UDIELLE VERMELHO LACERDA

GREEN TEA KOMBUCHA: CHEMICAL CHARACTERIZATION, PHENOLIC PROFILE, BIOACTIVE PROPERTIES AND STABILITY DURING STORAGE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de Magister Scientiae.

Orientador: Frederico Augusto R. de Barros

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Orientador

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"Quem olha para fora sonha, quem olha para dentro desperta."

Carl Jung

RESUMO

LACERDA, Udielle Vermelho, M.Sc. Universidade Federal de Viçosa, fevereiro de 2024. **Green tea kombucha: chemical characterization, phenolic profile, bioactive properties and stability during storage.** Orientador: Frederico Augusto Ribeiro de Barros. Coorientadora: Monique Renon Eller.

A kombucha é uma bebida à base de chá que é fermentada por um consórcio simbiótico de bactérias e leveduras denominado SCOBY, no qual se utiliza como substrato o chá de *Camellia sinensis* açucarado. O consumo regular de kombucha pode gerar benefícios à saúde devido às suas propriedades bioativas, como, por exemplo, atividade anticâncer, antimalárica, anti-hipertensiva, modulação da microbiota intestinal, entre outros. Contudo, não foram encontrados trabalhos que avaliem o potencial bioativo e a vida de prateleira de kombucha utilizando chá verde *(Camellia sinensis)* produzido em território brasileiro. Assim, os objetivos do presente estudo consistiram em realizar uma revisão de literatura (capítulo 1), caracterização físico-química, microbiológica, perfil de fenólicos, fenólicos totais e atividades bioativas *in vitro* (capítulo 2), além de avaliar as alterações físico-químicas, microbiológicas, teor de açúcares residuais (sacarose, glicose, frutose) e fenólicos totais da kombucha de chá verde mantida sob refrigeração a 4º C por 120 dias (capítulo 3). Um total de 92 compostos fenólicos foram identificados na kombucha de chá verde, sendo a maioria pertencente à classe dos flavonoides. Esse rico perfil de fenólicos auxiliou a explicar as propriedades bioativas verificadas. A kombucha exerceu atividade antimalárica contra cepas de *P. falciparum* sentíveis (3D7) e não sensíveis (W2) ao medicamento cloroquina. Ela também apresentou atividades antiproliferativas contra células epiteliais de adenocarcinoma (A549), células de carcinoma de cólon humano (HCT8), células de câncer de fígado humano (HepG2) e baixa toxicidade para células endoteliais da veia umbilical humana (HUVEC) e fibroblastos pulmonares humanos normais (IMR90). Além disso, desempenhou uma proteção dose-dependente aos eritrócitos, evitando sua hemólise, e foi capaz de reduzir a formação de espécies reativas de oxigênio (ERO) intracelular. Durante a vida de prateleira (capítulo 3), em geral as propriedades da bebida permaneceram estáveis nos padrões da legislação. Entretanto, a acidez volátil aumentou ao longo desse período possivelmente devido à contribuição de outros ácidos minoritários, ou até mesmo acetato de etila. Ao longo dos 120 dias ocorreu hidrólise de 50% da sacarose residual, refletindo em um aumento da glicose, mas de forma interessante com a frutose constante, o que indica o consumo desta. Os microrganismos presentes na kombucha

tiveram uma redução significativa que foi atribuída à temperatura e ao ambiente de armazenamento. De forma geral, a kombucha produzida com chá verde *(Camellia Sinensis)* cultivado em território brasileiro se mostrou promissora ao desempenhar de forma satisfatória todas as propriedades bioativas testadas e ao mesmo tempo, a bebida seguiu os padrões de identidade e qualidade pela legislação vigente ao longo dos 120 dias. Assim, pode-se concluir que a troca do chá importado pelo chá nacional pode ser uma alternativa para reduzir os custos de produção da bebida sem causar perdas no potencial bioativo e/ou seguimento na legislação vigente.

Palavras-chave: Kombucha; Compostos fenólicos; Propriedades bioativas; Vida de prateleira.

ABSTRACT

LACERDA, Udielle Vermelho, M.Sc. Federal University of Viçosa, February 2024. **Green tea kombucha: chemical characterization, phenolic profile, bioactive properties and stability during storage.** Supervisor: Frederico Augusto Ribeiro de Barros. Co-supervisor: Monique Renon Eller.

Kombucha is a tea-based beverage fermented by a symbiotic culture of bacteria and yeast (SCOBY), using sweetened *Camellia Sinensis* tea as a substrate. Regular consumption of kombucha may offer health benefits due to its bioactive properties, such as anticancer, antimalarial, antihypertensive, and modulation of intestinal microbiota, among others. However, there is a lack of studies evaluating the bioactive potential and shelf life of kombucha using green tea *(Camellia Sinensis)* produced in Brazilian territory. The objectives of this study included a literature review (chapter 1), physical-chemical and microbiological characterization, phenolic profile, total phenolics, and in vitro bioactive activities (chapter 2). Additionally, the study aimed to assess the physical-chemical changes, microbiological aspects, residual sugar content (sucrose, glucose, fructose), and total phenolics of green tea kombucha kept under refrigeration at 4°C for 120 days (chapter 3). A total of 92 phenolic compounds were identified in green tea kombucha, with the majority belonging to the flavonoid class. This rich phenolic profile helped explain the observed bioactive properties. Kombucha exhibited antimalarial activity against sensitive (3D7) and non-sensitive (W2) strains of P. falciparum, as well as antiproliferative activities against adenocarcinoma epithelial cells (A549), human colon carcinoma cells (HCT8), and human liver cancer cells (HepG2), with low toxicity to human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblasts (IMR90). Additionally, it provided dose-dependent protection to erythrocytes, preventing hemolysis, and reduced intracellular reactive oxygen species (ROS) formation. Throughout the shelf life (chapter 3), the beverage's properties generally remained stable within legal standards. However, volatile acidity increased, possibly due to the contribution of other minor acids or even ethyl acetate. Over the 120 days, 50% of residual sucrose underwent hydrolysis, resulting in increased glucose, while fructose remained constant, indicating its consumption. The microorganisms present in kombucha experienced a significant reduction attributed to temperature and storage conditions. In summary, kombucha produced with green tea *(Camellia Sinensis)* grown in Brazilian territory showed promising results, satisfactorily displaying all tested bioactive properties. Simultaneously, the beverage complied with identity

and quality standards set by current legislation over the 120 days. Therefore, replacing imported tea with domestically grown tea could be an alternative to reduce production costs without compromising bioactive potential or compliance with current regulations.

Keywords: Kombucha; Phenolic compounds; Bioactive properties; Shelf life.

1. INTRODUÇÃO GERAL

A kombucha é uma bebida obtida da fermentação do chá verde ou preto da planta *Camellia sinensis* por um consórcio simbiótico de bactérias e leveduras denominado SCOBY (Jayabalan et al, 2007; Tran et al., 2020). Nos últimos anos o consumo de kombucha vem aumentando consideravelmente devido a uma maior preocupação das pessoas em desenvolver hábitos de vida mais saudáveis que culmina na busca pelo consumo de alimentos e bebidas com propriedades bioativas principalmente após a COVID-19, como é o caso da kombucha (Chong et al, 2023). Estima-se que o mercado mundial de kombucha em 2028 atinja 4,26 bilhões de dólares (Mordor intelligence, 2023). No Brasil, o consumo de kombucha também é crescente, fato este que culminou na criação da Instrução Normativa 41 de 2019 que estabelece os padrões de identidade e qualidade da kombucha produzida em território nacional (Brasil, 2019). Nas gôndolas de supermercados e lojas de produtos naturais a presença e abundância de marcas que comercializam kombucha no Brasil também vem aumentando. Porém, não existe uma padronização no modo de produção da bebida fato este que se torna um empecilho com relação à percepção do consumidor e pode dificultar, inclusive o seguimento às normas regulamentadoras (de Oliveira et al., 2023),

Vários estudos demonstraram benefícios para a saúde devido ao consumo de kombucha tais como: melhora do metabolismo de glicose, combate ao estresse oxidativo, potencial para reduzir dislipidemias, atividade anti-inflamatória, antimalárica, anticâncer, entre outros (Cardoso et al., 2020, 2021; Costa et al., 2021, 2022; de Noronha et al., 2022). O potencial antiproliferativo de kombuchas de chá verde e preto foi avaliado contra adenocarcinoma epitelial de pulmão e adenocarcinoma colorretal epitelial e ambas as kombuchas desempenharam um potencial antiproliferativo (Cardoso et al., 2020). A kombucha de chá preto apresentou atividade antimalárica contra cepas de *Plasmodium falciparum* sensíveis e não sensíveis à cloroquina em estágios diversos do desenvolvimento do *Plasmodium* (de Noronha et al, 2022). Ratos que consumiram kombucha de chá verde e preto regularmente tiveram diminuição da: inflamação sistêmica, esteatose hepática de grau 2 para 1 e do tecido adiposo total mesmo após consumirem uma dieta rica em frutose e gordura por algumas semanas (Cardoso et al, 2021). Em outro trabalho foi constatado que o consumo regular de kombucha de chá verde e preto foi capaz de melhorar a saúde intestinal de ratos alimentados com uma dieta rica em frutose e gordura, por meio do aumento da abundância de bactérias benéficas, tais

como *Adlercreutzia*, além de ter havido aumento na concentração de propionato (Costa et al., 2022). Essas propriedades bioativas citadas podem ser explicadas pela rica composição em compostos fenólicos encontrados na kombucha (Cardoso et al., 2020; Costa et al., 2022).

A kombucha também possui ácidos orgânicos como o acético, lático, glicônico e glicurônico, além de etanol, açúcares residuais, amonoácidos, compostos minoritários como alguns minerais e vitaminas do complexo B e C (Cardoso et al., 2020; Dartora et al., 2023 a; de Noronha et al., 2022; Dinh et al., 2019; Jayabalan et al., 2007; Villarreal-Soto et al., 2018). Com relação aos compostos fenólicos, foram identificados quase 130 em kombuchas de chá verde e preto, sendo os cinco mais abundante: galocatequina 3-O-galato/epigalocatequina 3-O-galato; isômero 2 de galocatequina/epigalocatequina; catequina; isômero 2 da quercetina 3-O-ramnosil-ramnosil-glicosídeo e a quercetina 3-O-glucosil-ramnosilgalactosídeo isômero 2 (Cardoso et al., 2020).

A composição microbiológica da kombucha é variável. De acordo com Costa et al (2022), dentre as leveduras a espécie *Dekkera bruxellensis* correspondeu a mais de 99% dos achados, seguida por *Saccharomyces bayanus* tanto em kombuchas de chá verde e preto, quanto no SCOBY (Costa et al., 2022). Leveduras, geralmente ocorrem em menor abundância que as bactérias, especialmente: *Brettanomyces bruxellensis , Saccharomyces cerevisiae* , *Schizosaccharomyces pombe , Zygosaccharomyces bailii* e *Zygosaccharommyces rouxii* (Dufresne; Farnworth, 2000; jayabaln et al., 2017). Com relação às bactérias, o filo Proteobacteria foi o predominante tanto no SCOBY, quanto nas kombuchas de chá verde e chá preto. De forma distinta, filos como Firmicutes, Bacteroidetes *e* Actinobacteria tiveram uma abundância significativa nas kombuchas, porém não tiverem nos respectivos SCOBYs, (Costa et al., 2022). Isso demonstra que, embora alguns microrganismos estejam presentes no SCOBY não necessariamente estarão na respectiva kombucha. Outros trabalhos apontam que gêneros como *Acetobacter, Gluconoacetobacter, Lactobacillus, Leuconostoc, Komagataeibacter* são os principais encontrados relacionados a bactérias do ácido acético (BAA) (Jayabalan et al, 2017; Martínez Leal 2018, 2018). Em alguns casos, ainda podem ser encontradas bactérias produtoras de ácido lático (BAL). A interação entre as bactérias do ácido acético e as leveduras pode favorecer ou inibir o crescimento de algumas espécies e, assim, modificar a composição físico-química e características sensoriais da bebida (Villarreal-Soto et al., 2018).

Por ser uma bebida fermentada, o perfil de compostos voláteis que engloba ácidos voláteis, aldeídos, cetonas, ésteres e a formação de alguns metabólitos pode ser um ponto chave na definição da qualidade da bebida uma vez que estão diretamente relacionados às características sensoriais e aroma (Coton et al, 2017; Dartora et al., 2023b). A kombucha contém microrganismos variáveis que podem se manter viáveis ao longo do tempo e assim a composição da bebida pode se alterar ao longo da vida de prateleira (Fu et al., 2014). Este processo pode influenciar na estabilidade da bebida e até mesmo em qual categoria ela se enquadra, uma vez que para ser considerada não alcoólica o teor de etanol não pode ser superior a 0,05% (v/v) (Brasil, 2019). Embora os estudos com esta bebida tenham aumentado nos últimos anos, não foram encontrados trabalhos que avaliem a estabilidade, o perfil de compostos fenólicos e propriedades bioativas como atividade antimalárica e potencial antiproliferativo de kombucha produzida com chá verde brasileiro. O uso do chá verde produzido em território nacional pode ser uma alternativa para reduzir os custos de produção e ainda incentivar o seu cultivo em território nacional. Além disso, a mudanças no tipo de chá, neste caso o chá foi cultivado em território brasileiro, requer novos estudos para avaliar o potencial biológico da nova bebida desenvolvida uma vez que alteração em parâmetros como umidade, precipitação, composição do solo, entre outros, impactam na composição fenólica do chá (Santos et al., 2023) e consequentemente, no potencial bioativo. Dessa forma, o objetivo do presente estudo foi caracterizar a kombucha de chá verde nacional em termos físico-químicos, microbiológicos, perfil de fenólicos, atividades bioativas *in vitro* e avaliação da vida de prateleira ao longo de um período de 120 dias, sob refrigeração a 4 ºC.

CAPÍTULO I - REFERENCIAL TEÓRICO

1 Histórico e mercado

A kombucha é uma bebida milenar fermentada tendo sua origem relatada no leste da Ásia em 220 A.C. No período das grandes navegações começou a ser introduzida em outros locais como Rússia e Europa Ocidental. Durante a Segunda Guerra Mundial foi difundida na França e norte da África, onde teve o seu uso popularizado (Jayabalan et al., 2014). Nos países ocidentais seu consumo vem crescendo nos últimos anos, principalmente nos Estados Unidos. Esse crescimento está associado ao efeito hepatoprotetor, efeito hipocolesterolêmico, antidiabético e antioxidante em ratos alimentos com kombucha; redução na propagação de câncer, melhora das condições imunológicas, gastrointestinais, possível modulação da microbiota intestinal de ratos, entre outros (Cardoso et al., 2020; Chakravorty et al., 2019; Costa et al., 2021, 2022; Martínez Leal et al., 2028; Yang et al., 2014).

Nos últimos anos houve um crescimento tanto do consumo quanto na produção e abundância de marcas que comercializam kombucha. Dados do *Mordor Inteligence* (2022) apontam que em 2028 o mercado de kombucha irá arrecadar aproximadamente US 4.3 bilhões. No Brasil, o consumo da bebida também é crescente, culminando na criação no ano de 2019 da Instrução Normativa nº41, que estabelece os Padrões de Identidade e Qualidade da bebida produzida no âmbito nacional (Brasil, 2019).

2 Produção da kombucha

A kombucha é obtida através do processo fermentativo de chá verde ou preto, da planta *Camellia sinensis,* adoçado. Neste é adicionado um consórcio simbiótico de bactérias e leveduras denominado SCOBY, que atua como cultura starter e, após a fermentação, deixa a bebida com características agridoce (Brasil, 2019; Jayabalan et al., 2014). Este consórcio simbiótico tem a aparência de um disco de material celulósico que é produzido via metabolismo bacteriano e flutua na superfície da bebida (Ramírez Tapias et al., 2022; Villarreal-Soto et al., 2018). De forma geral, a cultura mais jovem vai se formando por cima da cultura mais velha (Marsh et al., 2014). Na figura 1 são demonstradas as interações e produtos do metabolismo microbiano envolvidos na fermentação da kombucha.

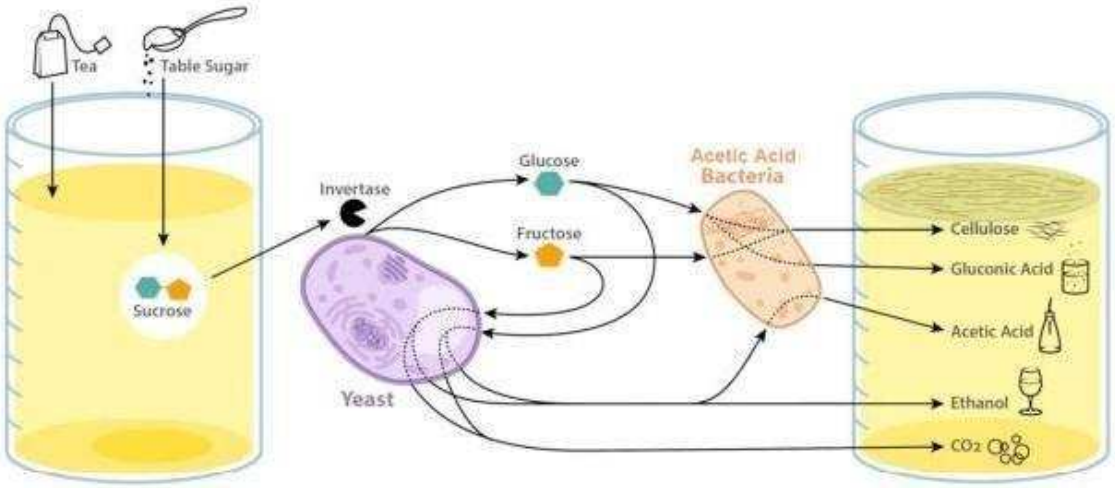

Figura 1: Metabolismo microbiano, interações e principais produtos

Fonte: Tran et al. (2020)

Num primeiro momento, a sacarose é hidrolisada pela enzima invertase produzida pelas leveduras em glicose e frutose. Estes monossacarídeos podem ser utilizados pelas leveduras e gerar etanol através da fermentação alcoólica (Jayabalan et al., 2007). Assim, ocorre uma relação de simbiose, onde as leveduras fornecem as fontes de energia metabolizáveis pelas bactérias do ácido acético que posteriormente irão gerar ácidos orgânicos, principalmente o acético (Chen; Liu, 2000; Jayabalan; Waisundara, 2019). Além disso, pode ocorrer a oxidação do etanol, formando ácido acético e o ácido glicônico, os quais podem também ser obtidos através da glicose. A acidificação do meio e redução do pH, presença de etanol, ácidos orgânicos e algumas vitaminas contribuem para causar uma inibição do crescimento de microrganismos patogênicos em virtude de estresse ambiental e pressão seletiva no meio (Jayabalan et al, 2014; Neffe-Skocińska et al., 2017). Paralelo a isso, ainda ocorre a formação de um biofilme com aspecto gelatinoso, composto principalmente por celulose oriunda do metabolismo de algumas bactérias, em especial, *Komagataeibacter xylinus* (ZhanG et al., 2018). Vale destacar que a composição do SCOBY é variável em termos de substrato, condições ambientais e até mesmo localização geográfica, o que impacta diretamente nos produtos gerados pela fermentação (de Filippis et al., 2018).

Tradicionalmente ao meio utilizado na produção de kombucha são adicionados, além do SCOBY, sacarose em torno de 10% m/v para atuar como fonte de energia para os microrganismos realizarem seu metabolismo, além de 10 a 15% (v/v) de kombucha de uma batelada anterior, que tem como função causar a redução no pH inicial do chá e, assim, propiciar um melhor desenvolvimento dos microrganismos presentes na cultura

starter (Jayabalan et al., 2008, 2014). A fermentação pode durar entre 7 a 14 dias e a temperatura recomentada é de 25 a 30 ºC. O controle desta temperatura é essencial para garantir um correto crescimento dos microrganismos, além é claro, de influenciar no perfil sensorial da bebida no que diz respeito ao balanço entre acidez e doçura (Crum et al., 2016; Dufresne; Farnworth, 2000). A fermentação é influenciada por diversos parâmetros como temperatura, pH, composição do meio, disponibilidade de oxigênio, dióxido de carbono, geometria do fermentador no qual é realizada, entre outros (Marsh et al., 2014).

Na kombucha são encontrados açúcares como sacarose, frutose e glicose, fibras, ácidos orgânicos, etanol, polifenóis do chá, aminoácidos como lisina e teanina, além de minerais, vitaminas hidrossolúveis, dióxido de carbono, entre outros que auxiliam no correto funcionamento do organismo, porém conforme relatado, esta composição irá variar a depender das condições e substratos usados na fermentação (Bortolomedi et al., 2022; Jayabalan et al., 2014).

