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MOLECULAR CHARACTERIZATION OF THE MICROBIOTA AND PHYSICAL-CHEMICAL EVALUATION OF THE FERMENTATIVE PROCESS OF KOMBUCHA

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COSME DAMIÃO BARBOSA

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COSME DAMIÃO BARBOSA

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“ Abrir o ângulo, fechar o foco sobre a vida
Transcender pela lente do amor.”

(Lente do amor, Gilberto Gil)

“No caminho dessa cidade passarás por Turmalina
Sonharás com Pedra Azul, viverás em Diamantina.”

(Itamarandiba, Milton Nascimento)

ABSTRACT

Kombucha is an Asian beverage obtained by the alcoholic and acetic fermentation of *Camellia sinensis*. Aimed characterize the microbial diversity and the physical-chemical parameters of the fermentative process of Kombucha. The analysis of the microbiota revealed low diversity of yeast and acetic bacteria species. *Zygosaccharomyces (Z. bailii)* and *Komagataeibacter (K rhaeticus, K, hansenii, K. intermedius, K. europaues, and K. xylinus)* are the main genera prevalent in Kombucha, respectively. The physical-chemical parameters analysis showed similar results for black and green tea kombucha fermentation. The composition of the phenolics was different between the black tea and green tea kombucha samples, and they influenced the formation of the groups through the analysis of principal components. The use of chemometric tools and data fusion strategy proved to be feasible for differentiating between the two types of Kombucha. Both kombuchas analyzed, under natural and filtered conditions, were effective in inhibiting different *E. coli* serotypes. The present work provides relevant contributions regarding the composition of the microbiota and the formation of compounds during the fermentation of black tea and green tea for Kombucha's production. From the results obtained in this study regarding the kombucha microbiota, it was possible to know the microbial species' predominance. Also, recognizing important compounds presents in the substrates with the potential to bring health benefits.

Key-words: *Camellia sinensis*, acetic acid bacteria, yeast, phenols.

RESUMO

Kombucha é uma bebida asiática obtida pela fermentação alcoólica e acética de *Camellia sinensis*. Objetivou a caracterização da diversidade microbiana e avaliação físico-química do processo fermentativo de kombucha. A análise da microbiota revelou baixa diversidade de espécies de leveduras e bactérias acéticas. *Zygosaccharomyces (Z. bailii)* e *Komagataeibacter (K rhaeticus, K, hansenii, K. intermedius, K. europaues, and K. xylinus)* são os principais gêneros predominantes em Kombucha, respectivamente. A análise dos parâmetros físico-químicos demonstrou similaridade nas fermentações de kombucha de chá preto e chá verde. A composição dos fenólicos foi distinta entre as amostras de kombucha de chá preto e chá verde, e exerceram influência na formação dos grupos por meio da análise de componentes principais. A utilização de ferramentas quimiométricas e estratégia de fusão de dados mostrou-se viável para diferenciação entre os dois tipos de kombucha. Ambas as kombuchas analisadas, nas condições natural e filtrada, foram eficazes na inibição de diferentes sorotipos de *E. coli*. O presente trabalho fornece relevantes contribuições em relação a composição da microbiota, bem como da formação de compostos durante a fermentação de chá preto e chá verde para produção de kombucha. A partir dos resultados obtidos neste estudo referentes a microbiota de kombucha, foi possível ter o conhecimento sobre a predominância de espécies. Além disso, reconhecer compostos importantes presentes no substrato com potencial em trazer benefícios à saúde.

Palavras-chave: *Camellia sinensis*, bactérias ácido acéticas, leveduras, fenólicos.

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INTRODUCTION

Kombucha is a fermented beverage globally consumed since its emergence in the region of China and introduction in the European and American continents (DUFRESNE; FARNWORTH, 2000; JARRELL et al., 2000; JAYABALAN et al., 2014; POTHAKOS et al., 2016; COTON et al., 2017). The consumption of this beverage is due to the claims of its functional properties (GREENWALT el., 1998; SREERAMULU et al., 2000; CHUN; CHEN, 2006; BHATTACHARYA et al., 2011; ABSHENA et al., 2012; BHATTACHARYA et al., 2013; JAYABALAN et al., 2014; BHATTACHARYA et al., 2016; VITAS et al., 2018).

Kombucha fermentation is a spontaneous and complex process. Several factors may influence the microbial and physical-chemical composition. (JAYABALAN et al., 2014; MARSH et al., 2014; VITAS et al.; 2018; DE FILIPPIS et al., 2018). The inoculum's microbiota composition has been studied by dependent and independent cultivate techniques (TEOH et al., 2004, MARSH, et al., 2014; CHAKROVROTY et al., 2016; COTON et al., 2017; ARIKAN et al., 2020; VILLARREAL-SOTO et al., 2020).

The research of microorganisms has deepened since the 19th century with the emergence of in vitro culture techniques (ROSSELLO; AMANN, 2001). The growth of microorganisms in synthetic nutritive media enabled the morphological, biochemical, and physiological characterization of the organisms (KAMPFER; GLAESER, 2012). The frequent use of these characteristics allowed the emergence of numerical taxonomy, which grouped microorganisms by similarity based on the analysis of a large number of morphological and metabolic data (ROSSELLO; AMANN, 2001).

After the middle of the twentieth century the discovery of DNA and the development of new analytical techniques allowed the fusion of genetic and morphological characteristics as tools for microbial classification (GUPTA; GRIFFTI, 2002). The improvement of molecular biology techniques allowed a refinement in the classification of bacteria, one of which was discovering the genes that encode the ribosomal subunit. The ribosome is a cellular organelle involved in protein synthesis and comprises three subunits (5S, 16S, and 23S) (ROSSELLO; AMANN, 2001). The classification based on the 16s rRNA subunit compares the nucleotides' sequence in this gene between organisms. The similarity in the sequences between the organisms can determine the evolutionary proximity between them. Due to the ease and speed

in this gene's sequencing, this sequence comparison technique has become the most comprehensive and used classification method in prokaryotes (ROSSELLO; AMANN, 2001; GUPTA; GRIFFTI, 2002; SCHLEIFER, 2009; TINDAL et al., 2010). For fungi, the regions generally sequenced are the D1/D2 domain (28 S) or the Internal Transcribed Space -ITS region (LANCHANCE et al., 1999).

The requirement for cultivation became an obstacle in the study microorganisms. There is an estimate that less than 1% of prokaryotes are cultivable (RAJENDRHARAN; GUNASEKARAN, 2008; SCHLOSS; HANDELSMAN, 2003; STREIT; RA, 2004). Thereat, techniques are developed to allow the extraction, amplification, and sequencing of genetic material directly from the microbial community without the need for cultivation, thus establishing the metagenomics technique.

Metagenomics can be defined as a technique of functional and genomic analysis of a community (REISENFELD et al., 2004). In this technique, it is common to use sequences with known primers that will amplify the 16s rRNA subunit's genes and with microbial identification taking place from the analysis and comparison of the sequences (BRUCE et al., 2010).

The composition and concentration of organic acids produced during kombucha fermentation can vary depending on the microbiota and the temperature used in the process (DE FILIPPIS et al., 2018). The physical-chemical characterization of the process is vital to elucidate the biochemical processes and the contribution of microbial interactions in the beverage composition. Foods are complex matrices formed by different constituents. The quality of food is multifactorial and involves aspects of chemical, microbiological, and sensory perception. In the food area, the use of data fusion strategies allows a more comprehensive analysis of the characterization of the food or drink, since it uses data sources of different natures (BIANCOLILLO al., 2019).

Identifying microorganisms involved in the kombucha fermentation process can provide information about the microbial succession process and a possible relationship of species dominance in the process. The study of microbial relationships and the beverage composition is essential to promote methodologies for the conservation of the inoculum, standardization, and optimization of the fermentation process.

OBJECTIVES

The present study aimed to characterize the microbiota and the physicochemical parameters in the black and green tea kombucha fermentation.

Specific objectives

- Charaterize the microbial diversity of kombuch;
- Characterize kombucha fermentation through physical-chemical parameters (pH, ethanol, total reducing sugars, and total titratable acidity) and quantification of phenolics (chlorogenic acid, gallic acid, caffeine, catechin, rutin, and quercetin);
- Qualitatively evaluate the formation of compounds during fermentation by Gas Chromatography coupled with Mass Spectrometry (GC-MS);
- Evaluate kombucha fermentations from data obtained by NIR, physical-chemical analysis, and chemometric tools combined with a data fusion approach;
- Evaluate the antimicrobial activity of kombucha using the agar diffusion method.

LITERATURE REVIEW

1. Introdution

The global Kombucha market is the fastest-growing among the functional beverages category and is estimated at 1.8 million dollars in the year 2020 (MARKET AND MARKETS, 2020). The consumption of kombucha is ancient and originated in China in the year 220 BC Afterward, the kombucha was introduced in other regions of Asia, Europe, and North America (JAYABALAN et al., 2014; DUTTA; PAUL, 2019; LAUREYS et al., 2020). Kombucha can receive different names according to the region "Indischer teepilz," "tea fungus," "fungus japonicus," "Haipao," "theebier," "Cainii grib," "kambucha," "Manchurian mushroom," "Cainii kvass," or "Wolga jellyfish" (DUFRESNE; FARNWORTH, 2000; JARRELL et al., 2000; JAYABALAN et al., 2014; MARSH et al., 2014; POTHAKOS et al., 2016; COTON et al., 2017).

The consumption of Kombucha was associated with religious beliefs. The beverage was prepared in prayer areas that were kept clean and had less contamination and produced a liquid with pleasant sensory characteristics. The popularization of Kombucha consumption occurred in World War II due to the scarcity of tea and sugar that were highly demanded products in Eastern and Western Europe (DUTTA; PAUL, 2019).

Currently, Kombucha is globally consumed and represents a considerable market share of functional beverages (MARKET AND MARKETS, 2020). Traditionally in the production of kombucha, *Camellia sinensis* is used, a perennial, shrubby plant, originally from Asia and belonging to the Theacea family (LORENZI; MATOS, 2002; JAYABALAN et al., 2007, 2014; CHAKRAVORTY et al., 2016; VITAS et al., 2018). To prepare the infusion of *Camellia sinensis*, sprouts and leaves are used, with or without oxidation. From the *Camellia sinensis* treatment process, four distinct types of teas are obtained, namely: white tea, green tea, oolong tea, and black tea (SIMONETTI; SIMONETTI, 1990; MUKHTAR, 2000; CVETKOVIC; MARKOV, 2006; DUARTE; MERAMIN, 2006, MATSUBARA; RODRIGUEZ-AMAYA, 2006; LIMA ET AL., 2009; WINSTON; KUHN, 2012; BRAIBANTE et al., 2014). For the preparation of Kombucha, black tea or green tea is commonly used. The alcoholic and acetic fermentation of the *Camellia sinensis* infusion gives rise to Kombucha (JAYABALAN et al., 2007; CHAKRAVORTY et al., 2016; POTHAKOS et al., 2016; VITAS et al., 2018, TEOH et al., 2004; MALBASA et al., 2006; JAYABALAN et al., 2007; JAYABALAN et al., 2010;

AMARASINGHE et al., 2017; MARSH et al., 2014; COTON et al., 2017; NEFFE-SKOCINSKA et al., 2017; KAEWKOD et al., 2019).

New products were developed due to the high consumption of kombucha. Currently, ingredients such as oolong tea (WATAWANA et al., 2016; KAEWKOD et al., 2019), hibiscus, yarrow (*Achillea millefolium*) (VITAS et al., 2018), African mustard (*Brassica tournefortii*) (RAHMANI et al., 2019), artichoke extract (LONCAR et al., 2007), purple basil (YIKMIŞ et al., 2019), coconut water (WATAWANA et al., 2016), Jasmin (WATAWANA et al., 2016), rooibos (GÀGGIA et al., 2019), blackberry (WATAWANA et al., 2016) and *Satureja montana* L. (CVETKOVIĆ; MARKOV, 2005) are used.

Kombucha fermentation occurs between yeast and acetic bacteria. Yeasts, from sucrose metabolism, produce alcohol, and acetic bacteria convert glucose and fructose into gluconic, glucuronic, and acetic acid. The kombucha microbiota is present in the cellulosic matrix and the fermented broth. A deposit of cellulose forms the cellulosic matrix. The formation of this film allows greater availability of atmospheric oxygen for acetic acid bacteria (KOJIMA et al., 1998; LIMA et al., 2001; JAYABALAN et al., 2007; JAYABALAN et al., 2014; LONCAR et al., 2014; CHAKRAVORTY et al., 2016; MATSUSHITA et al. 2016; SEMJONOVS et al., 2017; MAY et al., 2019).

Some studies suggest that Kombucha has functional characteristics such as antimicrobial benefits, liver and gastrointestinal functions, immune stimulation, detoxification, antioxidant and anti-tumor properties, inhibits the development and progression of cancer, cardiovascular diseases, diabetes, and neurodegenerative diseases. According Kapp; Sumner (2018), most studies are conducted in vitro, and the effects on humans are still scarce. The purpose of this review is to carry out a general approach to the fermentation process, microbial ecology, and the functional properties of Kombucha.

1.1.Briefly history of Kombucha

Since the Tsin Dynasty period in Manchuria, China, tea has been known for its medicinal properties. It has been widespread with trade missions to the far east of China and Japan (250–538 AD) (HOLBOURN; HURDMAN, 2017). The spread of the tea trade in Asia is related to the first reports of Kombucha production.

One of Kombucha's origins is associated with the emergence of a drink with medicinal characteristics developed by an Asian doctor named Kombu, who introduced a tea ("cha" in

Japanese) in Japan (DUFRESNE; FARNWORTH, 2000; POTHAKOS et al., 2016). Another origin also accepted by historians is that Kombucha arose from a Japanese non-alcoholic beverage prepared with water and edible brown seaweed grown in countries like Japan, Korea, and China. This seaweed was called dashi kombu (*Saccharina japonica*) (JARELL et al., 2000; JAYABALAN et al., 2014; POTHAKOS et al., 2016; AMARASINGHE et al., 2017).

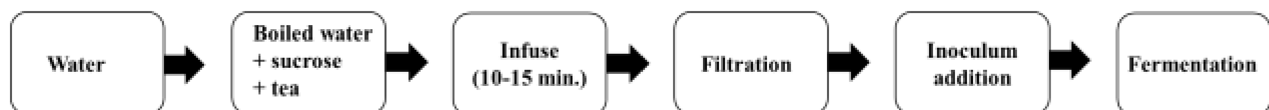
With the extension of the eastern trade routes, Kombucha was introduced in Russia and Eastern Europe. Kombucha became popular in Russia with a drink with metabolic medicinal effects (DUTTA; PAUL, 2019). During World War II, the consumption of Kombucha was extended to Western Europe and North Africa. Its consumption increased in the United States and expanded during the early 21st century (DUTTA; PAUL, 2019). With the popularization of the drink and the growth of consumption, large beverage companies started to produce Kombucha. Currently, the Kombucha producing market is a promising niche that moves 1.8 million dollars a year (MARKET AND MARKETS, 2020).

2. Microbial ecology and biochemistry of the fermentative process in the production of Kombucha

2.1 Preparation of Kombucha

The infusion of black tea or green tea is the basis for the fermentation of Kombucha. The figure 1 describes the flowchart for preparing the fermented beverage. The substrate of the fermentation by the filtered and cooled infusion is added with sucrose and the inoculum. (SIEVERS et al., 1995; CVETKOVIC ; MARKOV, 2006; MALBASA et al., 2008; NEFFE-SKOCINSKA et al., 2017; VITAS et al., 2018; RAHMANI et al., 2019; BARBOSA et al., 2020).

