regression) and correlation coefficient (R^2). BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the coculture of all three microorganisms.

All cultures presented a decrease in headspace oxygen during the six first hours of P2. However, different behaviors could be observed in contrast to the beginning of P1. Regression was performed on the headspace oxygen concentration curves (Figure I4). A striking difference was visible among monocultures with linear regression associated to yeasts (BB and HV) and polynomial regression to *A. indonesiensis* (Figure I4A). Yeasts coculture BBHV was also associated to a linear regression with a greater slope in absolute value (-0.51). However, HV and BBHV possessed poor correlation coefficient (0.78 and 0.72 respectively). We can hypothesize that this linear oxygen consumption consisted mainly in chemical oxidation as it was the case at the beginning of P1 (Tableau I2 and Figure I3). Nevertheless, the opposition yeast/acetic acid bacterium and linear/polynomial regression was also clearly illustrated in the yeast-acetic acid bacterium cultures and kombucha, which were all associated with polynomial regression. The fastest kinetics that induced anaerobiosis in the headspace (and thus, in the whole system) after three hours were kombucha and T. AI, BBAI and HVAI achieved this in six hours. This suggests a stimulating effect of the simultaneous presence of *B. bruxelensis* and *H. valbyensis* on *A. indonesiensis*' oxygen consumption kinetics. Consequently, the presence of acetic acid bacteria was the main driver of oxygen consumption during P2, and by extension during

P1 most probably. Moreover, the decrease in *A. indonesiensis* population observed in AI, BBAI, HVAI (Figure I2C) can be linked to the oxygen deprivation. It is remarkable that yeasts cultures (without acetic acid bacterium) did not achieve the same level of anaerobiosis during P2, with residual oxygen concentration above 1 kPa at day 12 (120 hours after capping). In conclusion, acetic acid bacteria were the main oxygen consumers during kombucha production and yeasts showed much lower consumption. Chemical analyses can bring elements about the microorganisms' metabolic activity in relationship with oxygen availability.

3.4 Effect of oxygen concentration on basic chemical parameters of kombucha production

Sucrose, glucose, and fructose variations suggested hydrolysis of sucrose and release of monosaccharides, yet no significant difference was detected between the days per sugar (Annexe SI1, SI2 and SI3).

Figure I5 shows the production kinetics of ethanol (Figure I5A) and total acidity (Figure I5B). Initial ethanol concentration at D0 was 0.15 g L^{-1}, probably due to the use of pure ethanol for the disinfection of the sieve during sugared tea preparation. Surprisingly, the production of ethanol quickly reached +1.54 g L^{-1} in only one day after inoculation for T, whereas other culture did not exceed +0.5 g L^{-1}. *A. indonesiensis* in monoculture did not produce meaningful amounts of ethanol during the process, as expect, and likely consumed the initial ethanol of the medium through oxidative metabolism (Lynch *et al.*, 2019; Tran *et al.*, 2020b) (Figure I5A). On the other hand, yeasts in monocultures produced ethanol during the process without exceeding 1 g L^{-1}. *B. bruxellensis* showed greater fermentative activity than *H. valbyensis*, which was reported in our previous study (Tran *et al.*, 2020b). This was especially true during P2 which suggests a stimulation of fermentation from oxygen exhaustion (Figure I4). This phenomenon was especially visible for the yeasts coculture BBHV with a brutal increase of ethanol production between day 7 and day 10 (from +0.11 to +3.36 g L^{-1}). Increase in ethanol production also occurred in T with a lower intensity (+0.57 g L^{-1}). This difference depending on the presence of acetic acid bacteria can be explained by their capacity to oxidize ethanol even with limited oxygen (Chouaia *et al.*, 2014; Tran *et al.*, 2020b). It is visible on the acidification kinetics

(Figure I5B). Total acidity increased in all cultures, but different behavior could be observed. Yeasts in monoculture (BB and HV) and the yeasts coculture BBHV did not show significant increase in total acidity production between day 3 and day 7. Oppositely, it was the case for cultures including *A. indonesiensis* (AI, BBAI, HVAI, T) and kombucha. As expected, acidification occurred during P1. However, as suggested by the ethanol production kinetics, acidification also took place during P2. Three groups could clearly be statistically distinguished: a low acidification group with HV and HVAI, an intermediate acidification group with AI, BB and BBHV and a higher acidification group with BBAI, T and kombucha. T reached significantly higher total acidity increase with an average value of 30.7 meq L^{-1}.

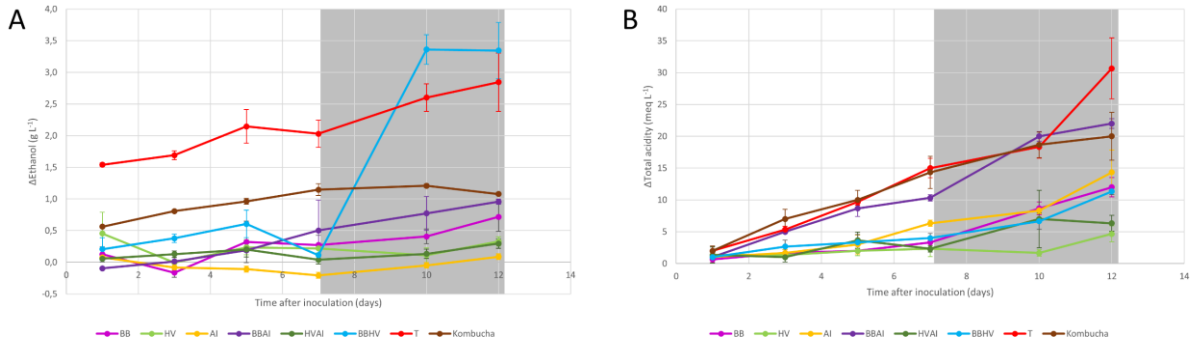

Figure I5 : Difference in (A) ethanol and (B) total acidity in cultures between day 0 and the endpoint (day 1, 3, 5, 7, 10 or 12) during the two-phase kombucha production. *B. bruxellensis* (BB), (B) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds to the coculture of all three microorganisms. Curves are meant to ease the reading. The dark background corresponds to the closed vessel phase. Error bars correspond to confidence intervals (α = 0.05).

To get more insight into the acidification process, individual organic acids were analyzed in the samples (Tableau I4). Acetic and succinic acids were detected in all cultures, whereas gluconic acid was only detected in cultures including acetic acid bacteria (AI, BBAI, HVAI, T and kombucha), as expected (Lynch *et al.*, 2019). Cultures with low acidification capacity (HV and HVAI) showed no significant increase in any of the organic acids. Indeed, in our previous study, *H. valbyensis* was characterized with low invertase and fermentative activity, which prevented the release of substrate for acetic acid bacteria and thus an efficient acidification (Tran *et al.*, 2020b). The intermediate group cultures

(BB, AI and BBHV) performed significant acidification between day 7 and day 12 through significant production of acetic acid (from no detection to +0.36 g L^{-1} for BB, from +0.46 to +0.82 for AI and from +0.14 to +0.51 g L^{-1} for BBHV). As it is expected from *A. indonesiensis* to produce acetic acid, *B. bruxellensis* is also known to produce acetic acid, particularly in aerated conditions due to Custer effect (Aguilar Uscanga *et al.*, 2003; Custer, 1940; Steensels *et al.*, 2015). *A. indonesiensis* in monoculture also produced more gluconic acid, which was also reported previously (Tran *et al.*, 2020b). Finally, the group with high acidification capacity included BBAI, T and more specifically, kombucha, with a significant increase in acetic acid between day 7 and day 12 (from +1.27 to + 1.80 g L^{-1}). This might imply a production of other organic acids during P2 for BBAI and T. Acidification during P1 in kombucha was mainly due to acetic acid production from acetic acid bacteria during P1. However, acidification from acetic acid bacteria oxidative metabolism carried on regardless of limited oxygen of the headspace, which was consumed in few hours. As stated in our previous study, production of acetic acid could occur from pathways that are not dependent on oxygen (Prust *et al.*, 2005; Tran *et al.*, 2020b). Moreover, oxygen limitation did not only stimulate fermentation but also production of organic acids that participated in the acidification during P2.

Tableau I4 : Difference in organic acids in cultures between day 0, day 7 and day 12 (average values, n = 3). *B. bruxellensis* (BB), (B) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds to the coculture of all three microorganisms. nd = not detected. Common letters correspond to no significant differences between values of the same time of analysis according to ANOVA (α = 0.05). Bold police highlight significant difference between day 7 and day 12 for a given culture.

	Acetic acid (g L^{-1})		Gluconic acid (g L^{-1})		Succinic acid (g L^{-1})	
	Day 7	Day12	Day 7	Day12	Day 7	Day12
BB	nd	**0.36cdef**	nd	nd	0.25a	0.29a
HV	0.05fg	0.17defg	nd	nd	0.02a	0.88a
AI	**0.46cde**	**0.82b**	**0.26b**	**0.35a**	0.10a	0.06a
BBAI	0.24cdefg	0.39cdef	0.10c	0.05c	0.17a	0.22a
HVAI	0.22cdefg	0.35cdef	0.07c	0.03c	0.07a	0.08a
BBHV	**0.14efg**	**0.51cd**	nd	nd	0.54a	1.05a
T	0.29cdefg	0.38cdef	0.08c	0.07c	0.34a	0.47a
Kombucha	**1.27c**	**1.80a**	0.21b	0.23b	0.93a	1.10a

To summarize, after inoculation of kombucha in sugared tea, antioxidant tea compounds (presumably polyphenols) spontaneously consumed oxygen within 24 hours (Tableau I2, Figure I3). At this point, oxygen consumption was mainly chemical since microbial population and activity were very limited in the first hours following inoculation. Once anaerobiosis was achieved, the liquid phase remained an anaerobic medium for the rest of the production process. This is expected to orientate yeasts toward fermentative metabolism and thus the release of ethanol (Figure I5). Together with monosaccharides, those compounds were converted into organic acids by acetic acid bacteria through oxidative metabolism (Tableau I4). This metabolism can be optimally performed only at the surface of the liquid, independently of the biofilm formation (Figure I1 and I2). The biofilm's function would be as a catalyser by keeping yeasts and bacteria at the same location (Tran *et al.*, 2021b), but it is not the driver of anaerobiosis in the liquid, as previously suggested (May *et al.*, 2019). So, this might explain the bacteria domination in the aerobic biofilm and the yeast domination in the anaerobic liquid. Our results suggested

that bacteria's oxidative metabolism was the main factor maintaining anaerobiosis in the liquid during P1 and the rapid consumption (few hours) of the headspace oxygen at the beginning of P2 (Figure I4), leading to complete anaerobiosis of the system. This stimulated ethanol production by yeasts and lead to ethanol accumulation due to inhibition of oxidative metabolism of acetic acid bacteria. However, this inhibition was partial and acidification from acetic acid bacteria remained active. The genomic study of acetic acid bacteria suggests that the capacity to thrive at low oxygen concentration is necessary for them to act as a symbiote in the insects' gut (Chouaia *et al.*, 2014). Our data also showed that P2 stimulated the production of organic acids by yeasts (namely acetic acid) from *B. bruxellensis*, that could contribute to the acidification during P2. At the end of P2, the decrease in population of *A. indonesiensis* and *H. valbyensis* could point at a delayed negative effect of P2 on their populations (Figure I1).

5 Conclusions

This study provides a better understanding of the fate of oxygen in the context of kombucha production. It brings useful information on the oxygen consumption of kombucha yeasts and acetic acid bacteria and their kinetics of consumption. These data can be linked to both microbial dynamics and activities and can help kombucha brewers to manage oxygen input during fermentation. In the context of traditional static production, this study highlights the importance of Specific Interfacial Surface (SIS), meaning the ratio between the liquid surface and the liquid volume in terms of access to oxygen since the liquid is an anaerobic medium except at the surface during the first phase of production. This study also confirms that the biofilm is not the origin of the liquid's anaerobiosis. The biofilm's location at the liquid/air interface is imposed by the matrix and microbial activity's oxygen consumption since it is the zone where oxidative metabolism is the most active. By extension, the matrix also sets conditions to orientate microbial dynamics and activities. Finally, more insights could be obtained about the second phase of production, which remains under-investigated and pose issues of reproducibility during production. Complementary to oxygen, the management of carbon dioxide and gas pressure would be very useful to enhance knowledge on natural carbonation of kombucha. Also, it could be interesting to determine to what extent maintaining an anaerobic environment could

protect beneficial compounds of kombucha that are sensitive to oxidation, typically tea polyphenols.

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Declaration of interest

Authors declare no conflict of interest.

Author contributions

Thierry Tran took the lead of the writing of this article, but all other authors provided critical and complementary elements to the manuscript. Thierry Tran performed microbiological and chemical analyses, as well statistical treatments. François Verdier and Antoine Martin provided the kombucha cultures used in the experiments.

J Chapitre 8 : Mise en lumière de la formation et de la structure du biofilm de kombucha à l'aide de la microscopie biphotonique.

L'article " *Shedding Light on the Formation and Structure of Kombucha Biofilm Using Two-Photon Fluorescence Microscopy*" publiée dans le journal *Frontiers in Microbiology* braque les projecteurs sur la mère de kombucha, également appelée biofilm ou SCOBY (*Symbiotic Culture of Bacteria and Yeast*).

La formation *de novo* du biofilm cellulosique de kombucha a été suivie par microscopie biphotonique durant les trois premiers jours suivant l'inoculation de thé sucré avec une phase liquide de kombucha originale. La structure ainsi formée a pu être appréciée et comparée à un biofilm formé uniquement par des bactéries acétiques isolées de la kombucha.

Ces observations ont révélé au jour 1 l'implication des levures organisées en agrégats, suggérant un potentiel rôle d'échafaudage sur lequel la cellulose s'est accumulée pour la formation du biofilm. En effet, en leur absence, la consistance du biofilm s'en est trouvée affaiblie. Cette fondation initiale aboutit au jour 2 à une structure laminaire composée d'épaisses couches cellulosiques superficielles peu riches en micro-organismes et d'une couche intermédiaire riche en biomasse. Cette dernière est suspectée constituer un site microbiologiquement actif, foyer de l'activité microbienne associée aux interactions entre micro-organismes, pouvant notamment optimiser par un rapprochement spatial les interactions levures-bactéries acétiques. Au jour 3, la structure laminaire a gagné en épaisseur en conservant les structures observées au jour 2. La couche inférieure faisant face au liquide est constituée d'un réseau cellulosique dans lequel sont retenues biomasses et cellules bactériennes viables.

Ce chapitre fait écho aux précédents en mettant en évidence un potentiel rôle du biofilm comme agent structurant permettant les interactions levures-bactéries acétiques mises en lumière dans les précédents chapitres. L'interaction entre levures et bactéries est également soulignée lors des premières étapes de formation du biofilm de par l'incorporation des cellules levuriennes dans le maillage cellulosique produit par les bactéries acétiques.

Shedding light on kombucha biofilm's formation and structure using two-photon fluorescence microscopy

Running title: Formation and structure of kombucha biofilm

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Abstract

Kombucha pellicles are often used as inoculum to produce this beverage and have become a signature feature. This cellulosic biofilm produced by acetic acid bacteria (AAB) involves yeasts, which are also part of kombucha *consortia*. The role of microbial interactions in the *de novo* formation and structure of kombucha pellicles was investigated during the three days following inoculation, using two-photon microscopy coupled with fluorescent staining. Aggregated yeasts cells appear to serve as scaffolding to which bacterial cellulose accumulates. This initial foundation leads to a layered structure characterized by a top cellulosic-rich layer and a biomass-rich sublayer. This sublayer is expected to be the microbiologically active site for cellulose production and spatial optimization of yeast-AAB metabolic interactions. Pellicles then grow in thickness while expanding their layered organization. Comparison with pellicles grown from pure AAB cultures shows differences in consistency and structure that highlighting the impact of yeasts on kombucha pellicles' structure and properties.

Keywords

Kombucha, biofilm, cellulose, interaction, two-photon fluorescence microscopy.

1 Introduction

Starting as a traditional fermented beverage, kombucha is nowadays getting increasing popularity as a bottled beverage produced at industrial scale (Kim and Adhikari, 2020). Nevertheless, the image that one generally has from kombucha is rather those of a kitchen jar containing the fermented sugared tea in which floats a macroscopic cellulosic mat that bears different names such as “pellicle”, “mother”, “tea fungus” or more recently “SCOBY” (Symbiotic Culture of Bacteria and Yeasts) (Greenwalt *et al.*, 2000; Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). Indeed, kombucha is a tea-based beverage engendered by a microbial consortium including yeasts, acetic acid bacteria (AAB), and in some cases lactic acid bacteria (Dufresne and Farnworth, 2000; Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2018). Metabolic interactions between yeasts and AAB allow sugared tea infusion to be transformed into a sparkling sour beverage, similar to a soda with often lower sugar content (Blanc, 1996; Chen and Liu, 2000; Jayabalan *et al.*, 2010; Tran *et al.*, 2020b). Kombucha is produced by inoculating a sugared tea infusion with a kombucha culture using its broth, the pellicle or both. The acidification of the broth occurs in an open vessel to ensure access to oxygen to microorganisms, acetic acid bacteria in particular (Tran *et al.*, 2020a) while a new pellicle forms at the surface. Sucrose is converted by yeasts into monosaccharides (glucose and fructose) and ethanol through invertase activity and alcoholic fermentation, respectively. These compounds serve as substrates for AAB, with the production of gluconic and acetic acids through oxidative metabolism (Jayabalan *et al.*, 2014; Liu *et al.*, 1996; Lynch *et al.*, 2019; Tran *et al.*, 2020b; Villarreal-Soto *et al.*, 2018). Natural carbonation can then be performed by bottling, after discarding the pellicle (Tran *et al.*, 2020b, 2020a). As previously stated, kombucha pellicle is used as a solid inoculum to be added to a fresh batch of sugared tea that could be shared and spread, hence the large diffusion of home-made kombucha across the world (Zagrabinski, 2020a, 2020b). The cellulosic structure is formed by AAB and can also be grown in pure AAB cultures (Gullo *et al.*, 2018; Iguchi *et al.*, 2000).

Komagataeibacter xylinus is the model bacteria used to elucidate the formation mechanism of cellulose by AAB. *K. xylinus* can use a range of compounds to produce cellulose including hexoses, glycerol, pyruvate, and dicarboxylic acids. These compounds are involved in gluconeogenesis leading indirectly to the cellulose precursor: uridine diphosphate glucose. Polymerization of glucose units bound by β1→4 linking using this precursor is catalyzed by cellulose synthase (Ross *et al.*, 1991). This transmembrane enzyme of 400-500 kDa can be seen as 50 to 80 pores organized spatially in row along the cell for the extrusion of cellulose. Cellulose synthase takes uridine diphosphate from the cytosol and extrudes 2-4 nm protofibrils into the extracellular medium (Chawla *et al.*, 2009; Iguchi *et al.*, 2000). Protofibrils associate into ribbon shaped microfibril of 80 x 4 nm (Chawla *et al.*, 2009). Under static conditions, as it is the case for kombucha, the result is a cellulosic mat floating at the air/liquid medium interface (Czaja *et al.*, 2004; Iguchi *et al.*, 2000). Intensive research was carried out because of the remarkable rheological properties of bacterial cellulose, which is purer than plant cellulose and can potentially be used as biocompatible material for health applications (Esa *et al.*, 2014; Iguchi *et al.*, 2000; Zhu *et al.*, 2014).

Cellulosic pellicles hosting microorganisms from kombucha and vinegar can be considered as biofilm despite their macroscopic scale. Biofilms are aggregates of microorganisms embedded in self-produced matrix of extracellular polymeric substances (Vert *et al.*, 2012), and several research works include sessile microbial structures without a solid surface in the definition of a biofilm (Alhede *et al.*, 2011; David-Vaizant and Alexandre, 2018; Zara *et al.*, 2005). These resistance forms limit the diffusion of toxins, the impact of brutal changes and extreme conditions in environmental parameters such as pH, temperature or hygrometry and facilitates the access to nutrients and microbial communication *via* quorum-sensing signalization in some bacterial species (Carlier *et al.*, 2015; Davies, 1998; Flemming *et al.*, 2016). It has been hypothesized that AAB formed floating cellulose mats to facilitate their access to oxygen as obligate aerobes and that the extracellular matrix could protect them against ultraviolet rays, other microorganisms and heavy metals (Iguchi *et al.*, 2000; Schramm and Hestrin, 1954). It has also been suggested that AAB cellulosic biofilm was the matrix supporting cellular communication as it has been observed with model bacteria (Cooper *et al.*, 2014; Gullo *et al.*, 2018).

Several of those putative functions of cellulosic biofilms are suspected to be appliable to kombucha pellicle and to play an important role in microbial interaction (May *et al.*, 2019). Very little investigation has been carried out about kombucha pellicle, especially through the lens of spatial organization and microbial interactions (Betlej *et al.*, 2020; Coton *et al.*, 2017; Goh *et al.*, 2012a; Mayser *et al.*, 1995; Sharma and Bhardwaj, 2019; Zhu *et al.*, 2014). The study of Coton *et al.* (2017) reported differences in microbial composition in proportion of genera between the liquid phase and the biofilms for yeasts and bacteria using non-culture dependent method, thus highlighting the impact of two different environments in terms of planktonic and sessile state or oxygen and nutrient access on microbial dynamics (Coton *et al.*, 2017). A recent study showed that synthetical consortia made of one yeast and one AAB species isolated from the same kombucha culture were able to perform the elaboration of kombucha-like beverages without the presence of a consistent biofilm, especially during acidification phase (Tran *et al.*, 2020b).

This pioneer study aims to investigate the relationship binding the structure of kombucha biofilm with microbial interactions and related functions, namely at the early-stage biofilm formation. Besides macroscopic observations, chemical and microbiological analyses, this study was based on two-photon microscopy. This technique is non-invasive and allows a better penetration in thick samples compared to confocal fluorescence microscopy and is therefore adapted to the study kombucha cellulosic pellicles (Helmchen and Denk, 2005). Two-photon microscopy can provide not only visual information on the structure of an object, but also about its composition when coupled with fluorescence staining. The staining of polysaccharides and nucleic acids was used to visualize the cellulose structure and cells according to their physiological state.

2 Article types

Original Research

3 Materials and Methods

3.1 Generation of kombucha pellicle samples

The production of sugared black tea and traditional kombucha was carried out according to previous work (Tran *et al.*, 2020b). Briefly, after steeping 1% (m/v) of black tea, 60 g L^{-1} of sucrose was dissolved and left to cool before inoculation with 12% (v/v) of 14 days old black tea kombucha broth only, no pellicle was added. So, *de novo* formation of a pellicle could be investigated. To ensure a physiological state of microorganism comparable to regular industrial scale production, a primary inoculum was produced using the same procedure, except that it was inoculated using a mother culture. The mother culture is a kombucha culture provided by Biomère (Paris, France) that was refreshed monthly with sugared black tea. All cultures were carried out in triplicate in 123 mL Boston flasks with a Specific Interfacial Surface (SIS) of 0.01 cm^{-1}. Bottlenecks were loosely covered with tin fold to allow gas exchanges. Incubation time was 3 days at 26°C in static conditions. The observation of the very first three days of biofilm formation was chosen to ensure low biofilm thickness and focus on the early formation steps.

