UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

TESE DE DOUTORADO

PADRONIZAÇÃO DO PROCESSO PRODUTIVO DE KOMBUCHA PELA APLICAÇÃO DE TÉCNICAS DE BIOTECNOLOGIA E BIOPROCESSO

MARIANA FENSTERSEIFER FABRICIO

PORTO ALEGRE, 2022.

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**PADRONIZAÇÃO DO PROCESSO PRODUTIVO DE KOMBUCHA PELA
APLICAÇÃO DE TÉCNICAS DE BIOTECNOLOGIA E BIOPROCESSO**

Tese apresentada ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos como requisito para obtenção do título de Doutora.

Orientador: Prof. Dr. Marco Antônio Záchia Ayub
Co-orientadora: Profa. Dra. Simone Hickmann Flôres

PORTO ALEGRE, 2022.

AGRADECIMENTOS

Ao meu orientador, Marco Antônio Záchia Ayub, o qual eu admiro como pessoa, professor e orientador, por ter confiado na minha capacidade de executar o trabalho e por me ensinar tanto.

À minha co-orientadora, Simone Flôres, por todo auxílio na execução da pesquisa.

À minha mãe, pelo apoio financeiro e emocional, por me inspirar e me motivar a ir em busca dos meus objetivos.

Ao meu pai e minha irmã, por torcerem por mim e me encorajarem a tentar o meu melhor.

Aos meus amigos e amigas, por me apoiarem incondicionalmente e torcerem por mim sempre.

Aos professores, professoras e colegas do PPGCTA, pelos ensinamentos e trocas.

Aos colegas do BIOTECLAB, em especial à Bruna Vargas, pela amizade, paciência e colaboração na pesquisa.

À banca de qualificação, pelas contribuições ao longo do doutorado.

Ao professor Roger Wagner, por disponibilizar o seu laboratório de compostos voláteis na UFSM e também pela dedicação e entusiasmo em ajudar e ensinar.

Ao professor Jeverson Frazzon e Michele Mann, pela ajuda e disponibilidade nas análises de microbioma.

À professora Bruna Tischer, pelo auxílio nas análises de antioxidantes.

À Caroline Kothe, pela ajuda, pelos ensinamentos e por me motivar a buscar novas oportunidades.

À minha bolsista Julia Ramos, pela colaboração e entusiasmo durante a participação na pesquisa.

À CAPES, pelo apoio financeiro.

E por fim, à todos e todas que fizeram parte desta trajetória.

me levanto

sobre o sacrifício

de um milhão de mulheres que vieram antes

e penso

o que é que eu faço

para tornar essa montanha mais alta

para que as mulheres que vierem depois de mim

possam ver além

legado – Rupi Kaur

RESUMO

Kombucha é uma bebida obtida através da fermentação de uma infusão de *Camellia sinensis* por uma cultura simbiótica de microrganismos e vem ganhando cada vez mais popularidade, em função de seus possíveis efeitos benéficos à saúde. A composição microbiológica, predominada por bactérias ácido acéticas e leveduras, é complexa e muito diversa entre kombuchas e bateladas. Isso resulta em processos e produtos finais muito diferentes e normalmente em concentrações alcoólicas acima dos limites permitidos pela legislação. As variáveis do processo, como temperatura, pH, concentração de substratos, geometria do tanque de fermentação e tempo de cultivo, contribuem para o controle da fermentação, no entanto não são suficientes para garantir a padronização do processo. Em função da dificuldade de controle do processo fermentativo, o objetivo geral do presente trabalho foi estudar o perfil microbiológico de kombucha, avaliando a interação de sua microbiota com os produtos formados durante a fermentação, e desenvolver uma cultura starter a partir de microrganismos isolados, para a obtenção de um produto padronizado, com boa aceitação sensorial e dentro das exigências da legislação. Para isso, o perfil de bactérias e leveduras de uma cultura starter tradicional de kombucha do Rio Grande do Sul foi avaliado através de sequenciamento de nova geração. A cultura starter foi processada de três maneiras diferentes (controle, cultura starter centrifugada e cultura starter liofilizada) para avaliar as mudanças na composição microbiológica, bioquímica e sensorial da kombucha. Foi possível identificar sete gêneros de bactérias na cultura starter, incluindo *Komagataeibacter, Gluconacetobacter, Gluconobacter, Acetobacter, Liquorilactobacillus, Ligilactobacillus* e *Zymomonas*, e três gêneros de levedura, *Dekkera/Brettanomyces, Hanseniaspora* e *Saccharomyces*. Apesar de não ter diferido significativamente no teste de aceitação sensorial, diferentes processamentos da cultura starter tradicional resultaram em produtos com diferentes composições bioquímicas e microbiológicas. Embora tenhas sido possível liofilizar a cultura starter, esta não garantiu a padronização do produto final. Em função disso, foram realizados mais estudos visando a padronização do produto e controle da produção de álcool, desta vez através do desenvolvimento de uma cultura starter com microrganismos de interesse isolados e selecionados. Foi desenvolvido um inóculo com a bactéria acética *Komagataeibacter saccharivorans* e as leveduras *Dekkera anomala* e *Kluyveromyces marxianus fragilis* (probiótica). O tempo de fermentação utilizando essa combinação de microrganismos foi reduzido para 48 h e dois métodos de carbonatação foram testados (natural e forçada) e submetidos a uma análise de vida de prateleira. As kombuchas obtidas foram comparadas com duas marcas comerciais em relação aos metabólitos, compostos voláteis, aceitação sensorial e atividade antioxidante. A kombucha obtida através da carbonatação forçada obteve a mesma aceitação que as bebidas comerciais e o teor alcoólico permaneceu dentro dos limites permitidos pela legislação por 60 dias. Em relação aos compostos voláteis, os ésteres foram a classe que mais impactou a composição e diferenciou as kombuchas. Este trabalho trouxe novas informações a respeito da composição microbiológica, perfil aromático e outras características de kombuchas, além de uma nova abordagem para a produção de uma bebida padronizada e estável.

Palavras-chave: cultura starter; microbioma; alimentos fermentados; compostos voláteis; análise sensorial.

ABSTRACT

Kombucha is a beverage obtained through the fermentation of *Camellia sinensis* infusion performed by a complex symbiotic culture of microorganisms and has been rising in popularity, due to its possible beneficial health effects. The microbial composition, dominated by acetic acid bacteria and yeasts, is very complex and diverse among commercial kombuchas and fermentation batches. It results in a highly variable process and end products and, usually, the alcohol content surpasses the legal limits. Process variables, such as temperature, pH, the concentration of substrates, vessel geometry, and time, help to control the fermentative process but are not sufficient to assure the standardization of kombucha production. Due to the difficulty of controlling the fermentation process, the objective of the present work was to study the microbiological profile of kombucha, evaluating the interaction of its microbiota with the products formed during fermentation, and to develop a starter culture from isolated microorganisms, in order to obtain a standardized product, with satisfactory sensory acceptance and within the requirements of the legislation. For this, the profile of bacteria and yeasts of a traditional kombucha starter culture from Rio Grande do Sul was evaluated through next-generation sequencing. The starter culture was processed in three different ways (control, centrifuged starter culture, and freeze-dried starter culture) to assess changes in the microbiological, biochemical, and sensory characteristics of the kombucha. We identified seven genera of bacteria, including *Komagataeibacter, Gluconacetobacter, Gluconobacter, Acetobacter, Liquorilactobacillus, Ligilactobacillus,* and *Zymomonas,* and three genera of yeasts, *Dekkera/Brettanomyces, Hanseniaspora*, and *Saccharomyces*. Although there were no statistically significant differences in the acceptance test in sensory analysis, different starter cultures resulted in products showing different microbial and biochemical compositions. Even though it was possible to freeze-dry the starter culture, it did not assure the standardization of the final product. As a result, further studies were carried out to standardize the product and control the production of alcohol, through the development of a starter culture with isolated and selected microorganisms of interest. A starter culture was developed using the acetic acid bacteria *Komagataeibacter saccharivorans*, and the yeasts *Dekkera anomala*, and *Kluyveromyces marxianus fragilis* (probiotic). The fermentation time of the best combination of microbes was optimized for 48 h and two carbonation methods were tested (forced and natural carbonation) and the shelf-life was evaluated. The kombuchas were compared to two commercial brands regardings their metabolites, volatile compounds, sensory acceptance, and antioxidant activity. The acceptance of the forced carbonated Kombucha was similar to the commercial brands, and the alcohol content remained within legal limits during 60 days of storage. Regarding volatile compounds, the esters were the most impactful compounds that differentiated the kombuchas. This work brings new information about the microbial composition, aromatic profile, and other characteristics of kombuchas, in addition to a new approach for the production of a standardized and stable beverage.

Keywords: Starter culture; microbiome; fermented foods; volatile compounds; sensory analysis.

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INTRODUÇÃO

A população mundial tem demonstrado cada vez mais interesse em alimentos funcionais, em razão dos efeitos positivos à saúde e ao bem-estar físico e emocional (BIRCH; BONWICK, 2019; DINI, 2019). Esta categoria de alimentos inclui aqueles que contém compostos capazes de melhorar a saúde em geral ou reduzir o risco de doenças específicas (KAPSAK *et al.*, 2011). É neste contexto que a kombucha, uma bebida tradicional obtida através da fermentação de chá verde e/ou preto, está ganhando popularidade (DE FILIPPIS *et al.*, 2018). Caracterizada como um produto ácido, levemente doce e gaseificado, a kombucha é composta por açúcares, ácidos orgânicos, vitaminas, aminoácidos, minerais e componentes oriundos do chá (DE FILIPPIS *et al.*, 2018; VITAS *et al.*, 2018).

A fermentação da kombucha acontece a partir de uma associação simbiótica de microrganismos, conhecida como SCOBY (*Symbiotic Colony of Bacteria and Yeast*), composto por celulose, bactérias acéticas e leveduras (DE FILIPPIS *et al.*, 2018; MARSH *et al.*, 2014). Os principais gêneros que compõe o SCOBY são *Acetobacter e Komagataeibacter* (anteriormente *Gluconacetobacter*) para bactérias e *Zygosaccharomyces e Dekkera/Brettanomyces* para leveduras (ARıKAN *et al.*, 2020; CHAKRAVORTY *et al.*, 2016; COTON *et al.*, 2017; MARSH *et al.*, 2014; VILLARREAL-SOTO *et al.*, 2020; WATAWANA *et al.*, 2016). A celulose, que compõe o SCOBY, é produzida a partir da glicose por alguns gêneros de bactérias, como *Aerobacter*, *Agrobacterium*, *Azotobacter*, *Rhizobium*, e *Gluconacetobacter* (MOHITE; PATIL, 2014).

A produção da kombucha é realizada através da infusão de folhas de chá verde e/ou preto adoçado, inoculado com uma película de celulose e uma porcentagem de kombucha pronta, contendo os microrganismos fermentadores (VILLARREAL-SOTO *et al.*, 2018). A fermentação ocorre em temperatura ambiente e a duração varia entre 7 e 14 dias (GREENWALT *et al.*, 1998). Durante o processo, as leveduras hidrolisam a sacarose em glicose e frutose e utilizam esses monossacarídeos para produção de etanol e dióxido de carbono. As bactérias oxidam o etanol produzido a ácido acético e a glicose à ácido glucônico (GREENWALT *et al.*, 1998; SIONEK *et al.*, 2017).

Em relação aos efeitos benéficos à saúde, alguns autores sugerem que o consumo de kombucha pode reduzir a pressão sanguínea, aliviar artrite, aumentar

imunidade, longevidade e melhorar a resposta contra câncer (DUFRESNE; FARNWORTH, 2000; HARTMANN *et al.*, 2000). Um dos responsáveis por esses benefícios seria o ácido glicurônico, que pode ser produzido durante a fermentação e é conhecido por seus efeitos desintoxicantes (NGUYEN *et al.*, 2015). Além dos ácidos orgânicos, os antioxidantes oriundos do chá e da fermentação também são responsáveis pelos efeitos positivos para saúde humana (JAYABALAN *et al.*, 2014; MALBAŠA *et al.*, 2011). Apesar de todos os benefícios citados na literatura, não há estudo algum realizado em humanos, e, portanto, não há evidência científica que comprove tais afirmações (MARTINI, 2018; MORALES, 2020; VARGAS *et al.*, 2021).

Ao final do processo de fermentação, o líquido contém microrganismos viáveis, que podem conferir benefícios à microbiota intestinal. No entanto, por ser um processo artesanal, a composição destes microrganismos varia muito entre kombuchas em geral, mesmo entre lotes de um mesmo fabricante e, portanto, o produto final pode ou não conter as características benéficas desejadas. As evidências científicas são limitadas a respeito deste assunto (MARTINI, 2018; VARGAS *et al.*, 2021).

Apesar de todos os benefícios para saúde sugeridos na literatura, produtos preparados a partir de fermentações espontâneas como a kombucha resultam em processos e produtos finais altamente variáveis, apresentando composição microbiológica e bioquímica muito diversa (DE FILIPPIS *et al.*, 2018; TRAN *et al.*, 2020; VILLARREAL-SOTO *et al.*, 2018). O processo fermentativo e o produto final variam muito em função dos microrganismos utilizados, das condições utilizadas (pH, temperatura, tempo) e das concentrações e tipos de substratos (DE FILIPPIS *et al.*, 2018; JAYABALAN *et al.*, 2014).

No Brasil, a kombucha é um produto relativamente novo e teve sua legislação criada em 2019, que determina os padrões de identidade e qualidade da bebida (BRASIL, 2019). No entanto, as indústrias têm muita dificuldade em controlar o processo, sendo a principal preocupação o limite de álcool produzido e as perdas na produção por bateladas que originam compostos voláteis desagradáveis.

Com base na variabilidade que esse tipo de bebida pode sofrer, é de grande importância o desenvolvimento de um estudo aprofundado que normalize os parâmetros de produção, promovendo a padronização do produto final e garantindo os benefícios à saúde. Diversos outros produtos de fermentação como vinho, cerveja e iogurte passaram pelo processo de desenvolvimento de culturas *starters*

com composição conhecidas e hoje em dia são totalmente controlados. A padronização de culturas *starters* que garantam a segurança e qualidade do produto são cruciais em todos os níveis de fermentação, sejam estas caseiras, artesanais ou ao nível industrial (CAPOZZI *et al.*, 2017) A adição de culturas starters seletivas neste tipo de processo controlam a fermentação, reduzindo o risco de perda de bateladas e aumentando a segurança, estabilidade e qualidade sensorial do produto final (VINICIUS DE MELO PEREIRA *et al.*, 2020).

Com isso, buscou-se estudar a influência da cultura starter em kombucha a fim de desenvolver uma tecnologia que garanta um produto sensorialmente agradável, com as características bioquímicas exigidas pela legislação e a padronização dos metabólitos produzidos.

OBJETIVOS

Objetivo geral

O objetivo geral deste trabalho foi estudar o perfil microbiológico de kombucha, avaliando a interação de sua microbiota com os metabólitos formados durante a fermentação, e desenvolver uma cultura *starter* padronizada e segura, que resulte em um produto boa aceitação sensorial e com características probióticas.

Objetivos específicos

- Caracterizar o perfil microbiológico de uma amostra comercial de kombucha produzida no Rio Grande do Sul;
- Avaliar a interação entre os microrganismos identificados no SCOBY e no líquido com os metabólitos formados durante a fermentação;
- Avaliar a possibilidade de liofilização da cultura *starter* de kombucha como forma de padronização de processo;
- Selecionar bactérias acéticas e leveduras isoladas para a elaboração de uma cultura *starter*, a fim de obter controle total do processo tecnológico de produção de kombucha;
- Testar a adição de microrganismos probióticos à cultura *starter*;
- Avaliar, através de cinética, a formação de ácidos orgânicos, etanol e atividade antioxidante a partir de diferentes inóculos;
- Elaborar uma kombucha dentro dos limites legais de concentração de ácido acético e etanol, através de cultura *starter* sintética, com tempo de fermentação reduzido;
- Realizar análise sensorial das kombuchas produzidas, comparando com marcas comerciais;
- Analisar os compostos voláteis formados durante a fermentação;
- Avaliar a influência do método de carbonatação no produto final e na estabilidade das kombuchas;
- Avaliar a vida de prateleira das kombuchas produzidas.

CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

1.1 História e definição

Embora não se saiba a origem exata da kombucha, supõe-se que teve origem na Rússia ou na China (DE FILIPPIS *et al.*, 2018; MAYSER *et al.*, 1995). A bebida, que vem sendo consumida há mais de 2000 anos, é produzida através da fermentação de chá e açúcar por uma associação simbiótica de microrganismos, conhecida como SCOBY (*Symbiotic Colony of Bacteria and Yeast*) (DE FILIPPIS *et al.*, 2018). A fermentação resulta na formação de uma película de celulose (Fig. 1) e em um produto final com sabor ácido e levemente adocicado (VILLARREAL-SOTO *et al.*, 2018). Segundo a legislação brasileira, pela portaria nº 41, de 17 de setembro de 2019, kombucha é definida como uma "bebida fermentada obtida através da respiração aeróbia e fermentação anaeróbia do mosto obtido pela infusão ou extrato de *Camellia sinensis* e açúcares por cultura simbiótica de bactérias e leveduras microbiologicamente ativas (SCOBY)." (BRASIL, 2019). Seu consumo é frequentemente associado a efeitos benéficos para saúde, mas, como tais propriedades não são comprovadas cientificamente, a legislação não permite alegação de produto funcional (BRASIL, 2018).

Figura 1. Kombucha fermentada contendo película de celulose.

Fonte: Mgarten (Wikimedia Commons, 2007)

1.2 Produção de kombucha

O processo de produção da kombucha é geralmente realizado conforme fluxograma apresentado na Figura 2.

Figura 2. Fluxograma do processo de produção da kombucha.
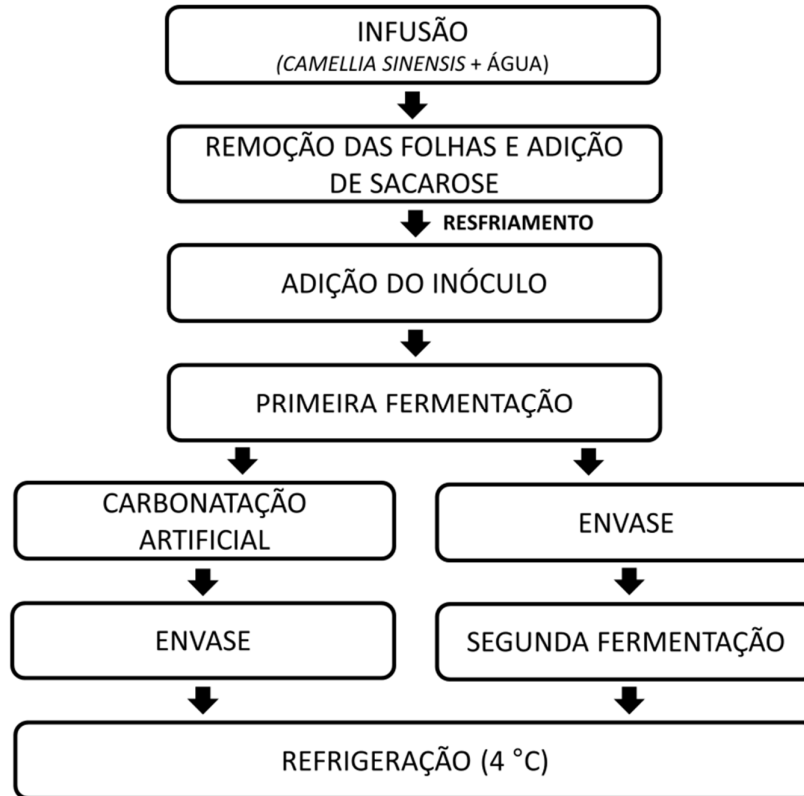
Fonte: autor, 2022.

A primeira etapa da produção é o preparo da infusão de chá adoçado, que quando atinge temperatura ambiente é inoculado. As concentrações de chá e sacarose variam bastante na literatura, sendo o mais comum utilizar entre 6 e 10 g.L^{-1} de chá e 100 g.L^{-1} de sacarose (DE FILIPPIS *et al.*, 2018; SIONEK *et al.*, 2017). O inóculo da kombucha consiste na adição da película de celulose e uma porcentagem de uma kombucha já fermentada. As concentrações destes depende de cada formulação, sendo que algumas preparações utilizam apenas o líquido fermentado como inóculo (MALBAŠA *et al.*, 2008).

A fermentação da kombucha é realizada em duas etapas. A primeira é feita em frasco de vidro ou inox, tapado com um pano que permita a passagem de ar, e a duração é bem variável, normalmente entre 7 e 14 dias (CRUM *et al.*, 2016). Após esta etapa, sempre é gerada uma nova película de celulose (SCOBY) que pode dar origem a uma nova fermentação ou ser descartada (VILLARREAL-SOTO *et al.*, 2018). Após a primeira fermentação, as películas de celulose são removidas e acondicionadas com uma quantidade de líquido fermentado, que servirá de inóculo

na próxima fermentação. Após esta etapa, pode ser feita uma segunda fermentação, que é opcional e realizada após o envase, em garrafas hermeticamente fechadas. Nesta etapa a bebida fermenta por mais 24 a 72 h, com intuito de obter carbonatação (CRUM *et al.*, 2016; GREENWALT *et al.*, 1998). Uma alternativa à segunda fermentação é a carbonatação artificial/forçada, que é permitida pela legislação brasileira e é uma tecnologia que facilita o processo, evitando riscos como explodir garrafas ou produzir altas concentrações de etanol.

Após a primeira fermentação a kombucha pode ser saborizada, através da adição de sucos de frutas, especiarias, entre outros (CRUM *et al.*, 2016). A temperatura de fermentação das kombuchas depende das concentrações de inóculo e substrato. Quando feita artesanalmente, a kombucha é fermentada a temperatura ambiente. Em nível industrial a temperatura é controlada e varia de 20 a 28 ºC (DE FILIPPIS *et al.*, 2018; VILLARREAL-SOTO *et al.*, 2018). A última etapa da produção das kombuchas é a refrigeração, que deve ser mantida até o consumo.

1.3 Composição microbiológica

A fermentação da kombucha acontece através de uma associação simbiótica de leveduras e bactérias, responsáveis pela formação de um biofilme de celulose (SCOBY), hidrólise da sacarose e formação de metabólitos. Além disso, a simbiose pode ser capaz de inibir o crescimento de bactérias contaminantes no produto (DUFRESNE; FARNWORTH, 2000; VILLARREAL-SOTO *et al.*, 2018).

Os microrganismos encontram-se dispersos no líquido e no SCOBY e a composição microbiológica pode variar entre fermentações e regiões demográficas (CHAKRAVORTY *et al.*, 2016; MARSH *et al.*, 2014). Apesar disso, alguns gêneros de microrganismos predominam, aparecendo na maioria das culturas de kombucha.

Os primeiros estudos para identificação de microrganismos em kombucha foram realizados através do isolamento de cultura em placas, o que torna os resultados limitados, devido à dificuldade de isolamento de algumas espécies e erros de identificação (Raspor and Goranovic, 2008). Nos últimos anos, diversos autores utilizaram o sequenciamento de nova geração como ferramenta para a identificação de microrganismos, reforçando as diferenças entre as comunidades microbianas em kombuchas (ARıKAN *et al.*, 2020; CHAKRAVORTY *et al.*, 2016; MARSH *et al.*, 2014; REVA *et al.*, 2015; VILLARREAL-SOTO *et al.*, 2020).

Os gêneros das bactérias predominantes na kombucha mencionados na literatura (Tabela 1) são *Acetobacter, Komagataeibacter* e *Gluconacetobacter,* os quais possuem a capacidade de produzir celulose, que dará origem ao SCOBY (VILLARREAL-SOTO *et al.*, 2018). Em relação às leveduras (Tabela 1), predominam os gêneros *Zygosaccharomyces e Dekkera/Brettanomyces* e *Saccharomyces.*

Tabela 1. Gêneros de bactérias e leveduras que compõem a população microbiana da kombucha.

Microrganismo	Referências
Bactéria	
Acetobacter	(COTON *et al.*, 2017; FABRICIO *et al.*, 2022; LIU *et al.*, 1996; MARSH *et al.*, 2014; SIEVERS *et al.*, 1995; SUHRE *et al.*, 2021; TRAN *et al.*, 2020; WATAWANA *et al.*, 2016)
Lactobacillus	(CHAKRAVORTY *et al.*, 2016; COTON *et al.*, 2017; MARSH *et al.*, 2014; WATAWANA *et al.*, 2016; YANG *et al.*, 2022)
Komagataeibacter	(ARıKAN *et al.*, 2020; CHAKRAVORTY *et al.*, 2016; FABRICIO *et al.*, 2022; GAGGÌA *et al.*, 2018; SUHRE *et al.*, 2021; TRAN *et al.*, 2020; VILLARREAL-SOTO *et al.*, 2020; WATAWANA *et al.*, 2016; YANG *et al.*, 2022)
Gluconacetobacter	(COTON *et al.*, 2017; FABRICIO *et al.*, 2022; MARSH *et al.*, 2014; SUHRE *et al.*, 2021; VILLARREAL-SOTO *et al.*, 2020; YANG *et al.*, 2010)
Gluconobacter	(CHAKRAVORTY *et al.*, 2016; COTON *et al.*, 2017; FABRICIO *et al.*, 2022; GAGGÌA *et al.*, 2018; SUHRE *et al.*, 2021; VILLARREAL-SOTO *et al.*, 2020; YANG *et al.*, 2022)
Lactococcus	(MARSH *et al.*, 2014; WATAWANA *et al.*, 2016)
Allobacalum	(MARSH *et al.*, 2014)
Bifidobacterium	(CHAKRAVORTY *et al.*, 2016; MARSH *et al.*, 2014; WATAWANA *et al.*, 2016; YANG *et al.*, 2022)
Ruminococcaceae Incertae Sedis	(MARSH *et al.*, 2014)
Thermus	(MARSH *et al.*, 2014)

Microrganismo	Referências
Bactéria	
Propioninbacterium	(MARSH *et al.*, 2014)
Enterobacter	(CHAKRAVORTY *et al.*, 2016)
Lyngbya	(CHAKRAVORTY *et al.*, 2016)
Collinsella	(CHAKRAVORTY *et al.*, 2016)
Weissella	(CHAKRAVORTY *et al.*, 2016)
Oenococcus	(COTON *et al.*, 2017; SUHRE *et al.*, 2021)
Levedura	
Zygosaccharomyces	(ARıKAN *et al.*, 2020; COTON *et al.*, 2017; GAGGÌA *et al.*, 2018; MARSH *et al.*, 2014; SIEVERS *et al.*, 1995; WATAWANA *et al.*, 2016)
Dekkera/Brettanomyces	(COTON *et al.*, 2017; FABRICIO *et al.*, 2022; GAGGÌA *et al.*, 2018; MARSH *et al.*, 2014; SUHRE *et al.*, 2021; TRAN *et al.*, 2020; VILLARREAL-SOTO *et al.*, 2020; WATAWANA *et al.*, 2016; YANG *et al.*, 2022)
Kazachtania	(MARSH *et al.*, 2014)
Pichia	(CHAKRAVORTY *et al.*, 2016; COTON *et al.*, 2017; MARSH *et al.*, 2014; SUHRE *et al.*, 2021; WATAWANA *et al.*, 2016)
Lachancea	(CHAKRAVORTY *et al.*, 2016; MARSH *et al.*, 2014; SUHRE *et al.*, 2021)
Meyerozyma	(CHAKRAVORTY *et al.*, 2016; MARSH *et al.*, 2014; YANG *et al.*, 2022)
Saccharomyces	(CHAKRAVORTY *et al.*, 2016; COTON *et al.*, 2017; FABRICIO *et al.*, 2022; MARSH *et al.*, 2014; SUHRE *et al.*, 2021; TRAN *et al.*, 2020; YANG *et al.*, 2022)
Candida	(CHAKRAVORTY *et al.*, 2016; COTON *et al.*, 2017; SUHRE *et al.*, 2021)
Kluyveromyces	(CHAKRAVORTY *et al.*, 2016)

Microrganismo	Referências
Levedura	
Debaryomyces	(CHAKRAVORTY *et al.*, 2016)
Hanseniaspora	(COTON *et al.*, 2017; TRAN *et al.*, 2020)
Torulaspora	(COTON *et al.*, 2017)

Fonte: adaptado de KUMAR e JOSHI (2016)

A simbiose entre bactérias e leveduras na fermentação da kombucha é de extrema importância (Fig. 3), visto que as leveduras presentes na kombucha são responsáveis por hidrolisar a sacarose em glicose e frutose (SIEVERS *et al.*, 1995). Além disso, as leveduras também são responsáveis pela conversão anaeróbia dos açúcares em etanol, que é posteriormente convertido em ácido acético, através de oxidação, pelas bactérias ácido acéticas (KUMAR; JOSHI, 2016). Além do ácido acético, outros metabólitos são produzidos durante a fermentação, como ácido glicurônico, ácido glucônico, ácido lático, ácido cítrico e glicerol (JAYABALAN *et al.*, 2007; SIEVERS *et al.*, 1995; SIONEK *et al.*, 2017).

As bactérias ácido acéticas, predominantes na kombucha, têm papel fundamental no processo de fermentação, pois são responsáveis pela produção da película de celulose e por metabolizar o álcool produzido em ácidos orgânicos (SIONEK *et al.*, 2017).

Figura 3. Representação esquemática dos processos bioquímicos que ocorrem durante a fermentação da kombucha.
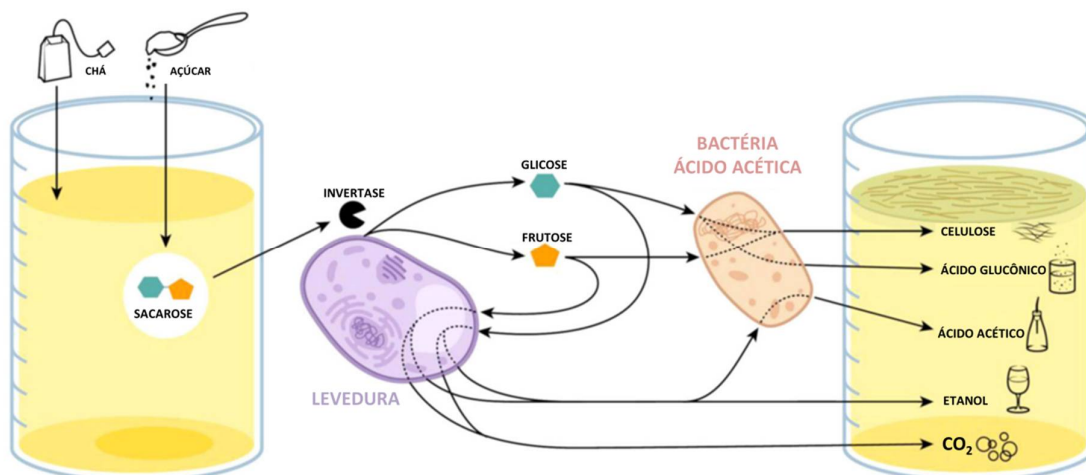
Fonte: adaptado de TRAN *et al.* (2020).

1.4 Composição química

A composição e as concentrações de metabólitos na kombucha dependem da composição microbiana do inóculo, concentração e tipo de substrato, condições de pH, temperatura e tempo de fermentação (JAYABALAN *et al.*, 2007; SIONEK *et al.*, 2017).

A kombucha apresenta em sua composição diversos ácidos orgânicos, açúcares, vitaminas, aminoácidos, etanol, minerais e dióxido de carbono (JAYABALAN *et al.*, 2014). Dentre os ácidos orgânicos estão o ácido acético, glicurônico, lático, cítrico, málico, oxálico (SIONEK *et al.*, 2017), glucônico (DE FILIPPIS *et al.*, 2018) e succínico (VITAS *et al.*, 2018). Dentre os açúcares estão a sacarose, a frutose e a glicose. A kombucha pode apresentar diversos aminoácidos essenciais e não essenciais, entre eles isoleucina, leucina, lisina, metionina, fenilalanina, valina, alanina, cisteína, ácido aspártico, ácido glutâmico, glicina, histidina, prolina, entre outros (JAYABALAN *et al.*, 2010). As vitaminas que podem ser encontradas na kombucha são B1, B2, B6 (BAUER-PETROVSKA; PETRUSHEVSKA-TOZI, 2000) e vitamina C (LONČAR *et al.*, 2006). Também podem ser encontrados polifenóis oriundos do chá (JAYABALAN *et al.*, 2007) e minerais como manganês, ferro, níquel, zinco, cobalto e cromo (BAUER-PETROVSKA; PETRUSHEVSKA-TOZI, 2000).

1.5 Fatores que influenciam a fermentação

Além da microbiota presente no SCOBY, outros diversos fatores influenciam a fermentação, como temperatura, pH, transferência de oxigênio, tipo e concentração de substrato (JAYABALAN *et al.*, 2014; MARSH *et al.*, 2014). Esses fatores influenciam diretamente o produto final, refletindo nas características sensoriais, nutricionais e físico-químicas (VILLARREAL-SOTO *et al.*, 2018).

1.5.1 Substrato

A kombucha é obtida a partir da fermentação de chá verde e/ou preto adoçados (JAYABALAN *et al.*, 2014). Esses substratos, quando metabolizados pelos microrganismos, podem resultar em diferentes produtos finais, dependendo do seu tipo e concentração (JAYABALAN *et al.*, 2014; MARSH *et al.*, 2014). REISS (1994),

avaliou a fermentação a partir de sacarose, glicose, frutose e lactose em diferentes concentrações (entre 30 e 150 g.L^{-1}) e obteve produtos finais com concentrações completamente diferentes de etanol e ácido lático, além de valores de pH entre 3,8 e 5,6 após 6 dias de fermentação.

Alguns autores testaram fermentações a partir de outros substratos, como sucos de fruta de romã, cereja, maçã (AKBARIRAD *et al.*, 2017), uva (AYED *et al.*, 2017), infusão de crisântemo, madressilva, hortelã (ZHANG *et al.*, 2021) e mil em rama (VITAS *et al.*, 2018). No Brasil, a legislação em vigor permite o uso de infusões de qualquer espécie vegetal, contanto que esteja associada à espécie *Camellia sinensis* (BRASIL, 2019).

1.5.2 Tempo de fermentação

O tempo de fermentação tem relação direta com mudanças na atividade antioxidante (CHU; CHEN, 2006), concentração de etanol, ácidos orgânicos e decréscimo do valor de pH na kombucha (LONČAR *et al.*, 2006). Embora haja interesse no aumento da capacidade antioxidante do produto final, que pode ocorrer em longas fermentações, cultivos muito longos acumulam ácidos orgânicos em níveis muito altos para o consumo e, portanto, não são recomendadas (VILLARREAL-SOTO *et al.*, 2018). O período de fermentação depende das características sensoriais desejadas para o produto final e normalmente varia entre 7 e 14 dias (GREENWALT *et al.*, 1998; VILLARREAL-SOTO *et al.*, 2018).

1.5.3 Taxa de transferência de oxigênio

A taxa de transferência de oxigênio para o líquido em fermentação influencia na composição da kombucha, visto que as bactérias ácido acéticas, predominantes no líquido, são aeróbias e convertem monossacarídeos e etanol em ácidos orgânicos (SIONEK *et al.*, 2017; VILLARREAL-SOTO *et al.*, 2018). A oxidação completa de 1 mol de etanol (46 g) em ácido acético requer um mol de oxigênio (32 g) e, portanto, a taxa de transferência de oxigênio do ar para o meio de cultura controla a atividade das bactérias ácido acéticas (VILLARREAL-SOTO *et al.*, 2018). Além disso, a geometria do vaso de fermentação tem influência na taxa de transferência de oxigênio, que reflete nas concentrações de celulose e metabólitos produzidos. Um

estudo avaliou a influência de dois vasos com diferentes razões entre superfície/altura, mas com a mesma relação superfície/volume e concluiu que a maior razão entre superfície/altura do vaso favorece a produção de etanol, enquanto a menor razão favorece a produção de ácido acético (VILLARREAL-SOTO *et al.*, 2019).