Figure 1: Generic kombucha production flowchart.



Sucrose is the most widely used carbohydrate in Kombucha fermentation (JAYABALANA et al., 2014). There are different sources of sucrose such as honey (AL - KALIFAWI, 2014; WATAWANA et al., 2016; DEĞIRMENCIOĞLU et al., 2020), molasses, or brown sugar (MALBASA et al., 2008; AMARASINGHE et al., 2017) and commercial or table sugar (CVETKOVIC; MARKOV, 2006; JAYABALAN et al., 2007; AL-KALIFAWI, 2014; TEOH

et al., 2014; WATAWANA et al., 2016; NEFFE-SKOCINSKA et al., 2017; BARBOSA et al., 2020).

The inoculum used in Kombucha fermentation comprises two parts: cellulosic matrix (film) and fermented (liquid). The cellulosic matrix contains acetic bacteria and yeasts immobilized on cellulose. In the fermented beverage, the cells of the microorganisms are present in the free form. The process occurs statically and in the presence of oxygen, due to the fact of AAB. The fermentation time described in the literature varies from 7 to 60 days (SIEVERS et al., 1995; CHEN; LIU, 2000; CVETKOVIC; MARKOV; 2006; DUTTA; GAUCHHUI, 2006; JAYABALAN et al., 2007; MALBASA et al., 2008; BATTIKH et al., 2012; JAYABALAN et al., 2014; LONCAR et al., 2014; TEOH et al., 2004; CHAKRAVORTY et al., 2016; NEFFE-SKOCINSKA et al., 2017; VITAS et al., 2018; RAHMANI et al., 2019).

Kombucha is a sweet, slightly acidic, and naturally carbonated beverage. However, the inoculum's origin can alter the sensory characteristics, and drinks with longer fermentation times are less sweet and more acidic (JAYABALAN et al., 2014; NEFFE-SKOCINSKA et al., 2017; VITAS et al., 2018).

2.2 Microorganisms involved in kombucha fermentation

The microbial ecology present in the kombucha is complex and composed mainly of yeast species and acetic acid bacteria and with a lower incidence of lactic acid bacteria (JARRELL et al., 2000; MO et al., 2008; JAYABALAN et al. 2014, COTON et al., 2017). Studies indicate that the microbiota that makes up the cellulosic matrix and the fermented broth can vary according to the origin of the inoculum, with its handling and the fermentation temperature (JAYABALAN et al., 2014; CHAKRAVORTY et al., 2016; DE FILIPPIS et al., 2018).

At the end of the Kombucha fermentation process, the bacteria populations are approximately 4 log10 CFU. mL^{-1} and yeast are 6log10 UFC. mL^{-1} (CHEN, LIU, 2000; GOH et al., 2012; TEOH et al., 2004; MARSH et al., 2014). The proportion of microbial cells is higher in the fermented broth than in the cellulosic matrix. After the tenth day of fermentation, there is a reduction in the viability of the microbial population due to a decrease in oxygen concentration and an increase in the acidity of the medium (CHEN, LIU, 2000; TEOH et al., 2004; COTON et al., 2017).

2.2.1. Yeasts

Yeasts are eukaryotic organisms, single-celled fungi, have generally spherical, oval, or cylindrical cells. They present mostly asexual reproduction by budding and still by binary fission (KURTZMAN et al., 2011). Some yeast species perform sexual reproduction; in this process, the fusion of two cells occurs with subsequent formation of the zygote and ascospores inside the fused cell (MADINGAN et al., 2004; TORTORA et al., 2003, KURTZMAN et al., 2011).

Different yeast species show similarities in central carbon metabolism's metabolic pathways with substantial differences in the mechanisms for nutrient absorption, isoenzymes, and regulation of fermentation and respiration processes (FLORES et al. 2000, RODRIGUES et al., 2006).

In nature, yeast species have a vast carbon source (organic acids, alcohols, amino acids, polyols) to support their growth, but they have a predilection for metabolizing sugars (RODRIGUES et al., 2006). There is a great deal of information about the metabolism of different carbon sources in yeasts, with sugars being the most widely described (RODRIGUES et al., 2006). The metabolism of hexoses and disaccharides share the same pathway (RODRIGUES et al., 2006).

Yeasts' metabolic versatility has a significant role in the food industry, especially concerning fermented foods (HITTINGER et al., 2018; COPPETTI, 2019). Yeasts are responsible for the development of sensory characteristics by the production of metabolites such as higher alcohols, esters (FLEET, 2003; MAS et al., 2014; COPPETTI, 2019), volatile fatty acids, and carbonyls (MAS et al., 2014), alcohols, polyols, aldehydes, ketones, volatile sulfur compounds (FLEET, 2003). Yeasts can be spontaneously present or added as starter cultures, accelerating the fermentation process and standardizing fermented food (COPPETTI, 2019)

Yeasts are the organisms most used in obtaining biotechnological products (MARQUES et al., 2015). Among the yeast species, *Saccharomyces cerevisiae* is the primary yeast used in biotechnology (KURTZMAN et al., 2011; MARQUES et al., 2015) and alcoholic fermentation processes (COPPETTI, 2019). The addition of *Saccharomyces* as starter cultures in fermented beverages helps to form the sensory profile of drinks (COPPETTI, 2019).

The yeast genera most commonly found in kombucha fermentation are *Zygossacharomyces*, *Brettanomyces*, *Saccharomyces*, *Candida*, *Dekkera*, *Pichia*, and *Torulaspora* (LIU et al., 1996; KURTZMAN et al., 2001; TEOH Et al., 2004; MARSH et al.,

2014; REVA et al., 2015; CHAKRAVORTY et al., 2016; COTON et al., 2017; GAGGÌA et al., 2019; ARIKAN et al., 2020; VILLARREAL-SOTO et al., 2020). The high concentration of sugars, acids, and ethanol in Kombucha favors the predominance of osmotolerant yeast species, such as *Zygosaccharomyces bailii* (DANG et al., 2009). Table 1 lists the main genera and species of yeast isolated from kombucha.

Table 1. Species of yeast found from fermented kombucha identified by dependent and independent culture methods

Genus	Species	Identification Method	Reference
Brettanomyces	*Brettanomyces* sp	Culture-dependent and culture-independent	LIU et al., 1996; TEOH et al., 2004; REVA et al., 2015, GAGGIA et al., 2019
	Brettanomyces bruxelensis	Culture-dependent and culture-independent	LIU et al., 1996; TEOH et al., 2004; REVA et al., 2015, GAGGIA et al., 2019
Candida	*Candida* sp.	Culture-dependent and culture-independent	TEOH et al., 2004; CHAKRAVORTY et al., 2016 ; COTON et al., 2017
	Candida boidinii	Culture-dependent and culture-independent	COTON et al., 2017
	Candida parapsilosis	Culture-independent	CHAKRAVORTY et al., 2016
	Candida stellata	Culture-dependent	TEOH et al., 2004
	Candida stellimalicola	Culture-independent	CHAKRAVORTY et al., 2016
	Candida tropicalis	Culture-independent s	CHAKRAVORTY et al., 2016
Davidiella	*Davidiella* sp.	Culture-independent	MARSH et al., 2014
Debaryomyces	*Debaryomyces* sp.	Culture-independent	CHAKRAVORTY et al., 2016
	Debaryomyces hansenii	Culture-independent	CHAKRAVORTY et al., 2016
Dekkera	*Dekkera* sp.	Culture-dependent and culture-independent	MARSH et al., 2014; Coton et al., 2017
	Dekkera anomala	Culture-dependent and culture-independent	COTON et al., 2017
	Dekkera bruxelensis	Culture-dependent and culture-independent	COTON et al., 2017
Eremothecium	*Eremothecium* sp.	Culture-independent	CHAKRAVORTY et al., 2016
	Eremothecium cymbalariae	Culture-independent	CHAKRAVORTY et al., 2016

Table 1. Continuation

Genus	Specie	Identification Method	Reference
Hanseniospora	*Hanseniospora* sp.	Culture-dependent and culture-independent	COTON et al., 2017
Herbaspirillum	*Herbaspirillum* sp.	Culture-independent	REVA et al., 2015
Kazachstania	*Kazachstania* sp.	Culture-independent	CHAKRAVORTY et al., 2016
Kluyveromyces	*Kluyveromyces* sp.	Culture-independent	MARSH et al., 2014 ; CHAKRAVORTY et al., 2016
	Kluyveromyces marxianus	Culture-independent	CHAKRAVORTY et al., 2016
Lachancea	*Lachancea* sp	Culture-independent	MARSH et al., 2014; CHAKRAVORTY et al., 2016
	Lachancea fermentati	Culture-independent	CHAKRAVORTY et al., 2016
	Lachancea thermotolerans	Culture-independent	CHAKRAVORTY et al., 2016
Leucosporidiella	*Leucosporidiella* sp	Culture-independent	MARSH et al., 2014
Merimbla	*Merimbla* sp	Culture-independent	CHAKRAVORTY et al., 2016
Meyerozyma	*Meyerozyma* sp	Culture-independent	CHAKRAVORTY et al., 2016
	Meyerozyma caribbica	Culture-independent	CHAKRAVORTY et al., 2016
Naumoyozyma	*Naumoyozyma* sp	Culture-independent	MARSH et al., 2014
Pichia	*Pichia* sp	Culture-dependent and culture-independent	MARSH et al., 2014; CHAKRAVORTY et al., 2016 ; COTON et al., 2017
	Pichia anomla	Culture-dependent and culture-independent	COTON et al., 2017
	Pichia membranifaciens	Culture-dependent and culture-independent	COTON et al., 2017
	Pichia mexicana	Culture-independent	CHAKRAVORTY et al., 2016
Rhodotorula	*Rhodotorula* sp	Culture-dependent	TEOH ET AL., 2004
	Rhodotorula, mucilaginosa	Culture-dependent	TEOH ET AL., 2004

Table 1. Continuation

Genus	Specie	Identification Method	Reference
Saccharomyces	*Saccharomyces* sp	Culture-dependent and culture-independent	CHAKRAVORTY et al., 2016; COTON et al., 2017
	Saccharomyces cerevisiae	Culture-dependent and culture-independent	LIU et al., 1996; COTON et al., 2017
	Saccharomyces uvarum	Culture-dependent and culture-independent	COTON et al., 2017
Schizosaccharomyces	*Schizosaccharomyces* sp.	Culture-dependent	TEOH et al., 2004
	Schizosaccharomyces pombe	Culture-dependent and culture-independent	TEOH et al., 2004 ; VILLARREAL-SOTO et al., 2020
Sgiyamaella	*Sgiyamaella* sp	Culture-independent	CHAKRAVORTY et al., 2016
Sporopachydermia	*Sporopachydermia* sp	Culture-independent	CHAKRAVORTY et al., 2016
Torulaspora	*Torulaspora* sp	Culture-dependent and culture-independent	TEOH et al., 2004 ; Coton et al., 2017
	Torulaspora delbreuckii	Culture-dependent	TEOH et al., 2004
	Torulaspora microellipsoides	Culture-dependent and culture-independent	COTON et al., 2017
Wallemia	*Wallemia* sp.	Culture-independent	MARSH et al., 2014
Zygosaccharomyces	*Zygosaccharomyces* sp	Culture-dependent and culture-independent	LIU et al., 1996; KURTZMAN e tal., 2001; TEOH et al., 2004; MARSH et al., 2014; COTON et al., 2017; GAGGIA et al., 2019; ARIKAN et al., 2020
	Zygosaccharomyces bailli	Culture-dependent and culture-independent	TEOH et al., 2004 ; COTON et al., 2017
	Zygosaccharomyces parabailii	Culture-dependent and culture-independent	LIU et al., 1996; GAGGIA et al., 2018
Zygotorulaspora	*Zygotorulaspora* sp	Culture-dependent and culture-independent	COTON et al., 2017
	Zygotorulaspora florentina	Culture-dependent and culture-independent	COTON et al., 2017
Zygowilliopsis	*Zygowilliopsis* sp	Culture-independent	CHAKRAVORTY et al., 2016

2.2.2. Acetic acid bacteria

Acetic acid bacteria (AAB) are microorganisms belonging to the Acetobacteraceae family. They are ellipsoidal rods, Gram-negative, strictly aerobic, polar or peritrich flagella, not spore-forming, positive catalase, and negative oxidase (YAMADA; YUKPAN 2008). The optimal pH range for multiplication is 5.0 to 6.5. They are tolerant to acidic conditions and can multiply up to pH 3.0 and 4.0. The optimum growth temperature is 25 to 30 ° C (HOLT et al., 1994). They are ubiquitous microorganisms and can be isolated from flowers, fruits, beer, Kombucha, wine, chocolate, tequila, vinegar (YAMADA; YUKPAN 2008; MATSUSHITA et al., 2016).

The *Acetobacteraceae* family comprises 17 genera *Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea, Nguyenibacter*, *Saccharibacter*, *Swaminathania*, *Swingsia*, *Tanticharoenia* (YAMADA, 2016).

Recently the BAA nomenclature was modified, and the genres were reclassified. Some species previously belonging to the genera *Acetobacter* and *Gluconacetobacter* were grouped into a new genus *Komagataeibacter* (MATSUSHITA et al., 2016). The genera most reported in kombucha fermentation are *Komagataeibacter* followed by *Acetobacter* (REVA et al., 2015; CHAKRAVORTY et al., 2016; COTON et al., 2017; DE FILIPPIS et al., 2018; GAGGÌA et al., 2018, MAY et al., 2019; ARIKAN et al., 2020; VILLAREAL-SOTO et al., 2020). Table 2 shows the main BAA species isolated in Kombucha.

Acetic acid bacteria oxidize ethanol and carbohydrates to organic acids for energy. This process is known as oxidative metabolism and is catalyzed by dehydrogenases and ubiquinones. The oxidation of ethanol to acetic acid is called acetic fermentation (BARJA et al., 2016; MATSUSHITA; MATSUTANI, 2016; OKAMOTO-KAINUMA; ISHIKAWA, 2016). This fermentation may be related to the phylogeny of acetic acid bacteria. The genus *Gluconobacter* has a high affinity for substrates such as D-glucose, D-gluconic acid, D-sorbitol and glycerol, and ethanol. On the other hand, *Acetobacter* and *Komagataeibacter* species have more affinity for ethanol than for carbohydrates. Besides, these two genera can hydrolyze acetic acid and convert it to carbon dioxide and water in the absence of ethanol (MATSUSHITA et al., 2016).

Some *Komagataeibacter* species (*K. europaeus*, *K. xylinus*, *K. intermedius*, and *K. obodediens*) are tolerant of high acetic acid concentrations, and are commonly found in vinegar (BARJA et al., 2016).