3.2 Generation of pellicle samples from pure acetic acid bacteria culture

To investigate the role of yeasts in the formation and structure of pellicles, kombucha biofilms were compared with pure AAB biofilm in sugared black tea. Modified sugared black tea was inoculated with two strains of AAB isolated from black tea kombucha with sucrose being substituted with 1:1 amount of glucose and fructose. *Acetobacter indonesiensis* and *Komagataeibacter saccharivorans* isolated from kombucha (Tran *et al.*, 2020b) were inoculated at the rate of 1.10^5 CFU mL^{-1} from a 3 days preculture in liquid De Man Rogosa and Sharpe (MRS) medium at 28°C using the same procedure as in previous work (Tran *et al.*, 2020b). Cultivation then occurred in the same conditions as kombucha samples but with an extended incubation duration 14 days, time required under these conditions for pellicle production. Consequently, the comparison will focus on the biofilm's structure at endpoint and purposely avoid any dependence of AAB regarding yeast invertase activity by having monosaccharides available in the medium.

3.3 Macroscopic observation and chemical analyses

Macroscopic observation of pellicles was made from the time of inoculation (D0) and during the next three days of production (D1, D2 and D3) for kombucha, and after 14 days for pure AAB cultures.

Variations of fresh pellicle weight was determined with biological triplicates that were sacrificed for each measurement. Determination of dry weight content was also made for each measurement point in triplicates using gravimetric methods after treating the samples at 102°C in oven during 24h.

Samples were kept frozen at -20°C and were centrifuged prior to chemical analyses (3000g, 15 minutes, 10°C). After thawing, pH was determined using a Mettler Toledo Five Easy pH meter coupled with a LE498 probe. Total acidity was determined by titration with 1 N NaOH and 0.2% phenolphthalein as color indicator (OIV, 2009).

3.4 Microbiological analyses of kombucha

Microbial composition of kombucha culture was performed by isolating 12 yeast colonies per morphotype and eight bacteria colonies were isolated on Wallerstein Lab (WL) agar medium (Green and Gray, 1951) and MRS agar medium (pH 6.2) respectively. WL agar allows the discrimination of yeasts according to the shape and color of the colonies (Pallmann *et al.*, 2001). Isolates were restreaked on appropriate agar and incubated at 28°C during 5 days for fluorescence microscopic observation and DNA extraction. DNA extraction, amplification and sequencing were performed according to a previous work (Tran *et al.*, 2020b). The 26S and 16S PCR were used to amplify DNA extracts from yeasts and bacteria respectively (Maoura *et al.*, 2005; Wang and Qian, 2009). For yeasts, the 26S rDNA region ribosomal non transcribed spacer 2 (NTS 2) was amplified using the following primers: NL1 (50-GCATATCAATAAGCGGAGGAAAAG-30) and NL4 (50-GTCCGTGTTTCAAGACGG-30) (Maoura *et al.*, 2005). For bacteria, 16S ribosomal DNA was amplified using the following primers: E517F (50-GCCAGCAGCCGCGGTAA-30) and E106R (50-CTCACGRCACGAGCTGACG-30) (Wang and Qian, 2009). Both strands Sanger sequencing was performed on amplified DNA by Genewiz® (Leipzig, Allemagne) using corresponding primers for yeasts and bacteria. The sequences were analyzed using

Geneious R7 (version 7.1.5) and NCBI's Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), thus returning genus and species names. Identities were determined based on the lowest E-value.

3.5 Fluorescence imaging microscopy

Two-photon imaging microscopy was performed to achieve 2D and 3D (z-stacks) representations of the kombucha pellicle. Images were collected on a Nikon A1-MP scanning microscope equipped with a Plan APO IR 60x objective (NA, 1.27; Water Immersion, Nikon) at a scanning speed of 1 frame s^{-1}. An IR laser (Chameleon, Coherent) was used to provide excitation at 800 nm. Fluorescence emission was collected on four detection channels: FF01-492/SP (400-492 nm), FF03-525/50 (500-550 nm), FF01-575/25 (563-588 nm) and FF01-629/56 (601-657 nm) (Semrock). Images provided in the manuscript are obtained by merging these four detection channels without any other spectral selection. All images were processed the same way to optimize contrast between channels.

Pellicles were simply withdrawn from the liquid surface using pliers and placed between glass slide and coverslip after labelling. Pellicle fragments at D1 were withdrawn at the surface of the liquid using an enlarged pipet tip. A drop of 1 µL of each fluorescent label was deposed directly on the pellicle. Cellulose was labelled using 1 mM calcofluor from Merck (Darmstadt, Germany) (Haigler *et al.*, 1980). Yeast and bacteria were labeled using propidium iodide (PI) and SYTO9 (green fluorescent nucleic acid stain) simultaneously, a widely used mix for cell viability assay (Kirchhoff and Cypionka, 2017; Stiefel *et al.*, 2015). Nucleic acids of viable cells were stained using 5 mM SYTO9 from Thermo Fischer Scientific (Carlsbad, USA) which possesses a green fluorescence (emission maximum at 503 nm). Nucleic acids in dead or damaged cells and in extracellular medium were stained using 0.2 µM propidium iodide in distilled water from Merck (Darmstadt, Germany). Propidium iodide possesses a stronger affinity to nucleic acids than SYTO9 and possesses a red fluorescence (emission maximum at 636 nm). Unspecific fluorescent labelling was made using fluorescein from Merck (Darmstadt, Germany) that is used at D1 to strengthen the visualization of cells at a concentration of 1 nM and possesses a green fluorescence, therefore, it was not used in combination with SYTO9. Different

control tests have been carried out. No autofluorescence appeared on the images made with the microscope settings detailed above. Individual labelling of each fluorochromes has been tests as well as every combination of simultaneous labelling. The presence of SYTO9 aggregates has been observed and could be due to the low pH value (below 4) of the liquid phase in which the pellicle forms. It has also been observed that the addition of propidium iodide alone caused a slight outline of yeasts and this signal got stronger with increasing concentration.

4 Results

4.1 Visual and physical chemical parameters of pellicle formation during kombucha elaboration

The macroscopic aspect of kombucha pellicles was followed at its early stages of formation after inoculation with broth only, and no pellicle (Figure J1). Starting from a plain liquid suspension in sugared black tea at D0 (Figura 1-A), biofilm fragments could be observed floating at D1 (Figure J1-B). A consistent pellicle was formed at D2 and was very smooth and flexible, yet consistent and could be easily manipulated using pliers (Figure J1-C). The next and final day of the experiment, the pellicle got more rigid and looked rougher on the upper surface (Figure J1-D). Pellicles grown from pure AAB cultures in modified sugared tea were producted. Firstly, their growth rate was much longer since no floating particle could be seen before 7 days of incubation, thus preventing any comparison at D3. The present photographs were taken after 14 days of incubation (Figure J1-E and J1-F). The aspect of these pellicles was very different from the ones produced by the kombucha culture with a smooth aspect, low thickness and poor consistence, preventing any manipulation by breaking into smaller fragments. This could result from the difference in inoculation method with only addition of cells in the case of pure cultures as opposed to the addition of 12 % (v/v) of kombucha from previous batch in the other case. The resources in nitrogen, quantitatively and qualitatively, probably different and the absence of ethanol could play a role in the rate of cellulose production (Betlej *et al.*, 2020; Mamlouk and Gullo, 2013; Nguyen *et al.*, 2008). Another hypothesis would lie in the presence of microbial interactions that come with the use of a consortium.

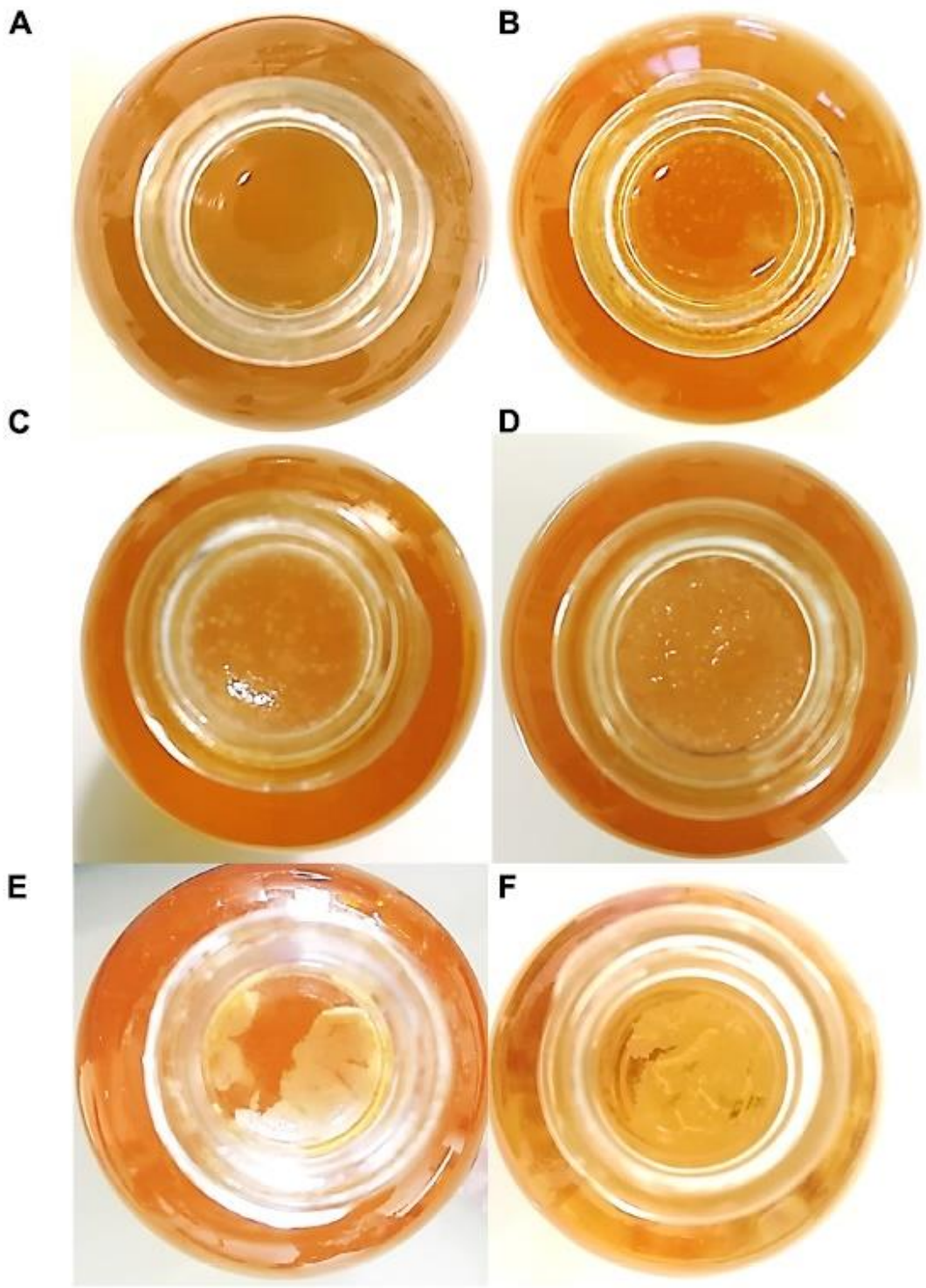

Figure J1 : Macroscopic aspect of pellicles from top view in Boston bottle. Kombucha grown pellicles in sugared tea at D0 (A), D1 (B), D2 (C), D3 (D). Pellicles grown by pure acetic acid bacteria culture in modified sugared tea at D14 (E) by *Acetobacter indonesiensis* and (F) by *Komagataeibacter saccharivorans*. Diameter of bottlenecks and pellicles is 10 mm.

During kombucha elaboration, pH value of sugared tea dropped from 6.66 to 4.27 following the addition of the inoculum (previous batch) with total acidity of 86 ± 4.3 meq L^{-1} in elaborate product (Tableau J1). pH value decreased between D0 and D3 until 3.67 ± 0.02 and total acidity increased from 7.7 ± 1.0 to 19.7 ± 1.3 meq L^{-1}, proof that organic acid production, the central process of kombucha elaboration, occurred. Fresh and dry weight increased significantly between D2 and D3 by around 3-fold, while the dry weight content did not change significantly (10 % average). This value was reported for pellicles grown by AAB and it is supposed that the dry weight rate measured is greatly influenced by microbial biomass rather than cellulose itself (Schramm and Hestrin, 1954).

Tableau J1 : Physical chemical parameters of liquid phase and pellicle during kombucha elaboration (n=3, average ± confidence interval with α = 0.05).

Sample	pH_{liquid}	Total $acidty_{liquid}$ (meq L^{-1})	Fresh $weight_{pellicle}$ per tea volume (mg L^{-1})	Dry $weight_{pellicle}$ per tea volume (mg L^{-1})	Dry $weight\ content_{pellicle}$ (%)
Sugared black tea	6.66 ± 0	< 1			
Inoculum	2.75 ± 0	86 ± 4.3			
D0 (after inoculation)	4.27 ± 0	7.7 ± 1.0		Not applicable	
D1	3.92 ± 0.07	14.7 ± 2.8			
D2 (samples 1, 2 and 3)	3.84 ± 0.06	17.7 ± 0.5	178.7 ± 115.3	16.5 ± 2.8	9.3 ± 1.6
D3 (samples 4, 5 and 6)	3.67 ± 0.02	19.7 ± 1.3	462.9 ± 446.3	51.7 ± 6.4	11.2 ± 0.4

4.2 Morphology and identification of yeasts and bacteria from kombucha consortium.

Cultures of the liquid phase samples at D0 on differential and selective agar media allowed the quantification of yeast and bacteria populations. Species identities obtained by biomolecular method were characterized according to colony morphotype and microscopic aspect (Tableau J2).

Tableau J2 : Identification and description of yeast and bacteria in the liquid phase at D0.

Microorganism	PCR type	E-value	Colony morphotype
Yeasts		0	
Brettanomyces bruxellensis	26S	0	Small white colonies appearing after the other yeast colonies (3 days after)
Hanseniaspora valbyensis	26S	0	Green colonies
Saccharomyces cerevisiae	26S	0	Large white colonies
Bacteria			
Acetobacter papayae	16S	0	Translucid colonies. Iridescent aspect when directly exposed to light

As reported previously (Tran *et al.*, 2020b), the identity of yeast colonies grown on WL agar medium were easy to determine based on their aspect and the time of apparition. This allowed the quantification of yeasts subpopulations according to the species. As an example, *B. bruxellensis* colonies appeared after several days and are small and white, whereas *H. valyensis* colonies appeared quicker and are green. This approach could not be applied to acetic acid bacteria, whose colonies had very similar aspects. The iridescence observed when light is directly projected on these colonies might be due to a diffraction phenomenon cause by the crystallinity of bacterial cellulose (Goh *et al.*, 2012a). At D0, total yeast population was larger than total bacteria population with $4.6.10^5$ and $1.6.10^5$ CFU mL^{-1} respectively. Yeasts population was dominated by the species *Hanseniaspora valbyensis* with $4.1.10^5$ CFU mL^{-1}. The second largest yeast population was *Brettanomyces bruxellensis* with 5.10^4 CFU mL^{-1} and finally the lowest population detected was *Saccharomyces cerevisiae* with $2.7.10^3$ CFU mL^{-1}. Since the pellicle formed from the inoculum sample was not added to the fresh sugared tea, those 3 yeast species were expected to be part of the newly formed biofilm together with the two species of acetic acid bacteria identified: *Acetobacter papayae* and *Gluconacetobacter takamatsuzukensis*. Focus was put on acetic acid bacteria and therefore, no anaerobic culture was carried out. Consequently, isolation of aero anaerobic lactic acid bacteria might have been overlooked.

At microscopic scale, distinct cell morphologies could be characterized from colonies grown on agar plates using two-photon microscopy and fluorescent labelling (Figure J2). Calcofluor labels polysaccharides including cellulose or cell wall compounds and PI stains nucleic acids when not blocked by non-permeable functional membranes.

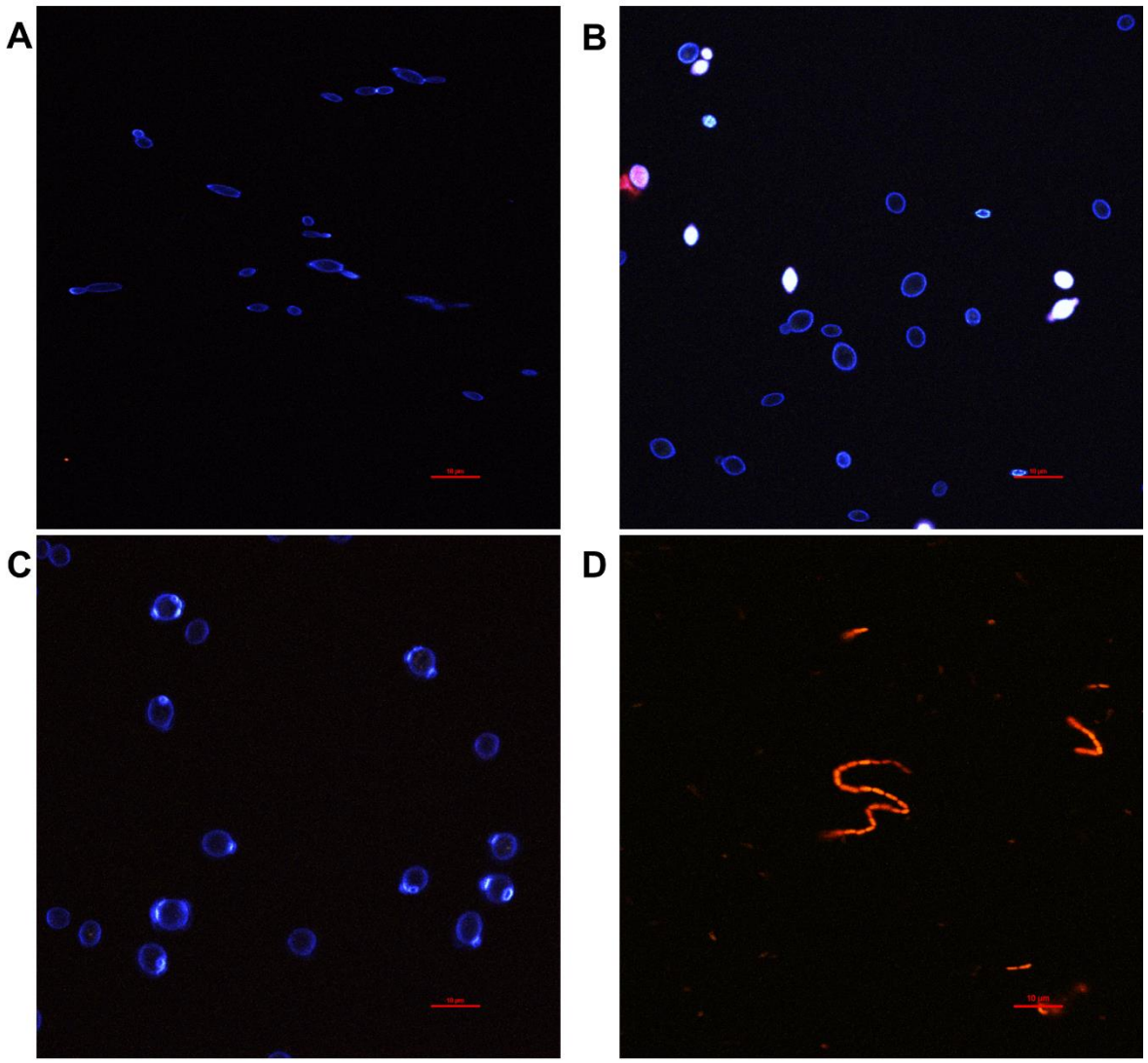

Figure J2 : Fluorescence microscopy observation of yeasts and bacteria isolates from agar plate cultures stained with calcofluor (blue), propidium iodide (red) and fluorescein (green), channels are mixed. (A) *Brettanomyces bruxellensis*, (B) *Hanseniaspora valbyensis*, (C) *Saccharomyces cerevisiae* and (D) *Acetobacter sp*.

Calcofluor staining (blue) highlighted the yeast cell walls whereas no staining could be observed for AAB (Figure J2). Instead, bacteria were fully stained with propidium iodide (PI), which is a sign of a permeable cell membrane. It should be noted that staining inconsistencies have been reported regarding the PI staining of bacteria, leading to false positive staining regarding the viability of cells (Kirchhoff and Cypionka, 2017). On the contrary, PI staining seemed to discriminate better permeable and non-permeable yeast cells with complete staining of the cytosol of permeable cells or even leaking intracellular material into the outer medium. Yeast cell morphologies were very different across species whereas it was very consistent between AAB species. Images of the latter AAB species were not displayed as they were redundant with the aspect observed for *Acetobacter sp*. *B. bruxellensis* was characterized by high polymorphism with cell size ranging between 3 and 10 µm and diverse shapes ranging from "rice grain"-like shape to elongated or round (Figure J2-A). Bud scars were also very visible. *H. valbyensis* cells were more homogenous with average cell size between 5 and 10 µm and characteristic polar budding leading to round or "lemon"-like shapes without visible bud scars (Figure J2-b). *S. cerevisiae* cells were about 7-10 µm and recognizable by their regular round shape with visible circular bud scars (Figure J2-C). Finally, AAB cells were about 1 µm long and were mostly organized by pairs although chains of more than 30 cells can be observed (Figure J2-D).

4.3 Observation of kombucha pellicle formation by fluorescence microscopy.

Due to low cell density at D0, no meaningful image could be acquired by two-photon microscopy. The floating biofilm fragments observed at D1 (Figure J1-B) could be withdrawn and observed (Figure J3). Bacteria stained by PI (red) were spotted aggregated in globular filamentous structures of cellulose stained by calcofluor (blue) (Figures J3-A and J3-D at the bottom of the image). Other structures involved what appeared to be yeasts, based on their size (Figures J3-B, J3-C, and J3-D). Cells with their cytosol stained by PI were supposedly non-viable while cells that were simply outlined by PI were supposedly viable. Yeasts cells appeared aggregated, but it is unclear whether yeasts cells were simply aggregated or if they were in pseudo-mycelium form. All around those aggregates floated smaller globular cellulose particles that appeared to accumulate at the surface of yeast aggregates, thus increasing their cohesion. The diversity in size

and shape of observed entrapped yeasts cells could point at *B. bruxellensis* or the association of different species. It can thus be hypothesized that the further aggregation process, facilitated by the entrapping action of accumulating cellulose produced by AAB, should lead to the formation of a consistent pellicle as observed at D2 (Figure J1-C).