1.5.4 Temperatura

Geralmente, a fermentação da kombucha acontece em temperaturas entre 20 e 30 ^{o}C (DE FILIPPIS *et al.*, 2018; VILLARREAL-SOTO *et al.*, 2018). O efeito dessa variável tem grande importância, pois, quando otimizada, influencia positivamente no crescimento microbiano e na atividade das enzimas envolvidas no processo fermentativo (VILLARREAL-SOTO *et al.*, 2018).

A temperatura pode favorecer o metabolismo de diferentes espécies de microrganismos, resultando na formação de diferentes ácidos no produto final, como ácido glucônico e glicurônico (DE FILIPPIS *et al.*, 2018). Além disso, temperaturas mais altas favorecem a síntese de metabólitos e vitamina C para uma determinada composição microbiológica (LONČAR *et al.*, 2006).

A diferença na temperatura durante a fermentação pode mudar a simbiose entre os microrganismos presentes na cultura *starter* da kombucha, resultando em um produto final com características sensoriais e bioquímicas muito variáveis entre diferentes e sucessivos lotes, o que dificulta a padronização do processo biotecnológico de obtenção da kombucha de qualidade padronizada (DE FILIPPIS *et al.*, 2018; LONČAR *et al.*, 2006; SIONEK *et al.*, 2017).

1.5.5 pH

O pH é um fator que controla a fermentação e, além de ser utilizado para determinar o final do processo, também influencia na ativação ou inibição de microrganismos desejáveis ou indesejáveis (MALBAŠA *et al.*, 2008; MO *et al.*, 2008). A concentração ideal de íons de hidrogênio para cada microrganismo varia dependendo da espécie. Bactérias ácido acéticas toleram pH na faixa de 3,6 e 6,3 (SIONEK *et al.*, 2017). Além de ter influência fundamental no crescimento dos microrganismos e no sabor, o pH também altera a estabilidade das catequinas

presentes no chá, podendo resultar em diferenças na atividade antioxidante da bebida (CHU; CHEN, 2006).

Durante a fermentação da kombucha ocorre um decréscimo no valor do pH, devido à formação de ácidos orgânicos (LONČAR *et al.*, 2006; SIEVERS *et al.*, 1995). LONČAR *et al.* (2006) sugerem que o valor mínimo aceitável para kombucha deve ser 3, no entanto diversos autores obtiveram valor de pH abaixo de 3 a partir do terceiro dia de fermentação e consideraram essa acidez aceitável (CHAKRAVORTY *et al.*, 2016; SIONEK *et al.*, 2017; YANG *et al.*, 2010). Na legislação brasileira, o pH da kombucha deve estar entre 2,5 e 4,2 para comercialização (BRASIL, 2019).

1.6 Propriedades biológicas

A propriedade antioxidante da kombucha é esperada pela infusão do chá *Camellia sinensis* e é uma das características que rotula o produto como uma bebida saudável, uma vez que o consumo de alimentos ricos em antioxidantes protege o organismo humano dos radicais livres e está relacionado com aumento de imunidade, alívio de inflamações e artrite (CHU; CHEN, 2006; LOBO *et al.*, 2017). O processo fermentativo pode aumentar ou diminuir a capacidade antioxidante da kombucha devido à formação ou bioconversão de compostos bioativos, como ácidos orgânicos, minerais e vitaminas hidrossolúveis. Além disso, a variação destes compostos está fortemente relacionada com as variáveis do processo fermentativo, como tipo e concentração de chá, comunidade microbiana e tempo de fermentação (CARDOSO *et al.*, 2020; JAYABALAN *et al.*, 2014; MALBAŠA *et al.*, 2011; SUN *et al.*, 2015).

A kombucha tem sido relacionada com aumento da resistência contra câncer, prevenção de doenças cardiovasculares, promoção da digestão, aumento da imunidade e redução de inflamações (DUFRESNE; FARNWORTH, 2000). HARTMANN *et al.* (2000), em estudos com ratos, verificaram que a ingestão de kombucha provoca inibição do aumento de peso e aumento da longevidade. Ao mesmo tempo que efeitos benéficos foram observados, também se verificou um aumento no tamanho dos fígado e baço dos ratos.

Um dos responsáveis pelos possíveis benefícios da ingestão de kombucha é o ácido glicurônico, um dos principais ácidos orgânicos oriundos da fermentação

(JAYABALAN *et al.*, 2007). Este ácido, resultante da oxidação da glicose, tem sido estudado nos últimos anos, devido às suas propriedades benéficas à saúde (SIONEK *et al.*, 2017). Por ser produzido pelo fígado humano, o ácido glicurônico apresenta efeitos desintoxificantes através da ligação com xenobióticos e fenóis, permitindo a sua excreção de forma mais eficiente pelos rins. Além disso, o ácido é precursor da biossíntese da vitamina C (NGUYEN *et al.*, 2015). Apesar de todos os benefícios citados na literatura, não é possível afirmar que todas as kombuchas produzidas irão possuir estas características, visto que a produção dos ácidos benéficos à saúde depende das variáveis do processo citadas anteriormente, como temperatura, microbiota, entre outros (DE FILIPPIS *et al.*, 2018; MARTINI, 2018). Além disso, faltam evidências científicas para as alegações de saúde da kombucha, visto que os estudos publicados foram realizados *in vitro* ou *in vivo* em modelos animais (MORALES, 2020).

Além dos possíveis benefícios à saúde citados na literatura, diversos autores têm estudado a atividade antimicrobiana de kombucha em relação a microrganismos patogênicos (GREENWALT *et al.*, 1998; JAYABALAN *et al.*, 2014; SREERAMULU *et al.*, 2000). STEINKRAUS *et al.* (1996) verificaram que kombucha fermentada com 4,36 g.L^{-1} de chá e 10 % de sacarose não apresentou atividade antibiótioca, além daquela resultante da presença de ácido acético, contra *Helicobater pylori, Escherichia coli, Staphylococcus aureus* e *Agrobacterium tumefaciens.* GREENWALT *et al.* (1998) testaram atividade antimicrobiana de kombucha contendo 33 g.L^{-1} de ácido total (7 g.L^{-1} de ácido acético) e obtiveram resposta negativa contra *Candida albicans* e positiva contra *Agrobacterium tumefaciens, Bacillus cereus, Salmonella choleraesuis* serotype *Typhimurium, Staphylococcus aureus* e *Escherichia coli.* Ao contrário do resultado obtido por GREENWALT *et al.* (1998), o efeito da kombucha na inibição de *Candida albicans* foi verificado por outros autores (BATTIKH *et al.*, 2012; SREERAMULU *et al.*, 2000).

As pesquisas realizadas com kombucha demonstram seu potencial antimicrobiano contra patógenos e essa capacidade é atribuída à presença de ácidos orgânicos, principalmente ácido acético, e catequinas (SREERAMULU *et al.*, 2000). É importante ressaltar que essas propriedades não estarão necessariamente presentes nas kombuchas, pois o produto final é dependente das variáveis do bioprocesso, matérias-primas e cultura starter. Em concordância, a legislação

brasileira não permite expressões que atribuam características de qualidade superlativas e propriedades funcionais não aprovadas em legislação, como artesanal, elixir da vida, energizante, probiótico, entre outros (BRASIL, 2019).

1.7 Kombucha como bebida probiótica

Segundo a Organização Mundial da Saúde (OMS), probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro (FAO/WHO, 2006). Para isso, as preparações ou produtos que contêm estes microrganismos definidos e viáveis devem possuir quantidade adequada, tornando-os capazes de alterar a microbiota intestinal por meio de implantação ou de colonização (SCHREZENMEIR; DE VRESE, 2001). A kombucha é mundialmente conhecida como uma bebida probiótica por conter microrganismos vivos. No entanto, não há evidência científica de que esses microrganismos estão em quantidade suficiente e trazem algum benefício específico à saúde (MARTINI, 2018). Os produtos que utilizam microrganismos probióticos a fim de conferir benefícios à saúde do consumidor devem indicar a posologia e o tempo necessário de uso para obtenção do efeito desejado, com base em evidências científicas e conforme aprovado no país de venda. Tais evidências devem ser obtidas a partir de estudos realizados *in vitro*, em animais e em humanos (FAO/WHO, 2006).

No Brasil, a ANVISA (Agência Nacional de Vigilância Sanitária) é responsável pela avaliação dos produtos probióticos e utiliza diversos requisitos para aprovação da alegação funcional (geral ou específica) dos produtos. A requisição é feita através de um dossiê técnico-científico constando comprovações de identidade e segurança da linhagem e do benefício à saúde. Para a caracterização do microrganismo como probiótico são necessários estudos *in vivo* ou *in vitro* de caracterização da linhagem que comprovem que a mesma resista à passagem pelas principais barreiras químicas (ex. temperatura, acidez) e biológicas (ex. enzimas salivares, ácido biliares) do corpo e atinge o intestino na forma viva (ANVISA, 2021). Para comprovação do efeito benéfico é necessário demonstrar uma casualidade entre o consumo do probiótico e o efeito alegado. Para esta confirmação, conforme a RDC 241/2018, é requisito fundamental dispor de estudos realizados com humanos,

sendo que estudos em animais ou *in vitro* podem ser utilizados para complementar a sustentação de uma alegação (BRASIL, 2018).

Com isso, sabe-se que é errônea a alegação de qualquer kombucha como probiótica, visto que não se sabe a composição e concentração dos microrganismos presentes na bebida. Essa característica pode se tornar viável através do desenvolvimento de uma cultura *starter* conhecida para a fermentação de kombucha.

O artigo científico de revisão bibliográfica intitulado "Health effects and probiotic and prebiotic potential of Kombucha: A bibliometric and systematic review" (Apêndice A) foi publicado no periódico Food Bioscience e aprofunda as propriedades biológicas da kombucha, esclarecendo os aspectos de alegação probiótica e prébiotica da bebida.

1.8 Desenvolvimento de uma cultura *starter*

Apesar de o SCOBY ser largamente utilizado como cultura *starter* para produção de kombucha, sua aplicação na produção industrial não é adequada, visto que a composição microbiológica varia muito ao longo das fermentações (WANG *et al.*, 2020). Para aplicação industrial, o desenvolvimento de uma cultura *starter* a partir de microrganismos conhecidos é essencial. Para isso, devem-se levar em consideração diversos critérios de avaliação, como seleção de linhagens, metabólitos desejados, tolerância ao estresse e parâmetros tecnológicos, conforme representação na Figura 4 (VINICIUS DE MELO PEREIRA *et al.*, 2020).

Figura 4. Representação esquemática dos critérios analisados no desenvolvimento de uma cultura *starter* para a indústria de alimentos.
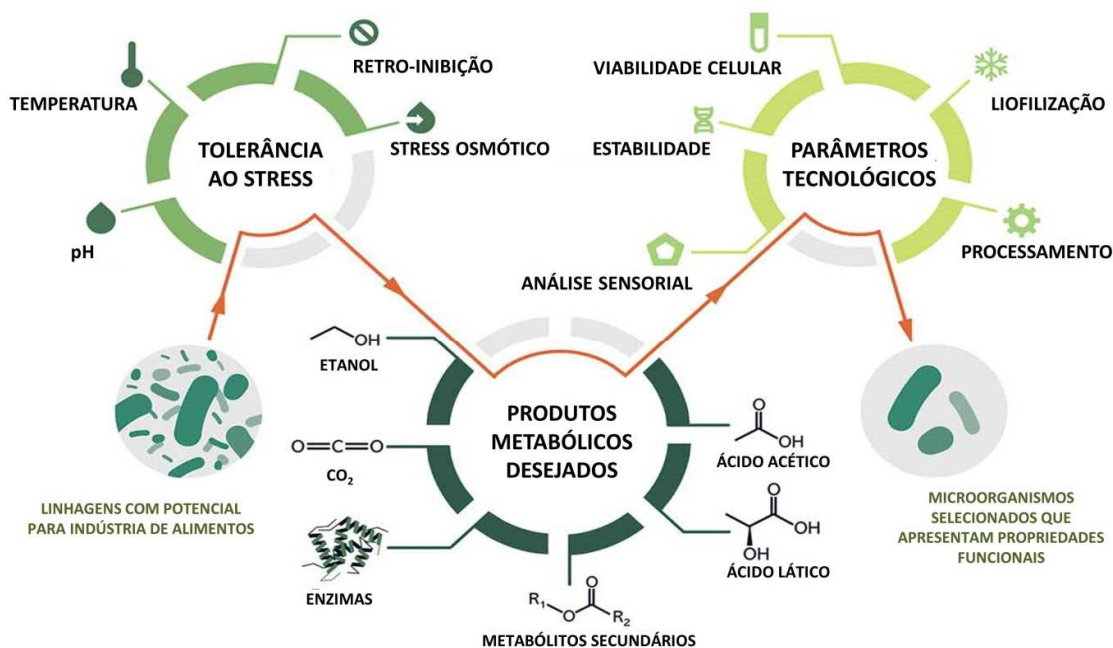
Fonte: adaptado de VINICIUS DE MELO PEREIRA *et al.* (2020)

Para seleção de uma cultura *starter* é necessário avaliar os metabólitos desejados no produto final e selecionar linhagens de microrganismos capazes de produzi-los. Além disso, é necessário que os microrganismos selecionados sejam classificados como GRAS (Generally Recognized as Safe) (VINICIUS DE MELO PEREIRA *et al.*, 2020). No caso da kombucha, os microrganismos predominantes já são conhecidos, portanto indicando a seleção de linhagens isoladas de bactérias ácido acéticas, ácido láticas e leveduras (CHAKRAVORTY *et al.*, 2016; MARSH *et al.*, 2014). Essas linhagens devem apresentar tolerância à glicose, ácido acético e etanol, ser capazes de oxidar etanol a ácido acético e produzir celulose (VINICIUS DE MELO PEREIRA *et al.*, 2020). Além disso, microrganismos probióticos ou produtores de aromas seriam desejáveis, a fim de melhorar a qualidade do produto final.

Após seleção das linhagens é necessário avaliar a tolerância das mesmas em relação a fatores de estresse, como pH, temperatura e retro-inibição. Se as linhagens forem resistentes a estes fatores, avaliam-se através de fermentações os metabólitos produzidos, com estudos que envolvem diferentes concentrações de inóculo. Ao final dos experimentos de otimização da fermentação pela cultura *starter*

são avaliadas a qualidade sensorial do produto e parâmetros tecnológicos de processamento, liofilização e estabilidade (VINICIUS DE MELO PEREIRA *et al.*, 2020).

1.9 Legislação brasileira

A kombucha é um produto relativamente novo no Brasil e sua legislação foi criada pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) através da portaria nº 14 de 17 de setembro de 2019, que estabelece o padrão de identidade e qualidade da kombucha em território nacional.

Os parâmetros analíticos estipulados pela legislação determinam que o pH esteja entre 2,5 e 4,2 e a acidez volátil entre 30 e 130 (mEq.L^{-1}). Para o teor alcoólico, a legislação classifica a kombucha como bebida não alcoólica quando apresenta o teor máximo de 0,5 % (v/v) de álcool, o equivalente a 4 g.L^{-1}. Quando o teor ultrapassa este valor, a bebida é caracterizada como alcoólica e o teor máximo permitido é 8 % (v/v) (BRASIL, 2019). Em relação à composição do produto, a portaria exige a kombucha seja fermentada a partir de infusão ou extrato aquoso de *Camellia sinensis* que pode estar associada à outras espécies vegetais.

A legislação permite a adição de gás carbônico (CO_2) puro para carbonatação da kombucha. Nesta situação, a pressão (atm a 20 °C) da bebida deve estar entre 1,1 e 3,9 psi. Também é autorizada a aplicação de processos tecnológicos como pasteurização, filtração, ultracentrifugação, entre outros (BRASIL, 2019).

Na portaria consta que "é vedado o uso de alegações funcionais e de saúde não autorizadas pela legislação específica da Agência Nacional de Vigilância Sanitária (ANVISA)", como por exemplo, o termo probiótico (BRASIL, 2019). Isso significa que o uso de probióticos em alimentos requer prévia avaliação da ANVISA, a qual envolve comprovação da identidade da linhagem do microrganismo, de sua segurança e de seu efeito benéfico (BRASIL, 2018). Além disso, a portaria não permite a adição de microrganismos após o processo de fermentação, o que impede que os produtores pasteurizem o produto e adicionem uma linhagem probiótica comercial, com intuito de rotular a bebida com alegações funcionais (BRASIL, 2019).

CAPÍTULO II – EFFECT OF FREEZE-DRIED KOMBUCHA CULTURE ON MICROBIAL COMPOSITION AND ASSESSMENT OF METABOLIC DYNAMICS DURING FERMENTATION

O capítulo II, intitulado "Effect of freeze-dried kombucha culture on microbial composition and assessment of metabolic dynamics during fermentation", está apresentado na forma de artigo científico publicado no periódico Food Microbiology.

O trabalho versa sobre o estudo da comunidade microbiana presente em uma amostra de kombucha do Rio Grande do Sul e sua interação com os metabólitos produzidos durante a fermentação, a fim de elucidar a correlação entre microbiota e produto final. Além disso, estudou-se a possibilidade de liofilização da cultura *starter* para fins de padronização do processo.

Effect of freeze-dried kombucha culture on microbial composition and assessment of metabolic dynamics during fermentation

Mariana Fensterseifer Fabricio[a], Michelle Bertoni Mann[b], Caroline Isabel Kothe[c], Jeverson Frazzon[a], Bruna Tischer[a], Simône Hickmann Flôres[a], Marco Antônio Záchia Ayub*[a]

[a] Food Science and Technology Institute, Federal University of Rio Grande Do Sul, Av. Bento Gonçalves, 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil

[b] Postgraduate Program in Agricultural and Environmental Microbiology, Basic Health Sciences Institute, Federal University of Rio Grande Do Sul, Porto Alegre, RS, Brazil

[c] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France

*Corresponding author:

Tel.: +55 51 3308 6685; fax: +55 51 3308 7048

E-mail address: mazayub@ufrgs.br (M. A. Z. Ayub)

Highlights

• Freeze-dried starter culture resulted in kombucha with lower content of alcohol.

• The results suggest that presence of *Zymomonas* influenced the alcohol content of kombucha.

• Yeast populations were predominated by *Dekkera/Brettanomyces*.

• Fermentation inhibited the presence of *Enterobacteriaceae* in kombuchas.

Abstract

Kombucha is a traditional fermented beverage gaining popularity around the world. So far, few studies have investigated its microbiome using next-generation DNA sequencing, whereas the correlation between the microbial community and metabolites evolution along fermentation is still unclear. In this study, we explore this correlation in a traditionally produced kombucha by evaluating its microbial community and the main metabolites produced. We also investigated the effects of starter cultures processed in three different ways (control, starter culture without liquid suspension (CSC), and a freeze-dried starter culture (FDSC)) to evaluate changes in kombucha composition, such as antioxidant activity and sensory analysis. We identified seven genera of bacteria, including *Komagataeibacter, Gluconacetobacter, Gluconobacter, Acetobacter, Liquorilactobacillus, Ligilactobacillus,* and *Zymomonas,* and three genera of yeasts, *Dekkera/Brettanomyces, Hanseniaspora*, and *Saccharomyces*. Although there were no statistically significant differences in the acceptance test in sensory analysis, different starter cultures resulted in products showing different microbial and biochemical compositions. FDSC decreased *Zymomonas* and *Acetobacter* populations, allowing for *Gluconobacter* predominance, whereas in the control and CSC kombuchas the first two were the predominant genera. Results suggest that the freeze-drying cultures could be implemented to standardize the process and, despite it changes the microbial community, a lower alcohol content could be obtained.

Keywords: Kombucha production; microbiome; 16S; ITS; fermented foods; sensory analysis.

1. Introduction

Kombucha is a traditional beverage obtained by fermentation of sweetened green and black tea, sometimes in a mixture of both, which has been gaining popularity due to its potential health benefits (J. Kim and Adhikari, 2020). Kombucha is an acidic, slightly sweet, carbonated beverage whose chemical profile consists of sugars, organic acids, alcohols, vitamins, amino acids, minerals, and notably antioxidant molecules from tea, such as catechins (De Filippis et al., 2018; Villarreal-Soto et al., 2018; Vitas et al., 2018). Its fermentation is performed by a symbiotic association of microorganisms known as SCOBY (Symbiotic Colony of Bacteria and Yeast), composed of cellulose, acetic acid bacteria, yeasts, and, sometimes, lactic acid bacteria (Coton et al., 2017; De Filippis et al., 2018; Marsh et al., 2014). Cellulosic biofilm, which is synthesized from glucose by a few genera of acetic acid bacteria, floats in the fermented liquid and is generated in each batch of fermentation (Mohite and Patil, 2014; Nguyen et al., 2008; Villarreal-Soto et al., 2018).

Although regarded by many people as an artisanal beverage, being fermented locally and, therefore, showing a large variability of microbial composition, the main genera reported in kombucha beverage are the bacteria *Acetobacter, Komagataeibacter,* and *Gluconacetobacter*, and the yeasts *Zygosaccharomyces, Candida, Brettanomyces* and *Hanseniaspora* (Arıkan et al., 2020; Bueno et al., 2021a; Chakravorty et al., 2016; Coton et al., 2017; Gaggìa et al., 2018; Marsh et al., 2014; Villarreal-Soto et al., 2020).

Many studies on kombucha microbiome are performed through culture-dependent analysis, which might produce ambiguous results due to the variability of the species or because many microorganisms are uncultured on the restricted culture media tested, rendering the description of the whole community unreliable (Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Next-generation DNA sequencing (NGS) or high-throughput sequencing (HTS) methods are powerful tools for an in-depth understanding of microbial communities, allowing the identification of complete microbial populations (Jagadeesan et al., 2019). So far, some studies have used this technology to improve the knowledge of kombucha composition around the world and results showed that the microbial composition varies according to the geographic location, making comparison difficult across studies regarding chemical composition and health benefits (Arıkan et al., 2020; Chakravorty et al., 2016; Coton et al., 2017;

Gaggìa et al., 2018; Marsh et al., 2014; Reva et al., 2015; Villarreal-Soto et al., 2020). Kombucha elaboration process is artisanal, with poor control of the temperature and the number of viable microorganisms in the starter culture, resulting in a final product with heterogeneous properties (Marsh et al., 2014; Villarreal-Soto et al., 2018). This is an important limitation to industrial production because it has been demonstrated that kombucha microbiome changes not only according to the geographic location but also between batches of fermentation due to many environmental factors such as temperature, time of fermentation, substrate composition and concentration, vessels geometry, pH, amount of dissolved O2, and CO2 (De Filippis et al., 2018; Marsh et al., 2014; Tran et al., 2020).

Because studies on kombucha properties and characteristics of microbial composition and metabolites produced during fermentation are scarce among Brazilian producers, this study aimed to investigate the microbial composition of one traditional kombucha produced in Brazil by HTS and to analyze the metabolic products generated and sensory properties after fermentation. Additionally, as microorganisms play a key role in kombucha production, we evaluated the effects of different starter cultures, including one freeze-dried, in order to propose an improvement of the control over the industrial process, using a standardized inoculum. This standardization pursues the controlled production of a biofilm that presents always the same microbial composition and may be used as a starter culture in each batch, avoiding the use of the biofilm from a previous batch that might have suffered undesired modifications and also minimizing culture manipulation.

2. Material and Methods

2.1 Starter cultures design

Fresh kombucha starter cultures (cellulosic biofilm and liquid, which consists of a fermented kombucha from a previous batch) were kindly supplied by a local Company (Porto Alegre, RS, Brazil) and were kept refrigerated at 4 °C until use. The liquid composition of the received starter culture was determined as (in $g.L^{-1}$): sucrose 19.54, fructose 14.92, glucose 10.84, acetic acid 1.76, ethanol 17.36, glycerol 1.34, and lactic acid 0.6, (pH 3.4). Cellulosic biofilm was aseptically homogenized for 2 min, using a mixer (SB40, Black & Decker, USA), and then

divided for three different applications: two fermentations with fresh biofilm and one with freeze-dried biofilm.

In this work, we designed three different inocula to be used as starter cultures:

1) A control starter culture (Control), reflecting the commercial production, composed of 50 g.L^{-1} of fresh cellulosic layer and 100 mL.L^{-1} of liquid;
2) Centrifuged-starter culture (CSC), composed of 50 g.L^{-1} of fresh cellulosic layer and 100 mL.L^{-1} of liquid centrifuged and resuspended in sweetened tea (8 g.L^{-1} of green tea and 80 g.L^{-1} of sucrose);
3) Freeze-dried starter culture (FDSC): 50 g.L^{-1} of freeze-dried cellulosic layer and 100 mL.L^{-1} of liquid centrifuged and freeze-dried.

In procedures 2 and 3, the liquid was centrifuged in order to generate pellets of cells to be used as inoculum. This was evaluated because, as the liquid contains organic acids, sugars, and alcohols, we would like to establish whether the chemicals present in the liquid would be of importance as inoculum or whether only the microbial community by itself could characterize the fermentation process. A schematic representation of the process can be seen in the supplementary material Fig. S1 (Appendix A).

For freeze-drying, the cellulosic layer and cells from centrifuged liquid were frozen in an ultra-freezer at −80 °C for 2 h. Then, the drying process was carried out using a freeze-dryer (L101, Liobras, Brasil) at −68 °C, at vacuum pressure <20 μm Hg for 60 h. During kombucha preparation, both freeze-dried samples were resuspended in 200 mL of sweetened tea (8 g.L^{-1} of green tea, 80 g.L^{-1} of sucrose) and incubated for 24 h at 28 °C before inoculating.

2.2 DNA extraction and quantification

Total DNA was extracted from cellulosic biofilm and sour liquid phase of kombucha received from the local company and also from the liquid phase after fermentation. For the liquid samples, 50 mL were centrifuged to generate a pellet. For the biofilm extraction, 20 g were crushed with 80 mL of liquid nitrogen and a pre-treatment was employed, which consisted of adding 500 μL of cellulase (Sigma-Aldrich, Germany) and incubating for 1 h at 40 °C. Both samples were subjected to

enzymatic digestion with 400 U of zymolyase at 30 °C for 45 min (Zymo Research, USA). DNA extractions were performed using Purelink Genomic DNA kit (Invitrogen, USA) following the manufacturer protocol. Quantification of DNA extract of each sample was determined using Qubit ® 3.0 (Invitrogen, USA).

2.3 Library preparation, *16S rRNA* gene and Internal Transcribed Sequence (ITS) region sequencing

Amplification of the V4 region of the 16S rRNA gene was performed using primers 515 F and 806 R (Caporaso et al., 2011) with identified adaptors of the Illumina platform. Volume of amplification was 25 µL, consisting in genomic DNA (12.5 ng), MgCl2, (1.0 mM), 0.5 µM of each primer, dNTP (0.2 mM), PCR Buffer (1X) and Platinum Taq DNA (2U) (Life Technologies). PCR conditions used for 16S rRNA gene amplification were 94 °C denaturation for 2 min, 30 cycles of 94 °C for 45 s (denaturation), 55 °C for 45 s (annealing), and 72 °C for 1 min and 5 min (extension). Amplification of Internal Transcribed Sequence (ITS) region of the fungal rRNA was performed using primers ITS1 and ITS2 (White et al., 1990) with identified adaptors of Illumina platform. Volume of amplification was 25 µL, consisting in genomic DNA (12.5 ng), $MgCl_2$, (2.5 mM), 0.16 µM of each primer, dNTP (0.2 mM), PCR Buffer (1X) and Platinum Taq DNA (1U) (Life Technologies). PCR conditions used for ITS amplification were 95 °C denaturation for 5 min, 30 cycles of 95 °C for 45 s (denaturation), 56 °C for 45 s (annealing), and 72 °C for 1 min and 10 min (extension). Amplicons generated from PCR reactions were purified using Agencourt AMPure XP beads, following the manufacturer's instructions. Indexes were added to DNA libraries following the manufacturer's instructions (Illumina Inc., San Diego, California, USA). Sequencing was conducted on an Illumina MiSeq System with a v2 500- cycles kit.

2.4 Amplicon sequencing data analysis

Data analysis of 16S rRNA gene and ITS sequence amplicons were performed using FROGS (Find Rapidly OTUs with Galaxy Solution). First, the raw sequence data was quality filtered with FastQC (Andrews, 2010) and sequences

were imported into the FROGS pipeline (Escudié et al., 2018) to obtain the Operational Taxonomic Units (OTUs). Then, the sequences with amplicon size from 50 to 500 bp were filtered and pooled into OTUs with SWARM (Mahé et al., 2015) with the distance parameter of d = 1. The chimeras removal was performed with VSEARCH (Rognes et al., 2016) and OTUs were filtered to keep at least 0.1% of all sequences. Taxonomic affiliations were checked using EzBiocloud database (O.-S. Kim et al., 2012) for bacteria, delimited at 97% identity (Edgar, 2018) and UNITE database (Nilsson et al., 2019) for yeasts. Microbial diversity was analyzed using the phyloseq and ggplot 2 packages in R Studio v. 3.6.1 (v1.30.0) (McMurdie and Holmes, 2013) and the species relative abundance of samples was plotted with the plot_composition function. Sequencing data were deposited into the NCBI Sequence Read Archive and are publicly available under access number PRJNA627742.

2.5 Preparation of tea infusion and fermentation

All kombucha starters were cultivated on the same culture medium consisting of distilled water, 8 g.L^{-1} of organic green tea (Vemat, SC, Brazil), and 80 g.L^{-1} of organic demerara sugar (Native, SP, Brazil).

For the kombucha media preparation, 1.2 L of water added with 144 g of sucrose was sterilized at 121 °C for 15 min in the 2 L bioreactor vessels. Then, 14.4 g of green tea leaves were infused for 10 min in 600 mL of boiling water and then filtered using a membrane pore size of 0.22 μM. After cooling down to 30 °C, tea infusion was added to the sugar solution in the bioreactor, totalizing 1.8 L of sweetened tea. Fermentations were performed in 2 L-working volume tank bioreactors (Biostat B, B. Braun Biotech International, Germany), filled with 1.8 L of sweetened green tea, and equipped with airflow, pH, and temperature controls. The cultivation followed for 5 days, under the conditions of 28 °C, aeration of headspace with an air rate of 1 L.min^{-1}. The bioreactors were inoculated with three different starter cultures as mentioned before, totalizing 2 L of media. Phosphoric acid solution was used to set the initial pH to 3.8, set based on the control experiment, whose pH resulted from the addition of the liquid (described in item 2.1), which reflects the commercial preparation of kombucha. Subsequently, the pH of cultures was monitored but not controlled, leaving the natural change in pH as expected for

kombuchas. After 5 days of fermentation in the bioreactor, samples were placed in hermetic glass bottles (anaerobically) and incubated at 25 °C for 48 h (totalizing 7 days of fermentation), in order to create carbonation and obtain a pleasant beverage for sensory analysis.

2.6 Determination of substrate consumption and fermentation metabolites

Samples of 5 mL were collected every 24 h from bioreactors, centrifuged (3000 g, 15 min) and the supernatant was filtered through 0.22 µM membrane pore size and analyzed by HPLC to determine the concentration of sugars from substrate and fermentation products. Ethanol, glycerol, acetic, lactic, and succinic acids were quantified using a Bio-Rad Aminex 87H column, using sulfuric acid 5 mM as eluent, a flow rate of 0.6 mL.min^{-1}, and temperature of 45 °C. Sucrose, fructose, and glucose were determined using a Bio-Rad Aminex 87C column, using ultrapure water as eluent, a flow rate of 0.6 mL.min^{-1}, and a temperature of 85 °C.

2.7 Antioxidant activity

The antioxidant activities of all prepared kombuchas were determined using the Oxygen Radical Absorbance Capacity (ORAC- hydrophilic) method described by Huang et al. (2005) (adapted). This assay verifies the sequestering capacity of an antioxidant (standard Trolox or sample) against AAPH peroxyl radical at 37 °C. Working solutions of fluorescein (81 nM) and AAPH (152 mM) in phosphate buffer (75 mM, pH 7.4) were prepared. Experiments were conducted in ELISA microplates with 150 µL of fluorescein, 25 µL of the diluted sample (dilution factor: 200) or Trolox (standard curve), and 25 µL of AAPH. Fluorescence readings were taken every 60 s, during 90 min at 37 °C, using Enspire 2300 Multimode Plate Reader (PerkinElmer, USA). Emission and excitation wavelengths were 485 nm and 528 nm, respectively. The area under the curve (AUC) was used to determine sample concentrations and values were calculated through a standard curve of Trolox (from 8 to 96 µmol.L^{-1}). Results were expressed as µmol Trolox equivalents (TE) per mL of kombucha.

The AUC was calculated by Eq. (1) as:

$$\text{``}AUC = 1 + {f1}/{f0} + {f2}/{f0} + {f3}/{f0} + \dots {fn}/{f0}\text{''}$$ (1)

where:

$f0$ = initial fluorescence reading

fn = fluorescence reading at time of each cycle.

2.8 Sensory analysis

Sensory analysis of kombucha was conducted using an acceptance test, based on the evaluation of the following attributes: appearance, color, aroma, taste, acidity, and overall acceptance. A 5-point hedonic scale (1- dislike extremely; 5- like extremely) was applied to a panel of 52 untrained panelists, between 18 and 60 years old. Approximately 30 mL of each sample coded with a three-digit random number was served and consumers were asked if they intended to purchase any sample and if they had already tried kombucha before, since it is not a common beverage. The Acceptance Index (AI) was calculated by Eq. (2):

AI (%) = (Attribute media. 5^{-1}). 100 % (2)

The research had the permission of the University Ethical Committee (UFRGS, Protocol n: 18613419.8.0000.5347).

2.9 Statistical analysis

The statistics of experimental data were assessed by analysis of variance (ANOVA) at $p < 0.05$, followed by Tukey's test, using the software Statistica 12.5 (StatSoft Inc., USA).

3   Results and discussion

3.1 Metagenetic analysis of kombucha

The microbial community of the starter culture (biofilm and liquid) and the final liquid composition of control, CSC, and FDSC were analyzed. Data analysis of 16 S rRNA gene sequence revealed two bacterial phyla in all samples, including Proteobacteria and Firmicutes. Proteobacteria was the dominant phyla and in all samples with a relative abundance was up to 80%. Several other authors who analyzed kombucha have also reported this as the most abundant phyla (Arıkan et al., 2020; Chakravorty et al., 2016; Marsh et al., 2014).

Regarding the initial starter culture, major changes between biofilm and liquid microbiota were observed at family level (Fig. 1). Acetic acid bacteria, which belong to the *Acetobacteraceae* family, were more abundant in biofilm (97.1%) than in liquid (3.25%) since they are aerobic microorganisms. This family, whose bacterial representatives are Gram-negative and catalase-positive, is associated with the synthesis of cellulose biofilm, which is produced in the form of fibrils, attached to the bacterial cell (Campaniello and Sinigaglia, 2017; Gomes et al., 2018; Villarreal-Soto et al., 2018). The biofilm is produced extracellularly from a variety of carbon sources such as glucose, ethanol, and glycerol and remains attached to the cells (Campaniello and Sinigaglia, 2017; Villarreal-Soto et al., 2018). As in this study, other authors found *Acetobacteraceae* family above 80% of relative abundance in biofilm (Arıkan et al., 2020; Chakravorty et al., 2016; Villarreal-Soto et al., 2020). In contrast, the dominant family in initial liquid sample was *Sphingomonadaceae* (41.6%), followed by *Enterobacteriaceae* (35.2%). The family *Enterobacteriaceae* has over 30 genera and 120 species, some of which naturally inhabit the mammalian gut, while others include a wide range of potentially disease-causing microorganisms (Donnenberg, 2015; Rock and Donnenberg, 2014). Other than these, *Lactobacillaceae* was also presented in liquid with 19.9% of relative abundance.
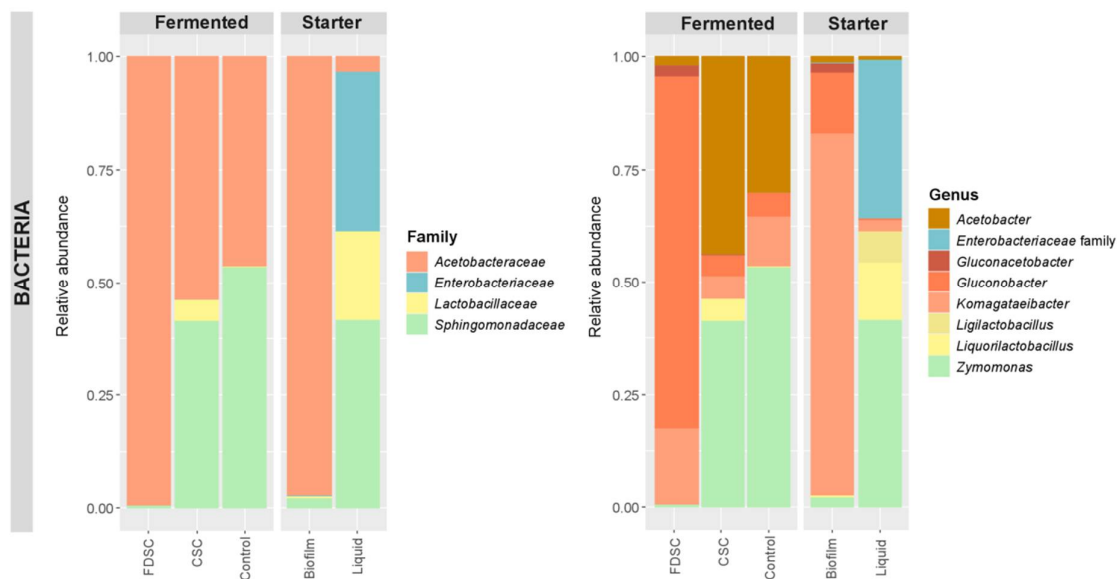
Figure 1. Relative abundance (%) of bacterial community at family and genus level in kombucha based on 16S rRNA gene metabarcoding.