3. Composição química da kombucha

A composição química da kombucha é diversa e inclui os ácidos orgânicos como acético, glicônico e glicurônico podendo apresentar também ácido cítrico, lático, málico, tartárico, oxálico, succínico, pirúvico, malônico, entre outros. Além disso há a presença de açúcares como sacarose, glicose e frutose, vitaminas do complexo B, vitamina C, aminoácidos, pigmentos, proteínas, lipídeos, etanol. enzimas hidrolíticas, compostos fenólicos, alguns minerais, produtos metabólicos de bactérias e leveduras, além do D-ácido 1,4-lactona (DSL), cafeína, teofilina e teobromina (de Roos; de Vuyst, 2018; Jayabaln et al., 2014; Villarreal-Soto et al., 2018). É importante destacar que o ácido glicurônico, além de possuir efeitos desintoxicantes, pode atuar como precursor na síntese de vitamina C (Jayabalan et al., 2014; Nguyen et al., 2015). O teor de ácido lático, por sua vez, é maior quando se utiliza o chá verde em comparação com o chá preto (Nguyen et al., 2015).

A interação entre as bactérias do ácido acético e as leveduras pode ser denominada metabiose, ou seja, as leveduras produzem os substratos que são oxidáveis e as culturas de bactérias do ácido acético podem gerar ácido acético na ausência das leveduras (Tran et al, 2020). Desse modo, as bactérias do ácido acético influenciam nos tipos e concentrações dos ácidos orgânicos e as leveduras nas características sensoriais da bebida

(Jayabalan et al., 2014; Villarreal-Soto et al., 2018). A geração destes ácidos orgânicos tende a causar uma redução do pH da bebida que pode variar entre 2,5 a 4,2 a depender das condições de fermentação. No geral, este pH tende a decair mais rapidamente ao longo dos três primeiros dias de fermentação e depois esse decaimento é menor. Esta faixa de pH inibi o crescimento de microrganismos não desejáveis (Malbaša et al., 2011; Muhialdin et al., 2019). Além disso, a autólise das leveduras pode fornecer metabólitos essenciais como vitaminas e aminoácidos importantes para o desenvolvimento de bactérias do ácido acético.

Além disso, o tipo de chá utilizado também influencia na composição química da kombucha. De forma geral, cerca de 1/3 da massa do chá (em base seca) é marcada pela presença de compostos fenólicos, porém a abundância e diversidade destes depende do tipo de chá e dos aspectos relacionados ao plantio do mesmo como solo, condições climáticas, entre outros (Dufresne; Farnworth, 2000; Feng et al, 2022; Dinh et al., 2019).

3.1 Compostos fenólicos

As pessoas estão cada vez mais em busca de um equilíbrio entre atividade física e dieta saudável. Neste sentido, os alimentos conhecidos por apresentar compostos bioativos, ou seja, que trazem potenciais benefícios à saúde têm ganhado mais destaque no comércio e consumo. Dentre estes compostos bioativos, os compostos fenólicos estão presentes de forma natural em frutas e hortaliças e a kombucha é uma bebida rica nesta classe devido à presença do chá verde ou preto (Cardoso et al., 2020; de Araújo et al., 2021). De forma simplificada, os compostos fenólicos são moléculas altamente reativas que possuem propriedades capazes de ativar enzimas antioxidantes, transferir elétrons para radicais livres ou ainda inibir a formação de espécies reativas do oxigênio (Heleno et al., 2015; Jayabalan et al., 2008).

Na kombucha, os compostos fenólicos presentes em maior quantidade geralmente são os flavonoides, entre eles, a catequina e ácidos fenólicos como o ácido gálico. Já foram reportados 127 compostos fenólicos sendo a maioria (70,8%) pertencente à classe dos flavonóides, além de ácidos fenólicos, lignanas e estilbenos (Cardoso et al., 2020). Entre os 10 compostos fenólicos mais abundantes, 60% deles estavam presentes em ambas as kombuchas sendo: galocatequina 3-O-galato/epigalocatequina 3-O-galato, galocatequina isômero 2/epigalocatequina, catequina, ácido 5-O-galoilquínico,

quercetina 3 -O-ramnosil-ramnosil-glicosídeo isômero 2 e quercetina 3-O-glicosil-ramnosil-galactosídeo (Cardoso et al., 2020).

Embora nas folhas do chá sejam encontrados os compostos fenólicos com notáveis efeitos bioativos atrelados à promoção da saúde, durante o processo fermentativo ocorrem uma série de biotransformações que podem melhorar a capacidade antioxidante da bebida (Cardoso et al., 2020; Morales, 2020). Vale destacar que o tipo de chá, quantidade de açúcar e condições de fermentação vão impactar diretamente no perfil dos compostos fenólicos (Cardoso et al., 2020; Coton et al., 2017; de Noronha et al., 2022; Watawaka et al., 2016). Um dos parâmetros que mais pode influenciar no incremento ou redução de compostos bioativos é o tempo no qual a fermentação é realizada, haja visto que a degradação, síntese ou transformação destes compostos ocorre ao longo dos dias (Bortolomedi et al., 2022).

De Noronha et al. (2022) demonstraram que ao longo do processo fermentativo na produção de kombucha de chá preto de *Camellia sinensis* ocorre a modificação do perfil de compostos fenólicos. Neste estudo, a classe dos flavonoides foi a principal reportada, correspondendo a cerca de 50% dentre 164 compostos os identificados. A concentração de ácido fenólicos aumentou com o tempo de fermentação (0-10 dias). Já os flavonoides reduziram ao longo dos quatro primeiros dias de fermentação e depois aumentaram (de Noronha et al., 2022). De acordo com os autores, o aumento no conteúdo de ácido fenólicos pode estar correlacionado às reações envolvendo enzimas microbianas capazes de atuar na degradação dos flavonoides. Alguns compostos como hesperidina, galocatequina e nepetina foram degradados e não foram identificados nos dias 5, 7 e 10 de fermentação enquanto a concentração de outros como ácido gálico, verbacosídeo e ácido cinâmico, além da diversidade de fenólicos, aumentou a partir dos 5 dias de fermentação, o que indicou a importância de se produzir a kombucha com pelo menos 5 dias de processo fermentativo (de Noronha et al., 2022).

É importante destacar que o tipo de SCOBY utilizado também ajuda a modular o perfil de compostos fenólicos encontrado. Ao comparar a kombucha de chá preto fermentada com SCOBY de três fornecedores foi constatado que o ácido gálico estava presente em ambas as kombuchas em concentrações similares, o ácido cafeico aumentou, já a catequina e epicatequina diminuíram após a fermentação (Vilarreal-Soto et al., 2020). Os ácidos cumáricos, ferúlicos e transcinâmico estavam presentes em baixas quantidades nas três kombuchas e, por outro lado, a teobromina, rutina e ácido clorogênico apresentou

maior teor em uma das amostras, provavelmente devido a algum microrganismo em específico que estava presente (Vilarreal-Soto et al., 2020).

4. Composição microbiológica da kombucha

Assim como ocorre com outros alimentos fermentados, a composição microbiológica da kombucha irá depender do tipo de inóculo utilizado, no caso da kombucha, do SCOBY adicionado. De acordo com Costa et al (2022) dentre os fungos, houve uma predominância superior a 99% de *Dekera bruxellensis e Saccharomyces bayanus* em torno de 0,5% de predominância, isso tanto nas kombuchas de chá verde e preto, quanto no SCOBY de ambas as bebidas. Filos bacterianos como Firmecutes, Bacteroidetes e Actinobactéria foram os principais identificados. Dentre as famílias, *Acetobacteraceae*, *Erysipelotrichaceae*, *Porphyromonadaceae*, *Rikenellaceae* e *Streptococcaceae* foram as cinco mais abundantes em ambas as kombuchas (Costa et al., 2022). Com relação ao gênero dominante no SCOBY foi reportado *Gluconacetobacter* e para as kombuchas foi *Acetobacter*. Os táxons pouco abundantes corresponderam a cerca de 13,5% para a kombucha de chá preto e 6,85% na kombucha de chá verde, estes dados sugerem maior diversidade microbiana na kombucha de chá verde (Costa et al., 2022). Um outro ponto interessante é que em ambas as kombuchas havia maior diversidade microbiana que nos respectivos SCOBYS. Essa diferença em termos de composição da microbiota ajuda, inclusive a explicar a maior acidez da kombucha de chá verde (Costa et al., 2022).

Kombucha produzida a partir de chá verde e outras ervas (malva, hortelã-pimenta, rosa-selvagem e limão verbeno) foi avaliada quanto ao perfil de leveduras e bactérias do ácido acético. Assim, foi realizada a amplificação via reação de PCR onde, de fato, as leveduras puderam ser melhor identificadas sendo elas: *D. anomala* e *S. cerevisiae* estas somadas chegam a 36 das 58, além de: *S. uvarum*, *S. uvarum* / *S. bayanus* / *S. bayanus* / *pastorianus*, *Zygosaccharomyces lentus* Z *ygosaccharomyces bailii* e *Zygosaccharomyces parabailii* (Grassi et al., 2022). Em um outro estudo com abordagem metagenômica e dependente de cultura *Komagataiebacter* foi o principal gênero bacteriano acético encontrado e *Zygosaccharomyces* foi o dominante entre as leveduras tanto em kombuchas de chá verde, quanto na de chá preto (Barbosa et al., 2021). No total foram isoladas 133 cepas de bactérias e leveduras após 15 dias de fermentação, havendo maior número de cepas na kombucha de chá preto quando comparada à de chá

verde. Do total de cepas cerca de 92,16% correspondeu a Bactérias do ácido acético (Barbosa et al., 2021). A explicação para estes dois gêneros predominantes se dá pelo fato de que melhor se adaptam às pressões seletivas do meio como baixo pH, altas concentrações de ácido acético e etanol (Marsh et al., 2014).

Na kombucha também podem estar presentes gêneros de bactérias do ácido lático como: *Lactobacillus* e *Lactococcus* além de leveduras como *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *B. bruxellensis,* entre outras (Coton et al., 2017; Marsh et al., 2014). Bactérias do ácido lático como *Lactococcus lactis, Lactobacillus parakefiri, Lactobacillus sp. wkB10, e Bifidobacterium sp*. foram identificadas (Li et al., 2022).

A espécie *K. xylinus*, se destaca como a principal produtora do biofilme celulósico que dá o aspecto visual ao SCOBY e forma ácidos orgânicos que ajudam a compor o perfil sensorial da bebida, além de possui maior resistência ao ácido acético quando comparada com outros microrganismos (Jayabalan et al., 2014; Subbiahdoss et al., 2022; Villarreal-Soto et al., 2018). Além disso, o gênero *Gluconacetobacter* pode atuar na produção de ácidos glicônico e glicurônico, e ácido D-sacárico-1,4-lactona durante a fermentação (Li et al., 2022).

5. Propriedades bioativas da kombucha

A rica composição da kombucha em substâncias bioativas corrobora para que pesquisadores a avaliem com relação a potenciais benefícios tanto *in vitro* quanto *in vivo* à saúde de quem a consome de forma regular (Morales, 2020). De forma simplificada, o consumo regular de kombucha auxilia na prevenção e tratamento da obesidade e tende a modular a microbiota intestinal, possui ação anti-inflamatória, auxilia no metabolismo da glicose, combate estresse oxidativo, pode auxiliar no combate a sepse, entre outros (Cardoso et al., 2021; Costa et al., 2021; 2022; Wang et al., 2021). Dentre os efeitos bioativos, a capacidade antioxidante da kombucha é a mais estudada, porém outras propriedades também tem sido relatadas, dentre elas efeito hepatoprotetor, antidiabético, atividade antimalárica, entre outros (Morales, 2020; Cardoso et al., 2020; de Noronha et al, 2022).

5.1 Capacidade antioxidante da kombucha

Os compostos fenólicos podem ser chamados de antioxidantes potentes devido sua capacidade de eliminar radicais livres, oxigênio singlete, radicais hidroxila, entre outros (Jayabalan et al., 2007). Na literatura é crescente o número de trabalhos envolvendo o estresse oxidativo que ocorre quando há um desequilíbrio entre a geração dos radicais livres e os mecanismos utilizados pelo corpo humano para combatê-los. Esse desequilíbrio pode gerar doenças como câncer, cardiovasculares e até mesmo degenerativas. Neste sentido, o uso de antioxidantes naturais provindos da dieta pode ajudar na correta manutenção da saúde (Morales et al., 2020).

Cinco kombuchas foram obtidas por infusão de chá branco, preto, oolong, pu-erh e verde e fermentaram por 15 dias na temperatura de 30ºC. Destas a de chá verde foi a que apresentou melhores resultados para capacidade antioxidante tanto pelo método do radical $ABTS^{\cdot}$ quanto pelo ensaio CUPRAC (que mede o potencial de redução do íon cúprico) (Değirmencioğlu et al., 2021). Em outro estudo, foram produzidas 3 kombuchas, cada qual a partir de um substrato (chá verde, preto e remanescentes do chá). Destas, a kombucha de chá verde apresentou maior capacidade antioxidante e isto ocorreu após 18 dias de fermentação. Uma possível explicação é devido ao fato de que o chá verde possui maior conteúdo de catequinas (Jayabalan et al., 2008). Quanto à capacidade inibitória na oxidação do ácido linoleico medida por radicais hidroxila, ela também foi maior na kombucha de chá verde. Os autores supracitados apontam que o aumento da atividade antioxidante irá depender do tempo de fermentação, tipo do chá e do SCOBY utilizado (Jayabalan et al., 2008).

Kombuchas de chá verde e preto utilizando diferentes SCOBY foram analisadas e a capacidade antioxidante da kombucha de chá verde foi maior quando inoculada por um SCOBY nativo e, por outro lado, a capacidade antioxidante da kombucha de chá preto foi superior quando utilizaram SCOBY com adição de determinadas cepas (Malbaša et al., 2011), o que sugere que em diferentes substratos prevalecem melhor determinados microrganismos.

5.2 Atividade antimicrobiana da kombucha

Com o aumento do número de microrganismos resistentes a antibióticos, pesquisadores têm buscado novos meios de inibir estas infecções e os compostos

antimicrobianos naturais e provindos da dieta podem ser essenciais para este fim (Morales et al., 2020) e neste contexto, tem-se a kombucha. A presença de ácido acético e outros ácidos orgânicos fracos em sinergia com outros metabólitos provindos do chá e da fermentação, corroboram para a ação antimicrobiana da kombucha (Morales et al., 2020).

A capacidade antimicrobiana de kombuchas de chá verde e preto foi verificada pelo método da concentração mínima inibitória contra 4 bactérias patogênicas sendo elas: *S. aureus, L. monocytogenes, E. coli* e *Salmonella* e a kombucha de chá verde inibiu o crescimento de ambas as bactérias, fato este que não ocorreu com a de chá preto (Cardoso et al., 2020). Uma possível explicação para essa melhor ação antimicrobiana da kombucha de chá verde se dá pelo fato de a bebida apresentar uma maior quantidade de catequinas conhecidas pelo potencial antimicrobiano, de possuírem exclusivamente verbascosídeo e apresentar uma acidez maior quando comparada à kombucha de chá preto (Cardoso et al., 2020).

As propriedades antifúngicas e antimicrobianas de kombuchas de chá verde e malba e seus respectivos chás foram testadas utilizando a concentração mínima inibitória contra os seguintes microrganismos patogênicos: *C. albicans, C. neoformans, P. brasiliensis, E. coli, S. aureus, L. monocytogenes* utilizando o método da concentração mínima inibitória para as atividades antifúngicas e antimicrobianas foram utilizados (Silva et al., 2021). Foi constatado que tanto o chá de malba quanto sua kombucha não apresentaram atividade antifúngica e antimicrobiana. Já o chá verde teve efeito fungicida sobre *P. brasiliensis* e inibitório contra *C. albicans, C. neoformans.* Já a kombucha de chá verde apresentou efeito fungicida e inibitório apenas para *P. brasiliensis.* Este ponto permitiu aos autores supracitados inferir que o processo fermentativo, que gera queda do pH, possa influenciar negativamente sobre alguns compostos fungicidas presentes no chá. Com relação à capacidade antimicrobiana, os chás e suas kombuchas não apresentaram esse efeito (Silva et al., 2021). Estes estudos são importantes para atestar esse potencial da kombucha em atuar como um possível agente de prevenção contra esses microrganismos deteriorantes e/ou patogênicos, principalmente em regiões endêmicas.

5.3 Potencial citotóxico, antiproliferativo e antimalárica da kombucha

O potencial *in vitro* de kombucha de chá preto contra malária foi verificado utilizando uma solução de 0,03 g/mL de kombucha chá preto liofilizada contra duas cepas de *P. falciparum* a W2 (resistente à cloroquina) e 3D7 (sensível à cloroquina) em

diferentes etapas do desenvolvimento do *P. falciparum*. Os resultados obtidos foram animadores pois a bebida se mostrou eficaz contra as 2 cepas analisadas e reduziu a viabilidade em até 35% a depender do estágio do plasmódio no caso da cepa W2 e para a cepa 3D7 os resultados obtidos foram independentes do estágio (de Noronha et al., 2022).

O efeito antiproliferativo *in vitro* da kombucha de chá preto mantida fermentando por até 21 dias a 25 ºC em dois vasilhames com diferentes geometrias foi testado contra linhagens celulares de câncer de cólon humano (HCT-116) e de câncer de mama humano (MCF-7). Os melhores resultados foram obtidos para as linhagens contra o câncer de cólon após 21 dias de fermentação. Um outro ponto que merece destaque é que a geometria do fermentador, bem como, a relação base e altura maior (s/h) causou uma cinética de fermentação favorecida, o que gerou como consequência melhores propriedades antiproliferativas e anti-inflamatórias, principalmente para a linhagem HCT-116 (Villarreal-Soto et al., 2019).

Em outro estudo a kombucha de chá verde e preto foram avaliadas em relação ao potencial antiproliferativo e citotóxico contra adenocarcinoma epitelial de pulmão, adenocarcinoma colorretal ileocecal, adenocarcinoma colorretal epitelial e a célula pulmonar normal. Ambas as kombucha ofereceram efeito antiproliferativo contra as linhagens testadas e baixa toxicidade contra as células sadias. Porém, a kombucha de chá verde apresentou melhores resultados de GI 50 que está relacionado com a concentração que inibe 50% da proliferação celular, estes resultados foram atrelados a uma maior presença de catequinas e verbacosídeos (Cardoso et al., 2020).

Na literatura não foram encontrados estudos que avaliem o potencial da kombucha na eliminação de espécies reativas de oxigênio, porém, há trabalhos que apontam que as catequinas (um dos principais compostos fenólicos presentes na kombucha de chá verde) são capazes de atuar inibindo a expressão de genes relacionados a processos inflamatórios e que a presença de alguns compostos como vitamina C e E, pode atuar de forma sinérgica com as catequinas atuando de maneira a controlar a geração de espécies reativas do oxigênio (Queiroz et al., 2017; Zaveri, 2006).

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CAPÍTULO II

Artigo I

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Green tea kombucha is rich in phenolic compounds and has antioxidant, antiproliferative, antibacterial and antimalarial activities

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Short version of title: Green tea kombucha bioactive properties

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ABSTRACT: Green tea kombucha, produced using a green tea (*Camellia sinensis)* grown in Brazil, was characterized and its *in vitro* bioactive properties were evaluated. Overall, 92 phenolic compounds were identified (70.7% flavonoids, 25% phenolic acids, 2.2% lignans, and 1.1% other polyphenols), contributing to the observed high antioxidant capacity. The major phenolics identified were gallocatechin, catechin 5-O-gallate and epicatechin. Green tea kombucha exhibited antibacterial activity against all tested bacteria, being more effective against *Salmonella spp*. In addition, green tea kombucha demonstrated antimalarial activity against both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum,* and antiproliferative activity against cancer cell lines A549, HCT8, HepG2, and HUVEC. Additionally, it presented antioxidant properties by effectively reducing the generation of reactive oxygen species (ROS) and provided protection to erythrocytes against AAPH-induced oxidative stress. Thus, green tea kombucha is rich in antioxidants and has interesting bioactive properties that can be explored by the food and pharmaceutical industries.

Keywords: bioactive compounds, biological activity, phenolic profile, UPLC- MS^E

1 Introduction

Kombucha is a beverage obtained from the fermentation of green or black tea from the *Camellia sinensis* plant, in which a symbiotic consortium of bacteria and yeast, known as SCOBY is inoculated (Jayabalan et al., 2007; Tran et al., 2020). According to *Global Market Insights* data (2021), an annual growth in kombucha consumption of over 15% is expected between 2022 and 2030. Additionally, it is estimated that the kombucha market will reach $4.26 billion in 2028 (Mordor Intelligence, 2023). This market growth is associated to increased public concern for healthier lifestyles and a search for food and beverages with bioactive properties, especially post-COVID-19, as seen in the case of kombucha (Chong et al., 2023). In Brazil, kombucha consumption is also increasing, leading to the creation of Normative Instruction 41 in 2019, which establishes identity and quality standards for kombucha produced in the country (Brazil, 2019).

The bioactive properties of kombucha can be attributed to its rich chemical and microbiological composition, which varies based on tea type, fermentation parameters such as time and temperature, and microorganisms present in SCOBY (Jayabalan et al., 2007; Villarreal-Soto et al., 2018). Kombucha contains organic acids such as acetic, lactic, gluconic, and glucuronic acids, various vitamins, and phenolic compounds, known for their antioxidant potential and free radical scavenging abilities (Cardoso et al., 2020; de Noronha et al., 2022; Jayabalan et al., 2007, 2008). Phenolic compounds found in green tea kombucha include catechins, epigallocatechin, various isomers, quercetin, phenolic acids, among others, derived from tea and the fermentation process, which undergoes numerous biotransformations, leading to a diversified phenolic profile compared to the original tea (Cardoso et al., 2020; de Noronha et al., 2022).