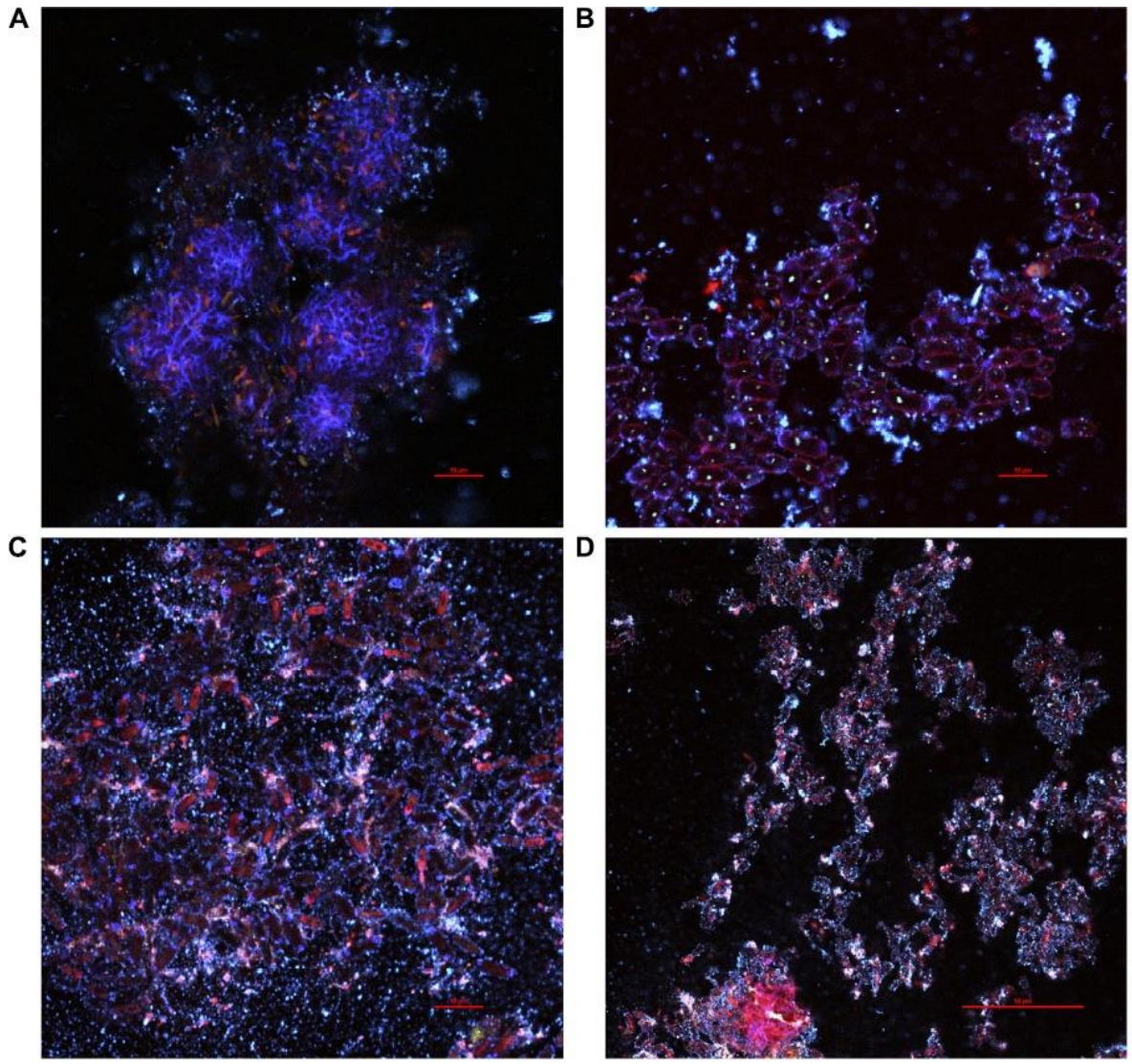

Figure J3 : Various pellicle fragments observed at D1 during kombucha elaboration sampled from the surface of the liquid phase using two-photon microscopy, featuring mainly (A) bacteria, (B, C) yeasts, or both (D). Fluorescence labeling of cellulose with calcofluor (blue) and of nucleic acids outside or inside of damaged cells with propidium iodide (red) and unspecific labeling with fluorescein (green).

The pellicles produced at D2 (samples 1,2 and 3) could be observed along their z-axis (thickness). Based on acquired z-stacks (Figures J4 and J5), the thickness of pellicles was superior to 25 µm. The penetration depth of two-photon microscopy is dependent on the material analyzed for a given laser power. The maximal penetration was obtained for the sample 1 (Figure J4). Fluorescent staining highlighted the presence of different layers: a thick top layer and a thin bottom layer with predominance of cellulose and a middle layer heavily stained by PI (Figures J4-B, J4-D, and J4-F). The surface of the top layer consisted in filamentous cellulose in which bacteria stained by SYTO9 (green) dwell (Figure J4-A). Under the immediate surface, the upper area mainly consists in cellulose organized in fibrils of different sizes (Figure J4-C). The middle layer stained by PI hosted viable bacterial cells arranged individually or in chains surrounded by biomass stained by PI responsible for the overall red fluorescence of the layer (Figure J4-E). Because of the quality of the staining and the laser power attenuation caused by the depth, it is difficult to precisely determine the nature of this biomass. However, it can be expected to be cell debris, potentially remaining of yeast cell walls.

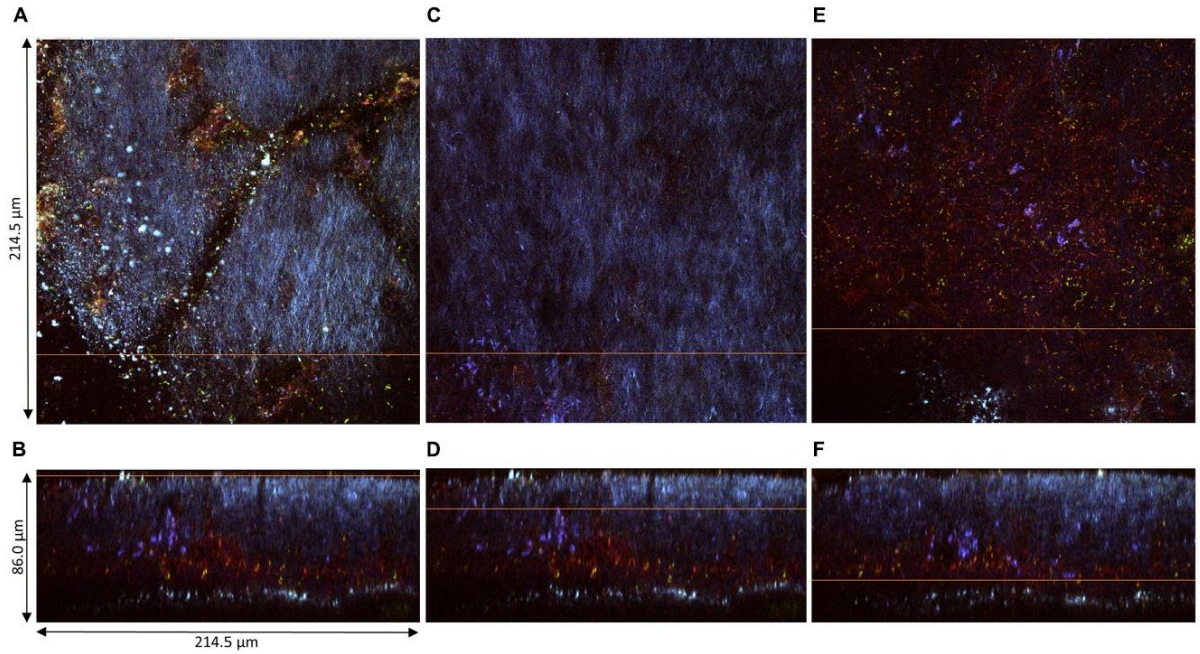

Figure J4 : Pellicle (sample 1) observed at D2 during kombucha elaboration using two-photon microscopy. Cross section of (A) top region, (C) middle region, (E) bottom region with the orange line defining the position of longitudinal section (dimensions: 214.5 x 214.5 µm). Longitudinal section (B), (D) and (F) with the orange line defining the position of corresponding cross section (214.5 x 86.0 µm). Fluorescence labelling of cellulose with calcofluor (blue), of permeable cells and external nucleic acids with propidium iodide (red) and viable cells using SYTO9 (green).

The structures of samples 2 and 3 were also organized in layers but showed dissimilarities (Figure J5). The middle PI-stained layer was thicker in sample 2 (Figure J5-B) and on the contrary was not present in sample 3 (Figure J5-D). The surface of the top layer hosted in all cases viable and non-viable cells dwelling in cellulose fibrils (Figures J5-A and J5-C). Yeast microcolonies could be seen at the bottom of Figure J5-A and Figure J5-C (white dotted circles). Non-viable yeast cells were stained with PI with whole cytosol stained and viable cells show slight nucleus stain with SYTO9. On the same plane as the yeast microcolony on Figure J5-A, a PI-stained biomass belonging to the middle layer appeared that points rather at remaining yeast cell walls rather than whole cells. The top layer surface of sample 3 exhibited numerous viable and fewer non-viable bacteria cells and unstained yeast cells (black holes) (Figure J5-C). Issues with fluorescence labelling of non-model bacteria using SYTO9 / PI combination were reported and they may also be

appliable to non-model yeasts (Stiefel *et al.*, 2015). Another limitation dwells in the difficulty to control the stains concentration at each location of the solid biofilm. Overall, the structure of pellicles at D2 seemed very variable.

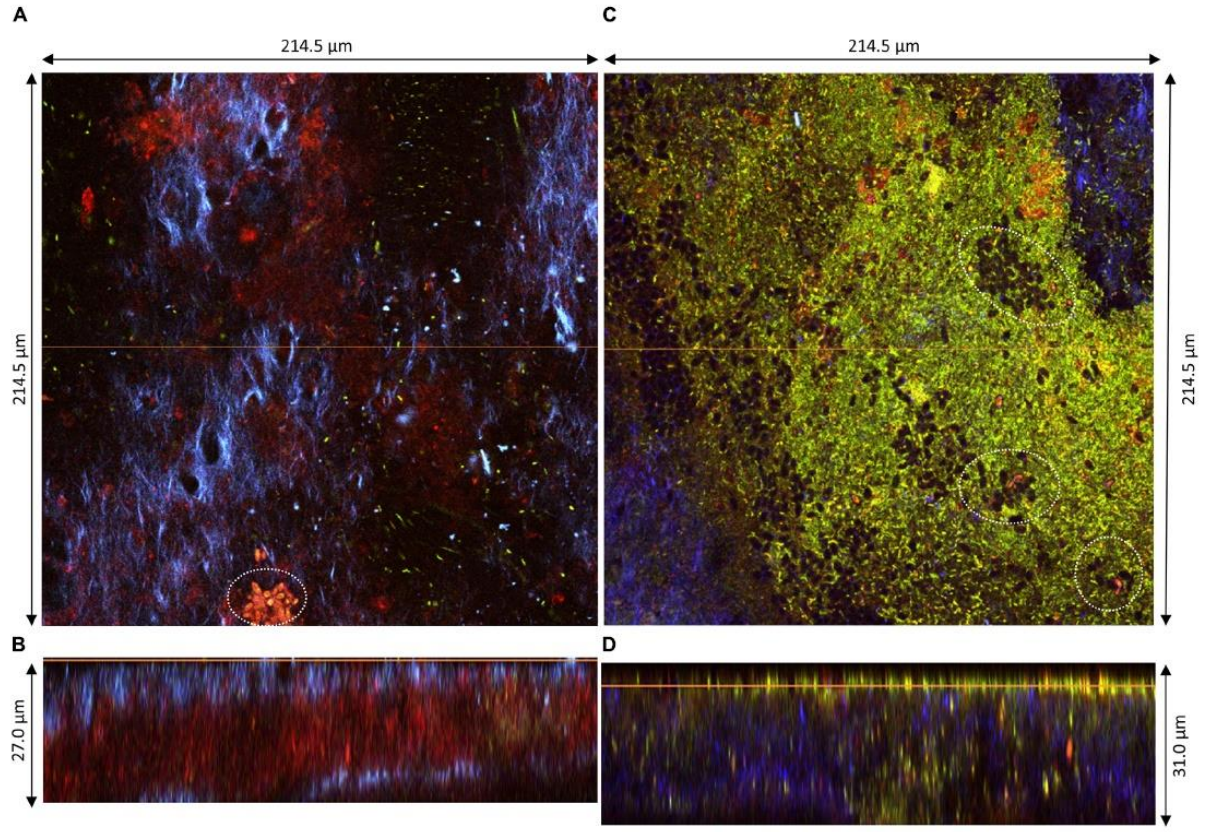

Figure J5 : Pellicles observed at D2 during kombucha elaboration using two-photon microscopy. Longitudinal sections of (A) sample 2 (214.5 x 27.0 µm) and (C) sample 3 (214.5 x 31.0 µm), with the orange line defining the position of corresponding cross section (B) and (D) respectively. Cross sections of top region of (B) sample 2 and (D) sample 3 with the orange line defining the position of longitudinal section (dimensions: 214.5 x 214.5 µm). Fluorescence labelling of cellulose with calcofluor (blue), of permeable cells and external nucleic acids with propidium iodide (red) and viable cells using SYTO9 (green). White dotted circles highlight examples of yeasts microcolonies.

The samples 4, 5 and 6 obtained at D3 were much thicker than those at D2, hence z-stacks ranging between 40 and 143 µm (Figure J6). Samples at D3 clearly conserved their layered organization with a common pattern across samples: a top layer consisting of cellulose fibrils and a sublayer stained by PI containing viable bacteria cells and

supposedly yeast debris similarly to sample 1 (Figure J3). The top layer had variable thickness from less than 20 µm (Figure J6-A) to more than 100 µm (Figure J6-B and J6-C). In all samples, viable cells could be observed at the top surface like samples at D2 (Figures J4 and J5) and unstained strata also appear inside the top layer (Figures J6-B and J6-C). Despite the good diffusion of fluorescent probes, the PI-stained sublayer appears to act as a barrier for laser penetration which could be due to its physical property. Therefore, penetration and detection of photons was strongly lowered beyond this layer. So, pellicles were without a doubt thicker than the z-stacks, hence the double acquisitions made from the top surface (Figure J6) and from the bottom surface (pellicle upside down on the glass slide) (Figure J7).

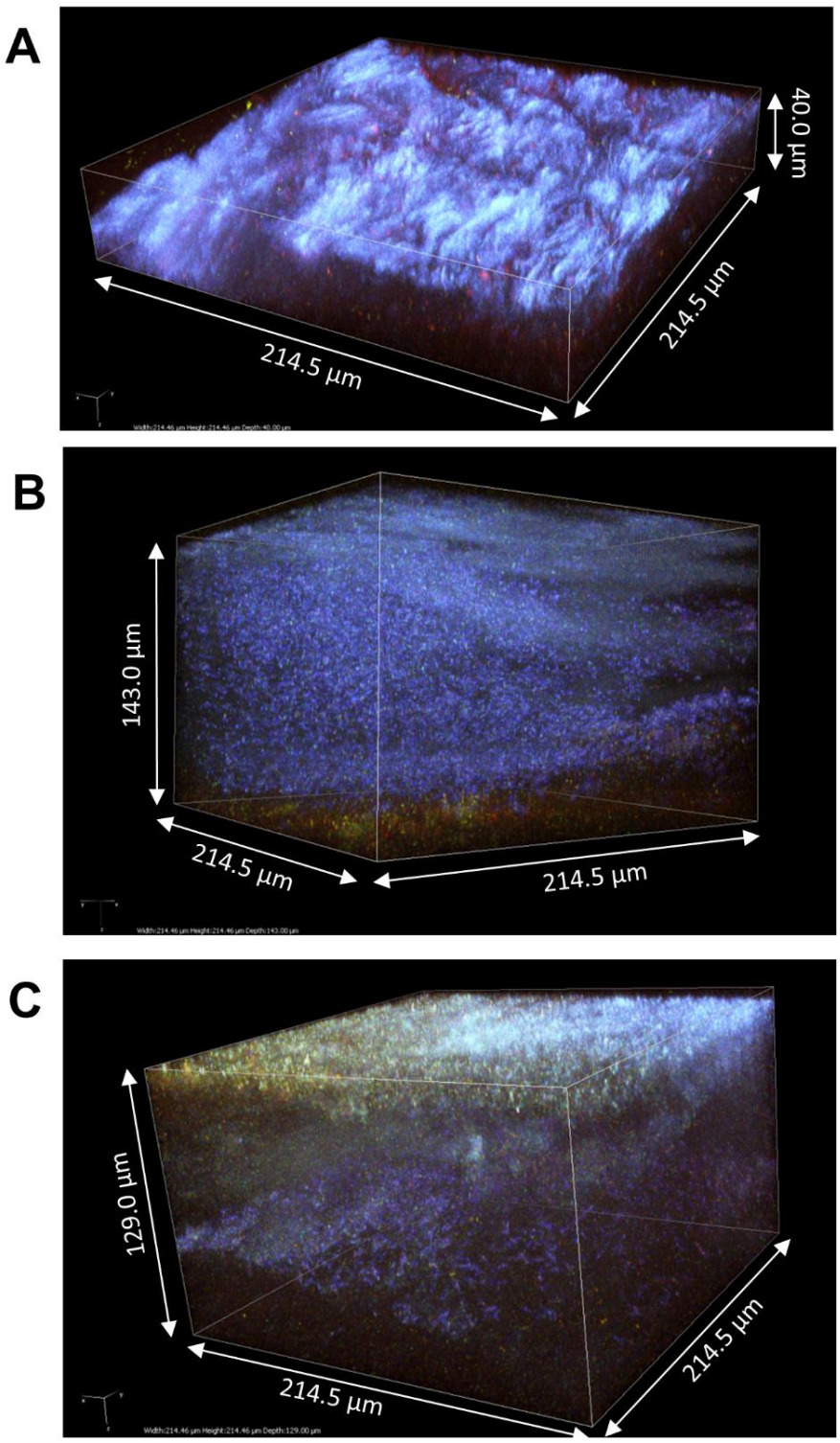

Figure J6 : 3D modelling of z-stack of upper region of pellicles observed at D3 during kombucha elaboration using two-photon microscopy, (A) sample 4 (214.5 x 214.5 x 40.0 µm), (B) sample 5 (214.5 x 214.5 x 143.0 µm) and (C) sample 6 (214.5 x 214.5 x 129.0 µm). Fluorescence labelling of cellulose with calcofluor (blue), of permeable cells and external nucleic acids with propidium iodide (red) and viable cells using SYTO9 (green).

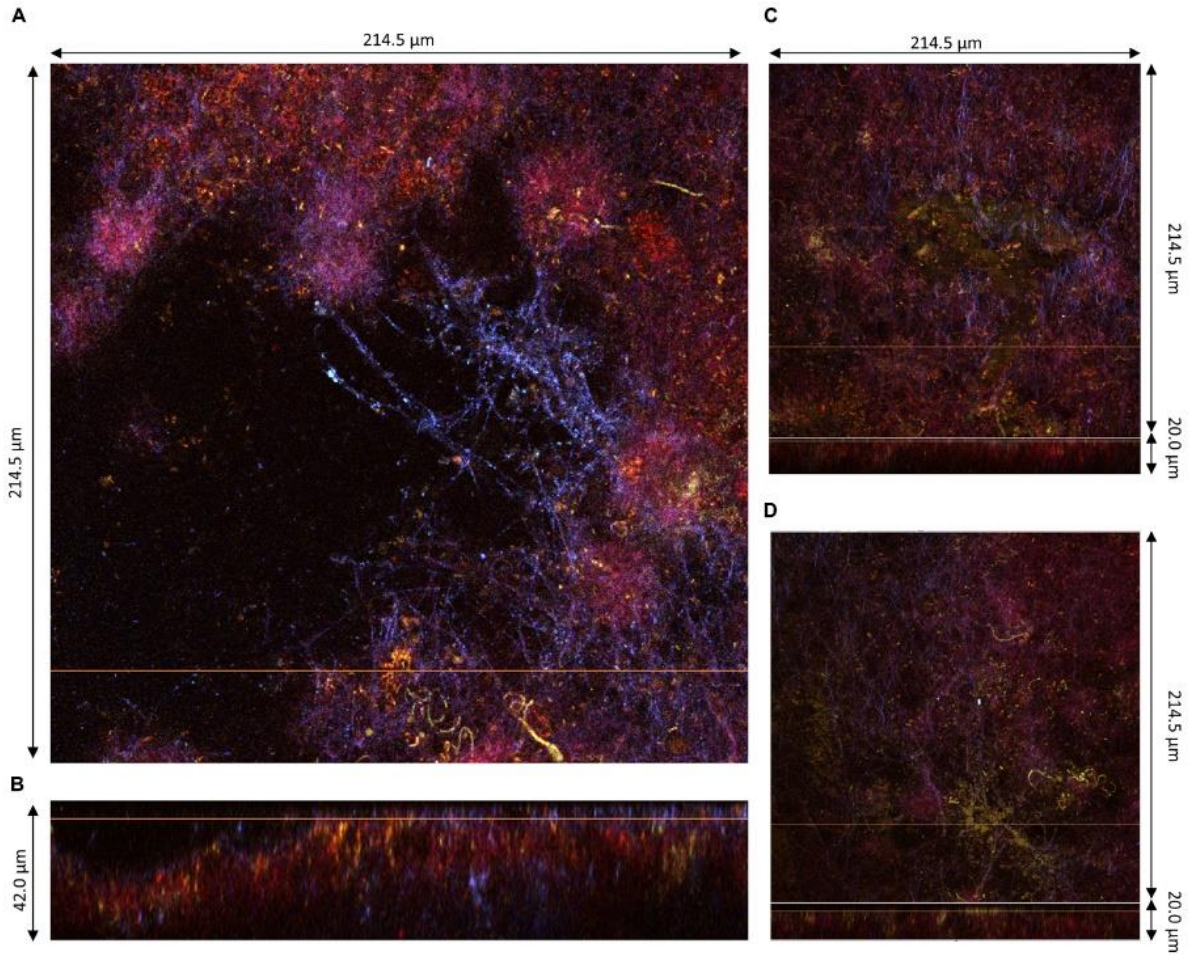

Figure J7: Lower region of pellicles observed at D3 during kombucha elaboration using two-photon microscopy. (A) Cross sections of bottom surface of sample 5 with the orange line defining the position of longitudinal section (dimensions: 214.5 x 214.5 µm). (B) Longitudinal section of sample 4 (214.5 x 42.0 µm) with bottom facing up and the orange line defining the position of corresponding cross section. (C) and (D) are assembled images of cross section on top and longitudinal sections with bottom facing up at the bottom of sample 5 (214.5 x 214.5 x 20.0) and sample 6 (214.5 x 214.5 x 20.0) respectively. Fluorescence labelling of cellulose with calcofluor (blue), of permeable cells and external nucleic acids with propidium iodide (red) and viable cells using SYTO9 (green).