Concerning the bacterial composition at genus level, *Komagataeibacter* was predominant in the biofilm, representing 80.3% of relative abundance, whereas in the liquid this genus represented only 2.3%. Liquid showed higher diversity of bacterial genera than biofilm (Fig. 1), mainly represented by *Zymomonas* (41.57%). The presence of this genus in kombucha was reported for the first time in the literature by Marsh et al. (2014), despite having already been identified in other fermented beverages such as water kefir (Hsieh et al., 2012; Marsh et al., 2013). *Zymomonas* species are able to produce high amounts of ethanol, fructooligosaccharides (FOS), sorbitol, and gluconic acid, depending on the carbon source. In this study, this genus was represented by the species *Zymomonas mobilis*, a gram-negative and facultative anaerobic strain. *Z. mobilis* presents potential probiotic activity, being effective in regulating intestinal transit (Tallyne de Aguiar Silva et al., 2020) and also presenting immunomodulatory effects by protecting mice against sepsis (Campos et al., 2013). In addition, this species can produce FOS from sucrose, a prebiotic that is efficient in reducing cholesterol (Tallyne de Aguiar Silva et al., 2020).

The genus *Liquorilactobacillus* was also found in kombucha initial samples, representing 13% of the bacterial population in liquid and less than 1% in biofilm.

This genus has been identified in kombucha, water kefir, and cocoa fermentation before (Bueno et al., 2021b; Coton et al., 2017; Verce et al., 2021). The presence of LABs is interesting due to their potential to confer probiotic properties and promote health through the production of prebiotics, such as polysaccharides produced by *Liquorilactobacillus satsumensis* (i.e soluble dextran and water-insoluble glucan) (Bueno et al., 2021b; Côté et al., 2013; Nguyen et al., 2015; Paiva et al., 2016). Another LAB identified in the initial liquid was *Ligilactobacillus* with 6.9% of relative abundance.

After seven days of fermentation, differences in the bacterial community were observed in all samples. An important change was observed in control, CSC, and FDSC regarding the presence of *Enterobacteriaceae* that disappeared at the end of fermentation. This shift was extremely positive because this family, that includes genera such as *Escherichia, Salmonella, Shigella, Klebsiella,* and *Serratia*, has been used as an indicator of contamination in food safety and quality (Halkman and Halkman, 2014; Rock and Donnenberg, 2014).

Other changes were observed in the samples at the end of fermentation, with FDSC presenting the most significant differences compared to initial bacterial community. *Zymomonas mobilis*, which represented 41.6% in the initial liquid, decreased to 0.5% in FDSC, increased to 53.62% in the control, and remained the same (41.4%) in CSC. An increase in this species is desirable due to its potential symbiotic activity, acting as a probiotic and producing prebiotics in the broth (Tallyne de Aguiar Silva et al., 2020).

Control and CSC samples presented a similar microbial community, except for the presence of *Liquorilactobacillus* in CSC (4.6%). This genus represented less than 1% in control and completely disappeared in FDSC. Control and CSC also presented an increase in the relative abundance of the genus *Acetobacter* that shifted from less than 2% in liquid and biofilm to 29.9% and 43.1% in control and CSC, respectively. *Gluconobacter* genus was also presented in the initial liquid (<1%) and biofilm (13.4%) increasing to 75.5% in FDSC, remaining low in control and CSC. The simple change of centrifuging the liquid of starter culture, as done in experiment CSC, reflected in changes in the microbial composition of kombucha. The metabolites present in the liquid, such as ethanol, acetic acid, lactic acid, and glycerol may serve as a carbon source for some genera, while may harm others.

Compared to bacteria, yeast diversity was considerably lower and results were similar in biofilm and liquid from initial starter culture. Data analysis of ITS amplicons revealed that both initial samples were dominated by Ascomycota phyla and Saccharomycetales order. *Dekkera/Brettanomyces* was the predominant yeast genus in biofilm (98.8%) and liquid (99.9%), belonging to the family *Pichiaceae* (Fig. 2). This is one of the main genera of yeasts reported to be found in kombucha, besides *Zygosaccharomyces*, and *Candida* (Chakravorty et al., 2016; Coton et al., 2017; Gaggìa et al., 2018; Marsh et al., 2014). The genus *Saccharomyces* was present in very low concentration in biofilm (1.2%) and did not appear in the liquid composition. After fermentation, *Dekkera* remained the predominant genus in samples, despite the analysis also revealing the presence of the genus *Hanseniaspora* in control (25.7%) and CSC (15.8%) samples. For the FDSC samples, there was no yeast DNA amplification, thus it was not possible to analyze the final yeast diversity in this sample, if they were present. This was not on account of some technical problem, but rather as a consequence of the completely different bacterial profile obtained in this sample (Fig. 1). We hypothesize that the yeasts were present in such a low concentration that no yeast DNA amplification could be detected. It is possible that the predominance of *Gluconobacter*, which has shown to present some antifungal activity, rendered yeast growth, as it has been demonstrated by Bevardi et al. (2013).
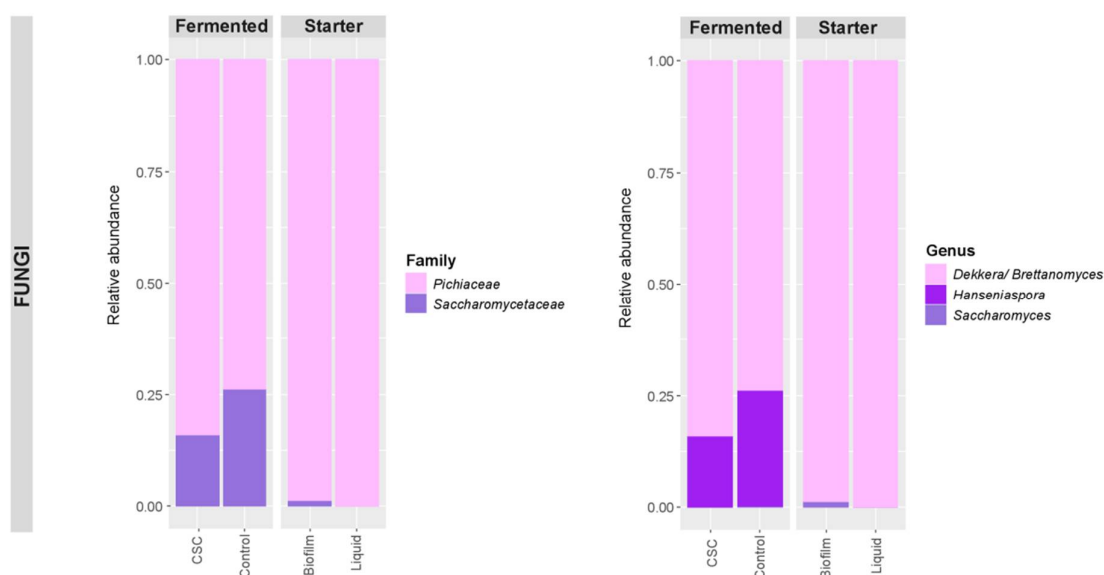
Figure 2. Relative abundance (%) of yeast community at family and genus level in kombucha based on ITS amplicons.

In samples where yeasts were detected, we could identify *Dekkera anomala*, *Dekkera bruxellensis,* and *Hanseniaspora guilliermondii*. *Dekkera/Brettanomyces* genus has the characteristic to produce ethanol under slow fermentation rates, in comparison with other fermenting yeasts such as *Saccharomyces cerevisae*, which is an interesting characteristic for kombucha (Ciani and Comitini, 2014). This genus can produce acetic acid, depending on the oxygen availability, and may also produce undesirable compounds, such as volatile phenols and tetrahydropyridines, considered to be a spoilage microorganism in wine, beer, and cider (Conterno et al., 2010). *Dekkera bruxellensis*, that represented 26.4% of control and 38.1% of CSC, presents high invertase activity, which is very important in kombucha fermentation, in order to release fructose and glucose to be metabolized by those microorganisms which are not able to hydrolyze sucrose.

Hanseniaspora guilliermondii is an apiculate yeast able to produce ethyl acetate and β-phenylethyl acetate, which contribute to 'rose', 'honey', 'flowery' and 'fruity' aromas (Martín et al., 2018; Rojas et al., 2001). This yeast represented <1% of the population in the starter culture samples, but after fermentation increased to 25.76% in control and 15.8% in CSC and may have improved the aroma profile of the final product.

3.2 Kombucha fermentation kinetics

Even though the decreasing pH pattern of FDSC, which presented a higher relative abundance of acetic acid bacteria, was more accentuated among the experiments, the final pH was similar to the Control and CSC, most likely due to the formation of other organic acids during fermentation, which were not analyzed in this work, such as gluconic acid. Therefore, kombucha beverage samples inoculated with three different inocula presented decreasing patterns of pH (Fig. 3), with the final value being similar among samples. This variable is associated with microbial growth, breakdown and stability of phytochemicals, polyphenols deprotonation, and, consequently, antioxidant activity (Hur et al., 2014).
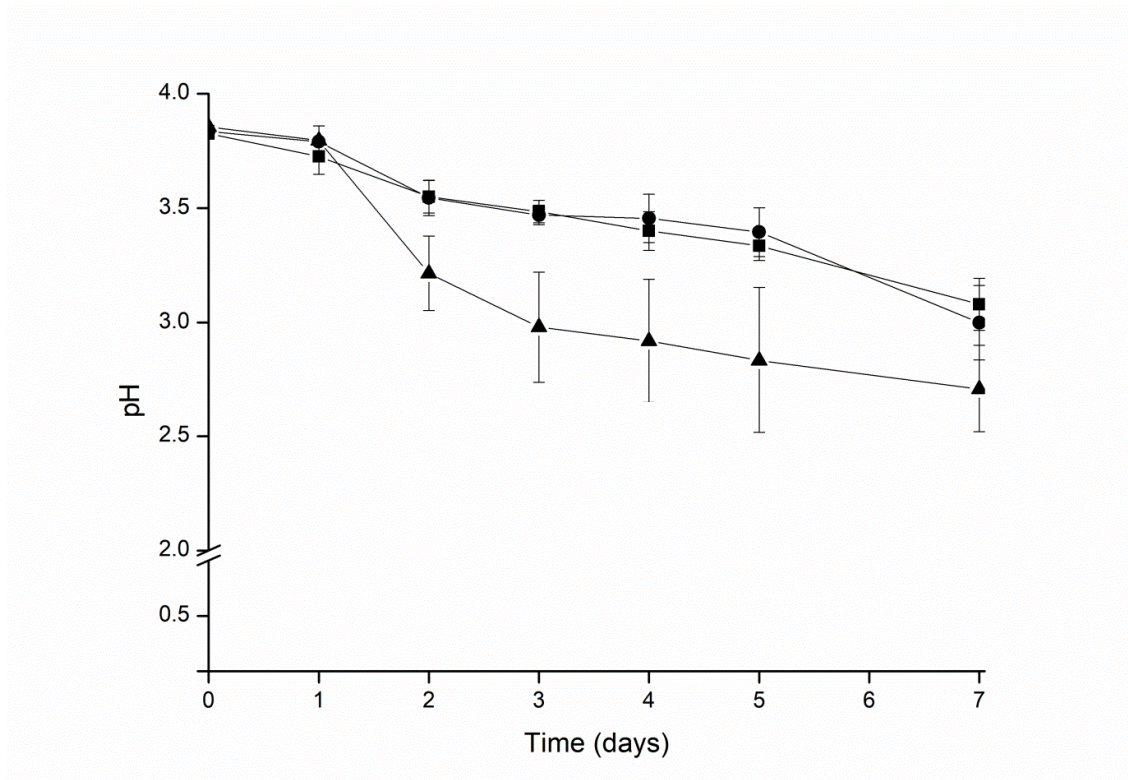
Figure 3. pH changes during fermentation of kombucha: Control (■), CSC (●), FDSC (▲). Experiments are the mean of duplicates.

During the fermentation process, sucrose was hydrolyzed into glucose and fructose by yeast invertase (β-fructofuranosidase). The release of reducing sugars from hydrolysis was higher on FDSC than in the other inocula formulations (Fig. 4A), clearly showing that the process of freeze-drying the inoculum, in order to propose a standardized process, is possible, without reducing the enzymatic capability of cells. As expected, although the sucrose hydrolysis rate was higher on FDSC, the fructose and glucose released were not totally consumed, which is important to keep the sweetness characteristic of kombucha (Fig. 4B).

Although both reducing sugars were used by bacteria and yeasts, fructose values were higher than glucose in all experiments, which shows that microorganisms have a preference to metabolize the latter sugar as a carbon source. This preference was observed in another study on kombucha fermentation (Kallel et al., 2012).
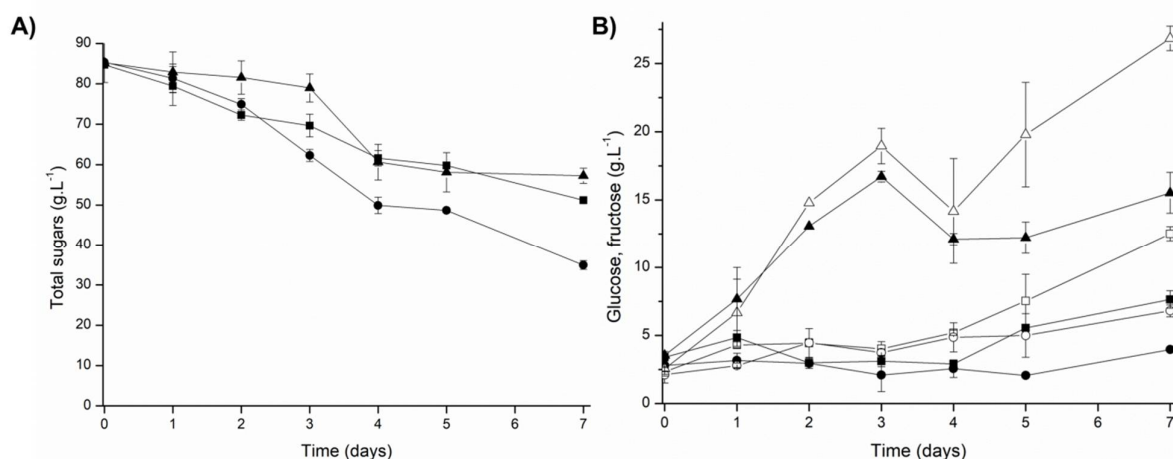
Figure 4. Sugar concentration (g.L^{-1}) during kombucha fermentation. A) Total sugars; B) Fructose (open symbols) and Glucose (solid symbol). Control (■), CSC (●), FDSC (▲). Experiments are the mean of duplicates.

Released sugars from yeast invertase were converted into products such as acetic acid, ethanol, and glycerol. Neither lactic nor succinic acid was found in cultivations during 5 days of fermentation. The genera *Komagataeibacter, Acetobacter, Gluconobacter,* and *Gluconacetobacter* present in the biofilm and in the initial liquid, are responsible for acetic acid production. These acetic acid bacteria utilize two membrane-bound enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, that catalyze the oxidation of ethanol into acetic acid (Gomes et al., 2018; Iida et al., 2008). Acetic acid was produced in all different experiments (Fig. 5) reaching a maximum of 5.56 ± 0.01 g.L^{-1} in control. FDSC showed the lowest production of acetic acid (3.57 ± 0.14 g.L^{-1}) and that may be caused by the changes in microbial dynamics. In the literature, values of acetic acid in kombucha fermented for 7 days were found to range from 1.16 g.L^{-1} to 5.72 g.L^{-1} (Chakravorty et al., 2016; Gaggìa et al., 2018; Sionek et al., 2017) and this wide range can be explained by the variables of the process, such as substrate concentration and temperature. Our results showed that sucrose was more effectively broken into monosaccharides and less acetic acid was produced in the kombucha made of the FDSC and its related to the freeze-drying process that changed the dominant species dynamics.
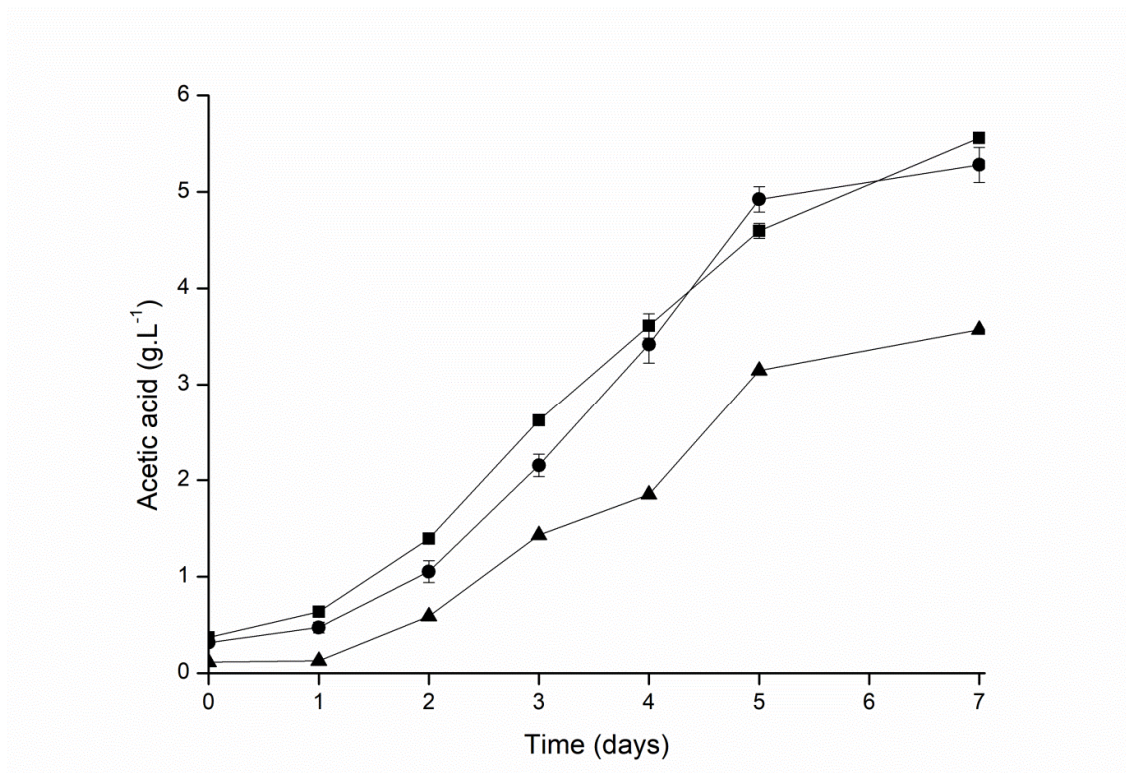
Figure 5. Acetic acid production (g.L^{-1}) during kombucha fermentation. Control (■), CSC (●), FDSC (▲). Experiments are the mean of duplicates.

Ethanol production, another key parameter regarding kombucha quality, was observed in all conditions, reaching a maximum of 10.77 ± 0.07 g.L^{-1} in the control, after 7 days (Fig. 6). Ethanol concentration reported by other authors presents large variability, between 0.28 g.L^{-1} and 7.8 g.L^{-1} (Chakravorty et al., 2016; Gaggìa et al., 2018; Sionek et al., 2017; Tran et al., 2020). Usually, a decrease in ethanol concentration is expected due to its conversion into acetic acid (Chakravorty et al., 2016; Chen and Liu, 2000), which was clearly observed for the control culture, from day 4–5, and in CSC from day 3–4. FDSC presented the lowest amount of ethanol in the final product (4.39 ± 0.09 g.L^{-1}) compared to control and CSC and it may be related to the absence of *Zymomonas*, an ethanol producer, in the final composition of FDSC. Another reason for the lower production of ethanol and acetic acid could be a delay in the fermentation kinetics of FDSC, due to the freeze-drying process. The relation between ethanol production and the presence of *Zymomonas* is reinforced by control and CSC experiments, that presented 53.62% and 41.4% of *Zymomonas* and produced 10.77 ± 0.07 g.L^{-1} and 6.3 ± 0.14 g.L^{-1} of ethanol, respectively. In many countries, the legal limit for non-alcoholic beverages is 0.5% of alcohol by

volume (AVB), but it is known that many commercial kombuchas contain higher levels of ethanol and the consuming public is now aware of that (Talebi et al., 2017). Without standardization of kombucha fermentation, the alcohol level may be harmful to those who avoid its consumption. In Brazil, regulation for kombucha production emphasizes the importance of alcohol limit and the clear information in the label, and so classifies kombucha as a non-alcoholic (AVB <0.5%) or alcoholic (AVB from 0.5% to 8%) beverage (BRAZIL, 2019). In this study, only the FDSC presented an AVB <0.5%, reinforcing that the process standardization by freeze-drying the culture is possible and useful to obtain a product within legal limits. Also, it was possible to observe from CSC that inoculating without liquid containing ethanol and acids may help to maintain lower levels of alcohol in the final product.

Along with ethanol production, glycerol accumulation was observed and FDSC presented approximately five-fold higher concentration of this important alcohol than in the other treatments (Fig. 6). Compared to wine, the amount of glycerol was low and probably has no effect on sensory characteristics (Nurgel and Pickering, 2005).
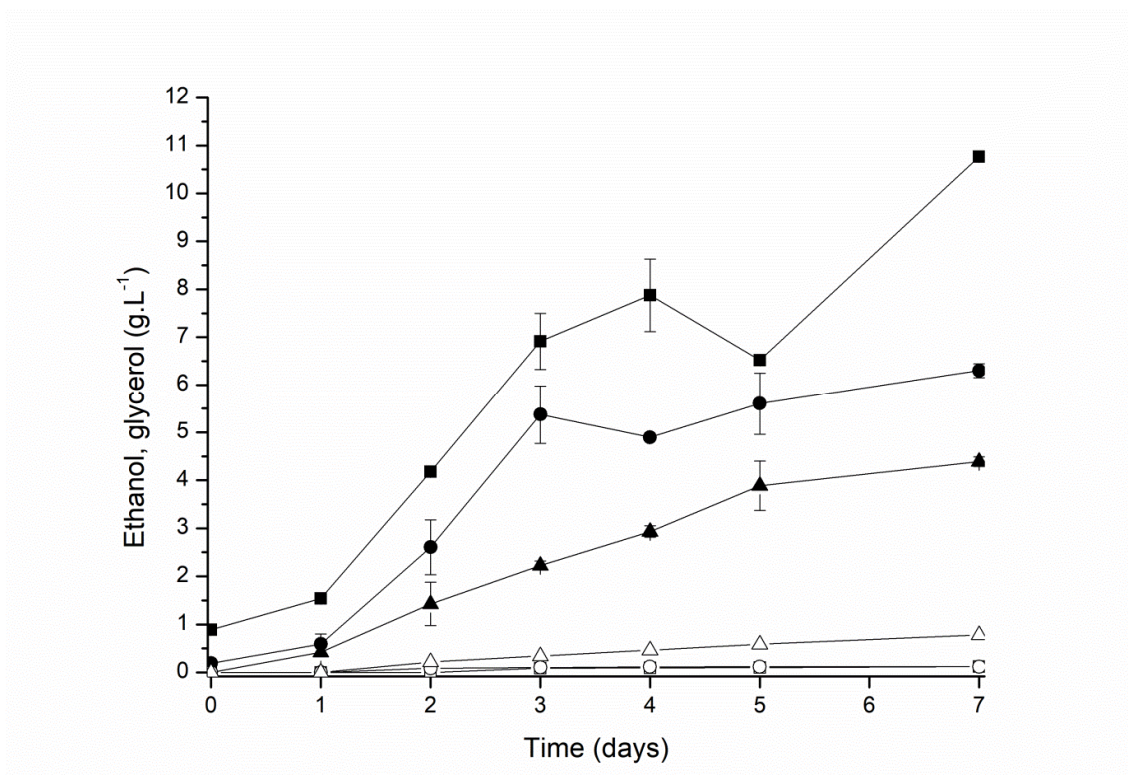
Figure 6. Production (g.L^{-1}) of ethanol (solid symbols) and glycerol (open symbols) during kombucha fermentation. Control (■), CSC (●), FDSC (▲). Experiments are the mean of duplicates.

After bottling the obtained kombucha beverages, yeasts remained hydrolyzing sucrose into glucose and fructose and an increase in acids and alcohol formation was observed. The control sample produced lactic acid in a concentration of 0.24 ± 0.02 g.L^{-1} and FDSC produced 0.11 ± 0.01 g.L^{-1} of succinic acid, which were not observed before bottling, showing that the anaerobic conditions of incubation benefit the metabolism of some genera. Although lactic and succinic acids are sometimes found in kombucha, their presence is not mandatory (Jayabalan et al., 2007; Vitas et al., 2018). However, the presence of these acids may improve the sour taste of kombucha, reducing the sensorial perception of acetic acid aroma and providing beneficial health effects (Sui et al., 2018).

The differences in the FDSC composition are due to the overall distinction in the microbial community after the freeze-drying process.

 3.3 Antioxidant activity

The kombucha antioxidant property is expected from *Camellia sinensis* tea infusion and it is one of the characteristics that labels kombucha as a healthy beverage, since consumption of food rich in antioxidants protects the human organism from free radicals (Lobo et al., 2017). As shown in Fig. 7, antioxidant activity, expressed as µmol TE.mL^{-1}, there were no differences between the control sample and CSC, whereas a small decrease was observed in the FDSC process. Some studies have reported a progressive increase in antioxidant activity of kombuchas, measured by ORAC, DPPH, and/or ABTS radical scavenging methods, with fermentation time (Chu and Chen, 2006; Jayabalan et al., 2008a, 2008b; Sun et al., 2015). The antioxidant activity of kombucha is related to polyphenols and flavonoids present in green tea and the fermentation process may increase or decrease this property due formation or bioconversion of bioactive compounds such as organic acids, minerals, and water-soluble vitamins. In addition, the compound variation is strongly correlated with the significant number of possible variables of the process, such as the type of tea and microbial community (Cardoso et al., 2020; Lobo et al., 2017; Malbaša et al., 2011; Sun et al., 2015). Starter culture of kombucha has an influence on antioxidant capability and a study has shown that changing yeast strains, for instance, from *S. cerevisae* to *Zygosaccharoyces spp.*, had a negative

influence on DPPH radical antioxidant activity (Malbaša et al., 2011). For instance, it has been shown that *D. anomala*, the predominant yeast in the starter culture in this work, is known as being capable of consuming p-coumaric, ferulic acid, and caffeic acid (Harris et al., 2008). The reason for antioxidant activity in FDSC presenting different behavior than for control and CSC might be explained by the altered symbiosis after freeze-drying. Decreases in ORAC values in kombucha have been reported before (Sun et al., 2015), but the final antioxidant activity was still high in FDSC, as compared to other kombuchas brewed using black tea (Sun et al., 2015), making this product acceptable concerning claims of antioxidant activity.
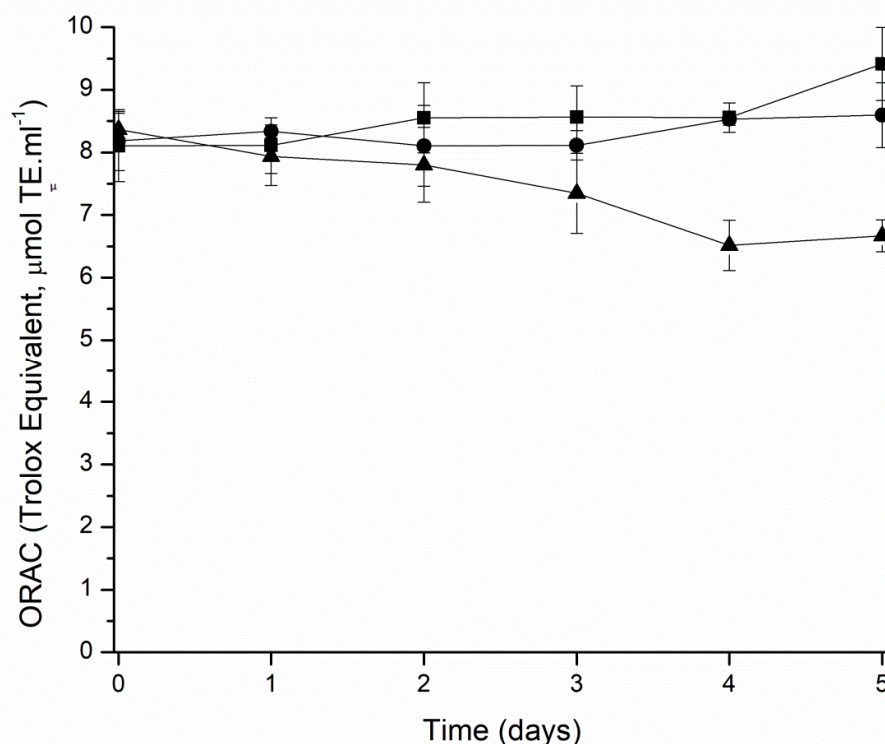
Figure 7. Changes in oxygen radical absorbance capacity (ORAC, µmolTE.mL^{-1}) during fermentation of kombucha: Control (■), CSC (●), FDSC (▲). Experiments are the mean of duplicates.

3.4 Sensory analysis

The acceptance test was performed in order to evaluate if changes in inocula would impact the main sensory attributes of kombucha. In our work, all attributes evaluated in the acceptance test showed no significant difference ($p > 0.05$) among

kombucha samples and all samples reached about 70% of overall acceptance (Table 1). In addition, when panelists were questioned about purchase intent, 73% would buy one or more of the samples. Control sample would be purchased by 45.4% of panelists, while CSC and FDSC would be purchased by 40.9 and 13.6%, respectively, a difference that we cannot explain. However, it is important to mention that 24 out of 52 panelists had never tasted kombucha beverage before since this product is still uncommon in Brazil.

Table 1. Acceptance of sensory attributes of Control, CSC and FDSC samples of kombucha.

	Appearance	Color	Aroma	Taste	Acidity	Overall acceptance
Control	3.83 ± 0.81^a	3.79 ± 0.78^a	3.18 ± 0.96^a	3.61 ± 1.05^a	3.52 ± 1.07^a	3.48 ± 1.00^a
CSC	3.86 ± 0.86^a	3.81 ± 0.81^a	3.30 ± 0.98^a	3.52 ± 1.09^a	3.29 ± 1.23^a	3.42 ± 1.04^a
FDSC	3.81 ± 0.97^a	3.81 ± 0.89^a	2.94 ± 0.85^a	3.04 ± 1.14^a	2.81 ± 1.12^a	2.98 ± 0.98^a

*Different letters in the same column are significantly different as determined by Tukey test ($p \leq 0.05$).

4 Conclusions

This study showed that biofilm and liquid from kombucha differ regarding the bacterial community and presented higher diversity compared to yeasts. The fermentation process was able to eliminate *Enterobacteriaceae*, showing the great importance of this ancient process in food safety. Control and CSC presented similar microbial and metabolites composition after 7 days of fermentation, except for the presence of *Liquorilactobacillus* in CSC and the final concentration of ethanol, higher in control. This suggests that the production of kombucha without liquid of starter culture may help to maintain lower amounts of alcohol in the final product. Additionally, the process of freeze-drying the starter culture resulted in a shift in the microbiota that maintained lower amounts of ethanol and produced lower concentrations of acetic acid, which may be positive for sensorial acceptance. Using a new freeze-drying starter culture for each batch, produced under controlled conditions, improved standardization of the process, avoiding natural fluctuations or even the possibility of pathogen contaminations since the microbial symbiosis would remain the same and the manipulation from the producer would be minimal. The

difference between final products in this work, after fermentation under identical conditions except for inocula preparation, suggests that freeze-dried starter culture, as prepared by us, changes symbiosis between microorganisms. It is important to perform an advanced investigation of this and other techniques in order to achieve full control of the fermentation process in kombucha, as it was done for other fermented beverages and yogurt. It is then possible to envisage a practical microbial inoculum for industrial applications and standardize the expected metabolites of the final product, resulting in a safe product that can be regarded as beneficial for health.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

The following is the Supplementary data to this article:
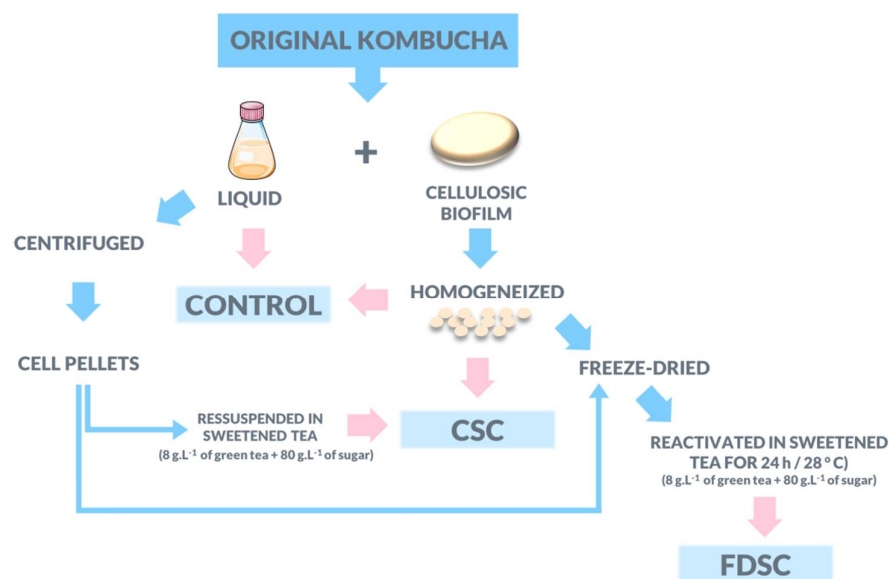
Figure S1. Schematic representation of the experimental approaches for kombucha fermentation in this work.

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CAPÍTULO III - DEVELOPMENT OF SYNTHETIC MICROBIAL CONSORTIA FOR KOMBUCHA FERMENTATION: ASSESSMENT OF METABOLITES AND VOLATILE COMPOUNDS PRODUCTION

A partir dos estudos demonstrados no capítulo anterior foi possível conhecer mais sobre a microbiota que compõe a kombucha, que é uma informação essencial para a padronização do inóculo deste produto. Além disso, observou-se a obtenção de produtos bioquimicamente diferentes através de um mesmo inóculo processado de maneiras diferentes. A diferença observada nas fermentações sugere que a simbiose dos microrganismos presentes na bebida é alterada com a manipulação da cultura *starter*, como a liofilização, que não foi suficiente para garantir a padronização do produto final. Com isso, se torna essencial o estudo do desenvolvimento de uma cultura *starter* conhecida e padronizada, a partir de microrganismos isolados, que será abordada neste capítulo.