Table 2. Species of Acetic acid bacteria found from fermented kombucha identified by dependent and independent culture methods

Genus	Specie	Identification Method	Reference
Acetobacter	*Acetobacter* sp	Culture-dependent and culture-independent	EL-SALAM, 2012; MARSH et al., 2014; CHAKRAVORTY et al., 2016; COTON et al., 2017
	Acetobacter aceti	Culture-dependent	LIU et al, 1996; EL-SALAM, 2012
	Acetobacter lovaniensis	Culture-dependent and culture-independent	COTON et al., 2017
	Acetobacter nitrogenifigens	Culture-dependent	DUTTA and GACHHUI, 2006
	Acetobacter okinawensis	Culture-dependent and culture-independent	COTON et al., 2017
	Acetobacter pasteurianus	Culture-dependent	LIU et al, 1996
	Acetobacter peroxydans	Culture-dependent and culture-independent	COTON et al., 2017
	Acetobacter syzygii	Culture-dependent and culture-independent	COTON et al., 2017
	Acetobacter tropicalis	Culture-dependent and culture-independent	COTON et al., 2017
Gluconacetobacter	*Gluconacetobacter* sp	Culture-dependent and culture-independent	MARSH et al., 2014; CHAKRAVORTY et al., 2016; COTON et al., 2017
	Gluconacetobacter entanii	Culture-dependent	GAGGÌA et al., 2018
	Gluconacetobacter liquefaciens,	Culture-dependent and culture-independent	COTON et al., 2017
	Gluconacetobacter saccharivorans	Culture-dependent and culture-independent	COTON et al., 2017 ; DE FILIPPIS et al., 2018
Gluconobacter	*Gluconobacter* sp	Culture-dependent and culture-independent	REVA et al., 2015; CHAKRAVORTY et al., 2016 COTON et al., 2017; GAGGIA et al., 2018 ; VILLARREAL-SOTO et al., 2020
	Gluconobacter cerinus	Culture-dependent and culture-independent	COTON et al., 2017
	Gluconobacter oxydans	Culture-dependent and culture-independent	COTON et al., 2017 ; VILLARREAL-SOTO et al., 2020

Table 2. Continuation

Genus	Specie	Identification Method	Reference
Komagataeibacter	*Komagataeibacter* sp	Culture-dependent and culture-independent	REVA et al., 2015; CHAKRAVORTY et al., 2016 GAGGIA et al., 2018, ARIKAN et al., 2020
	Komagataeibacter europaeus	Culture-dependent and culture-independent	COTON et al., 201 ; DE FILIPPIS et al., 2018 ; VILLARREAL-SOTO et al., 2020
	Komagataeibacter hansenii	Culture-dependent and culture-independent	COTON et al., 2017
	Komagataeibacter intermedius	Culture-dependent and culture-independent	GAGGIA et al., 2018 ; VILLARREAL-SOTO et al., 2020
	Komagataeibacter kombuchae	Culture-dependent	GAGGIA et al., 2018
	Komagataeibacter oboediens	Culture-dependent and culture-independent	COTON et al., 2017
	Komagataeibacter rhaeticus	Culture-dependent and culture-independent	COTON et al., 2017 ; GAGGIA et al., 2018 ; VILLARREAL-SOTO et al., 2020
	Komagataeibacter xylinus	Culture-dependent and culture-independent	COTON et al., 2017 ; VILLARREAL-SOTO et al., 2020
Tanticharoenia	*Tanticharoenia sakaeratensis*	Culture-dependent and culture-independent	COTON et al., 2017

2.2.3. Other microorganisms present in kombucha fermentation

The microbiota dominant in the fermentation of Kombucha is composed of yeasts of acetic bacteria. However, some studies report the presence of lactic acid bacteria. The main species are *Lactobacillus kefiranofaciens*, *Lactobacillus nagelli*, *Lactobacillus satsumensis*, *Lactococcus* sp. (MARSH et al. 2014; COTON et al., 2017) and *Oenococcus* (COTON et al., 2017).

2.3. Ecological and biochemical interactions of the kombucha fermentation process

Biochemical and ecological interactions during kombucha fermentation are complex and still poorly understood (KALLEL et al., 2012; JAYABALAN et al., 2014; MARSH et al., 2014; NEFFE-SKONCINSKA et al., 2017). In the fermentation processes conducted by VILLARREAL-SOTO et al. (2020), the genus *Komagataeibacter* predominates over the genera among the acetic bacteria *Gluconacetobacter* and *Gluconobacter*. For yeasts, the predominant families are *Saccharomycetaceae* and *Schizosaccharomycetaceae*. During fermentation, sucrose is hydrolyzed to glucose and fructose by yeasts. After hydrolysis, carbohydrates are converted into ethanol and organic acids, both by yeast and acetic bacteria (figure 2).

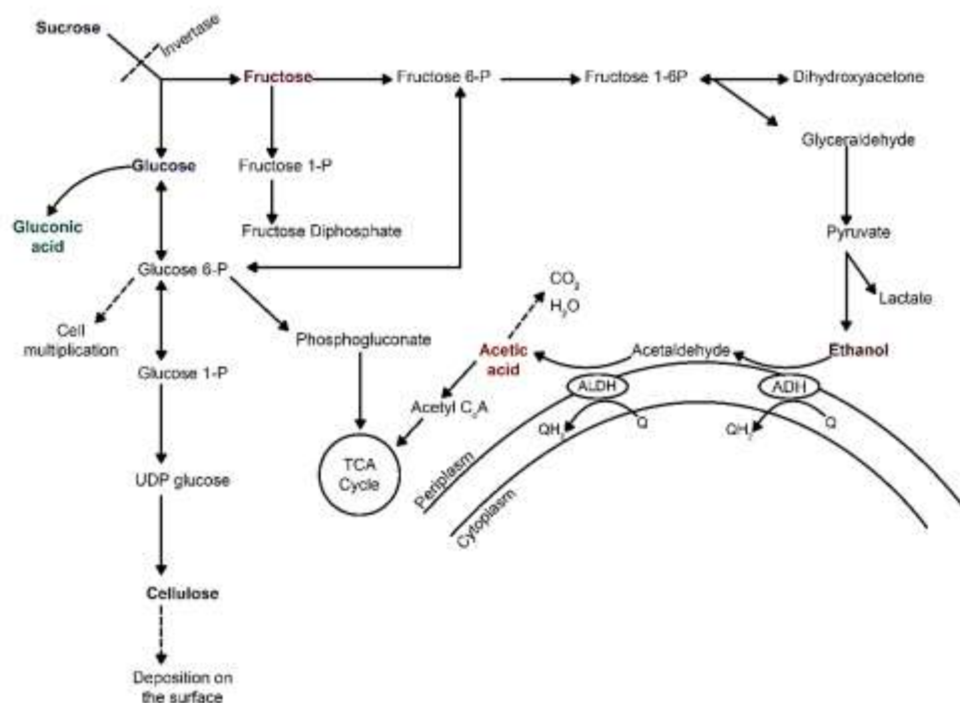


Figure 2. Main biochemical processes involved in the production of kombucha (NAKANO; EBISUYA, 2016; ARAI et al., 2016 and TONOUCHI, 2016).

The main biochemical processes for Kombucha production are alcoholic and acetic fermentation (JAYABALAN et al., 2014; CHAKRAVORTY et al., 2016). Yeasts play a fundamental role in the fermentation process, starting with the production of invertase. Invertase is an extracellular enzyme produced by yeasts that hydrolyzes sacarose into two monosaccharides: glucose and fructose that will later be transported into cells and metabolized (BATISTA et al., 2004).

The glycolytic pathway is the metabolic route used by yeasts for the metabolism of sugars. The metabolic reactions of the glycolytic pathway occur in the cytoplasm of cells and involve a sequence of reactions catalyzed by specific enzymes (FLORES et al., 2000; LIMA et al., 2001; RODRIGUES et al., 2006). The reactions that are catalyzed by cytoplasmic enzymes can be affected by the concentration of nutrients and minerals, changes in pH and temperature, and also by the presence of metabolites (FLORES et al., 2000; LIMA et al., 2001; RODRIGUES et al., 2006). In addition, during alcoholic fermentation, the use of alternative metabolic routes, by yeast, produces different compounds such as glycerol, organic acids, higher alcohols and acetaldehyde (FLORES et al., 2000; LIMA et al., 2001; CIANI et al., 2008).

The glucose into the in cell is phosphorylated by the kinase into glucose 6-phosphate and then isomerized to fructose 6-phosphate by the action of phosphoglucose isomerase. The next stage consists of new phosphorylation by the enzyme phosphofructokinase, subject to regulation by various metabolites, forming fructose 1,6-bisphosphate. The other enzymes involved in glycolysis are aldolase, trisphosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase. Fructose 1-6 bisphosphate will give rise to 2 compounds of 3 carbons each, being dihydroxy ketone and glyceraldehyde 3-phosphate. By the action of enzymes, glyceraldehyde 3-phosphate will be oxidized to pyruvate, generating ATP, and NADH (KRUCKEBERG; DICKISON, 2004; RODRIGUES et al., 2016).

In addition to ethanol production, yeast autolysis and death plays a significant role in the metabolism of acetic bacteria. Yeast cell lysis releases vitamins and other nutrients that stimulate the growth of BAA. This process is an amensalistic relationship of interaction that can understand this phenomenon of coexistence (VILLARREAL-SOTO et al., 2018).

The main products of acetic bacteria's metabolism are organic acids, cellulose polymers, vitamins, and amines (JAYABALAN et al., 2014; LONCAR et al., 2014; CHAKRAVORTY et al., 2016; MATSUSHITA et al. 2016).

Acetic fermentation is a reaction of ethanol's oxidation to acetic acid with the incorporation of oxygen, production of water, and release of energy carried out by acetic acid bacteria (PALMA et al., 2001). The process is catalyzed by the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (figure 2). ADH converts ethanol to acetaldehyde, removing hydrogen atoms, while ALDH converts acetaldehyde to acetic acid by incorporating oxygen atoms (ZILIOLI, 2011; MATSUSHITA, 2006).

Gluconic and glucuronic acids result from glucose oxidation, while acetic acid is produced by the oxidation of ethanol from alcoholic fermentation carried out by yeasts (JAYABALAN et al., 2007; LONCAR et al., 2014; MATSUSHITA et al. 2016, NEFFE-SKOCINSKA et al., 2017). Besides, carbohydrate metabolism promotes the synthesis of cellulose polymers, forming a matrix responsible for maintaining aerobic microorganisms on the surface. The immobilization provided by the cellulosic matrix is essential to favor AAB's aerobic metabolism and acetic acid formation. (KOJIMA et al., 1998; LIMA et al., 2001; JAYABALAN et al., 2007; JAYABALAN et al., 2014; LONCAR et al., 2014; MATSUSHITA et al. 2016; SEMJONOVS et al., 2017; MAY et al., 2019).

Acetic bacteria of the genus *Acetobacter* synthesize acetic acid and ethanol for energy and convert it into carbon dioxide and water (SETO et al., 1997; CHU; CHEN, 2006; JAYABALAN et al., 2014; CHAKRAVORTY et al., 2016; MATSUSHITA et al., 2016).

The species of the genus *Komagataeibacter* synthesize gluconic acid, glucuronic acid, and cellulose from glucose, and acetic acid is produced from ethanol (CHANG et al., 2001; DANG et al., 2009). Two species of the genus *Komagataeibacter*, *Komagataeibacter xylinus* [*Acetobacter xylinum* (YAMADA et al., 2012)] and *Komagataeibacter rhaeticus*, are the most responsible for the production of cellulose during the fermentation of Kombucha (MIKKELSEN et al., 2009; STRAP et al., 2011; MARSH et al., 2014; SANTOS et al., 2014; SEMJONOVS et al., 2017).

The organic acids most commonly produced in kombucha fermentation through glucose oxidation and microbiological processes are: acetic, glucuronic, gluconic, lactic, succinic, malic, oxalic, pyruvic, tartaric, citric, and malonic (JAYABALAN et al., 2014; NEFFE-SKOCINSKA et al., 2017), with acetic acid being the main one (JAYABALAN et al., 2007). The concentration of glucuronic acid in the fermentation will depend on the yeast species and acetic acid bacteria and the conditions used, being of fundamental importance the selection of

these microorganisms and the control of the fermentation process (MALBASA et al., 2011; NGUYEN et al., 2015; NEFFE-SKOCINSKA et al., 2017).

2.3.1. Main chemical compounds present in Kombucha

Kombucha's chemical composition varies with the origin of the inoculum, with the concentration of sugar and tea (VILLARREAL-SOTO et al., 2018). The main compounds present in Kombucha (Table 3) are organic acids, sugars, vitamins, amino acids, alcohols, biogenic amines, polyphenols, pigments, macromolecules (BLANC 1996; CHEN; LIU, 2000; LONCAR et al., 2000; PETROVSKA; TOZI (2000); TEOH et al., 2004; MALBASA et al., 2005; CHU; CHEN, 2006; JAYABALAN et al. 2007; MALBASA et al., 2011; BATTIKH et al., 2012; BHATTACHARYA et al., 2013; NGUYEN et al., 2015; JAYABALAN et al., 2014; CHAKRAVORTY et al., 2016; NEFFE-SKOCIŃSKA et al., 2017; WATAWANA et al. 2017; VITAS et al., 2018, BARBOSA et al., 2020).

Table 3. Chemical compounds in Kombucha

	Coumpound	**Reference**
Organic acids	Acetic Acid	SIEVERS et al., 1995; CHEN ; LIU., 2000; JAYABALAN et al., 2007; MALBASA et al., 2008 ; CHAKRAVORTY et al., 2016 NEFFE-SKOCINSKA et al., 2017; KHOSRAVI et al., 2018; KAEWKOD et al., 2019; RAHMANI et al., 2019
	Gluconic Acid	SIEVERS et al., 1995; CHEN; LIU., 2000; CHAKRAVORTY et al., 2016 NEFFE-SKOCINSKA et al., 2017; DE FILIPPIS et al., 2018 ; KHOSRAVI et al., 2018; KAEWKOD et al., 2019.
	Glucoronic Acid	CHEN ; LIU., 2000; LOCAR et al., 2000 ; JAYABALAN et al., 2007; DE FILIPPIS et al., 2018, KAEWKOD et al., 2019.
Alcohols	Ethanol	SIEVERS et al., 1995; LONCAR et al., 2014 ; CHAKRAVORTY et al., 2016 NEFFE-SKOCINSKA et al., 2017; RAHMANI et al., 2019 BARBOSA et al., 2020
Carbohidrats	Sugars (sucrose, glucose and fructose)	SIEVERS et al., 1995; CHEN ; LIU., 2000; MALBASA et al., 2002 , 2008 ; LONCAR et al., 2014 ; CHAKRAVORTY et al., 2016 NEFFE-SKOCINSKA et al., 2017 ; RAHMANI et al., 2019 ; BARBOSA et al., 2020.

Table 3. Continuation

	Coumpound	Reference
Phenols	(Caffeine ; Catechin and fractions ; Chlrogenic acid, Gallic acid, Rutin, Quercetin , Theaflavin, Thearubigin, flavonols)	JAYABALAN et al., 2007; CHAKRAVORTY et al., 2016; DE FILIPPIS et al., 2018 KHOSRAVI et al., 2018; ZHAO et al., 2018 ; IVANISOVÁ et al., 2019 ; BARBOSA et al., 2020.
Vitamin	Vitamin C	BAUER-PETROVSKA; PETRUSHEVSKA-TOZI, 2000; VITAS et al., 2018
Minerals		BAUER-PETROVSKA ; PETRUSHEVSKA-TOZI, 2000; KUMAR et al., 2008; JAYABALAN et al., 2010
Amino acids		JAYABALAN et al., 2010

The conduction of the fermentation process, the composition of the substrate, and the environmental conditions of the process can influence the final composition of the Kombucha (SIEVERS et al., 1995; CHEN; LIU, 2000; GREENWALT et al., 2000; TEOH et al., 2004 CVETKOVIĆ; MARKOV, 2005; LONCAR et al., 2007; ABSHENAS et al., 2012; BATTIKH et al., 2012; WANG et al., 2014; LONCAR et al., 2014; CHAKRAVORTY et al., 2016; KABIRI; SERTOKI, 2016; DE FILIPPIS et al., 2018; KAEWKOD et al., 2019).