The bottom surface of all samples was dominated by a PI staining of around 10 µm thickness and seemed established in the preexisting bottom cellulose layer (Figures J4-B, J5-B, and J5-B). This liquid / biofilm interfacial region hosted numerous viable bacteria sometimes organized in chains. It seems like PI-stained biomass was trapped by cellulose fibrils. Those fibrils have a different aspect than on the top surface with a spider-net pattern rather than parallel, with a compact fur-like aspect (Figures J4-A and J6-A).

4.4 Comparison of kombucha pellicles with pellicles from pure acetic acid bacteria cultures

To investigate the impact of yeasts on the structure of pellicles, z-stacks of pure AAB grown pellicles were also acquired (Figure J8). These pellicles were composed of viable bacteria cells and cellulose fibrils. Overall, the aspect of pure AAB pellicles was much homogenous. The shape of cellulose fibrils of *A. indonesiensis* appeared shorter than that of *K. saccharivorans*. Also, *A. indonesiensis* cells tend to arrange more often in chains compared to *K. saccharivorans*. Despite 14 days of incubation, the thickness of those biofilms (22.5 and 39.0 µm; Figure J8-A and J8-B respectively) appeared to be smaller than those in kombucha at D3 (above than 80 µm) (Figure J6). However, the thickness was more similar to that of kombucha pellicles at D2 between 27 and 86 µm (Figures J4 and J5). But even with similar thickness, pure culture pellicles showed poor consistence at macroscopic scale (Figure J1-E and J1-F). When comparing with D2 kombucha pellicles, the striking difference lies in the lack of alternance between cellulosic layer and IP-stained middle layer with viable cells and biomass (Figures J4, J5 and J6). As a result, the absence of yeasts limited the growth rate of the pellicle and led to the absence of the biomass-rich layer. Instead, AAB cells can be found in any part of the pure culture pellicle. However, the characteristic laminar structure might be observed after further incubation time, since it has been reported in static AAB pure cultures (Gullo *et al.*, 2018). Finally, the results pointed clearly at the role of yeasts as enhancers of pellicle formation and in the early establishment of the biomass-rich layer stained by PI.

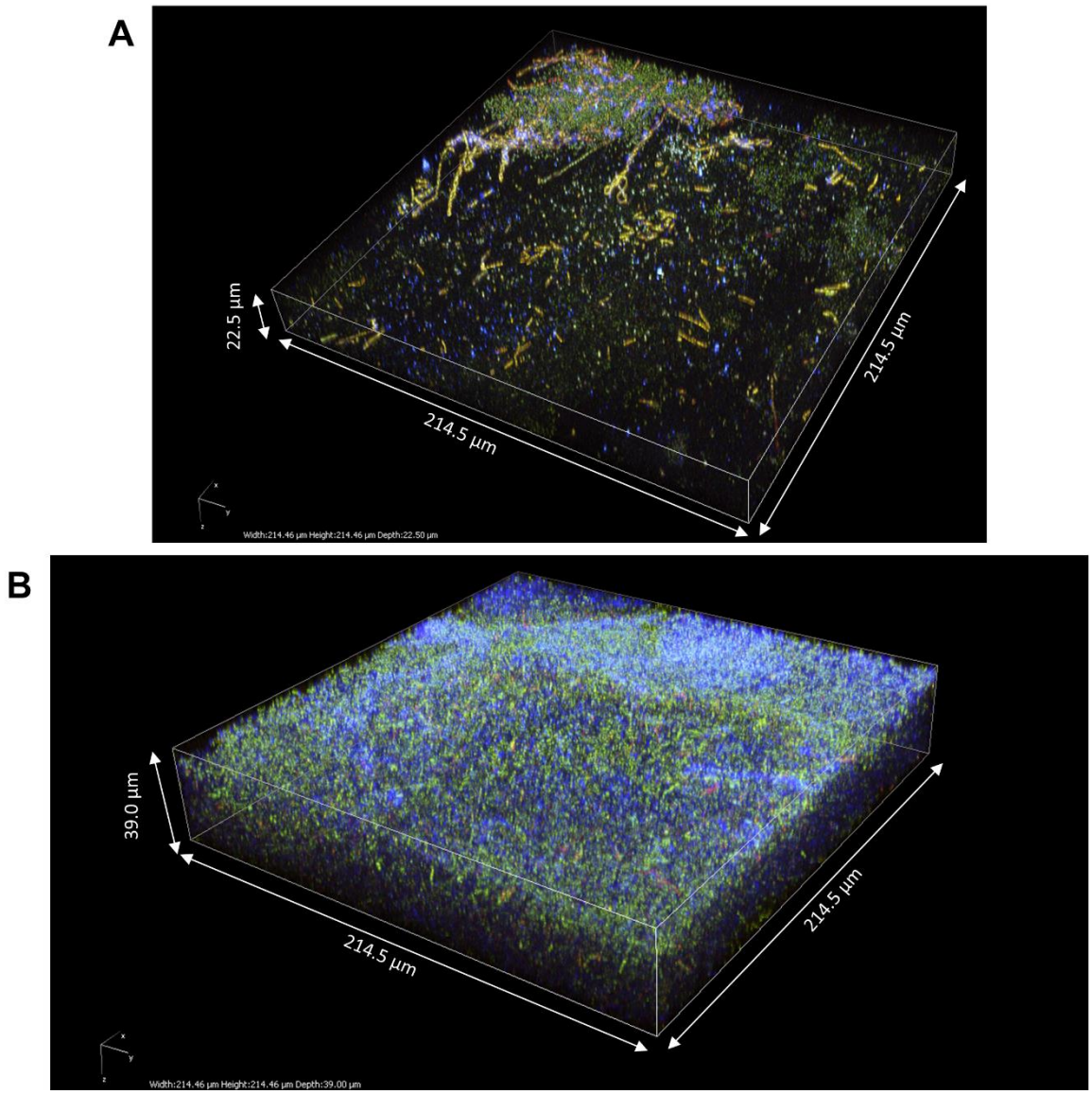

Figure J8 : 3D modelling of z-stack of upper region of pure acetic acid bacteria pellicles observed after 14 days cultures in modified sugared tea using two-photon microscopy, (A) *Acetobacter indonesiensis* grown pellicle (214.5 x 214.5 x 22.5 µm), (B) *Komagataeibacter saccharivorans* grown pellicle (214.5 x 214.5 x 39.0 µm). Fluorescence labelling of cellulose with calcofluor (blue), of permeable cells and external nucleic acids with propidium iodide (red) and viable cells using SYTO9 (green).

5 Discussion

The layered structure of kombucha pellicles observed in this study echoes the theoretical structure of AAB pellicle given by Gullo *et al.* (2018). Based on the gathering of previous experimental and modelling data, the authors described a putative cellulosic pellicle structure that has not yet been confirmed by microscopic observation, to our knowledge. This structure is organized in layers with an active cellulose-producing layer located between 50 and 100 µm under the top surface of the pellicle. The reason of this location can be explained by the evaporative pressure at the very top of the pellicle and an optimal balance between oxygen and substrate gradients, with the latter being the limiting factor after establishment of the pellicle (Verschuren *et al.*, 2000). The biomass-rich layers hosting viable bacteria cells could be observed in the present study beneath a top surface cellulosic layer between 10 and 120 µm and seems to fulfil this function. Also, filamentous cellulose on the bottom surface was also observed at microscopic scale as mentioned by the above-cited review (Gullo *et al.*, 2018). Generally, the observation made on kombucha pellicle are coherent with the model proposed for pellicles produced by AAB, but the presence of yeasts clearly impacted the formation of the biofilm.

Based on comparison with pure AAB biofilm grown in sugared tea, yeasts boosted the formation of the pellicle and induced the presence of the heavily PI-stained middle layer that may be caused by the release of nucleic acids and other intracellular material from dead yeasts cells, although bacteria could also release plasmids as part of horizontal gene transfers (Van Meervenne *et al.*, 2014). The review of Gullo *et al.* (2018) also mentions the possibility for this layer to be the support of microbial communication and nutrition. Moreover, yeast aggregates during the very early steps of pellicle formation could play a role of scaffold by supporting the networking of cellulose fibrils. It can be suspected that yeasts aggregates include filamentous or pseudo-hyphal forms as reported in strawberry vinegar biofilm (Valera *et al.*, 2015). Namely, formation of pseudo-mycelium was reported in wine for *B. bruxellensis* and *S. cerevisiae* and is suspected to be triggered by nutritional depletion in carbohydrate and nitrogen in the growth medium, but also by metabolites produced by other yeasts such as fusel alcohols (Dickinson, 2008; Louw *et al.*, 2016; Uscanga *et al.*, 2000). The formation of pseudo-mycelium by yeast in the context of

kombucha could therefore be linked to low nitrogen content of sugared tea and / or by microbial interaction that are yet to be determined.

The entrapment of yeasts by bacterial cellulose could benefit AAB by keeping their main source of substrate available in a confined space. Whether entrapped yeasts are alive and providing monosaccharide and ethanol or releasing assimilable nitrogen through autolysis, they would be used as nutrient storage. This way, the positive action of yeast activity on AAB observed previously in the liquid phase could occur in an optimized space inside the biofilm (Tran *et al.*, 2020b). This would imply that the pellicle, particularly within the active biomass-rich layer, acts as a catalyzer for the organic acids production of AAB occurring during the aerobic acidification phase of kombucha production. This metabolic boost could then also stimulate the production of cellulose during and after the early steps of biofilm formation. Due to the lack of labelled viable yeasts inside the pellicle, it is difficult to conclude on the benefits for yeasts of being embedded in the cellulose matrix in terms of protection against biotic and abiotic perturbation. Nevertheless, research works on the molecular interaction of cellulose show that it is able to bind polyphenols and heavy metals, which could inhibit their toxicity towards microorganisms dwelling in the pellicle (Najafpour *et al.*, 2019; Phan *et al.*, 2015).

6 Conclusions

Kombucha biofilm presents similarities to cellulosic pellicles grown by pure AAB in sugared black tea but differs by the presence of yeast, which are involved from the early steps of the biofilm formation. Based on observations, the formation model consists in an initial trapping and aggregation of yeasts (possibly in pseudo-mycelium form) in bacterial cellulose. With accumulation of cellulose and cells, a consistent layered pellicle is formed and from then, grows in thickness. The top layer at the interface with air is made of parallel cellulose fibrils and hosts yeasts and bacteria, the bottom surface at the interface with liquid is made of cellulose network colonized by bacteria and where biomass accumulates. Finally, a middle layer located under the top cellulosic layer is filled with biomass and viable bacterial cells that are suspected to be the active agents of pellicle growth. This region is thought to play a nutritional function for bacteria by taking advantage of entrapped yeast metabolism and autolysis, thus revealing an aspect of microbial interactions in kombucha.

This study indicates that yeast-AAB interactions in kombucha act on the structure and building of the pellicle, which could in turn enhance other types of interactions, including the metabolic interplay necessary for optimal kombucha production. The function of the pellicle as catalyzer of the biological acidification of kombucha could be further investigated to help optimize its production. Namely, the method used in this study could benefit from improvements regarding the penetration and concentration adjustments of fluorescent labels. Also, optimization of laser compensation would be used to obtain a better signal. Further investigation should focus on the discrimination of acetic acid bacteria from lactic acid bacteria using fluorescence *in situ* hybridization (FISH) labelling for the determination of localization differences in the structure, for example regarding the access in oxygen. Also, the contribution of yeasts in exopolymers inside the cellulosic matrix itself could be of interest using relevant probes.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Thierry Tran took the lead of the writing of this article, but all other authors provided critical and complementary elements to the manuscript. Pascale Winckler gave access and support to two photons microscopy through the Dimacell imaging facility. François Verdier and Antoine Martin provided the kombucha cultures used in the experiments.

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Contribution to the Field Statement

Observation of kombucha biofilm formation at microscopic scale was performed for the first time using two-photon microscopy, revealing the involvement of both yeasts and acetic acid bacteria and that interkingdom interactions play a role in the structure of the biofilm. Previous work on cellulosic pellicles involved biofilm produced in pure acetic acid bacteria cultures and not on complex cultures including indigenous yeasts and bacteria. Specifically regarding kombucha pellicles, their functionality was investigated based on the study of microbial dynamics based on cell counts or metagenomics. However, their location within the structure was not observed previously and the existence of an active site where living acetic acid bacteria dwell clarifies the functionality of this biofilm. Also, no element on the early steps of this biofilm formation was available. Observations show the involvement of yeasts cells on top of cellulose production by acetic acid bacteria in the formation of the biofilm, which is another form of interkingdom microbial interaction. The understanding of the biofilm's structure helps clarify its function for the microbial symbiosis occurring in kombucha and its functionality for the beverage's production.

K Discussion

Cette partie a pour objectif de relier les résultats présentés dans les chapitres précédents et de les mettre en perspective vis-à-vis de la littérature scientifique. En combinaison avec des approches analytiques chimiques distinctes, les plans d'expérience employés dans les chapitres 3 à 7 sont identiques en termes de composition microbienne et de conditions de cultures (composition du milieu, temps et conditions d'incubation). Cela permet une comparaison et un recoupement des résultats fiables. Plus particulièrement, les échantillons utilisés pour les analyses chimiques pour les chapitres 4, 5 et 6 sont strictement identiques.

K.1 Rôle des levures et bactéries acétiques dans les interactions microbiennes de la kombucha

Les résultats obtenus grâce aux différentes études présentées nous renseignent sur le comportement et la fonctionnalité des levures et des bactéries au sein de la kombucha. Ils montrent également des effets d'interactions entre micro-organismes qui sont à la base des transformations subies par la matrice.

K.1.1 Rôle des levures

La consommation du saccharose engendrant une acidification de la matrice constitue la transformation première et primordiale intervenant dans la fabrication de kombucha. Il a été établi que le métabolisme levurien était le paramètre principal influençant l'acidification de la matrice (Blanc, 1996 ; Jayabalan *et al.*, 2014 ; Villarreal-Soto *et al.*, 2018). De fortes activités invertase et fermentaire levuriennes permettent la mise à disposition efficace de substrats (glucose, fructose et éthanol) aux bactéries acétiques pour la production d'acides organiques (acides gluconique et acétique). L'étude présentée dans le chapitre 2 (Tran *et al.*, 2020b) montre que les espèces *B. bruxellensis* et *S. cerevisiae* isolées d'une kombucha thé noir satisfont à ce rôle. A l'inverse, *H. valbyensis* n'est pas adaptée pour remplir ce rôle étant donné sa faible activité invertase consécutive à son évolution. Cette espèce fait en effet partie d'une lignée de levures *Hanseniaspora* à évolution plus rapide ayant subi une perte de gènes dont certains sont impliqués dans le cycle cellulaire, la réparation de l'ADN, l'activité invertase (*SUC*) et la glycolyse précédant la voie de la fermentation alcoolique (Steenwyk *et al.*, 2019 ; Valera *et al.*, 2020). Il faut noter que dans le cas de la kombucha, la répression catabolique par le glucose ne s'applique pas comme au cours de la fermentation de moûts de raisin ou de malt dont les

matrices présentent des concentrations élévées en glucose (>100 g/L). Néanmoins la concentration en glucose, issu de l'hydrolyse du saccharose dans la matrice kombucha, peut mener à l'instauration de la répression catabolique, diminuant l'expression du gène *SUC2* comme observé chez *S. cerevisiae*. Cette régulation entraîne une diminution de l'activité invertase pour des concentrations en glucose >1%. De ce fait, au fur et à mesure de l'hydrolyse du saccharose, se crée un feedback régulant la synthèse d'invertase lorsque la concentration en glucose atteint un certain seuil(Marques *et al.*, 2016 ; Özcan *et al.*, 1997). A noter que pour de très faibles concentrations en glucose, une induction de l'expression du gène *SUC2* a été observée chez *S. cerevisiae* mais aucune étude à ce jour ne décrit ce phénomène chez *Brettanomyces* (Özcan *et al.*, 1997). Ce phénomène est observé chez *S. cerevisiae* en monoculture en condition d'incubation ouverte (Chapitre 2 ; Figure D4). Ce comportement n'a en revanche pas été observé chez *B. bruxellensis* dont l'activité invertase est équivalente en condition d'incubation ouverte et en condition fermée. Dans tous les cas, l'anaérobiose induite par la mise en bouteille initiant la seconde phase de prise de mousse impose l'utilisation du métabolisme fermentaire (Chapitres 2 et 7 ; Figures D4 et I4).

Au niveau des composés volatils (Chapitre 5), les levures étudiées (*B. bruxellensis* et *H. valbyensis*) sont à l'origine de la production d'éthanol, d'alcool supérieurs (comme l'isobutanol) de manière spécifique et d'acide gras saturés de manière moins spécifique (Tableau G2). Comparé à *B. bruxellensis*, *H. valbyensis* produit davantage d'esters (en particulier l'acétate d'éthyle) de manière similaire à ce qui est observé chez les levures du genre *Hanseniaspora* dans les conditions œnologiques (Díaz-Montaño and de Jesús Ramírez Córdova, 2009 ; Martin et al., 2018). De ce fait, le rôle technologique de *H. valbyensis* est la production d'esters volatils associés à des arômes fruités, qui peuvent être désirés dans le vin par exemple. Par opposition, les altérations rencontrées dans le vin avec la production de phénols volatils par *B. bruxellensis* n'ont pas été relevées dans les présent travaux sur la kombucha et s'expliquent par l'absence des acides phénoliques précurseurs (Horžić *et al*., 2009 ; Pagliosa et *al.*, 2010b). Ainsi, les composés volatils produits par les levures sont contributeurs d'arômes fruités tels que « fruits blancs » (Chapitre 5 ; Figure G3).

K.1.2 Interactions levure-levure entre B. bruxellensis et H. valbyensis

L'étude fine des interactions levuriennes inter-espèces a été réalisée avec *B. bruxellensis* et *H. valbyensis* isolées du même consortium de kombucha (Chapitre 1). Ce choix a été réalisé sur la base d'oppositions en termes de comportements : activité invertase, rendement fermentaire

et vitesse initiale de croissance opposés. *B. bruxellensis* est caractérisée par une croissance lente (Smith, 2011b), tandis que *H. valbyensis* est caractérisée par une croissance rapide qui est une conséquence d'un cycle de division cellulaire raccourci en lien avec son appartenance à une branche évolutive marquée par la perte de gènes (Steenwyk *et al.*, 2019). Il est effectivement observé dans le chapitre 7 que la phase de latence de *H. valbyensis* est plus courte (moins d'un jour) que celle de *B. bruxellensis* en monoculture (moins de deux jours) (Figure I2). En revanche, cette dernière est raccourcie à moins d'un jour lorsque les deux espèces sont en coculture. Il semble donc que *H. valbyensis* induise une croissance plus rapide de *B. bruxellensis*. Il a pu être observé que la teneur en FAN (Free Amino Nitrogen) était supérieure lors des monocultures de *H. valbyensis* comparées aux monocultures de *B. bruxellensis* ou *A. indonesiensis*, suggérant un relargage d'acides aminés (Chapitre 6, Figure H1). Ces nutriments disponibles pourraient être utilisés par les autres micro-organismes dans le cadre d'un commensalisme. De plus, l'analyse métabolomique menée dans le chapitre 4 suggère l'inclusion de peptides parmi les métabolites signature de *H. valbyensis* ainsi que leur consommation en coculture (Figures F2 et F3). Ainsi, ces peptides relargués par *H. valbyensis* pourraient jouer un rôle dans la modification de la croissance de *B. bruxellensis*. Des mécanismes inter-espèces de communication de type *quorum sensing* pourraient être évoqués avec beaucoup de prudence (Albuquerque and Casadevall, 2012 ; Sieuwerts *et al.*, 2008). En retour, l'activité de *B. bruxellensis* permet un accès facilité aux monosaccharides pour *H. valbyensis* à faible activité invertase (Chapitre 2). Cela pourrait expliquer la croissance plus rapide de *H. valbyensis* en coculture avec *B. bruxellensis* (Chapitre 7 ; Figure I2).

K.1.3 Rôle des bactéries acétiques

Les bactéries acétiques isolées de kombucha thé noir ont montré des propriétés plus homogènes selon les espèces comparées à la diversité observée pour les levures (Chapitre 1 ; Tableau D1). Leur rôle principal dans la production de kombucha réside dans la production d'acides organiques à partir des substrats fournis efficacement par certaines levures. Dans le produit final, les différences observées entre espèces concernent le profil en acides organiques produit. Ces différences sont principalement dues à la capacité de certaines espèces, en particulier *K. saccharivorans* à maintenir une production d'acide acétique en condition anaérobie (Chapitres 2 et 7 ; Figures D7 et I5), modifiant ainsi le rapport acétate/gluconate (Chapitre 2 ; Tableau D3). Au niveau des composés volatils, *A. indonesiensis* ne se distingue pas sur la production de composés spécifiques, mis à part l'acide acétique (Chapitre 5 ; Tableau G2). Ce composé impliqué dans l'arôme de vinaigre a également montré un rôle dans la perception

d'arômes fruités tels que « jus de pomme » et « fruits exotiques » en association avec des alcools supérieurs et esters d'origine levurienne (Chapitre 5 ; Figure G3).

L'étude de consommation de l'oxygène décrite dans le chapitre 7 montre que les bactéries acétiques consomment l'oxygène plus rapidement que les levures. Elles jouent donc un rôle prépondérant dans le maintien de l'anaérobiose du liquide lors de la première phase, puis dans la consommation complète de l'oxygène dans l'espace de tête lors de la seconde phase de production (Figure I4). Ceci induit des conditions anaérobies inhibant leur métabolisme oxydatif, mais inhibant également chez les levures le métabolisme respiratoire et l'activité de la succinate déshydrogénase (entraînant une accumulation de succinate (Camarasa *et al.,* 2003)). Il est intéressant de mentionner que les conditions anaérobies rencontrées au cours de la phase en bouteille fermée n'ont pas induit de mortalité, comme cela peut être le cas pour les bactéries issues de vinaigrerie (Barja *et al.,* 2016) (Figures D1, D2 et I2). Cette forte mortalité liée à l'anoxie semble spécifique aux souches adaptées aux apports élevés en oxygène, comme c'est le cas lors de procédé d'acétification submergé. L'analyse du génome de souches de bactéries acétiques isolées de l'appareil digestif de drosophiles suggère la possibilité pour certaines bactéries acétiques de se maintenir et de se développer en condition micro-aérée (Chouaia *et al.,* 2014). Par conséquent les souches issues de la kombucha sont plus faciles à manipuler en laboratoire comparé aux souches issues de vinaigrerie.