Esta etapa do trabalho, que teve como objetivo o desenvolvimento de uma cultura *starter* selecionada para a fermentação de kombucha, será apresentada em forma de artigo científico, submetido ao periódico International Journal of Food Microbiology, intitulado "Development of synthetic microbial consortia for kombucha fermentation: assessment of metabolites and volatile compounds production". O trabalho versa sobre a seleção de algumas linhagens de bactérias e leveduras para a fermentação de kombucha como forma de padronizar e garantir uma bebida com características exigidas pela legislação, principalmente em relação ao teor de álcool. Foram avaliados os impactos nos aspectos tecnológicos, como tempo de fermentação, e microbiológicos e bioquímicos, como viabilidade celular, metabólitos produzidos, vida de prateleira, perfil de compostos voláteis, aceitação sensorial e potencial antioxidante. Alguns experimentos preliminares foram realizados para o desenvolvimento deste artigo e constam no Apêndice B.

Development of synthetic microbial consortia for kombucha fermentation:

assessment of metabolites and volatile compounds production

Mariana Fensterseifer Fabricio[1], Bruna Krieger Vargas[1], Bruna Tischer[1], Roger Wagner[2], Stephanie Reis Ribeiro[2], Nereida Cordeiro[3,4], Simône Hickmann Flôres[1], Marco Antônio Záchia Ayub*[1]

[1]Food Science and Technology Institute, Federal University of Rio Grande do Sul; Av. Bento Gonçalves, 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil.

[2]Department of Food Science and Technology, Federal University of Santa Maria, Roraima Avenue 1000, Camobi, Santa Maria 97105-900, RS, Brazil.

[3]LB3, Faculty of Exact Science and Engineering, University of Madeira, 9020-105 Funchal, Portugal

[4]CIIMAR-Interdisciplinary Centre of Marine and Environmental Research, University of Porto, 4450-208 Matosinhos, Portugal

*Corresponding author:

Tel.: +55 51 3308 6685; fax: +55 51 3308 7048

E-mail address: mazayub@ufrgs.br (M. A. Z. Ayub)

Abstract

The kombucha market has been growing fast as consumers are more interested in fermented, healthy products and suitable. Although many studies refer to Kombucha as a health beverage, it is not possible to generalize that information as the quality of final products depends mainly on the starter culture's microbial composition. However, there is a high variability of microbial composition. One of the major problems in the fluctuation of final products is the alcohol content, which usually surpasses the legal limits. This work aimed to develop a synthetic starter culture for kombucha fermentation to enhance quality and standardize the final product. The microbial starter studied include the bacteria *Acetobacter aceti* ATCC 15973, *Gluconacetobacter hansenii* ATCC 23769 and *Komagataeibacter saccharivorans*, and the yeasts *Dekkera anomala* UFMG-CM-Y4734, and *Kluyveromyces marxianus fragilis* BO399, a probiotic yeast. The fermentation time of the best combination of microbes was optimized, and two carbonation methods were tested (forced and natural carbonation). The kombuchas were analyzed regarding substrate consumption and fermentation metabolites, antioxidant activity, sensory acceptance, and volatile compounds profile, which were compared with two commercial brands. The shelf-life was evaluated to set up the stability of the beverages. It was possible to obtain a kombucha in 48 h of fermentation using the symbiosis of three microorganisms: *K. saccharivorans*, *D. anomala*, and *K. marxianus fragilis*. The acceptance of the forced carbonated Kombucha was similar to the commercial brands, and the alcohol content remained under 0.5 % ABV (alcohol by volume) during 60 days of storage. Regarding volatile compounds, the esters were the most impactful compounds that differentiated the kombuchas, such as ethyl 3-methyl butanoate, phenetyl acetate, ethyl hexanoate, ethyl 2-hydroxypropanoate and 2-methyl-1-propyl acetate. This work brings new information about the aromatic profile and other characteristics of kombuchas, which are still unclear in the literature, and a new approach for the production of a standardized beverage regarding its health benefits.

Keywords: Kombucha fermentation; starter culture; acetic acid bacteria; probiotic yeast; sensory analysis; aroma profile

1.   Introduction

Scientists, physicians, and the general population are showing an increased interest in functional foods, looking for positive effects on health and wellness (Lončar et al., 2006). This food category includes components associated with the improvement of overall health or the prevention of specific diseases (Kapsak et al., 2011). Among several beverages labelled as functional, the interest in Kombucha is thriving, since it may be prepared at home, and many health claims about it have been shared on popular media, despite the limited scientific evidence in support of its beneficial effects on health (Greenwalt et al., 1998; Lončar et al., 2006; Martini, 2018).

Kombucha is obtained by fermentation of sweetened green and/or black tea infusions by a symbiotic microbiota presented in a cellulosic pellicle, known as SCOBY (Symbiotic Culture of Bacteria and Yeast) (Jayabalan et al., 2014). This diverse culture is predominated by acetic acid bacteria (AAB), which are responsible for the production of the cellulosic pellicle and organic acids, such as acetic and glucuronic acids (Tran et al., 2020). The microbial composition depends on the origin of the culture, but among several strains that have been reported in the literature, *Komagataeibacter*, *Acetobacter*, and *Gluconacetobacter* are the main bacteria, whereas *Brettanomyces/Dekkera* and *Zygosaccharomyces* are the commonest yeast strains (Arıkan et al., 2020; Chakravorty et al., 2016; Fabricio et al., 2022; Marsh et al., 2014).

The literature on Kombucha reports that microbial and biochemical compositions are diverse between products, even for commercial sources of this beverage (De Filippis et al., 2018; Fabricio et al., 2022; Marsh et al., 2014), because products prepared from spontaneous fermentation result in highly variable processes and end products (Vinicius De Melo Pereira et al., 2020). Moreover, natural consortia of microorganisms may produce inhibitory and/or toxic metabolites that interfere with the microbiota (Che and Men, 2019). The process variables, such as temperature, pH, the concentration of substrates, vessel geometry, and time, help control the fermentative process but are not sufficient to assure the standardization of kombucha production (Abaci et al., 2022). The alcohol content of kombuchas is the main concern in terms of industrialization, and some studies showed the difficulty of

maintaining the levels of alcohol by volume (ABV) below 0.5 %, as required by several countries' regulations (Suhre et al., 2021; Talebi et al., 2017).

Considering these obstacles and challenges, a starter culture technology is crucial to assure food safety and quality at all levels of production of fermented foods: households, traditional, and industrial scale (Capozzi et al., 2017). In view of this, the aim of this work was to develop a synthetic starter culture of bacteria and yeasts suitable for kombucha fermentation, as an alternative to replace the traditional inoculation process (cellulose pellicle + fermented liquid from previous fermentations). To evaluate the suitability of the microbial consortia, we analyzed the impact on metabolites production, sugar consumption, antioxidant activity, and volatile compounds. A sensorial analysis was also carried out to assess the impact of the fermentation process over the product.

2. Material and methods

2.1 Development of starter culture for kombucha fermentation

2.1.1 Strains, cell maintenance, and pre-inocula

Three acetic acid bacteria (AAB) and two yeasts were used in this work in co-cultures. The AAB *Acetobacter aceti* ATCC 15973 and *Gluconacetobacter hansenii* ATCC 23769 were obtained from André Tosello Research and Technology Foundation (FAT, Campinas, Brazil), and the *Komagataeibacter saccharivorans* was kindly supplied by the University of Madeira (UMa, Funchal, Portugal). The yeast *Dekkera anomala* UFMG-CM-Y4734 was kindly provided by the Collection of Microorganisms and Cells of the Federal University of Minas Gerais (UFMG, Minas Gerais, Brazil) and the *Kluyveromyces marxianus fragilis* BO399 was kindly supplied by Turval Company (Udine, Italy). Cells were kept frozen at -80 ^{o}C, in a 40 % glycerol solution-cell suspension. For immediate use, cells of *A. aceti* and *G. hansenii* were kept on plates containing Mannitol agar (25 g/L mannitol, 3 g/L peptone, 5 g/L yeast extract, 15 g/L agar). *K. saccharivorans* was kept on MRS agar (De Man et al., 1960), and yeasts were cultivated on YM agar (10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract, 20 g/L agar). The same culture medium, without agar, was used for each microorganism pre-inocula. Microorganisms were selected based on the most common reports about Kombucha in the literature (Coton et al., 2017; Fabricio et al., 2022; Marsh et al., 2014; Sui et al., 2018; Tran et al., 2020). The yeast

K. marxianus fragilis was selected based on its high invertase activity, fast growth, and probiotic properties, such as immune system stimulation and gut colonization (Maccaferri et al., 2012).

Pre-inocula were prepared by transferring 0.5 mL of glycerol-solution cell suspension of each microorganism to 250 mL conical flasks containing 50 mL of each respective cultivation medium and incubated in a rotary shaker at 37 ºC and 180 rpm for *K. marxianus fragilis* and 30 °C and 120 rpm for the other microorganisms, until reaching desired cell concentration. The cell pellets were washed twice with sterile distilled water, centrifuged at 3 000 *g* for 15 min, and resuspended in a sweetened green tea infusion to be used as the starter culture.

2.1.2 Preparation of tea infusion and fermentation conditions

All Kombuchas were fermented on the same culture medium consisting of distilled water, 8 g/L of organic green tea (Vemat, SC, Brazil), and 60 g/L or 50 g/L of organic demerara sugar (Native, SP, Brazil). Fermentations were performed in 250 mL beakers filled with 251 mL (specific interfacial area (SIS) of 0.132) of sweetened green tea, covered with sterile gauze and cheesecloth to create aerobic conditions. Different batches of Kombucha were fermented with each starter culture.

For the preparation of sweetened tea, a solution of distilled water added with sucrose was sterilized at 121 °C for 15 min. Green tea leaves were infused for 10 min in boiling water and then filtered using a membrane pore size of 0.22 μm After cooled, tea infusion was added to the sugar solution, and 251 mL of sweetened tea was placed in each beaker. A phosphoric acid solution (1 M) was used to set the initial pH to 4.5.

2.1.3 Starter culture design

To determine the best suitable combination of microorganisms to obtain Kombucha, a Plackett-Burmann (PB) design was performed based on four microbial strains (variables): *A. aceti*, *G. hansenii*, *K. saccharivorans*, and *D. anomala*. The yeast *K. marxianus fragilis* was used as a fifth, fixed ingredient because this yeast has probiotic properties and high invertase activity, necessary to hydrolyse sucrose. Cell concentration, determined based on data from the literature and previous

experiments (data now shown), was 1×10^7 CFU/mL for bacteria and 1×10^5 CFU/mL for yeasts. An 8-run PB design (Table 1) was used to evaluate the survival of each strain and production of acetic acid and ethanol after fermentation, the two key products in Kombucha.

For the PB experiments, the beakers were inoculated with the different starter cultures, and cultivations were incubated for 10 days at 28 °C. After 10 days of fermentation, samples of 20 mL were collected from beakers for analysis of microbial growth, pH, sugars, and fermentation metabolites (see 2.2).

2.1.4 Growth kinetics of kombuchas fermented with the selected starter culture

The best suitable mixture of microorganisms obtained in the PB design (1×10^7 CFU/mL of *K. saccharivorans*, 1×10^5 CFU/mL of *D. anomala,* and 1×10^5 CFU/mL of *K. marxianus fragilis*) was further studied. Kinetics of fermentation was performed to observe the evolution of metabolites production and sugar consumption. Then, the same starter culture with a higher concentration of the yeast *K. marxianus fragilis* (1×10^6 CFU/mL) was performed with the aim of obtaining a higher invertase activity, a possible probiotic activity in the beverage, and a shorter fermentation time. After observing that it was possible to shorten fermentation time to 3 days, the concentration of 50 g/L of sucrose was tested to obtain a kombucha in less time, with residual sugar similar to commercial brands. Preparation of all tea infusions and fermentation were performed as described for the PB design (described in items 2.1.3 and 2.1.4). Samples were collected for specific periods of time to evaluate pH, microbial growth, sugar consumption, and acids and alcohols production.

The best condition for the fermentation of Kombucha by the synthetic consortia (sugar concentration and time of fermentation) was subjected to the carbonation process, as it is performed in industries. The fermented Kombucha was bottled in sterile hermetic glass bottles, and two different processes of carbonation were carried out. A natural carbonation process was performed, in which the yeasts were responsible for converting sugars into CO_2 and consisted in bottling and fermenting at 28 °C for 48 h. The second process was forced carbonation, consisting in infusing CO_2 into the liquid from a gas cylinder. In this case, the Kombucha was refrigerated to 4±0.5 °C, and then CO_2 was injected at constant agitation and pressure of 1 bar

until the system stabilized and saturated. This stage aimed to evaluate the most suitable process for kombucha carbonation, as well as to compare the influence of the carbonation method on the final product characteristics and shelf-life. The natural and forced carbonated kombuchas' antioxidant activity, sensory acceptance, and volatile profile were assessed and compared with two commercial brands. Antioxidant activity and volatile compounds of non-fermented tea were also evaluated.

2.1.5 Shelf-life study design

A shelf-life study of the natural and forced carbonated kombuchas was performed. Samples of Kombucha were refrigerated (4 °C) after carbonation, and pH, microbial growth, sugars, and metabolites were analyzed after 10, 20, 30, 45, 60, and 90 days.

2.2 Fermentation monitoring

2.2.1 Enumeration of microorganisms

Microorganisms were enumerated by surface inoculation on agar, incubated at 30 °C, and CFU were counted. To inhibit yeast growth, *A. aceti* and *G. hansenii* were plated on Mannitol agar containing 128 µg/mL of fluconazole to inhibit yeast growth. *K. saccharivorans* was cultivated on MRS agar added of 128 µg/mL of fluconazole, to inhibit yeast growth, and 0.1 % (v/v) of cysteine-HCl to inhibit *A. aceti* and *G; hansenii* growth. To inhibit bacterial growth, yeast enumeration was performed on YM agar containing 34 µg/mL chloramphenicol (Sigma-Aldrich, Germany).

2.2.2 Determination of substrate consumption and fermentation metabolites

Collected samples were centrifuged (3 000 × g, 15 min), and the supernatant was filtered through 0.22 µm membrane pore size to determine the concentration of glucose, sucrose, fructose, glycerol, ethanol, acetic, lactic, and succinic acids. Analyses were performed in HPLC using Bio-Rad Aminex 87C for sugars and Bio-Rad Aminex 87H for organic acids and alcohols. HPLC assay conditions used for each column were run according to a previous publication (Fabricio et al., 2022).

2.2.3 Volatile compounds profile

The volatile compounds (VC) were extracted by headspace solid-phase microextraction (HS-SPME) using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) 2 cm - 50/30 µm covering fibre (SupelcoTM, Darmstadt, Germany). For analysis, 5 ± 0.1 mL of Kombucha were transferred into 20 mL glass vials (hermetically closed with a silicone/PTFE cap), added with 1.6±0.1 g of NaCl and 10 µL of 3-octanol (internal standard - IS, 8.5 µg/mL). The vials were then immersed in a thermostatic water bath at 35 °C for 10 min, followed by a 45 min exposure for adsorption of the compounds. Each treatment was done in triplicate. The VC analysis was run in a gas chromatograph coupled to a mass spectrometer (GC/MS) (Shimadzu GC/MS-QP 2010 Plus, Kyoto, Japan). The fibre was thermal desorbed into the injector at 250 °C for 10 min in splitless mode (1 min split-off). Helium was the carrier gas at a constant flow of 1.3 mL/min. The VC were separated using a polar phase fused silica capillary column (ZB-Wax, Phenomenex, USA; 60 m × 0.25 mm; 0.25 µm of thickness film). The initial oven temperature was set at 35 °C for 1 min, followed by a 3 °C/min temperature ramp to 180 °C and then, increasing 5 °C/min up to 230 °C, remaining for 2 min. The GC/MS interface and the ionization source were kept at 250 °C and 230 °C, respectively. The MS data were collected in the electron impact ionization mode at +70 eV, using mass range scanning of 35-350 m/z. The identification of VC was performed by comparing the mass spectrum available in the National Institute of Standards and Technology (NIST) library, the linear retention index (LRI) from literature, with those experimentally obtained data. The experimental LRI was obtained through a series of n-alkanes at the same GC conditions. The VC concentration was determined by internal standardization, using the equivalent of 3-octanol IS solution. The response factor between IS and each analyte was assumed as one. All analyses were carried out in triplicate and the results were expressed as mg/L.

2.3 Antioxidant activity

The ability to inhibit peroxyl radicals was evaluated using the Oxygen Radical Absorbance Capacity (ORAC- hydrophilic) method described by Huang et al. (2005) (adapted). Kombucha samples were diluted 1:200 in potassium phosphate buffer (75 mM), and Trolox was used as the control standard (from 8 to 96 µmol/L). Experiments were conducted in ELISA microplates with 150 µL of fluorescein

solution (81 nM), 25 µL of the diluted sample or Trolox, and 25 µL of AAPH (2,2-azobis(2-amidinopropane) dihydrochloride) solution (152 mM). The assay was carried out on a fluorescence reader (Enspire 2300 Multimode Plate Reader, Perkin Elmer, USA), and readings were taken every minute for 90 min at 37 °C, with excitation and emission wavelengths of 485 and 528 nm, respectively. Results were calculated using the area under the curve (AUC) and expressed as µmol Trolox equivalents (TE) per mL of Kombucha. The AUC was calculated by Equation (1) as:

$$AUC = 1 + f1/f0 + f2/f0 + f3/f0 + ... fn/f0 \qquad (1)$$

where $f0$ was the initial fluorescence reading and fn was the fluorescence reading at each cycle.

2.4 Sensory analysis

The kombuchas produced in this work (natural and forced carbonated) and two Brazilian commercial brands were subjected to an acceptance test using a 9-point hedonic scale (1- dislike extremely; 9- like extremely). Panelists evaluated the attributes of appearance, aroma, taste, acidity, and overall acceptance. The Acceptance Index (AI) was calculated by Equation (2):

$$AI (\%) = (Attribute\ media \times 9^{-1}) \times 100 \qquad (2)$$

A panel of 100 untrained panelists, between 18 and 60 years old, were served with randomized samples of 30 mL coded with a three-digit random number. The research had the permission of the University Ethical Committee (UFRGS, Protocol n: 18613419.8.0000.5347).

2.5 Statistical analysis

The results obtained from fermentations were submitted for analysis of variance (ANOVA), and the means were compared using Tukey's test ($p < 0.05$). The significant results of volatile compounds analysis and sensory evaluation were subjected to principal component analysis (PCA) using the Statistica 12.5 software

(StatSoft Inc., USA). For this, each variable was auto-scaled to obtain the same weight for all variables (mean = 0 and variance = 1) before PCA analysis.

3 Results and discussion

3.1 Selection of the microorganisms for kombucha fermentation

The PB experimental design matrix and the responses are shown in Table 1. The purpose of the Plackett-Burman experiments was to understand metabolic interactions between strains and select the most suitable combination of microorganisms capable of fermenting sweetened green tea into a product similar to that produced with the traditional, artisanal kombucha starter culture (SCOBY). The responses expected for ethanol and acetic acid were based on the Brazilian legal limits for Kombucha. To be labelled as a non-alcoholic beverage, the legal limit of alcohol in Brazil and many other countries is 0.5 % ABV (alcohol by volume). The regulatory concentration of acetic acid must be between 1.8 and 7.8 g/L (BRAZIL, 2019). The concentration of acetic acid expected was the lowest allowed since this acid imparts an unpleasant aroma when present in excess.

Table 1. Process variables and experimental results of the 8-run Plackett-Burmann design to study the impact of co-cultured strains on kombucha fermentation. Results are expressed in g/L.

Run	AA	GH	KS	DA	Acetic acid	Ethanol
1	-1	-1	1	-1	0.00 ± 0.00	0.00 ± 0.00
2	1	-1	-1	-1	0.31 ± 0.01	9.08 ± 0.15
3	-1	1	-1	-1	0.29 ± 0.06	8.60 ± 0.17
4	1	1	1	-1	0.33 ± 0.02	9.08 ± 0.21
5	-1	-1	1	1	3.39 ± 0.67	0.27 ± 0.02
6	1	-1	-1	1	0.32 ± 0.02	6.22 ± 0.48
7	-1	1	-1	1	0.31 ± 0.04	6.74 ± 0.12
8	1	1	1	1	5.03 ± 0.34	0.28 ± 0.07

AA: *A. aceti;* GH: *G. hansenii;* DA: *D. anomala;* KS; *K. saccharivorans.* (-1) absence of variable; (1) presence of variable.

Results showed that the bacteria *A. aceti* and *G. hansenii* were not suitable for kombucha fermentation in co-culture because both strains presented very few cells or no viable counts (Fig. 1), respectively, after 10 days of fermentation, consequently producing low amounts of acetic acid. Another result of this drop in viability of

bacteria in runs 2, 3, 4, 6, and 7 was the final concentration of alcohol above legal limits (Table 1), once *K. marxianus fragilis* in co-culture with *A. aceti* and/or *G. hansenii* produced high concentrations of ethanol and was able to produce succinic acid, which was not observed in the consortia with *K. saccharivorans*. The final pH values were correlated with bacterial growth and acetic acid production, *i.e.* fermentations with *A.aceti* and *G. hansenii* presented final pH of 3.3 and less than 0.5 g/L of acetic acid. In contrast, experiments with *K. saccharivorans* presented a pH of 2.5 and acetic acid production between 3.5 and 4.9 g/L (Fig. 1). The pH results showed that, unlike other yeasts, *K. marxianus fragilis* and *D. anomala* are tolerant to low pH, and this emphasizes that those strains are robust microorganisms for kombucha fermentation.

It is important to observe the influence of the yeast *D. anomala* in the microbial consortia that, although did not influence the production of ethanol, changed the symbiosis between *K. saccharivorans* and *K. marxianus fragilis*. When *D. anomala* was absent, *K. saccharivorans* remained viable, but did not produce acetic acid (Table 1; run 1 *vs* run 5).
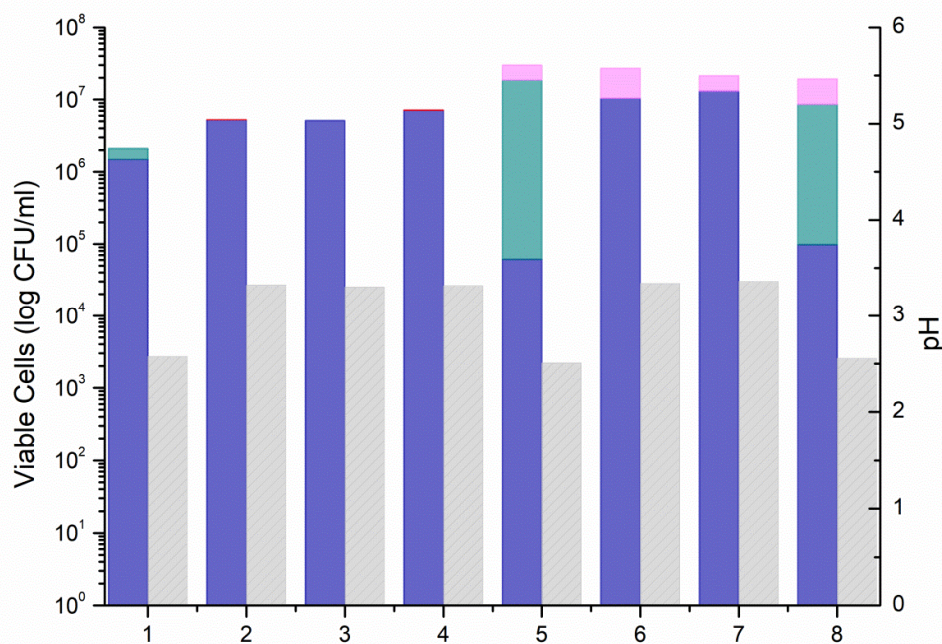
Figure 1. pH and viable counts of bacteria and yeast populations after 10 days of fermentation with different starter culture designed by PB experiments. Blue: *K. marxianus fragilis*; Green*: K. saccharivorans*; Pink: *D. anomala*; Red: *G. hansenii* and *A. aceti*.

The most favorable combination of microorganisms for kombucha fermentation was run 5, composed of *K. saccharivorans*, *D. anomala* and *K.marxianus fragilis*. Under this condition, *K. saccharivorans* maintained the same viability and produced acetic acid within the legal limits. Although results of run 8 presented good values for ethanol and acetic acid, the microbial combination included two acetic acid bacteria that were not viable after 10 days of fermentation (*A. aceti* and *G. hansenii*). The combination of run 5 was further studied in the subsequent experiments. The microbial interaction in this consortium probably corresponds to mutualism, which refers to the cross-feeding between strains through the exchange of metabolic products, which is beneficial to both partners (Che and Men, 2019). In this case, the yeasts produced ethanol that was oxidized by bacteria to acetic acid. The yeasts also hydrolyzed sucrose into glucose and fructose for bacterial growth and production of metabolites.

3.2 Kinetics of Kombucha fermented using the selected starter culture

To understand the metabolism of the chosen starter culture, 10-days kinetics was performed under the same conditions of previous experiments with the most potential consortia (Table 1, run 5). The microbial growth, pH, production of metabolites, and sugar consumption are present in Fig. 2. As expected, a decline in pH value was observed during fermentation, which is related to microbial growth and acid production. From the start of fermentation, the pH decreased from 4.5 to 2.85 in 3 days, taking another 7 days to drop to 2.44. Concerning microbial growth, *K. marxianus fragilis* increased on day 3 and then slightly decreased until day 10. The cell concentration of this yeast is responsible for ethanol production because it has a faster growth rate compared to *D. anomala*. Most of the ethanol produced was oxidized to acetic acid by *K. saccharivorans*, which population remained stable through 10 days of fermentation. The probiotic yeast *K. marxianus fragilis* is able to produce lactic acid from lactose (Tabanelli et al., 2016), however, it did not produce this acid from glucose and fructose, as the main products metabolized were ethanol and glycerol. The yeast *D. anomala* increased cell counts to 7.14 log CFU/mL after 10 days of fermentation, suggesting that this strain has tolerance toward low pH environments. After day 8 of fermentation, the kombucha pH was lower than the minimum legal limits (pH 2.5), which means the fermentation should be interrupted at day 8 at most. The total sugar consumption was low, as 53.96 g/L of the total sugar

still remained on day 10. Not surprisingly, the residual concentration of fructose was higher than glucose at the end of fermentation because yeasts and bacteria prefer to metabolize the latter as a substrate.
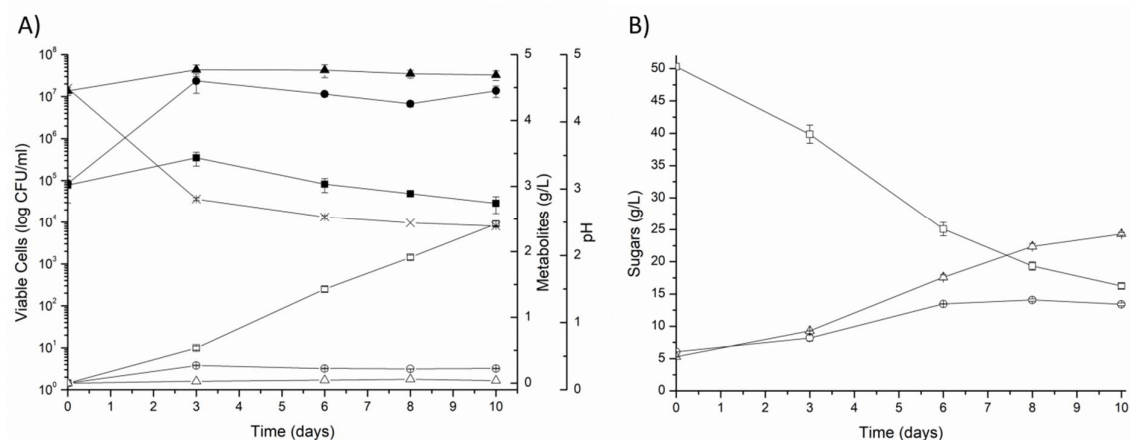
Figure 2. 10-days kinetics of kombucha fermentation with the synthetic consortia (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *D. anomala,* and 10^5 CFU/mL of *K. marxianus fragilis*) from PB experiments. A) metabolites: pH (x), ethanol ($\triangle$), glycerol ($\circ$), acetic acid ($\square$); and viable counts: *K. saccharivorans* (▲), *K. marxianus fragilis* (●), and *D. anomala* (■). B) Sugars: sucrose ($\square$), glucose ($\circ$) and fructose ($\triangle$). Experiments are mean of duplicate.

Modifying the starter culture composition individually is essential to make the synthetic consortia cost-effective, stable, and robust (Che and Men, 2019). A shorter fermentation would be an economic advantage for making the process more efficient for the industry. To accelerate the kombucha fermentation process, kinetics using a higher initial cell concentration of *K. marxianus fragilis* to 1×10^6 CFU/mL was performed. The aim of this step was, in addition to fermenting Kombucha faster, to obtain a higher concentration of the probiotic yeast in the final beverage. The results showed a higher production of ethanol and glycerol and, consequently, acetic acid production (Fig. 3). Even with the higher ethanol concentration, the acetic acid bacteria were able to maintain the final level of alcohol very low, as in the previous experiment (less than 0.4 g/L). After 10 days of fermentation, acetic acid concentration was excessive (6.26 ± 0.01 g/L), and the pH was extremely low (under 2.5). It means that this condition had a positive impact on fermentation time, and it would result in the same product as the previous experiment in only 3 days. On day 3, it was observed the higher viable counts of the probiotic yeast, *K. marxianus fragilis*. It was possible to observe the influence of the cell concentration on sugar

hydrolysis, resulting in lower amounts of sucrose and higher concentration of glucose and fructose in the final product. The total sugar consumption in both 10 days kinetics was around 11 % of the initial sugar, and for this reason, the following experiments were performed for 3 days using 50 g/L of sucrose instead of 60 g/L.
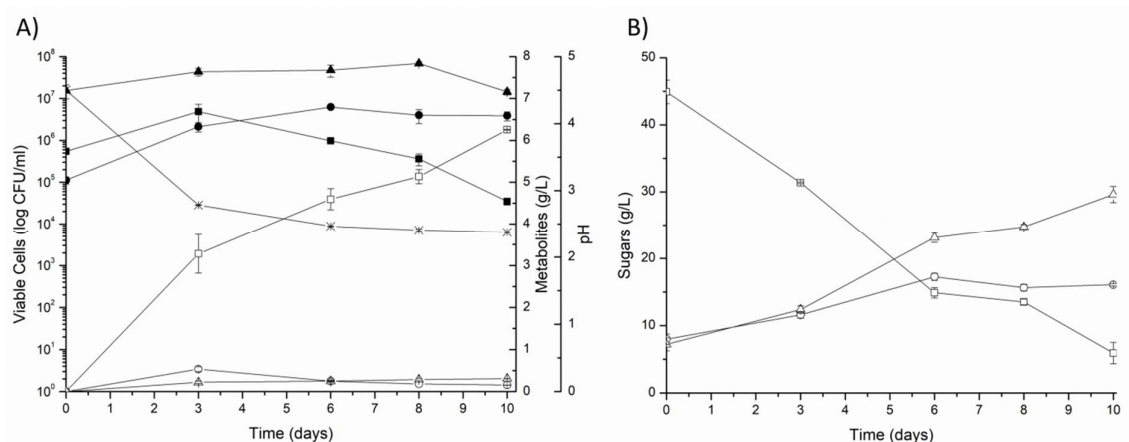
Figure 3. 10-days kinetics of kombucha fermentation with the synthetic consortia (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *D. anomala,* and 10^6 CFU/mL of *K. marxianus fragilis*. A) pH (x), ethanol (Δ), glycerol ($\circ$), acetic acid ($\square$), viable counts of *K. saccharivorans* ($\blacktriangle$), *K. marxianus fragilis* ($\bullet$), and *D. anomala* ($\blacksquare$). B) Sugars: Sucrose ($\square$), glucose ($\circ$) and fructose (Δ). Experiments are mean of duplicate.

The 3-day kinetics results (Fig. 4) showed that the consortia had a remarkable ability to ferment Kombucha, and it would be possible to interrupt fermentation as early as 48 h to obtain a product with low amounts of alcohol and acetic acid, respecting the legal limits.
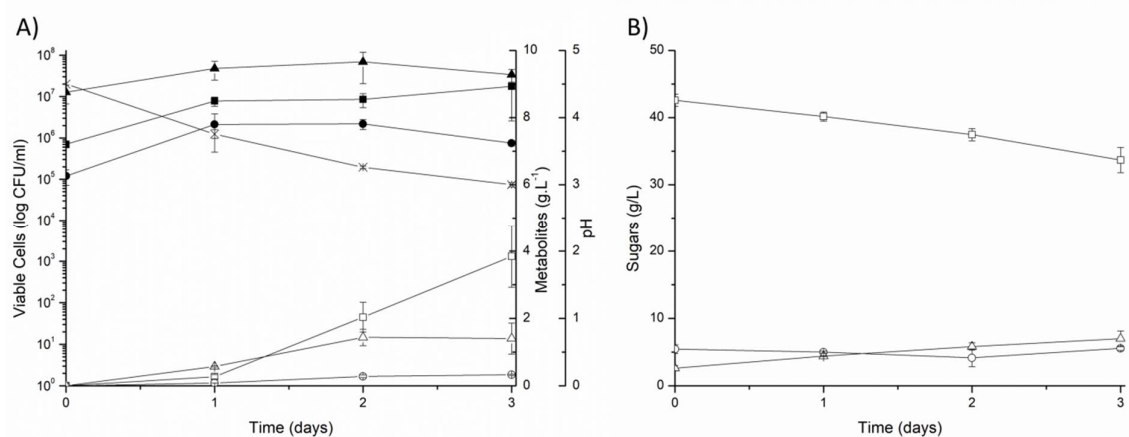
Figure 4. 3-days kinetics of kombucha fermentation with the synthetic consortia (10^r CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *D. anomala,* and 10^6 CFU/mL of *K. marxianus fragilis*. A) pH (x), ethanol (Δ), glycerol ($\circ$), acetic acid ($\square$), viable counts of *K. saccharivorans* ($\blacktriangle$), *K. marxianus fragilis* ($\bullet$), and *D. anomala* ($\blacksquare$). B) Sugars: Sucrose ($\square$), glucose ($\circ$) and fructose (Δ). Experiments are mean of triplicate.

The next step was to test the carbonation of the Kombucha after 48h of fermentation and to analyze its antioxidant properties, sensory acceptance, and volatile compounds. The results of organic acids, pH, and alcohols of final products made by natural carbonation (NC), forced carbonation (FC), and two commercial brands of Kombucha (CB1 and CB2) are shown in Table 2. The brands were chosen regarding the production process, as CB1 is produced by natural fermentation and CB2 is produced using the forced carbonation method.

Table 2. Concentrations of sugars, organic acids, and pH of kombuchas made by NC and FC and two commercial brands (CB1 and CB2). Results are expressed in g/L.

Sample	NC	FC	CB1	CB2
Acetic acid	1.88 ± 0.07^b	1.74 ± 0.05^b	5.74 ± 1.10^a	3.03 ± 0.34^b
Glycerol	0.45 ± 0.05^{ab}	0.28 ± 0.14^b	0.53 ± 0.13^a	0.06 ± 0.02^c
Ethanol	4.83 ± 1.32^b	1.96 ± 0.21^b	18.24 ± 3.35^a	2.01 ± 0.10^b
Lactic acid	0	0	1.05 ± 0.25^a	0
Succinic acid	0	0	0.14 ± 0.01^a	0
pH	3.20 ± 0.01^b	3.20 ± 0.01^b	3.39 ± 0.06^a	3.18 ± 0.06^b
Sucrose	23.37 ± 1.08^b	37.46 ± 0.05^a	2.05 ± 0.85^c	32.77 ± 1.86^a
Glucose	6.06 ± 0.29^c	3.39 ± 0.01^d	13.52 ± 0.26^a	10.17 ± 0.66^b
Fructose	9.99 ± 1.40^b	3.78 ± 0.01^c	22.63 ± 1.50^a	9.27 ± 1.07^b

NC: natural carbonation; FC: forced carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same row are significantly different as determined by the Tukey test ($p \leq 0.05$).