3. Functional aspects of Kombucha

3.1. Heath Benefits

There are some reports of consumers associating Kombucha's use and beneficial health effects (JAYABALAN et al., 2014). However, studies that prove these benefits are still scarce. These studies attribute the health benefits to Kombucha's compounds. (VIJAYARAGHAVAN et al., 2000; MARSH et al., 2014; JAYABALAN et al., 2014). Table 4 summarizes the main functional effects associated with the consumption of Kombucha.

Table 4. Functional aspects of Kombucha

Biological activity	Effects	Experimental	Reference
Antioxidant			
	Ingestion of kombucha completely reversed the changes induced by oxidative stress in rats.	Rat	RAM et al. (2000)
	Both kombuchas analyzed (*in vitro*) showed the ability to eliminate free radicals and inhibit the oxidation of low-density lipoprotein (LDL).	Mice	YANG et al. (2009)
	Kombucha showed significant anti-glycemic potential, repair of pathophysiological changes, and activation of caspase-3 in the pancreas.	Rat	BHATTACHARYA et al. (2013)
	Showed reducing power for in vitro methods (DPPH, superoxide anions, and hydroxyl radical).	*In vitro*	FU et al. (2014)
	Kombuchas prepared with extract showed antioxidant activity.	*In vitro /* cells	VITAS et al. (2018)
	Kombucha showed antioxidant activity.	Microorganisms/cells	KAEWKOD et al. (2019)
Hepatoprotection			
	Kombucha performs hepatoprotective activity against paracetamol.	Mice	ABSHENAS et al. (2012)
	Kombucha modulated cellular apoptosis induced by oxidative stress in hepatocytes in a murine model	Rat	BHATTACHARYA et al. (2013)
	Kombucha showed hepatoprotective activity	Mice	WANG et al. (2014)

Table 4. Continuation

Biological activity	Effects	Experimental	Reference
Anticarcinogenic			
	Kombucha showed an anticarcinogenic effect on 786-O, U2OS, and A549 cells	Cells	JAYABALAN et al. (2011)
	Kombuchas prepared with infusion showed r anticarcinogenic and antimicrobial activity	*In vitro* / cells	VITAS et al. (2018)
	Kombucha presented toxicity in colorectal cancer cells (Caco-2)	Microorganisms/cells	KAEWKOD et al. (2019)
Anti-glycemic			
	Kombucha showed inhibition of α-amylase and lipase activities in plasma and pancreas. It reduced blood glucose levels. It delayed the absorption of LDL cholesterol and efficiently protected the liver-kidney functions of diabetic rats	Rats	ALOULOU et al., 2012;
	Decreased glycemia and glycated hemoglobin with increased plasma insulin, hemoglobin, and glycogen. Normalization of the activity of gluconeogenic and glycolytic enzymes	Rats	SRIHARI et al. (2013)
	Kombucha significantly reduced fasting plasma glucose levels, improved oxidative stress, lipid profile, pancreatic tissues, and pancreatic β cells	Rats	ZUBAIDAH et al.(2019)

Table 4. Continuation

Biological activity	Effects	Experimental	Reference
Neuroprotection			
	Kombucha showed protective effects on ischemia and reperfusion-induced brain damage	Rats	KABIRI; SETORKI (2016)
Hypocholesterolemic			
	In vivo tests showed that the hypocholesterolemic effect did not differ between kombuchas.	Mice	YANG et al. (2009)
Cicatrization			
	The kombuchas (black and green tea) analyzed can effectively cure gastric ulceration	Mice	BANERJEE et al. (2010)

There is still a gap about direct evidence to support the benefits of kombucha for human health. To date, there are no studies in humans that claim several reported benefits of kombucha for human health (KAPP; SUMNER, 2019).

3.1.1. Antimicrobial Activity

Some studies report Kombucha's antimicrobial activity (BATTIKH et al., 2012; BHATTACHARYA et al., 2016; GREENWALT et al., 1998; SREERAMULU et al., 2000). However, the mechanisms involved in the inhibition of pathogens are still divergent, and few elucidated.

GREENWALT et al. (1998) evaluated the antimicrobial activity of black tea and green tea kombucha in different concentrations. The authors tested the antimicrobial potential against E. coli serotype H10 (NRRL B-2207), *E. coli* serotype H48 (NRRL B-3704), *Salmonella enterica* serovar Typhimurium (NRRL B-4420), *Staphylococcus aureus* (NRRL B-1317), and *S. aureus* (NRRL B-1318). The results showed that the pathogen *Candida albicans* was not inhibited by green tea and black tea kombucha. The neutralized Kombucha had no antimicrobial activity against the pathogens tested. The authors emphasize that the antimicrobial activity is related to acetic acid and the acidic pH of Kombucha.

SREERAMULU et al. 2000, analyzed the inhibitory activity of black tea kombucha during 14 days of kombucha fermentation against 16 pathogenic microorganisms. Were tested fermented beverages, neutralized with sodium hydroxide (pH 7.0), and heat-treated (80 ° C for 30 minutes) and controls with an acetic acid solution (pH 2.3) and unfermented black tea. The authors observed kombucha inhibitory activity against *E. coli*, *Shigella sonnei*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Campylobacter jejuni* even in neutralized drinks (pH 7.0) and in the heat-treated beverage. The results of this study suggest the presence of an antimicrobial agent possibly produced by the bacteria and/or yeasts responsible for the fermentation of Kombucha. This agent is different from acetic acid, large proteins, and catechins in Kombucha.

BATTIKH et al. (2012) analyzed the antimicrobial activity of *Camellia sinensis'* infusions under different conditions: unfermented infusion, infusion fermented for 21 days without pH adjustment (traditional Kombucha), neutralized fermented infusion (neutralized Kombucha), acidified infusion with acetic acid to pH 2.6 and the thermally treated infusion (120 ° C for 20 minutes). Antimicrobial activity was tested against seven pathogenic microorganisms *Staphylococcus epidermidis*-CIP 106510; *Staphylococcus aureus*-ATCC 25923, *Micrococcus luteus*-NCIMB 8166, *Salmonella* Typhimurium-LT2, *Escherichia coli*-

ATCC 35218, *Listeria monocytogenes*-ATCC 19115, *Pseudomonas aeruginosa*-ATCC 27853. In addition to the bacteria, the authors tested seven species of *Candida* species. Among them, *C. albicans*. The tested antimicrobial extracts showed antimicrobial activity against all tested microorganisms. Fermented infusions (traditional Kombucha) and heat-treated Kombucha had the most significant effect.

4. Final considerations

Kombucha's industrial production presents challenges related to the inoculum's stability, uniformity, viability, and harmlessness. Also, bioreactors' capacity, temperature control, aeration, and post-fermentation beverage stability challenges. Another critical point in the fermentation of Kombucha is related to the microbiota's composition, which interferes with the final chemical composition of the drink (DE FILIPPIS et al., 2018; JAYABALAN et al., 2007; 2011; 2014).

In kombucha fermentation, the cellulosic matrix formed is used as a starter culture in the fermentation process. There is a need to develop a method of preserving this film or creating a starter culture for the Kombucha fermentation process.

The presence of a compound with functional characteristics indicates that Kombucha may play an essential role in human health. It can also be a source of other components for biotechnological or industrial use, such as gluconic acid, glucuronic acid, and cellulose.

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CHAPTER I

Integrating analyses of the microbiota and physicochemical parameters of kombucha and a new perspective for a starter microbial culture

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Integrating analyses of the microbiota and physicochemical parameters of kombucha and a new perspective for a starter microbial culture

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Abstract

Since kombucha is a drink that results from a spontaneous fermentation, with many human health claims, many studies have been performed to unveil its microbiological and physicochemical aspects. The integration of these results is fundamental to better understand and discuss the biological activities attributed to kombucha. In this sense, the our study carried out the isolation of bacteria and yeasts involved in the fermentation of kombucha produced with green (GK) and black (BK) teas, as well as the amplicon metagenomic of the microbial communities (16S and ITS) along 0, 3, 5, 10 and 15 days of fermentation, at 28°C. Microbial communities were linked to key biochemical parameters that were monitored during fermentation such as pH, total titratable acidity, total reducing sugars, polyphenols, acetic acid, and ethanol production. Ordination analysis (PCA) showed a clear separation of GK and BK groups during the fermentation process. Caffein, gallic acid, and chlorogenic acids were the chemical features that most influenced the separation of GK and BK. Additionally, the presence of *Komagataeibacter* spp. and catechins seemed to exert selective pressure against contaminating microorganisms. Therefore, based on both the literature and on our study, a proposal of microbial composition for a kombucha starter culture is suggested.

Keywords: *Camellia sinensis*, *Zygosaccharomyces*, PCA, GC-MS, UPLC.

1. INTRODUCTION

Kombucha is a green or black tea (*Camellia sinensis*) sugared beverage, fermented by a complex culture of yeast and acetic acid bacteria. Many health effects are claimed, and studies have shown that fermentation has an important role in their production. Thus, substrate tea composition, environmental parameters, and inoculum origin certainly should also be taken into account (Jayabalan et al., 2007; Jayabalan et al., 2011; Jayabalan et al., 2014; De Filippis et al., 2018). Also, kombucha is a habitat with a strong environmental pressure mainly due to pH, organic acids, phenols, ethanol and a complex microbial interaction (Malbaša et al., 2004; Battikh et al., 2012; Jayabalan et al., 2014; Neffe-Skocińska et al., 2017).

Culture-independent and -dependent methods for microbial community analysis pointed out *Acetobacteraceae* members and yeasts as the main microorganisms present in kombucha. Furthermore, according to current taxonomic classification, *Komagataeibacter* is the genus most reported in kombucha fermentation, and a more variable composition of dominant yeasts are found (Table 01).

Table 01 - Studies on culture-dependent and -independent methods of microbial kombucha microbiota indicating acetic acid bacteria (AAB) and yeasts that are found in kombucha fermentation.

Culture-dependent methods		
Taxa	**Species or Genus**	**Reference**
AAB		
	Acetobacter aceti	Liu et al., 1996; El-Salam, 2012
	A. pasteurianus	Liu et al., 1996
	A. nitrogenifigens	Dutta and Gachhui, 2006
	Komagataeibacter sp.	Gaggìa et al., 2018
	Komagataeibacter kombuchae	Dutta and Gachhui, 2007; Yamada et al., 2012

Taxa	Species or Genus	Reference
	K. intermedius, *K. rhaeticus, Ga. entanii*	Gaggìa et al., 2018
Yeasts		
	Zygosaccharomyces bailli, Torulaspora delbreuckii, Schizosaccharomyces pombe, Rhodotorula mucilaginosa	Teoh et al., 2004
	Brettanomyces bruxelensis and *Candida stellata*	
	Candida sp and *Lachancea* sp.	Chakravorty et al., 2016
	Brettanomyces bruxelensis	Liu et al., 1996; Gaggìa et al., 2018
	Z. parabailii	Liu et al., 1996; Gaggìa et al., 2018
	Saccharomyces cerevisiae subsp. *cerevisiae*	Liu et al., 1996

Culture-independent methods

Taxa	Species or Genus	Reference
AAB		
	Komagataeibacter oboediens, *K. xylinus, K. rhaeticus, K. europaeus, K. hansenii, K. intermedius, Acetobacter aceti, A. lovaniensis, A. peroxydans, A. tropicalis, A. syzygii, A. okinawensis, Gluconobacter cerinus, G. oxydans, Gluconacetobacter liquefaciens, Ga. saccharivorans, Tanticharoenia sakaeratensis*	Chakravorty et al., 2016; Coton et al., 2017; De Filippis et al., 2018; Podolich et al., 2016; Arikan, et al., 2020
Yeasts		
	Zygosaccharomyces	Marsh et al., 2014; Arikan et al., 2020
	Dekkera and *Hanseniaspora*	Coton et al., 2017
	Candida, *Pichia*, *Torulaspora*, *Brettanomyces, Rhodotorula, Saccharomyces* and *Schizosaccharomyces*	Jankovic and Stojanovic, 1994; Chen and Liu, 2000; Liu et al., 1996; Markov et al., 2001; Teoh et al., 2004, Jayabalan et al., 2014, Marsh et al., 2014, Coton et al., 2017
	Saccharomycetaceae	Chakravorty et al., 2016

Therefore, knowledge and research on the kombucha microbiota, and physicochemical parameters that interfere in the succession of microbial communities along fermentation is essential to understand the biotechnological kombucha properties, applications, and suggestions for starter cultures.

The study aimed to carry out an integrative analysis of the microbial diversity and physicochemical features in the black and green tea kombucha fermentation.

2. Material and Methods

2.1 Kombucha inoculum, fermentation process, and sampling

A spatial-temporal block experimental design (2 x 3 x 3 x 5) was performed consisting of two blocks, one for black tea, and one for green tea. In each block, there were three fermentation bioreactors (biological replicates). The fermentation process was carried out with black and green tea leaves (*Camellia sinensis*) as described by Barbosa et al. (2020), including three technical replicates for each bioreactor that were sampled in five times: 0 (infusion just after the addition of the kombucha inoculum), 3, 7, 10, and 15 days.

For all sampled time points, aliquots of 50 mL of sour broth and 50 g of floating cellulose pellicle layer were collected from each bioreactor. The sour broth samples, 30 mL were refrigerated (4 °C) for further analysis of physicochemical parameters. A total of 25 g (mixed of sour broth and cellulosic matrix) was used for the isolation of microorganisms in fresh material, and the remaining of each sample (cellulosic pellicle) were stored separately at -20 °C for amplicon sequencing analysis.

2.2 Culture-dependent approach

2.2.1 Isolation of culturable Acetic Acid Bacteria (AAB), Lactic Acid Bacteria (LAB), and yeasts

25g of each sample were homogenized and diluted with 225 mL of peptone saline water and serial dilutions were performed. AAB detection was performed using double layer agar culture media (1.5 and 0.9% of agar - superior layer and inferior layer, respectively) of RAE (Sokollek and Hammes, 1997), and MYP (De Ley et al., 1984), and then incubated at 30 °C for 5 days at 95% relative humidity according to Oliveira et al. (2010) and Spinosa (2002). LAB were detected on MRS medium according to Coton et al. (2017). Yeasts were detected in GYMP and Sabouraud agar, both containing 0.05% of chloramphenicol, and plates were incubated at 30 °C for 72-96 h (Entani et al., 1985; Spinosa, 2002). Twenty colonies of bacteria and fungi were randomly isolated from each and then purified from each sample in MYP and GYMP, respectively (Spinosa, 2002; Oliveira et al., 2010).

2.2.2 DNA extraction, purification and amplification of bacteria

DNA extraction, purification, and amplification were performed according to Ruiz et al. (2000) with some modifications such as: (i) use of GRS Genomic DNA and PCR & Gel Band Purification (GRiSP Research Solutions) for extraction and purification DNA; (ii) use of primers 16S-F (5'-GCTGGCGGCATGCTTAACACAT-3') and 16S-R (5'-GGAGGTGATCCAGCCGCAGGT-3'); (iii) for the amplification, the samples were incubated for 5 min at 94 °C to denature the target DNA, and cycled 35 times at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min.; (iv) PCR amplification product was not digested with restriction endonucleases.

2.2.3 Sequencing and analysis of 16S rDNA of bacteria

For bacteria, conventional Sanger 16S rDNA sequencing was performed with amplicons previously purified with GenElute PCR Clean-Up Kit (Sigma) (Ruiz et al., 2000; Vaz-Moreira et al., 2009). Sequences obtained from an automatic DNA sequencer were subjected to BLAST analysis. The percent identity was determined using the National Center of Biotechnology

Information databases (http://www.ncbi.nlm.nih.gov). All the raw sequences were deposited in NCBI SAR under BioProject PRJNA665807.