Les bactéries acétiques sont également productrices de cellulose qui est l'exo-polymère principal du biofilm de kombucha (Chawla *et al.,* 2009 ; Goh *et al.,* 2012a ; Savary *et al.,* 2021 ; Tran *et al.,* 2021b). Il est important de mentionner que seule la kombucha original a permis la production d'un biofilm consistant. Les cultures en thé sucré, autre que celles réalisées avec le consortium de kombucha original, incluant des bactéries acétiques sélectionnées au cours de ce travail ont produit des biofilms lacunaires et de faible consistance, que les bactéries soient cultivées en présence ou non de levures (Chapitres 2, 4, 5, 6, 7). Ceci pourrait s'expliquer par l'étape d'isolement réalisé en laboratoire qui a pu induire la sélection de souches non productrices de cellulose (Schramm and Hestrin, 1954). Toutefois, l'absence de biofilm de qualité équivalente à celui formé dans la kombucha originale n'a pas entravé le processus d'acidification (Chapitres 2, 5 et 6 ; Figures D7 et I5B).

K.1.4 Interactions levure(s)-bactérie acétique

Les résultats présentés dans les différents chapitres de thèse montrent clairement des interconnections entre les métabolismes levurien et bactérien. L'exemple le plus reconnu concerne la conversion des sucres en acides organiques abordée dans les parties K.1.1 et K.1.3. Les différentes études menées au cours de ce présent travail ont évalué la capacité d'acidification de *consortia* minimaux (levures + bactéries acétiques) et ont montré clairement que l'association de *B. bruxellensis* et *A. indonesiensis* (avec ou sans *H. valbyensis*) stimulait la production d'acides organiques comparée à ce qui est observé dans une monoculture de bactérie acétique (Chapitres 2, 5 et 7 ; Figures D7 et I5B). De plus, dans le cas de *S. cerevisiae*, la présence de bactérie acétique a entrainé l'augmentation de son activité invertase et de son métabolisme fermentaire (Chapitre 2 ; Tableau D2). Ce comportement fait partie d'une stratégie pour la consommation rapide des sucres et l'accumulation d'éthanol dans le but de contrecarrer l'opportunisme de micro-organismes « *cheaters* » (Celiker and Gore, 2012). Cette stratégie échoue partiellement en présence de bactéries acétiques puisqu'elles peuvent dégrader l'éthanol et profiter de ce substrat qu'elles peuvent spécifiquement et efficacement utiliser pour se développer (Chapitre 1). Au niveau des composés volatils, les cocultures incluant *H. valbyensis* produisent moins d'esters spécifiques à cette espèce, qu'il s'agisse des cocultures avec *B. bruxellensis* ou *A. indonesiensis* (Chapitre 5). Pour l'alcool isoamylique et l'heptanol, cela suggère l'effet d'une compétition pour les substrats précurseurs en amont de la production de composés volatils, puisque la diminution intervient indifféremment en présence de *B. bruxellensis* ou de *A. indonesiensis*. Toutefois, il apparait également que la présence de *A. indonesiensis* induit la diminution de certains alcools supérieurs (isobutanol, hexanol et nonanol) produits par l'action d'activités enzymatiques encore non identifiées (Chapitre 5).

En termes d'impact sur les populations, des effets différents sur *A. indonesiensis* ont été observés selon la présence de *B. bruxellensis* ou *H. valbyensis*, avec respectivement une inhibition et une stimulation de la croissance de la bactérie (Chapitre 7 ; Figure I2). Les analyses métabolomiques montrent également des effets opposés sur la production de métabolites (Chapitre 4 ; Figures F4 et F5). En effet, la coculture avec *B. bruxellensis* a induit la production de métabolites (acides gras et peptides), alors que la coculture avec *H. valbyensis* a induit la consommation de composés, tels que des acides aminés ou acides gras d'origine levurienne. La bactérie acétique a pu bénéficier de ces métabolites dans le cadre d'un commensalisme, ce qui expliquerait sa meilleure croissance (Chapitres 4 et 7 ; Figures F4, F5 et I2). On remarque par ailleurs que l'impact négatif de *B. bruxellensis* sur *A. indonesiensis* n'a pas inhibé la production

d'acides organiques, bien au contraire, ce qui souligne bien l'importance du métabolisme levurien (Chapitres 2, 5, 7 ; Figures D7, I5B). Le cas de la coculture en trio, elle a engendré une inhibition de la croissance de *A. indonesiensis.* La présence de *B. bruxellensis* a annulé l'effet bénéfique de *H. valbyensis* sur la croissance de la bactérie acétique mais également sur celle de *B. bruxellensis* (Chapitre 7 ; Figure I2). L'analyse métabolomique révèle que la plupart des composés produits par l'interaction entre *B. bruxellensis* et *H. valbyensis* sont absents en présence de *A. indonesiensis.* Ceci pourrait avoir un lien avec l'absence de stimulation de croissance de *B. bruxellensis* par *H. valbyensis*, du fait de l'absence de production ou la consommation des métabolites impliqués dans ce phénomène (Chapitres 4 et 7 ; Figures F6 et I2).

De manière opposée, l'impact de la présence de *A. indonesiensis* n'a pas révélé de différences aussi importantes sur les croissances levuriennes (Chapitre 7 ; Figure I2). De manière similaire, l'impact sur la production de métabolites par la bactérie acétique est similaire quelle que soit la levure associée, avec notamment la consommation de l'acide déhydroquinique (Chapitre 4 ; Figures F4, F5 et F6). Ce métabolite universel connectant les voies de la glycolyse et de la biosynthèse des acides aminés a la particularité d'être produit à partir de l'acide quinique *via* la quinoprotéine quinate déshydrogénase liée à la membrane plasmique et dont le site actif est situé à l'extérieur de la cellule bactérienne (Adachi *et al.*, 2003). Ce métabolite utilisable par les levures et bactéries est donc accessible et cela suggère une stratégie d'attraction de la part de la bactérie acétique pour d'autres micro-organismes. Une étude a par ailleurs suggéré que la présence des déshydrogénases et oxydases identifiées chez les bactéries acétiques et chez d'autres bactéries suggère l'existence de telles stratégies ayant donné lieu à des transferts de gènes (Matsushita and Matsutani, 2016).

K.2 Rôle du biofilm

Comme montré dans le Chapitre 8, le biofilm est composé de biomasses levuriennes et bactériennes vivantes et mortes et de cellulose produite par les bactéries acétiques. Dès les premières étapes de formation du biofilm, des agrégats levuriens sur lesquels la cellulose semble s'accumuler ont pu être observés (Figure J3). Le rassemblement de ces agrégats est suspecté de mener à la formation du biofilm à proprement parlé, ce qui semble être corroboré par mesure rhéologique dans une récente étude (Bertsch *et al.*, 2021). Par la suite, le biofilm se structure en couches hétérogènes, dont l'une d'entre elles, riche en biomasse, représente un site d'intense activité microbiologique (Figures J4, J5 et J6). La position de cette couche s'explique

par d'étroites conditions de développement imposées par le gradient en oxygène (depuis la surface) et le gradient en substrat (depuis le liquide) (Gullo *et al.*, 2018). Il est fait l'hypothèse que cette couche permette l'optimisation des interactions microbiennes grâce à un rapprochement spatial. Une autre étude récente (Savary *et al.*, 2021) a montré la présence de clusters séparés de levures et de bactéries acétiques dans des biofilms de 7 à 27 jours par microscopie de fluorescence confocale et par microscopie électronique à balayage. Cela suggère une différentiation spatiale au fur et à mesure du développement du biofilm en lien avec des conditions physico-chimiques adaptées à telle ou telle population. Le biofilm de kombucha est suspecté d'avoir d'autres fonctions de résistance caractéristiques contre la dessication, les ultra-violets, l'activité antimicrobienne des polyphénols ou les contaminations microbiennes ou chimiques (comme les métaux lourds par exemple) (Goh *et al.*, 2012a ; Lee *et al.*, 2021 ; May *et al.*, 2019 ; Najafpour *et al.*, 2019 ; Phan *et al.*, 2015).

Les résultats obtenus dans le chapitre 7 contredisent l'hypothèse que le biofilm induise une anaérobiose dans le liquide (à la manière d'un couvercle) ou qu'il se forme pour garantir l'accès à l'oxygène aux bactéries (à la manière d'une bouée) (May *et al.*, 2019). En effet, les observations suggèrent davantage que la formation de biofilm est réalisée à la surface de liquide parce que c'est la seule zone où l'oxygène est disponible (Chapitre 7 ; Figure F4). Or, l'oxygène est le substrat clé du métabolisme oxydatif des bactéries acétiques. Le rendement de production de cellulose, dont la voie de synthèse n'est pas directement dépendante de l'oxygène, est lié à l'activité du métabolisme des sucres. Il semble que ce métabolisme, qui lui dépend de l'oxygène, fournisse l'énergie nécessaire à la synthèse de la cellulose. Par conséquent, la présence d'oxygène stimule le catabolisme et par extension la production de cellulose (Chawla *et al.*, 2009 ; Rezazadeh *et al.*, 2020 ; Ross *et al.*, 1991). Comme développé dans la partie K.1.3, le biofilm n'est pas obligatoire pour la réalisation de l'acidification de la matrice par les bactéries acétiques, et donc pour la production de kombucha (Chapitres 2, 5, 7). En revanche, le biofilm représente un support pour la rémanence des micro-organismes avec une biodiversité supérieure à celle observée dans le liquide. Il fait office d'inoculum solide facile à disséminer (Chapitre 1).

K.3 Rôle de la matrice thé sucré

L'utilisation d'infusion de thé sucré a plusieurs conséquences sur la production et la qualité de la kombucha. Premièrement, il a été montré que l'oxydation de composés extraits (principalement phénoliques) induisait une consommation totale de l'oxygène dans le liquide

au début du procédé (Chapitre 7 ; Figure F3). Cela a pour conséquence une spatialisation des micro-organismes et de leurs activités selon leur dépendance à l'oxygène. Comme évoqué dans la partie K.2, cela détermine la position du biofilm. Le maintien de l'anaérobiose dans le liquide engendré par la consommation microbienne de l'oxygène permet de soulever l'hypothèse d'une protection des composés phénoliques contre l'oxydation. L'assimilation de composés phénoliques antioxydants peut constituer un intérêt pour la santé (Scalbert *et al.*, 2002). De plus, les analyses métabolomiques ont montré d'importantes transformations des composés phénoliques au cours de la fabrication (Chapitre 3 ; Figures E3 et E4). Ces changements sont plus prononcés en cas d'utilisation de thé noir et durant la première phase de production (Figures E5 et E6), en concordance avec l'étude de Cardoso *et al.*(2020). Plus spécifiquement, la libération d'acide gallique semble provenir de l'hydrolyse de liaisons esters de groupement gallates de polyphénols polymériques (Chapitre 3). De telles transformations sont suspectées améliorer la biodisponibilité des polyphénols (Scalbert *et al.*, 2002), améliorant d'autant plus le potentiel nutritionnel de la kombucha.

Le faible rapport C/N (rapport entre la quantité de sucre sur la quantité d'azote assimilable) du thé sucré entraine plusieurs conséquences. Pour les micro-organismes, il induit une limitation en termes de production de protéines et de croissance (Chapitre 6 ; Figures H1 et H2) (Weiping Zhang *et al.*, 2018). Cette limitation en azote pourrait expliquer également la lente consommation des sucres comparée à d'autres fermentations (Bisson, 1999) provoquée par les systèmes de régulation transcriptionnelles (par exemple GAAC) (Bianchi *et al.*, 2019). La kombucha originale est la seule condition présentant un maintien des ressources en azote de manière concomitante avec un maintien de la teneur en protéine (Chapitre 6 ; Figures H1 et H2). Cette stratégie mutualiste laisse supposer l'existence de mécanismes de régulation et potentiellement de communications inter-espèces (Sieuwerts *et al.*, 2008). Une autre conséquence d'un faible rapport C/N est un déterminisme de la composition en composés volatils. L'étude présentée dans le Chapitre 5 montre que la plupart des composés volatils fermentaires analysés peuvent être produits indépendamment de la voie de Ehrlich et donc d'un apport en acides aminés à partir des voies du métabolisme du glucose (Figure G2). Il est cependant fait l'hypothèse que les composés volatils variétaux influencent le profil olfactif des kombuchas selon le type de thé utilisé (Figure G4). En définitive, la matrice thé sucré conditionne fortement l'activité microbienne avec des conséquences importantes sur les paramètres de fabrication (durée, apport en oxygène) et le profil sensoriel de la kombucha.

K.4 Mutualisme et éco-évolution

L'étude du Chapitre 2 s'achève sur l'idée que les bactéries acétiques seraient des parasites non-obligatoires des levures vis-à-vis de l'utilisation des sucres et fait l'hypothèse qu'il pourrait en être de même pour *H. valbyensis*. Cela est concordant avec une spécialisation de cette espèce sur les matrices basées sur le saccharose, en lien avec sa dépendance pour l'activité invertase (Leech *et al.*, 2020). Les bactéries acétiques et *H. valbyensis* ont pour point commun la perte de gènes en lien avec des voies métaboliques centrales comme la glycolyse ou cycle de Krebs, diminuant ainsi la taille de leur génome (Deppenmeier and Ehrenreich, 2009 ; Ochman, 2006 ; Steenwyk *et al.*, 2019 ; Valera *et al.*, 2020). Or, ce type d'évolution est caractéristique des organismes parasites, puisqu'ils tirent parti des fonctions biologiques ou métaboliques de leur cible (Steenwyk *et al.*, 2019). C'est bien ce que l'on observe pour les micro-organismes cités vis-à-vis des levures comme *B. bruxellensis* ou *S. cerevisiae*. Toutefois, le recoupement des résultats obtenus montre l'existence d'une mise à disposition de métabolites/substrats des micro-organismes « parasites » vers leur cible, comme des acides aminés, des acides gras (acide hydroxy stéarique), l'acide déshydroquinique, des alcools supérieurs (pour les bactéries acétiques), avec parfois des bénéfices visibles sur la croissance des « levures cibles » (par exemple entre *H. valbyensis* et *B. bruxellensis*) (Chapitres 4, 5, 6, 7). On constate bien ici la complexité des interactions microbiennes et la difficulté de les classer en fonction des mécanismes envisagés. Il ressort de l'étude des interactions entre micro-organismes en conditions modèles l'existence de nombreuses imbrications métaboliques avec des effets plus ou moins positifs sur les populations. Au-delà de l'aspect purement métabolique, la production de biofilm semble jouer un rôle déterminant dans le maintien des consortia de kombucha, et a par ailleurs permis leur dissémination au sein des communautés humaines sous forme d'inoculum solide. Par conséquent, ces résultats permettent de qualifier le système de globalement mutualiste. Au sein de ce système, les interactions envisagées individuellement peuvent paradoxalement relever de commensalisme, ou même de compétition. En comparaison avec le consortium de kombucha original, cet ensemble ou système d'interactions peut expliquer, sans toutefois complètement l'élucider, la symbiose entre levures et bactéries. Ces micro-organismes ont probablement éco-évolué vers la mise en place d'un système d'interactions globalement mutualiste, sous la pression imposée par un environnement pauvre en composés azotés (l'infusion de thé) et doté d'un substrat carboné non-assimilable directement (le saccharose) (Chapitres 1 et 2) (Harrington and Sanchez, 2014 ; Sanchez and Gore, 2013). Autrement dit, des conditions de vie difficile imposent la coopération. La

sélectivité des genres et espèces isolés dans la kombucha et leur rémanence sont d'autres éléments corroborant cette hypothèse (Chapitre 1 ; Tableau C3).

L Conclusions et perspectives

Les travaux de thèse ont permis de répondre aux différentes problématiques énoncées en introduction (partie A.5). Ils ont permis de placer le rôle des interactions microbiennes, du biofilm et de la matrice thé sucré vis-à-vis des phénomènes microbiologiques, chimiques et sensoriels impliqués durant la fabrication de la kombucha.

Eléments clés reliant les interactions microbiennes et la matrice thé sucré avec la maîtrise de la production de kombucha

Les rôles des micro-organismes dans la fabrication de la kombucha et dans leurs interactions ont pu être définis. L'association d'une levure à hautes activités invertase et fermentaire, telle que *B. bruxellensis* avec une bactérie acétique représente la base pour l'acidification efficace de la matrice. L'association levure-bactérie acétique est également à l'origine du profil olfactif caractéristique de la kombucha, en particulier l'arôme de jus de pomme ou cidre. Des levures telles que *H. valbyensis* sont susceptibles d'impacter positivement le profil olfactif par l'apport d'esters volatils associés à des arômes fruités. Les activités microbiennes engendrent également la protection et la transformation des composés phénoliques antioxydants issus du thé, avec pour possible conséquence l'augmentation de leur biodisponibilité. La matrice thé sucré, de part sa composition riche en sucre et en polyphénols et faible en nutriments azotés, détermine très fortement l'activité microbienne, la composition chimique et le profil sensoriel du produit. Ce sont les composés du thé qui établissent la surface du liquide comme seule zone d'accès à l'oxygène, ce qui a pour conséquence la formation du biofilm à l'interface air/liquide. Le faible rapport C/N expliquerait la lente consommation de sucres et serait à l'origine de l'éco-évolution ayant amené les micro-organismes de la kombucha à développer un système mutualiste d'interactions. L'abondance de la ressource en sucre comparée à celle en acides aminés détermine les composés volatils fermentaires produits. Etant donné que la composition en acides aminés n'influence que peu le profil volatil, ce sont les composés volatils du thé qui sont susceptibles de distinguer deux kombuchas produites avec des thés différents (pour un consortium donné). Le type de thé influence de fait la composition initiale du produit. Toutefois, la composition phénolique finale du produit est dépendante de transformations induites par l'activité microbienne et qui sont plus poussées en cas d'utilisation de thé noir comparé à du thé vert. Ces transformations ont lieu principalement durant la première phase de production. En revanche, la sucrosité du produit tend à masquer l'amertume et l'astringence des composés du thé. Par conséquent le choix du thé se fera plus judicieusement en fonction du profil

aromatique. Enfin, le biofilm de kombucha semble concentrer en son sein ce système mutualiste d'interactions en impliquant clairement levures et bactéries au cours de sa formation. Il aménage également des environnements adaptés aux besoins des différents micro-organismes et pourrait favoriser les interactions trophiques. D'un point de vue technologique il pourrait servir de catalyseur aux transformations ayant lieu dans la phase liquide sans toutefois être obligatoire.

Les résultats soulignent le caractère mutualiste des interactions microbiennes ayant lieu au cours de la production de kombucha, en particulier entre les levures et les bactéries acétiques. L'hypothèse d'une éco-évolution de ces organismes dans la matrice thé sucré argumente en faveur d'une définition de la kombucha traditionnelle incluant d'une part : une communauté microbienne composée de levures et de bactéries acétiques (sous réserve que le métabolisme levurien soit adapté) et d'autre part : une infusion de thé supplémentée en saccharose, dont la gestion et l'assimilation judicieuse des substrats requierent une collaboration microbienne. Ces éléments pourraient contribuer à la conception de réglementations sur la kombucha, qu'elle soit produite traditionnellement ou selon des procédés innovants tout en distinguant ces cas de figure. Les résultats obtenus dans le cadre de cette thèse contribuent à la connaissance fondamentale des interactions microbiennes dans les aliments fermentés et met en évidence les spécificités de la kombucha en termes de dynamique microbienne. Ils posent également des bases méthodologiques pour la conception de consortia minimaux visant à non seulement à améliorer la maîtrise du procédé d'élaboration de kombucha, comme dit précédemment, mais également à établir un modèle d'études robuste dans le but de comprendre les phénomènes d'interactions entre micro-organismes.

Dans le cadre de la conception d'un consortium microbien minimal et contrôlé pour la fabrication de kombucha, le choix de la levure ou des levures sera déterminant. L'attention devra en particulier être portée sur l'activité invertase, le rendement fermentaire et la production de composés volatils. Toutefois, les résultats présentés montrent également l'importance de prendre en considération les interactions microbiennes pouvant avoir lieu avec les autres levures et les bactéries acétiques. Ces interactions sont susceptibles de moduler mutuellement leurs métabolismes. L'utilisation de micro-organismes issus de la kombucha peut s'avérer judicieux étant donné la mise en évidence d'une éco-évolution des micro-organismes favorisant des interactions mutualistes et assurant la stabilité des communautés microbiennes. L'utilisation d'un consortium minimal comme inoculum maîtrisé et caractérisé est applicable dans le contexte de production. Elle peut permettre une meilleure maîtrise et l'optimisation du procédé

de fabrication, assurant la qualité du produit. Un consortium minimal caractérisé représente également un outil intéressant en recherche. En se servant d'un consortium de kombucha simplifié avec une composition microbienne maitrisée, on s'affranchit du caractère « boîte noire » intrinsèquement lié à l'utilisation d'un consortium original. Comme souligné dans les résultats, un consortium minimal, bien que se rapprochant globalement de l'original sur sa composition microbiologique, ne se substitue pas strictement au consortium original en termes de comportement (notamment concernant la production de biofilm). Par conséquent, l'utilisation des deux consortia (minimal et original) semble être l'option à privilégier pour de futures expérimentations sur la matrice kombucha.

Au sein de la thématique des interactions microbiennes dans la kombucha, le rôle des bactéries lactiques et les interactions bactérie-bactérie (acétique ou lactique) n'ont pas été abordés dans ces travaux. Ces axes de recherches sont très larges et peuvent être approchés de diverses manières, en incluant les méthodologies décrites dans cette thèse, par exemple. Le choix a donc été fait de développer deux perspectives aux thématiques mieux circonscrites, et partant d'hypothèses formulées dans le cadre des travaux de thèse.