The secondary fermentation of Kombucha is carried out to obtain a sparkling beverage through the production of ethanol and carbon dioxide in the anaerobic environment of the bottle. Under this condition, the metabolism of acetic acid bacteria is inhibited, and the yeasts are responsible for alcoholic fermentation through the conversion of residual sugars. The process of natural carbonation is the most commonly used by industries. However, the control of the fermentation is hampered by many variables, such as the addition of fruits or juices as flavoring agents, which adds more sugar and other microorganisms to the beverage and may interfere with the fermentation, risking spoiling an entire batch of kombucha production. Also, the production of carbon dioxide increases the pressure of the bottles, risking the rupture of glass bottles (Kim and Adhikari, 2020). Another critical issue about natural carbonation is the amount of ethanol produced since the acetic acid bacteria are not

able to oxidize the ethanol into acetic acid in the anaerobic environment of the bottle. In this work, it was possible to observe that natural carbonation resulted in a 3-fold increase in ethanol content, exceeding the legal limits. Given these difficulties, forced carbonation is a useful way to avoid excess ethanol production and fluctuations between batches. Although second fermentation is widely used, little investigation has been performed on this topic (Tran et al., 2020).

Monitoring pH is usually a parameter used to determine the end of kombucha fermentation. However, the results of this work showed that, besides very similar pH, the kombuchas presented very different compositions, and it shows the importance of controlling and standardizing the production of this beverage beyond pH control.

CB1 kombucha labelling indicates its alcohol content as 0.9 % ABV, however, the results obtained in this work revealed that the concentration is more than twice as high (2.31 % ABV) as the indicated concentration. This increase in the alcohol content may be due to the storage period, which is a lacking topic of investigation in the literature. NC kombucha slightly exceeded the legal limits (0.61 % ABV), reinforcing that natural carbonation is the most critical step in controlling ethanol during kombucha fermentation. FC kombucha suggests that forced carbonation is, indeed, a very useful alternative for controlling the process and ensuring the quality and safety levels of Kombucha.

3.3 Oxygen Radical Absorbance Capacity (ORAC)

Kombucha is considered a drink with high antioxidant potential, and this characteristic depends on the type and concentration of tea, fermentation time, and starter culture used (Jakubczyk et al., 2020; Malbaša et al., 2011). This potential is derived from the tea, which is rich in catechins-theaflavin and tearubigin, characteristic that is the most related to the health benefits of Kombucha (Cardoso et al., 2020; Jakubczyk et al., 2020). The results of antioxidant activity of the non-fermented broth, FC, NC, CB1, and CB2 are presented in Table 3.

Table 3. Oxygen radical absorbance capacity (ORAC) of non-fermented tea and NC, FC, CB1, and CB2 kombuchas.

	ORAC (µL TE/mL)
Non-fermented tea	10.81 ± 1.47^a
NC	10.66 ± 0.80^a
FC	11.11 ± 0.55^a
CB1	8.22 ± 0.75^{ab}
CB2	5.13 ± 1.42^b

NC: natural carbonation; FC: forced carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same column are significantly different as determined by the Tukey test ($p \leq 0.05$).

The fermentation can directly affect the antioxidant power of a product since this process involves the formation or bioconversion of bioactive compounds such as organic acids, responsible for combating reactive oxygen species (ROS) (Fabricio et al., 2022; Malbaša et al., 2011). The fermented kombuchas (FC and NC) did not differ significantly from the non-fermented tea, which is positive as green tea presents high antioxidant potential (Khan and Mukhtar, 2019). Also, it was possible to observe that natural carbonation, obtained in an anaerobic environment for an extra 48 h of fermentation, did not affect the ORAC values. The results obtained for FC and NC showed that the synthetic starter culture using *K. marxianus fragilis, D. anomala*, and *K. saccharivorans* is suitable for kombucha fermentation and does not negatively impact the antioxidant compounds, as it may occur in some cases, as demonstrated by Malbasa et al. (2011), which used *S. cerevisae* and *Zygosaccharoyces* spp. as starter culture and observed a decrease in the antioxidant activity (measured through the DPPH radical). On the other hand, some studies have shown that fermentation may increase the antioxidant capacity of Kombucha (Ahmed et al., 2020; Cardoso et al., 2020; Vargas et al., 2021). For example, Wang et al. (2020) developed a synthetic microbial community with *Acetobacter pasteurianus*, *Gluconacetobacter xylinus* and *Zygosaccharomyces bailii* that resulted in an increase in the total phenol and flavonoid content during fermentation of Kombucha, achieving maximum values in the day 8 of the process. Throughout the starter culture development in the present work, the kombucha fermentation period was reduced to 2 days (4 days in the case of NC kombucha), and the stability of the antioxidant power may be attributed to the short fermentation process. The ORAC assay is a method that, in addition to being adaptable for several matrices, also is relevant to *in vivo* conditions, as it measures the radical chain-breaking ability of antioxidants using

a peroxyl radical, which is the predominant free radical in human biology (Prior, 2015; Zhong and Shahidi, 2015). However, only a few studies have assessed the kombucha antioxidant capability using this assay. Fabricio et al. (2022) found ORAC values from 8.1 to 9.4 µmol/mL in kombuchas fermented from 8 g/L of green tea for 5 days, whereas Sun et al. (2015), fermented Kombucha using 10 g/L of black tea for 12 days and found values of approximately 4 to 6.42 µmol/mL. Silva Junior et al. (2021) also evaluated the potential antioxidant of Kombucha by ORAC assay. They found even lower values, varying from 1.82 to 3.71 µmol/mL, in traditional Kombucha fermented for 7 days using 125 g/L of green tea as substrate. All those works that performed the ORAC analysis in kombuchas presented lower amounts in comparison to FC and NC kombuchas obtained in this work.

Besides CB1 and CB2 labels showing they were also produced from green tea, the CB2 presented the lowest antioxidant capacity and differed ($p < 0.05$) from FC and NC. This difference may be related to the microorganisms involved in the fermentation process of CB1 Kombucha and possibly to the concentration of green tea used in the infusion by producers.

3.4 Sensory analysis of kombuchas

There is still no standard for the sensory profile of Kombucha except for its vinegary sour characteristic (Tran et al., 2020). Also, in the literature, there are no research publications that focus only on the sensory or consumption of Kombucha, and the few works that performed sensory analysis only evaluated a small number of participants (from 8 to 50) (Fabricio et al., 2022; Neffe-Skocińska et al., 2017; Tran et al., 2020). In this work, the sensory test was performed to evaluate if consumers accepted the kombuchas produced by synthetic consortia. The scores for all attributes, given by the 100 participants, ranged from 4.61 to 7.21 and kombuchas presented significant statistical differences ($p < 0.05$) (Table 4). Compared to other samples, the NC kombucha had the lowest scores for all attributes, except for appearance. Concerning aroma and overall acceptance, the CB1 and CB2 had higher scores and differed statistically ($p < 0.05$) from FC and NC.

Table 4. Acceptance of kombuchas' sensory attributes evaluated by 100 participants.

	Natural carbonation		Forced carbonation	
	CB1	NC	FC	CB2
Appearance	6.63 ± 1.72[ab]	6.93 ± 1.54[a]	7.19 ± 1.54[a]	7.22 ± 1.82[a]
Aroma	6.37 ± 2.02[a]	4.64 ± 1.97[b]	5.15 ± 1.89[b]	6.22 ± 1.63[a]
Flavor	6.47 ± 1.96[ab]	5.56 ± 1.97[c]	6.00 ± 2.03[bc]	6.68 ± 1.76[a]
Acid flavor	6.47 ± 1.90[ab]	6.12 ± 1.91[b]	6.20 ± 1.83[b]	6.81 ± 1.80[a]
Overall acceptance	6.62 ± 1.69[a]	5.63 ± 1.78[b]	6.06 ± 1.97[b]	6.90 ± 1.57[a]

NC: natural carbonation; FC: forced carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same row are significantly different as determined by the Tukey test ($p \leq 0.05$).

Kombucha is still not widely consumed in Brazil, as it is sold mainly in specialty stores or health food stores at high prices. For this reason, the participants were asked if they had already tried Kombucha and if they considered themselves consumers of such beverages. Of 100 participants, 53 had already tried and liked Kombucha. Analyzing the data from those 53 participants, the averages of acceptance ranged from 4.72 to 7.51, and the samples differed statistically regarding aroma and overall acceptance attributes ($p < 0.05$) (Table 5).

Table 5. Acceptance of kombuchas' sensory attributes evaluated by the 53 participants that had already tried Kombucha before.

	CB1	NC	FC	CB2
Appearance	6.75 ± 1.80[a]	7.02 ± 1.68[a]	7.30 ± 1.54[a]	7.51 ± 1.81[a]
Aroma	6.68 ± 2.04[a]	4.72 ± 2.00[b]	5.17 ± 1.88[b]	6.09 ± 1.75[a]
Flavor	6.58 ± 1.91[a]	5.94 ± 1.78[a]	6.42 ± 1.88[a]	6.66 ± 1.70[a]
Acid flavor	6.34 ± 2.02[a]	6.13 ± 1.92[a]	6.49 ± 1.79[a]	6.66 ± 1.91[a]
Overall acceptance	6.79 ± 1.72[a]	5.85 ± 1.74[b]	6.38 ± 1.94[ab]	6.85 ± 1.51[a]

NC: natural carbonation; FC: forced carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same row are significantly different as determined by the Tukey test ($p \leq 0.05$).

Regarding aroma, the commercial samples presented higher averages ($p < 0.05$) compared to FC and NC. The differences observed may be explained by the volatile profile of kombuchas (discussed below), as CB1 and CB2 presented higher amounts and diversity of esters (Table 6), which are responsible for fruity notes. Besides the high concentration of acetic acid in CB1, the acceptance for aroma e overall quality was higher than for the kombuchas fermented with synthetic consortia. A likely explanation for this would be the presence of esters in CB1, which is reinforced by the comments left by consumers such as "apple aroma" and "green grape aroma". Also, this sample was the only one that presented lactic acid in its

composition, which, as well as the presence of esters, helps to mask the perception of "vinegar smell".

Besides the differences between the kombuchas produced in this work and commercial brands, the Acceptance Index for FC, CB1 and CB2 were greater than 70 %, which indicates they represent potential for commercialization. Since FC kombucha had adequate acceptance, total control of fermentation, and low concentrations of alcohol, results show it would be a very suitable consortium for kombucha industrial production. Forced carbonation seems to be a recommended or suitable method for controlling the ethanol concentration in Kombucha, as the secondary fermentation (anaerobic) is avoided.

3.5 Volatile profile of kombuchas

The aroma of kombuchas originated from the tea, and the volatile metabolites produced during fermentation have been widely described as "vinegary" and "cidery" (Tran et al., 2020). The first is related to acetic acid concentration, and the second is associated with yeast activity during the production of superior alcohols and esters. In this work, 102 volatile compounds were detected in the sweetened tea and kombuchas by HS-SPME-GC/MS (Table 6). They belonged to 12 chemical classes: acids, alkanes, alcohols, aldehydes, amines, ketones, esters, ethers, lactones, phenols, sulphurs, and terpenes.

Table 6. Volatile compounds of non-fermented sweetened tea and kombuchas.

Compounds	Non-fermented tea	FC	NC	CB1	CB2
Acids					
Propionic acid	nd	184.46 ± 9.22^a	181.57 ± 61.9^a	93.08 ± 3.82^{ab}	154.73 ± 28.3^a
2-Methyl propanoic acid	8.87 ± 2.43^{ab}	3.15 ± 0.33^c	3.52 ± 0.26^c	7.49 ± 0.38^b	11.92 ± 0.56^a
Butanoic acid	nd	115.45 ± 30.8^a	145.28 ± 11.88^a	111.17 ± 18.09^a	nd
3-methyl butanoic acid	7.23 ± 1.47^a	0.79 ± 0.08^c	0.87 ± 0.03^c	1.36 ± 0.06^{bc}	2.75 ± 0.37^b
Hexanoic acid	3.56 ± 0.73^c	15.5 ± 1.14^b	14.09 ± 0.61^b	3.54 ± 0.1^c	28.08 ± 3.83^a
Ethyl hexanoic acid	12.20 ± 3.38^d	53.3 ± 1.84^{ab}	46.07 ± 1.94^b	30.68 ± 4.73^c	63.13 ± 10.25^a
Heptanoic acid	8.81 ± 1.15^b	26.41 ± 1.82^b	31.29 ± 3.7^b	25.21 ± 1.87^b	93.02 ± 27.07^a
Octanoic acid	2.29 ± 0.51^{bc}	4.24 ± 0.20^b	4.15 ± 0.59^b	0.34 ± 0.01^c	8.44 ± 2.00^a

(continue)

Compounds	Non-fermented tea	FC	NC	CB1	CB2
2-Heptenoic acid	13.73 ± 3.98[a]	nd	nd	nd	nd
Nonanoic acid	2.15 ± 0.30[b]	5.77 ± 0.64[b]	7.97 ± 2.37[b]	6.81 ± 2.82[b]	16.13 ± 4.76[a]
2-Octenoic acid	37.49 ± 20.25[a]	nd	nd	nd	nd
Decanoic acid	3.86 ± 0.87[b]	6.29 ± 0.98[ab]	4.05 ± 1.12[b]	0.45 ± 0.06[c]	7.32 ± 1.63[a]
9-Decenoic acid	nd	338.22 ± 84.83[a]	162.16 ± 65.74[b]	13.43 ± 1.99[bc]	nd
Geranic acid	nd	327.84 ± 18.59[a]	253.62 ± 85.49[a]	42.55 ± 7.1[b]	302.46 ± 83.82[a]
3-Decenoic acid	nd	nd	nd	21.36 ± 1.82[b]	189.02 ± 20.97[a]
(E)-2-Decenoic acid	20.27 ± 4.22[c]	nd	52.36 ± 6.12[b]	114.15 ± 12.58[a]	nd
Benzoic acid	72.47 ± 20.54[a]	nd	nd	nd	nd
Dodecanoic acid	9.69 ± 1.83[c]	29.05 ± 6.32[ab]	27.5 ± 9.52[ab]	10.07 ± 2.74[c]	36.52 ± 7.22[a]
Tetradecanoic acid	11.57 ± 2.23[b]	41.44 ± 6.05[a]	49.44 ± 17.43[a]	23.98 ± 6.99[ab]	52.81 ± 10.24[a]
Pentadecanoic acid	3.34 ± 0.88[c]	10.99 ± 1.45[ab]	18.51 ± 10.34[ab]	7.12 ± 2.78[ab]	20.53 ± 9.34[a]
Alkanes					
Cyclohexane	nd	nd	nd	1.83 ± 0.31[a]	1.97 ± 0.28[a]
m-xylene	21.22 ± 0.98[b]	110.56 ± 14.83[ab]	185.62 ± 90.09[a]	203.78 ± 45.29[a]	146.8 ± 24.56 [ab]
Alcohols					
2-Methyl-1-propanol	39.73 ± 21.11[a]	2.88 ± 0.35[b]	2.34 ± 0.24[b]	6.1 ± 0.27[b]	26.03 ± 2.88[ab]
1-Butanol. 2-methyl-	17.28 ± 2.22[a]	2.29 ± 0.24[b]	2.06 ± 0.24[b]	2.31 ± 0.14[b]	22.03 ± 5.28[a]
1-Butanol. 3-methyl-	8.48 ± 2.23[a]	0.46 ± 0.05[b]	0.37 ± 0.02[b]	0.26 ± 0.01[b]	2.31 ± 0.08[b]
1-Hexanol	30.60 ± 12.45[c]	147.42 ± 20.86[b]	299.09 ± 33.82[a]	75.61 ± 14.65[c]	83.66 ± 2.10[c]
cis-3-hexen-1-ol	nd	Nd	nd	9.26 ± 0.43[b]	320.44 ± 26.16[a]
2-Ethyl 1-hexanol	3.17 ± 0.47[b]	5.5 ± 0.93[b]	5.02 ± 1.49[b]	4.67 ± 0.37[b]	15.4 ± 1.12[a]
1.11-Undecanediol	3.37 ± 1.91[a]	53.97 ± 43.22[a]	Nd	25.27 ± 8.39[a]	52.23 ± 50.65[a]
1.3-Butanediol	nd	Nd	Nd	48.34 ± 14.95[a]	nd
(E)-2-Octen-1-ol	5.17 ± 1.32[a]	Nd	nd	nd	nd
1-Nonanol	7.75 ± 1.15[b]	Nd	nd	nd	25.01 ± 5.68[a]
1-Decanol	22.79 ± 3.68[b]	177.18 ± 9.78[a]	196.91 ± 57.89[a]	62.86 ± 5.2[b]	nd
1-undecanol	nd	Nd	nd	nd	198.9 ± 66.94[a]
β-Phenylethanol	28.62 ± 11.06[a]	1.54 ± 0.16[b]	0.67 ± 0.02[b]	0.46 ± 0.01[b]	2.15 ± 0.33[b]
1-Dodecanol	13.42 ± 5.48[b]	56.62 ± 28.8[ab]	54.35 ± 15.16[ab]	47.15 ± 22.18[b]	106.34 ± 14.51[a]
Hexadecanol	52.21 ± 13.2[c]	269.72 ± 38.51[a]	235.52 ± 14.99[ab]	167.17 ± 44.33[b]	258.37 ± 36.68[a]

Compounds	Non-fermented tea	FC	NC	CB1	CB2
Aldehydes					
Acetaldehyde	8.04 ± 2.01^{ab}	24.64 ± 17.73^{ab}	21.19 ± 7.92^{a}	nd	Nd
Isobutyraldehyde	nd	Nd	nd	nd	60.51 ± 54.73
2-methyl butanal	144.46 ± 4.94^{a}	Nd	2514.18 ± 2262.39^{a}	nd	nd
3-methyl butanal	nd	Nd	429.39 ± 192.27^{a}	nd	nd
Hexanal	5.33 ± 0.30^{c}	82.00 ± 22.3^{bc}	128.01 ± 65.46^{ab}	192.08 ± 20.98^{a}	80.05 ± 6.65^{bc}
Octanal	9.74 ± 2.95^{ab}	40.76 ± 20.39^{ab}	80.31 ± 54.09^{a}	nd	nd
(Z)-2-Heptenal	4.43 ± 1.13^{c}	61.42 ± 14.72^{b}	nd	127.68 ± 32.99^{a}	86.11 ± 22.85^{ab}
Nonanal	2.97 ± 1.71^{a}	34.57 ± 31.80^{a}	28.50 ± 22.15^{a}	16.34 ± 1.35^{a}	48.93 ± 29.87^{a}
Benzaldehyde	nd	95.64 ± 6.53^{b}	99.00 ± 55.58^{b}	56.95 ± 10.69^{b}	262.19 ± 47.58^{a}
(E)-2-Dodecenal	nd	218.70 ± 18.08^{a}	195.27 ± 124.06^{a}	132.36 ± 12.32^{ab}	nd
Butylated hydroxytoluene aldehyde (BHT)	nd	Nd	nd	147.17 ± 66.63^{b}	376.17 ± 74.12^{a}
Amines					
Ethylacetamide	nd	Nd	nd	109.27 ± 17.24^{a}	nd
Ketones					
2-Butanone	105.34 ± 68.65^{a}	Nd	nd	nd	nd
(E)-3-penten-2-one	18.63 ± 2.42^{b}	177.62 ± 31.57^{ab}	333.14 ± 189.24^{a}	120.62 ± 22.23^{ab}	23.49 ± 1.75^{b}
5-Methyl 3-heptanone	2.05 ± 0.23^{b}	84.01 ± 31.07^{a}	110.32 ± 14.45^{a}	34.92 ± 5.11^{b}	83.85 ± 2.63^{a}
3-Hydroxy 2-butanone	nd	Nd	nd	11.91 ± 0.12^{b}	17.77 ± 4.34^{a}
Octen-3-one	4.60 ± 0.68^{b}	66.17 ± 10.60^{ab}	211.01 ± 63.94^{a}	nd	116.71 ± 28.67^{b}
5-Octen-2-one	nd	97.76 ± 23.73^{a}	77.92 ± 14.06^{a}	30.14 ± 1.34^{b}	Nd
6-Methyl-5-heptene-2-one	6.15 ± 1.06^{c}	98.72 ± 5.56^{a}	51.44 ± 18.32^{b}	54.93 ± 1.96^{b}	80.77 ± 3.69^{a}
3.5-Octadien-2-one	10.51 ± 2.34^{d}	263.90 ± 17.09^{a}	nd	82.80 ± 4.14^{c}	180.01 ± 21.94^{b}
Esters					
Ethyl acetate	1.22 ± 0.18^{a}	1.20 ± 0.18^{a}	0.87 ± 0.04^{b}	nd	0.70 ± 0.06^{b}
2-Methyl-1-propyl acetate	15.52 ± 2.62^{c}	40.90 ± 7.91^{b}	29.67 ± 5.98^{bc}	19.54 ± 1.69^{c}	90.17 ± 3.19^{a}
Ethyl butanoate	2.31 ± 0.20^{c}	31.83 ± 6.62^{ab}	41.27 ± 15.47^{a}	42.35 ± 4.61^{a}	13.02 ± 0.27^{bc}
Ethyl 3-methyl butanoate	nd	nd	225.89 ± 19.79^{a}	61.9 ± 8.66^{b}	247.95 ± 29.65^{a}
3-Methyl butyl acetate	0.71 ± 0.10^{c}	6.47 ± 1.18^{a}	4.76 ± 2.17^{ab}	2.37 ± 0.34^{bc}	5.61 ± 0.04^{ab}

(continue)

Compounds	Non-fermented tea	FC	NC	CB1	CB2
Ethyl 3-methyl-2-butenoate	Nd	nd	nd	nd	1095.32 ± 209.69[a]
Ethyl hexanoate	Nd	nd	nd	16.52 ± 2.46[b]	128.32 ± 15.14[a]
3-Methylbutyl butyrate	Nd	nd	nd	nd	183.16 ± 35.21[a]
3-Methylbutyl isovalerate	Nd	nd	nd	nd	153.38 ± 41.67[a]
Ethyl 2-hydroxypropanoate	Nd	nd	nd	8.30 ± 0.61[b]	205.10 ± 13.41[a]
Ethyl 2-hydroxy-3-methylbutanoate	8.60 ± 2.16[b]	nd	nd	347.65 ± 87.36[a]	Nd
Diethyl butanedioate	Nd	nd	nd	82.37 ± 11.43[a]	Nd
Ethyl (Z)-4-decenoate	Nd	nd	nd	69.32 ± 19.36[a]	Nd
Decyl methacrylate	Nd	nd	nd	nd	36.53 ± 3.38[a]
Benzyl ethanoate	Nd	nd	nd	155.38 ± 27.34[a]	Nd
Ethyl phenylacetate	Nd	nd	nd	22.43 ± 0.90[a]	Nd
Phenethyl acetate	Nd	13.49 ± 2.49[b]	0.46 ± 0.06[c]	8.93 ± 0.74[b]	30.55 ± 4.97[a]
Ethyl dodecanoate	Nd	nd	nd	47.79 ± 5.26[a]	Nd
Methyl ethyl tetradecanoate	36.75 ± 14.25[a]	84.13 ± 33.66[a]	nd	88.43 ± 63.89[a]	Nd
Hexyl salicylate	Nd	158.5 ± 63.59[b]	nd	nd	666.40 ± 295.18[a]
Methyl Dihydrojasmonate	24.11 ± 12.33[bc]	nd	121.68 ± 40.02[ab]	94.2 ± 69.90[bc]	207.82 ± 21.68[a]
Diisooctyl adipate	Nd	nd	nd	75.19 ± 12.23[b]	141.11 ± 5.99[a]
Diethyl Phthalate	14.27 ± 4.18[d]	62.27 ± 10.29[bc]	66.74 ± 4.55[b]	47.55 ± 10.33[c]	101.96 ± 1.93[a]
Ether					
1.1-Diethoxyethane (Capsicum annuum)	2.39 ± 0.37[a]	nd	nd	nd	nd
Phenols					
Phenol	nd	94.15 ± 14.21[a]	45.60 ± 16.74[b]	nd	nd
Phenol. 2-ethyl-	nd	42.55 ± 3.60[b]	14.56 ± 2.26[c]	18.44 ± 3.31[bc]	178.24 ± 19.36[a]
2.6-di-t-Butylphenol	3.56 ± 1.31[b]	16.52 ± 5.09[ab]	14.56 ± 7.89[ab]	8.08 ± 2.04[b]	28.05 ± 7.39[a]
Lactones					
Butyrolactone	nd	62.95 ± 9.69[bc]	80.14 ± 11.10[ab]	5.57 ± 0.88[c]	144.17 ± 56.91[a]
Sulfurs					
Dimethyl sulfide	nd	nd	Nd	7.81 ± 1.46[a]	nd
3-Methylsulfolane	nd	nd	Nd	nd	227.04 ± 11.28[a]

(continue)

Compounds	Non-fermented tea	FC	NC	CB1	CB2
3-(Methylthio) 1-propanol	nd	nd	26.56 ± 6.09[b]	82.06 ± 20.39[a]	nd
Terpenes					
Eucalyptol	nd	nd	Nd	nd	226.76 ± 18.20[a]
Cardene	nd	nd	Nd	20.51 ± 2.27[b]	577.37 ± 94.24[a]
(+)-4-Carene	nd	nd	774.81 ± 113.86[a]	nd	nd
β-Cyclocitral	29.93 ± 5.26[a]	nd	Nd	nd	nd
Menthol (isomer)	21.31 ± 5.44[b]	131.04 ± 29.9[a]	102.45 ± 46.03[ab]	52.06 ± 0.75[ab]	108.63 ± 32.89[ab]
α-Terpinol	36.49 ± 12.80[a]	30.58 ± 4.97[ab]	16.59 ± 0.93[bc]	7.83 ± 0.19[c]	13.43 ± 1.50[c]
Citronellol (isomer)	nd	nd	Nd	10.32 ± 0.39[ab]	348.2 ± 271.69[a]
β-Damascenone	53.84 ± 3.35[a]	nd	Nd	nd	nd
β-Ionone	11.00 ± 2.69[c]	78.89 ± 11.92[b]	53.61 ± 8.39[bc]	28.96 ± 1.45[bc]	169.29 ± 42.2[a]
Geranylacetol	nd	52.17 ± 9.21[b]	36.52 ± 3.19[b]	95.3 ± 10.13[b]	303.99 ± 95.25[a]
β-Ionone epoxide	9.37 ± 3.47[b]	85.63 ± 6.57[a]	82.58 ± 25.44[a]	nd	nd
Ethylguaiacol	nd	45.1 ± 6.10[b]	11.46 ± 1.25[c]	110.69 ± 12.45[a]	nd
Nerolidol	nd	211.39 ± 58.21[a]	73.03 ± 29.12[ab]	65.58 ± 16.57[ab]	206.08 ± 96.51[a]
(E)-Ethyl cinnamate	nd	nd	nd	193.44 ± 12.35[a]	nd

NC: natural carbonation; FC: forced carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Results are expressed as mean peak areas ($\times 10^5$) ± standard deviation; (nd = not detected;). The values followed by different letters in the same row differed significantly in the Tukey test ($p \leq 0.05$). The results are expressed by the mean of the triplicates.

The non-fermented sweetened tea was analyzed to detect which volatile compounds were consumed and produced by the synthetic consortia of microorganisms. Among the 56 volatile compounds found in non-fermented tea, the most abundant group were acids (n = 15), followed by alcohols (n = 12). The most abundant compounds were 2-methyl butanal, 2-butanone, benzoic acid, and *β*-damascenone. 2-Methyl butanal is derived from amino acid degradation (Pripis-Nicolau et al., 2000) and is responsible for almond, cocoa, fermented, hazelnut, and malt notes (Kim et al., 2016). This compound was fully consumed in FC kombucha while increased in NC kombucha, being the most abundant compound in this sample. The terpenic ketone *β*-damascenone has a low odor threshold and is considered one of the most potent flavor constituents in teas (Yang et al., 2013). The *β*-damascenone was fully consumed during the fermentation time in both FC and NC kombuchas. Other tea volatile compounds such as 1-nonanol, 2-heptenoic acid,

benzoic acid, 2-octen-1-ol, ethyl 2-hydroxy-3-methylbutanoate, and *β*-cyclocitral were totally consumed during fermentation. The metabolization of some volatile compounds and the production of others explain why kombucha flavors remarkably differ from tea.

Even with the same starter culture, the carbonation method used resulted in some differences between kombuchas. The anaerobic in-bottle fermentation in NC kombucha produced more ethanol and different volatile compounds, such as (E)-2-decenoic acid, 2-methyl butanal, 3-methyl butanal, ethyl 3-methyl butanoate, methyl dihydrojasmonate, and 3-(methylthio)-1-propanol. The latter, a volatile compound also found in CB1, is a sulfur flavor found in wine and soy sauce, which imparts off-flavor cauliflower-like and potato-like and has a low odor threshold of 1 to 3 ppm (Lwa et al., 2015).

Esters and acids were the main chemical classes found in the fermented kombuchas and both classes have great importance in the volatile profile and in the acceptance of the beverage. The aldehydes hexanal, nonanal, benzaldehyde, and E-2-decenal were detected in all fermented samples, and those compounds are related to off-flavors, such as rancid, fat, and green odor descriptors (Kim et al., 2016). However, due to synergistic effects, the perception of some off-flavors may be masked or enhanced by the presence of other volatile compounds (Savary et al., 2021). Many of those aldehydes have been detected in kombucha before (Tran et al., 2022).

The mixture of higher alcohols produced in the fermentative process by yeasts is known as fusel alcohols and imparts off-flavors at high concentrations. Those alcohols, such as isoamyl alcohol (3-methyl butanol), are derived from amino acids and are mainly attributed to the Ehrlich pathway in wine (Hazelwood Lucie et al., 2008). The fusel alcohols, produced through the transamination of an amino acid, can be further esterified to produce volatile esters and, at low concentration with their esters, contribute to the aroma of the final product (Hazelwood Lucie et al., 2008; Tran et al., 2022). The fusel alcohols may also be produced independent of the Ehrlich pathway, as the amino acids that originate those alcohols may be produced using amino acids biosynthesis pathways (Tran et al., 2022). The understanding of the metabolic pathways helps further studies directed to the production of desirable compounds.

All fermented kombuchas also presented the esters 2-methyl-1-propyl acetate (fruit, apple, and banana notes), ethyl butanoate (fruit and pineapple notes), 3-methyl butyl acetate (banana notes), and phenethyl acetate (rose, honey, and tobacco notes), and the terpenes menthol (peppermint notes), α-Terpinol (anise, mint notes), geranylacetol (magnolia and green notes), and nerolidol (wood and flower notes), compounds which contribute positively to the aroma. CB1 and CB2 differed on the profile of esters and presented higher diversity than FC and NC. Those esters may have contributed to masking the off-flavors found in the kombuchas, reflecting in the higher scores for aroma on sensory analysis.

The comments left by participants on the sensory evaluation are strongly related to the volatile compounds. CB1 was related to apple, and green grape aroma by consumers on the sensory analysis, and those notes are related to the compounds ethyl (Z)-4-decenoate, ethyl 2-hydroxy-3-methylbutanoate, and ethyl hexanoate, which were found in this sample.

A principal component analysis (PCA) was performed in order to explore and visualize groupings and discrimination in the volatile profile and sensory analysis. For that, we analyzed the results of the volatile compounds that differed significantly ($p < 0.05$) in the fermented samples (NC, FC, CB1, and CB2) and the sensory attributes (overall acceptance, aroma, acid flavor, and flavor). Principal components PC 1 and PC 2 explained 50.25 and 40.29 % of the variation in the data, respectively (Fig 5a). PC 1 separated CB2 from the other 3 samples, while PC 2 separated CB1 and CB2 from the other samples (FC and NC). The right-hand upper quadrant suggests a similar volatile composition between NC and FC kombuchas, which was expected since the difference between both was the carbonation process. The loadings plot (Fig 5b) showed that CB2 was characterized by having higher concentrations of 2-methyl-1-propyl acetate, an ester with apple and banana sensory notes, which are in accordance with the comments of the participants in the sensory analysis. Also, the attributes of the sensory evaluation are more correlated to commercial brands because of the higher scores of those samples and their higher concentrations of fruity esters and terpenes. Regarding terpenes, (E)-Ethyl cinnamate (honey, cinnamon) was found only in CB1, eucalyptol (mint, sweet) exclusively in CB2, and FC and NC presented β-Ionone epoxide (fruit, wood). The FC and NC kombuchas were mainly correlated with aldehydes, such as

acetaldehyde (originated from sugars) and octanal (originated from lipid oxidative process), and this may explain the lower aroma scores on the sensory analysis since those compounds are associated with rancid, pungent, and fat aroma sensory descriptors. At the same time, the presence of esters that helps to mask those off-flavors was lower compared to commercial brands.
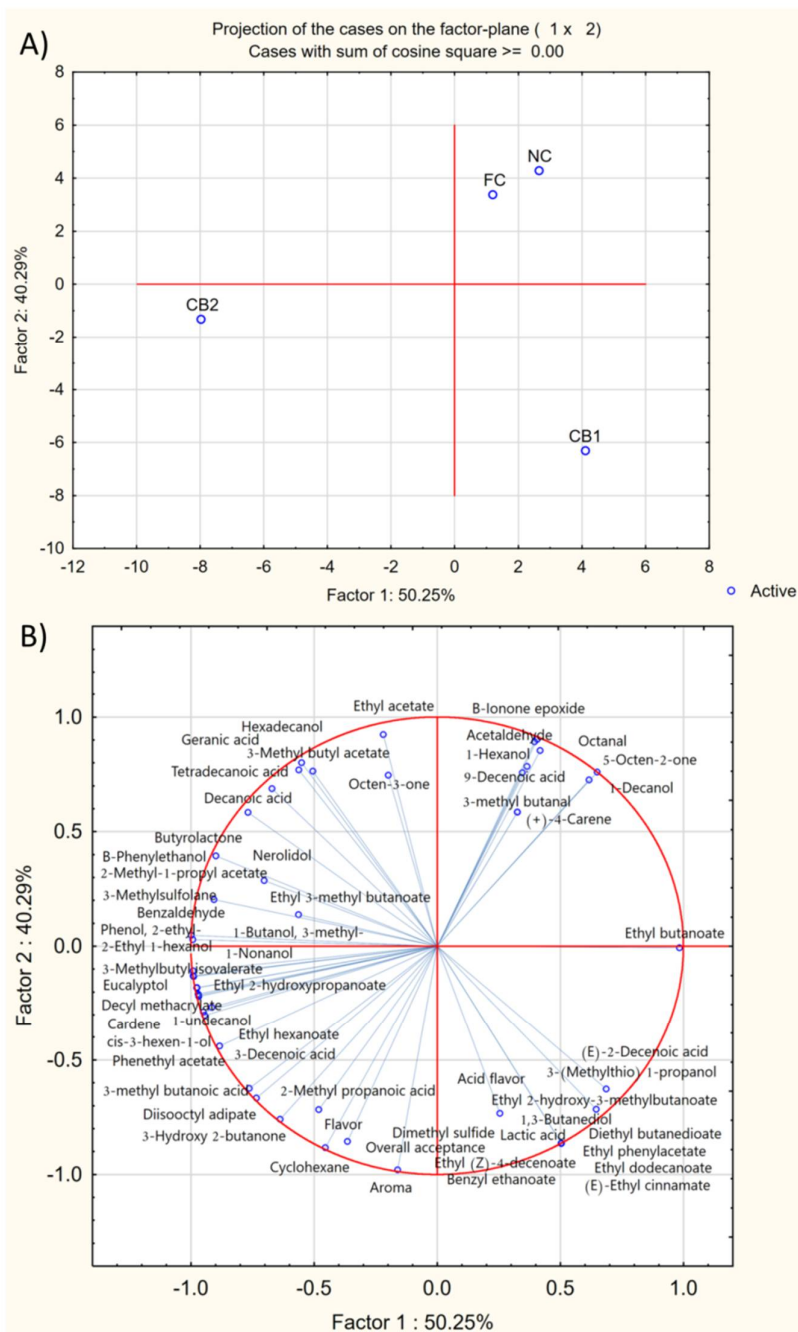
Figure 5. Principal Component Analysis plots of volatile compounds and sensory attributes found in fermented kombuchas (FC, NC, CB1, and CB2). A) Score plot. B) Loadings plot (volatile compounds, sensory attributes and organic acids).

Since there is no expected profile of volatile compounds in Kombucha and only a few studies were performed, more studies like this are necessary to define which volatile compounds are essential in Kombucha and what are their origins (Savary et al., 2021; Tran et al., 2022; Zhang et al., 2021). The use of a synthetic starter culture helps control the production of volatile and non-volatile metabolites, making it possible to produce high quality and standardized Kombucha.