Moreover, there are studies indicating bioactive properties of kombucha, due to its composition rich in phenolics, such as anticancer, antimalarial, improvement in glucose metabolism, oxidative stress reduction, potential to reduce dyslipidemia, modulation of intestinal microbiota, etc (Cardoso et al. 2020, 2021; Costa 2021, 2022). The antiproliferative potential of green tea kombucha has been evaluated against lung and colorectal cancers, yielding satisfactory results (Cardoso et al., 2020). The black tea kombucha demonstrated antimalarial activity against strains of both chloroquine-sensitive and chloroquine-resistant Plasmodium falciparum at various stages of *Plasmodium* development (de Noronha et al., 2022).

Although research on kombucha has increased in recent years, there are no studies evaluating the antibacterial activity and bioactive potential, specifically antimalarial and antioxidant properties, of green tea kombucha. Moreover, the use of green tea produced in Brazil may be an alternative to reduce production costs and promote its cultivation in Brazil, which will motivate the kombucha industry, since imported teas are more expensive. Thus, the aim of this study was to characterize and to evaluate antibacterial and bioactive activities of a green tea kombucha.

2 Materials and Methods

2.1 Kombucha production

Kombucha was produced from green tea *(Camellia sinensis)* leaves produced in Brazil according to the methodology described by Cardoso et al. (2020), with some modifications. Green tea was obtained from the Amaya brand, grown in the city of Registro, São Paulo, Brazil. Preliminary tests were carried out to define the infusion time and temperature binomial, according to recommendations of the manufacturer, 70 ºC for 1 min. Before initiating fermentation, a starter tea from a previous batch of kombucha was added, ensuring that the initial pH remained in the range of 4.4 to 4.2.

2.1.1 Fermentation

Kombucha fermentation was carried out in three batches at 25 °C for five days. The kombucha was stored in plastic containers (fermenters) with a capacity of 20L and kept in an incubator (BOD). At the end of fermentation, aliquots were collected through a tap located at the bottom of the fermenter and the SCOBY was removed. Kombucha samples were tested for counts microbiological, pH and total acidity. Aliquots were also collected and transferred to tubes centrifuged at 10,000 rpm for 10 min and stored at -18 °C until analyses (total phenolics, volatile acidity, antioxidant capacity, sugars (glucose, fructose, sucrose, ethanol and acetic acid). Some of the kombucha was freeze-dried, and then used to evaluate its *in vitro* bioactive properties and phenolic profile.

2.2 Kombucha characterization

2.2.1 Total acidity, volatile acidity and pH

Total acidity was determined by titration with standardized 0.01 N NaOH and phenolphthalein as indicator, and the result was expressed as % (w/v) acetic acid (IAL, 2005). The pH was determined by a previously calibrated pH meter. To determine volatile acidity, the methodology of the Adolfo Lutz Institute (IAL) (2005) was followed.

2.2.2 Determination of sugars, acetic acid and ethanol

The quantification of sucrose, fructose and glucose, ethanol and acetic acids were carried out using a High-Performance Liquid Chromatograph (HPLC), SHIMADZU brand. For this purpose, the methodology of Siegfried et al. (1984) was followed, with some modifications. The HPX87H column (BIO-RAD) and pre-column of the same phase were used, oven temperature: 32 ºC; flow rate: 0.6 mL. min^{-1} and for the mobile phase H_2SO_4 was used at 5 mM. The obtained results were expressed in g/L.

2.2.3 Microbiological counting

The kombucha samples were serially diluted and plated on GYC agar (Merck, Germany) for acetic acid bacteria counting. Lactic acid bacteria counting was performed on MRS agar (Merck, Germany) supplemented with bromocresol indicator (0.004%), with colonies identified as lactic acid bacteria being those that were yellow (acid producers), catalase-negative, and gram-positive. PDA agar (potato dextrose agar, Merck, Germany) was used for yeast counting. Plates were incubated at 30°C for 3 days under aerobic conditions. Lactic acid bacteria, on the other hand, were incubated under microaerophilic conditions. All results were expressed in CFU/mL.

2.2.4 Antibacterial activity

The antibacterial activity of the kombucha was tested against the following pathogenic bacteria: *Salmonella* enterica subsp. enterica (ATCC 13076), *Escherichia coli*

(ATCC 25922), *Staphylococcus aureus* (ATCC 6538), and *Listeria monocytogenes* (ATCC 49594). The antibacterial activity was determined by calculating the percentage of inhibition using the broth microdilution method with a 96-well microtiter plate (CLSI, 2012), with some modifications. Initially, the cultures were activated in BHI broth at 35 °C/24 h, in two steps. After activation, the inoculum was standardized to approximately 1.0×10^8 CFU/mL using the McFarland scale of 0.5. Serial dilutions were prepared (250 µL/mL to 0.9765 µL/mL) by adding 100 µL of kombucha samples to 100 µL of quadruple concentrated Mueller-Hinton broth. To assess the effect of phenolic compounds and eliminate the influence of low pH on microbial inhibition, kombucha neutralization was performed. Thus, 1M NaOH was used in the original kombucha until reaching a pH of 7, measured with a pH meter. All wells were inoculated with 100 µL of each standardized bacterial culture, except for negative control wells (containing only 200 µL of Mueller-Hinton broth). The final concentration of bacteria in each well was approximately 5.0×10^5 CFU/mL. As a positive control, 100 µL of double-concentrated Mueller-Hinton broth and 100 µL of the respective standardized bacterial culture were added to the wells. To assess the action of the original kombucha and the neutralized kombucha (both with a final concentration of 40% relative to the volume of kombucha aliquot added), 50 microliters of seven times concentrated Mueller-Hinton and 200 microliters of kombucha were added. Subsequently, 150 microliters of this mixture (kombucha and culture medium) were discarded, and 100 microliters of each standardized culture were added. The plates were incubated at 35 °C/24 h, and the percentage of microbial inhibition was calculated based on optical density, measured at 625 nm using a spectrophotometer (CLSI, 2012). Here is the formula for calculating the percentage of inhibition.

$$\%inhibition = \left(\frac{(P_c - N_c) - (K_{40\%} - N_c)}{(P_c - N_c)}\right) * 100$$

P_c represents the OD_{625}nm value of the positive control, N_c represents the OD_{625}nm value of the negative control and $K_{40\%}$ represents the OD_{625nm} value that treated by 40% kombucha.

2.2.5 Total phenolics

The concentration of total phenolics was determined according to the Folin-Ciocalteau colorimetric method, using gallic acid as standard (Singleton; Rossi, 1965).

The absorbance was measured at 760 nm. The results were expressed as *mg* of gallic acid equivalent per mL of kombucha (mg GAE/mL).

2.2.6 Antioxidant capacity

The antioxidant capacity was determined by its ability to inhibit the ABTS+ radical. (2,2'-azinobis-3-ethyl-benzothiazoline-6-sulfonate), according to the methodology of (Re et al., 1999). Trolox was used as standard and results were expressed as μmol of Trolox equivalent per milliliter of kombucha (μmol TE/mL).

2.2.7 Phenolic profiling using UPLC – MSE

The phenolic profile was performed in an ultra-performance liquid chromatography system coupled to mass spectrometry using an electrospray source and multiplex acquisition method (UPLC-MS^E) follow Cardoso et al. (2020) methodology with some modifications. Green tea kombucha was lyophilized and reconstituted in 3 mL of 2% methanol (LC-MS grade), 5% acetonitrile (LC-MS grade), and 93% Milli-Q water. The reconstituted extracts were filtered through a 0.22 μm hydrophilic PTFE filter (Analytical) and stored in vials. A mixture of analytical standards (Sigma Aldrich) of phenolic compounds was prepared at a final concentration of 10 ppm. This solution was injected in triplicate before sample injection, using the same parameters described to ensure instrument reproducibility and to be used as confirmation of the identified phenolic compounds in the samples. Five μL of each sample were injected into the UPLC Acquity system (Waters Co., USA) coupled to the Xevo G2S Q-Tof (Waters Co., England) equipped with electrospray ionization (ESI) source and quadrupole time-of-flight (QTof) mass analyzer. For chromatographic separation, a UPLC HSS T3 C18 column (100 mm × 2.1 mm, 1.8 μm particle diameter; Waters) was used, maintained at 30 °C with a flow rate of 0.5 mL/min for mobile phases: ultrapure water containing 0.3% formic acid and 5 mM ammonium formate (mobile phase A); and LC-MS grade acetonitrile containing 0.3% formic acid (mobile phase B), following the gradient: 0 min − 97% A; 11.80 min − 50% A; 12.38 min − 15% A; 14.23 min −15% A; 14.70 min − 97% A. Data were acquired in MS E mode using argon as collision gas, applying low and high collision energy with a ramp from 25 to 55 V. Acquisitions were performed in negative and centroid mode

between m/z 50 and 1000. Ionization conditions were applied: cone voltage 30 V, capillary voltage 3.0 kV; desolvation gas (N2) 1,200 L/h at 600 °C; cone gas 50 L/h and source temperature at 150 °C. All acquisitions were performed using leucine enkephalin (Leu-Enk, m/z 554, 2615, [M-H] -) for lock mass calibration. The compounds annotated were also classified into phenolic classes before carrying out the semi-quantification by using representative standards of phenolic compounds analyzed under the same experimental conditions. Processed data were exported to XLSTAT software (Addinsoft, France), where abundance values obtained from ion mass spectra were used for relative quantification and statistical evaluation of the data (One-way Anova, Tukey post-test, $p < 0.05$).

2.3 *In vitro* bioactive potential

2.3.1 Cell lines

The lung adenocarcinoma epithelial cells (A549), human colon carcinoma cells (HCT8), human liver cancer cells (HepG2), human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblast (IMR90) cell lines were obtained from the Rio de Janeiro cell bank (Rio de Janeiro, Brazil) and maintained in Dulbecco's Modified Eagles' Medium/Nutrient Mixture F-12 Ham (DMEM) supplemented with a heat-inactivated fetal bovine serum to final concentration of 20% (IMR90) and 10% (A549, HCT8, Hep-G2, HUVEC). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3.2 Cytotoxicity profiling of green tea kombucha

The cytotoxicity and proliferation assays of kombucha phenolic compounds were determined by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The cells are seeded into 96-well plates at 5×10^3 cells/well (A549), 6×10^3 cells/well (IMR90), 1×10^4 cells/well (HCT8, HUVEC), 2×10^4 cells/well (HepG2), 100 µL/well, for 24 h. After 24 h, the cells were treated with the freeze-dried kombucha at different concentrations, based on total phenolic content (0.5, 5, 50, 100, 200, 500 µg GAE/mL) diluted in culture medium for 48 h. Then, 10 µL of MTT (0.5 mg/mL) was

added to each well and incubated for 4 h at 37 °C. The metabolically active cells reduced the MTT to blue formazan crystals, which are dissolved in 100 µL of DMSO/well. The absorbance (570 nm) was measured using a microplate reader (SynergyTM H1, Biotek), and the dose response was determined by nonlinear regression (curve fitting) using GraphPad Prism® software (GraphPad Software, Inc., San Diego, CA, USA). According to the method described by Escher et al. (2018), the IC 50 (50% cell viability inhibition), GI 50 (50% growth inhibition), and LC 50 (50% cell death) parameters were obtained. The kombucha selective index (SI), which indicates the selectivity of the sample to the cell lines tested, was calculated by the ratio IC 50 (normal cell line)/IC 50 (cancer cell lines). Any sample that has an SI value higher than 3 will be considered to have high selectivity (Boechat et al., 2014).

2.3.3 Intracellular reactive oxygen species (ROS) activity

We assessed the intracellular ROS using the DCFH-DA assay (Escher et al., 2018) with modifications. First, the cells (HUVEC and HCT8) were seen in a 96-well plate at 6 x 10 4 cells/well with culture medium. After 24 h to adhesion, the cells were treated with different concentrations of freeze-dried kombucha at different concentrations, based on total phenolic content (10, 50, and 100 µg GAE/mL), and H_2O_2 (15 µmol/L) for the positive control, and incubated for 1 h at 37 °C. After the treatment, the cells were carefully washed with PBS and received HANKS solution containing H_2O_2 (15 µmol/L) as a post-treatment. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm, with a microplate reader (SynergyTM H1, Biotek). The data were expressed as a percentage of fluorescence intensity relative to the untreated group (negative control) and were analyzed using the One-Way ANOVA test followed by the Tukey test using GraphPad Prism® software (GraphPad Software, Inc., San Diego, CA, USA).

2.3.4 Erythrocyte cellular antioxidant activity and protection

The oxidative stress of erythrocytes was induced by AAPH, according to Liao et al. (2014), with adjustments. Red blood cells (RBC, hematocrit 20% in PBS; 100 µL) were mixed with 100 µL PBS (negative control) or quercetin (25 – 50 µg/mL dissolved

and diluted in PBS), or freeze-dried kombucha extracts (50 to 150 µg GAE/mL dissolved and diluted in PBS) and incubated for 20 min (37 ºC, 150 rpm). 200 µL of AAPH (200 mM, in PBS) was added and the microtubes were incubated at 37 °C for 2h. Then, the mixture was centrifuged at 1200 xg for 3 min, to obtain the supernatant (SN) and the precipitate (PT). To assess the effect of Kombucha in RBC hemolysis and hemoglobin oxidation, an aliquot of SN or PBS as a blank (100 µL) was added to a 96-well plate and mixed with 200 µL of PBS. The hemolysis was recorded at 523 nm and the hemoglobin oxidation rate (%) was calculated by the ratio between the 630 nm and 540 nm absorbance. To evaluate the ROS generation, PT was washed with 400 µL of PBS and centrifuged (1200 g, 3 min) before adding 400 µL of DCFH-DA solution at 10 µmol/L. Then, 300 µL was transferred to a 96- well microplate and incubated at 37 ºC for 20 min in the dark. The fluorescence intensity was measured using a microplate fluorometer (Synergy™ H1, Biotek, Waltham, MA, USA) at 485 and 520 nm for excitation and emission, respectively. Quercetin was used as the standard. The results are expressed as a percentage.

2.3.5 Antimalarial Properties

The anti-plasmodial effect of kombucha phenolic compounds was performed according to do Carmo et al. (2020), using chloroquine-resistant (W2) and sensible (3D7) strains. *Plasmodium* strains were cultivated in RPMI culture medium, with 10% albumax II and 4% hematocrit, incubated at 37 °C using the candle jar method. The parasites were diluted and incubated in 96- well plates with freeze-dried kombucha at different concentrations, based on total phenolic content (5, 50, 100, and 200 µg GAE/mL) or culture medium as a positive control. After 48h, the supernatant was removed and, subsequently added 100 µL of lysis buffer solution (20 mM, pH 7.5), EDTA (5 mM), saponin (0.008%; w/v), and Trition X-100 (0.08%; v/v), in addition to 0.2 µL/mL Sybr Safe. The microplates were incubated in the dark for 30 min and the reading was done in a microplate reader (Synergy™ H1, Biotek, Waltham, MA, USA) with excitation at 485 nm and emission at 535 nm.

2.4 Target fishing analysis

Compounds with relative abundance above 10^6 were selected for SEA webserver Keiser et al. (2007), predictions, covering the twelve most abundant compounds present in the kombucha sample. The molecular structures were retrieved from PubChem database in the SMILE format and each compound was submitted to the SEA webserver. The predicted human/plasmodium targets were examined. In this sense, this server predicts based on the Tanimoto similarity between the compound and datasets tested against different targets (please see the literature for more information on how the predictions work (Keiser et al., 2007). This target prediction strategy was based on previous reported works on the literature (Adnan et al., 2022; Azevedo et al., 2022; de Noronha et al., 2022; MA et al., 2022; Paes et al., 2024; Serafim et al., 2019). as method to suggest potential mechanism of action of compounds and mixtures (e.g. extracts).

2.5 Statistical analysis

The results were expressed as mean ± standard deviation. The differences between the means were analyzed using Student's t test, with $p < 0.05$ level. All statistical analyzes were performed using the Rstudio program, version 4.3.1.

3 Results and discussion

3.1 Total acidity, volatile acidity and pH

The green tea kombucha had a pH of 3.41 ± 0.09 and total acidity of 0.20 % ± 0.02 (w/v acetic acid). The increase in acidity over time, leading to a subsequent reduction in pH, is caused by the production of organic acids throughout the fermentation process, with an emphasis on acetic acids (Cardoso et al., 2020; Jayabalan et al., 2014). Some studies suggest that green tea kombucha has higher acidity when compared to black tea kombucha under the same fermentation time and temperature conditions (Cardoso et al., 2020). This fact can be explained by the difference in terms of microbiological composition present in green tea and black tea kombuchas. Bacteria of the *Acetobacter* genus, for example, were more abundant in green tea kombuchas and are closely related

to the production of acetic acid, which may account for the lower pH and higher acidity (Costa et al., 2022).

In our study, the kombucha exhibited volatile acidity of 35.71 mEq/L, which is also an important parameter related to the quality of the beverage. According to Normative Instruction 41 from the Ministry of Agriculture, Livestock, and Supply in Brazil, the volatile acidity of kombucha should be in the range of 30 to 130 mEq/L of acetic acid (Brasil, 2019). In another study, green tea kombucha also produced in Brazil and fermented at 25 ºC for four days reached a volatile acidity of 126.7 mEq/L, and from the fifth day onwards, it was already outside Brazilian standards (Dartora et al., 2023a; Brazil, 2019). Standardizing the initial pH, along with the microbiological composition of the SCOBY, may have contributed to keeping the final pH of the beverage and volatile acidity within the limits recommended by regulations.

3.2 Sugars, acetic acid and ethanol

The green tea kombucha had 22.24 g/L of sucrose. In the preparation of green tea kombucha, 50 g/L of sucrose was added, meaning that approximately 55.5% of the sucrose, equivalent to 27.56 g/L, was consumed by the microorganisms during the five days fermentation period. At the beginning of fermentation, sucrose is hydrolyzed by invertase produced by yeast into its constituent monomers, glucose, and fructose, for metabolism. Yeasts, in turn, can metabolize both glucose and fructose, producing ethanol through alcoholic fermentation (Wang et al., 2022). On the other hand, acetic acid bacteria can use both ethanol or sugars to generate acetic acid (Dufresne & Farnworth, 2000).

The residual glucose and fructose concentrations were 11.49 g/L and 12.57 g/L, respectively. Similar by to sucrose, this remaining amount contributes to the sensory aspects of the beverage. In a previous study, green tea kombucha was prepared using 50g/L of sucrose, and after 10 days of fermentation at 25°C, lower values were obtained for the concentration of glucose and fructose (Cardoso et al., 2020). One possible explanation for this difference in concentration is due to the longer fermentation time (10 days), allowing microorganisms to consume these sugars to maintain their activities.

The acetic acid content in this study was consistent with the findings of (Cardoso et al., 2020), and higher than that found by Ivanišová et al. (2020). It is worth noting that these differences are expected due to the combination of fermentation time and

temperature, microorganisms present in the SCOBY, type of tea, among other factors (Barros Santos et al., 2019; Jayabalan et al., 2008). The ethanol content was 4.7 g/L. Thus, our green tea kombucha can be classified as a non-alcoholic beverage according to Brazilian Normative Instruction, as the ethanol content is below 5.0 g/L (Brazil, 2019). Kombuchas from different brands were evaluated, revealing discrepancies in residual sugar levels, alcohol content, as well as organic acids. All these factors impact the sensory characteristics and potential bioactive properties that these commercial kombuchas may exhibit (Anderson et al., 2022). Therefore, standardizing fermentation conditions, sugar content, among other factors, can be a key piece in developing a beverage that caters to the market niche unwilling to consume alcoholic beverages.

3.3 Microbiological counting

For the microbiological counting of green tea kombucha, lactic acid bacteria, acetic acid bacteria, and yeast were assessed, and their populations were estimated at 7.29 log CFU/mL, 7.02 log CFU/mL, and 7,17 log CFU/mL, respectively. Similar results were found in a study on kombucha obtained from fractions of sweetened green and black tea (100 g/L), fermented for 10 days, with populations also around 10^7 CFU/mL (Neffe-Skocińska et al., 2017). It is evident that, despite having twice the added sugar and fermentation time compared to the present study, this factor did not impact in microbial count. Even with a shorter fermentation time of 5 days, a higher microbial growth was obtained compared to other studies where these populations ranged between 10^5 to 10^6 CFU/mL (Cardoso et al., 2020).

3.4 Phenolic compounds and antioxidant capacity

The kombucha had a total phenolic content of 0.32 ± 0.007 mg GAE/mL, about half of that found in green tea kombucha produced using imported green tea and longer fermentation time (Cardoso et al., 2020). Additionally, it is worth noting that the infusion time and water temperature (75 ºC for 2 min) during the tea preparation for our study were lower (70 ºC for 1 min), which may have impacted the reduction in phenolic compound extraction.

The antioxidant capacity of green tea kombucha was 3.24 ± 0.43 μmol TE/mL. Antioxidant capacity is correlated with the content of phenolic compounds (major contributor), however, other metabolites present in kombucha also contribute to this antioxidant capacity, such as vitamin C (Antolak et al., 2021). The antioxidant activity of each phenolic compound depends on its chemical characteristics, degree of polarity, and stability in the environment where it will react, as they can easily participate in redox reactions (Antolak et al., 2021; Jayabalan et al., 2008).