<u>Perspectives de recherches sur la nutrition azotée au sein du consortium</u>

Des analyses complémentaires par chromatographie liquide sont en cours, permettant de quantifier les variations des teneurs en chaque acide aminé au cours du procédé d'élaboration. Ceci permettra de mieux caractériser les phénomènes de consommation et relargage. L'étude approfondie des protéines mises en évidence dans les surnageants par protéomique pourrait révéler des protéines clé impliquées dans des mécanismes d'interaction. L'hypothèse envisagée serait la production de protéines susceptibles de modifier l'activité d'autres micro-organismes (comme le prion [GAR+] (Ramakrishnan *et al.*, 2016)) ou ayant pour fonction l'établissement de résistances face à un stress (comme la résistance à l'acide acétique (Andrés-Barrao *et al.*, 2012 ; Nakano and Ebisuya, 2016)). Les analyses métabolomiques ont suggéré de potentiels rôles des peptides dans les interactions. Ainsi, l'analyse des protéines pourrait être complémentée par une approche peptidomique qui permettrait d'identifier des molécules impliquées dans la communication (Albuquerque and Casadevall, 2012) ou dans des relations d'amensalisme (peptides *killers* (Albergaria *et al.*, 2010)). Ces expérimentations pourraient être réalisées à l'aide de micro-organismes isolés de la kombucha afin de privilégier des particularités génétiques consécutives à l'évolution des micro-organismes dans cette matrice. Toutefois, l'étude peut être facilitée si le choix des micro-organismes se porte sur des espèces

dont le génome est connu. Cela permettrait d'envisager à terme une analyse transcriptomique chez les différents partenaires microbiens impliqués. Il pourrait être également envisagé de séquencer le génome des souches d'études issues de la kombucha que nous avons plus particulièrement étudiées. Une telle approche permettrait d'aborder également la question de l'évolution des micro-organismes de la kombucha.

Perspectives d'études sur l'évolution des micro-organismes en lien avec la matrice kombucha

Des études de génomique comparative incluant les souches de références et des souches issues de la kombucha permettraient de mieux caractériser l'influence de la matrice sur l'évolution des micro-organismes (par exemple entre souches de bactéries acétiques issus de vinaigrerie et celles issues de la kombucha). Cette approche nécessite un certain niveau de représentativité qui doit se traduire par un échantillonnage important de consortia de kombucha. Une telle collection a déjà pu être rassemblée par une équipe de microbiologistes de l'Université d'Oregon aux Etats-Unis. Dans une récente étude (Harrison and Curtin, 2021), des analyses de métagénomique ont été réalisées sur 103 échantillons de kombuchas issus d'Amérique du Nord. Une telle collection pourrait faire l'objet d'une étude de génomique comparative pour investiguer l'évolution des micro-organismes de kombucha et potentiellement vis-à-vis d'autres matrices fermentaires (vins, bières, cidres et kéfirs). Chez les levures, l'analyse du génotype par différentes techniques, dont la RAPD (*Random Amplified Polymorphic DNA*), l'AFLP (*Amplified Fragment Length Polymorphism*) et l'analyse par microsatellite a permis de comparer des souches de *B. bruxellensis* ou de *H. uvarum*. L'analyse des microsatellites chez *B. bruxellensis* a révélé un lien entre des groupes génétiques et la matrice d'origine des souches (vin, bière, kombucha, tequila et bioéthanol) (Albertin *et al.*, 2016, 2014 ; Avramova *et al.*, 2018). Les souches de *B. bruxellensis* issues de la kombucha semblent d'ailleurs caractérisées par un état diploïde. Chez les bactéries lactiques et acétiques, des techniques telles que la RFLP (*Restriction Fragment Length Polymorphism*), ERIC-PCR (*Enterobacterial Repetitive Intergenic Consensus-PCR*) et REP-PCR (*Repetitive Extragenic Palindromics-PCR*) ont été appliquées en contexte œnologique (Gonzalez *et al.*, 2004 ; González *et al.*, 2005). Si cela est possible, l'établissement de groupes génétiques pourrait mettre en évidence l'influence de la matrice sur l'évolution de différentes espèces endogènes de la kombucha. Des investigations plus poussées des génomes de représentants par groupe pourraient alors être envisagées afin de caractériser les particularités génétiques en lien avec l'évolution et le phénotype des micro-organismes. Par exemple, il serait intéressant de réaliser une comparaison de gènes codant des invertases parmi différentes espèces levuriennes et bactériennes afin de mettre en évidence des

duplications de gènes (comme pour *S. cerevisiae* (Naumov and Naumova, 2010)) ou des transferts de gènes (comme cela semble être le cas pour *B. bruxellensis* à partir de bactéries acétiques (Roach and Borneman, 2020)).

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N Annexes

Annexe SC1 : Composition of liquid and agar media

Yeast Peptone Dextrose (YPD): 2 % (m/v) glucose, 0.5 % (m/v) yeast extract, 1 % (m/v) bactopeptone, 0.02 % (m/v) chloramphenicol, 2 % (m/v) agar pH 6.5.

Differential Wallerstein Laboratory nutrient agar medium from ThermoScientific (Waltham, USA) (Hall, 1971; Pallmann *et al.*, 2001): 0.4 % (m/v) yeast extract, 0.05 % (m/v) tryptone, 0.5 % (m/v) glucose, 550 ppm (m/v) dihydrogen potassium phosphate, 425 ppm (m/v) potassium chloride, 125 ppm (m/v) calcium chloride, 125 ppm (m/v) iron (III) chloride, 2,5 ppm (m/v) magnesium sulphate, 220 ppm (m/v) bromocresol green, 0.02 % (m/v) chloramphenicol, 0.05 ppm (m/v) natamycin (Delvocid®), 0.063 ppm (m/v) penicillin, 1.5 % (m/v) agar, pH = 6.5.

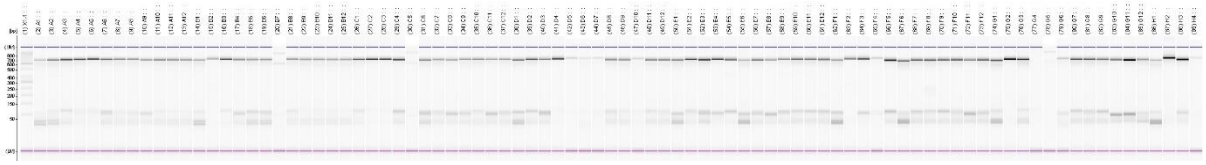
Mannitol medium: 2.5 % (m/v) mannitol, 1 % (m/v) extrait de levure, 0.05 ppm (m/v) natamycine (Delvocid®), 0.063 ppm (m/v) penicillin, 2 % (m/v) agar, pH 6.5.

MRS medium: 0.2 % (m/v) dextrose, 0.1 % (m/v) bactopeptone, 0.8 % (m/v) beef extract, 0.5 % (m/v) sodium acetate, 0.2 % (m/v) yeast extract, 0.2 % (m/v) dipotassium phosphate, 0.2 % (m/v) ammonium citrate, 0.1 % (v/v) Tween 80, 0.2 % (m/v) magnesium sulphate, 0.005 % (m/v) manganese sulphate, 0.05 ppm (m/v) natamycin (Delvocid®), 2 % (m/v) agar, pH = 6.2.

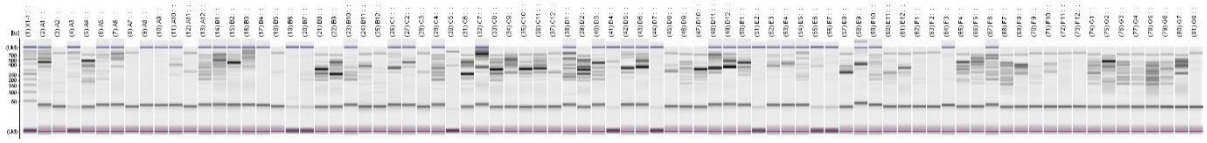
LAC medium: 7.8 % (v/v) white grape juice from Casino®, 3.3 % (m/v) yeast extract, 0.06 % (v/v) Tween 80, 0.008 % (m/v) manganese sulphate, 0.05 ppm (m/v) natamycin (Delvocid®), 2.5 % (m/v) agar, pH = 5.1.

M17 medium: 0.5% (m/v) tryptone, 0.5% (m/v) soy peptone, 0.5% (m/v) meat infusion, 0.25% (m/v) yeast extract, 0.05% (m/v) ascorbic acid, 0.025% (m/v) magnesium sulphate, 1.9% (m/v) disodium glycerophosphate, 1.1% (m/v) agar, pH = 6.9.

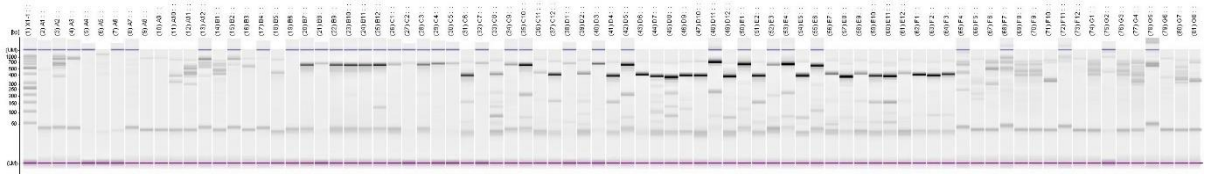
Annexe SC2 : Examples of electrophoresis profile for 26S PCR samples.



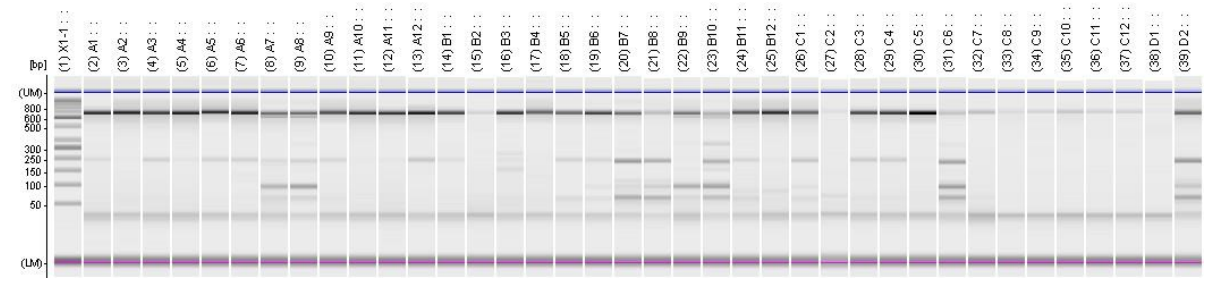
Annexe SC3 : Examples of electrophoresis profile for RAPD PCR samples targeting acetic bacteria.



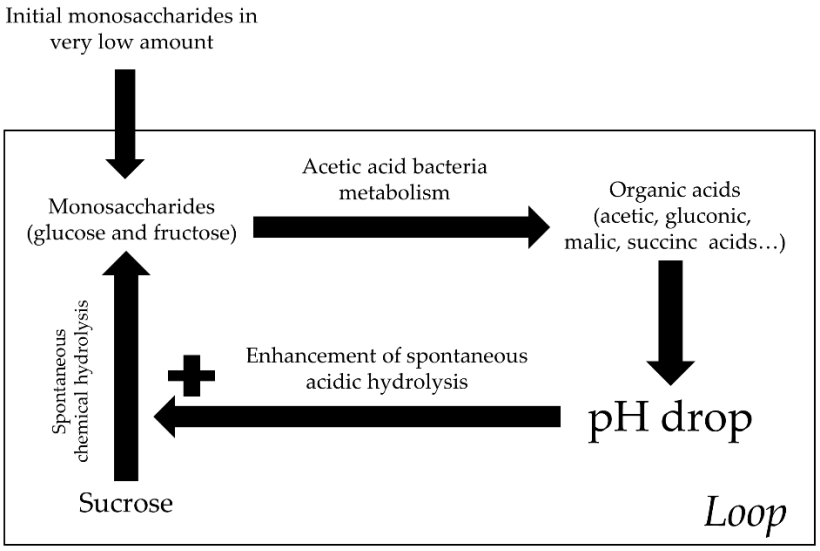
Annexe SC4 : Examples of electrophoresis profile for RAPD PCR samples targeting lactic acid bacteria.



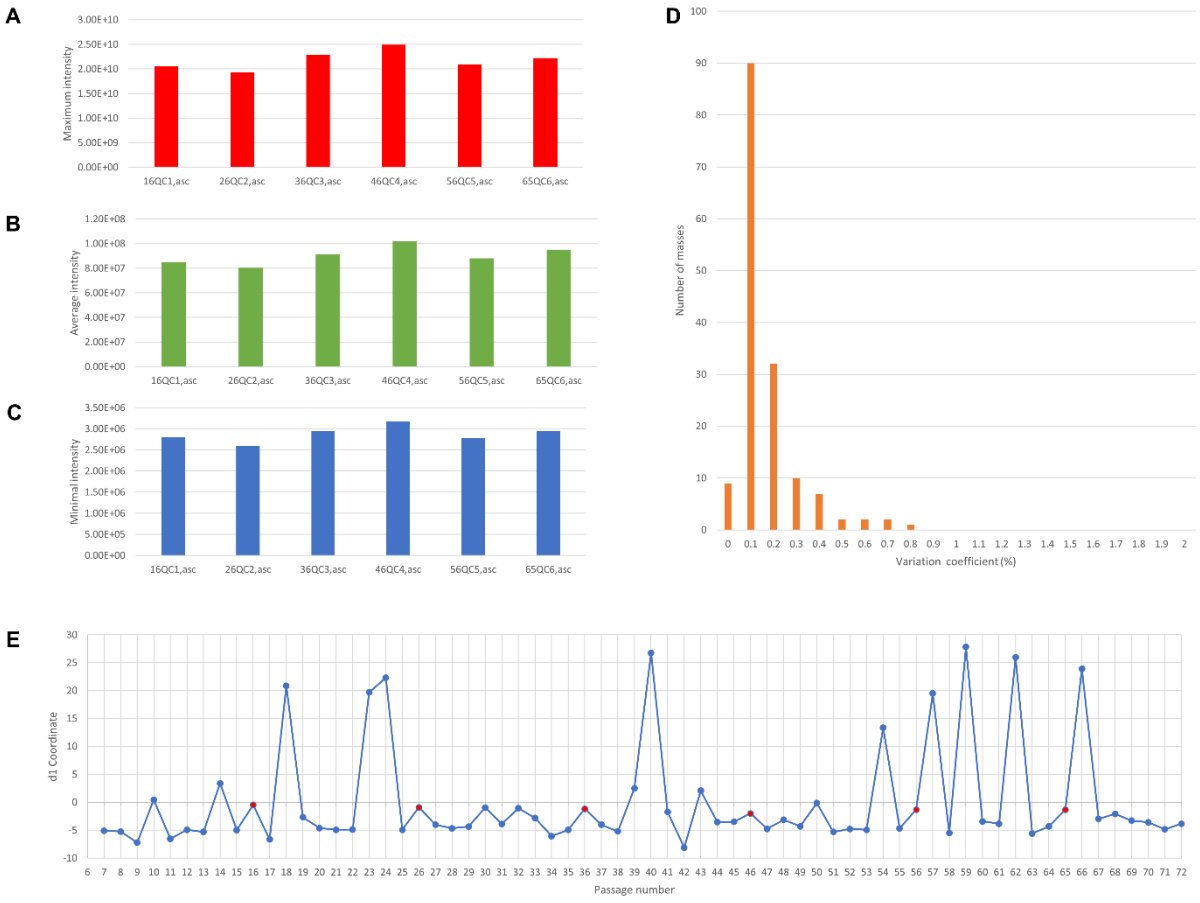
Annexe SC5 : Examples of electrophoresis profile for 16S PCR samples.



Annexe SD1 : Hypothetical "snowball effect" explaining sucrose hydrolysis by acetic acid bacteria.



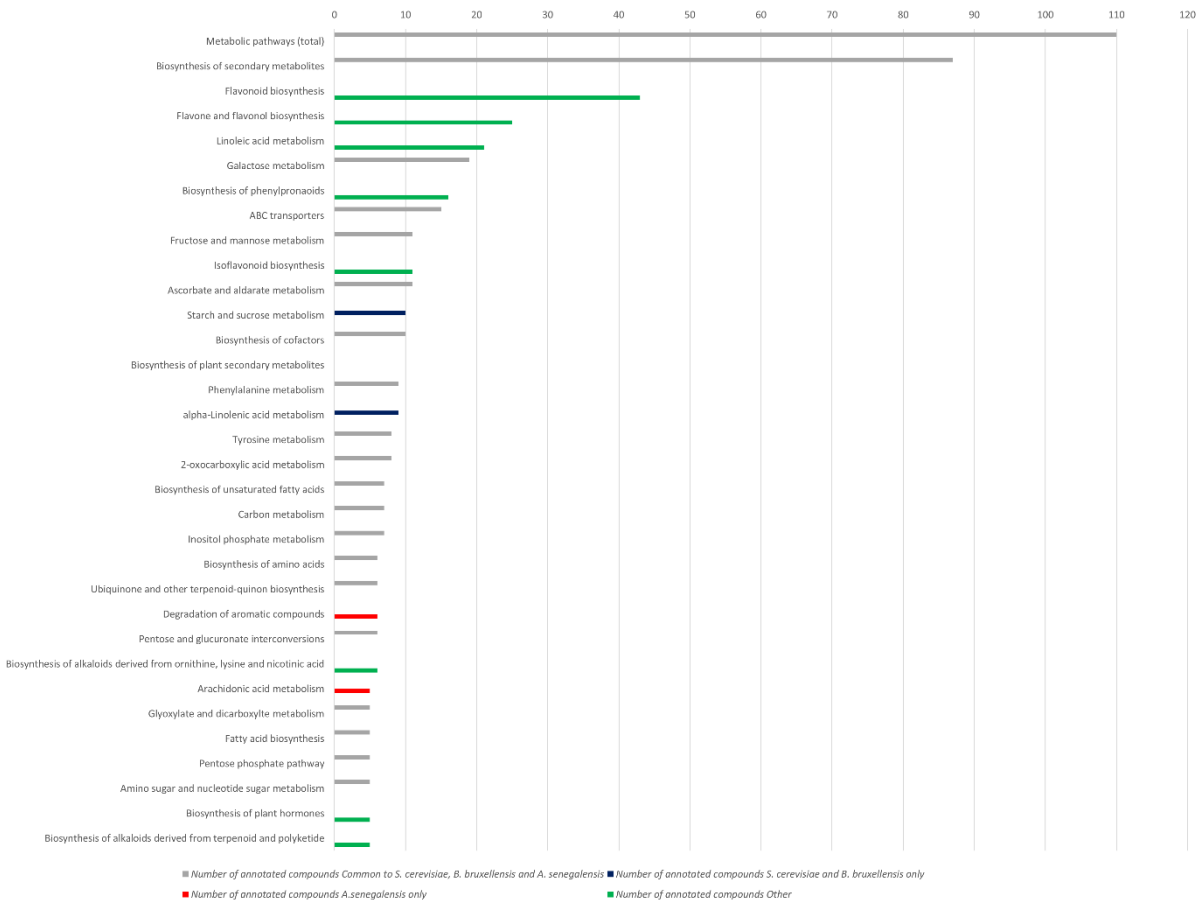
Annexe SE1 : Visualization of Quality control (QC) samples. (A) maximum, (B) average and (C) minimal ion intensities measured in QC samples. (D) Distribution of mass number according to variation coefficient of QC samples. (E) Principal Component Analysis d1 coordinate of samples according to passage number, with QC samples signalized in red.



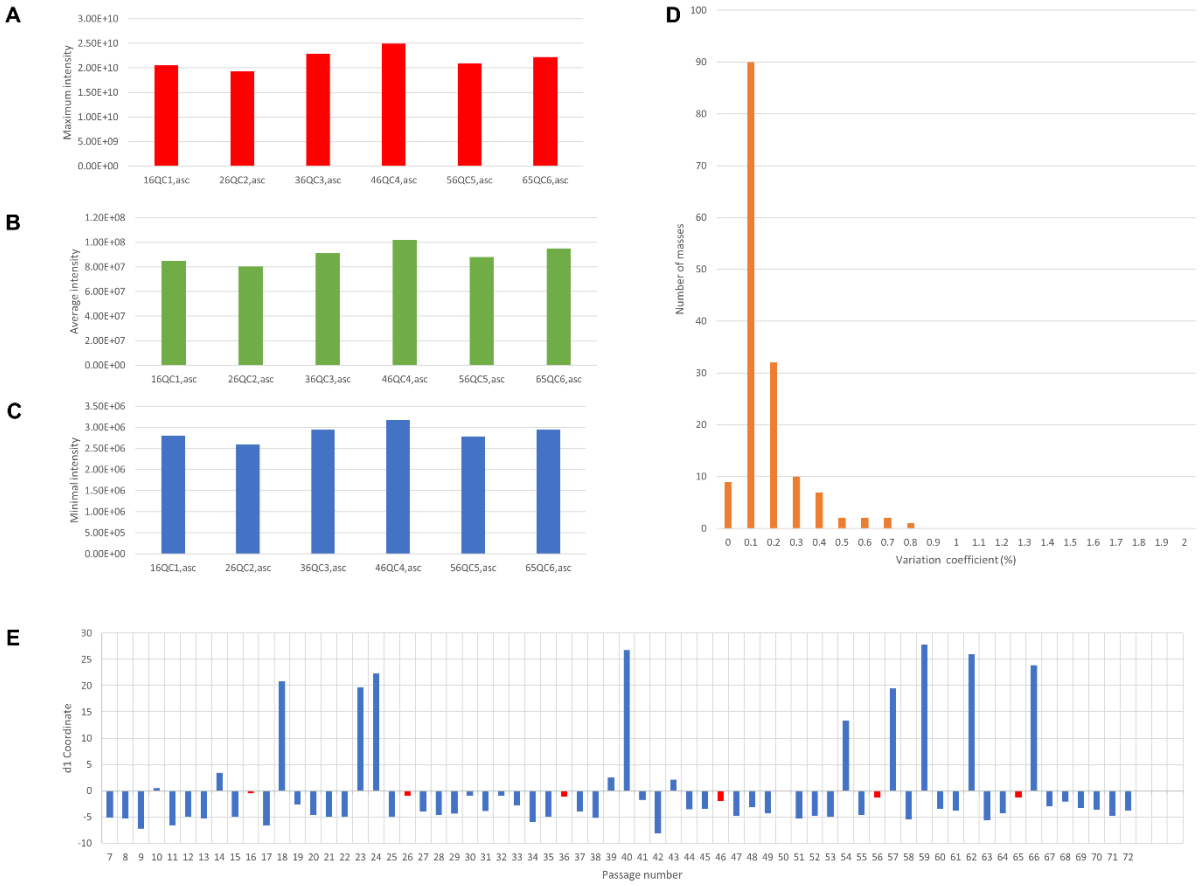
Annexe SE2 : Database annotation of markers

Mass (average)	Formula	Database annotation
169.01427	$C_7H_6O_5$	Gallic acid
173.00917	$C_6H_6O_6$	aconitic acid
179.05612	$C_6H_{12}O_6$	Glucose or Fructose
191.01973	$C_6H_8O_7$	Citric acid
195.05103	$C_6H_{12}O_7$	Gluconic acid
253.21727	$C_{16}H_{30}O_2$	Palmitoleic acid
255.23292	$C_{16}H_{32}O_2$	Palmitic acid
273.07682	$C_{15}H_{14}O_5$	Epiafzelechin
281.24859	$C_{18}H_{34}O_2$	Oleic acid
283.26424	$C_{18}H_{36}O_2$	Stearic acid
289.07175	$C_{15}H_{14}O_6$	Epicatechin
305.06669	$C_{15}H_{14}O_7$	Epigallocatechin
341.10891	$C_{12}H_{22}O_{11}$	Sucrose
425.08788	$C_{22}H_{18}O_9$	Epiafzelechin gallate
441.08278	$C_{22}H_{18}O_{10}$	Epicatechin gallate
503.16189	$C_{18}H_{32}O_{16}$	Dextrin
535.15180	$C_{18}H_{32}O_{18}$	1,4-bêta-D-Glucan

Annexe SE3 : Distribution of annotated compounds using MASSTRIX database according to metabolic pathways according to KEGG Mapper Color.