2.2.4 Classical and molecular identification of yeasts

Yeasts were identified according to methods described by Rademaker and De Bruijn (2003), González et al. (2004), Kurtzman et al. (2011), and Gomes et al. (2015). The isolates were subjected to PCR using the microsatellite primer $(GTG)_5$ as described by Lieckfeldt et al. (1993) and Gomes et al. (2015). The clustering was performed by similarity based on the molecular profile of the isolates. One strain of each molecular profile was carried for D1/D2 rRNA sequencing (Lachance et al., 1999) using the primers NL1 and NL4 according to (White et al., 1990; O'Donnell et al., 1993; Kurtzman and Robnett, 1998; Lachance et al., 1999 and Rosa et al., 2009). The sequences obtained were subjected to BLAST analysis, and percent identity with known bacterial species were determined using the National Center of Biotechnology Information databases (http://www.ncbi.nlm.nih.gov).

2.3 Culture-independent approach – Amplicon metagenomics techniques

2.3.1 DNA extraction

The DNA extraction of floating cellulosic matrix samples of the kombuchas was carried according to Marsh et al. (2014) with some changes such as (i) use of DNeasy® PowerFood® Microbial Kit (Qiagen); (ii) was used 1mL of cellulase; (iii) mechanical lyse by two cycles of 40 sec in a FastPrep (MP Biomedicals); (iv) The solution was incubated for 2 h at 40 °C; (v) enzymatic digestion with enzymes mutanolysin (100 $U.mL^{-1}$) and lysozyme (50 $mg.mL^{-1}$) at 37 °C for 1 h. Extraction was optimized with a 10-min 70 °C incubation (Quigley et al., 2012).

2.3.3 Sample preparation, PCR, amplicon sequencing of 16S rRNA gene and ITS2

Samples were prepared for Illumina Sequencing for 16S rRNA gene and ITS1 region amplification of the microbial communities. DNA was amplified using KAPA HiFi HotStart

PCR Kit according to manufacturer instructions, 0.3 µM of each PCR primer: forward primer F1 and reverse primer R5 and forward primer ITS1F and reverse primer ITS2R (Marsh et al., 2014) and 12.5 ng of template DNA in a total volume of 25 µL. The PCR conditions were a 3-min denaturation at 95 °C, followed by 30 cycles (bacterial DNA)/25 cycles (fungal DNA) of 98 °C for 20 s, at 55 °C (bacterial DNA)/60 °C (fungal DNA) for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all the amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled, and paired-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Sequence data were processed at Genoinseq, and raw reads were extracted from Illumina MiSeq® System in fastq format.

2.4 Physicochemical analyses of kombucha fermentation

Prior to the physicochemical analyses, all the samples were centrifuged at 2240 *g* for 5 min, and the supernatant was used for the analyses. The concentrations of ethanol (E) (Salik and Povoh, 1993), total reducing sugars (TRS) (Miller, 1959), total titratable acidity (TTA) (IAL, 2008), and pH (AOAC, 2007) were measured as described by Barbosa et al. (2020).

2.4.1 Chromatographic methods

For chromatographic analysis, all the samples were filtered through a 0.22 µm millipore membrane.

2.4.1.1 Phenols detection by UPLC analysis

The polyphenols identification was carried out using a UPLC system (Acquity, Waters) with a diode array detector, according to previous works (Chisté et al., 2012; Eça et al., 2015) and described by Barbosa et al. (2020).

2.4.1.2 Qualitative determination of compounds by GC-MS

The determination of compounds was performed on a gas chromatograph (Agilent 7890B) with a mass spectrometry detection system (Agilent 5977A -MSD) and a quadrupole type mass analyzer. A column CP - WAX 52 CB capillary type (Polyethylene glycol, 30 m x 0.25 mm x 0.25 µm internal diameter) was used for chromatographic separations. The compounds were identified by comparison with the mass, and fragmentation profile of the peaks were compared with the spectrum bank of the National Institute of Standards and Technology (NIST) library. From the peaks obtained and their respective retention times, those with the highest signal were selected, and their identification was at least 50% (Rigobello et al., 2015).

2.5. Bioinformatics and statistical analysis of the microbiome, quantitative and qualitative physicochemical variables, and their integration

We performed a customized workflow for bioinformatic analyses, using VSEARCH v.2.7.1 (Rognes et al., 2016) and the databases Silva (for Bacteria) (Quast et al., 2012) and UNITE (for Fungi) (Nilsson et al., 2018), as well as customized scripts in Python that were applied for amplicon metagenomic analysis.

In brief, VSEARCH was used to merge pairs, filter, dereplication, pre-clustering, quimera removal and clustering. erging pairs was done using **fastq_mergepairs** function, (ii) filtering was done using **fastq_filter** function with the flag fastq_maxee and fastq_minlen with parameter 0.5 and 300, respectively, (iii) two scripts (join_files and abundance) were built to join all the samples in one file and to create abundance table, (iv) BLAST was used to identify

all the centroids in cluster analysis. We used 90% coverage and 97% identity with the flags -qcov_hsp_perc 90.0 and -perc_identity 97.0, respectively.

In order to integratively analyzing all the data of amplicon metagenomics with physicochemical attributes, we used the multivariate statistics ordination method of Principal components analysis (PCA), which finds hypothetical components, accounting for as much as possible of the variance in the data (Legendre and Legendre, 1998). A normalized variance-covariance matrix was constructed with the multivariate data to be the input of Singular Value Decomposition (SVD) algorithm. Furthermore, we also performed a pairwise correlation analysis, using the parametric correlation coefficient (Pearson's r), with all the quantitative variables of both amplicon metagenomics with physicochemical traits (Schilling, 1984). Ordination (PCA) and correlation analyses were carried out in Past 4.03 (Hammer et al. 2001).

2.6 Statistical methods for physicochemical variables

Each variable (E, Q, R, etc) were deregressed according to the block effects (week effects). These deregressed variables were regressed on time (0, 3, 7, 10, and 15) by applying multiple linear regression with dummy variables (green tea - GT and black tea - BT). Multiple linear regression models of 1° and 2° degree for time were analyzed. Analysis of variance for regression was also performed at 1% probability of error type I, and 95% confidence intervals were estimated for each treatment (GT and BT) and time with "effects" package (Fox et al., 2019). The statistical analyses were performed in R software (R Core Team, 2020).

3. RESULTS AND DISCUSSION

3.1. Microbiological Analysis

Komagataeibacter and *Zygosaccharomyces* were the dominant genera of bacteria and yeast, respectively, during all the fermentation of both kombucha (GK and BK), considering culture and amplicon metagenomics approaches (Supplementary Table 01). The double layer of RAE (Sokollek and Hammes, 1997), MYP (De Ley, Gosselé, Swings; 1984), and incubation at 95% of relative humidity (Spinosa, 2002; Oliveira et al., 2010), a well-known methodology for isolating AAB, favored the isolation of *Komagataeibacter*. GYMP and Sabouraud especially favored the isolation of *Zygosaccharomyces* yeast (Supplementary Table 01).

Regarding the same methodology (isolation or amplicon sequencing), usually, the number of isolates and reads in GK and BK along 15 days of fermentation were quite similar, for both bacteria and fungi (Figure 1).

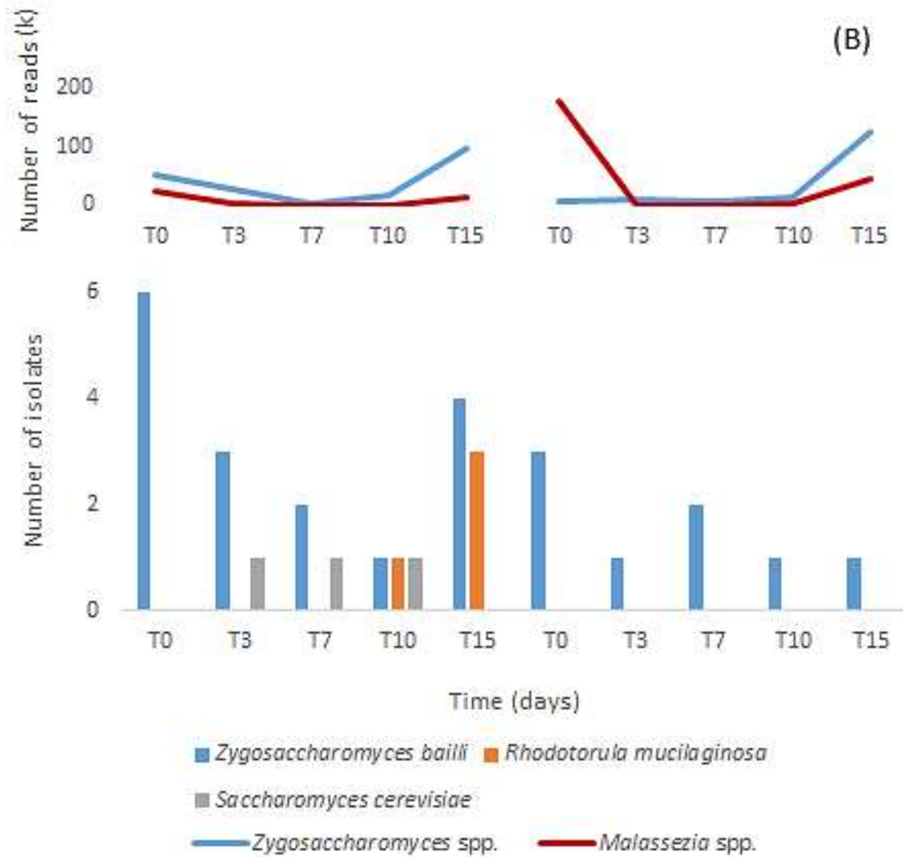

Figure 01 - Number of isolates and reads in green (GK) and black (BK) kombucha along 15 days of fermentation, at 28°C. (A) Bacteria, (B) Fungi. (COLOR SHOULD BE USED)

Both bacteria (48.19% and 51.45%) and fungi (0.13% and 0.23%) displayed similar relative abundances in GK and BK (Supplementary Table 01). Nonetheless, the number of reads and the relative abundance of bacteria were much higher than fungi in both kombucha fermentations (Figures 2 and 3).

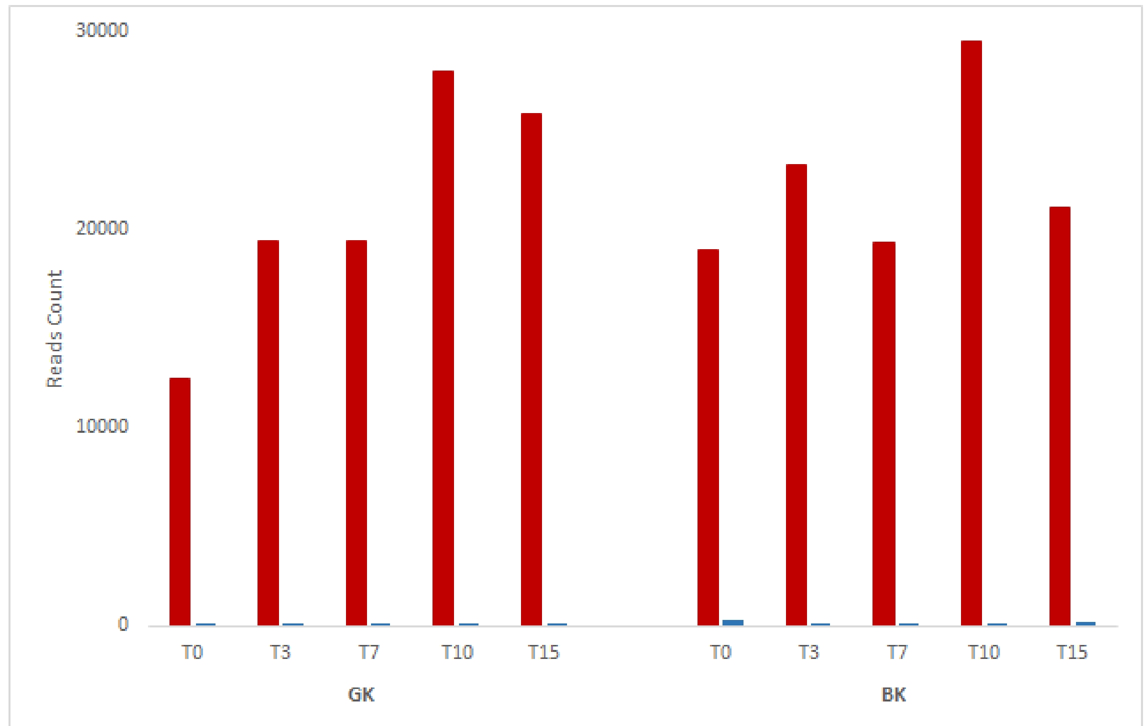

Figure 2 - Total reads count of bacteria (red) and fungi (blue) in green kombucha (GK) and black kombucha (BK) along 15 days of fermentation achieved by amplicon sequencing. (COLOR SHOULD BE USED)

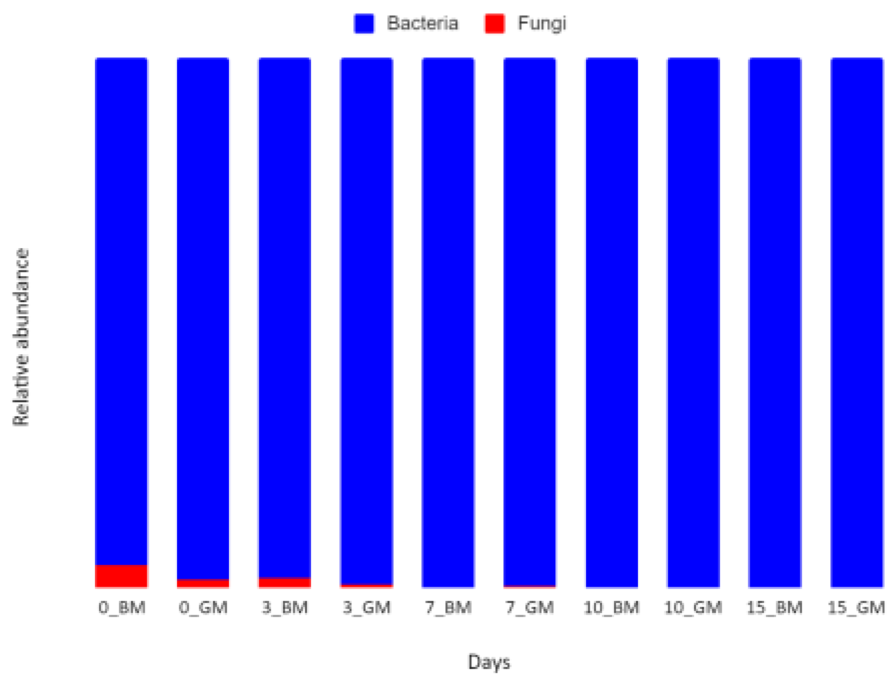

Figure 3- Relative abundance of reads of fungi and bacteria in cellulosic matrix of green (GK) and black (BK) kombucha during 15 days of fermentation retrieved by amplicon sequencing. (COLOR SHOULD BE USED)

Considering all the fermentation time (0-15 days), the total number of isolates of bacteria and fungi was 102 and 31, respectively. By the isolation method, a total of 102 bacterial isolates were identified by sequencing the 16S rDNA, and 31 yeast isolates were initially grouped by GTG_5 and then had the ITS region sequenced. The total number of those taxonomic groups of microorganisms was higher in GK (63 bacteria and 23 fungi) than in BK (39 bacteria and 8 fungi) along all the fermentation (Figures 01-03; Supplementary Material Table 01).