The green tea kombucha phenolic content was evaluated using UPLC-MS^E after 5 days of fermentation, revealing the presence of 92 phenolic compounds (Table S1). The majority (70.7%) belonged to the class of flavonoids, followed by phenolic acids (25%), lignans (2.2%), and other polyphenols (1.1%). Among the 92 compounds, the ten most abundant are presented (Figure 1).

In other studies that assessed the phenolic compound profiles of green and black tea kombuchas, the flavonoid class also emerged as the predominant one, followed by phenolic acids (Cardoso et al., 2020; de Noronha et al., 2022). Comparing the phenolic profile obtained here with other studies on green tea kombuchas, it is evident that there was some similarity in terms of the most abundant phenolics (Cardoso et al., 2020; Mizuta et al., 2020). As shown in (Fig. 1), epigallocatechin 3-O-gallate was the most abundant phenolic compound found, which also occurred in both green tea and black tea kombuchas according to Cardoso et al. (2020). The presence of catechin, epicatechin, epicatechin gallate, and epigallocatechin is even reported for kombucha analogs using oak (Vázquez-Cabral et al., 2017).

Generally, green tea kombucha exhibits a lower diversity and abundance of phenolic compounds compared to black tea kombucha (Cardoso et al., 2020). However, more than half of these compounds were found in both beverages (Cardoso et al., 2020). Fermentation is a process that promotes the diversification of phenolic compounds, as various biotransformations can occur due to the presence of microbial enzymes (de Noronha et al., 2022). Among them is the hydrolysis of high-molecular-weight flavonoids into lower-weight ones, contributing to an increase in phenolic acids over time (de Noronha et al., 2022; Jayabalan et al., 2007). Approximately one-third of the tea mass (dry basis) is composed of phenolic compounds, and optimizing this content through the fermentation process is valuable, given the well-known health benefits of these compounds (Dinh et al., 2019).

Gallocatechin gallate consumption improves the cognitive function of elderly rats and inhibits advanced glycation end products, helping combat diabetic complications (Ahn et al., 2022; Wu et al., 2020). Green tea catechins, especially epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), and gallic acid-3-gallate (GCG), controled B16F10 melanoma cells (Wu et al., 2020). Meanwhile, quercetin-3-O-rutinoside can prevent gastrointestinal injuries, oxidative stress, and inflammation (Dutta; Dahiya, 2023; Sharma et al., 2023). The rich phenolic composition of kombucha contributes to its bioactive potential.

3.5 Antibacterial properties

The green tea kombucha was inoculated at 40% after mixing the aliquot of kombucha with the culture medium and adding the tested microorganisms. The beverage inhibited the four tested pathogenic microorganisms (Table 1).

Among the analyzed microorganisms, kombucha was able to act more satisfactorily against Salmonella and, to a lesser extent, against E. coli, gram-negative bacteria. The neutralization of kombucha did not result in a significant decrease ($p > 0.05$) in its antimicrobial potential compared to kombucha at its normal pH, around 3.3. These results suggest that the antimicrobial activity of the beverage is not solely attributed to its low pH (presence of organic acids). One possible explanation for the antimicrobial mechanism of action is that during the fermentation process, microorganisms can generate compounds such as bacteriocins, proteins, enzymes, among others, capable of creating selective pressure in the environment and inhibiting the development of certain microorganisms (Al-Mohammadi et al., 2021; Barbosa et al., 2021; Bhattacharya et al., 2016). Additionally, compounds such as isorhamnetin and catechin have been previously identified as capable of increasing the permeability of microorganisms' membranes and consequently causing cellular dysfunction (Bhattacharya et al., 2018; 2016). In another study, kombucha prepared from lemon balm tea *(Melissa officinalis L.),* also neutralized with NaOH solution, demonstrated antimicrobial activity against gram-negative bacteria *E. coli* and *Salmonella sp*., but was not effective against *L. monocytogenes* (Velićanski, 2014).

3.6 *In vitro* bioactive properties of green tea kombucha

3.6.1 Antiproliferative properties of green tea kombucha

Cancer emerges as a primary contributor to global public health issues, causing the demise of countless individuals. It is estimated that one in every five individuals will be affected by this disease at some point in their lives (Sung et al., 2021). Lung cancer holds the second position among the most prevalent cancers, followed by colorectal cancer in third place. In Brazil, it is projected that more than 700 thousand new cancer cases will arise between 2023-2025 (INCA, 202). Due to the potential for numerous side effects associated with cancer treatment, there is a growing body of research dedicated to evaluating the therapeutic efficacy of natural products in combating this disease (Yang et al., 2019).

Green tea kombucha was tested against various cell types, including lung adenocarcinoma epithelial cells (A549), human colon carcinoma cells (HCT8), human liver cancer cells (HepG2), human umbilical vein endothelial cells (HUVEC), and normal human lung fibroblasts (IMR90). The results of these tests are presented in Figure 2.

The green tea kombucha showed a cytotoxic profile in all tested cancer cell lines, evidenced by growth inhibition (GI50), decreased cell viability (IC50), and lethal concentration (LC50). The A549 cell line appeared to be more sensitive to the effects of the extract. In line with this, we observed that the extract presented a selective index (A549 = 2.84; HCT8 = 1.74; HepG2 = 1.4, and HUVEC = 1.23) concerning the normal cell lineage, as IMR90 was not affected by treatment (IC50, IG50, and LC50 > 500 μg GAE/mL). When analyzed separately, the LC50 showed better effects for the Hep-G2 cell line at 424.4 μg/mL, followed by HCT8 at 431.2 μg/mL, while A549 required the highest kombucha concentration, 480.3 μg/mL, and IMR90 > 500 μg/mL to cause a lethal effect on the cells.

Cardoso et al. (2020) assessed the antiproliferative and cytotoxic potential of green and black tea kombuchas against HCT8 (ileocecal colorectal adenocarcinoma), CACO-2 (colorectal epithelial adenocarcinoma), using IMR90 cells as a control for healthy cells. It was found that, for all these, green tea kombucha was more efficient than black tea kombucha. This higher potential of green tea kombucha was attributed to the action of its phenolic compounds, which had previously been reported in the literature for their anticancer activities (Cardoso et al., 2020).

In our study, the cytotoxic potential obtained for IMR90 cells (IC50, GI50, and LC50 > 500 µg GAE/mL) was lower than that reported by Cardoso et al. (2020). According to the classification of the National Cancer Institute, a compound is considered non-toxic when it presents IC50 > 500 (Geran, 1972). Thus, it is possible to infer that green tea kombucha acted satisfactorily on cancer cells but without affecting healthy cells, confirming non-toxicity in the latter and an antiproliferative effect on the target cells (A549, HCT8, and HepG2). It is worth noting that the toxic effect of a compound on the cell, whether healthy or not, depends on the exposure time and the characteristics of that compound (Di Nunzio et al., 2017). Green, black, and oolong tea kombuchas were tested for their antiproliferative potential in colorectal cancer cells (CACO-2). Of these, only green tea kombucha and black tea kombucha showed high toxicity to CACO-2 cells and low toxicity to healthy cells (Kaewkod et al., 2019).

Some findings suggest that kombucha could act by inhibiting certain functions of cancer cells, such as IL-8, VEGF, COX-2, HIF-1α, MMP-2, and MMP-9, leading to apoptosis (Srihari et al., 2013). Among the phenolic compounds present in green tea kombucha, flavonoids were the most abundant class, comprising more than 70%. Therefore, understanding how they act as anticancer agents is crucial. Dietary flavonoids act pleiotropically in the tumor environment by controlling various pathways such as PI3K/Akt, NF-κB, JNK/STAT, p38/MAPK, and VEGF, thus preventing the development of tumor cells and the chance of metastasis (Duan et al., 2023). In general, the pro-oxidant action of bioactive compounds, especially phenolic compounds, is important to assist in cellular apoptosis (Cheng et al., 2020). The structure of the phenolic compound also affects the efficiency of cellular apoptosis; for example, the structure of pyrogallol in the B-ring is a key factor in its occurrence (Saeki et al., 2000). Although epigallocatechin gallate has the highest anticancer potential, it is known that the presence of others, such as epicatechin gallate, epigallocatechin, and epicatechin, can potentiate the bioactive effect, as their bioavailability in the body is increased (Cheng et al., 2020). A noteworthy point is the need for studies that create strategies to increase the bioavailability of these compounds through the use of nanotechnology, liposomes, among other emerging technologies, to improve their overall health effects (Almatroodi et al., 2020). Since kombucha contains phenolic compounds from tea, it is believed that the mechanisms by which tea acts can also be applied to a possible action of kombucha.

3.6.1.1 Target fishing analysis: Human Targets Related to Cancer Treatment

Most of the compounds (83.3%) were predicted to interact with ELAV-like protein 3 as a target. This target is a member of the ELAV-like protein family, playing an important role post-transcriptional process. The four isoforms of this family predominantly work positively regulating gene expression, and their dysregulation is related to different diseases, such as inflammation and cancer (Nasti et al., 2017). Additionally, 6-phosphogluconate (6PGD) dehydrogenase was predicted as a target for 58.3% of the compounds. This enzyme is capable of mediating biological functions, for example, redox homeostasis, metastasis, and proliferation of cells. The 6PGD enzyme is involved in the oxidative pentose phosphate pathway, and its overexpression is described in multiple cancers. The upregulation of this enzyme is already described in colorectal tumors, breast carcinoma, hepatocellular cancer, lung carcinoma, and others (Sarfraz et al., 2020).

Abnormal protein glycosylation is associated with malignant transformation in cells (Costa et al., 2020; Wang, 2005), and following this mechanism, two target groups were predicted by the target fishing approaches: the alpha-(1,3)-fucosyltransferase (FUT) family and sialyltransferases. The FUT family are enzymes responsible for synthesizing fucosylated oligosaccharides involved in interactions between cells (Cheng et al., 2013). Both FUT4 and FUT7 were predicted by 58.3% of the compounds, and these two promote neoplastic cell proliferation and hepatocellular carcinoma cell growth (Cheng et al., 2013). The same frequency was found for the CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase (SIAT6), an enzyme from the sialyltransferases family. This protein is expressed in different cancers (Sjöstedt et al., 2020; https://www.proteinatlas.org/ENSG00000126091-ST3GAL3), nevertheless it is not a well-established molecular target for small molecule drug design. Another frequent target (58.3%) was the phosphoglycerate mutase 1 (PGAM1), an enzyme involved in the glycolysis pathway catalyzing the conversion of 2-phosphoglycerate into 3-phosphoglycerate. PGAM1 is involved in the upregulation of the pentose phosphate pathway and is overexpressed in different cancers (Yang et al., 2022). Carbonic anhydrase (CA) protein family members were also predicted as targets for compounds (-)-epicatechin, (+)-catechin, and Quercetin 3-O-glucoside. They play important roles in

facilitating transport of carbon dioxide and protons in the intracellular space, across biological membranes and in the unstirred layers of the extracellular space (Potter; Harris, 2003), thus, controlling the pH in the tumor microenvironment (Mboge et al., 2018). From the twelve CA, active isoforms CA9 and CA12 are considered molecular targets for anticancer therapy (Mboge et al., 2018). The (-)-epicatechin (+)-catechin, and quercetin 3-O-glucoside displayed a Tanimoto coefficient equals to 1 against CA9 and CA12 isoforms, and they already have experimentally validated activity against these targets (Karioti et al., 2015, 2016). CA enzymes were already predicted to be potential targets of flavonoids and phenolic acids from sorghum flours with anticancer properties (Paes et al., 2024). The metabolites present in the kombucha display potential activity for different targets related to cytotoxicity activity, and some compounds have been previously described, highlighting the complexity of the sample composition. Furthermore, the minority compounds were not considered along with synergistic effects between the components. The complete description of the predicted targets is described in Table S2.

3.3.6 Measurement of Intracellular ROS

Reactive oxygen species (ROS) are generated by aerobic metabolism, and they are highly reactive against various biological targets. These molecules are associated with oxidative stress but can also serve as indicators of biological processes (Schieber; Chandel, 2014). In recent years, there has been increasing concern about oxidative stress and ways to mitigate it. In this context, phenolic compounds are molecules with high antioxidant potential due to their chemical structure containing aromatic rings. Consequently, they can transfer electrons to free radicals, inhibit the formation of ROS, and remain stable due to the resonance chemical process (Heleno et al., 2015; Jayabalan et al., 2008; Queiroz et al., 2017).

As kombucha is a beverage rich in phenolic compounds, it was evaluated for its ability to inhibit ROS generation. The kombucha phenolic extract demonstrated a protective and antioxidant effect against ROS induced by H_2O_2, reducing the levels of oxidation under basal conditions in both tested cell lines (Figure 3).

It can be observed that both HCT8 and HUVEC cells, when treated with 10 µg GAE/mL of green tea kombucha without the addition of hydrogen peroxide, showed protection against the generation of reactive oxygen species (ROS), as the treated cells

exhibited a lower concentration compared to cells under normal conditions. HCT8 cells treated with kombucha concentrations between 10 and 100 µg GAE/mL, despite the induction of ROS formation due to the presence of hydrogen peroxide, did not show a significant difference compared to the negative control. In contrast, in HUVEC cells, only at the highest concentration, in this case, 100 µg GAE/mL, there was no significant difference compared to the negative control. This demonstrates that kombucha was effective in combating the generation of reactive oxygen species in both cell types.

Bovine mammary cells treated with green tea showed a reduction in hydrogen peroxide-induced oxidative damage, attributed to the activation of pathways such as NFE2L2, HMOX1, and NQO1. This activation led to an increase in endogenous antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), resulting in a decrease in levels of malondialdehyde and reactive oxygen species (Ma et al., 2022). The NFE2L2 pathway is linked to the activation of antioxidant enzymes, playing a fundamental role in combating potential oxidative damage (Merry; Ristow, 2016). Thus, catechin can act directly as an antioxidant or, concurrently, by activating or increasing the activity of antioxidant enzymes (Nobari et al., 2021). Additionally, the presence of multiple compounds with antioxidant characteristics, such as phenolic compounds and some vitamins, enhances the effect on the elimination of ROS (Queiroz et al., 2017). The mechanism of ROS generation and combat is multifactorial, and green tea kombucha has demonstrated its ability to control ROS generation.

3.3.7 Effects of kombucha in the Protection of RBC against AAPH-induced Stress

The 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) is a highly unstable molecule capable of generating various free radicals. When these radicals interact with lipids present in the erythrocyte plasma membrane, they can lead to the failure of this membrane, causing hemolysis. Erythrocytes, in turn, are highly sensitive to oxidative damage and, at the same time, have a metabolism that is easily understood (Halliwell, 2012; Liao et al., 2014). As green tea kombucha contains phenolic compounds in its composition, it was tested for its ability to protect erythrocytes against potential damage caused by oxidative stress induced by AAPH. For all the parameters, an antioxidant effect of green tea kombucha extract was observed, as the levels of hemolysis and ROS

generation were reduced in a dose-dependent manner, and hemoglobin oxidation was decreased in all concentrations tested, conferring RBC protection (Figure 4). Quercetin was used as a standard to compare the protective effects of kombucha extracts against the effects of quercetin, a potent flavonoid with strong antioxidant properties.

It can be observed that, at a concentration of 150 μg GAE/mL, kombucha was able to inhibit the formation of ROS at values equivalent to the negative control, completely neutralizing the effects of AAPH on the cells. Green tea kombucha also acted preventively against hemolysis at lower concentrations (50 μg GAE/mL) without a significant difference compared to the negative control. Demonstrating that even with the stress induced by AAPH, the erythrocyte was able to reach values similar to a cell that was not exposed to this harmful molecule. In another study, at the same concentration of 50 μg/ml, both green tea extract and quince leaf extract were able to prevent hemolysis to the extent of matching the negative control (Costa et al., 2009). Flavonoids, by interacting with the erythrocyte membrane, make it more organized, preventing hemolysis. Additionally, the site of interaction between flavonoids and the membrane is crucial for their action to be greater or lesser (Chaudhuri et al., 2007; Zheng et al., 2022). In a simplified manner, dietary antioxidants can convert the peroxyl radical into another form that is non-reactive (Banerjee et al., 2008).

Regarding hemoglobin oxidation, the increase in green tea kombucha concentration did not cause a significant improvement in preventing this phenomenon (Figure 4). Das et al. (2020) pointed out that catechin can act directly on the heme group of hemoglobin, potentially oxidizing Fe (II) to Fe (III), exhibiting pro-oxidant action at higher doses. This fact may help explain why the increase in kombucha concentration did not improve hemoglobin oxidation. Similarly, the polyphenols from green and black tea showed a protective effect on red blood cells at concentrations of up to 10 μg/ml, and beyond this point, no significant observation was made (Grinberg et al., 1997). This protective effect on erythrocytes mediated by the action of green tea kombucha helps to explain the antimalarial activity that was verified for both sensitive (3D7) and non-sensitive (W2) strains to the chloroquine drug.

3.3.8 Antimalarial Activity

Malaria is a disease that affects millions of people worldwide, caused by *Plasmodium falciparum, P. vivax*, and *P. malariae,* which can infect the Anopheles mosquito (Boniface; Ferreira, 2019; Lima et al., 2015; Puttappa et al., 2017). The life cycle of *Plasmodium* encompasses phases in the Anopheles mosquito and the human host. In the intraerythrocytic cycle of *Plasmodium,* invasion, growth, and replication of erythrocytes occur. In the ring stage, *Plasmodium* is in its youngest form, which is associated with drug resistance, as these parasites can undergo a temporary growth arrest. Among commonly used medications, most target the parasite in later blood stages, not addressing the ring stage (Platon & Ménard, 2024). In Brazil, malaria is endemic in the Amazon region, and the resistance of some strains of *Plasmodium* to commonly used drugs is alarming (Lima et al., 2015). Consequently, studies have emerged to evaluate the efficacy of alternative plant-based treatments against malaria (Devi et al., 2023).

Kombucha green tea demonstrated toxicity against the W2 and 3D7 strains of *P. falciparum*, with the 3D7 strain showing greater sensitivity (IC50 = 4.2 µg GAE/mL) to treatment than W2 (IC50 = 26.7 µg GAE/mL), as illustrated in (Figure 5).

The results obtained were encouraging and better than those found in the study by (de Noronha et al., 2022), as the IC50 values obtained were lower. This suggests that a smaller amount of kombucha is needed to act against *P. falciparum*, depending on the IC50 values. Flavonoids can be classified from highly active to inactive based on IC50 values (Boniface; Ferreira, 2019). Following this classification, green tea kombucha for the W2 strain is highly active (IC50 <10), and for 3D7, it is moderately active (20 < IC50 <50).

The presence of phenolic compounds in black tea kombucha, notably catechin, quercetin, and epigallocatechin gallate, has been attributed to its ability to combat the spread of *P. falciparum* (de Noronha et al., 2022). Furthermore, an increase in their presence is directly proportional to a decrease in the concentration required to cause a 50% reduction in parasitemia (IC50) (do Carmo et al., 2020). It is believed that the action of black tea kombucha on *P. falciparum* involves the rupture of its membrane, affecting fatty acid synthesis, and impairing the action of proteases (de Noronha et al., 2022). Plasmodial lipids play a crucial role in signaling, subcellular transport, and even protein anchoring in the membrane, as changes in lipid structure can alter substrate hydrophobicity and prevent the binding of proteins essential for *Plasmodium* survival (Counihan et al., 2022). Among the mechanisms by which drugs act on *Plasmodium* are

the blockade of pyrimidine biosynthesis, reduction in mitochondrial membrane function, or even through the neutralization of the heme group (Platon & Ménard, 2024).

It is believed that the mechanism of action of catechin against *P. falciparum* is due to the generation of oxidative stress, leading to a redox imbalance and inhibiting its growth (Abdulah et al., 2017). Among flavonoids, for example, epigallocatechin gallate was able to reduce the ability of *Plasmodium* to adhere to the ICAM-1 receptor, essential for the development of cerebral malaria with a high mortality rate (Patil et al., 2011). A noteworthy point is that catechins EGCG and ECG, when administered together with artemisinin, one of the main drugs used in malaria treatment, intensify the drug's antiplasmodial action (Sannella et al., 2007). Since green tea kombucha contains several phenolic compounds, it is believed that the antimalarial effect is caused by synergy between different bioactive compounds through various pathways.