3.6 Shelf-life evaluation

A shelf-life study of 90 days was carried out to evaluate the stability of kombuchas produced in this work, and the results are presented in Table 7. The microbial counts of *K. marxianus fragilis* during the shelf-life of both kombuchas were lower than 1.10^7 viable cells, which is the dosage that delivers beneficial effects defined in previous studies (Lisotti et al., 2013; Maccaferri et al., 2012). Further studies with higher cell concentrations or other probiotic strains would be of great interest.

Although, the natural carbonation process increased *K. marxianus fragilis* growth, followed by a decrease in viable counts along the storage period. The viable counts of *K. saccharivorans* have severely dropped on day 90 in both FC and NC kombuchas. Despite being viable for 60 days, the bacteria were not able to oxidize the ethanol produced by yeasts in acetic acid because of the absence of oxygen in the bottle. For this reason, an increase in ethanol concentration of NC and FC kombuchas during storage was observed. Along with ethanol concentration, the pH slightly increased in both samples. The ethanol in NC kombucha exceeded the legal limits (0.5 % ABV) and reached 1.2 % ABV in 45 days of storage. FC kombucha, which showed potential industrial application, remained stable as the parameters were within legal limits for 60 days under refrigeration.

Results showed that regardless the kombuchas were stored at 4 °C, the fermentation process continued at a low pace. The invertase activity of yeasts continued to hydrolyze the sucrose, which decreased during the 90 days of storage. Nonetheless, the glucose and fructose released were little consumed. To the best of our knowledge, there are no studies about kombucha's shelf-life impacts on metabolites and cell concentration in literature.

Table 7. The effect of storage time on NC (natural carbonation) and FC (forced carbonation) kombuchas fermented with a synthetic microbial starter culture.

Time (days)	Microbial counts (log CFU/mL)			Metabolites (g/L)			Sugars (g/L)		
	KMF	DA	KS	Acetic acid	Ethanol	pH	Sucrose	Glucose	Fructose
NC									
0	7.24^a	6.48^a	6.45^a	1.88 ± 0.07^a	4.83 ± 1.32^c	3.20 ± 0.01^{bc}	23.37 ± 1.08^a	6.06 ± 0.29^g	9.99 ± 1.40^e
10	6.60^b	6.23^b	6.71^a	1.99 ± 0.11^a	5.64 ± 0.84^{bc}	3.16 ± 0.01^c	19.41 ± 0.78^b	7.95 ± 0.04^f	11.41 ± 0.09^e
20	6.83^{ab}	6.67^a	6.64^a	2.34 ± 0.34^a	8.42 ± 0.96^{ab}	3.24 ±0.08^{abc}	16.15 ± 0.08^c	9.54 ± 0.12^e	14.46 ± 0.46^d
30	6.56^{bc}	6.60^{ab}	5.24^c	2.04 ± 0.06^a	8.01 ± 0.08^{ab}	3.25 ± 0.13^{abc}	10.97 ± 0.11^d	10.89 ± 0.17^d	16.25 ± 0.09^{cd}
45	6.59^b	6.30^{ab}	5.71^{bc}	2.23 ± 0.09^a	9.51 ± 0.39^a	3.31 ± 0.07^{ab}	8.27 ± 0.31^e	12.02 ± 0.04^c	17.99 ± 0.02^{bc}
60	6.70^b	6.41^{ab}	6.18^{ab}	2.00 ± 0.01^a	8.58 ± 0.42^a	3.36 ± 0.01^a	4.90 ± 0.10^f	13.42 ± 0.11^b	19.54 ± 0.06^{ab}
90	6.06^c	6.56^{ab}	2.05^d	1.95 ±0.02^a	8.77 ± 0.29^a	3.37 ± 0.01^a	3.46 ± 0.11^f	14.72 ± 0.13^a	20.35 ± 0.22^a
FC									
0	6.71^{abc}	6.40^a	7.16^a	1.76 ± 0.01^c	1.96 ± 0.21^b	3.20 ± 0.01^b	37.46 ± 0.05^a	3.39 ± 0.01^e	3.78 ± 0.01^e
10	6.96^{ab}	6.01^b	7.21^a	1.77 ± 0.04^{bc}	2.28 ± 0.04^b	3.20 ± 0.01^b	31.99 ± 1.04^b	5.62 ± 0.38^{de}	8.09 ± 1.83^d
20	7.11^a	6.44^a	6.67^b	2.17 ± 0.02^a	3.12 ± 0.02^b	3.15 ±0.21^b	28.79 ± 0.45^c	7.40 ± 0.13^{cd}	9.71 ± 0.35^d
30	7.05^{ab}	6.40^a	6.83^b	2.16 ± 0.05^a	4.18 ± 0.05^{ab}	3.25 ± 0.08^{ab}	22.17 ± 0.07^d	9.89 ± 0.23^{bc}	13.25 ± 0.17^c
45	6.77^{abc}	6.18^{ab}	6.54^b	1.92 ± 0.06^b	4.22 ± 0.06^{ab}	3.32 ± 0.07^{ab}	17.73 ± 0.01^e	12.14 ± 1.09^b	15.48 ± 0.48^{bc}
60	6.46^{bc}	6.33^a	5.25^c	1.93 ± 0.03^b	3.83 ± 0.03^{ab}	3.33 ± 0.01^{ab}	12.10 ± 0.16^f	15.15 ± 0.07^a	18.33 ± 0.24^{ab}
90	6.43^c	6.14^{ab}	2.65^d	1.83 ±0.01^{bc}	5.65 ±0.01^a	3.42 ± 0.04^a	9.21 ± 0.98^g	15.69 ± 1.62^a	19.95 ± 0.62^a

KMF: *K. marxianus fragilis;* DA: *D. anomala;* KS; *K. saccharivorans.* Different letters in the same column are significantly different as determined by the Tukey test ($p \leq 0.05$).

4. Conclusion

The synthetic microbial consortia developed in this work (*K. saccharivorans*, *D. anomala*, and *K. marxianus fragilis*) was efficient and suitable for kombucha fermentation as a new alternative for the use of traditional starter culture. The replacement of the old and artisanal method used by producers (spontaneous fermentation through back slopping process) is considered an innovative way to control the process, guaranteeing the food safety and consistency of final products between batches and over the years. Moreover, the use of a known and controlled starter culture, as suggested in the present work, provides a new approach to further studying the health benefits of Kombucha as it makes it possible to reproduce the fermentation process anywhere. Further studies are required, however, using other yeast and bacteria strains to improve the volatile profile of Kombucha, and, consequently, the sensory acceptance. In addition, the use of other strains with evidence of delivering health benefits may allow the possibility of producing a probiotic-certified kombucha.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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CAPÍTULO IV – CONSIDERAÇÕES FINAIS

Este trabalho teve como objetivo estudar as interações entre microrganismos e formação de metabólitos em kombucha, a fim de desenvolver uma solução biotecnológica para a produção padronizada desta bebida. Existem muitos desafios para a produção de uma kombucha que respeite os padrões de qualidade exigidos pela legislação, devido ao grande número de variáveis que influenciam sua composição final. Como a composição microbiana da cultura *starter* varia entre bateladas, tanto em relação à concentração celular, quanto ao perfil de linhagens presentes, a definição de uma cultura *starter* para ser utilizada nos experimentos foi o primeiro desafio. Por essa razão, a primeira etapa desta pesquisa, apresentada no capítulo II, foi avaliar a composição microbiológica de uma kombucha local, utilizando sequenciamento de nova geração, e verificar a possibilidade de liofilização da película de celulose e dos microrganismos presentes no líquido inicial, a fim de verificar se esses liofilizados poderiam ser utilizados como inóculo padronizado. Para isso, uma amostra comercial local de kombucha (película + líquido) foi analisada em relação ao perfil de bactérias e leveduras e foi submetida a diferentes processos: tradicional (controle); líquido inicial centrifugado e ressuspendido em chá adoçado; centrifugação e liofilização. Após serem submetidas ao mesmo processo fermentativo, foram avaliadas as mudanças no perfil microbiológico das kombuchas e também foram feitas análises de metabólitos produzidos, consumo de açúcares, atividade antioxidante e análise sensorial. Foram observadas mudanças à nível de gênero de bactérias e leveduras em todas as amostras, inclusive no controle, reforçando as evidências sobre as mudanças entre bateladas de kombucha e a dificuldade de padronização do processo produtivo dessa bebida. Este estudo, além de trazer informações sobre a composição microbiana de mais uma kombucha para a literatura, quando ainda eram escassos os trabalhos utilizando sequenciamento de nova geração, também demonstrou que o teor alcoólico da bebida depende diretamente da composição da cultura *starter*. Além disso, foi possível concluir que o densenvolvimento de uma cultura sintética, em conjunto com aspectos tecnológicos, seria a solução mais adequada para a padronização do processo fermentativo.

Em busca da solução ideal para atingir os padrões de qualidade da kombucha, a segunda etapa desta pesquisa, apresentada no capítulo III, teve como

objetivo selecionar microrganismos de interesse e estudá-los em conjunto, com intuito de desenvolver um inóculo para produção da bebida. Para isso, primeiramente foram realizados alguns experimentos preliminares em biorreator utilizando a bactéria acética *A. aceti,* a bactéria lática *L. plantarum* e a levedura *D. anomala.* Os resultados desta etapa não foram satisfatórios, visto que a viabilidade da bactéria acética decaiu muito já no segundo dia de fermentação. Como resultado, a concentração de ácido acético produzida foi menor que o limite mínimo necessário para caracterizar a bebida como kombucha, conforme a legislação. Além disso, foi possível observar que a levedura *D. anomala* apresentou baixa atividade invertase e que a fermentação em biorreator ocasionou a evaporação de parte do líquido, dificultando a análise de consumo de açúcares e produção de metabólitos. Portanto, nas etapas seguintes, uma levedura com rápido metabolismo e alta atividade invertase foi selecionada e os experimentos foram conduzidos em beckers cobertos com um voal, conforme é feito no processo de produção tradicional. A levedura selecionada (*K. marxianus fragilis)*, a qual possui atividade probiótica, foi utilizada como variável fixa no planejamento Plackett-Burmann, utilizado para selecionar a melhor mistura de microrganismos. Para o planejamento foram utilizadas três bactérias acéticas (*K. saccharivorans*, *G. hansenii* e *A. aceti*) e a levedura *D. anomala* como variáveis independentes. Para diminuir as variáveis desta etapa, decidiu-se não adicionar a bactéria lática, visto que esta não é essencial no processo fermentativo. Das três bactérias acéticas, apenas a *K. saccharivorans* foi capaz de permanecer viável e produzir ácido acético em simbiose com as demais leveduras. O resultado da combinação de *K. saccharivorans*, *K. marxianus fragilis* e *D. anomala* foi satisfatório em relação à produção de etanol e ácido acético. O tempo de fermentação utilizando este consórcio foi otimizado, através da concentração celular inicial, passando de 10 para 2 dias. Além disso, nesta etapa, foram testados dois aspectos tecnológicos para carbonatação da bebida (natural e forçada), onde a carbonatação forçada se mostrou uma ótima opção para controle de produção e estabilidade do teor alcoólico durante a vida de prateleira da kombucha. A utilização da cultura starter sintética juntamente com a aplicação da tecnologia de carbonatação artifical se mostrou uma ótima alternativa para produzir kombucha de forma padronizada, garantindo a qualidade da bebida independente da sazonalidade.

O perfil de compostos voláteis da kombucha ainda é pouco investigado na literatura, e, portanto, ainda não há um perfil de compostos esperados para esse tipo de bebida. Esta pesquisa colaborou com os trabalhos já existentes, investigando a variedade de compostos voláteis encontrados em kombuchas comerciais e nas kombuchas produzidas através do inóculo com microrganismos isolados. O perfil de compostos voláteis da kombucha desenvolvida e carbonatada artificialmente, comparado com amostras comerciais, apresentou menor diversidade e concentração de ésteres frutados, que ajudam a mascarar compostos de odor desagradável e conferem aromas desejáveis na bebida. Apesar disto, a aceitação da bebida não diferiu das amostras comerciais para os consumidores de kombucha. Mesmo assim, como perspectivas, é interessante o estudo das variáveis do processo, como tempo, temperatura e concentração de substrato, para a obtenção de um produto ainda mais refinado.

CAPÍTULO V - CONCLUSÕES

Com os resultados obtidos nesta pesquisa foi possível entender mais sobre a importância e interação da cultura starter da kombucha com os produtos finais. Além disso, foi alcançado o objetivo principal de produzir uma kombucha através da aplicação de bioprocessos aliados a uma tecnologia que permite fidelidade entre os lotes produzidos, características sensoriais específicas e garantia de um produto conforme os padrões de qualidade exigidos pela legislação ao longo da vida de prateleira.

PERSPECTIVAS

Através dos resultados obtidos neste trabalho e do conhecimento adquirido ao longo da pesquisa, podemos sugerir algumas perspectivas para futuros desenvolvimentos:

- Testes de diferentes variáveis do processo (tempo, temperatura, pH, concentração e tipo de substratos) com intuito de aumentar a diversidade e concentração de compostos voláteis de interesse, como ésteres frutados.
- Adição de outros microrganismos e/ou indutores para a produção direcionada de compostos voláteis de interesse.
- Adição de bactérias ácido láticas com propriedade probiótica comprovada, a fim de aumentar as características funcionais da bebida e produzir ácido lático como composto de interesse sensorial.
- Testes de secagem dos microrganismos pelas técnicas de liofilização ou *spray drying* e aplicação em maior escala.

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APÊNDICES

Apêndice A. Artigo de revisão bibliográfica intitulado "Health effects and probiotic and prebiotic potential of Kombucha: A bibliometric and systematic review" publicado no periódico *Food Bioscience*.

Este artigo, apresentado aqui como material suplementar, será conteúdo da tese de doutorado da Bruna Vargas.

Health effects and probiotic and prebiotic potential of Kombucha: A bibliometric and systematic review

Bruna Krieger Vargas[a,1], Mariana Fensterseifer Fabricio [a,1], Marco Antônio Záchia Ayub[a]

[a] Laboratory of Biotechnology (BiotechLab), Food Science and Technology Institute, Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, ZC, 91501-970, Porto Alegre, Rio Grande do Sul, Brazil.

Highlights

• Many health benefits attributed to Kombucha make it popular as a functional beverage.

• LAB are important for probiotic claims, but they are not always present in kombucha.

• Kombucha microbial community has high potential for isolation of probiotic strains.

• Kombucha prebiotic-like compounds may stimulate probiotic strains in gut microbiota.

• Kombucha fermentation and its microorganisms may release post-biotics substances.

Graphical abstract
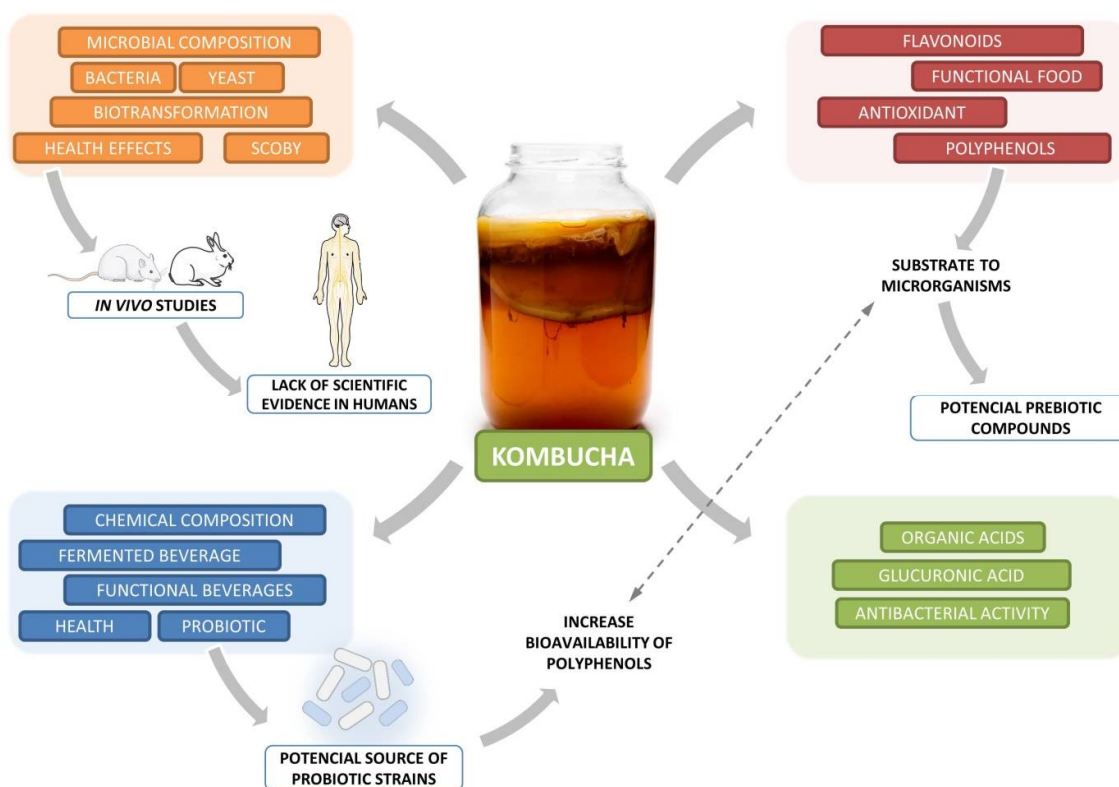
Abstract

Kombucha is a fermented beverage composed of a range of natural compounds such as sugars, ethanol, organic acids, and complex microbial communities of bacteria and yeasts. Based on this several biological properties are attributed to this drink. However, the production of kombucha is not standardized and the final composition of the beverage is highly dependent on the raw materials used and the physicochemical parameters adopted in the process. As a consequence, kombuchas not only vary from one producer to another but also from different batches of the same producer, making the assumptions of quality and properties questionable. In this review, we explore the largely unchecked relations between kombucha and its claimed health benefits. A systematic review was also performed to specifically discuss the potential probiotic and prebiotic effects of kombucha. Although several studies report that kombucha present antimicrobial, antioxidant, detoxifying, and hepatoprotective activities, among others, whereas others classify kombucha as a probiotic drink, there is a lack of scientific evidence about the content of probiotics in this drink and its possible role in the intestinal microbiota. These facts highlight the opportunities in researching and modifying the microbiome composition of kombucha, possibly improving the general qualities of this so-called functional drink.

1. Introduction

Kombucha is an ancient beverage obtained by the infusion or extract of *Camellia sinensis* and sugars fermented by a symbiotic culture of microbiologically active bacteria and yeasts (SCOBY) (Greenwalt et al., 2000). Kombucha is believed to have originated in China, in the Manchuria region, being consumed for more than 2000 years (Dorothy et al., 2020; Jayabalan et al., 2014). Traditionally, kombucha was homemade, based on a very simple preparation. Initially, the tea leaves and sugar are added to boiling water, leaving the mixture to rest for a few minutes. After the cooling of this solution, a starter culture, consisting of a cellulosic pellicle (known as SCOBY) and a percentage of the previously fermented batch of kombucha is added to perform the fermentation of the sweetened tea. In this way, kombucha tea fermentation comprises two distinct portions, as demonstrated in Fig. 1: a liquid tea phase and a floating microbial cellulose pellicle layer (Chen & Liu, 2000). This fermentation stage usually takes from 7 to 14 days; at the end of it, the SCOBY is removed from the kombucha along with a small volume of the ready drink to be used as a starter culture in the next fermentations (Dufresne & Farnworth, 2000).
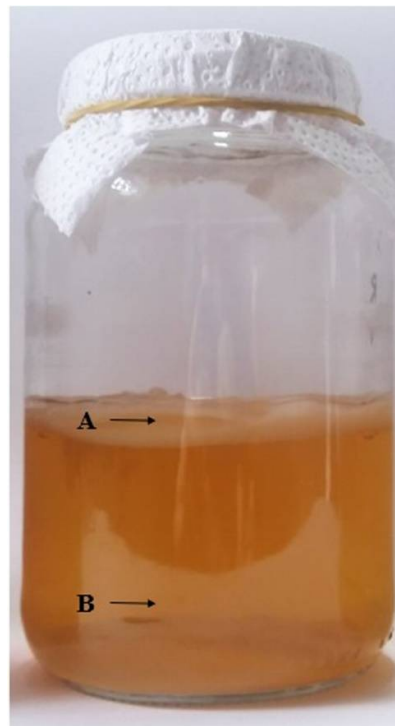

Fig. 1. Two portions of kombucha fermentation process. (A) Floating microbial cellulose pellicle layer and (B) liquid tea phase.

Currently, kombucha is commercially available, its consumption has been growing in several countries and it is considered one of the most popular low-alcohol beverages worldwide, mainly because of claims of its functional properties and health appeals (Watawana et al., 2015).

The production process of kombucha has not been standardized and the final composition of the beverage will heavily depend on the parameters adopted, such as type and concentration of tea and sugar (Reva et al., 2015b; Shahbazi et al., 2018; Watawana et al., 2016), fermentation time, and temperature (De Filippis et al., 2018; Tran et al., 2020), and in particular on the composition of the starter culture (Nguyen et al., 2015; Villarreal-Soto et al., 2018). The latter is a complex multi-species microbial ecosystem, mostly composed of acetic acid bacteria and yeasts. Several studies show that this community of microorganisms varies in great stance based on the geographical origin (Chakravorty et al., 2016; Coton et al., 2017; De Filippis et al., 2018) and also over time between batch fermentations of the same producer (Marsh et al., 2014). However, some genera prevalently appear in most starter cultures, such as *Komagataeibacter* and *Acetobacter*, among bacteria, and *Saccharomyces, Zygosaccharomyces, Brettanomyces/Dekkera,* and *Candida* for yeasts (Arıkan et al., 2020; Jayabalan et al., 2014). It is important to note that in some kombuchas, lactic acid bacteria (LAB) may also be present, such as *Lactobacillus* and *Lactococcus*. However, despite the importance of LAB for the probiotic properties of kombucha, this group of bacteria is not always present in the microbial consortium of this drink (Laureys et al., 2020; Morales, 2020; Murphy et al., 2018).

The set of microbial genera included in the kombucha is responsible for reducing the initial pH of the beverage, inhibiting the proliferation of possible contaminating bacteria, and also for carrying out the fermentation process (Neffe-Skocińska et al., 2017). Fermentation is initiated by yeasts that hydrolyze sucrose, converting it into glucose and fructose, ultimately producing ethanol, glycerol, and carbon dioxide. In the sequence, the bacteria oxidize ethanol, producing acetic acid and transforming glucose into glucuronic acid (Gaggìa et al., 2018). After the fermentation process, a refreshing, slightly acidic, and carbonated drink is produced, containing a diversified content of components, such as sugars (sucrose, glucose, and fructose remaining from fermentation), ethanol, amino acids, organic acids (mainly acetic, gluconic, and glucuronic acids), polyphenols, hydrolytic enzymes, and

micronutrients, such as B-complex and C vitamins, and minerals (zinc, copper, iron, manganese, and cobalt) (Jayabalan et al., 2014; Kaczmarczyk and Lochyński, 2014). This nonetheless interesting composition is perhaps the basis for claims of several biological properties attributed to kombucha (Kapp and Walton, 2019).

This article reviews the information available in the literature on the relationship between kombucha composition, consumption, and the health effects and probiotic potentials attributed to it. The contribution of this bibliometric and systematic review is to gather data about the biological properties of kombucha, improving knowledge about this so-called functional beverage.

2. Methodology

This study was performed to present a review of health effects associated with kombucha consumption and employed methods such as a bibliometric and systematic review, according to Fig. 2. The review was organized through a search for studies published to date in the Scopus (scopus.com) database using the following terms: (a) "kombucha" AND "health"; (b) "kombucha" AND (health" AND "effect" OR "benefit*" OR "claim"); (c) "kombucha" AND "pr*biotic", according to the supplementary materials. Boolean Operators were used to find the publications containing the terms of interest on the title, abstract, or keywords of the articles available on Scopus in the years.
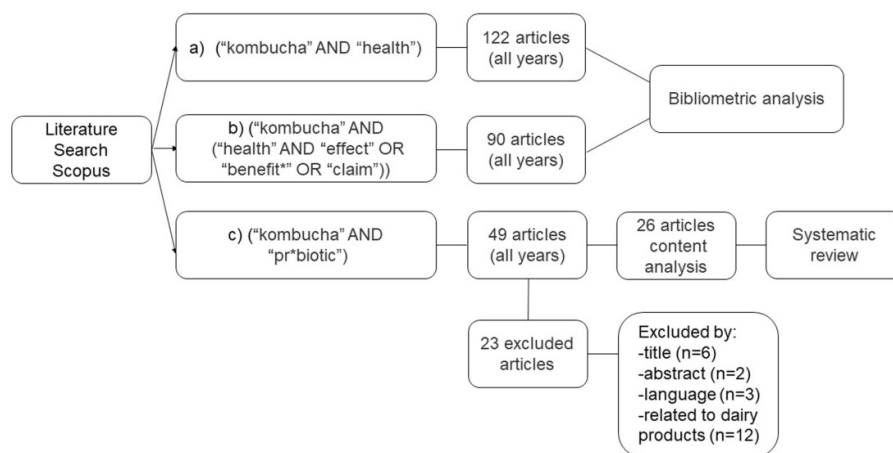
Fig. 2. Methodological flowchart performed in this work.

The bibliometric review was conducted to present an overview of scientific production on the topic kombucha and health over the years and to identify the 50 most incident words between title, keywords, and summary among all the articles found. All publications found with the terms "a" and "b" in the English language until March of 2021 were exported in BibTex format and imported into Rstudio software (RStudio Team 2010) through the Bibliometrix package (bibliometrix.org). Fig. 3 and Fig. 4 were generated by bibliometric analysis. Fig. 3 was elaborated through 122 articles found using the term "a" analyzed according to the year of publication and Fig. 4 was developed using thematic maps (Aria & Cuccurullo, 2017), generated through the 90 articles found in the term "b".
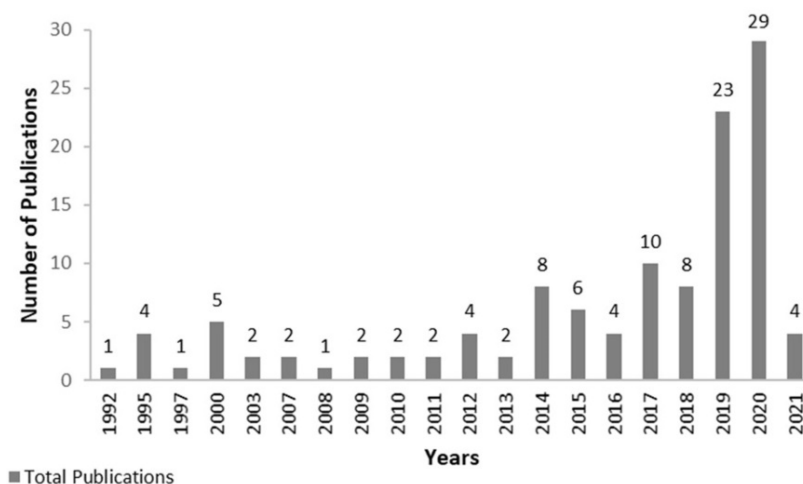
Fig. 3. Scientific production over the years from articles found on Scopus with the terms "kombucha" AND "health".
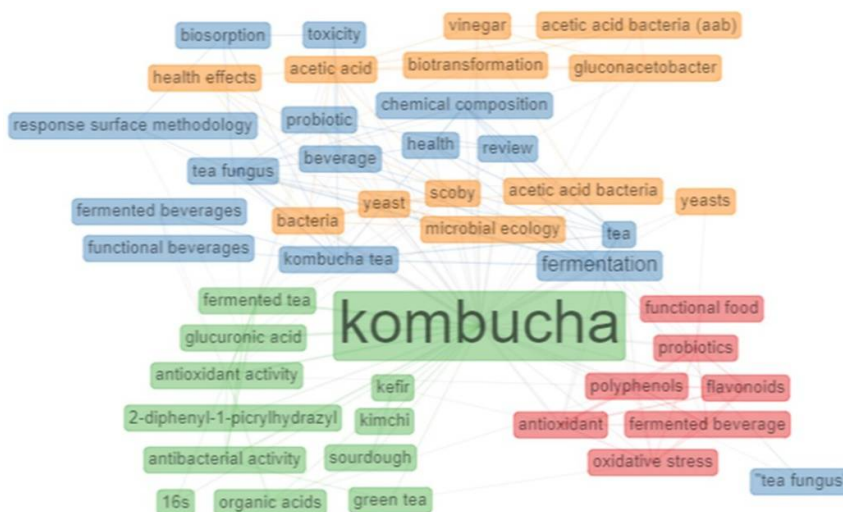
Fig. 4. Thematic map generated on Bibliometrix from articles found on Scopus with the terms "kombucha" AND ("health" AND "effect" OR "benefit*" OR "claim").

The systematic review was performed to explore, specifically, the general aspects in the context of the probiotics and prebiotics effects of kombucha and to respond to these questions:

● Is kombucha a probiotic beverage in the technical definition of the term?

● What is the evidence of this possible probiotic and/or prebiotic effect?

A content analysis of all articles found in the database from terms "c" was performed to discuss and deeply explore this topic.

3. Results and discussion

3.1 Kombucha and its health effects

To guide the discussion about kombucha and its health effects, Fig. 4 was generated through bibliometric analysis and presents the thematic map from the analysis and grouping of the 50 most recurrent words found in the literature with the term "c" (Cobo et al., 2011). It is possible to observe in Fig. 4 the occurrence of four main clusters, among which we can find the words "tea", "kombucha tea" and "fermented tea" in two of these clusters. This results from the important association of tea in the effects of kombucha.

The biological activities performed by kombucha were primarily attributed to the composition of the tea itself used in the preparation of this beverage. *Camellia sinensis* is a species in the Theaceae family, which includes pu-erh, jasmine, white, oolong, yellow, green, and black tea (Chakravorty et al., 2019). Tea is historically recognized as a healthy beverage due to the presence of antioxidants (Wang et al., 2020). The distinction between these types of tea of the species *Camellia sinensis* is related to the degree of oxidation in the processing of tea leaves, which can significantly impact on its content and health benefits (de Almeida Souza et al., 2020).

Traditionally, black tea is the most used for preparing kombucha. Along with the refined white sugar (sucrose), are considered the best ingredients to the proper content of the kombucha (Jakubczyk et al., 2020; Kaczmarczyk and Lochyński, 2014). Black tea is the most processed and susceptible to oxidation. The major compounds found in this type of tea are alkaloids (caffeine, theobromine, and

theophylline), and polyphenols, such as catechins, theaflavins, and thearubigins (Anal, 2019). On the other hand, green tea undergoes little to no processing and does not suffer oxidation reactions. The term green tea can be found in the thematic map (Fig. 4), configuring it as one of the most used in the articles. Gaggìa et al. (2018) in their study comparing black, green, and rooibos teas found that green tea presented the most significant antioxidant properties, whereas black tea showed the lowest values. In addition, green tea kombucha showed less degradation of epicatechin isomers compared to black tea kombucha (Jayabalan et al., 2007). A great number of studies evaluated the choices of other types of tea for fermentation, instead of the traditional black tea, in order to enhance the functional properties of kombucha (Gaggìa et al., 2018; Jakubczyk et al., 2020; Jayabalan et al., 2007).

Over the years, investigations began to be carried out to understand the impact of tea fermentation by the microorganisms of the kombucha and the influence of this process on its beneficial health effects (Baschali et al., 2017; Dufresne & Farnworth, 2000). This can be seen in Fig. 4 where, among the most cited words in the title, keywords, and summary of all articles analyzed, are the terms "fermentation", "fermented tea", "fermented drink" and "tea fungus", also known as SCOBY, appearing in 3 of the 4 clusters on the thematic map.

The production of fermented foods is an ancient practice among many cultures and remains as the earliest biological method of food processing and preservation (Anal, 2019; Marco et al., 2017). Fermentation is considered one of the first known applications of biotechnology (de Almeida Souza et al., 2020). The fermentative process in kombucha drink has several advantages. We can cite initially the reduced risk of contamination, which is prevented by compounds formed during fermentation, such as organic acids, ethanol, and bacteriocins (Marco et al., 2017). Notwithstanding, the fermentation process is also responsible for many biochemical modifications of the drink (Chakravorty et al., 2016). Some of these improve the sensorial profile of the beverage, developing new and complex tastes and desirable flavors, characterizing kombucha, and differentiating it from the initial vegetable matrix (Marco et al., 2017).

Besides, fermented foods and beverages are an important nutritional source. This is because these products carry potential beneficial microorganisms and their metabolites. Some of these can be genetically similar to probiotic strains, providing

microbial stability and changes in the digestibility of nutrients, enhancing the nutritional content (de Almeida Souza et al., 2020; Marco et al., 2017; Villarreal-Soto et al., 2018). Fermented products have been attracting the attention of consumers mainly because of the health benefits associated with their consumption (Marco et al., 2017). Today, Kombucha is a ready-to-drink beverage easily found in several markets and has been related to various functional effects such as helping to establish the balance of the organism and avoiding the appearance of chronic non-communicable diseases like obesity and diabetes. The claims of "functional beverages" and "functional food" are related to kombucha in two different clusters in the thematic map (Fig. 4). In the functional beverages cluster, the association of terms "fermentation" and "fermented beverages" with the word "chemical composition" was observed, demonstrating that the components generated in this drink during fermentation influence its functional potential. The term "fermented beverage" also appears in the functional food cluster, associated with the antioxidant compounds "polyphenols" and "flavonoids", both responsible for reducing oxidative stress and suggesting the functional appeal of kombucha.

In view of the growing consumer demand for functional beverages, food industries have been exploiting the fermentation process for the development of new functional products (Baschali et al., 2017).

Along with the growing interest of the population and the food industry in fermented foods, research in this area has also been expanding, especially concerning kombucha. The potential therapeutic properties of kombucha have become a field of interest (Murugesan et al., 2009), as shown in Fig. 3, which presents the increase in articles published with the terms "kombucha" and "health" over years. It can be noticed that, in the Scopus database, the publications in 2019 exceeded the number of 20 articles, increasing in 2020 to almost 30 published works. Until March 2021, 4 articles were published on the same base. One of the first articles found using these terms, by Hartmann et al. (2000), investigated the effects of chronic kombucha ingestion during an *in vivo* pilot study using mice. They found positive differences in appetitive behaviors, gross body weight, and greater longevity in animals receiving kombucha compared to the control group. Moreover, the consumption-group had longer spleens and enlarged livers. These adverse effects need more biochemical analysis for a full understanding. The hepatomegaly effect

could be associated with some hepatotoxicity cases in humans, but the authors stated that comparable effects and mechanisms in humans were uncertain at the time. This shows kombucha may bring health or some undesirable outcomes by its consumption.

Regarding positive outcomes, several health benefits make Kombucha popular as a functional beverage or food (Mousavi et al., 2020; Villarreal-Soto et al., 2018). Table 1 presents some *in vitro* and *in vivo* studies showing the possible biological properties associated with the consumption or administration of kombucha. Vīna et al. (2013) reviewed the main physiological properties related to kombucha consumption, listing, among others, antioxidant reaction, detoxifying properties, promoting immunity, and energizing capacity. The latter may be related to the release of iron from the kombucha tea, increasing the hemoglobin level. As a result, tissue oxygenation is favored, stimulating ATP synthesis in the body.

Table 1. *In vivo* and *in vitro* biological properties associated with kombucha.