Among the 31 isolated of yeasts, *Z. bailli* exhibited the highest number (24), followed by *Rhodotorula mucilaginosa* (4) and, also, *Saccharomyces cerevisiae* (3) (Supplementary Material Table 01), whose presence has already been described in kombucha (Teoh et al., 2004; Chakravorty et al, 2016;) and are recognized as ethanol producers (Thomas and Davenport, 1985, Pitt and Hocking, 1997, Martorell et al., 2007; Kurtzman et al., 2011; Vajzovic et al., 2012; Jaiboon et al., 2016).

The genus *Zygosaccharomyces* is usually known as a spoiler of unprocessed food, and *Z. bailii* showed the most marked and diversified resistance characteristics, such as low pH and high sugar content (Thomas and Davenport, 1985; Erickson and McKenna, 1999), but many studies have shown its participation in spontaneous fermentation of kombucha (Liu et al., 1996; Teoh, 2004; Coton et al., 2007), vinegar (Solieri et al., 2006), wine (Garavaglia et al., 2015), Chinese Maotai (Wu et al., 2013), Mezcal, and other products such as candied fruits, nougat, and syrups (Martorell et al., 2005).

Studies described that *Zygosaccharomyces bailii* metabolizes sucrose to produce glucose and fructose. *Z. bailii* preferentially metabolizes fructose to produce ethanol, even when glucose is present in the growth medium as a carbon source (Merico et al., 2003; Kurtzman et al., 2011).

Fructose is transported into yeast cells by a specific high-capacity system while glucose is transported with higher affinity and lower capacity system, which is partially activated by fructose. In addition, fructose is able to use the glucose transporter, by competing with glucose (Sousa-Dias, 1996). Moreover, *Z. bailii* shows osmotolerance and resistance to different acids and ethanol (Kurtzman et al., 2011). In *Z. bailii*, the existence of two separate transport systems, the glucose system with higher affinity and lower capacity than the fructose system, seems to be related to the fact that this yeast is a strong spoilage agent of food substrates rich in fructose.

Amongst 102 isolated bacteria, 94 of them corresponded to AAB (92.16%) and only 8 to the genus *Sphingomonas* (7.84%). The main bacterial isolated species were *K. hansenii* (41.2%), *K. europaeus* (23.5%) and *K. xylinus* (14.7%) (Supplementary Material Table 01), a species frequently isolated from kombucha fermentation (Marsh et al., 2014, Gaggìa et al., 2018).

Understanding that taxonomic updates are important information in the analysis of the microbiota for a correct evaluation of the results, the updating of the nomenclature for *Komagataeibacter* genus studied are shown in Table 02.

Table 02 - Updating of the nomenclature for *Komagataeibacter* studied: current nomenclature, basonym and synonymous

Current nomenclature	Basonym	Synonymous
Komagataeibacter hansenii	*Acetobacter hansenii* (Gosselé et al., 1983)	*Gluconacetobacter hansenii* (Gosselé et al., 1983; Yamada et al. 1998) *Gluconacetobacter kombuchae* (Dutta and Gachhui, 2007)
Komagataeibacter xylinus	*Acetobacter aceti* subsp. *xylinus* (Brown, 1886; De Ley and Frateur, 1974)	Gluconacetobacter *xylinus* (Brown, 1886; Yamada et al., 1998)
Komagataeibacter europaeus	*Acetobacter europaeus* (Sievers et al. 1992)	*Acetobacter europaeus* (Sievers et al., 1992), *Gluconacetobacter europaeus* (Sievers et al., 1992; Yamada et al., 1997)
Komagataeibacter rhaeticus	*Gluconacetobacter rhaeticus* (Dellaglio et al., 2005)	*Gluconacetobacter rhaeticus* (Dellaglio et al. 2005)
Komagataeibacter intermedius	*Acetobacter intermedius* (Boesh et al., 1998; Yamada, 2000)	*Gluconacetobacter intermedius* (Boesch et al. 1998) Yamada 2000, *Acetobacter intermedius* (Boesh et al., 1998)

Komagataeibacter rhaeticus was less expressive than the other *Komagataeibacter* species (7.8%), and the same percentage of isolates was also obtained for *Sphigomonas melonis*, a species that could cause brown spots on melon fruits (Buonaurio et al., 2002). *Sphingomonas melonis* was especially isolated in GK, but only at T0 and T15 (Supplementary Material Table 01). Some species of *Sphingomonas* are also described as gellan and welan gum (exopolysaccharides) producers and have industrial applications (Kang et al., 1982; Wang et

al., 2006; Kaur et al., 2014). Thus, *Sphingomonas* isolates may also be related with polysaccharide production in our study. *Sphingomonas melonis* was also isolated by Ivanišová et al. (2019) who studied only black tea kombucha.

Komagataeibacter xylinus is a species frequently cited in kombucha microbiota studies as dominant in fermentation and responsible for the production of cellulosic pellicle (Marsh et al., 2014). Nevertheless, in our study, *K. xylinus* accounted for less than 15% of the total isolates in observed that cellulosic film was present, and it was not detected by amplicon sequencing. Other species of *Komagateibacter* were also able to produce the cellulosic pellicle (Komagata, Iino, and Yamada, 2014), and this in fact, occurred in both kombuchas studied. The low presence of *K. xylinus* could be explained by the temperature used in our work (28 °C). De Filippis et al. (2018) indeed stated that the fermentation temperature can alter the microbial composition of the kombucha. In that study, the authors reported the dominance of *K. xylinus* in fermentation at 20 °C and *K. saccharivoran*s multiplying abundantly at 30°C.

Amplicon sequencing approach generated a much higher number of total reads of bacteria (217.618) than fungi (775). Sample sufficiency was observed by the analysis of the rarefaction curve (Supplementary Material Figure 01), and the total microbial diversity (Shannon index) was similar between both kombuchas, GK and BK (data not shown). Conversely to the culture-dependent method findings, the total number of bacterial and fungi reads was higher in BK (112.374 and 492, respectively) than GK (105.244 and 283, respectively).

A total of 11 OTUs of fungi were detected, and two of them belong to *Malassezia* genus and were considered as only one in analyses (Supplementary Material Table 01). *Zygosaccharomyces* was the most represented OTU considering the total number of fungal reads (352), followed by *Malassezia* (262), and *Rhodotorula* (58). *Zygosaccharomyces* were detected along all the fermentation process while *Malassezia* occurred only in the beginning

and in the final times of fermentation. Seven OTUs of bacteria were found, and six of them belong to the *Komagataeibacter* genus, and *Stenotrophomonas* was only detected at T07 in BK (Supplementary Material Table 01).

In general, our results are similar to those observed in the studies by Marsh et al. (2014) and Arıkan et al. (2020) that also applied a very similar DNA isolation method and found 90% of bacteria belonging to the genus *Gluconacetobacter*, which formerly included *Komagataeibacter* (Yamada et al., 2012), and 95% of the total fungi belonging to the genus *Zygosaccharomyces*.

The presence of bacterial reads was much higher than fungal reads during all the fermentation time in both kombucha (GK and BK). Despite the total number of bacterial reads being similar between GK and BK, the number of reads in GK was mostly increasing, while in BK the number of the bacterial reads oscillated along the fermentation (Figures 01-03; Supplementary Material Table 01).

Our results showed that the chemical composition of GK and BK does not interfere with the relative total abundance of bacteria and yeasts during kombucha fermentation. Coton et al. (2017) reported differences in the relative abundance only for bacteria but not for fungi when comparing green and black tea kombucha. Additionally, the microbial diversity, especially the fungal one found by Coton et al. (2017) was quite different from our work.

Amongst taxonomical composition of yeasts, the genera frequently cited are *Saccharomyces*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Brettanomyces*, *Rhodotorula*, *Candida*, *Hanseniaspora*, *Lachancea* and *Pichia*. Regarding bacteria, *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, and *Komagataeibacter*, belonging to the AAC group, are often mentioned as well as *Lactobacillus* and *Oenococcus* in the LAB group are also reported in kombucha fermentation (Greenwalt et al. 2000; El-Salam 2012; Teoh,

Heard, Cox 2004; Jayabalan et al., 2014; Marsh et al., 2014a; Chakravorty et al., 2016; Coton et al., 2017; Arıkan et al., 2020).

Regarding the comparison of the fungal community in kombucha obtained by culture-dependent approach and the amplicon sequencing. The 31 fungal isolates were grouped by GTG_5, resulting in 27 profiles (data not shown), and the sequencing of the ITS regions also pointed out the genus *Zygosaccharomyces* as the most abundant in the samples (45.4%), followed by *Malassezia* (33.8 %), and *Rhodotorula* (7.5%) (Supplementary Material Table 01).

Studies reported that *Zygosaccharomyces* as the most abundant isolated fungal genus in kombucha (Teoh et al., 2004; Marsh et al., 2014; Arikan et al., 2020), and the presence of the genus *Rhodotorula* was also reported (Teoh et al., 2004; Marsh et al., 2014). Rodrigues et al. (2012) pointed out that *Z. bailii* (also isolated in our study) is benefited from an additional energy source of respiratory metabolism of acetic acid, even under glucose-fermentative conditions, using the co-metabolism of glucose and acetic acid.

The presence of *Malassezia* was previously supposed to be limited to the dermis of humans and animals and intricately linked to pathogenicity, but, in fact, molecular analysis of environmental sequences showed that members of *Malassezia* are among the most widespread fungi in the world. *Malassezia* has been found in fermented food, marine environments in both pelagic and benthic habitats, and other substrates (Luo et al., 2020; Góes-Neto et al., 2019; Amend, 2014; Gao et al., 2008).

In our study, *Rhodotorula* and *Malassezia* and other amplicon sequences were detected especially at the beginning and at the end of fermentation (Supplementary Material Table 01). The larger population of *Rhodotorula* and *Malassezia* and other ethanol non-producers yeasts in BK in competition with *Zygosaccharomyces* probably contributed to the lower ethanol production (Figure 04).

In our study, AAB isolates were also dominant in GK and BK along all the fermentation process, considering isolation and metagenomics tools (Figure 1; Supplementary Material Table 01), and *Komagataeibacter* is the most cited genus (Chakravorty et al., 2016; Reva et al., 2015; Arikan et al., 2020). Coton et al. (2017) identified in industrial kombucha fermentation of black and green teas a high diversity of species of AAB, and, a among them, *Komagataeibacter* emerged as a dominant genus, and to a lesser extent *Lactobacteriaceae*. Amplicon sequencing indicated that the total reads of the bacteriome (217,618 reads) is basically composed of *Komagataeibacter* species (99.8% total reads) (Figure 1; Supplementary Material Table 01). Only 0.02% (47) of the reads belonged to the *Stenotrophomonas*, a genus of ubiquitously bacteria distributed in the environment, mainly of plant-associated origin (Ryan et al., 2009). All the *Stenotrophomonas* reads were present in BK, and only at sample T07 (Supplementary Material Table 01).

Few studies report the presence of LAB in kombucha fermentation (Marsh et al. 2014; Coton et al., 2017), and in our study LAB were not detected in any kombucha tea.

The microbial isolation method as well as the amplicon sequencing approach, clearly indicated the genera *Komagataeibacter* and *Zygosaccharomyces* as dominant bacteria and fungi, respectively, in GK and BK. This reinforces that these genera are adapted to environmental selective pressures in kombucha tea fermentations, such as low pH, high acetic acid and ethanol concentrations, and the possible positive interactions between them (Teoh et al., 2004; Dang et al., 2009; Kurtzman et al., 2011; Marsh et al., 2014; Reva et al., 2015; Pothakos et al., 2016; Nakano and Ebisuya, 2016; Matsushita et al., 2016; Arikan et al., 2020).

3.2. PhysicoChemical Analysis and Microbiota integration

For most of the analyzed parameters, changes were observed during all the fermentation time and indicated changes in chemical dynamics during fermentation in both kombucha (Figure 04 and Supplementary Material Tables 02-05). Regarding kombucha fermentation kinetics, the variables were better adjusted in the quadratic linear regression (Supplementary Material Figure 02).

The concentration of **total reducing sugars** decreased along all the fermentations, indicating their consumption (Figure 01) by the kombucha microbial community. (Figure 04; Supplementary Material Figure 02; Supplementary Material Table 02). The presence of sugar is important to microbial growth and to produce bacterial cellulose matrix (Tonouchi, Naoto, 2016). Additionally, *Komagataeibacter* is able to use sucrose, glucose, and fructose (Komagata et al., 2014) to obtain energy and to grow, competing with yeasts for sugar since the beginning of fermentation. Among other sugars, *Komagataeibacter* is able to produce acid from glucose and ethanol, but not from fructose (Komagata et al., 2014). A recent study showed that among acetate, ethanol, fructose, glucose, lactose, mannitol, or sucrose, *Komagataeibacter* sp. W1 reached the highest yield of bacterial cellulose matrix in fructose (Wang et al., 2018).

The **pH** variation throughout the fermentation was slightly decreasing in both kombuchas (GK and BK), as a result of an increase of organic acid production. The maximum and minimum pH values detected were 3.8 and 3.1, respectively (Figure 04; Supplementary Material Table 02), which means a low initial pH of the infusions and a small decline until the end of the fermentation for both kombuchas.

Although the initial (T0) concentration of reducing sugars was similar in GK and BK (74.5 and 73.2 gL^{-1}, respectively), the total concentration of **ethanol** detected along all the fermentation process was higher in GK when compared to BK, especially at times T7 and T10. In BK fermentation, no statistical difference was observed in ethanol production throughout the fermentation (Figure 04; Supplementary Material Table 02; Supplementary Material Figure

02). The microbial composition, especially yeasts, may have contributed to this result and will be discussed further. The lowest concentrations of **ethanol** are in T0 and T15, times when a wide range of microorganisms grow (Figures 01 and 04; Supplementary Material Table 01). Yeasts belonging to *Zygosaccharomyces* genus show the ability to ferment hexose sugars, such as glucose and fructose (Thomas and Davenport, 1985; Pitt and Hocking, 1997; Martorell et al., 2007; Wang et al., 2018). Therefore, in the beginning of the kombucha fermentation, the fermenting yeasts *Zygosaccharomyces* transform the available sugar into ethanol, and acetic acid bacteria *Komagataeibacter* metabolize ethanol and produce acetic acid mostly.

Usually, the **total acidity** was also similar for both kombuchas (Table 03). The values of the total titratable acidity concentration were also increasing in both fermented teas throughout all the fermentation time (from 2.5 to 24 gL^{-1} in BK) and were always higher in GK (from 4.5 to 27.3 gL^{-1}) (Figure 04; Supplementary Material Table 02). According to literature on kombucha fermentation, weak acids such as acetic, glucuronic and gluconic acids are produced by AAB in high concentrations (Jayabalan et al., 2014). Acetic acid may induce stimulatory effects on growth and fermentative metabolism of *Z. bailii* (Prudencio et al., 1998). Furthermore, *Z. bailii* seems to be more resistant than *S. cerevisiae* to the inhibitory effects of ethanol in the presence of acetic acid that allowed them to re-ferment sugars in wines (Fernandes et al., 1997).

Antimicrobial activity of **polyphenols** of plants has been extensively investigated and shows a wide range against zoopathogenic and phytopathogenic microorganisms. In our study, the concentration of most of the detected polyphenols (gallic acid, caffeine, quercetin and rutin) were higher in BK than GK during all the fermentation times. Nonetheless, carboxylic acid was the only polyphenol higher in GK than BK (Supplementary Material Figure 02; Supplementary Material Figure 02). Those polyphenols can show some influence in microbial composition when GK and BK are compared, which could explain some differences among the read counts

of bacteria by amplicon sequencing (Figure 1A; Supplementary Material Table 03). The presence of polyphenols in kombuchas, especially gallic acid and caffeine in black tea, probably interfered negatively in the microbial composition and growth during the fermentation process (Figure 5; Supplementary Material Table 03).