3.3.8.1 Target fishing analysis: Antimalarial Targets

Most compounds were predicted to interfere in the fatty acid biosynthesis of *Plasmodium sp* as inhibitors of 3-oxoacyl-acyl-carrier protein reductase (83.3%), beta-hydroxyacyl-ACP dehydratase (83.3%), 3-oxoacyl-[acyl-carrier-protein] reductase (58.3%), and enoyl-acyl-carrier protein reductase (58.3%). Specifically, (-)-epigallocatechin, and (+)-gallocatechin displayed a Tanimoto coefficient equal to 1 against the enoyl-acyl-carrier protein reductase suggesting that both compounds have already been reported in the literature with experimental activities against those targets. The first one is already described as an inhibitor of the plasmodial enoyl-acyl-carrier protein reductase along with other flavonoids (Tasdemir et al., 2006; Wang; Tian, 2001). Nevertheless, biological data for (+)-gallocatechin was not found; this could be related to the fingerprint used to measure the Tanimoto similarity not being sensitive to distinguish between (+)-gallocatechin and their optical isomers. Previously reported works also called attention to flavonoids present in kombucha samples and their correlation to the antimalarial activity. These findings reinforce the importance of flavonoids targeting the Plasmodium falciparum fatty acid biosynthesis to the antimalarial activity (de Noronha et al., 2022b). Enoyl reductase enzymes from Plasmodium were already predicted to be potential targets of flavonoids and phenolic acids from sorghum flours with anticancer properties (Paes et al., 2024) as well as other

Kombucha samples (de Noronha et al., 2022). Also, (-)-epicatechin and (+)-catechin displayed the maximum Tanimoto coefficient value (Tc=1) for the M18 aspartyl aminopeptidase (+)-gallocatechin inhibitors, and these compounds were already tested against this target (Wang et al., 2012). This enzyme is an exopeptidase, involved in the host's hemoglobin degradation (Rout; Mahapatra, 2019; Sivaraman et al., 2012). In this sense, the in vitro activity observed in the kombucha sample could be related to the parasite membrane biosynthesis disruption and in in vivo assays, the sample could have additional mechanisms of action. We also do not discard the influence of other compounds with minor concentrations that were not predicted by SEA webserver as well as possible synergistic effects within the kombucha constituents. The complete description of the predicted targets is described in Table S2.

4 Conclusions

Ninety-two phenolic compounds were found in green tea kombucha, with 70.7% being flavonoids and 25% phenolic acids. This rich phenolic profile contributes to the beverage's excellent antioxidant capacity. Green tea kombucha exhibited antibacterial activity against all tested strains. The phenolic compounds were largely responsible for the bioactive properties of the kombucha, which showed antimalarial activity against sensitive strains of *P. falciparum* (3D7) and strains not sensitive to chloroquine (W2). The beverage provided dose-dependent protection to erythrocytes, preventing their hemolysis even when subjected to 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), and reduced intracellular ROS formation. Additionally, the kombucha demonstrated antiproliferative activities against epithelial cells of adenocarcinoma (A549), human colon carcinoma cells (HCT8), human liver cancer cells (HepG2), and showed low toxicity to human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblasts (IMR90). Along with these bioactive properties, the green tea kombucha met all parameters of Brazilian regulation that outline the identity and quality criteria that kombucha produced in Brazil must meet.

Conflicts of Interest

The authors declare no conflict of interest.

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Tables

Table 1 - Antimicrobial inhibition percentage of neutralized and non-neutralized green tea kombucha

Amostra	***S. aureus***	***L. monocytogenes***	***Salmonella sp***	***E. coli***
Non-neutralized	56.82 ± 14.96[a]	54.24±19.43 [a]	72.82±17.34 [a]	41.98±1.13 [a]
Neutralized	34.37± 13.77 [a]	48.95±15.82 [a]	66.23±0.17 [a]	47.91±17.66 [a]

Equal letters indicate that there was no significant difference by the t-test ($p > 0.05$) between the original and the neutralized kombucha.

Figures

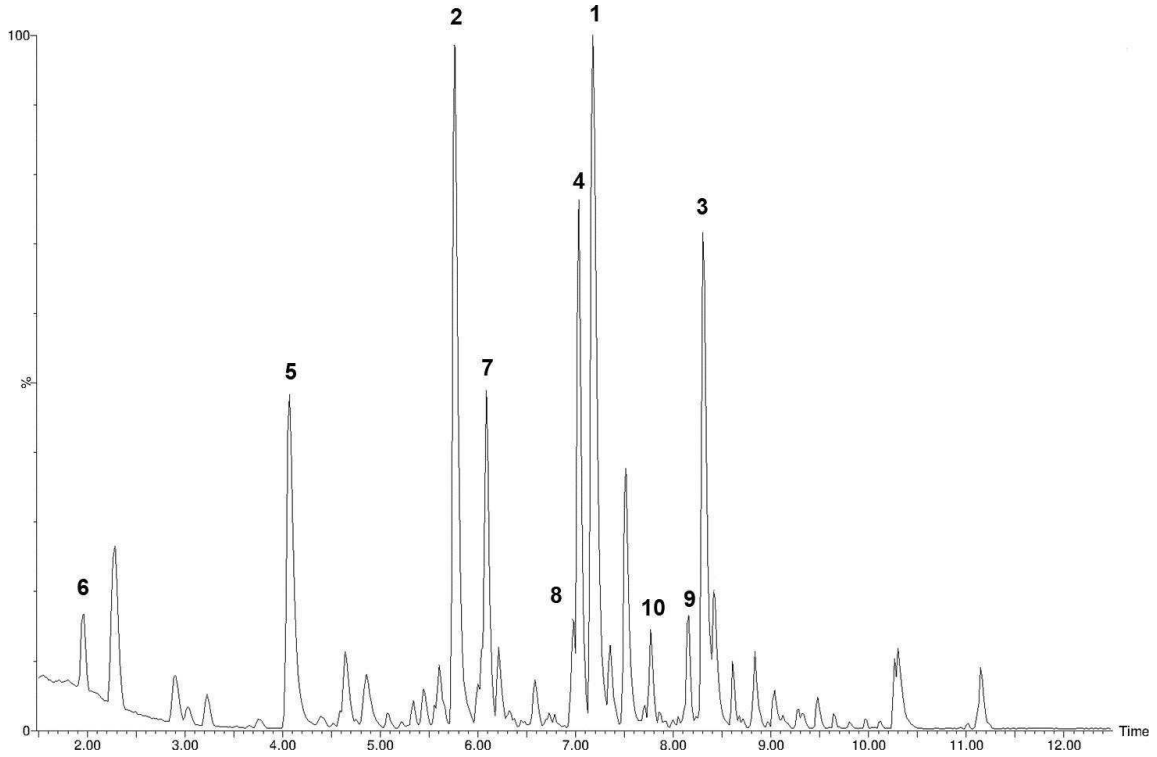

Figure 1 – Raw chromatogram of the ten most abundant phenolic compounds found in green tea kombucha
1 (+)-gallocatechin 3-O-gallate/(-)-epigallocatechin 3-O-gallate ; **2** (+)-gallocatechin ; **3** Catechin 5-O-gallate ; **4** (-)-epicatechin ; **5** (-)-epigallocatechin ; **6** 5-O-Galloylquinic acid ; **7** catechin; **8** 5-p-coumaroylquinic acid ; **9** Quercetin 3-O-rutinoside ; **10** Myricetin 3-O-glucoside

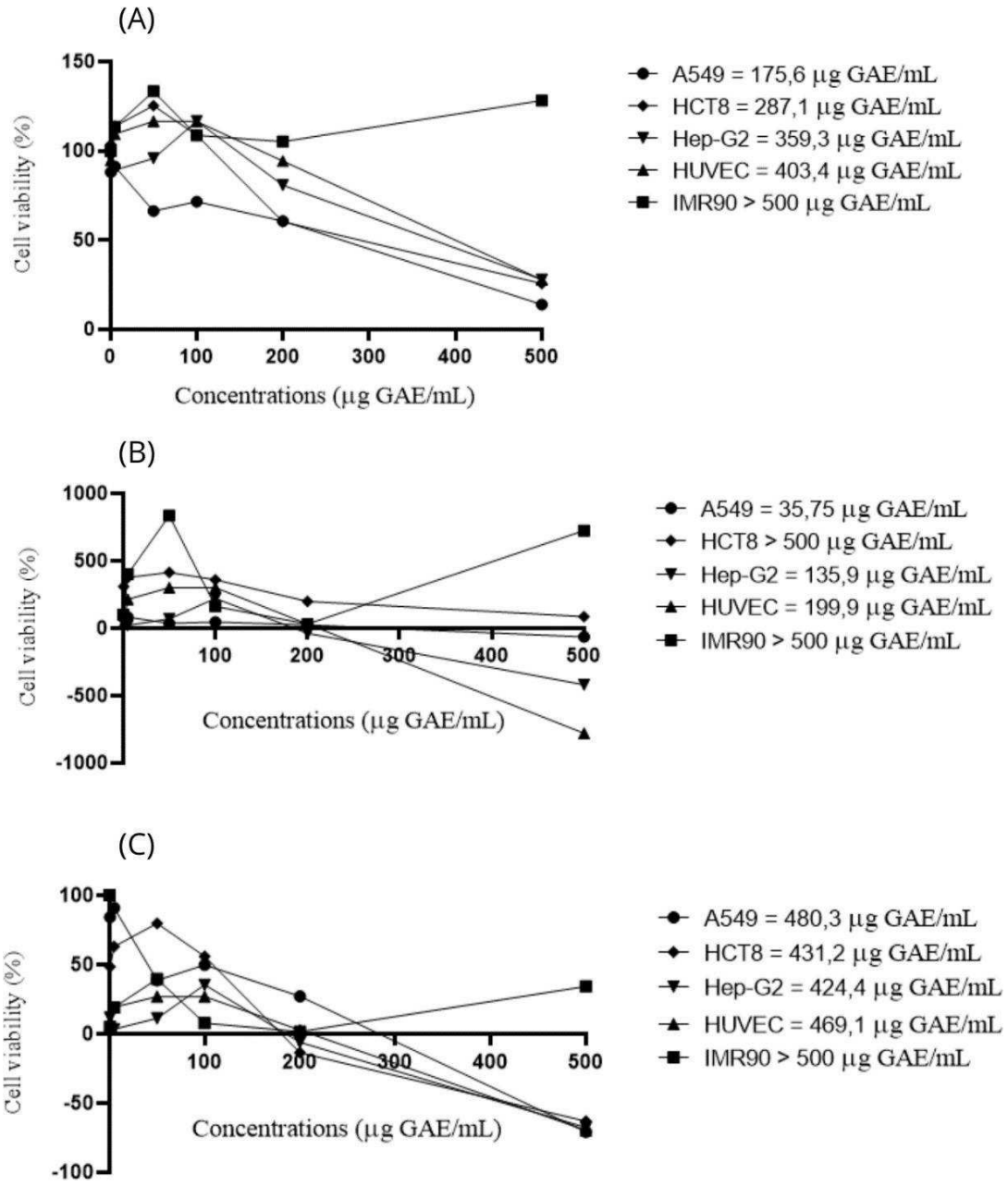

Figure 2 - (A) IC_{50} (50% cell viability inhibition); (B) GI_{50} (50% growth inhibition) and (C) LC_{50} (50% cell death) of green tea kombucha

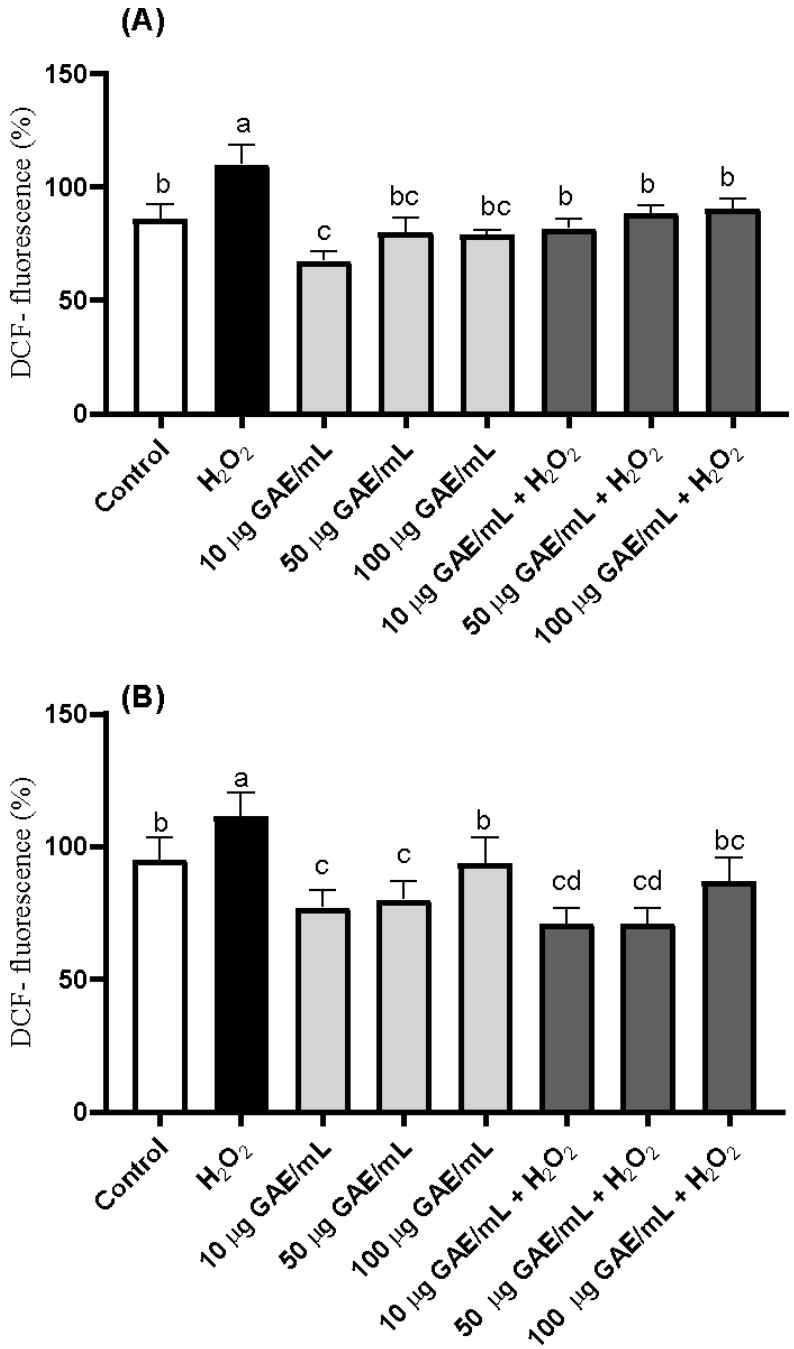

Figure 3 - Intracellular ROS measurement in HCT8 (A) and HUVEC (B) cells by spectrofluorimetry that received treatment with kombucha extract at 10, 50, and 100 µg/mL. Quantitative data are the mean ± standard deviation (n = 4). Different letters represent statistically significant differences ($p \leq 0.05$).

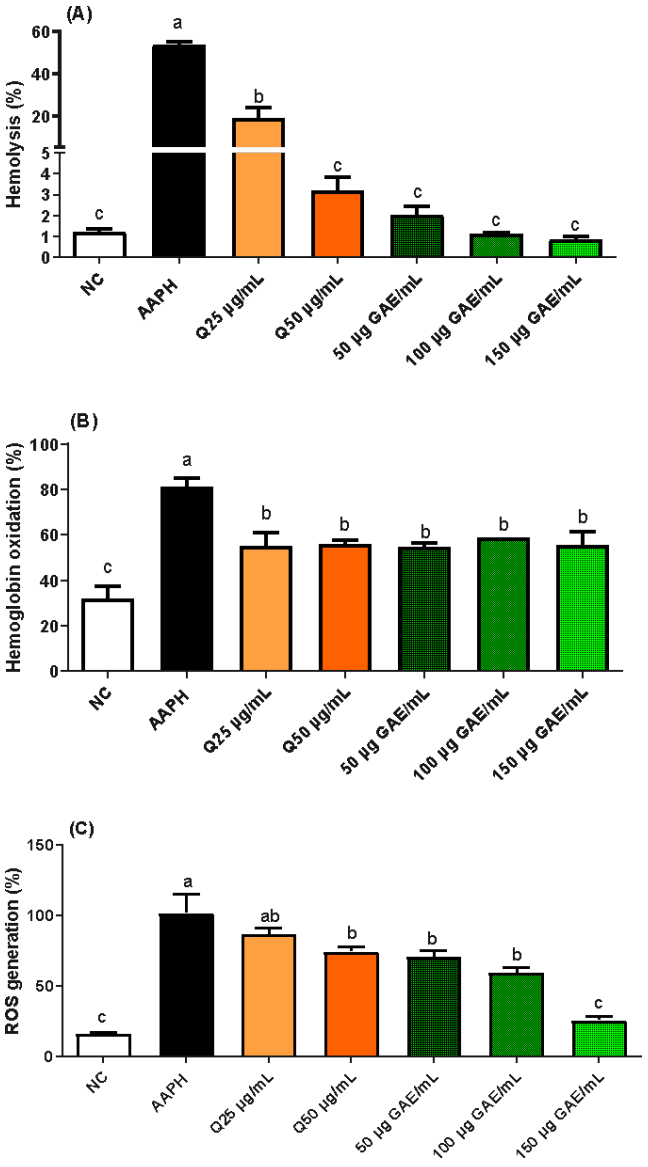

Figure 4 - (A) Effect of kombucha extract on blood hemolysis induced by2'-azobis(2-amidinopropane) dihydrochloride (AAPH); (B) Effect of kombucha extract on hemoglobin oxidation induced by AAPH and (C) Effect of kombucha extract on intracellular reactive oxygen species (ROS) induced by AAPH. Note: NC: negative control; AAPH: Positive control; Q: quercetin. Quantitative data are the mean ± standard deviation (n=4). Different letters represent statistically significant differences ($p \leq 0.05$).

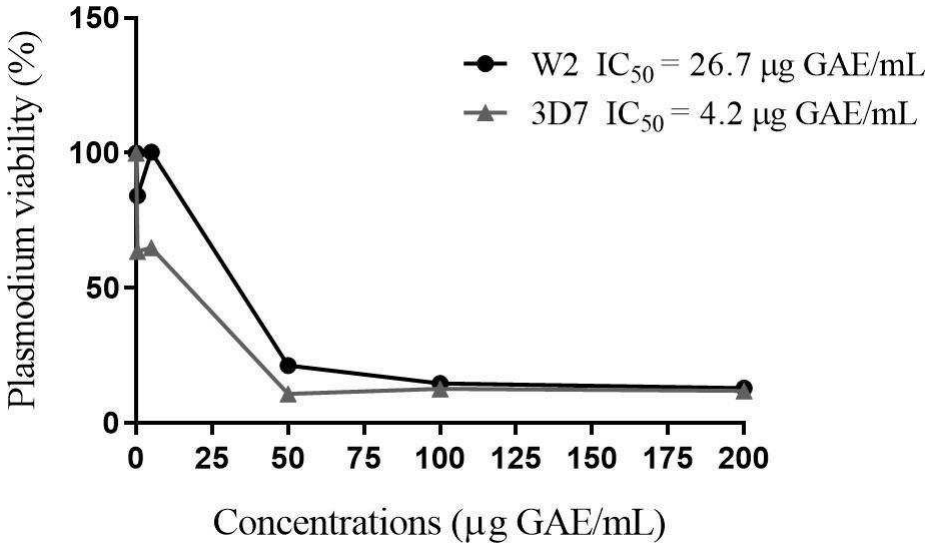

Figure 5 - Antiplasmodial activity and cytotoxicity of kombucha phenolic extract, against chloroquine resistant strain (W2) and chloroquine sensitive strain (3D7).