Type of assay	Biological property	Method/Experimental model	References
In vitro	Antibacterial	*S.epidermidis, S. aureus, Mi. luteus, E. coli, P. aeruginosa, S. typhimurium, L. monocytogenes*	(Battikh, Bakhrouf, and Ammar 2012)
		E. coli, H. influenzae	(Ivanišová et al. 2020)
		E. coli	(Mulyani et al. 2019)
		E. coli, S. sonnei, S. typhimurium, S. enteritidis, C. jejuni	(Sreeramulu, Zhu, and Knol 2000)

Type of assay	Biological property	Method/Experimental model	References
In vitro	Antibacterial	*E. coli, S. aureus. B. cereus, S. dysenteriae*	(Valiyan, Koohsari, and Fadavi 2021)
		E. coli, Salmonella	(Ma et al. 2019)
		S. aureus, K. pneumoniae, E. coli, P.vulgaris, P. mirabilis, B. subtilis	(Vitas et al. 2018)
		E. coli, S. typhimurium, M. luteus, S. epidermidis	(Deghrigue et al. 2013)
		E. coli, Salmonella, S. aureus, L. monocytogenes	(Cardoso et al. 2020)
	Antifungal	*C. glabrata, C. tropicalis, C. sake, C. dubliniensis, C. albicans*	(Battikh, Bakhrouf, and Ammar 2012)
		C. krusei C. glabrata, C. albicans, C. tropicalis	(Ivanišová et al. 2020)
		C. albicans, A. niger	(Vitas et al. 2018)
	Anti-carcinogenic	Human cell lines A549 (lung carcinoma), U2OS (osteosarcoma), 786-O (renal carcinoma)	(Jayabalan et al. 2011)
		Human cell lines A549 (lung carcinoma), Hep-2 (epidermoid carcinoma)	(Deghrigue et al. 2013)
		Human cell lines RD (rhabdomyosarcoma), Hep2c (cervix carcinoma-HeLa derivative) and murine cell line L2OB (fibroblast)	(Vitas et al. 2018)

Type of assay	Biological property	Method/Experimental model	References
In vitro	Anti-carcinogenic	Human cell lines HCT-116 (colon cancer) and MCF7 (breast cancer)	(Villarreal-Soto et al. 2019; Villarreal-Soto et al. 2020)
		Human cell line PC-3 (prostate cancer)	(Srihari et al. 2013)
		Cell lines A549 (lung adenocarcinoma epithelial), HCT8 (ileocecal colorectal adenocarcinoma), CACO-2 (colorectal adenocarcinoma epithelial)	(Cardoso et al. 2020)
	Anti-inflammatory	Human monocytic cells (THP-1) differentiated to macrophages	(Vázquez-Cabral et al. 2017)
		5- lipoxygenase enzyme	(Villarreal-Soto et al. 2019)
		15-lipoxygenase enzyme	(Villarreal-Soto et al. 2020)
	Antioxidant	ABTS radical scavenging	(Ivanišová et al. 2019; Cardoso et al. 2020; Jafari et al. 2020; Aung and Eun 2021; Gamboa-Gómez et al. 2017)
		DPPH (1,1-diphenyl 2-picrylhydrazyl) radical scavenging	(Ivanišová et al. 2020; Mulyani et al. 2019; Villarreal-Soto et al. 2019; Villarreal-Soto et al. 2020; Gamboa-Gómez et al. 2017; Gaggìa et al. 2018; Hoon et al. 2014; Yang et al. 2009; Aung and Eun 2021; Jakubczyk et al. 2020; Bhattacharya, Gachhui, and Sil 2013)
		Oxygen Radical Absorbance Capacity (ORAC)	(Vitas et al. 2018; Gamboa-Gómez et al. 2017)

(continue)

Type of assay	Biological property	Method/Experimental model	References
In vitro	Antioxidant	Ferric reducing antioxidant power (FRAP)	(Aung and Eun 2021; Gaggìa et al. 2018; Jakubczyk et al. 2020)
		Reactive oxygen species induced by H_2O_2	(Yang et al. 2009; Vázquez-Cabral et al. 2017)
		Superoxide radical (O_2^-) scavenging ability	(Hoon et al. 2014; Yang et al. 2009; Bhattacharya, Gachhui, and Sil 2013)
		Cupric ion reducing antioxidant capacity (CUPRAC)	(Jafari et al. 2020)
		Nitric oxide scavenging (NO)	(Gamboa-Gómez et al. 2017)
	Anti-hyperglicemic	Glucose diffusion	(Gamboa-Gómez et al. 2017)
	Radioprotective (ionizing radiation)	Human peripheral lymphocytes	(Cavusoglu and Guler 2010)
	Cytotoxicity	Human peripheral blood lymphocytes	(Mrdanović et al. 2007)
		Human cell IMR90 (normal lung cell)	(Cardoso et al. 2020)
In vivo	Anti-hypercholesterolemic	White rabbits	(Alaei, Doudi, and Setorki 2020)
		Alloxan diabetic rats	(Aloulou et al. 2012)
		Wistar rats	(Bellassoued et al. 2015)
		Mice	(Yang et al. 2009)
	Anti-hyperglycemic	Alloxan diabetic rats	(Aloulou et al. 2012)

Type of assay	Biological property	Method/Experimental model	References
In vivo	Anti-hyperglycemic	Female C57BL/6 mice	(Gamboa-Gómez et al. 2017)
		Swiss albino male rats	(Bhattacharya, Gachhui, and Sil 2013)
		Streptozotocin-induced diabetic rats	(Zubaidah et al. 2019)
		Normal and alloxan-induced diabetic mice	(Shenoy 2000)
	Radioprotective (electromagnetic radiation)	Male albino rats	(Gharib 2014)
	Brain damage prevention	Ischemic rats	(Kabiri and Setorki 2016)
	Anti-hyperuricemic	Rats	(Sukrama 2015)
	Hepatoprotective	Mice	(Wang et al. 2014)
		Male albino rats	(Jayabalan, Baskaran, et al. 2010)
		Mice	(Lee et al. 2019)
	Microbiome modulation	Male C57BLKS db/db mice	(Jung et al. 2018)
	Longevity	Mice	(Hartmann et al. 2000)
	Antioxidant	Male albino rats	(Sai Ram et al. 2000)
		Mice	(Yang et al. 2009)
	Prevention of myocardial injury caused by isoproterenol	Male albino Wistar rats	(Lobo, Chandrasekhar Sagar, and Shenoy 2017)
	Antiviral	Swine	(Fu et al. 2015)

Regarding the antioxidant capacity, several studies show the association of kombucha consumption with the improvement of antioxidant protection to the cells,

helping in the reduction of the damage caused by oxidative stress to the body (de Almeida Souza et al., 2020). The terms "antioxidant activity", "antioxidant" and "oxidative stress" appear in two clusters of the thematic map (Fig. 4) associated with the words "polyphenols" and "flavonoids". Antioxidant capacity is one of the best-known actions performed by phenolic compounds, and kombucha is a beverage rich in these components. For instance, Cardoso et al. (2020) screened the phenolic profile of green and black tea and their resulting kombuchas and observed that black tea presented a higher abundance of phenolic compounds. Also, this kombucha showed a greater diversity of these components after the fermentative process at 25 °C for 10 days. Differences in the profile of phenolic compounds were observed by ultra-performance liquid chromatography coupled with an electrospray ionization quadrupole time-of-flight mass spectrometry operating in MSE mode (UPLC-QTOF-MSE) analysis wherein 127 phenolics were detected in the green and black tea kombuchas (70.2% flavonoids, 18.3% phenolic acids, 8.4% other polyphenols, 2.3% lignans, and 0.8% stilbenes). At least 103 of these phenolic components were detected for the first time in kombuchas. Among them are pelargonidin 3-O-glucoside, gardenin B, lithospermic acid, and oleuropein, found exclusively in black tea kombucha. These bioactive substances are known for their activities, such as antitumoral and hypotensive effects and positive gut microbiota modulation (Cardoso et al., 2020). Ivanišová et al. (2019) also compared the phenolic profile of black tea and its kombucha after fermentation at 22 °C for 7 days. They found higher content of total polyphenols in kombucha (0.42 mg gallic acid equivalent (GAE)/mL), flavonoids (0.13 mg quercetin equivalent (QE)/mL), and phenolic acids (0.19 mg caffeic acid equivalent (CAE)/mL) than in black tea (0.18 mg GAE/mL; 0.02 mg QE/mL; 0.05 mg CAE/mL, respectively). The same author further demonstrated that black tea kombucha has a significantly higher value in total polyphenol content (412.25 mg (GAE)/L) compared to black tea (180.17 mg GAE/L) (Ivanišová et al., 2020). Similarly, Wang et al. (2020) reported an increase in the concentrations of total phenol and flavonoids of kombucha during fermentation, boosting its health benefits. Sai Ram et al. (2000) carried out an *in vivo* study, showing that phenolic compounds in kombucha, in addition to reducing oxidative stress, could decrease the immunosuppression caused by this stress condition. Based on these studies, kombucha would contribute to immunity and reduce inflammatory foci and cell damage, preventing the development of chronic non-communicable diseases (Vīna

et al., 2013). Despite that, the phenolic content of kombucha is variable and changes according to the chemical composition of the tea before fermentation. It will also depend on the concentration of carbon source, pH, temperature, inoculum size, and microbial community of kombucha (Jafari et al., 2020). The bacteria and yeasts present in kombuchas are responsible for releasing enzymes that determine the metabolites generated during its fermentation, as well as degrading the polyphenols in smaller molecules (de Almeida Souza et al., 2020; Jakubczyk et al., 2020). Ivanišová et al. (2019) revealed kombucha has a high concentration of phenolic metabolites, such as gallic acid, chlorogenic acid, protocatechuic acid, *p*-coumaric acid, ellagic acid, rutin, vitexin, and resveratrol. Other compounds, such as catechins, epicatechin, and flavonoids are also produced by the microorganisms through biotransformation (Villarreal-Soto et al., 2018; Jayabalan, Malini, et al., 2010; Ivanišová et al., 2020; Mousavi et al., 2020).

When analyzing the thematic map (Fig. 4), it can be identified as a cluster that reunites the words "bacteria", "acid acetic bacteria", "yeast", "SCOBY", "microbial ecology". In the same cluster, the terms "biotransformation" and "health effects" appear. Thereby, it can be noticed the importance of the microbial community of kombucha in the generation of products with better biological action than in unfermented tea (Bhattacharya et al., 2013).

As demonstrated by many authors, the fermentative process is responsible for the increase of the bioactivity of these phenolic components. Chakravorty et al. (2016) demonstrated that fermentation of kombucha gradually enhanced its ability to eliminate radicals, peaking on the seventh day of fermentation. Ivanišová et al. (2020) measured the antioxidant activity of kombucha and observed significantly higher values (1318.56 mg Trolox equivalent antioxidant capacity (TEAC)/L) when compared with unfermented black tea (345.59 mg TEAC/L). Similarly, Bhattacharya et al. (2013) found that despite black tea shows potent antioxidant power itself, the scavenging activities of fermented black tea on parameters such as 2,2-diphenyl-1-picryl hydrazyl (DPPH), hydroxyl, and superoxide radicals increased 18.9%, 17.2% and 14.97% respectively. Ivanišová et al. (2019) also observed that kombucha after 7 days of fermentation at 22 °C showed higher antioxidant capacity by ABTS and phosphomolybdenum method (1.16 mg TEAC/mL and 2.04 mg TEAC/mL, respectively) when compared to black tea (0.67 and 0.81 mg TEAC/mL, respectively).

Abuduaibifu and Canan Ece Tamer (2019) verified that along with the enhancement of the protective effect against oxidative stress of three kombuchas samples (black tea, black goji berry, and red goji berry kombucha), the bioaccessibility properties also increased. The total phenolic content of all samples was higher in post-digestion (gastric, intestinal) than in pre-digestion. In the same way, Aloulou et al. (2012) demonstrated by histological analysis that kombucha tea had better protective actions on the pancreas and liver-kidney functions on rats, compared to black tea. These *in vivo* improvements and the greater protective capacity of the kombucha is due to the large number of phenolic compounds and its products, which are responsible for inhibiting the formation of reactive species and strengthening endogenous enzyme defenses (Mulyani et al., 2019; Murugesan et al., 2009; Sai Ram et al., 2000; Vázquez-Cabral et al., 2017). Furthermore, this characteristic may even promote curative effects, for example, in conditions of hyperglycemia and hypercholesterolemia (Aloulou et al., 2012; Bellassoued et al., 2015; de Almeida Souza et al., 2020).

In relation to the fermentation process, the products synthesized by microorganisms in kombucha may play an important function in human health caused by their detoxifying action (Anal, 2019). As for the products that are formed during the fermentative process, the most relevant and often reported is glucuronic acid, the main organic acid resulting from the oxidation of glucose in kombucha (Neffe-Skocińska et al., 2017). This acid is naturally synthesized in the human liver and has detoxifying properties from its potential of binding toxin molecules and by carrying out the excretion of xenobiotics and phenols through the kidneys or intestines. Thus, kombucha assists in the elimination of excess metabolites and may bring relief in situations related to the accumulation of toxins in the body, such as kidney stones, rheumatism, or arthritis (Dufresne & Farnworth, 2000; Kaczmarczyk and Lochyński, 2014). The relevance of this organic acid can be noted by its presence on the thematic map (Fig. 4).

The production of this important organic acid is directly related to predominant bacteria in the starter culture, which can vary largely depending on the variables of the process, such as pH and temperature. De Filippis et al. (2018) characterized bacterial populations in kombucha fermented at 20 °C and 30 °C and evaluated glucuronic acid production, finding substantial differences between the final products.

Fermentation at 20 °C favored *Komagataiebacter xylinus* (formerly named *Gluconacetobacter xylinus*) growth and resulted in approximately 45 mg/L of glucuronic acid, whereas fermentation at 30 °C favored *Komagataeibacter saccharivorans* (formerly named *Gluconacetobacter saccharivorans*), resulting in a production of 28 times higher glucuronic acid concentrations (approx. 1300 mg/L). These results empathize the importance of not generalizing information about kombucha, since one product may be completely different from another, even when using the same starter culture.

Other compounds with health-modulating potential may result from the fermentation, such as B vitamins, including folate, riboflavin, and B_{12} (Chamlagain et al., 2015; Martínez; Leal et al., 2018). Nevertheless, these microbial-derived products are strain-dependent and are synthesized by certain bacteria in plant and dairy foods (Russo et al., 2014). Therefore, as the microbial community of the kombucha is variable, not all kombuchas will necessarily produce these vitamins. In this sense, Wang et al. (2014) identified the functional strains and quantified the functional components with hepatoprotective effects in kombucha tea (KT). The authors concluded that *Gluconacetobacter sp.* A4 was the microorganism able to produce significantly high values of the D-saccharic acid-1,4-lactone (DSL) in the modified KT (fermented by single *G. sp.* A4) when compared to original kombuchas, fermented by tea fungus. DSL is believed to be the compound responsible for hepatoprotection conferred by kombucha by assisting the glucuronic acid to exert some properties like anti-tumor, detoxifying, and antioxidant (Baschali et al., 2017). These results encourage the standardization of inocula in the production of kombucha, with microorganisms selected in the starter culture, in order to synthesize compounds of interest.

Finally, antibacterial activity also stands out on the thematic map (Fig. 4), being one of the most reported effects in the literature in relation to kombucha. The antimicrobial action of this drink has already been reported for several microorganisms, including Gram-negative and Gram-positive bacteria (Dufresne & Farnworth, 2000), the most frequently reported being *Escherichia coli, Staphylococcus aureus*, and *Helicobacter pylori*. The latter being the cause of gastric ulcers and other complications of the digestive system (Kaczmarczyk and Lochyński, 2014). The antibacterial activity of kombucha is related to the microbial community of

its starter culture, acting in a symbiotic way to prevent potential contaminations, and also to the metabolites formed during the fermentation, such as catechins, ethanol, and organic acids, especially acetic acid (Kaczmarczyk and Lochyński, 2014; Watawana et al., 2015). This acid is reported to control the growth of pathogenic bacteria, avoiding contamination in the production of kombucha and having antifungal and antiviral properties as well (Fu et al., 2015; Mousavi et al., 2020; Watawana et al., 2015). It should also be recalled that low pH values contribute to this antimicrobial action.

Although several benefits are regarded to the consumption of kombucha, it is important to highlight that it may be harmful when not safely brewed and also may lead to metabolic acidosis in some individuals when consumed in excess (Martini, 2018). Adverse effects from kombucha consumption have been reported, such as allergies, jaundice, nausea, and head and neck pain (Srinivasan et al., 1997). Also, kombucha should be avoided by lactating and pregnant women (Jayabalan et al., 2014).

Nevertheless, kombucha fulfills the consumer's demand for healthy beverages, reflecting in a market growth that is partially based on widespread information of health-promoting effects (Kapp and Walton, 2019; Kim & Adhikari, 2020). Recently, some scientific studies are using kombucha as a vehicle to reduce the pathogens associated with human illnesses (Mousavi et al., 2020), while many researchers have reported the different activities of kombucha and demonstrated its potential health benefits. This wide range of advantages has led to investigating the role of the microbiome on health, where it is proposed that kombucha may have the ability to act as a probiotic agent (Kapp and Walton, 2019; Kim & Adhikari, 2020; Martínez Leal et al., 2018; Mann et al., 2017).

3.2 Probiotic and prebiotic potential

Recent studies associate the consumption of kombucha as influencing and modifying the gastrointestinal microbiota. The human gut microbiota is complex and colonized by approximately $1 \cdot 10^{13}$ to $1 \cdot 10^{14}$ microorganisms (Li et al., 2016). Studies report that the phyla most identified in the colon are: Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Proteobacteria (Zmora et al., 2019). When this

microbial community is in dysbiosis, that is, pathogenic species are in greater concentration, an imbalance situation arises, and different pathologies can originate in individuals. Because of the association between the deregulated intestinal microbiome and the genesis of illnesses, there is a growing interest in the modulation of microbial genera (Morales, 2020).

In this context, with a currently strong link between intestinal health and its direct role in human health, new treatment approaches and pharmaceutical interventions for disease prevention are being investigated. Thus, one of the main therapies that has been implemented is the use of beneficial microbes, such as probiotics in functional foods (de Almeida Souza et al., 2020; Heinen et al., 2020). According to the World Health Organization (WHO), probiotics are "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2006, p. 85; Gibson et al., 2017; Hill et al., 2014). Probiotics are said to maintain the intestinal barrier integrity, improve digestion and modify and select the host-microbiota (Kozyrovska et al., 2012; Watawana et al., 2015). There are different mechanisms in which these microorganisms perform these actions. First, they stimulate the growth of beneficial resident gut microorganisms by supplying some substances generated by their metabolism. Second, directly inhibit the growth of pathogenic genera through niche competition, bacteriocins, or a decrease in pH. Finally, some microorganisms can indirectly interact with the host epithelium and the epithelial immune system. As a result, they impact the host-microbiota by controlling pro-inflammatory cytokines (Derrien and van Hylckama Vlieg, 2015; Marco et al., 2017). The agents most associated with the probiotic function are lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium*, non-lactic acid species such as *Bacillus cereus* and *Propionibacterium freudenreichii*, and yeasts of the genus *Saccharomyces* (species *boulardii* and *cerevisiae*) (Kozyrovska et al., 2012; Holzapfel et al., 2001). These microorganisms can assist in the generation of a healthy microbiome, restoring homeostasis in the body (Kozyrovska et al., 2012).

Human diets are capable of changing the composition of gut microbiota. Modifications in the food intake pattern, even for a short period of time, may increase the amount of some bacteria, while reducing the number of others, altering the whole microbial community (Heinen et al., 2020). In this sense, it is observed that a typical

western diet, with high fat and sugar intakes, is associated with increased Firmicutes and reduced Bacteroides. On the other hand, plant-based diets, with high consumption of fibers such as fruits and vegetables, lead to a higher abundance of Bacteroidetes. Thereby, the consumption of a diversified diet is the key to microbial homeostasis (Million et al., 2018; Zmora et al., 2019).

The delivery of beneficial microorganisms to the gut microbiota is dependent on the food matrix. Many fermented foods and beverages are related to the modification in the indigenous microbiota. The regular ingestion of these fermented products possibly enhances up to $1 \bullet 10^4$ times the microorganisms resident in the colon (Lang et al., 2014). Although fermented products demonstrate being a potential vehicle to provide probiotic strains, some of these are processed in a way that those beneficial microorganisms are absent when consumed (Marco et al., 2017).

Kombucha is known worldwide as a probiotic drink, as it contains live microorganisms in its composition. This beverage represents the fastest growing product in the probiotics market. It is possible to find kombucha labels in several countries with the appeals of "natural", "organic", "raw", "living culture", "non-dairy probiotics", "healthy for your gut", among other claims (Kim & Adhikari, 2020). However, some of these statements are mostly unfounded or at least unproved. So much so, that in most parts of Europe the use of claims such as "probiotics" or "contain probiotics" is not allowed on labels (Marco et al., 2017). The presence of probiotic strains is inconsistent in kombuchas. Normally, they are not present or remain in low concentrations, mainly after storage (Coton et al., 2017; Fu et al., 2014; Matei, Salzat, et al., 2018).

Preparations or products that contain probiotic microorganisms must have an adequate quantity, making them capable of altering the intestinal microbiota through implantation or colonization (Schrezenmeir and de Vrese, 2001; Kapp and Walton, 2019). So far, there is no evidence that these probiotic microorganisms are in sufficient quantity in kombucha and bring some specific health benefit (Martini, 2018). Furthermore, products that use probiotic microorganisms in order to confer health benefits to the consumer must indicate the dosage and the time needed to obtain the desired effect, based on scientific evidence obtained from studies carried out *in vitro* and *in vivo* (in animals and humans) (FAO/WHO, 2006, p. 85). Moreover, legislation

from the country of sale must be observed and may vary from place to place (FAO/WHO, 2006, p. 85).

In this research, many articles mentioned kombucha as a "probiotic-containing food", without presenting scientific evidence that the microbial community indeed delivers the benefits to the host (Chandrakala et al., 2019, pp. 591–616; Dikeocha et al., 2020; Fu et al., 2014; Onur et al., 2019; Ranjan et al., 2020; Salafzoon et al., 2018; Vilela, 2019). This possibly inadequate use of the term *probiotic* frequently appears and may be related to the fact that several articles have reported the benefits of kombucha to health and also owing to the presence of live microorganisms in the drink (Kapp and Walton, 2019; Martínez Leal et al., 2018). However, the studies suggest that the benefits come from the tea and metabolites from fermentation, such as organic acids, polyphenols, and vitamins (Kapp and Walton, 2019). To the best of our knowledge, no studies have reported health benefits from the whole microbial community of kombucha. Notwithstanding, the microbial culture of kombucha has been used in other raw materials in order to develop new *probiotic* drinks (Soares et al., 2021). Mulyani et al. (2019), in their study, aimed to produce an allegedly probiotic beverage made from beat (Beta vulgaris L.) fermented with kombucha culture to prevent digestive infections. The results showed that the drink had antibacterial activity against *E. coli*. The authors, however, did not identify or quantify the probiotic strains in the drink, and regarding antibacterial activity, it is not known to what extent this effect would be observed in vivo gastrointestinal tract (Lavefve et al., 2019).

Fermented probiotic products are produced from a complex and unstable set of microorganisms, which can be modified because they remain in open environments and are susceptible to variations according to the growing conditions and substrates available for their development (Reva et al., 2015a). The studies reporting yeasts and bacteria communities in kombucha show that there exists a huge variability between products, although there are some genera that appear more frequently. In the case of bacteria, the most predominant genera are *Komagataeibacter* (formerly assigned to *Gluconacetobacter*) (Arıkan et al., 2020; Chakravorty et al., 2016; Gaggìa et al., 2018; Marsh et al., 2014; Reva et al., 2015a; Villarreal-Soto et al., 2020), and *Acetobacter* (Coton et al., 2017). *Komagataeibacter* species are described as bionanocellulose producers, showing a large phenotypic

strain diversity, strongly dependent on carbon source affinities, bionanocellulose syntheses rates, and composition, and strain stability (Ryngajłło et al., 2019). In relation to its potential probiotic ability, it is assumed that the set of central microorganisms of the kombucha could recruit some environmental bacteria, particularly of the genus *Lactobacillus*. Possibly, this whole microbial community favors the fermentation of kombucha for the development of a probiotic characteristic (Reva et al., 2015a). However, only a few genera of LAB may be found in this beverage, such as *Lactobacillus* (Chakravorty et al., 2016; Coton et al., 2017; Marsh et al., 2014; Reva et al., 2015a), *Bifidobacterium* (Chakravorty et al., 2016; Marsh et al., 2014), and *Lactococcus* (Marsh et al., 2014), all appearing to a lesser amount than acetic acid bacteria. Yeast genera are more variable than bacteria, with *Brettanomyces/Dekkera* being the prevalent species (Coton et al., 2017; Gaggìa et al., 2018; Reva et al., 2015a; Villarreal-Soto et al., 2020), followed by *Zygosaccharomyces* (Arıkan et al., 2020; Coton et al., 2017; Marsh et al., 2014; Villarreal-Soto et al., 2020), *Candida* (Chakravorty et al., 2016; Villarreal-Soto et al., 2020), and *Hanseniaspora* (Coton et al., 2017).

Among the most predominant bacteria and yeasts found in kombucha, some have been studied regarding their potential probiotic properties. Matei, Salzat, et al. (2018) isolated nine yeast species from pollen fermented kombucha and identified them by PCR-ITS RFLP technique, aiming isolation of prospective probiotic yeasts. It was possible to identify the species *Dekkera bruxellensis*, but no studies on probiotic potential were performed. The same research group has studied the possible probiotic effect of five LAB isolated from kombucha (Matei, Salzat, et al., 2018). The isolates of *Pediococcus* pentosaceus species were subjected to on-plate screening for bacteriocin production, wherein three of five strains were positive. Furthermore, isolates were tested for bile-salt resistance, with three strains showing tolerance to 3% and 6% of bile salts. Among the five isolates, the "L5" strain showed higher potential as a probiotic because it was the only bacterium that showed both bacteriocin production and bile salt resistance (Matei, Salzat, et al., 2018). This strain was further studied regarding the potential of adhesivity to Caco-2 intestinal cells and the authors suggested a probiotic effect since L5 adhered to intestinal cells in a time-dependent manner (Utoiu et al., 2018). *Pediococcus* is not a common genus found in kombucha and it has not been identified in studies that used next-sequencing

generation (Arıkan et al., 2020; Bueno et al., 2021; Chakravorty et al., 2016; Coton et al., 2017; Gaggìa et al., 2018; Marsh et al., 2014; Villarreal-Soto et al., 2020).

The variability in the kombucha microbial community demonstrates how complex is the process of this fermented beverage. Although some other fermentations contain only a few dominant taxa, the fluctuations in the microorganisms of kombucha may affect the quality of this drink (Marco et al., 2017; Reva et al., 2015a). With the increase in kombucha consumption, consequently, its homemade production evolved to an industrial scale (Soares et al., 2021). While SCOBY can be useful as a starter culture for homemade kombucha, in the industrial sector this type of inoculum is not suitable because of the unrepeatable and uncontrollable qualities of the process, leading to variations in the organoleptic properties, affecting the final product (Marco et al., 2017; Wang et al., 2020). The beverage industries are investing in food safety and improvements in the biotechnological properties of kombucha production. Standard process conditions and a stable set of microorganisms throughout fermentations result in kombuchas showing stable characteristics. The consistency of end products is a primordial factor to produce high-quality food (Marco et al., 2017; Mousavi et al., 2020). Thus, efforts are being made to characterize the essential microbiome of kombucha in an attempt to standardize its production and also to identify the best mixture of microorganisms to improve kombuchas yield (Mousavi et al., 2020; Wang et al., 2020). Wang et al. (2020) developed a symbiotic microbial community for kombucha with three main microorganisms, *Acetobacter pasteurianus*, *Komagataeibacter xylinus* (previously named *Gluconacetobacter xylinus*), and *Zygosaccharomyces bailii*. The authors verified that this microbial set could accelerate the fermentation process of kombucha and generate a beverage with desirable qualities. Furthermore, the ability to select species of bacteria and yeasts is also an important approach to determining the metabolites generated through the fermentation process. Thereby, the modification of the kombucha inoculum can also be fundamental in improving the functional properties of this drink (Kozyrovska et al., 2012). Wang et al. (2020) also concluded in their study that the designed starter culture was capable of enhancing the antioxidant content of kombucha. Similarly, Nguyen et al. (2015) carried out a screening of ideal strains for high production of glucuronic acid, in order to improve the antioxidant, antimicrobial and detoxifying activities of the kombucha. The authors

found that the best ratio would be an initial combination of *Dekkera bruxellensis* KN89 and *Komagataeibacter intermedius* KN89 (formerly named *Gluconacetobacter intermedius*) in a 4:6 parts, respectively.

Another alternative strategy to obtain a probiotic kombucha could be the addition of well-known and studied probiotic strains into the fermentation process, as was shown in a few studies reported in this systematic review. Al-Dulaimi et al. (2018) developed two kombuchas using black tea and skim milk, adding in each formulation a probiotic culture starter, composed of two species of *Lactobacillus* (*delbruekii* and *fermentum*). Investigating the beneficial effects of both kombuchas on some physiological and biochemical parameters in rats, the authors verified that animals that were treated with probiotic kombuchas had decreased concentrations of total lipid profile, ALT, AST, ALP hepatic enzymes, and serum glucose, when compared to the control group. The reduced serum glucose levels can be addressed to the activity of probiotic bacteria since they use glucan compounds to carry out fermentation. Thus, this beverage could present some beneficial actions on the liver and human body health in general. In another study using the incorporation of probiotics Bueno et al. (2021) developed a coffee kombucha added of two probiotic strains: *Lactobacillus rhamnosus* (LR) and *Lactobacillus casei* (LC). After fermentation and removal of the biofilm, the kombucha fermented from coffee was added with LR or LC, or no probiotics (control) and stored for 15 days at 4 °C. The authors evaluated the survival of probiotics in the kombuchas and the microbial viabilities under simulated gastric and intestinal conditions. The authors also identified the microbial diversity through next-sequencing generation on the fifteenth day of storage. Results were very interesting and showed LC viability in coffee kombucha remained without significant changes at the end of storage, whereas LG presented a slight reduction of viable counts, allowing to suggest that LC and LG survive in the gastric and intestinal environment. Microbial community analysis revealed that the addition of *Lactobacillus* shifted the predominance of Acetobacteraceae family to Lactobacillaceae and, although the species added was *L. rhamnosus* and *L. casei*, the predominant genus in each kombucha was *Lactobacillus zeae* for LR and *Lactobacillus paracasei* for LG, both strains documented as probiotics (Bueno et al., 2021).

On the other hand, Fu et al. (2014) evaluating the survival of specific strains in kombucha after 14 days of refrigerated storage did not obtain results as positive as in the previous upper study. The authors developed a low-cost green tea kombucha (LGTK) inoculated with 5% of starter culture composed of *Saccharomyces cerevisiae Meyen ex Hansen* (10^8 CFU/mL), *Komagataeibacter* sp. (10^8 CFU/mL), and *Lactobacillus plantarum* (10^8 CFU/mL), in a ratio of 1:1:1. At the end of the study they verified that, under refrigeration at 4 °C, acetic acid bacteria moderately decreased up to ten days of storage, whereas the survival rate of lactic acid bacteria was only 0.98% on the eighth day of storage. These results reinforce the importance of the choice of the microorganisms contained in the starter inoculum and their influence on the final probiotic characteristics of the beverage.

Some beverage companies do not pasteurize the industrial scale kombuchas in order to maintain the drink's natural microbiome, in such a way that keeping the product raw would allow the development of a prebiotic or probiotic drink. In another approach, other companies proceed with the pasteurization process in their production and subsequently add recognized probiotic strains, such as *Bacillus coagulans*, *Saccharomyces boulardii* and *Lactobacillus rhamnosus*, to the final kombucha formulation (Kim & Adhikari, 2020). It is important to note that this strategy of adding probiotics is useful depending on the country of sale regulation. In the case of Brazil, for instance, the Normative Instruction for Quality and Identity Standards for Kombucha does not allow the addition of strains after fermentation, as it must be added at the beginning of the process (BRAZIL, 2019).

The criteria for probiotic strain selection involve functional, safety, technological and physiological aspects (Terpou et al., 2019). Among these aspects, the kombucha microbial community presents high potential as a probiotic because it is non-pathogenic (Arıkan et al., 2020; Chakravorty et al., 2016; Gaggìa et al., 2018), presents competitive inhibition of undesirable microbiota (Ayed et al., 2017; Kaewkod et al., 2019; Sreeramulu et al., 2000), and tolerance to acidic environments (Villarreal-Soto et al., 2018). It is important to note that most probiotic products are of dairy origin. Plant-based probiotic foods and beverages are less available in the market (Muhialdin et al., 2021). However, with the growing knowledge about their health properties, including their supposed role in boosting immunity, taking into account the recent Covid-19 pandemic, the demand for plant-based probiotics has

increased (Antunes et al., 2020). Kombucha has this advantage because it is a non-dairy drink. Its vegetable matrix used in its preparation guarantees that it can be consumed by vegans, people with lactose intolerance, or by people who are reducing the consumption of products of animal origin (Panghal et al., 2018; Reva et al., 2015a).

Furthermore, some authors have stated that kombucha not only can be a probiotic beverage but also acts as a prebiotic (Dufresne & Farnworth, 2000; Kozyrovska et al., 2012). Prebiotics are food-containing components that serve as a substrate for beneficial microorganisms (Gibson et al., 2017; Hill et al., 2014). These prebiotic agents are responsible for nourishing the probiotic strains, thus selectively stimulating the growth and/or activity of the microbiota resident in the human gut (Heinen et al., 2020; Marco et al., 2017).

Lavefve et al. (2021) studying the microbiota modulation demonstrated that kombucha and especially the kombucha supernatant were able to significantly enhance the *in vitro* growth of *Bifidobacterium* and *Collinsella*. These preliminary results showed that kombucha stimulates these beneficial microorganisms, acting in a prebiotic manner.

Among the possible prebiotic components present in kombucha are phenolic compounds. Some studies describe these phytochemicals as substances that exert prebiotic-like effects, being a class of bioactive most associated with the promotion of a healthy microbiome (Million et al., 2018; Dueñas et al., 2015; Tomás-Barberánet al., 2016). This association occurs because most of the ingested polyphenols pass intact through the small intestine, reaching the large intestine where they will be used as a substrate by beneficial genera of the microbiota. In a reciprocal interaction, these microorganisms increase the bioavailability of the polyphenols metabolizing them into smaller molecules (Hollister et al., 2014; Ozdal et al., 2016). Therefore, there is an enhancement of the detoxifying enzymes and improvement in the antioxidant response of fermented products, protecting the organism from oxidative stress and other possible chemical damages (Marco et al., 2017; Senger et al., 2016).

Other classes of food components showing prebiotic properties are some polysaccharides. The cellulosic pellicle of kombucha, present in the SCOBY, could

help in the growth and maintenance of the beneficial microbes in the beverage by the presence of insoluble fibers in its composition (Kozyrovska et al., 2012; Marco et al., 2017). These substances could be fermented by the microbial community of kombucha and then release some secondary metabolites, mainly short-chain fatty acids (SCFAs), such as acetate, propionate, butyrate, lactate, and succinate (Salazar et al., 2016; Sonnenburg & Fischbach, 2011). These compounds are known to provide some health benefits to the host. Particularly, butyrate is known to strengthen the intestinal epithelial barrier and to interact with inflammatory pathways, promoting the differentiation and maintenance of the defense cells, boosting immunity (Kozyrovska et al., 2012; Kim and de La Serre, 2018). Lactate, in turn, is said to reduce the reactive oxygen species in intestinal enterocytes (Kahlert et al., 2016). Therefore, if a portion of the lactic acid present in fermented kombucha reaches human metabolism, that organic acid may provide this health protection. Additionally, some ingested microorganisms produced in fermented foods might synthesize some vitamins, amino acids, and growth factors, such as exopolysaccharides, which could also compete as antioxidants, stimulating the immune system and preventing the adhesion of pathogens to the intestinal mucosa (Gerritsen et al., 2011; Makino et al., 2016; Thomas et al., 2017).

All these metabolites produced by the intestinal microbiota, such as SCFA, polyphenols, vitamins, and other functional compounds derived from the diet have recently been called post-biotics (Lavefve et al., 2021). Post-biotic is a new term used in the area of food and therefore there is no consensus on this concept yet. However, post-biotics can be defined in a wide spectrum as products or by-products generated or released by microorganisms during growth and fermentation in complex microbiological culture, food, or intestine (Moradi et al., 2021). These metabolites could resolve the intestinal imbalance, acting in the reestablishment of the microbiota symbiosis. Thus, post-biotics can offer positive physiological effects to the host, helping to promote their health (Cuevas-González et al., 2020; Klemashevich et al., 2014).