Except for **catechin**, significant different concentrations of all the other phenolic compounds of GK and BK analyzed by UPLC (gallic acid, caffeine, chlorogenic acids, rutin and quercetin) were detected (SupplementaryMaterial Figure 02, Supplementary Material Table 03). An increasing concentration of **gallic acid** was observed in both teas; however, in BK the concentration (average of 68.6 mg. L^{-1}) was at least 8x higher than in GK (average of 7.6 mg. L^{-1}) (Supplementary Material Table 03). The **caffeine** concentration was at least twice higher in BK (ranging from 177.4 to 205 mg. L^{-1}) than in GK (from 69.1 to 78.2 mg. L^{-1}) at all times analyzed (Supplementary Material Table 03). The variation in the concentrations of **rutin** and **quercetin** were smaller when considering the different times of the same kombucha. Among the kombuchas (GK and BK); however, significant differences were recorded. The concentrations of quercetin (averages of 0.226 and 1.16 mg. L^{-1}, for GK and BK, respectively) and rutin (mean of 16, 5 and 27.8 mg. L^{-1}, for GK and BK, respectively) have always been bigger in BK (Supplementary Material Table 03).

Chlorogenic acids were the only phenolic compound detected in higher concentration in GK than in BK. But, in this case, the concentration was at least 25 times higher in GK (average of 65 mg. L^{-1}) than in BK (average of 2.5 mg.L^{-1}) at all analyzed times (Supplementary Material Table 03).

It is important to mention the difference in the processing of green tea and black tea, which may lead to different chemical constitutions of the products, and possibly, the microbial dynamics during fermentation. Both teas are produced from the *Camellia sinensis* plant. After harvesting the leaves, green tea is readily dried with or without a fixation (drying) step to

inactivate enzymes (Hara et al., 1995). Black tea is the result of the oxidation of leaf polyphenols through heat dehydration and a multi-stage enzymatic process. And, thus, new polyphenol molecule complexes are formed during the processing of black tea (Hara et al., 1995).

The molecular identification of compounds related to the inoculum (prepared in green tea) and the fermentations of GK and BK were determined by CG-MS. A total of 18 compounds were identified, 16 in BK, 13 in GK, and 12 in the inoculum (Supplementary Material Table 05). Five compounds were detected exclusively in BK (Acetal, Glycolaldehyde, Hydroquinone, Paromomycin, Furyl hydroxymethyl ketone and D-Melezitose). It is worth noting that Paromomycin and Furyl hydroxymethyl ketone are, most probably, products of fermentation in BK since they were detected only from T3 of BK (Supplementary Material Table 06).

In addition to the presence of ethanol, organic acids reducing sugars caffeine, and other polyphenols that were quantitatively determined previously in our study, the presence of products related to furfural formation/degradation during kombucha fermentation was also observed by GC-MS (Supplementary Material Table 05). **Furfural** is naturally found in many fruits and leaves, and malt (Nonato, 1999; National Center for Biotechnology Information - PubChem, 2018). It is mainly derived from pentose dehydration and **5-(hydroxymethyl)-2-furaldehyde (HMF)** is formed from dehydration of hexoses during sugar degradation in an acid treatment (Boopathy et al., 1993). **HMF** can be transformed into furfural and then **furan**, and **formic acid** (Yan et al., 2014). There is a conversion between **furfural** and **furfuryl alcohol**, and both were detected in all the samples (inoculum, GK, and BK), and time points analyzed. In beer, furfural and HMF are originated from the Maillard reaction between reducing sugars (hexose/pentose) and theanine (Baert et al., 2012). The **5-Acetoxymethyl-2-furaldehyde** is a derivative of HMF and was not detected in the inoculum, but it was in GK and BK along the fermentation process, indicating that the presence of this compound may be

more related to the chemical composition of the prepared sugared teas (green and black) than to the microbial fermentation process. The 5-Acetoxymethyl-2-furaldehyde has been described in traditional balsamic vinegar (TBV) final product (Pinuh et al., 2016). It was initially described as a sweet taste enhancer in TBV and generated by esterification of the HMF with acetic acid originated from acetic acid fermentation (Hillmann et al., 2012).

Interestingly, **the furyl hydroxymethyl ketone**, was detected only in BK samples from T3-T15 (but not at T0), indicating that its presence may be related, besides to the presence of its precursors, to the metabolism of furfural degradation by the BK microbiota (Supplementary Material Tables 05 and 06). Similarly to **furyl hydroxymethyl ketone**, other 3 compounds were detected only in BK: **2-hydroxyacetaldehyde (Glycolaldehyde), benzene-1,4-diol (hydroquinone), and paromomycin.**

Glycolaldehyde is a stronger growth inhibitor than other reported major fermentation inhibitors such as 5-hydroxymethyl furfural (5-HMF) and furfural (Klinke et al., 2004; Jayakody et al., 2011). It is involved in the Maillard reaction, and is much stronger than that of glucose (Hayashi and Namiki, 1986). In a model wine system, glycolaldehyde was able to induce browning of (+)-catechin (Flamini and Vedova, 2003), which indicates its participation in the development of the brown coloration of the cellulose pellicule in BK. **2-hydroxyacetaldehyde (Glycolaldehyde)** (detected only in BK) can be turned into **acetaldehyde** and then subsequently into **acetic acid** (Lin, 2013; Wawrzetz et al., 2010).

Hydroquinone is a major constituent of several food wheat products, pears, and coffee and tea, red wine and broccoli (Deisinger, 1996). In our study, hydroquinone was found **only in BK** (Supplementary Material Table 05 and 06)**.**

Paromomycin is an aminoglycoside antibiotic described as produced by *Streptomyces rimosus* var. *paromomycinus*, with antimicrobial and amoebicidal activity (National Center for

Biotechnology Information- PubChem, 2018). It was detected in BK fermentation at 3, 7 and 15 days (Supplementary Material Table 05 and 05). Further studies should be conducted to verify the origin of this antibiotic, and this is the first report of its presence in kombucha fermentation **(BK)**.

D-Melezitose was detected **only in GK** (Supplementary Material Table 05 and 06)**.** Melezitose is a non-reducing trisaccharide sugar produced by many insects that feed on plant sap (Fischer and Shingleton, 2001). Another sugar detected was **dihydroxyketone**, a simple three-carbon sugar resulting from the oxidation of glucose by the action of acetic acid bacteria of the genus *Acetobacter* (Buckley, 1989). As *Komagataeibacter* spp. this compound was present in all the samples.

The presence of **propanol** was detected in the inoculum and in GK, but not in BK (Supplementary Material Table 05 and 06). It appears that the presence of 1-propanol is related to the raw material. In addition, fermentation conditions such as wort aeration, low pH (3.0 to 4.2) and fermentation temperatures between 28 to 30° C (Nonato, 1999) also need to be considered, as well as the microbiota.

1,1-Diethoxyethane (acetal, acetaldehyde diethyl acetal), was detected only in inoculum and BK (Supplementary Material Table 05 and 06). It is formed by the reaction of acetaldehyde with ethanol, and because of herbaceous odor, it is considered an important aromatic compound (Peinado et al., 2004; Christoph and Christoph, 2007; Karimali et al., 2020).

1,3-dihydroxypropan-2-one, an intermediate product of fructose metabolism, is cited as produced via retro-aldol fragmentation (Tuteja et al., 2012). It can be converted to **1-hydroxypropan-2-one**, and then to **propan-1-ol** (Gandarias et al., 2010). In our study, **1,3-dihydroxypropan-2-one** and **1-hydroxypropan-2-one** were detected by GC-MS in both

kombuchas (GK and BK), but **propan-1-ol** was detected only in inoculum and GK (Supplementary Material Table 05 and 06). Since **ethanol** can be formed from the hydrogenation of **propanol** (Lin, 2013), maybe it could contribute to the higher ethanol concentration in GK (Figure 04; Supplementary Material Table 02) than BK.

3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one has been found in flavonoid fraction of plants (Prabhu et al., 2020) and has antimicrobial and anti-inflammatory properties (Kannan et al., 2012).

In general, the compounds identified by GC-MS during kombucha fermentation (GK and BK) are responsible for the sensory formation of the product and may have antimicrobial activity.

Based on integrated analyzes of the physicochemical and microbial results of GK and BK using principal component analysis (PCA), there is a clear separation of physicochemical parameters of GK from BK, but not by the microbiota itself (Figure 5). **Caffein, gallic acid, and chlorogenic acids** were the physicochemical variables that most contributed to the separation of GK and BK in two distinct groups (Supplementary Material Figure 03 A). Moreover, the **total reducing sugars and titratable total acidity** showed the highest contribution to separate the samples based on the fermentation time point (Supplementary Material Figure 03 B).

The physicochemical differences between the two kombuchas can be observed also during fermentation (Figure 4 and 5; Supplementary Material Table 02-06). These clear, distinct, and dynamic profiles of GK and BK fermentation seem to be the result of the intrinsic characteristics of each type of tea (green and black teas), as well as the contribution of the kombucha microbial metabolism (Figure 5).

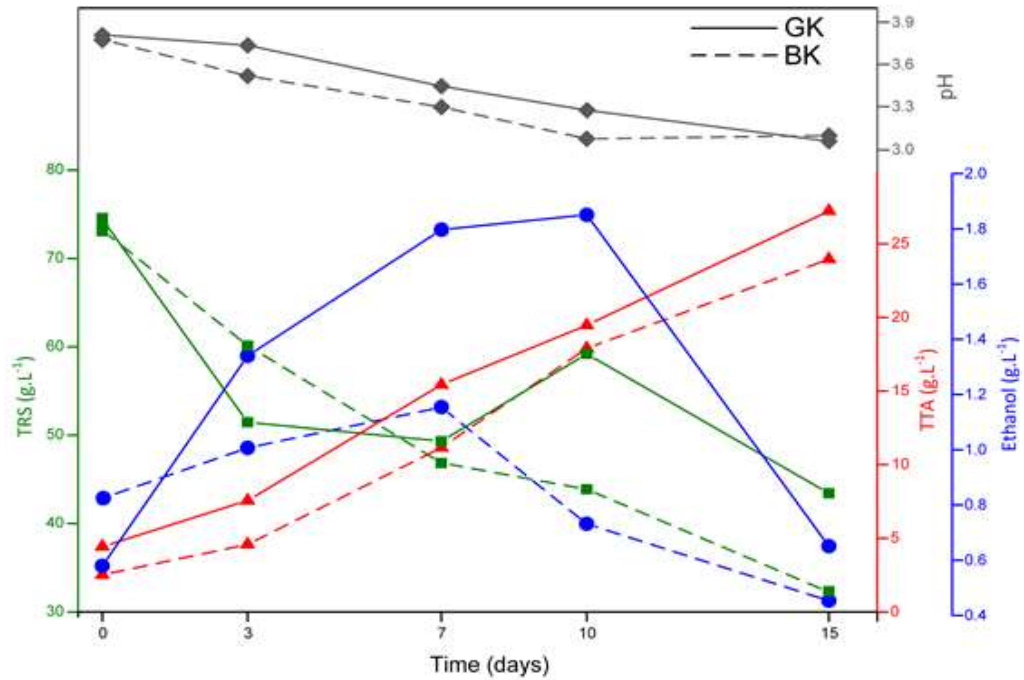

Figure 04 - pH and ethanol, organic acid, and total reducing sugar concentrations for 15 days of monitoring of kombucha fermentation process produced with green (GK) (filled lines) and black (BK) (dashed lines) teas, both at 28 °C. (COLOR SHOULD BE USED)

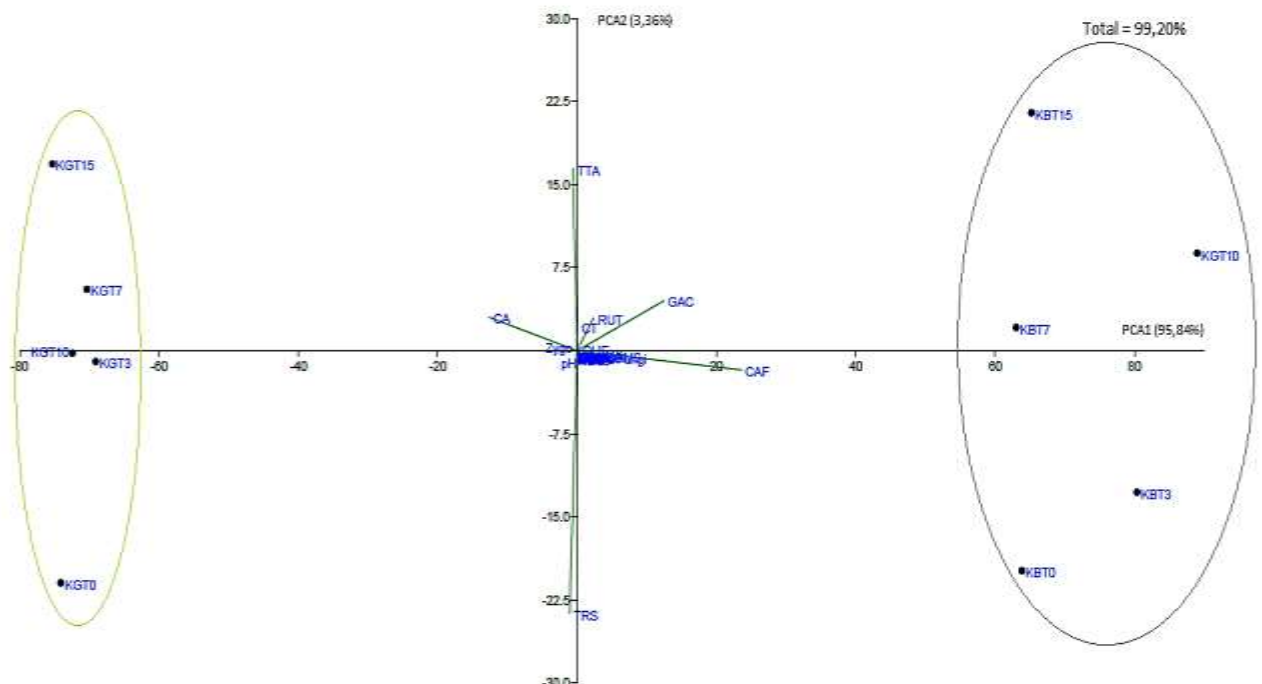

Figure 05 - Principal Component Analysis (PCA) of bacterial and fungal reads at genera level and physicochemical variables of GK and BK kombucha fermentations in different times (T0, T3, T7, T10, T15). (COLOR SHOULD BE USED)

Legend: TRS: Total Reducing Sugars; ETOH: Ethanol detected quantitatively; pH: hydrogen potential; TTA: Total Titratable Acidity; GAC: Gallic Acid; CT: Catechin detected quantitatively; CA: Chlorogenic Acid; CAF: Caffein; RUT: Rutin; QUE: Quercetin; Komag: *Komagataeibacter* spp.; Zygo: *Zygosaccharomyces* spp.; Malas: *Malassezia* spp.; Rhodo: *Rhodotorula* spp.; Other Fungi: other fungi; Acet: Acetal; EtOH: Ethanol detected by GC-MS; PpO: Propanol; HQ: Hydroxyacetone; GlyAl: Glycolaldehyde; AA: Acetic acid; FurfAL: Furfural; FA: Formic acid; furfYL: Furfuryl alcohol; DHA: Dihydroxyacetone; 5A2F: 5-Acetoxymethyl-2-furaldehyde; HMF (5-hydroxymethylfurfural); HQ: Hydroquinone; CAF-GCMS: Caffeine detected by GC-MS; Paro: Paromomycin; FurHMK: Furyl hydroxymethyl Ketone; 4HPyran: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; Mel: D-Melezitose.