APPENDIX A

Table S1 - Phenolic compounds identified in green tea kombucha by UPLC– MS^E

Name of compound	Molecular formula	*m/z*	RT (min)	Score (%)	FS (%)	Error (ppm)	IS (%)	Class
Sesamolinol	C20H20O7	371,1169	0,55	39,0	15,9	8,86	88,91	L
Didymin/poncirin	C28H34O14	593,1912	0,73	35,5	0,0	6,11	84,60	F
1-Acetoxypinoresinol	C22H24O8	415,1437	1,26	36,7	0,0	9,37	93,57	L
Caffeic acid	**C9H8O4**	**179,0355**	**1,98**	**39,4**	**4,4**	**2,98**	**96,06**	**PA**
5-O-Galloylquinic acid	C14H16O10	343,0655	2,26	57,6	93,8	-4,65	99,53	PA
(-)-epigallocatechin	**C15H14O7**	**305,0649**	**4,05**	**52,0**	**67,2**	**-5,90**	**99,52**	**F**
Caffeoylquinic acid isomer 1	C16H18O9	353,0857	4,23	57,5	98,1	-6,06	96,39	PA
Caffeoylquinic acid isomer 2	C16H18O9	353,0860	4,64	57,7	94,9	-5,13	99,51	PA
Cyanidin 3-O-(6"-caffeoyl-glucoside)/delphinidin 3-O-(6"-p-coumaroyl-glucoside)	C30H27O14+	610,1295	4,67	36,4	0,0	-5,33	88,23	F
Caffeoylquinic acid isomer 3	C16H18O9	353,0856	5,57	37,8	0,0	-6,30	96,36	PA
Procyanidin dimer isomer 1	C30H26O12	577,1338	5,62	39,5	12,9	-2,30	87,47	F
(+)-gallocatechin	C15H14O7	305,0651	5,75	51,2	62,4	-5,23	99,47	F
p-coumaric acid 4-O-glucoside	C15H18O8	325,0904	5,80	36,6	0,0	-7,61	91,46	PA
Procyanidin dimer isomer 2	C30H26O12	577,1337	5,89	37,7	0,0	-2,47	91,54	F
Caffeoylquinic acid isomer 4	C16H18O9	353,0859	6,03	44,2	28,1	-5,24	98,97	PA
Feruloylquinic acid isomer	C17H20O9	367,1011	6,05	39,9	11,8	-6,45	94,94	PA
(+)-catechin	**C15H14O6**	**289,0700**	**6,07**	**46,0**	**37,6**	**-6,20**	**99,41**	**F**
7,3',4'-Trihydroxyflavanone	C15H12O5	271,0585	6,08	47,0	55,4	-9,94	90,65	F
Caffeoylquinic acid isomer 5	C16H18O9	353,0856	6,21	56,2	89,3	-6,20	98,64	PA
Eriodictyol 7-O-neohesperidoside	C27H32O15	595,1650	6,52	45,5	33,5	-3,10	97,69	F

Procyanidin dimer isomer 3	C30H26O12	577,1337	6,68	39,0	0,0	-2,58	97,92	F
Caffeoylquinic acid isomer 6	C16H18O9	353,0855	6,71	40,9	13,2	-6,52	98,62	PA
Feruloyl glucose	C16H20O9	355,1060	6,79	35,8	0,0	7,11	87,14	PA
Luteolin 7-O-glucuronide	C21H18O12	461,0708	6,82	40,1	18,9	-3,82	86,26	F
Chicoric acid	C22H18O12	473,0707	6,87	38,2	0,0	-3,90	95,77	PA
Eriodictyol 7-O-rutinoside	C27H32O15	595,1641	6,87	45,2	44,3	-4,53	87,13	F
5-p-coumaroylquinic acid	C16H18O8	337,0912	6,96	52,6	69,8	-4,98	99,18	PA
(-)-epicatechin	**C15H14O6**	**289,0701**	**7,01**	**47,0**	**42,5**	**-5,79**	**99,24**	**F**
Paeoniflorin	C23H28O11	479,1544	7,01	38,8	1,9	-3,14	95,84	PA
Pentahydroxyisoflavone isomer 1	C15H10O7	301,0325	7,07	40,2	22,1	-9,49	89,16	F
Eriodictyol 7-O-glucoside	C21H22O11	449,1063	7,07	37,0	2,9	-5,88	88,76	F
5,6,7-Trihydroxyflavone	C15H10O5	269,0432	7,16	48,1	58,1	-8,85	91,99	F
(+)-gallocatechin 3-O-gallate/(-)-epigallocatechin 3-O-gallate	C22H18O11	457,0766	7,16	53,6	73,1	-2,17	97,34	F
3-feruloylquinic acid	C17H20O9	367,1011	7,30	39,8	9,5	-6,30	96,82	F
Procyanidin trimer C1	C45H38O18	865,1970	7,37	37,4	2,4	-1,74	86,85	F
Apigenin 7-O-apiosyl-glucoside	C26H28O14	563,1396	7,53	49,2	51,1	-1,81	97,05	F
Procyanidin dimer isomer 4	C30H26O12	577,1296	7,59	33,9	0,0	-9,67	80,12	F
Flavonoid compound 1	C21H20O11	447,0912	7,61	55,0	84,1	-4,75	96,33	F
Quercetin 7,4'-O-diglucoside/quercetin 3,4'-O-diglucoside	C27H30O17	625,1397	7,62	57,3	93,6	-2,14	95,51	F
Myricetin 3-O-glucoside	C21H20O13	479,0823	7,77	57,6	91,6	-1,73	98,27	F
4,2',4',6'-tetrahydroxydihydrochalcone	C15H14O5	273,0747	7,78	38,6	5,4	-7,92	96,33	F
(+)-catechin 3-O-gallate	C22H18O10	441,0802	7,82	56,4	98,5	-5,66	90,12	F
Flavonoid compound 2	C27H30O17	625,1392	7,84	37,4	0,0	-2,85	90,25	F
Chrysoeriol 7-O-apiosyl-glucoside	C27H30O15	593,1502	7,85	57,1	90,8	-1,67	96,96	F
Quercetin 3-glucosyl(1-3)rhamnosyl(1-6)galactoside	C33H40O21	771,1983	7,91	39,1	0,0	-0,83	96,69	F
Naringenin 7-O-glucoside	C21H22O10	433,1115	7,98	41,9	27,0	-5,81	89,15	F

Oleuropein	C25H32O13	539,1750	7,98	36,0	0,4	-3,70	84,05	OP
Quercetin 3-O-rhamnosyl-rhamnosyl-glucoside	C33H40O20	755,2032	8,05	51,2	59,5	-1,08	97,99	F
Procyanidin dimer isomer 5	C27H30O14	577,1549	8,09	50,6	60,1	-2,32	95,43	F
Quercetin 3-O-rutinoside	**C27H30O16**	**609,1456**	**8,14**	**56,6**	**84,6**	**-0,79**	**99,10**	**F**
5,7,4'-Trihydroxyisoflavone 7-O-glucoside	C21H20O10	431,0966	8,19	49,0	51,3	-4,01	98,37	F
Dihydroquercetin 3-O-rhamnoside	C21H22O11	449,1062	8,21	54,1	97,2	-5,98	80,35	F
Apigenin 7-O-glucuronide	C21H18O11	445,0753	8,23	46,7	49,5	-5,27	90,14	F
Naringenin	C15H12O5	271,0588	8,30	47,7	49,6	-8,78	98,46	F
Catechin 5-O-gallate	C22H18O10	441,0817	8,30	54,3	74,4	-2,27	99,60	F
Ellagic acid	**C14H6O8**	**300,9962**	**8,32**	**37,8**	**0,0**	**-9,16**	**98,92**	**PA**
Quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer	C33H40O20	755,2024	8,34	47,3	42,2	-2,09	96,63	F
Quercetin 3-O-glucoside	C21H20O12	463,0870	8,37	58,3	94,8	-2,52	99,69	F
5,7,3',4'-Tetrahydroxyflavanone	C15H12O6	287,0534	8,46	35,6	0,0	-9,34	88,22	F
Isorhamnetin 3-O-glucoside	C22H22O12	477,1016	8,53	37,7	0,0	-4,81	94,06	F
Dicaffeoylquinic acid isomer 1	C25H24O12	515,1183	8,61	44,3	26,4	-2,34	98,14	PA
Luteolin 7-O-rutinoside	C27H30O15	593,1506	8,61	55,9	82,1	-1,05	98,47	F
Verbascoside	C29H36O15	623,1968	8,61	40,1	8,2	-2,20	94,94	PA
Phloridzin	C21H24O10	435,1274	8,64	36,5	0,0	-5,14	88,46	PA
Luteolin 7-O-glucoside	C21H20O11	447,0918	8,67	54,5	78,7	-3,23	97,44	F
Quercetin 3-O-arabinoside/quercetin 3-O-xyloside	C20H18O11	433,0759	8,69	48,5	50,2	-4,11	97,12	F
Quercetin 3-O-arabinoside/quercetin 3-O-xyloside isomer	C20H18O11	433,0758	8,80	49,7	56,3	-4,28	96,91	F
Dicaffeoylquinic acid isomer 2	C25H24O12	515,1182	8,80	58,1	95,0	-2,51	98,52	PA
Kaempferol 3-O-glucoside/kaempferol 3-O-galactoside	C21H20O11	447,0922	8,83	56,9	88,8	-2,53	98,66	F
Unidentified compound 1	C22H22O12	477,1013	8,89	35,8	0,0	-5,33	85,13	F

Pentahydroxyisoflavone isomer 2	C15H10O7	301,0329	8,94	40,9	20,6	-8,25	92,95	F
Dihydroquercetin	C15H12O7	303,0488	9,00	43,0	28,9	-7,32	94,48	F
5,7-Dihydroxyflavanone	C15H12O4	255,0639	9,04	46,9	54,1	-9,30	90,55	F
Caffeoylquinic acid isomer 7	C16H18O9	353,0853	9,04	47,2	55,3	-7,16	88,73	PA
Dicaffeoylquinic acid isomer 3	C25H24O12	515,1182	9,04	53,1	70,4	-2,53	97,87	PA
Rosmarinic acid	C18H16O8	359,0750	9,14	46,0	40,1	-6,23	96,90	PA
Luteolin 7-O-malonyl-glucoside	C24H22O14	533,0929	9,17	49,4	61,5	-1,52	87,45	F
6"-O-Acetylglycitin	C24H24O11	487,1228	9,23	37,0	0,0	-3,75	89,48	F
Tectoridin	C22H22O11	461,1065	9,25	56,1	98,6	-5,37	87,92	F
5,6,7,4'-Tetrahydroxyflavone	C15H10O6	285,0382	9,27	50,5	64,4	-7,88	96,85	F
Myricetin	**C15H10O8**	**317,0280**	**9,32**	**56,5**	**91,8**	**-7,05**	**98,50**	**F**
Apigenin 6-C-glucoside	C21H20O10	431,0957	9,43	37,2	0,0	-6,12	92,88	F
Quercetin	**C15H10O7**	**301,0329**	**9,48**	**49,3**	**57,3**	**-8,07**	**98,35**	**F**
Tetrahydroxyflavone isomer	C15H10O6	285,0382	9,71	55,1	95,2	-7,98	89,39	F
Theaflavin	C29H24O12	563,1182	9,94	35,8	0,0	-2,29	81,56	F
Unidentified compound 2	C18H18O6	329,1036	10,23	37,0	0,0	1,74	87,13	NI
Kaempferol	**C15H10O6**	**285,0383**	**10,26**	**46,9**	**43,1**	**-7,42**	**99,65**	**F**
Rhamnetin/isorhamnetin	C16H12O7	315,0487	10,34	37,0	0,0	-7,50	93,42	F
6-Hydroxydihydrodaidzein	C15H12O5	271,0588	10,94	56,1	95,2	-8,79	95,07	F
Apigenin	C15H10O5	269,0433	11,02	54,1	80,4	-8,21	98,96	F
Jaceosidin/3,7-dimethylquercetin	C17H14O7	329,0643	11,10	43,9	30,6	-7,16	96,79	F
Isotectorigenin	C16H12O6	299,0535	11,16	48,0	55,8	-8,81	93,74	F

m/z = mass/charge; RT= retention time; FS= fragmentation score; IS= isotope similarity; PA = phenolic acids; F= flonoids

Table S2 – Targets for prediction of compounds present in higher abundance in the tested kombucha sample

Compound	Target ID	P-Value	Max Tc	Cut Sum	Z-Score	Name	Description
(+)-gallocatechin 3-O-gallate	FUT4_HUMAN	2.85E-106	0.8542	0.8542	189.0372	FUT4	Alpha-(1,3)-fucosyltransferase 4
	Q8I2S7_PLAF7	2.85E-106	0.8542	0.8542	189.0372		3-oxoacyl-[acyl-carrier-protein] reductase
	Q965D6_PLAFA	2.16E-94	0.8542	3.1898	167.7088	fabG	3-oxoacyl-acyl-carrier protein reductase
	6PGD_HUMAN	7.16E-83	0.8542	3.1898	147.0269	PGD	6-phosphogluconate dehydrogenase, decarboxylating
	Q965D7_PLAFA	2.51E-78	0.8542	3.1898	138.8671	fabZ	Beta-hydroxyacyl-ACP dehydratase
	SIAT6_HUMAN	7.43E-68	0.8542	0.8542	120.0674	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
	ELAV3_HUMAN	2.65E-59	0.7885	1.5292	104.7133	ELAVL3	ELAV-like protein 3
	FUT7_HUMAN	5.30E-54	0.8542	1.2342	95.1971	FUT7	Alpha-(1,3)-fucosyltransferase 7
	PGAM1_HUMAN	3.05E-52	0.8542	0.8542	92.0371	PGAM1	Phosphoglycerate mutase 1
	T2R31_HUMAN	5.14E-48	0.3243	0.9464	84.4485	TAS2R31	Taste receptor type 2 member 31
	TPMT_HUMAN	9.89E-46	0.2951	1.1744	80.3483	TPMT	Thiopurine S-methyltransferase
	MDR1_HUMAN	6.29E-26	0.8542	20.5233	44.7939	ABCB1	ATP-dependent translocase ABCB1
	Q965D5_PLAFA	2.16E-25	0.8542	3.7283	43.8342	fabI	Enoyl-acyl-carrier protein reductase

	ANTR2_HUMAN	2.76E-20	0.3231	0.3231	34.666	ANTXR2	Anthrax toxin receptor 2
	PAI1_HUMAN	9.82E-19	0.4151	4.3193	31.8796	SERPINE1	Plasminogen activator inhibitor 1
	AMY1A_HUMAN	1.11E-16	0.3231	0.3231	28.6592	AMY1A	Alpha-amylase 1
(+)-gallocatechin	FUT4_HUMAN	1.18E-77	0.6222	0.6222	137.6617	FUT4	Alpha-(1,3)-fucosyltransferase 4
	Q8I2S7_PLAF7	1.18E-77	0.6222	0.6222	137.6617		3-oxoacyl-[acyl-carrier-protein] reductase
	Q965D6_PLAFA	2.24E-68	0.6222	2.3033	121.0021	fabG	3-oxoacyl-acyl-carrier protein reductase
	PLGF_HUMAN	6.57E-66	0.4717	1.3053	116.5733	PGF	Placenta growth factor
	6PGD_HUMAN	4.71E-60	0.6222	2.3033	106.0615	PGD	6-phosphogluconate dehydrogenase, Decarboxylating
	Q965D7_PLAFA	9.04E-57	0.6222	2.3033	100.1664	fabZ	Beta-hydroxyacyl-ACP dehydratase
	SIAT6_HUMAN	1.16E-49	0.6222	0.6222	87.4084	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
	ELAV3_HUMAN	1.89E-39	0.5294	1.0102	69.0718	ELAVL3	ELAV-like protein 3
	PGAM1_HUMAN	2.76E-38	0.6222	0.6222	66.9813	PGAM1	Phosphoglycerate mutase 1
	FUT7_HUMAN	1.24E-27	0.6222	0.6222	47.8562	FUT7	Alpha-(1,3)-fucosyltransferase 7
	Q965D5_PLAFA	1.46E-22	1	3.3033	38.7515	fabI	Enoyl-acyl-carrier protein reductase
	VEGFA_HUMAN	1.86E-21	0.4717	1.3053	36.7681	VEGFA	Vascular endothelial growth factor A

		BACE1_HUMAN	0.2858	1	4.9255	0.399	BACE1	Beta-secretase 1
catechin gallate	5-O-	Q965D6_PLAFA	3.28E-68	0.6316	2.2976	120.7056	fabG	3-oxoacyl-acyl-carrier protein reductase
		FUT4_HUMAN	1.06E-64	0.5172	0.5172	114.4085	FUT4	Alpha-(1,3)-fucosyltransferase 4
		Q8I2S7_PLAF7	1.06E-64	0.5172	0.5172	114.4085		3-oxoacyl-[acyl-carrier-protein] reductase
		PLGF_HUMAN	6.14E-62	0.4348	1.2256	109.4446	PGF	Placenta growth factor
		6PGD_HUMAN	6.57E-60	0.6316	2.2976	105.8014	PGD	6-phosphogluconate dehydrogenase, decarboxylating
		Q965D7_PLAFA	1.24E-56	0.6316	2.2976	99.9207	fabZ	Beta-hydroxyacyl-ACP dehydratase
		ELAV3_HUMAN	1.06E-41	0.6316	1.0691	73.1163	ELAVL3	ELAV-like protein 3
		SIAT6_HUMAN	1.98E-41	0.5172	0.5172	72.6266	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
		FUT7_HUMAN	2.28E-36	0.5172	0.8249	63.5382	FUT7	Alpha-(1,3)-fucosyltransferase 7
		PGAM1_HUMAN	5.72E-32	0.5172	0.5172	55.6407	PGAM1	Phosphoglycerate mutase 1
		VEGFA_HUMAN	3.43E-20	0.4348	1.2256	34.4957	VEGFA	Vascular endothelial growth factor A
		Q965D5_PLAFA	1.61E-19	0.6316	2.8467	33.2909	fabI	Enoyl-acyl-carrier protein reductase
		ANTR2_HUMAN	3.62E-18	0.2879	0.2879	30.8638	ANTXR2	Anthrax toxin receptor 2
		PAI1_HUMAN	2.22E-16	0.44	3.765	27.6655	SERPINE1	Plasminogen activator inhibitor 1

	T2R31_HUMAN	2.22E-16	0.3108	0.3108	27.5613	TAS2R31	Taste receptor type 2 member 31
(-)-epicatechin	PLGF_HUMAN	4.04E-80	0.5636	1.5903	142.0876	PGF	Placenta growth factor
	Q965D6_PLAFA	4.39E-72	0.6	2.4296	127.6597	fabG	3-oxoacyl-acyl-carrier protein reductase
	Q965D7_PLAFA	7.65E-60	0.6	2.4296	105.6829	fabZ	Beta-hydroxyacyl-ACP dehydratase
	FUT4_HUMAN	6.01E-59	0.4706	0.4706	104.0748	FUT4	Alpha-(1,3)-fucosyltransferase 4
	Q8I2S7_PLAF7	6.01E-59	0.4706	0.4706	104.0748		3-oxoacyl-[acyl-carrier-protein] reductase
	6PGD_HUMAN	6.99E-56	0.6	2.1412	98.5716	PGD	6-phosphogluconate dehydrogenase, decarboxylating
	SIAT6_HUMAN	9.02E-38	0.4706	0.4706	66.0576	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
	ELAV3_HUMAN	1.31E-37	0.6	0.9621	65.7679	ELAVL3	ELAV-like protein 3
	T2R31_HUMAN	1.51E-31	0.3125	0.6161	54.8847	TAS2R31	Taste receptor type 2 member 31
	PGAM1_HUMAN	3.67E-29	0.4706	0.4706	50.601	PGAM1	Phosphoglycerate mutase 1
	VEGFA_HUMAN	5.48E-26	0.5636	1.5903	44.9013	VEGFA	Vascular endothelial growth factor A
	Q965D5_PLAFA	1.19E-21	0.7368	3.1665	37.1157	fabI	Enoyl-acyl-carrier protein reductase
	FUT7_HUMAN	4.24E-21	0.4706	0.4706	36.1256	FUT7	Alpha-(1,3)-fucosyltransferase 7
	KLK2_HUMAN	1.15E-17	0.4407	0.4407	29.9636	KLK2	Kallikrein-2

	CAH3_HUMAN	2.41E-11	1	1.3333	18.6121	CA3	Carbonic anhydrase 3
	CAH4_HUMAN	1.25E-06	1	2.8998	10.1479	CA4	Carbonic anhydrase 4
	Q8I2J3_PLAF7	8.69E-06	1	1	8.6357		M18 aspartyl aminopeptidase
	CAH5B_HUMAN	1.06E-05	1	1.3333	8.4807	CA5B	Carbonic anhydrase 5B, mitochondrial
	CAH6_HUMAN	1.61E-05	1	1.3333	8.1556	CA6	Carbonic anhydrase 6
	CAH7_HUMAN	2.66E-05	1	2.8998	7.7649	CA7	Carbonic anhydrase 7
	CAH5A_HUMAN	7.47E-05	1	1.3333	6.9589	CA5A	Carbonic anhydrase 5A, mitochondrial
	PPBT_HUMAN	0.002625	1	1	4.1825	ALPL	Alkaline phosphatase, tissue-nonspecific isozyme
	CAH12_HUMAN	0.0786	1	2.8998	1.5013	CA12	Carbonic anhydrase 12
	CAH9_HUMAN	0.9643	1	1	-1.3888	CA9	Carbonic anhydrase 9
	CAH1_HUMAN	0.9836	1	1.3333	-1.5519	CA1	Carbonic anhydrase 1
	CAH2_HUMAN	0.9904	1	1.6218	-1.6482	CA2	Carbonic anhydrase 2
(-)-epigallocatechin	FUT4_HUMAN	1.18E-77	0.6222	0.6222	137.6617	FUT4	Alpha-(1,3)-fucosyltransferase 4
	Q8I2S7_PLAF7	1.18E-77	0.6222	0.6222	137.6617		3-oxoacyl-[acyl-carrier-protein] reductase
	Q965D6_PLAFA	2.24E-68	0.6222	2.3033	121.0021	fabG	3-oxoacyl-acyl-carrier protein reductase
	PLGF_HUMAN	6.57E-66	0.4717	1.3053	116.5733	PGF	Placenta growth factor

	6PGD_HUMAN	4.71E-60	0.6222	2.3033	106.0615	PGD	6-phosphogluconate dehydrogenase, decarboxylating
	Q965D7_PLAFA	9.04E-57	0.6222	2.3033	100.1664	fabZ	Beta-hydroxyacyl-ACP dehydratase
	SIAT6_HUMAN	1.16E-49	0.6222	0.6222	87.4084	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
	ELAV3_HUMAN	1.89E-39	0.5294	1.0102	69.0718	ELAVL3	ELAV-like protein 3
	PGAM1_HUMAN	2.76E-38	0.6222	0.6222	66.9813	PGAM1	Phosphoglycerate mutase 1
	FUT7_HUMAN	1.24E-27	0.6222	0.6222	47.8562	FUT7	Alpha-(1,3)-fucosyltransferase 7
	Q965D5_PLAFA	1.46E-22	1	3.3033	38.7515	fabI	Enoyl-acyl-carrier protein reductase
	VEGFA_HUMAN	1.86E-21	0.4717	1.3053	36.7681	VEGFA	Vascular endothelial growth factor A
	BACE1_HUMAN	0.2858	1	4.9255	0.399	BACE1	Beta-secretase 1
5-O-galloylquinic acid	FUT4_HUMAN	2.34E-49	0.3929	0.3929	86.8574	FUT4	Alpha-(1,3)-fucosyltransferase 4
	Q8I2S7_PLAF7	2.34E-49	0.3929	0.3929	86.8574		3-oxoacyl-[acyl-carrier-protein] reductase
	FUT7_HUMAN	3.02E-49	0.3929	1.1238	86.6597	FUT7	Alpha-(1,3)-fucosyltransferase 7
	Q965D6_PLAFA	5.21E-45	0.3929	1.507	79.053	fabG	3-oxoacyl-acyl-carrier protein reductase
	6PGD_HUMAN	1.47E-39	0.3929	1.507	69.2688	PGD	6-phosphogluconate dehydrogenase, decarboxylating
	Q965D7_PLAFA	2.07E-37	0.3929	1.507	65.4078	fabZ	Beta-hydroxyacyl-ACP dehydratase