All things considered, it is known that the claim of any artisanal kombucha as a probiotic is inaccurate since the composition and concentration of the microorganisms present in the drink are unknown and change among batches depending on the fermentation variables (De Filippis et al., 2018). However,

kombucha has been proven to be a potential source for isolation of probiotic bacteria in industrial or pharmaceutical applications (Matei, Salzat, et al., 2018; Sinir et al., 2019; Utoiu et al., 2018). Also, prebiotic potential and post-biotics compounds in kombucha have recently become research topics. In order to have a stated functional kombucha beverage, further studies are needed through the development of a defined starter culture for the fermentation of kombucha, as it happens for other fermented products such as yogurt, wine, or beer. Still, more studies are needed concerning the possible prebiotic and probiotic effects of kombucha.

4. Final considerations

Kombucha is an important source of bioactive compounds arising from raw material and increased by the fermentation process. These beneficial compounds change between kombuchas and depend on several variables, such as type of raw material, time of infusion, starter culture microbial community, and fermentation parameters. Therefore, kombucha health-promoting properties should not be generalized, since there are so many variables affecting final product composition. So far, artisanal kombucha is produced through an uncontrolled fermentation process and the microbial community is complex, diverse, and variable. Also, interactions between yeasts, bacteria and their impact on human microbiota have not been understood or properly and thoroughly investigated. For this reason, kombucha cannot be considered a probiotic or symbiotic drink, unless documented probiotic strains have been added in the process. As the demand for this product is increasing, scientific studies are necessary to standardize the starter culture with well-known microorganisms, in order to control the metabolites produced, standardize the final product, and develop further studies on biological activities of kombucha, establishing it as a functional symbiotic beverage.

Declaration of conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Apêndice B. Experimentos preliminares realizados com intuito de desenvolver uma cultura starter selecionada para fermentação de kombucha.

Em meio à pandemia do Covid-19, decidimos iniciar alguns experimentos para a criação da cultura *starter* em biorreatores, utilizando os microrganismos que já estavam disponíveis no laboratório, enquanto esperávamos a chegada dos demais microrganismos de interesse. Desta forma, foram utilizadas uma bactéria acética, uma bactéria lática e uma levedura.

MATERIAL E MÉTODOS

Linhagens e manutenção celular

Três microrganismos foram usados neste trabalho em co-cultura: uma bactéria ácido acética, uma bactéria ácido láctica e uma levedura. *Acetobacter aceti* ATCC 15973, foi obtida através da Fundação Tropical de Pesquisas e Tecnologia André Tosello (FAT, Campinas, Brasil), *Lactobacillus plantarum* BL011 foi obtido através da Coleção de Culturas Microbiológicas do BiotecLab (UFRGS, Porto Alegre, Brasil) e *Dekkera anomala* UFMG-CM-Y4734 foi gentilmente fornecida pela Coleção de Microrganismos e Células da Universidade Federal de Minas Gerais (UFMG, Minas Gerais, Brasil). Os microrganismos foram estocados em solução de glicerol a -80 ^{o}C. Para uso imediato, células de *A. aceti*, *L. plantarum* and *D. anomala* foram mantidas em placas contendo meio ágar manitol (25 g.L^{-1} manitol, 3 g.L^{-1} peptona, 5 g.L^{-1} extrato de levedura, 15 g.L^{-1} ágar), ágar MRS (DE MAN *et al.*, 1960) e ágar YM (10 g.L^{-1} glicose, 5 g.L^{-1} peptona, 3 g.L^{-1} extrato de levedura, 3 g.L^{-1} extrato de malte, 20 g.L^{-1} ágar), respectivamente. Os mesmos meios de cultura, sem ágar, foram utilizados para o pré-inóculo de cada microrganismo.

A primeira etapa do trabalho foi realizar curvas de crescimento relacionadas à densidade óptica (DO) para conhecer o comportamento de cada um dos microrganismos e facilitar o cálculo da quantidade de inóculo em cada experimento. O crescimento dos microrganismos foi analisado através do plaqueamento nos meios de cultura específicos para cada linhagem e os resultados expressos em unidades formadoras de colônia por mililitro (UFC.mL^{-1}).

Pré-inóculo e cultura *starter*

Duas concentrações diferentes de inóculo foram projetadas para a fermentação de kombucha em co-cultura usando *A. aceti, L. plantarum* e *D. anomala*. Em cada inóculo, a concentração celular de cada microrganismo foi idêntica (1: 1: 1) e foram realizados os seguintes experimentos:

- Cultura *Starter* 1 (CS1): 1×10^6 UFC de cada microrganismo.mL^{-1} de chá adoçado
- Cultura *Starter* 2 (CS2): 1×10^7 UFC de cada microrganismo.mL^{-1} de chá adoçado

Para os pré-inoculos, 0,5 mL da solução de células em glicerol foram transferidos para frascos Erlenmeyers de 500 mL, contendo 100 mL do meio de cultura respectivo para cada microrganismo. Os frascos foram mantidos sob agitação orbital a 30 °C e 120 rpm, até a obtenção da concentração celular desejada. As células obtidas foram centrifugadas a 3000 *g* por 15 min e lavadas duas vezes com solução salina 0,9 %. O pellet total dos microrganismos foi ressuspendido em 40 mL de chá adoçado (8 g.L^{-1} de chá verde e 80 g.L^{-1} de sacarose) e utilizado como cultura *starter*.

Preparo da infusão de chá e fermentação da kombucha

As kombuchas foram fermentadas em meio de cultura contendo: água destilada, 8 g.L^{-1} de chá verde orgânico (Vemat, SC, Brasil) e 80 g.L^{-1} de açúcar orgânico demerara (Native, SP, Brasil). As fermentações foram realizadas em biorreator de 2 L (Biostat B, B. Braun Biotech International, Alemanha), contendo 1 L de chá adoçado, e equipado com controle de pH, temperatura e aeração do *headspace*. Os experimentos foram realizados em duplicata.

Para o preparo do chá adoçado, 864 mL de água destilada com 76,8 g de sacarose foram esterilizados a 121 °C por 15 min nos biorreatores. Folhas de chá verde (7,68 g) foram infusionadas em 96 mL de água fervente durante 10 min. Após atingir temperatura ambiente, o líquido foi filtrado em membrana de poro 0,22 μm e adicionado ao biorreator, totalizando o volume de 960 mL. O pH inicial foi ajustado para 5, utilizando solução de ácido fosfórico. Os biorreatores foram inoculados com a cultura *starter* ressuspendida em 40 mL de chá adoçado, totalizando o volume de 1 L. A fermentação foi conduzida a 28 °C, durante 10 dias, com aeração do

headspace de 1 L de ar.min^{-1} com intuito de reproduzir a produção industrial de kombucha, mas sem interferência de contaminantes. Após 10 dias de fermentação nos biorreatores, as kombuchas foram transferidas para garrafas de vidro de 300 mL, com tampa hermética, e incubadas a 28 °C por 48 h, totalizando 12 dias de fermentação.

Amostras de 8 mL foram coletadas a cada 48 h para análise do crescimento celular, consumo de substrato e produção de metabólitos.

Análises microbiológicas

A enumeração dos microrganismos foi realizada através do plaqueamento em superfície, com incubação a 30 °C durante 24h para *A. aceti*, 48h para *L. plantarum* e 120 h para *D. anomala*. A bactéria ácido acética foi plaqueada em meio ágar Manitol contendo 128 µg.mL^{-1} de fluconazol e 3 U.mL^{-1} de penicilina para inibir o crescimento da levedura e da bactéria ácido lática, respectivamente. A bactéria ácido lática foi cultivada em meio ágar MRS adicionado de 128 µg.mL^{-1} de fluconazol e 0,1 % (vol/vol) de L-cisteína HCl para inibir o crescimento da levedura e da bactéria ácido acética, respectivamente. A enumeração da levedura foi feita em ágar YM, contendo 34 µg.mL^{-1} cloranfenicol para inibir o crescimento bacteriano. Os resultados foram expressos em UFC.mL^{-1}.

**Determinação do consumo de substrato e produção de metabólitos**

As amostras coletadas foram centrifugadas (3 000 *g*, 15 min), o sobrenadante foi filtrado em membrana de acetato celulose 0.22 µm e as análises foram realizadas através de cromatografia de alta eficiência (CLAE) (Shimadzu, Japão). Foram analisadas as concentrações de sacarose, glicose e frutose através da coluna Bio-Rad Aminex 87C, com vazão de 0,6 mL.min^{-1} a 85 °C, utilizando água ultrapura como fase móvel. Etanol, glicerol, ácido acético, lático e succínico foram analisados pela coluna Bio-Rad Aminex 87H, com vazão de 0,6 mL.min^{-1} a 45 °C, utilizando solução de ácido sulfúrico 5 mM como fase móvel.

RESULTADOS

A primeira etapa do trabalho foi avaliar a capacidade da co-cultura, com apenas três microrganismos, de transformar o chá adoçado em uma kombucha.

Os valores de pH foram avaliados ao longo da fermentação e em ambos os experimentos o comportamento e valores finais foram semelhantes, embora a concentração dos inóculos tenha sido diferente (Fig A-B 1).

Figura A-B 1. Evolução do pH ao longo da fermentação das kombuchas CS1 (●) e CS2 (■).
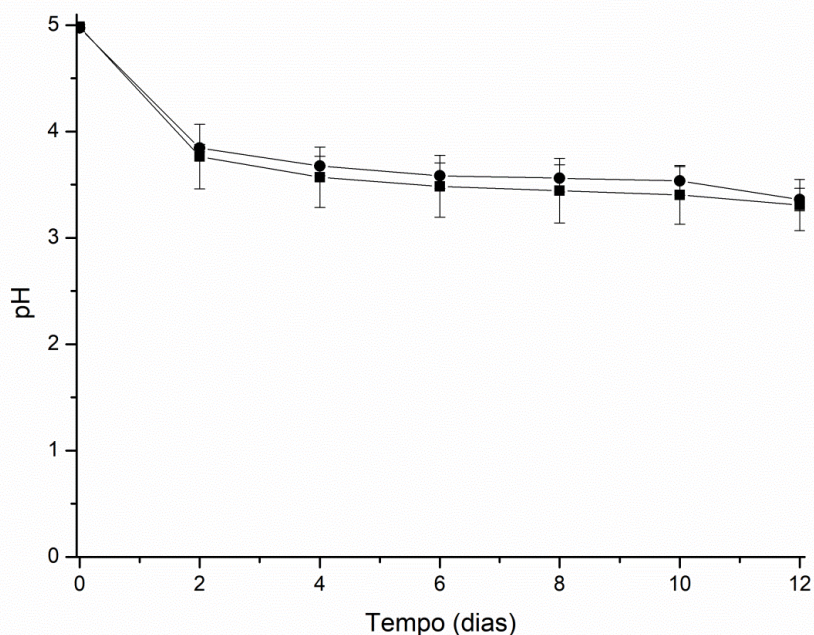
Em relação ao crescimento dos microrganismos, nos experimentos CS1 e CS2 o comportamento foi o mesmo (Figuras A-B 2 e A-B 3). O crescimento do *L. plantarum* aumentou nas primeiras 48 h, seguido por um decaimento constante até o fim da fermentação, chegando a valores de $3,25.10^5$ UFC.mL^{-1} e $5,10^5$ UFC.mL^{-1} para CS1 e CS2, respectivamente. *D. anomala* apresentou aumento na concentração celular nas primeiras 48 h e após, se manteve constante até o décimo segundo dia de fermentação, com $4,25.10^7$ UFC.mL^{-1} na CS1 e $9,10^7$ UFC.mL^{-1} na CS2.

Em ambos os experimentos foi possível observar uma queda importante da quantidade de células viáveis de *A. aceti* a partir do segundo dia de fermentação, sendo esse decaimento maior no experimento CS2. Isso pode ter ocorrido por diversos motivos, como pH, concentração de açúcar, falta de oxigênio ou nitrogênio.

Além disso, as bactérias do gênero *Acetobacter* não são capazes de metabolizar sacarose (MAMLOUK; GULLO, 2013), o que torna a simbiose com os demais microrganismos essencial, onde estes são responsáveis por hidrolisar a sacarose, liberando glicose e frutose no meio. Esta pode ter sido uma das razões para a diminuição da viabilidade da bactéria acética, visto que pouco açúcar foi metabolizado durante toda cinética (Tabela a-B 1). As kombucha produzidas (CS1 e CS2) não apresentaram a formação da película de celulose, como ocorre no processo com a cultura *starter* tradicional, o que nesse caso não é um problema, visto que na proposta de desenvolvimento de cultura *starter* a película se torna um resíduo da produção.

Figura A-B 2. Cinética de crescimento celular na fermentação da kombucha CS1. *A. aceti* (▲) *L. plantarum* (■) *D. anomala* (●).
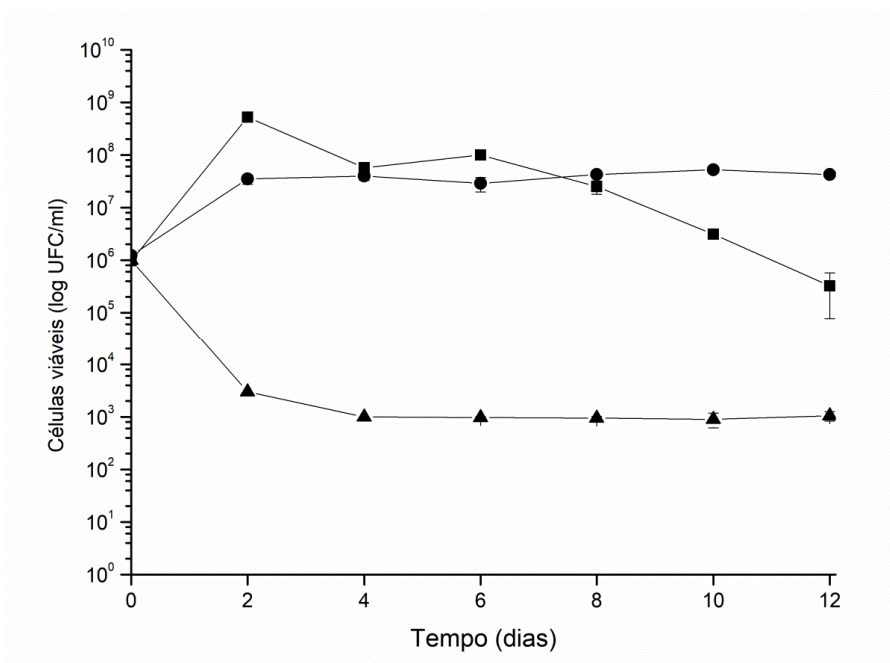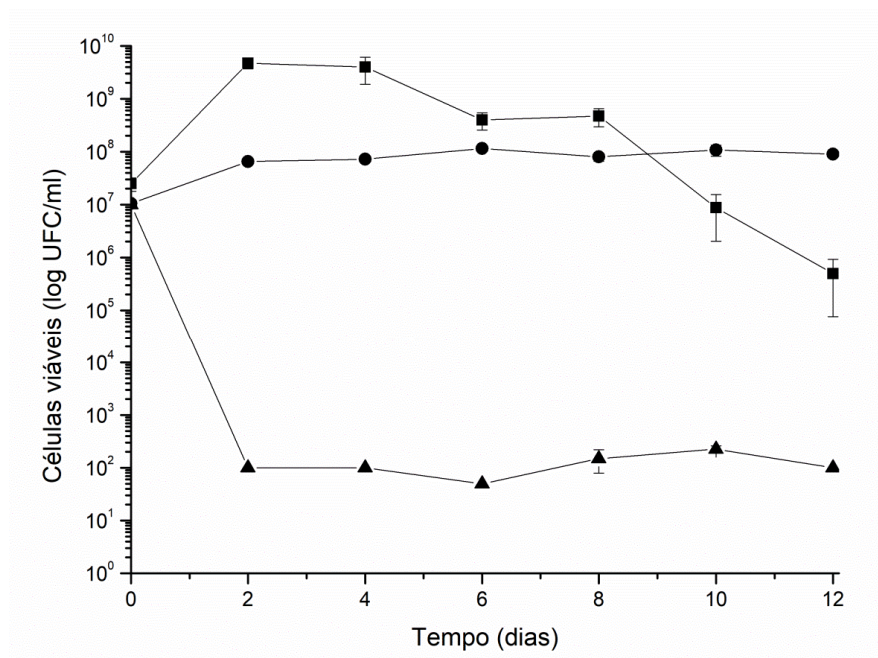
Figura A-B 3. Cinética de crescimento celular na fermentação da kombucha CS2. *A. aceti* (▲) *L. plantarum* (■) *D. anomala* (●).

Em relação aos açúcares, foi detectado um problema nos experimentos: os valores de sacarose aumentaram ao invés de diminuir (Fig A-B 4). A primeira hipótese levantada foi a produção de algum ácido orgânico que possuísse o mesmo tempo de retenção da sacarose, mas testando diversas outras colunas de CLAE percebeu-se que o valor permanecia alto. Então foi levantada uma segunda hipótese, que se confirmou: o volume do reator estava diminuindo, por evaporação, mesmo estando equipado com um condensador. No experimento CS2 foi possível ter um controle exato do volume amostrado e do volume final, onde foi constatado que nos biorreatores deveria conter 880 mL, mas havia apenas 630 mL e 670 mL (na duplicata).

Uma questão muito importante é que não foi encontrado relato algum na literatura que mencionasse a ocorrência da evaporação em seus trabalhos, no entanto, estima-se que o mesmo ocorre nas indústrias produtoras de kombucha, visto que a fermentação ocorre em tanques com passagem de ar na superfície.

Figura A-B 4. Concentração de sacarose ao longo da fermentação das kombuchas CS1 (■) CS2 (●).
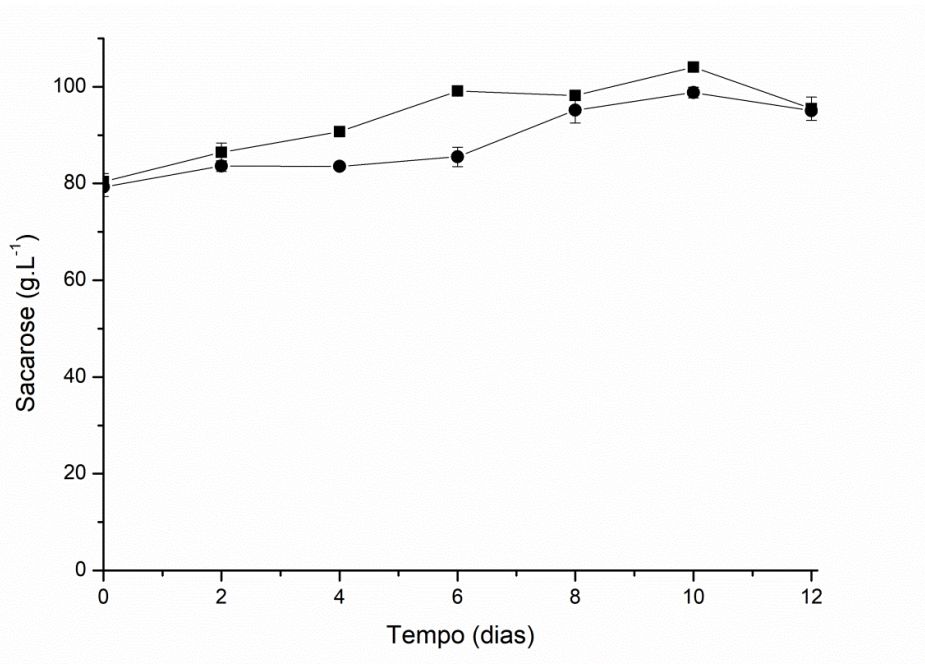
Os valores de glicose e frutose permaneceram baixos ao longo dos 12 dias de fermentação (Tabela A-B 1), o que indica que houve pouca hidrólise da sacarose pelos microrganismos. Em razão disso, além de diminuir a concentração de sacarose, busca-se empregar outros microrganismos nas próximas etapas, que tenham um metabolismo mais rápido, visando aumentar a disponibilidade de fonte de carbono para a bactéria acética e aumento da produtividade da bebida.

Tabela A-B 1. Concentrações de glicose e frutose ao longo das fermentações CS1 e CS2.

Tempo (dias)	Glicose (g.L^{-1})		Frutose (g.L^{-1})	
	CS1	CS2	CS1	CS2
0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
2	3,16 ± 0,79	1,58 ± 0,11	1,91 ± 0,62	1,77 ± 0,10
4	2,94 ± 0,28	1,60 ± 0,06	1,63 ± 0,06	1,77 ± 0,04
6	2,72 ± 0,26	1,64 ± 0,03	1,56 ± 0,07	1,81 ± 0,04
8	3,01 ± 0,05	2,71 ± 143	1,74 ± 0,25	2,18 ± 0,42
10	3,06 ± 0,24	2,35 ± 0,78	1,83 ± 0,05	2,28 ± 0,40
12	3,23 ± 0,27	1,79 ± 0,04	1,80 ± 0,07	1,99 ± 0,04

Em relação à produção de metabólitos, os experimentos apresentaram a produção de ácido acético, ácido lático, etanol e glicerol. Não foi observada a produção de ácido succínico. Os resultados estão apresentados em forma de cinética (Figuras A-B 5 e A-B 6), no entanto, assim como a sacarose, os valores estão superestimados em razão da evaporação do meio de cultura.

Figura A-B 5. Evolução dos metabólitos ao longo da fermentação da kombucha SC1. Ácido lático (■) Ácido acético (●) Etanol (▲) Glicerol (▼).
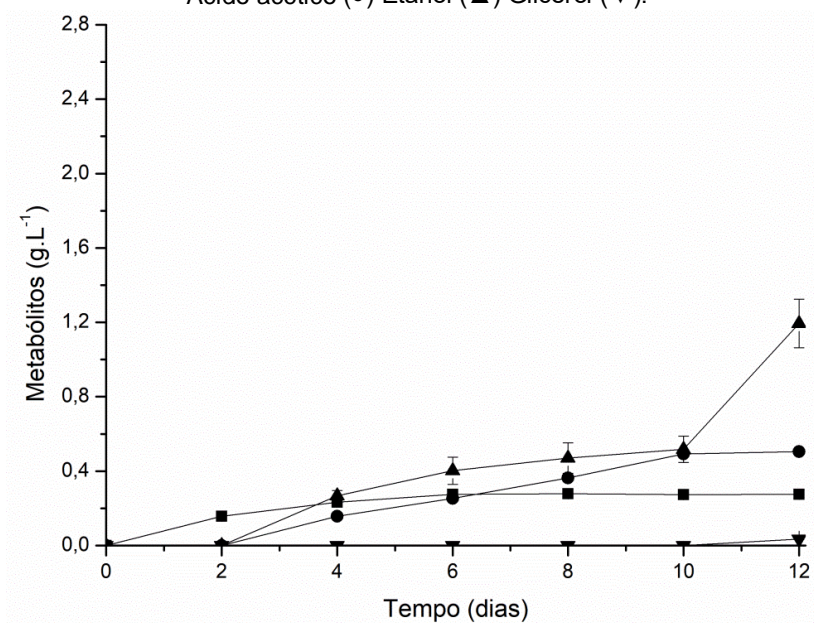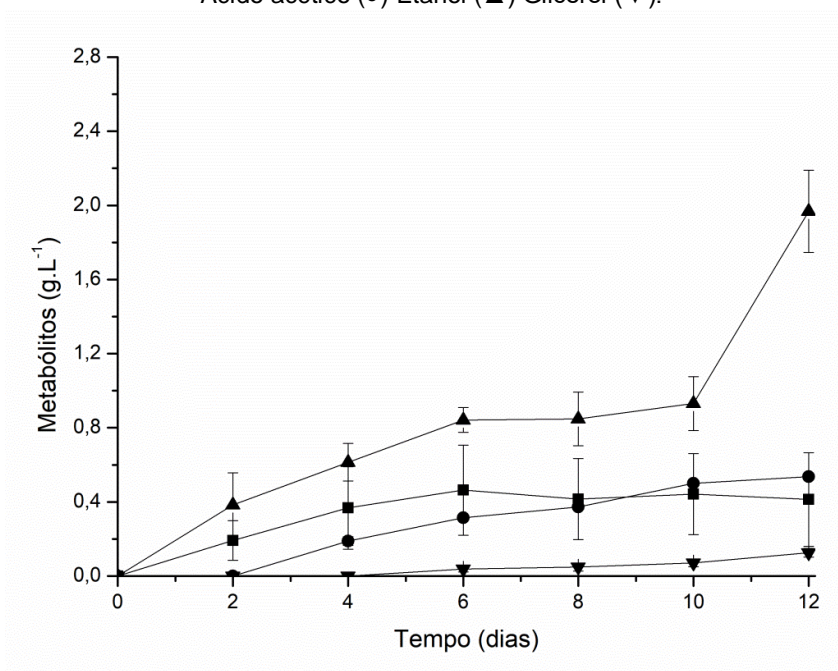
Figura A-B 64. Evolução dos metabólitos ao longo da fermentação da kombucha SC2. Ácido lático (■) Ácido acético (●) Etanol (▲) Glicerol (▼).

Através das cinéticas foi possível observar que a produção de ácidos orgânicos e álcoois foi baixa, mesmo com um aumento no inóculo de 10^6 para 10^7. Conforme a legislação, a kombucha deve apresentar no mínimo 1,8 g.L^{-1} de ácido acético em sua composição, portanto os dois produtos obtidos nesse estudo ficaram com valores inferiores aos exigidos pelo MAPA. Em relação à concentração de etanol, ambas bebidas se encaixam na legislação, aprensentando valores finais de 1,19 g.L^{-1} e 1,97 g.L^{-1} para CS1 e CS2, respectivamente. A produção de etanol, pela levedura *D. anomala*, vem acompanhada da síntese de glicerol em baixas concentrações, em ambos os experimentos.

Na preparação da cultura *starter* neste trabalho só foram adicionados microrganismos de interesse, que em simbiose não alcançaram as características necessárias para fermentação da kombucha (alta atividade invertase da levedura e produção de ácido acético conforme a legislação). Diversos fatores podem ter impactado a viabilidade da bactéria *A. aceti*, portanto essa linhagem ainda será estudada em conjunto com outros microrganismos. Nas próximas etapas foram ampliadas as linhagens de microrganismos e a concentração de sacarose foi reduzida. Com intuito de diminuir o número de variáveis, etapas seguintes foram realizadas sem a linhagem da bacteria lática, visto que estas nem sempre estão presentes na kombucha e não tem papel fundamental no processo tecnólogico e fermentativo.

Buscando a padronização da kombucha, as próximas etapas do trabalho buscaram estandardizar o processo de produção desta bebida, através da combinação ideal de microrganismos, garantindo um produto seguro, com no máximo 0,5 % ABV de etanol e que seja agradável sensorialmente. Para isso, o desenvolvimento da cultura *starter* já testada contou com a combinação de outras linhagens de microrganismos, entre eles *Gluconacetobacter hansenii, Komagataeibacter saccharivorans e Kluyveromyces marxianus fragilis* (resultados apresentados em formato de artigo no Capítulo II). A primeira etapa foi realizar a curva de crescimento de cada um dos microrganismos, seguida de diversas combinações de microrganismos para fermentação, a fim de obter um produto com as características exigidas pela legislação e com alta atividade invertase. Foram testadas diferentes condições de fermentação, a fim de otimizar a produtividade. Os produtos otimizados foram comparados com amostras comerciais e avaliados em

relação aos metabólitos e compostos voláteis formados, atividade antioxidante e análise sensorial.

Apêndice C. Participação no evento "Copenhagen Bioscience Conference: Microbial Foods 2022".

A seleção da melhor combinação de microrganismos para utilização como cultura *starter* em kombucha, parte do artigo "Development of synthetic microbial consortia for kombucha fermentation: assessment of metabolites and volatile compounds production", foi apresentada em formato de pôster no evento "Copenhagen Bioscience Conference: Microbial Foods 2022", que aconteceu entre os dias 8 e 12 de maio de 2022 na Dinamarca. A conferência foi financiada pela Novo Nordisk Foundation, que me concedeu uma bolsa viagem para participação no evento. O evento proporcionou a troca de conhecimento e ideias sobre alimentos fermentados e foi de grande valia para minha formação acadêmica.

O evento contou com pesquisadores e pesquisadoras de diversos países e trouxe discussões sobre os resultados científicos e tópicos mais recentes na área de fermentação. Alguns dos tópicos abordados foram: História da fermentação e fermentações tradicionais; Comunidades microbianas em alimentos fermentados; Novo organimos para fermentação de alimentos; Proteína sustentável produzida por novos organismos; Biomassa microbiana para alimentos e alimentação; Percepção humana e a interação com os alimentos fermentados.

Além de palestras, a programação também contou com *workshop* para produção de tempeh e momentos específicos para construção de novas colaborações, conversas com empreendedores, professores, editores de revista e pesquisadores da área.

Figura A-C 1. Resumo publicado no livro de resumos da Conferência Microbial Foods 2022.

C **Copenhagen Bioscience Conferences**
a novo nordisk foundation initiative

Microbial communities in fermented foods

017 | **Fensterseifer Fabricio, Mariana**

Federal University of Rio Grande do Sul
Food Science and Technology
Brazil

Co-authors:
Bruna Krieger Vargas, Simone Hickmann Flôres, Nereida Maria Abano Cordeiro, Marco Antônio Záchia Ayub

Notes

Synthetic microbial consortia in kombucha fermentation: Impacts on the final product

Kombucha is obtained through fermentation of sweetened green and/or black tea by a symbiosis of microorganisms, predominated by acetic acid bacteria and yeasts. The microbial composition is very diverse between kombuchas and fermentation batches. It results in highly variable process and end products and usually the alcohol content surpasses the legal limits. A starter culture technology is crucial to assure food safety and quality in fermented foods. Moreover, natural consortia may contain strains that produce inhibitory or toxic byproducts.

In this context, the aim of this work was to develop a synthetic starter culture for kombucha fermentation. The microorganisms selected were Acetobacter aceti, Gluconacetobacter hansenii, Komagataeibacter saccharivorans, Dekkera anomala and Kluyveromyces marxianus fragilis. In order to evaluate the best consortia of microorganisms, a Plackett-Burmann design was performed to analyse the responses of the survival of each strain, production of organic acids, ethanol and pH after 10 days of fermentation at 28 °C. Initial cell concentration, determined based on literature review, was 1.107 CFU/mL for bacteria and 1.105 CFU/mL for yeasts. The most suitable consortia for kombucha fermentation were composed of K. saccharivorans, D. anomala and K. marxianus fragilis, resulting in a product showing low amounts of ethanol and a concentration of acetic acid very similar to commercial kombuchas and within legal limits.

Results showed that some bacterial strains were not suitable for kombucha fermentation. Consortia modelling and improvement is fundamentally needed to use its potential to improve time of fermentation and final product properties.

Figura A-C 2. Pôster apresentado na Conferência Microbial Foods 2022.

SYNTHETIC MICROBIAL CONSORTIA IN KOMBUCHA FERMENTATION: IMPACTS ON THE FINAL PRODUCT

AUTHORS
Mariana Fensterseifer Fabricio[1], Bruna Krieger Vargas[1], Simone Hickmann Flôres[1], Nereida Maria Abano Cordeiro[2], Marco Antônio Záchia Ayub[1].

AFFILIATIONS
[1] Food Science and Technology Institute, Federal University of Rio Grande do Sul; Av. Bento Gonçalves, 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil.
[2] LB3, Faculty of Exact Science and Engineering, University of Madeira, 9020-105 Funchal, Madeira, Portugal

INTRODUCTION

Kombucha is a beverage obtained through fermentation of sweetened green and/or black tea by a symbiosis of microorganisms known as SCOBY (Symbiotic Culture of Bacteria and Yeast), predominated by acetic acid bacteria. The diverse microbial composition results in highly variable process and end products and usually the alcohol content surpasses the legal limits (0.5 % in Brazil and USA; 1.2 % in Europe) [1,2]. Process variables, such as temperature, pH, concentration of substrates, vessel geometry, and time, help to control the fermentative process, but are not sufficient to assure the standardization of kombucha production [3]. A starter culture technology is crucial to assure food safety and quality in fermented foods. Moreover, natural consortia may contain strains that produce inhibitory or toxic byproducts [4].
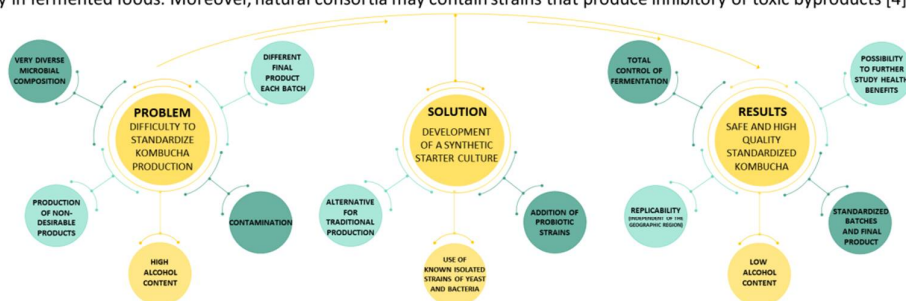
OBJECTIVE

The aim of this work was to select the most suitable consortia of microorganisms to be used as a synthetic starter culture for kombucha fermentation, as an alternative to the use of the traditional inoculation process (cellulose pellicle + fermented liquid from previous fermentations) and to test the possibility to produce high quality and standardized kombucha.

METHODOLOGY

8-RUN PLACKETT-BURMANN DESIGN

VARIABLES:
Acetobacter aceti (AA)
Gluconacetobacter hansenii (GH)
Komagataeibacter saccharivorans (KS)
Dekkera anomala (DA)

Initial cell concentration:
1.10^7 CFU.mL^{-1} for bacteria
1.10^5 CFU.mL^{-1} for yeasts

Fixed variable:
Kluyveromyces marxianus fragilis
PROBIOTIC YEAST

10 DAYS AT 28 °C

RESPONSES:
Acetic acid
Ethanol
Cell viability
pH

Media composition:
8 g.L^{-1} of organic green tea
(Vemat, SC, Brazil)

60 g.L^{-1} of organic demerara sugar
(Native, SP, Brazil)

Fermentations were performed in 250 mL beakers filled with 251 mL (specific interfacial area (SIS) of 0.132) of sweetened green tea, covered with sterile gauze and cheesecloth to create aerobic conditions.

RESULTS

The most satisfactory mixture of strains for kombucha fermentation was composed of *K. saccharivorans* and *D. anomala* (Table 1, run 5). Under this condition, *K. saccharivorans* maintained the same viability and produced acetic acid within the Brazilian legal limits (between 1.8 g.L^{-1} and 7.8 g.L^{-1}) (Fig 1).

Table 1. Process variables and experimental results of the 8-run Plackett-Burmann design to study the impact of co-cultured strains on kombucha fermentation. Results are expressed as means ± standard deviation (g.L^{-1}).

Run	AA	GH	DA	KS	Acetic acid	Ethanol
1	-1	-1	-1	1	0.00 ± 0.00	0.00 ± 0.00
2	1	-1	-1	-1	0.31 ± 0.01	9.08 ± 0.15
3	-1	1	-1	-1	0.29 ± 0.06	8.60 ± 0.17
4	1	1	-1	1	0.33 ± 0.02	9.08 ± 0.21
5	-1	-1	1	1	3.39 ± 0.67	0.27 ± 0.02
6	1	-1	1	-1	0.32 ± 0.02	6.22 ± 0.48
7	-1	1	1	-1	0.31 ± 0.04	6.74 ± 0.12
8	1	1	1	1	5.03 ± 0.34	0.28 ± 0.07

A. aceti
G. hansenii → Bacterial strains not suitable for kombucha fermentation in symbiosis with *K. marxianus fragilis*
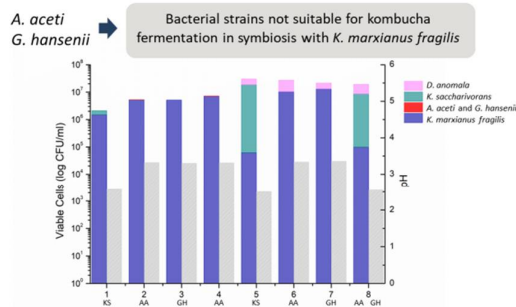
Figure 1. pH and viable counts of bacteria and yeast populations after 10 days of fermentation with different starter culture designed by PB experiments.

CONCLUSION

Results obtained in this work showed that a synthetic starter culture promotes the total control of the main metabolites in kombucha fermentation and low concentrations of alcohol, which shows potential for application in kombucha industrial production.

REFERENCES

UFRGS
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

BIOTECLAB
ICTA - UFRGS

CAPES

Contact: marianafenster@gmail.com