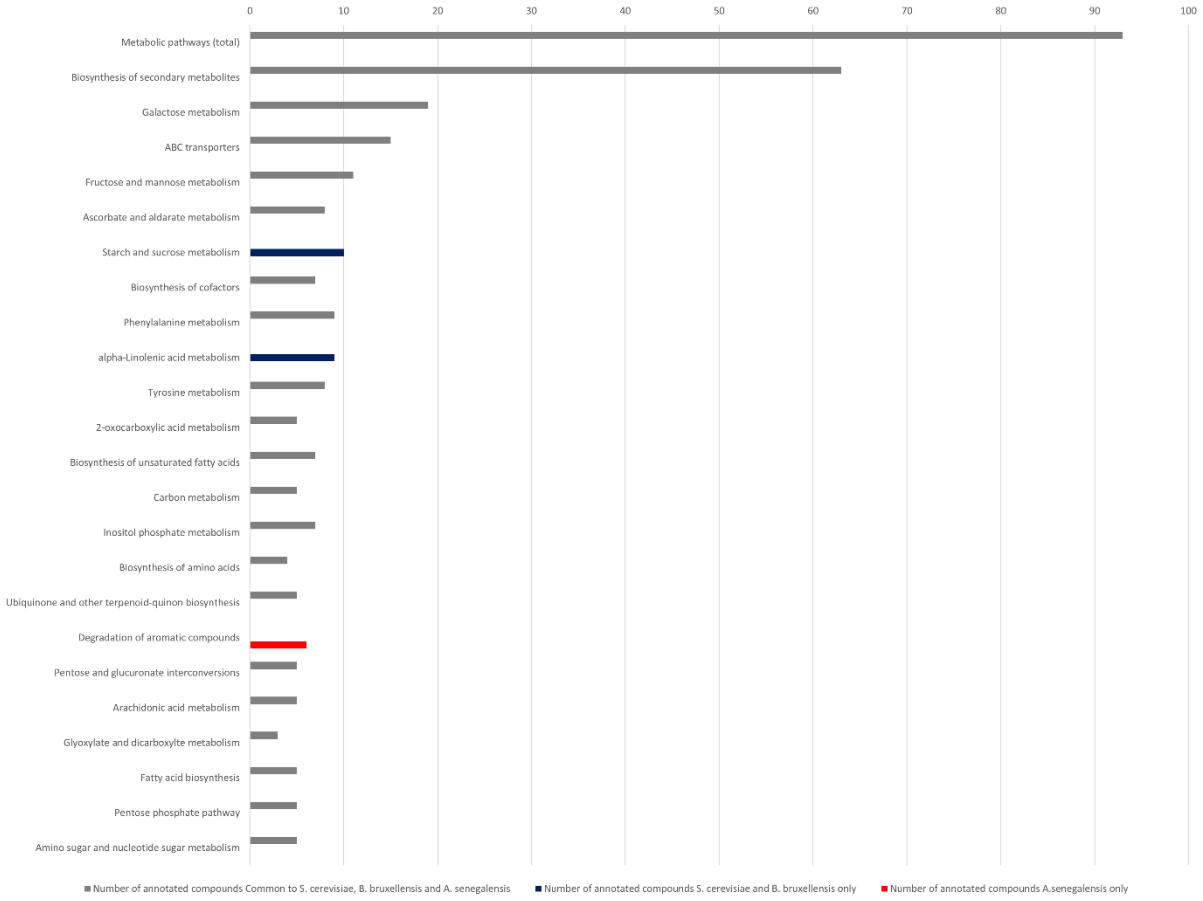
Annexe SF1 : Visualization of Quality control (QC) samples. (A) maximum, (B) average and (C) minimal ion intensities measured in QC samples. (D) Distribution of mass number according to variation coefficient of QC samples. (E) Principal Component Analysis d1 coordinate of samples according to passage number, with QC samples signalized in red.



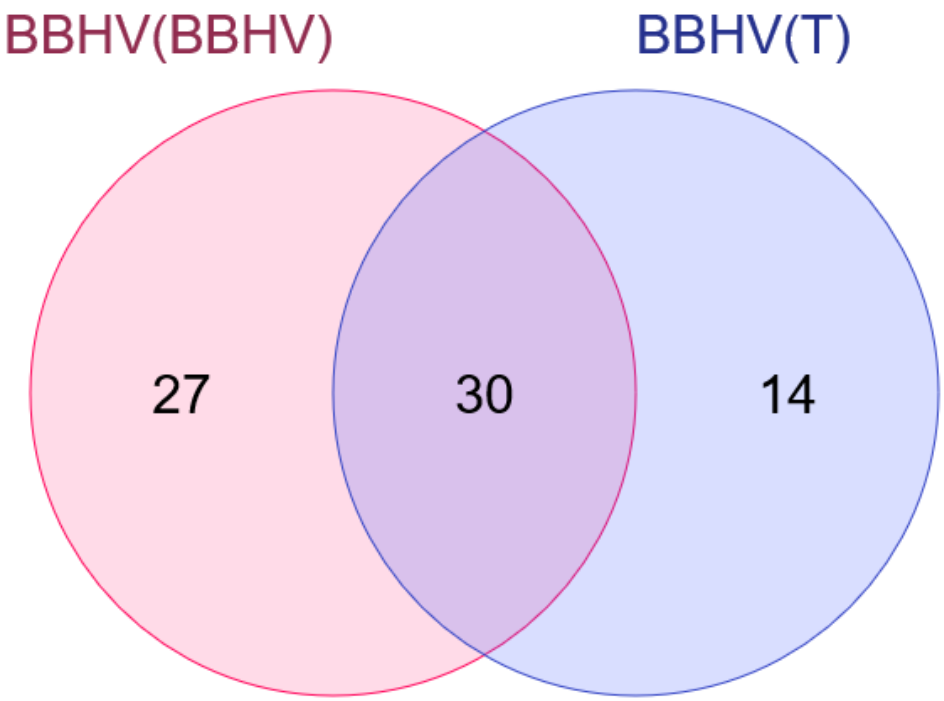
Annexe SF2 : Database annotation of markers

Mass (average)	Formula	Database annotation
163.04006	$C_9H_8O_3$	Phenylpyruvate
169.01427	$C_7H_6O_5$	Gallic acid
173.00917	$C_6H_6O_6$	aconitic acid
175.06121	$C_7H_{12}O_5$	Isopropyl malate
178.04774	$C_{10}H_6O_6$	Gluconolactone
179.05612	$C_6H_{12}O_6$	Glucose or Fructose
189.04046	$C_7H_{10}O_6$	Dehydroquinic acid
191.01973	$C_6H_8O_7$	Citric acid
195.05103	$C_6H_{12}O_7$	Gluconic acid
253.21727	$C_{16}H_{30}O_2$	Palmitoleic acid
255.23292	$C_{16}H_{32}O_2$	Palmitic acid
281.24859	$C_{18}H_{34}O_2$	Oleic acid
283.26424	$C_{18}H_{36}O_2$	Stearic acid
289.07175	$C_{15}H_{14}O_6$	Epicatechin
300.26645	$C_{18}H_{36}O_3$	Hydroxystearic acid
341.10891	$C_{12}H_{22}O_{11}$	Sucrose
441.08278	$C_{22}H_{18}O_{10}$	Epicatechin gallate
503.16189	$C_{18}H_{32}O_{16}$	Dextrin
535.15180	$C_{18}H_{32}O_{18}$	1,4-bêta-D-Glucan

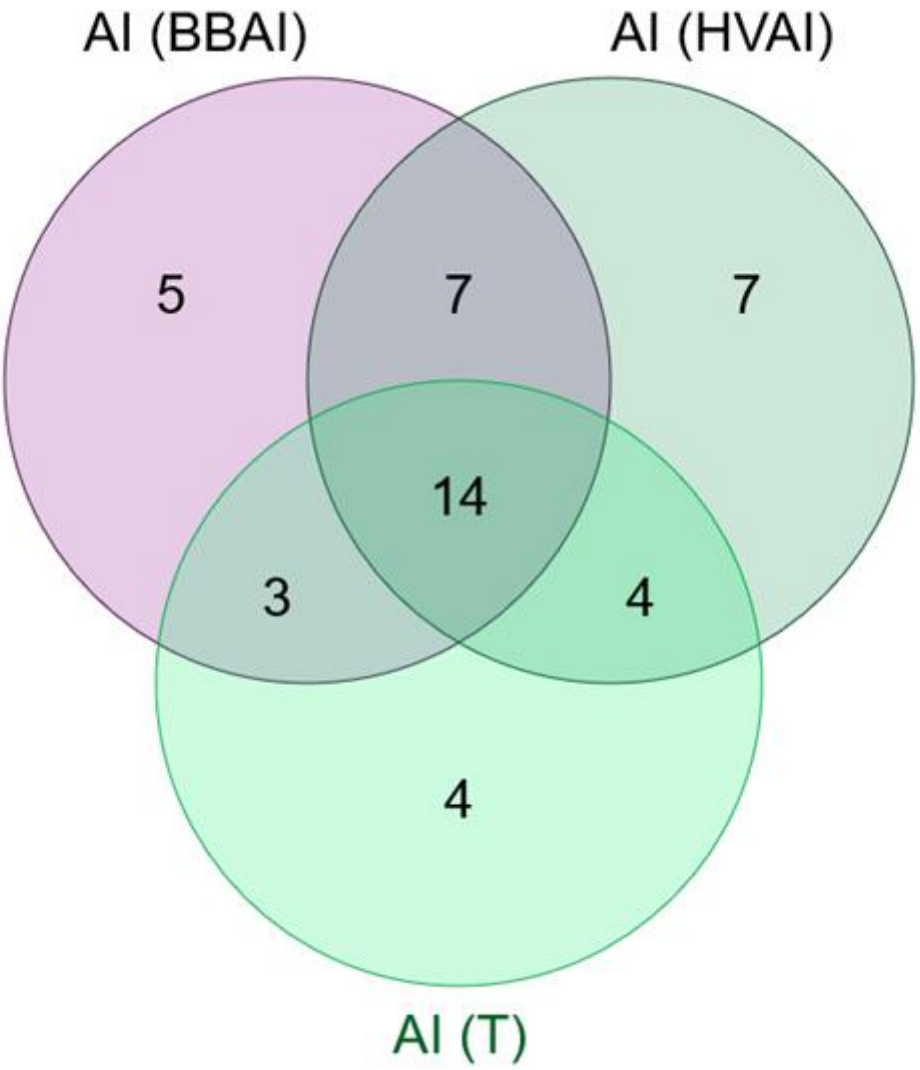
Annexe SF3 : Distribution of annotated compounds using MASSTRIX database according to metabolic pathways according to KEGG Mapper Color.



Annexe SF4 : Venn diagram showing the number of common and unique formulae between those produced in BBHV as part of the interaction between *B. bruxellensis* (BB) and *H. valbyensis* (HV) (labeled "BBHV(BBHV)" and those present in BBHV but inhibited by the presence of *A. indonesiensis* (AI) in the coculture gathering all three microorganisms (T) (labeled "BBHV(T)").



Annexe SF5 : Venn diagram showing the number of common and unique formulae between the lists of those associated with *A. indonesiensis* monoculture (AI), that were negatively impacted by the presence of yeast(s). The three cases involved *B. bruxellensis* alone (BBAI), *H. valbyensis* alone (HVAI) and the two yeasts simultaneously (T).



Annexe SG1 : Identification and quantification methods of volatile compounds by HS-SPME-GC/MS

Retention time	IUPAC name	Common name	CAS number	Identification	Quantification	RSD (%)
4.618	ethanal	acetaldehyde	75-07-0	1	a	14.5
6.467	methyl acetate	methyl acetate	79-20-9	1	b	6.4
8.229	ethyl acetate	ethyl acetate	141-78-6	2	b	2.0
9.345	2-methylbutanal	2-methylbutanal	96-17-3	1	a	ND
9.573	3-methylbutanal	isovaleraldehyde	590-86-3	2	a	ND
10.28	ethanol	ethanol	64-17-5	2	c	3.2
11.73	ethyl propanoate	ethyl propanoate	105-37-3	1	b	4.5
12.174	ethyl 2-methylpropanoate	ethyl isobutyrate	97-62-1	1	b	6.0
12.714	propyl acetate	propyl acetate	109-60-4	1	b	7.2
12.833	pentan-2-one	2-pentanone	107-87-9	1	a	13.7
13.039	butane-2,3-dione	diacetyl	431-03-8	2	c	8.2
19.206	hexanal	hexanal	66-25-1	2	d	ND
19.698	2-methylpropan-1-ol	isobutanol	78-83-1	2	d	6.9
21.643	3-methylbutyl acetate	isoamylacetate	123-92-2	1	b	3.7
25.428	2-Methyl-2-propanol	tert-butanol	75-65-0	1	d	3.9
26.357	3-methylbutan-1-ol	isoamyl alcohol	123-51-3	2	e	3.9
30.849	3-hydroxybutan-2-one	acetoin	53584-56-8	1	e	12.8
33.366	6-methyl-5-hepten-2-one	6-methyl-5-hepten-2-one	110-93-0	2	f	8.5
33.826	hexan-1-ol	hexanol	111-27-3	2	g	13.6
38.353	1-octen-3-ol	1-octen-3-ol	3391-86-4	2	h	6.2
38.562	heptan-1-ol	heptanol	111-70-6	1	g	19.9
42.141	benzaldehyde	benzaldehyde	100-52-7	2	g	ND
47.128	nonan-1-ol	nonanol	143-08-8,	1	g	15.5
51.111	2-phenylethyl acetate	2-phenylethyl acetate	103-45-7	1	i	7.8
52.501	2-phenylethan-1-ol	phenylethanol	60-12-8	2	i	9.1
53.574	phenol	phenol	108-95-2	2	i	5.8
40 385	acetic acid	acetic acid	64-19-7	2	j	9.0
49 609	3-methylbutanoic acid	isovaleric acid	72-18-4	2	k	7.6
53 233	pentanoic acid	valeric acid	109-52-4	1	k	12.2
55 272	octanoic acid	caprylic acid	124-07-2	2	l	15.9
56 279	nonanoic acid	nonanoic acid	112-05-0	2	l	14.3
57 369	decanoic acid	capric acid	334-48-5	2	m	14.6

1: tentatively identified based on the mass spectrum and the linear retention index; 2: identified by comparison with reference standard

Quantified using the response factor of a: 3-methylbutanal; b: ethyl acetate; c: butane-2,3-dione; d: 2-methylpropan-1-ol; e: 3-methylbutan-1-ol; f: 6-methyl-5-hepten-2-one; g: hexan-1-ol; h: 1-octen-3-ol; i: phenylethanol; j: acetic acid; k: 3-methylbutanoic acid; l: nonanoic acid; m: decanoid acid.

ND: not detected/not determined; RSD: analytical repeatability expressed as relative standard deviation (%)

Annexe SG2 : Descriptors and associated standards with concentrations for sensory analysis and panel training.

Descriptor	Standard	Concentrations
Sweetness	Sucrose	10-50 (g L^{-1})
Sourness	Lactic acid	0.045-0.36 (g L^{-1})
Bitterness	Caffein	1.5 (g L^{-1})
Astringent	Grape tannins	0.6 (g L^{-1})
Vinegar	Acetic acid	0.3-2.4 (g L^{-1})
Tea	Black and green tea infusion from Les jardins de Gaïa (Wittisheim, France) at room temperature	1% (m/v)
White fruit	As an example, quince standard #11 from “Le Nez du Vin”, Editions Jean Lenoir (Paris, France)	
Exotic fruit	As an example, pineapple standard #4 from “Le Nez du Vin”, Editions Jean Lenoir (Paris, France)	
Apple juice	Commercial apple juice from Casino (Saint Etienne, France)	
Cheesy	Organic Parmesan cheese from Casino (Saint Etienne, France)	

Annexe SG3 : Population levels expressed in CFU mL^{-1} in the different cultures at 7 (d7) and 12 days (d12) after inoculation (average values, n = 3).

Samples	*B. bruxellensis*	*H. valbyensis*	*S. cerevisiae*	*A. indonesiensis* or total bacteria (BTK and GTK)
BBd7	$6.5.10^5$ bc	nd	nd	nd
HVd7	nd	$1.4.10^6$ bcd	nd	nd
AId7	nd	nd	nd	$4.2.10^6$ a
BBAId7	$7.9.10^4$ c	nd	nd	$6.8.10^5$ b
HVAId7	nd	$7.1.10^5$ cd	nd	$6.0.10^6$ a
BBHVd7	$1.0.10^6$ bc	$2.0.10^6$ bc	nd	nd
Td7	$1.5.10^6$ bc	$2.4.10^6$ ab	nd	$7.2.10^5$ b
BTKd7	$2.5.10^6$ b	$1.7.10^5$ d	nd	$2.2.10^5$ b
GTKd7	$1.2.10^6$ bc	$3.8.10^5$ d	nd	$2.0.10^4$ b
BBd12	$1.3.10^6$ bc	nd	nd	nd
HVd12	nd	$3.6.10^5$ bcd	nd	nd
AId12	nd	nd	nd	$1.7.10^6$ b
BBAId12	$5.8.10^5$ bc	nd	nd	$4.7.10^5$ b
HVAId12	nd	$1.4.10^6$ bcd	nd	$9.7.10^5$ a
BBHVd12	$8.2.10^5$ bc	$3.3.10^6$ a	nd	nd
Td12	$1.9.10^6$ bc	$3.4.10^6$ a	nd	$1.8.10^6$ b
BTKd12	$9.2.10^6$ a	$1.8.10^5$ d	$9.0.10^3$ a	$2.4.10^5$ b
GTKd12	$8.3.10^6$ a	$7.6.10^5$ cd	$1.0.10^4$ a	$7.7.10^5$ b

nd = not detected, inferior to 1.10^3 CFU mL^{-1}. Common letters signify no significant differences between values of the same column according to ANOVA ($p < 0.05$). BB= *B. bruxellensis*, HV = *H. valbyensis*, SC = *S. cerevisiae*, AI = *A. indonesiensis*, BTK = black tea kombucha, GTK = green tea kombucha.

Annexe SG4 : Non-volatile chemical parameters in the different cultures at 7 (d7) and 12 days (d12) after inoculation (average values, n = 3).

Samples	Sucrose (g L^{-1})	Glucose (g L^{-1})	Fructose (g L^{-1})	pH	Total acidity (meq L^{-1})
SBT/SGT	58.3 ± 0.9	0.3 ± 0.4	0.4 ± 0.4	6.90 ± 0.10	<1
BBd7	54.4 a	0.5 b	0.4 a	4.75 a	4.7 f
HVd7	55.5 a	0.9 b	0.3 a	4.74 a	4.7f
AId7	54.4 a	2.9 ab	1.4 a	4.36 cde	5.0 f
BBAId7	55.3 a	0.7 b	0.6 a	4.43 bcd	9.7 ef
HVAId7	49.4 ab	1.0 b	0.5 a	4.51 abc	7.0 f
BBHVd7	50.3 ab	1.3b	0.7 a	4.68 ab	6.3 f
Td7	56.1 a	0.2 b	0.2 a	4.41 bcd	12.0 de
BTKd7	50.7 ab	0.7 b	0.4 a	4.09 cdef	20.7 c
GTKd7	50.0 ab	0.2 b	0.0 a	4.21 cdef	19.0 c
BBd12	45.1 ab	2.5 ab	1.1 a	4.17 defg	14.0 de
HVd12	50.7 ab	2.8 b	1.2 a	4.49abc	8.7 ef
AId12	43.6 ab	4.9 a	2.3 a	3.87 gh	13.7 c
BBAId12	39.1 b	1.1 b	0.0 a	4.04 fg	20.0 cd
HVAId12	46.3 ab	2.0 b	0.0 a	3.95 fg	15.7 cd
BBHVd12	52.2 ab	0.1 b	0.0 a	4.24 cde	14.0 de
Td12	52.0 ab	0.5 b	0.4 a	4.15 defg	20.0 cd
BTKd12	42.6 ab	1.6 b	0.3 a	3.69 h	40.7 a
GTKd12	47.6 ab	0.7 b	0.0 a	4.00 gf	28.3 b

Common letters signify no significant differences between values of the same column according to ANOVA ($p < 0.05$). SBT = sugared black tea, SGT = sugared green tea , BB= *B. bruxellensis*, HV = *H. valbyensis*, SC = *S. cerevisiae*, AI = *A. indonesiensis*, BTK = black tea kombucha, GTK = green tea kombucha.

Annexe SG5 : Concentration in volatile compounds in monocultures and sugared black tea (average values in µg/L, n = 3).