	SIAT6_HUMAN	1.13E-31	0.3929	0.3929	55.1126	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
	ELAV3_HUMAN	1.25E-28	0.3667	0.7273	49.6477	ELAVL3	ELAV-like protein 3
	PGAM1_HUMAN	1.74E-24	0.3929	0.3929	42.2041	PGAM1	Phosphoglycerate mutase 1
	ANTR2_HUMAN	9.99E-23	0.3636	0.3636	39.0475	ANTXR2	Anthrax toxin receptor 2
	PAI1_HUMAN	7.31E-21	0.5238	4.8219	35.701	SERPINE1	Plasminogen activator inhibitor 1
	KPCL_HUMAN	5.45E-20	0.3134	5.3115	34.1349	PRKCH	Protein kinase C eta type
	AMY1A_HUMAN	5.81E-19	0.3636	0.3636	32.2896	AMY1A	Alpha-amylase 1
	KPCE_HUMAN	4.44E-16	0.3134	5.6171	27.1401	PRKCE	Protein kinase C epsilon type
(+)-catechin	PLGF_HUMAN	4.04E-80	0.5636	1.5903	142.0876	PGF	Placenta growth factor
	Q965D6_PLAFA	4.39E-72	0.6	2.4296	127.6597	fabG	3-oxoacyl-acyl-carrier protein reductase
	Q965D7_PLAFA	7.65E-60	0.6	2.4296	105.6829	fabZ	Beta-hydroxyacyl-ACP dehydratase
	FUT4_HUMAN	6.01E-59	0.4706	0.4706	104.0748	FUT4	Alpha-(1,3)-fucosyltransferase 4
	Q8I2S7_PLAF7	6.01E-59	0.4706	0.4706	104.0748		3-oxoacyl-[acyl-carrier-protein] reductase
	6PGD_HUMAN	6.99E-56	0.6	2.1412	98.5716	PGD	6-phosphogluconate dehydrogenase, decarboxylating
	SIAT6_HUMAN	9.02E-38	0.4706	0.4706	66.0576	ST3GAL3	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase

	ELAV3_HUMAN	1.31E-37	0.6	0.9621	65.7679	ELAVL3	ELAV-like protein 3
	T2R31_HUMAN	1.51E-31	0.3125	0.6161	54.8847	TAS2R31	Taste receptor type 2 member 31
	PGAM1_HUMAN	3.67E-29	0.4706	0.4706	50.601	PGAM1	Phosphoglycerate mutase 1
	VEGFA_HUMAN	5.48E-26	0.5636	1.5903	44.9013	VEGFA	Vascular endothelial growth factor A
	Q965D5_PLAFA	1.19E-21	0.7368	3.1665	37.1157	fabI	Enoyl-acyl-carrier protein reductase
	FUT7_HUMAN	4.24E-21	0.4706	0.4706	36.1256	FUT7	Alpha-(1,3)-fucosyltransferase 7
	KLK2_HUMAN	1.15E-17	0.4407	0.4407	29.9636	KLK2	Kallikrein-2
	CAH3_HUMAN	2.41E-11	1	1.3333	18.6121	CA3	Carbonic anhydrase 3
	CAH4_HUMAN	1.25E-06	1	2.8998	10.1479	CA4	Carbonic anhydrase 4
	Q8I2J3_PLAF7	8.69E-06	1	1	8.6357		M18 aspartyl aminopeptidase
	CAH5B_HUMAN	1.06E-05	1	1.3333	8.4807	CA5B	Carbonic anhydrase 5B, mitochondrial
	CAH6_HUMAN	1.61E-05	1	1.3333	8.1556	CA6	Carbonic anhydrase 6
	CAH7_HUMAN	2.66E-05	1	2.8998	7.7649	CA7	Carbonic anhydrase 7
	CAH5A_HUMAN	7.47E-05	1	1.3333	6.9589	CA5A	Carbonic anhydrase 5A, mitochondrial
	PPBT_HUMAN	0.002625	1	1	4.1825	ALPL	Alkaline phosphatase, tissue-nonspecific isozyme

	CAH12_HUMAN	0.0786	1	2.8998	1.5013	CA12	Carbonic anhydrase 12
	CAH9_HUMAN	0.9643	1	1	-1.3888	CA9	Carbonic anhydrase 9
	CAH1_HUMAN	0.9836	1	1.3333	-1.5519	CA1	Carbonic anhydrase 1
	CAH2_HUMAN	0.9904	1	1.6218	-1.6482	CA2	Carbonic anhydrase 2
5-p-coumaroylquinic acid	AK1BA_HUMAN	1.74E-24	0.6939	3.5922	42.2071	AKR1B10	Aldo-keto reductase family 1 member B10
	G6PT1_HUMAN	3.33E-16	0.5625	0.5625	27.3069	SLC37A4	Glucose-6-phosphate exchanger SLC37A4
quercetin 3-O-rutinoside	PDIA1_HUMAN	6.54E-73	1	7.0511	129.144	P4HB	Protein disulfide-isomerase
	Q965D7_PLAFA	3.08E-54	0.4921	2.1991	95.6212	fabZ	Beta-hydroxyacyl-ACP dehydratase
	ELAV3_HUMAN	9.44E-51	0.4921	1.3056	89.3607	ELAVL3	ELAV-like protein 3
	Q965D6_PLAFA	4.21E-49	0.4921	1.6465	86.4005	fabG	3-oxoacyl-acyl-carrier protein reductase
	S28A3_HUMAN	1.67E-38	0.3049	0.3049	67.3701	SLC28A3	Solute carrier family 28 member 3
	CD22_HUMAN	4.61E-33	0.2991	2.0355	57.6033	CD22	B-cell receptor CD22
	IL2_HUMAN	2.13E-31	0.48	1.8188	54.6155	IL2	Interleukin-2
	NMUR2_HUMAN	3.94E-30	1	1	52.3409	NMUR2	Neuromedin-U receptor 2
	XDH_HUMAN	3.38E-26	0.6571	5.6584	45.2777	XDH	Xanthine dehydrogenase/oxidase
	CP1B1_HUMAN	3.03E-21	0.4921	4.7234	36.3876	CYP1B1	Cytochrome P450 1B1

	3MG_HUMAN	1.08E-18	0.3582	0.3582	31.8038	MPG	DNA-3-methyladenine glycosylase
	NOX4_HUMAN	1.99E-18	0.75	3.1144	31.3291	NOX4	NADPH oxidase 4
	AMYP_HUMAN	4.88E-18	0.2857	0.2857	30.6299	AMY2A	Pancreatic alpha-amylase
	KS6A3_HUMAN	7.31E-18	0.6567	10.0592	30.3146	RPS6KA3	Ribosomal protein S6 kinase alpha-3
	CBS_HUMAN	1.13E-17	0.4878	0.4878	29.9729	CBS	Cystathionine beta-synthase
	ADA2C_HUMAN	0.001543	1	1.75	4.597	ADRA2C	Alpha-2C adrenergic receptor
	ADA2A_HUMAN	0.1002	1	1	1.3028	ADRA2A	Alpha-2A adrenergic receptor
	ACES_HUMAN	0.1968	1	2.6882	0.7336	ACHE	Acetylcholinesterase
myricetin 3-O-glucoside	PDIA1_HUMAN	8.60E-63	0.8214	6.0638	110.9775	P4HB	Protein disulfide-isomerase
	Q965D7_PLAFA	9.68E-57	0.5283	2.3021	100.1135	fabZ	Beta-hydroxyacyl-ACP dehydratase
	S28A3_HUMAN	5.93E-51	0.4058	0.4058	89.7236	SLC28A3	Solute carrier family 28 member 3
	ELAV3_HUMAN	1.48E-50	0.5283	1.3005	89.0092	ELAVL3	ELAV-like protein 3
	Q965D6_PLAFA	1.85E-45	0.4237	1.5224	79.8606	fabG	3-oxoacyl-acyl-carrier protein reductase
	TYRO_HUMAN	6.82E-36	0.4286	3.6371	62.6841	TYR	Tyrosinase
	SC5A2_HUMAN	8.94E-36	0.4203	29.7347	62.4733	SLC5A2	Sodium/glucose cotransporter 2

	SC5A1_HUMAN	1.54E-32	0.4203	22.5688	56.6627	SLC5A1	Sodium/glucose cotransporter 1
	IL2_HUMAN	3.88E-31	0.5303	1.8034	54.1487	IL2	Interleukin-2
	XDH_HUMAN	2.07E-26	0.6667	5.7055	45.6626	XDH	Xanthine dehydrogenase/oxidase
	CP1B1_HUMAN	5.16E-21	0.5283	4.6709	35.9727	CYP1B1	Cytochrome P450 1B1
	3MG_HUMAN	1.33E-20	0.3966	0.3966	35.2358	MPG	DNA-3-methyladenine glycosylase
	NMUR2_HUMAN	6.54E-19	0.6176	0.6176	32.1971	NMUR2	Neuromedin-U receptor 2
	B4GT1_HUMAN	3.81E-18	0.2875	0.2875	30.8228	B4GALT1	Beta-1,4-galactosyltransferase 1
	FGF1_HUMAN	3.07E-17	0.3188	0.6009	29.1949	FGF1	Fibroblast growth factor 1
	KS6A3_HUMAN	4.15E-17	0.6833	9.6316	28.9614	RPS6KA3	Ribosomal protein S6 kinase alpha-3
	Q965D5_PLAFA	4.44E-16	0.5283	2.3319	27.1349	fabI	Enoyl-acyl-carrier protein reductase
	CBS_HUMAN	6.66E-16	0.4359	0.4359	26.7501	CBS	Cystathionine beta-synthase
quercetin 3-O-glucoside	PDIA1_HUMAN	3.90E-69	1	6.6827	122.3652	P4HB	Protein disulfide-isomerase
	Q965D7_PLAFA	4.14E-60	0.5357	2.4406	106.1621	fabZ	Beta-hydroxyacyl-ACP dehydratase
	ELAV3_HUMAN	7.33E-54	0.5357	1.3869	94.9439	ELAVL3	ELAV-like protein 3
	Q965D6_PLAFA	8.04E-53	0.5357	1.7732	93.0768	fabG	3-oxoacyl-acyl-carrier protein reductase

S28A3_HUMAN	5.32E-52	0.4143	0.4143	91.6039	SLC28A3	Solute carrier family 28 member 3
SC5A2_HUMAN	8.71E-44	0.4286	36.3822	76.8562	SLC5A2	Sodium/glucose cotransporter 2
IL2_HUMAN	4.25E-39	0.6349	2.2753	68.4384	IL2	Interleukin-2
TYRO_HUMAN	1.83E-38	0.4375	3.9023	67.3004	TYR	Tyrosinase
SC5A1_HUMAN	2.22E-36	0.4384	25.2358	63.5591	SLC5A1	Sodium/glucose cotransporter 1
XDH_HUMAN	2.54E-28	0.5507	6.1257	49.0936	XDH	Xanthine dehydrogenase/oxidase
CD22_HUMAN	5.78E-28	0.2929	1.7147	48.4507	CD22	B-cell receptor CD22
CBS_HUMAN	1.05E-26	0.4615	0.749	46.1927	CBS	Cystathionine beta-synthase
NMUR2_HUMAN	8.54E-23	0.75	0.75	39.1699	NMUR2	Neuromedin-U receptor 2
CP1B1_HUMAN	1.23E-22	0.5357	5.0395	38.8856	CYP1B1	Cytochrome P450 1B1
NOX4_HUMAN	4.16E-21	1	3.5805	36.1397	NOX4	NADPH oxidase 4
3MG_HUMAN	6.05E-20	0.3833	0.3833	34.0527	MPG	DNA-3-methyladenine glycosylase
KS6A3_HUMAN	4.78E-18	0.7167	10.1637	30.6454	RPS6KA3	Ribosomal protein S6 kinase alpha-3
FGF1_HUMAN	9.38E-18	0.3286	0.6197	30.1204	FGF1	Fibroblast growth factor 1
B4GT1_HUMAN	1.01E-17	0.2805	0.2805	30.0653	B4GALT1	Beta-1,4-galactosyltransferase 1

	CAH4_HUMAN	3.52E-17	1	7.6766	29.0892	CA4	Carbonic anhydrase 4
	PPAC_HUMAN	4.44E-16	0.3824	1.8989	27.0793	ACP1	Low molecular weight phosphotyrosine protein phosphatase
	CAH7_HUMAN	4.44E-16	1	8.9187	27.0234	CA7	Carbonic anhydrase 7
	AL1B1_HUMAN	5.55E-16	0.4783	0.4783	26.8837	ALDH1B1	Aldehyde dehydrogenase X, mitochondrial
	PPBI_HUMAN	6.99E-15	1	2.5278	24.9615	ALPI	Intestinal-type alkaline phosphatase
	ALDR_HUMAN	1.89E-14	1	7.6646	24.1888	AKR1B1	Aldo-keto reductase family 1 member B1
	CAH12_HUMAN	1.46E-11	1	15.9216	19.0056	CA12	Carbonic anhydrase 12
	CAH2_HUMAN	4.98E-05	1	12.0189	7.2754	CA2	Carbonic anhydrase 2
	ADA2C_HUMAN	0.001543	1	1.75	4.597	ADRA2C	Alpha-2C adrenergic receptor
caffeoylquinic acid isomer 4	SDF1_HUMAN	2.29E-49	0.3636	2.701	86.8737	CXCL12	Stromal cell-derived factor 1
	NR0B2_HUMAN	1.28E-48	0.3433	0.9585	85.5353	NR0B2	Nuclear receptor subfamily 0 group B member 2
	MYOC_HUMAN	1.85E-30	0.3774	0.3774	52.9304	MYOC	Myocilin
	AK1BA_HUMAN	1.28E-23	0.9111	3.4619	40.649	AKR1B10	Aldo-keto reductase family 1 member B10
	A4_HUMAN	3.29E-19	0.8333	13.0068	32.7322	APP	Amyloid-beta precursor protein

CAPÍTULO III

Artigo II

Artigo submetido na revista Desafios da Universidade Federal do Tocantins

Kombucha de chá verde mantém o padrão de identidade e qualidade sob estocagem refrigerada por até 120 dias

Green tea kombucha maintains its identity and quality standards under refrigerated storage for up to 120 days

El kombucha de té verde mantiene su identidad y estándares de calidad bajo almacenamiento refrigerado por hasta 120 días.

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RESUMO

A kombucha é uma bebida fermentada com microrganismos viáveis durante o armazenamento. Avaliar a estabilidade da kombucha de chá verde refrigerada é crucial para garantir qualidade e conformidade regulatória. Este estudo investigou uma kombucha de chá verde produzida no Brasil, armazenada a 4 °C por 120 dias. Ao longo desse período, a acidez total (0,197 - 0,261 g/100 mL), pH (3,41 a 3,32), ácido acético e fenólicos totais mantiveram-se estáveis, enquanto a acidez volátil aumentou para 37,62 mEq/L. A sacarose reduziu de 22,99 para 11,57 g/L, e as populações de bactérias lácticas (7,29 para 5,63 log UFC/mL), bactérias acéticas (7,02 para 5,09 log UFC/mL) e leveduras (7,17 a 5,42 log UFC/mL) diminuíram, o que foi atribuído à alta acidez, compostos tóxicos, limitação de oxigênio e baixa temperatura. A kombucha manteve suas propriedades originais, conforme regulamentações brasileiras para pH, etanol e acidez volátil. Estudos sensoriais são sugeridos para avaliar o impacto dessas mudanças na percepção dos consumidores.

Palavras-chave: vida de prateleira; compostos fenólicos; estabilidade

Abstract

Kombucha is a fermented beverage containing viable microorganisms during storage. Assessing the stability of refrigerated green tea kombucha is crucial to ensure quality and regulatory compliance. This study investigated green tea kombucha produced in Brazil, stored at 4°C for 120 days. Over this period, total acidity (0.197 - 0.261 g/100 mL), pH (3.41 to 3.32), acetic acid, and total phenolics remained stable, while volatile acidity increased to 37.62 mEq/L. Sucrose decreased from 22.99 to 11.57 g/L, and populations of lactic acid bacteria (7.29 to 5.63 log CFU/mL), acetic acid bacteria (7.02 to 5.09 log CFU/mL), and yeast (7.17 to 5.42 log CFU/mL) declined, attributed to high acidity, toxic compounds, oxygen limitation, and low temperature. The kombucha retained its original properties, complying with Brazilian regulations for pH, ethanol, and volatile acidity. Sensory studies are recommended to assess how these changes affect consumer perception.

Keywords: shelf life; phenolic compounds; stability

RESUMEN

La kombucha es una bebida fermentada que contiene microorganismos viables durante el almacenamiento. Evaluar la estabilidad de la kombucha de té verde refrigerada es crucial para garantizar la calidad y el cumplimiento regulatorio. Este estudio investigó la kombucha de té verde producida en Brasil, almacenada a 4°C durante 120 días. Durante este período, la acidez total (0,197 - 0,261 g/100 mL), el pH (3,41 a 3,32), el ácido acético y los fenoles totales se mantuvieron estables, mientras que la acidez volátil aumentó a 37,62 mEq/L. La sacarosa disminuyó de 22,99 a 11,57 g/L, y las poblaciones de bacterias lácticas (7,29 a 5,63 log UFC/mL), bacterias acéticas (7,02 a 5,09 log UFC/mL) y levaduras (7,17 a 5,42 log UFC/mL) disminuyeron, lo cual se atribuyó a la alta acidez, compuestos tóxicos, limitación de oxígeno y baja temperatura. La kombucha mantuvo sus propiedades originales, cumpliendo con las regulaciones brasileñas para pH, etanol y acidez volátil. Se recomiendan estudios sensoriales para evaluar cómo estos cambios afectan la percepción del consumidor.

Palabras clave: vida útil; compuestos fenólicos; estabilidade

1 INTRODUCTION

Kombucha is a beverage obtained through the fermentation of the sugary must resulting from the infusion or extract of *Camellia sinensis* by a symbiotic consortium of bacteria and yeast known as SCOBY (BRASIL, 2019; JAYABALAN; MARIMUTHUB.; SWAMINATHAN, 2007). Kombucha has a diverse composition, including acetic, lactic, gluconic, glucuronic acids, ethanol, some minerals and vitamins, carbon dioxide, volatile compounds, and other (CARDOSO et al., 2020; JAYABALAN; MARIMUTHUB.; SWAMINATHAN, 2007). This rich composition, especially regarding the phenolic compounds, is responsible for the health benefits associated with regular consumption of the beverage (TRAN et al., 2020). Kombucha presents antimicrobial, anticancer, antimalarial, anti-inflammatory activities, modulation of intestinal microbiota, combating oxidative stress, among others (CARDOSO et al., 2020, 2021; COSTA et al., 2022; DE NORONHA et al., 2022).

The kombucha market has been rapidly expanding in various countries, and it is expected to reach 4.26 billion dollars by 2028 (MORDOR INTELLIGENCE, 2023). In Brazil, a similar trend is observed, with growing consumption and various brands of kombucha available. This growth led to the publication of Normative Instruction 41, which establishes the standard of identity and quality for the beverages produced in Brazil (BRASIL, 2019). A produção de kombuchas dentro dos padrões estabelecidos é essencial para sua comercialização, definindo também a vida de prateleira da bebida. Entretanto, a manutenção das características é um desafio, especialmente considerando que kombucha contains variable microorganisms that can remain viable over time, potentially leading to changes in the composition of the beverage throughout its shelf life (FU et al., 2014). Thus, the objective of this study was to evaluate changes in physicochemical properties, total phenolics content and microbial count of green tea kombucha stored at 4 ºC for 120 days, simulating storage until consumption.

2 Methodology

2.1 Kombucha production

Green tea *(Camellia sinensis)* leaves cultivated in Brazil were purchased from Amaya brand grown in the city of Registro, São Paulo and used for kombucha production, as described by Cardoso et al. (2020), with some modifications. Infusion time and temperature follow the recommendations by the manufacturer (Amaya): 70 °C for one minute. Before fermentation, a starter tea from a previous batch of kombucha was added, ensuring that the initial pH remained between 4.2 and 4.4.

2.2 Fermentation

Forty-five liters of kombucha (fifteen liters for fermenters) was produced in plastic fermenters with a capacity of 20 L and kept in an incubator (BOD) at 25 ºC during 5 days. Three independent batches were performed. At the end of fermentation the SCOBY was removed and the beverages were tested for microbiological counts, pH, and total acidity. Additional aliquots were collected and transferred to tubes, centrifuged at

10,000 rpm (Eppendorf 5420 centrifuge) for 10 min, and stored at -18 °C for analyses of total phenolics, volatile acidity, sugars, ethanol and acetic acid content.

2.3 Shelf-life evaluation

Samples of 250 mL from each kombucha container (R1, R2, and R3), after 5 days fermentation, were aliquoted in 250 mL PET bottles previously sanitized with a peracetic acid solution at 2 ppm. The bottles containing the kombucha were labelled and stored in a refrigerator at 4 ºC. At 0, 7, 14, 21, 28, 60, 90, and 120 days of storage, one bottle of each replicate was left to defrost at room temperature and the content was analyzed for volatile acidity, ethanol, sugars, acid acetic and phenolic compounds.

2.4 Kombucha characterization

2.4.1 Total and volatile acidity

Total and volatile acidity were determined by titration, according to the methodology of the IAL (2008), and the result was expressed as % (w/v) acetic acid and meq/L of acetic acid, respectively (IAL, 2008).

2.4.2 Determination of sugars, ethanol and acetic acid

The quantification of sucrose, fructose and glucose, ethanol and organic acids were carried out using a High-Performance Liquid Chromatograph (HPLC), SHIMADZU, according to the methodology of Siegfried et al. (1984), with modifications. The HPX87H column (BIO-RAD) and pre-column of the same phase were used, oven temperature: 32 ºC; flow rate: 0.6 mL. min.-1 and for the mobile phase H2SO4 was used at 5 mM. The obtained results were expressed in g/L.

2.4.3 Total phenolics

The concentration of total phenolics was determined using the Folin-Ciocalteau colorimetric method, with gallic acid as standard (SINGLETON; ROSSI, 1965). The

absorbance was measured at 760 nm and the results were expressed as mg of gallic acid equivalent per mL of kombucha (mg GAE/mL).

2.5 Microbiological characterization

Aliquots of the kombucha samples were serially diluted and plated on GYC (Glucose,Yeast and CaCO3) agar (Merck, Germany) for acetic acid bacteria counting; MRS (Man-Rogosa and Sharpe) agar (Merck, Germany) supplemented with bromocresol indicator (0.004%) for counting of lactic acid bacteria; and PDA (potato dextrose agar, Merck, Germany) for yeast counting. Plates were incubated at 30 °C for 3 days under aerobic conditions, except for. Lactic acid bacteria, incubated under microaerophilic conditions. In this case, colonies identified as lactic acid bacteria (yellow - acid producers), were confirmed by catalase and Gram tests. All results were expressed in CFU/mL.

2.6 Statistical analyses

The data obtained from the analyses were tabulated using the means of three replicates for each shelf-life period. Outlier tests were initially conducted, followed by checks for normality and homoscedasticity. Subsequently, ANOVA was performed, and when necessary (in the case of significant ANOVA), regression analysis was carried out, fitting both linear and nonlinear models. The R software, version 4.3.1, was employed for the statistical data analysis.

3 Results and discussion

The kombucha produced from green tea cultivated in Brazil exhibited a composition in accordance with the quality required by Brazilian legislation (Table 1).

Table 1 - Composition of the kombucha produced during 5 days from green tea cultivated in Brazil, with infusion at 70 ºC for one minute.

Components	**Concentration**	**Range recommended in Legislation (IN 41)***
Ph	3.41 ± 0.09	2.5 - 4.2
Total acidity (g/100 mL acetic acid)	0.197 ± 0.020	Does not apply
Volatile acidity (meq/L acetic acid)	36.05 ± 0.60	30 – 130
Sucrose (g/L)	22.99 ± 1.90	Does not apply
Glucose (g/L)	11.49 ± 0.86	Does not apply
Fructose (g/L)	12.57 ± 2.16	Does not apply
Acetic acid (g/L)	3.43 ± 1.10	Does not apply
Ethanol (g/L)	4.70 ± 0.11	Maximum 5.0

*BRASIL, 2019.

During the 5-day fermentation period, the pH decreased from 4.3 ± 0.1 to 3.41 ± 0.09. Standardization of the initial pH helped ensure that, even after 5 days of fermentation, the pH remained at 3.41, making the beverage more palatable.

The total acidity of the beverages reached 0.2 ± 0.02% (w/v) acetic acid, primarily attributed to the production of organic acids by SCOBY bacteria (CARDOSO et al., 2020; JAYABALAN; MARIMUTHUB.; SWAMINATHAN, 2007). In kombucha, this parameter is mainly composed of volatile acidity, which significantly influences the sensory quality of the beverage (SPENCE, 2021). Moreover, it is directly related to the conversion of sugars into acetic acid by acetic bacteria, as well as other organic acids such as lactic, gluconic, citric, among others whose concentration changes over the course of fermentation (JAYABALAN; MARIMUTHUB.; SWAMINATHAN, 2007).

The volatile acidity of the produced kombucha reached 36.05 ± 0.6 meq/L acetic acid, or 0.31% acetic acid, while the concentration of acetic acid was 3.12 ± 1.1 g/L (Table 1). The volatile acidity of kombucha may depend on the time and temperature of fermentation, as well as the microorganisms present in the SCOBY and even the type of tea used. In another study, green tea kombucha was kept fermenting at 25°C for 14 days, and its volatile acidity after 4 days of fermentation was 126.4 mEq/L, close to the maximum limit established by Brazilian legislation. By the end of 14 days, it reached 384

mEq/L (DARTORA et al., 2023a). In this case, the high volatile acidity content may negatively impact the perception of flavor and aroma of the beverage (SPENCE, 2021). Therefore, it is important to properly monitor and control it within established limits.

Ethanol was present at only 4.8 g/L, confirming that it was a non-alcoholic beverage, according to the classification established by normative instruction 41 (BRAZIL, 2019). The production of non-alcoholic kombuchas in Brazil (with ethanol concentration < 5 g/L), and particularly maintaining this condition during storage, has been a challenge for producers, as the yeasts present in the SCOBY convert sugars into ethanol. Ethanol can also be produced by the transformation of acetic acid generated by lactic bacteria, as some strains of heterofermentative lactic bacteria can produce it (CARDOSO et al., 2020). An example of this would be the abundance of more than 60% among lactic bacteria of *Oenococcus oeni*, a heterofermentative lactic bacteria that was present in both green and black tea kombucha (COTON et al., 2017).

The wort used in the production of kombucha in this work was added with 50 g/L of sucrose, of which 44.28% (22.24 ± 1.9 g/L) remained unconsumed during fermentation, along with 11.49 ± 0.86 g/L and 12.57 ± 2.16 g/L of glucose and fructose, respectively. These residual sugars contribute to the sensory profile of the beverage (JAYABALAN et al., 2008; DARTORA et al., 2023b), and can also be metabolized by the microorganisms present in kombucha during storage, thus limiting the shelf life of the product.

3.1 Evaluation of pH, total acidity, volatile acidity and acetic acid during refrigerated storage of kombucha

Maintaining quality parameters throughout the shelf life of food products is essential for their commercialization, especially to ensure their safety for consumption, as microbial and enzymatic activity may continue to occur during storage, influencing the production of metabolites and even physical-chemical reactions (Morales-de la Peña et al., 2023). The pH and total acidity of the green tea kombucha produced in this study remained constant ($p > 0.05$) during 120 days of storage under refrigeration (Figure 1).

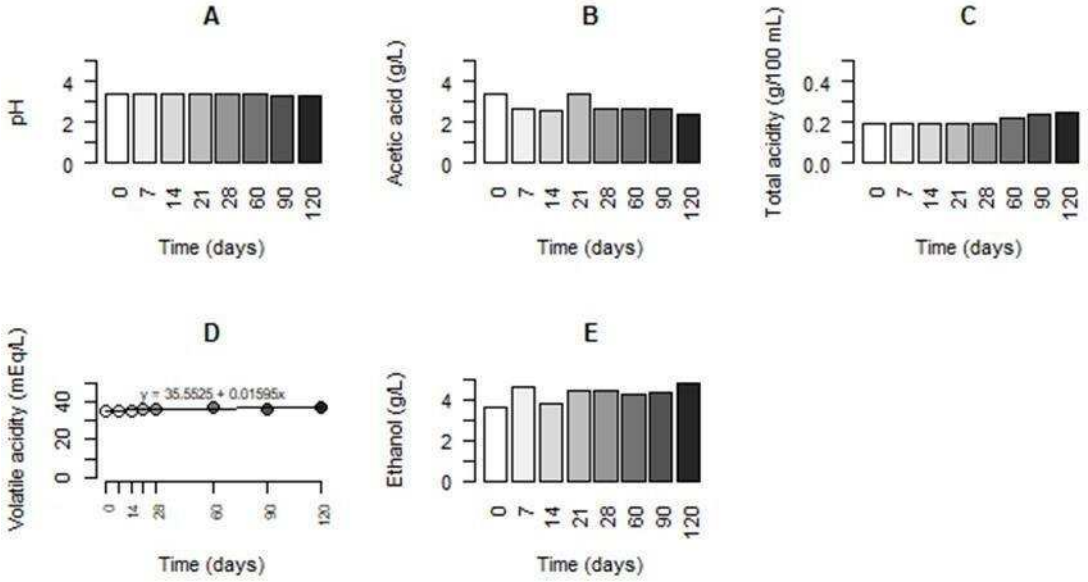

Figure 1 - Changes in pH (A), acetic acid (B), total acidity (C), volatile acidity (D) and ethanol (D) during the shelf-life.Bar charts (A, B, C, and E) showed no significant difference ($p > 0.05$) in the evaluated parameters. . Source: The authors

On the other hand, volatile acidity increased ($p \leq 0.05$) (Figure 1D), although it remained within the limits established by Brazilian regulations (30 - 130 mEq/L) (BRASIL, 2019). Storing green tea kombuchas added with aromatic herbs did not cause an increase in the beverages' pH, even when stored at room temperature. In this case, the pH remained in the range of 3.28-3.46 for 90 days (GRASSI et al., 2022).

Controlling volatile acidity is essential to ensure beverage quality, as excess can result in flavor or aroma issues, or even cause health problems for consumers (SPENCE, 2021; NUMMER, 2013). Although acetic acid is the main organic acid found in kombuchas, volatile acidity may consist of other substances such as carbonic acid, lactic acid, ethyl acetate, among others (WANG et al., 2023). This may help explain why volatile acidity increased over the analyzed period, even though total titratable acidity, pH, and acetic acid concentration remained stable.

3.2 Ethanol, glucose, fructose and sucrose concentration in kombuchas stored at refrigeration under 120 days

The ethanol concentration did not vary significantly (Figure 1E) ($p > 0.05$) during the storage of kombucha under refrigeration; thus, the beverage remained non-alcoholic (BRASIL, 2019). Controlling the ethanol content of kombuchas during storage is a

concern for producers; for example, pregnant women and children should not consume alcoholic beverages. The control of this parameter is hindered by the presence, in kombucha, of residual fermentable substrates from the fermentation process, especially sucrose, glucose, and fructose, and by the ability of some microorganisms to remain viable even under storage conditions. A noteworthy point is that some species of acetic acid bacteria may be capable of oxidizing ethanol, forming acetaldehyde, and this can be converted to acetic acid, which may help explain the maintenance of ethanol content (BUENO et al., 2021). In this work, despite the presence of residual substrates and active microorganisms (Table 1), storage of the beverages under refrigeration prevented the conversion of sugars into high ethanol levels for at least 120 days. Commercial kombuchas were evaluated over 60 days at two different temperatures, 4 and 22 ºC, and it was found that storage under refrigeration considerably reduced the alcoholic content compared to 22 ºC (TALEBI et al., 2017). Therefore, in logistics chains based on refrigerated conservation, strict temperature control throughout the chain is essential to ensure the maintenance of the quality of the products sold. However, refrigerated storage of the beverages was not able to prevent the transformation of residual sugars into organic acids by the present microorganisms (Table 2).

Table 2: - Composition of sugars of the green tea kombucha

Time (days)	Sucrose (g/L)	Fructose (g/L)	Glucose (g/L)
0	22.24 ± 1.90	12.71 ± 2.16	11.99 ± 0.87
7	20.94 ± 3.69	10.19 ± 0.50	13.43 ± 0.85
14	20.39 ± 3.97	10.65 ± 0.97	14.86 ± 0.87
21	19.35 ± 2.93	10.73 ± 1.31	14.63 ± 1.96
28	19.27 ± 1.47	12.13 ± 1.85	16.82 ± 1.82
60	15.21 ± 2.86	13.11 ± 0.59	18.07 ± 0.89
90	12.04 ± 4.23	13.27 ± 1.17	17.69 ± 1.91
120	9.31 ± 4.38	13.18 ± 2.70	17.71 ± 4.18

The sucrose concentration decreased by almost 50%, reaching 11.57 g/L ($p \leq 0.05$). For example, graviola kombuchas stored at 25 ºC and 4 ºC for 21 days also showed a significant reduction in sucrose for both storage conditions (TAN et al., 2020). Possibly, this conversion led to the production of organic acids different from acetic acid, justifying the observed increase in volatile acidity. Lactic acid bacteria, for example, can remain

viable at storage temperatures, and the lactic acid content tends to increase over time (PUTRI, SETIANI, WARYA, 2020). On the other hand, as also observed in the study of Tan et al. (2020), the glucose concentration increased over time, especially in the first 60 days, as a result of the hydrolysis of sucrose by bacterial and yeast enzymes (DUFRESNE; FARNWORTH, 2000). The maintenance of lower fructose concentrations compared to glucose may occur because, in an anaerobic or microaerophilic environment, heterofermentative lactic acid bacteria are predominant in the SCOBY and can use fructose as the final electron acceptor (CARDOSO et al., 2020; ORTIZ et al., 2013). The change in sugar concentrations (Table 2) during refrigerated storage of kombuchas may have a significant role in the acceptance of this product, leading to consumer rejection. Therefore, the increase in volatile acidity, despite maintaining values within the limits set by regulations, when associated with lower sucrose concentrations, should be evaluated in the sensory context for determining the shelf life of these beverages.

3.3 Stability of phenolic compounds throughout the shelf-life

The concentration of phenolic compounds in the beverage remained stable ($p > 0.05$) throughout the entire period of storage, what may be related to the anaerobic environment in the bottles, preventing the oxidation of those molecules (TRAN et al., 2022). Black tea kombucha stored at 4 ºC stability in total phenolics in the first 4 months, and from that point on, the phenolic content declined significantly (LA TORRE et al., 2021). Furthermore, the stability of phenolic compounds throughout the shelf life is very important, as they are directly related to the bioactive properties of the beverage (LA TORRE et al., 2021; TAN et al., 2020).

3.4 Changes in microbiological counting throughout storage time

A reduction in microbial populations was observed for all groups analyzed in this study (Figure 2).

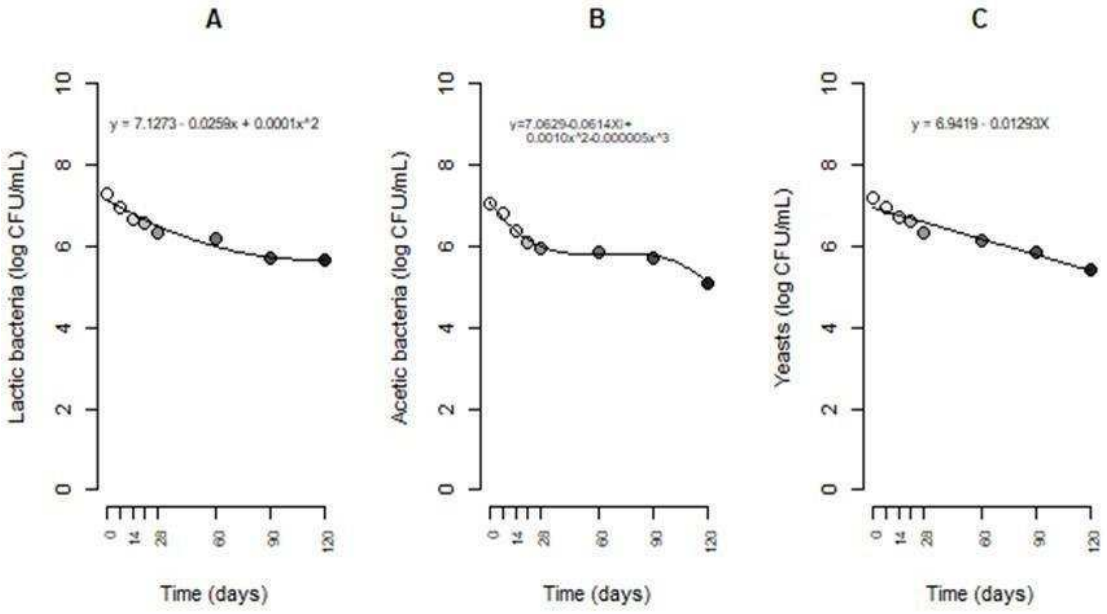

Figure 2 - Changes in lactic bacteria (A), acetic bacteria (B) and yeasts (C) during the shelf-life. Source: The authors

Yeasts exhibited the slowest reduction rate, decreasing by 1 logarithmic cycle after 60 days (Figure 2C). Although the anaerobic environment may favor yeast activity (BUENO et al., 2021; TRAN et al., 2022), it alone was not sufficient to prevent the observed population decline. In the absence or limited presence of oxygen, ethanol is synthesized from acetaldehyde, resulting in two moles of ATP. However, this process is not entirely efficient for cells (Puligundla et al., 2011), potentially causing a reduction in growth and cellular viability of this group of microorganisms over time.

For lactic acid bacteria, the same 1 logarithmic cycle reduction was observed after 28 days (Figure 2A). In another study, green tea kombucha added with other herbs, for example, did not exhibit detectable lactic acid bacteria by plate count after 20 days of storage at 4 ºC (GRASSI et al., 2022). However, the addition of these other herbs in kombucha preparation and the use of a different storage container, in that case, amber glass bottles, may have influenced the observed differences. The loss of viability of these populations may be attributed to adverse environmental conditions and the secretion of inhibitory substances, such as bacteriocins and reuterins (MANI-LÓPEZ, PALOU, LÓPEZ-MALO, 2014).

For acetic acid bacteria, after 28 days, the population had the greatest decline, decreasing by 1 logarithmic cycle (Figure 2B). An important point to note is that all the oxygen present in the bottle's headspace can be consumed within the

first 6 hours after bottling (TRAN; 2022). Since acetic acid bacteria are strictly aerobic, this lack of oxygen may explain the highest population reduction among the microorganisms evaluated over the 120 days.

It is worth noting that the viability patterns of lactic acid bacteria, acetic acid bacteria, and yeasts vary from one study to another, especially when dealing with complex fermentations involving a wide variety of microorganisms, different culture media used in the analyses, and even due to the type of packaging in which the beverage was stored (CABELLO-OLMO et al., 2020; LACERDA et al., 2022; TAN; MUHIALDIN; HUSSIN, 2020).

4 Conclusion

During 120 days of refrigerated storage (4 ºC), green tea kombucha remained within Brazilian standards for pH, volatile acidity, and ethanol content. A positive aspect observed was the stability of the phenolic compounds content over the 120 days, which is important to ensure the bioactive properties of the beverage. Brazilian green tea has proven to be promising for kombucha production. However, further studies on sensory properties during storage are recommended to determine if the changes may negatively impact consumer perception.

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Authors’ Contributions

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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CONCLUSÃO GERAL

Neste estudo, foi realizada uma completa caracterização físico-química, microbiológica, perfil de fenólicos, capacidade antioxidante e avaliação das propriedades bioativas da kombucha de chá verde brasileiro. Assim, foram identificados 92 compostos fenólicos (70.7% flavonóides, 25% ácidos fenólicos, 2.2% lignanas, e 1.1% outros polifenóis), que ajudam a explicar a boa capacidade antioxidante da bebida e propriedades bioativas verificadas. A kombucha de chá verde exerceu atividade antibacteriana, antimalárica, antitumoral, inibiu a geração de ERO intracelular e protegeu os eritrócitos contra estresse oxidativo induzido por AAPH. Para complementar esses achados, a kombucha se manteve dentro do preconizado pela legislação brasileira em termos de pH, acidez volátil e teor alcóolico para ser considerada como uma bebida não alcóolica.

Paralelamente a isso, esta mesma bebida foi avaliada ao longo de um período de 120 dias mantida sob refrigeração a 4ºC, simulando o modo de armazenamento das kombuchas comerciais. No estudo de acompanhamento da vida de prateleira ao longo dos 120 dias, a bebida continuou atendendo aos pré-requisitos em termos de legislação com relação ao pH, acidez volátil e teor de etanol. Além disso, seu conteúdo de fenólicos totais se manteve estável, o que é muito bom pois está diretamente relacionado com as propriedades bioativas da kombucha de chá verde brasileiro.

De forma geral, a utilização do chá verde nacional mostrou-se promissora como ingrediente para fabricação da kombucha, tanto em termos técnicos ao seguir o preconizado pela legislação brasileira, quanto ao mesmo tempo, sendo capaz de desempenhar boas propriedades bioativas. Os resultados obtidos sugerem que o consumo regular da bebida proposta neste estudo pode trazer benefícios à saúde dos indivíduos que a consumirem regularmente.