Sample	acetaldehyde	methyl acetate	ethyl acetate	2-methylbutanal	isovaleraldehyde	ethanol	ethyl propanoate	ethyl isobutyrate	propyl acetate	2-pentanone	diacetyl	hexanal	isobutanol	isoamylacetate	tert-butanol
SBT	nd	3.34	19.59b	1.2	3.7	2167.21de	nd	nd	nd	3.41	nd	149.87a	nd	nd	19.66
BBd7	13.56a	2.32	220.28b	8.04	1.56	25438.89b	nd	10.79b	nd	3.31	74.28	nd	240.86	10.32	463.61
HVd7	28.02a	13.5	11871.94a	nd	nd	8117.25c	77.37a	2.79b	19.77a	3.86	56.98	nd	214.54	1054.95	591.41
AId7	nd	nd	498.25b	nd	nd	190.074e	nd	2.37b	nd	2.8	51.02	40.36b	44.38	7.36	375.09
BBd12	7.82a	9.8	709.82b	nd	nd	34097.40a	nd	26.18a	nd	2.17	181.89	nd	311.81	0.37	367.17
HVd12	29.90a	5.78	11784.49a	nd	nd	11181.82c	45.73b	3.76b	16.91a	5	30.18	33.49b	367.06	1260.87	422.99
AId12	13.31a	4.46	450.39b	4.11	3.79	6533.66cd	nd	2.35b	nd	2.11	52.65	40.07b	336.72	16.5	418.46
p-value	0.04	0.347	<0.001	0.097	0.06	<0.001	<0.001	<0.001	<0.001	0.191	0.08	<0.001	0.212	0.095	0.062

Common letters signify no significant differences between values of the same column according to ANOVA ($p < 0.05$). BB = *B. bruxellensis*. HV = *H. valbyensis*. AI = *A. indonesiensis*. SBT = sugared black tea.

isoamyl alcohol	acetoin	6-methyl-5-hepten-2-one	hexanol	1-octen-3-ol	heptanol	benzaldehyde	nonanol	2-phenylethyl acetate	phenylethanol	phenol	acetic acid	isovaleric acid	valeric acid	caprylic acid	nonanoic acid	capric acid
7.63c	nd	5.46	0.87c	0.24c	0.40d	5.63	1.79c	35.54b	18.30b	18.78	nd	nd	nd	nd	nd	nd
556.34bc	33.5	4.72	3.32abc	1.03a	2.67ab	4.34	3.18c	27.25b	62.94ab	27.71	21697.41b	443.27b	88.76b	59	44.69b	17.07
1817.18ab	18.04	6.2	5.48a	0.76ab	3.51a	5.96	6.35ab	967.20a	139.24ab	31.77	40844.57b	311.31b	181.36ab	111	150.55a	49.11
74.67c	45.86	3.22	0.88c	0.26c	0.24d	0.7	1.32c	48.60b	34.25b	30.16	157346.82b	950.38ab	217.90ab	95.63	132.77a	29.19
981.60bc	100.38	3.66	2.30c	0.49bc	1.40cd	2.06	3.77bc	48.96b	199.21ab	26.57	55185.82b	1675.26a	222.97ab	182.67	136.23a	28.54
2246.82a	53.61	4.72	4.15ab	0.22c	3.85a	7.45	7.53a	197.78b	237.41a	15.43	29692.74b	403.35b	372.94a	47.07	50.11b	17.14
852.16bc	102.44	3.48	0.80bc	0.30c	1.70bc	nd	2.28c	123.82b	144.98ab	23.41	514361.63a	1598.37a	211.75ab	123.88	78.09ab	46.31
<0.001	0.298	0.259	0.007	0.001	0.1637	0.053	<0.001	<0.001	0.016	0.0576	<0.001	0.002	0.008	0.09	<0.001	0.141

Annexe SG6 : Concentration in volatile compounds in sugared teas, cocultures and kombuchas (average values in µg/L, n = 3).

Sample	acetaldehyde	methyl acetate	ethyl acetate	2-methylbutanal	isovaleraldehyde	ethanol	ethyl propanoate	ethyl isobutyrate	propyl acetate	2-pentanone	diacetyl	hexanal	isobutanol	isoamylacetate	tert-butanol
SBT	nd	3.34cd	19.59e	1.21a	3.70a	2167.20c	nd	nd	nd	3.41ab	nd	149.87a	nd	nd	19.66
SGT	nd	3.87cd	27.21e	2.41a	4.04a	2185.91c	nd	nd	nd	3.33ab	nd	314.99a	nd	nd	121.43
BBAId7	15.86a	8.36b	669.09e	2.57a	0.93b	4377.53c	nd	14.71cd	nd	2.19abc	33.67b	39.01c	207.06c	2.79d	462.44
HVAId7	9.46ab	10.58ab	11410.13a	nd	nd	1576.22c	33.97b	9.67d	17.90a	3.72a	31.76b	46.16c	66.76d	847.40b	330
Td7	9.32ab	5.30c	7867.73b	3.62a	3.38a	6833.60c	15.00c	20.23cd	3.67c	1.33abc	8.81b	37.47c	162.49cd	331.09c	282.97
BTKd7	11.53ab	2.32cd	1935.60cde	3.68a	1.88b	3467.12c	nd	19.91cd	1.36c	1.08abc	16.62b	56.85c	186.71cd	13.60d	518.39
GTKd7	13.77ab	1.96d	1390.46de	1.91a	0.71b	4596.26c	1.91c	35.45b	nd	3.70a	18.30b	61.78c	133.66cd	8.24d	184.42
BBAId12	11.28ab	12.48a	2026.11cde	3.21a	0.81b	19951.95b	nd	34.92b	nd	1.20bc	22.32b	37.87c	362.78b	19.02d	450.6
HVAId12	7.35b	4.18cd	11885.61a	nd	nd	5116.21c	82.63a	7.49d	11.88b	1.82abc	21.22b	34.93c	252.55bc	1967.11a	429.32
Td12	11.45ab	10.53ab	2981.79cd	2.71a	2.11a	16988.01b	7.00c	53.86a	0.56c	1.18abc	18.92b	42.06c	376.16b	74.70d	525.56
BTKd12	9.83ab	2.02d	3587.7c	2.41a	1.66b	26077.71a	nd	16.02cd	1.03c	0.58c	62.70a	32.40c	676.95a	51.07d	386.61
GTKd12	13.50ab	1.76d	2930.78cd	1.82a	1.70b	19997.98b	2.85c	29.90bc	0.54c	0.61c	5.26b	32.35c	348.55b	31.20d	633.12
p-value	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001	<0.001	0.197

Common letters signify no significant differences between values of the same column according to ANOVA ($p < 0.05$). SBT= sugared black tea. SGT = sugared green tea. BB = *B. bruxellensis*. HV = *H. valbyensis*. AI = *A. indonesiensis*. BTK

isoamyl alcohol	acetoin	6-methyl-5-hepten-2-one	hexanol	1-octen-3-ol	heptanol	benzaldehyde	nonanol	2-phenylethyl acetate	phenylethanol	phenol	acetic acid	isovaleric acid	valeric acid	caprylic acid	nonanoic acid	capric acid
7.64e	nd	5.46bc	0.87	0.24a	0.40b	5.63a	1.79b	35.54c	18.30e	18.78cd	nd	nd	nd	nd	nd	nd
13.46e	nd	13.59a	0.62	0.45a	0.38b	5.68a	1.22b	45.02c	38.61de	16.49d	nd	nd	nd	nd	nd	nd
218.97e	36.92abcd	3.42cd	2.3	0.53a	2.21a	4.84a	3.71b	77.30c	87.12cde	40.45a	866467.15b	1767.18b	386.57abc	167.04bcde	116.55a	32.84b
397.00de	56.76ab	4.25cd	0.88	0.40a	1.67ab	nd	2.34b	1179.14b	89.98cde	25.25bcd	691995.94b	4857.48ab	224.08c	72.65de	77.85a	37.05b
688.62cd	12.41cd	3.91cd	2.26	0.51a	1.83a	nd	nd	448.07c	129.15bcd	35.17ab	722200.34b	2453.10b	312.86c	233.41abcd	129.79a	49.18ab
436.05de	12.20cd	2.27d	1.44	0.25a	0.22b	2.29abc	nd	101.30c	166.74bc	28.65abcd	1602754.82a	3347.92b	346.92bc	298.35ab	144.61a	88.63a
391.56de	11.08cd	6.40b	1.48	0.50a	0.26b	nd	1.31b	27.51c	115.27bcde	33.81abc	1510188.16a	4038.12b	485.40ab	368.05a	186.14a	74.25ab
797.54cd	44.83abc	3.60cd	1.41	0.45a	1.67ab	nd	7.20a	139.84c	209.34b	20.81bcd	1130535.90ab	3182.02b	381.15abc	272.67abc	147.64a	50.54ab
939.80bc	60.54a	5.22bc	0.41	0.35a	1.60ab	1.08bc	1.75b	2280.02a	145.14bcd	27.46abcd	942225.85b	6399.95a	240.20c	97.41cde	116.02a	51.96ab
1244.57b	33.22abcd	4.37cd	2	0.28a	0.31b	nd	nd	157.78c	202.46b	26.58abcd	1050798.93ab	3317.22b	281.33c	189.12abcd	124.36a	39.53b
1751.50a	23.36bcd	2.36d	2.24	0.28a	0.80ab	3.59ab	3.53b	84.08c	460.51a	21.43bcd	1260493.82ab	3419.31b	464.93ab	377.71a	115.46a	67.46ab
1235.24b	5.19d	3.57cd	1.18	0.49a	2.21a	1.01bc	2.78b	100.32c	212.39b	14.56d	1219657.47ab	3023.21b	525.32a	339.76ab	158.59a	69.81ab
<0.001	<0.001	<0.001	0.035	0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001

K = black tea kombucha. GTK = Green tea kombucha.

Annexe SG7 : Concentration in volatile compounds in sugared black tea, cocultures and kombucha issued from minimal consortia (average values in µg/L, n = 3).

Sample	acetaldehyde	methyl acetate	ethyl acetate	2-methylbutanal	isovaleraldehyde	ethanol	ethyl propanoate	ethyl isobutyrate	propyl acetate	2-pentanone	diacetyl	hexanal	isobutanol	isoamylacetate	tert-butanol
SBT	nd	3.34	19.59b	1.2	3.70a	2167.21cd	nd	nd	nd	3.41a	0	149.87a	nd	nd	19.66b
BBd7	13.57ab	2.32	220.28b	8.04	1.56b	25438.89a	nd	10.79a	nd	3.31a	74.28	nd	240.86a	10.32c	463.61a
HVd7	28.03a	13.5	11871.94a	nd	nd	8117.25c	77.36ab	2.79a	19.77a	3.86a	56.98	nd	214.54a	1054.96a	591.41a
AId7	nd	0	498.25b	nd	nd	190.07d	nd	2.37a	nd	2.80a	51.02	40.37b	44.38bc	7.36c	375.08ab
BBAId7	15.86ab	8.36	669.09b	2.57	0.93b	4377.53cs	nd	14.71a	nd	2.19a	33.67	39.01b	207.06a	2.79c	462.44a
HVAId7	9.46ab	10.58	11410.14a	nd	nd	1576.22cd	33.97bc	9.68a	17.90a	3.72a	31.76	46.16b	66.76bc	847.40ab	330.00ab
BBHVd7	20.10ab	4.57	8148.93a	nd	nd	16056.90b	106.50a	20.52a	14.77a	1.67a	16.31	36.75b	166.22ab	515.87abc	491.91a
Td7	9.32ab	5.3	7867.73a	3.62	3.38a	6833.60cd	15.00bc	20.23a	3.67a	1.33a	8.81	37.47b	162.49ab	331.09bc	282.97ab
p-value	0.029	0.283	<0.001	0.1	<0.001	<0.001	0.004	0.023	0.031	0.021	0.44	<0.001	<0.001	0.002	0.016

Common letters signify no significant differences between values of the same column according to ANOVA ($p < 0.05$). SBT = sugared black tea. BB = *B. bruxellensis*. HV = *H. valbyensis*. AI = *A. indonesiensis*. BTK = black tea kombucha. G

isoamyl alcohol	acetoin	6-methyl-5-hepten-2-one	hexanol	1-octen-3-ol	heptanol	benzaldehyde	nonanol	2-phenylethyl acetate	phenylethanol	phenol	acetic acid	isovaleric acid	valeric acid	caprylic acid	nonanoic acid	capric acid
7.64c	nd	5.46	0.87b	0.24	0.40c	5.63	1.79ab	35.54b	18.30c	18.78	nd	nd	nd	nd	nd	nd
556.34bc	33.5	4.72	3.32ab	1.03	2.67ab	4.34	3.18ab	27.26b	62.94bc	27.71	21697.41c	443.27c	88.76bc	59.00b	44.69ab	17.07
1817.18a	18.04	6.2	5.48a	0.76	3.51a	5.96	6.35a	967.20a	139.24a	31.77	40844.57c	311.312c	181.36abc	111.00ab	150.55a	49.11
74.67c	45.86	3.22	0.88b	0.26	0.24c	0.7	1.32ab	48.60b	34.25c	30.16	157346.82c	950.38c	217.90ab	95.63ab	132.77a	29.19
218.97c	36.92	3.42	2.30b	0.53	2.21ab	4.84	3.71ab	77.30b	87.12b	40.45	866467.15a	1767.18b	386.57a	167.04ab	116.55a	32.83
397.00bc	56.72	4.25	0.88b	0.4	1.67bc	nd	2.34ab	1179.14a	89.98b	25.25	691995.94b	4857.48a	224.08ab	72.65ab	77.85ab	37.05
987.86b	6.05	4.85	4.25ab	0.71	1.88bc	6.34	6.96a	488.35b	146.21a	23.7	47234.42c	661.61c	168.30abc	148.50ab	56.79ab	38.4
688.62bc	12.41	3.91	2.26b	0.51	1.82bc	nd	nd	448.07b	129.16a	35.17	722200.34b	2453.10b	312.86a	233.41a	129.79a	49.18
<0.001	0.139	0.147	0.005	0.052	0.001	0.091	0.017	<0.001	<0.001	0.056	<0.001	<0.001	0.001	0.01	0.005	0.07

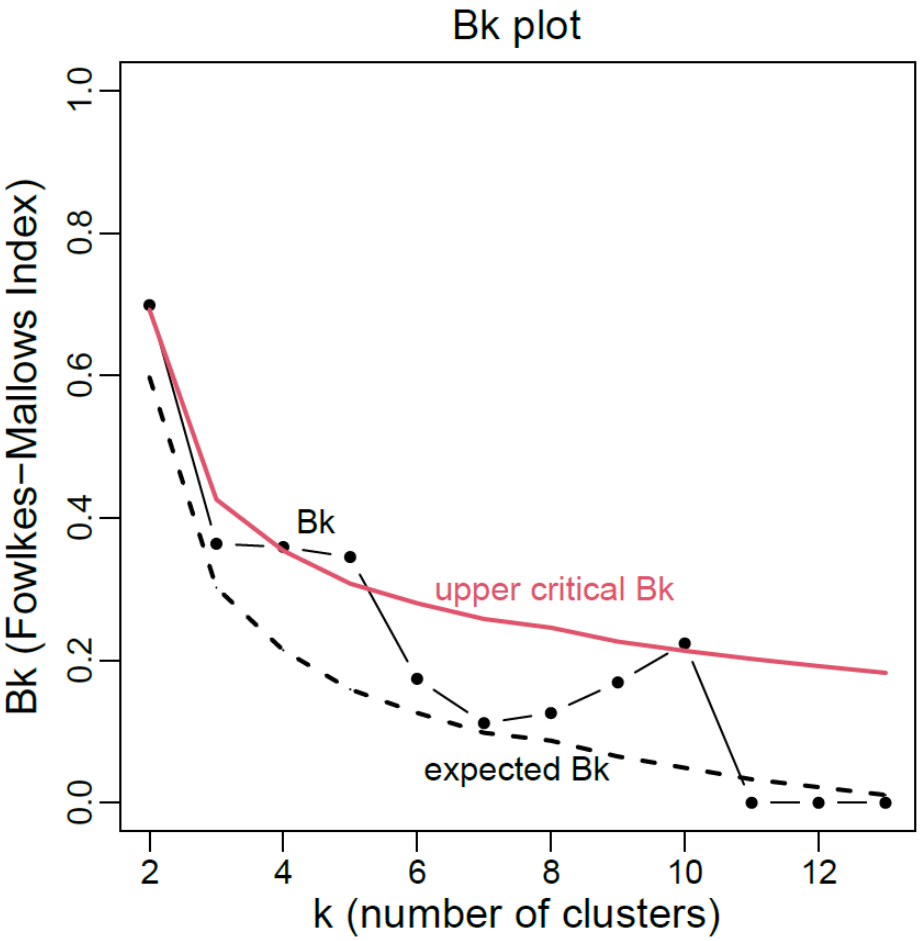
GTK = Green tea kombucha.

Annexe SG8 : Olfactive and gustative scores (/10) of samples per descriptor.

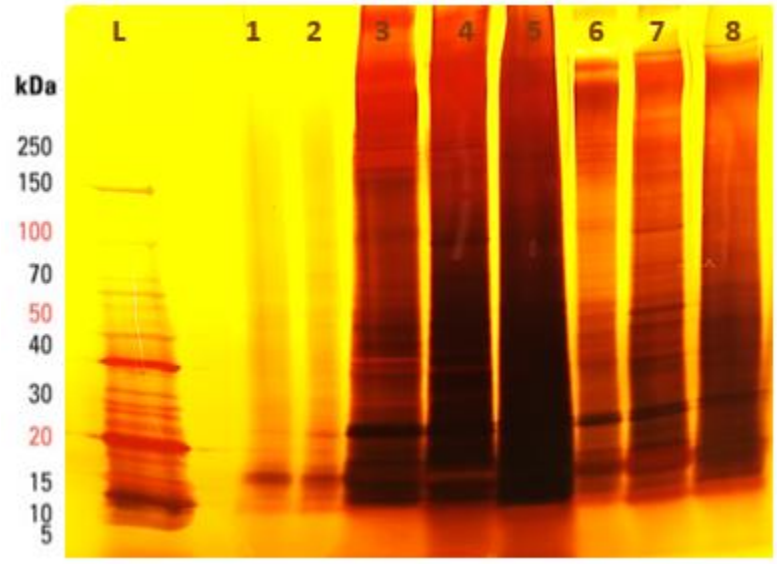
Sample	Tea	Vinegar	Apple Juice	White Fruits	Exotic Fruits	Cheesy	Sweetness	Sourness	Bitterness	Astringency
BBd7	1.3 cd	1.8	0.8 c	1.4 bc	0.8 b	7.4 a	not determined	not determined	not determined	not determined
HVd7	4.6 a	1.9	1.9 bc	3.4 abc	1.1 ab	2.3 b	not determined	not determined	not determined	not determined
AId7	1.0 d	3.3	1.8 bc	0.9 c	0.7 b	8.7 a	not determined	not determined	not determined	not determined
BBAId7	2.8 abcd	2.1	4.7 ab	4.5 a	2.5 ab	0.6 b	6.3	1.2 b	1.8	2.1
HVAId7	5.8 a	1.9	3.7 abc	4.5 ab	1.5 ab	0.8 b	5.6	1.6 b	1.0	1.6
BBHVd7	4.0 abc	2.4	2.8 abc	2.4 abc	0.8 b	1.4 b	not determined	not determined	not determined	not determined
Td7	3.4 abcd	3.4	3.9 ab	2.7 abc	1.1 ab	1.2 b	5.4	2.5 ab	1.3	2.3
BTKd7	2.8 abcd	2.8	4.9 ab	3.4 abc	1.4 ab	2.9 b	5.6	2.8 ab	1.9	2.5
GTKd7	4.4 ab	3.4	3.1 abc	2.7 abc	1.5 ab	2.4 b	5.8	3.3 ab	1.9	1.6
BBAId12	2.3 abcd	2.4	3.9 ab	2.8 abc	2.3 ab	2.0 b	3.9	4.1 a	1.5	1.7
HVAId12	3.0 abcd	3.3	5.4 a	4.1 ab	1.9 ab	1.8 b	4.0	5.1 a	2.6	3.3
Td12	2.0 abcd	3.6	4.0 ab	3.1 abc	3.1 a	2.2 b	4.1	4.6 a	1.7	2.0
BTKd12	1.9 abcd	4.9	3.6 abc	1.9 abc	2.5 ab	2.2 b	4.9	4.1 ab	1.6	2.3
GTKd12	1.5 bcd	3.0	1.9 bc	1.3 bc	0.9 ab	8.3 a	5.7	4.7 a	2.3	2.1
p value (Sample)	< 0.001	0.118	< 0.001	< 0.001	0.001	< 0.001	0.672	< 0.001	0.777	0.762

Common letters signify no significant differences between values of the same column according to ANOVA ($p < 0.05$). BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*, BTK = black tea kombucha, GTK = Green tea kombucha.

Annexe SG9 : Similarity index of the two dendrograms obtained from the sensory scores and the volatile metabolites concentrations for the 32 detected metabolites among the 14 samples as function of k clusters selected.



Annexe SH1 : SDS-PAGE electrophoretic protein profile of *Acetobacter indonesiensis* samples. L = Ladder. Lanes 1 and 2 = supernatant at day 7 and 12, respectively. Lanes 3 to 5 = repetitions of cytosolic material samples without dilution (70-170 µg/mL). Lanes 6 to 8 = repetitions of diluted cytosolic material (0.28-0.29 µg/mL).



Annexe SH1 : Difference in sucrose in cultures between day 0 and the endpoint (day 1, 3, 5, 7, 10 or 12) during the two-phase kombucha production. *B. bruxellensis* (BB), (B) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds the coculture of all three microorganisms. No significant differences were detected between average values (n=3) according to ANOVA (α=0.05).

Day	1	3	5	7	9	12
BB	-2.0	-1.6	-0.5	-2.8	-1.2	-3.3
HV	-7.8	-4.3	-5.9	-5.2	-15.1	-12.2
AI	-2.8	-18.5	-9.4	-1.3	-5.1	-9.8
BBAI	-3.8	-3.4	-2.8	-8.0	-14.0	-19.6
HVAI	-1.8	-7.1	-7.9	-6.2	-8.7	-11.0
BBHV	2.9	-6.9	-0.3	-14.0	-11.2	-15.5
T	-5.4	-8.0	-7.3	-14.3	-10.7	-24.0
Kombucha	-0.3	1.0	-2.4	-15.6	-14.8	-18.4

Annexe SH2 : Difference in glucose in cultures between day 0 and the endpoint (day 1, 3, 5, 7, 10 or 12) during the two-phase kombucha production. *B. bruxellensis* (BB), (B) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds the coculture of all three microorganisms. No significant differences between average values (n=3) according to ANOVA (α=0.05).

Day	1	3	5	7	9	12
BB	0.0	0.0	0.2	0.8	0.5	0.6
HV	-0.2	-0.3	-0.3	-0.3	-0.3	3.1
AI	0.2	0.7	0.3	0.8	1.1	2.0
BBAI	0.1	1.2	0.9	1.6	7.6	4.8
HVAI	-0.4	0.6	-0.1	-0.3	-0.3	2.8
BBHV	-0.2	0.3	-0.3	-0.2	0.3	3.0
T	0.3	1.6	0.5	0.2	1.7	6.2
Kombucha	0.8	0.6	0.6	0.3	0.4	0.7

Annexe SH3 : Difference in fructose in cultures between day 0 and the endpoint (day 1, 3, 5, 7, 10 or 12) during the two-phase kombucha production. *B. bruxellensis* (BB), (B) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds the coculture of all three microorganisms. No significant differences between average values (n=3) according to ANOVA (α=0.05).

Day	1	3	5	7	9	12
BB	0.5	0.1	1.3	0.4	0.8	0.7
HV	-0.2	-0.3	-0.3	-0.3	-0.1	-0.3
AI	0.4	1.0	0.6	0.9	0.7	1.4
BBAI	0.0	0.5	0.5	1.0	3.9	3.1
HVAI	0.0	1.2	-0.3	-0.2	-0.3	1.1
BBHV	0.2	0.4	0.2	0.2	0.3	1.4
T	0.1	0.7	0.4	1.2	4.4	3.2
Kombucha	0.8	0.6	0.6	2.7	2.5	2.7