Regarding microbial amplicon sequencing results, correlation analysis (Figure 06) showed that, in general, the presence of *Komagataeibacter* was positively correlated ($p<0.05$) with ethanol and catechins. For *Zygosaccharomyces*, no correlation was significant when paired with all variables studied ($p>0.05$). *Malassezia*, *Rhodotorula*, and other fungi were negatively related to *Komagataeibacter*, indicating that these bacteria are likely to play an important role in controlling the contaminating community of kombucha (non-*Zygosaccharomyces* fungi) (Figure 6).

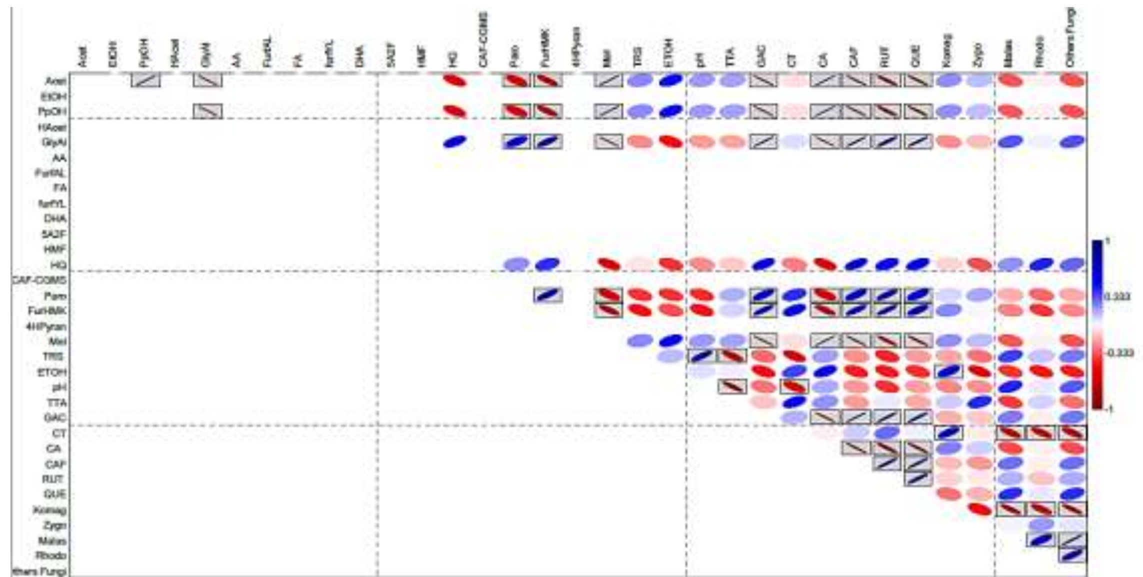

Figure 06 - Correlation analysis of bacterial and fungal reads at genus level and physicochemical variables in each stage from GK and BK fermentation sample groups. (COLOR SHOULD BE USED)

Legend: TRS: Total Reducing Sugars; ETOH: Ethanol detected quantitatively; pH: hydrogen potential; TTA: Total Titratable Acidity; GAC: gallic acid; CT: catechin detected quantitatively; CA: chlorogenic acid; CAF: caffein; RUT: rutin; QUE: quercetin; Komag: *Komagataeibacter* spp.; Zygo: *Zygosaccharomyces* spp.; Malas: *Malassezia* spp.; Rhodo: *Rhodotorula* spp.; Others Fungi: others fungi; Acet: Acetal; EtOH: Ethanol detected by GC-MS; PpO: propanol; HQ: Hydroxyacetone; GlyAl: glycolaldehyde; AA: Acetic acid; FurfAL: Furfural; FA: formic acid; furfYL: furfuryl alcohol; DHA: Dihydroxyacetone; 5A2F: 5-Acetoxymethyl-2-furaldehyde; HMF (5-hydroxymethylfurfural); HQ: hydroquinone; CAF-CGMS: caffeine detected by GC-MS; Paro: Paromomycin; FurHMK: Furyl hydroxymethyl ketone; 4HPyran: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; Mel: D-Melezitose. Blue ellipses are positive correlation, Red ellipses are negative correlations, and ellipses (either blue and red) inside grey boxes are statistical significant correlations ($p<0.05$). Note: As this test is not applicable to qualitative variables (e.g.: FurA, FAL, etc.), no ellipses are depicted in the plot for those qualitative variables.

Finally, kombucha seems to be a quite stable microbial system in which very adapted microorganisms are responsible for the low diversity (composition, richness, and abundance) specially along fermentation time. A little higher diversity was observed in the initial and end times (T0 and T15) of the fermentation of both kombuchas. At the beginning, they can reflect the plant and production process-associated microbiota, as well as the practices during harvesting and handling of tea leaves. Microorganisms that are able to remain viable, but not

detectable during fermentation, have the chance to reappear at the end of fermentation when selective pressures, such as ethanol concentrations in the system, decrease.

Several studies reported that the microbiota that make up the kombucha inoculants can vary according to the source and origin of the inoculum, the fermentation temperature, and the manipulation of the inoculum (Jarrell et al., 2000; Mo et al., 2008; Jayabalan et al., 2014; Marsh et al., 2014; De Filippis et al., 2018). Comparing our results with those in the literature, these results are in accordance with Amy et al. (2019) for kombucha bacterial diversity, which point that there is no apparent "canonical" species composition across all the substrates and all culture conditions is reinforced. Furthermore, the whole environment has an impact on the composition of community members. Nevertheless, considering the constant citation in the specialized literature, as well as the update in the nomenclature of some AAB groups, it seems that a frequent microbial core for kombucha may be the combination of *Komagataeibacter* and a yeast capable of carrying out alcoholic fermentation.

4. Conclusions

Komagataeibacter and *Zygosaccharomyces* genera were the most frequent bacterial and fungal genera that occurred in GK and BK kombucha during all the fermentation times, considering both dependent- and independent-cultive methods.

Ordination analysis (PCA) showed a clear separation of GK and BK groups during all the fermentation process, and the physicochemical features that most influenced in this separation were gallic acid, caffein, and chlorogenic acids. Additionally, *Komagataeibacter* and catechins seem to exert selective pressure against other microorganisms.

It should be noted that most of the compounds detected by GC-MS mentioned herein, whether from the tea itself or from the fermentative microbiota, have antimicrobial activity

already described in the literature. Their presence, allied to the increasing and high concentration of organic acids, low pH, and high concentrations of alcohols and phenolic compounds, make kombucha an extremely selective environment where only the very well adapted microorganisms can stand out.

This study provides important contributions to the knowledge about the profile of the integrated microbiota to the physicochemical results of the kombucha fermented in green tea and black tea. Based on our results and published literature on kombucha microbiota, we suggest, for future studies, the use of a starter culture for the production of kombucha composed of species of *Komagataeibacter*, producers of cellulose biofilm, combined with an acid resistant yeast, like *Z. bailii*, or another one that be able of carrying out alcoholic fermentation from sacarose, fructose and/or glucose.

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6. Declarations of interest: none.

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Supplementary Material

Supplementary Material Table 01 – Number and species of bacteria and fungi isolated, and OTUs (genus level) and reads of amplicons in kombucha in green tea (GK) and black tea (BK) during 15 days of fermentation.

Bacteria

	GK					BK					
Isolated species	**T0**	**T3**	**T7**	**T10**	**T15**	**T0**	**T3**	**T7**	**T10**	**T15**	Total of isolates /species
K. hansenii	14	6	3	1	5	7	1	0	1	4	42
K. europaeus	2	2	0	0	14	1	1	0	1	3	24
K. xylinus	2	0	1	1	7	0	0	1	0	3	15
K. rhaeticus	3	0	0	0	4	0	0	0	0	1	8
S. melonis	4	0	0	0	3	1	0	0	0	0	8
Komogataeibacter sp.	1	0	0	0	2	0	0	0	0	1	4
K. intermedius	0	0	0	0	0	0	0	0	1	0	1
Total	26	8	4	2	35	9	2	1	3	12	102
OTU/reads	**T0**	**T3**	**T7**	**T10**	**T15**	**T0**	**T3**	**T7**	**T10**	**T15**	Total of reads/ OTUs
Komagataeibacter spp.	12470	19464	19416	28029	25865	19022	23274	19390	29488	21153	217571
Stenotrophomonas sp.	0	0	0	0	0	0	0	47	0	0	47
Total	12470	19464	19416	28029	25865	19022	23274	19437	29488	21153	217618

Fungi

Isolated species	**T0**	**T3**	**T7**	**T10**	**T15**	**T0**	**T3**	**T7**	**T10**	**T15**	Total of isolates /species
Z. bailli	6	3	2	1	4	3	1	2	1	1	24
R. mucilaginosa	0	0	0	1	3	0	0	0	0	0	4
S. cerevisiae	0	1	1	1	0	0	0	0	0	0	3

	T0	T3	T7	T10	T15	T0	T3	T7	T10	T15	
Total	6	4	3	3	7	3	1	2	1	1	31

OTU/reads (Filo)	**T0**	**T3**	**T7**	**T10**	**T15**	**T0**	**T3**	**T7**	**T10**	**T15**	**Total of reads/ OTUs**
Zygosaccharomyces	51	26	3	16	96	7	9	6	14	124	352
*Malassezia**	22	2	0	0	12	177	0	0	4	45	262
Rhodotorula	2	0	0	0	29	23	0	0	0	4	58
Penicillium	0	0	0	0	3	17	0	2	0	0	22
Aspergillus	0	3	0	0	0	12	0	2	0	0	17
Fusicolla	5	0	0	0	0	12	0	0	0	0	17
Itersonilia	0	0	0	0	5	2	0	0	0	6	13
Cladosporium	0	0	0	0	8	4	0	0	0	0	12
Cyphellophora	0	0	0	0	0	11	0	0	0	0	11
Debaryomyces	0	0	0	0	0	0	0	0	0	11	11
Total	80	31	3	16	153	265	9	10	18	190	775

* 2 OTU of *Malassezia* genus were considered here as one unit.

Supplementary Material Table 02 – Physicochemical analyses of kombucha produced in green (GK) and black (BK) teas during 15 days of fermentation.

VARIABLES

	pH		**TTA** (g. L^{-1})		**TRS** (g. L^{-1})		**Ethanol** (g. L^{-1})	
Time (days)	**GK**	**BK**	**GK**	**BK**	**GK**	**BK**	**GK**	**BK**
T0	3.81	3.78	4.451	2.514	74.464	73.210	0.576	0.824
T3	3.73	3.52	7.577	4.582	51.489	60.131	1.341	1.007
T7	3.45	3.30	15.438	11.158	49.339	46.870	1.797	1.154
T10	3.27	3.07	19.478	17.902	59.215	43.883	1.852	0.731
T15	3.05	3.09	27.236	23.954	43.445	32.258	0.651	0.453

Legend: TTA: Total Titratable Acidity; TRS: Total Reducing Sugars.

Supplementary Material Table 03 - Phenolic compounds analyses (mg. L^{-1}) of kombucha produced in green (GK) and black (BK) teas during 15 days of fermentation.

	Phenolic compounds											
	Caffeine		**Chlorogenic acids**		**Catechin**		**Gallic acid**		**Quercetin**		**Rutin**	
Time (days)	**GK**	**BK**	**GK**	**BK**	**GK**	**BK**	**GK**	**BK**	**GK**	**BK**	**GK**	**BK**
T0	69.05	182.34	58.09	2.23	5.44	2.99	6.49	61.07	0.13	1.29	15.13	24.44
T3	78.22	198.57	66.44	2.58	6.01	6,61	7,58	69,00	0.29	1,11	16.69	27.11
T7	77.41	178.21	68.00	2.56	8.05	7.77	7.75	65.77	0.16	0.98	17.18	25.58
T10	74.50	204.82	66.42	2.68	7.67	8.28	7.80	76.21	0.42	1.20	16.96	31.61
T15	69.54	177.37	65.46	2.51	5.51	8.00	8.52	71.04	0.13	1.22	16.53	30.19

Supplementary Material Table 04 - Multiple linear regression for models of 1º and 2º degree from the variables analyzed in green tea (GK) and black tea (BK) in two confidence intervals.

	First Degree				Second Degree			
Variable	R^2	R^2adj	p 0.01	p 0.05	R^2	R^2adj	p 0.01	p 0.05
TRS	0.4372	0.4175	ns	**	0.4507	0.4180	ns	**
E	0.1252	0.0947	**	**	0.4365	0.4029	**	**
pH	0.8121	0.8056	**	**	0.8565	0.8479	**	**
TTA	0.8845	0.8805	**	**	0.8856	0.8787	**	**
GA	0.9900	0.9897	**	**	0.9920	0.9915	**	**
Ct	0.3278	0.3044	ns	ns	0.6103	0.5871	ns	ns
CA	0.9950	0.9948	**	**	0.9983	0.9982	**	**
Cf	0.9742	0.9733	**	**	0.9801	0.9789	**	**
R	0.9481	0.9463	**	**	0.9541	0.9514	**	**
Q	0.9265	0.9239	**	**	0.9518	0.9489	**	**

Supplementary Material Table 05 – GC-MS compounds detetion in the kombucha inoculum, and kombucha produced in green (GK) and black (BK) teas during 15 days of fermentation

Compound IUPAC*	Synonym	Inoc	GK	BK
1,1-diethoxyethane	Acetal	Y	N	Y
Ethanol	Ethanol	Y	Y	Y
propan-1-ol	propanol	Y	Y	N
1-hydroxypropan-2-one	Hydroxyacetone	Y	Y	Y
2-hydroxyacetaldehyde	glycolaldehyde	N	N	Y
Acetic acid	Acetic acid	Y	Y	Y
furan-2-carbaldehyde	Furfural	Y	Y	Y
Formic acid	formic acid	Y	Y	Y
furan-2-ylmethanol	furfuryl alcohol	Y	Y	Y
1,3-dihydroxypropan-2-one	Dihydroxyacetone	Y	Y	Y
(5-formylfuran-2-yl)methyl acetate	5-Acetoxymethyl-2-furaldehyde	N	Y	Y
5-(hydroxymethyl)furan-2-carbaldehyde	5-hydroxymethylfurfural	Y	Y	Y
benzene-1,4-diol	hydroquinone	N	N	Y
1,3,7-trimethylpurine-2,6-dione	caffeine	Y	Y	Y
(2*S*,3*S*,4*R*,5*R*,6*R*)-5-amino-2-(aminomethyl)-6-[(2*R*,3*S*,4*R*,5*S*)-5-[(1*R*,2*R*,3*S*,5*R*,6*S*)-3,5-diamino-2-[(2*S*,3*R*,4*R*,5*S*,6*R*)-3-amino-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-6-hydroxycyclohexyl]oxy-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl]oxyoxane-3,4-diol	Paromomycin	N	N	Y
1-(furan-2-yl)-2-hydroxyethanone	Furyl hydroxymethyl ketone	N	N	Y
3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Y	Y	Y
2-[4-hydroxy-2,5-bis(hydroxymethyl)-2-[(2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-	D-Melezitose	N	Y	N

6-(hydroxymethyl)oxan-2-yl]oxyoxolan-3-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol

Legenda: Inoc: inoculum; Y: detected; N: not detected under the conditions analysed.

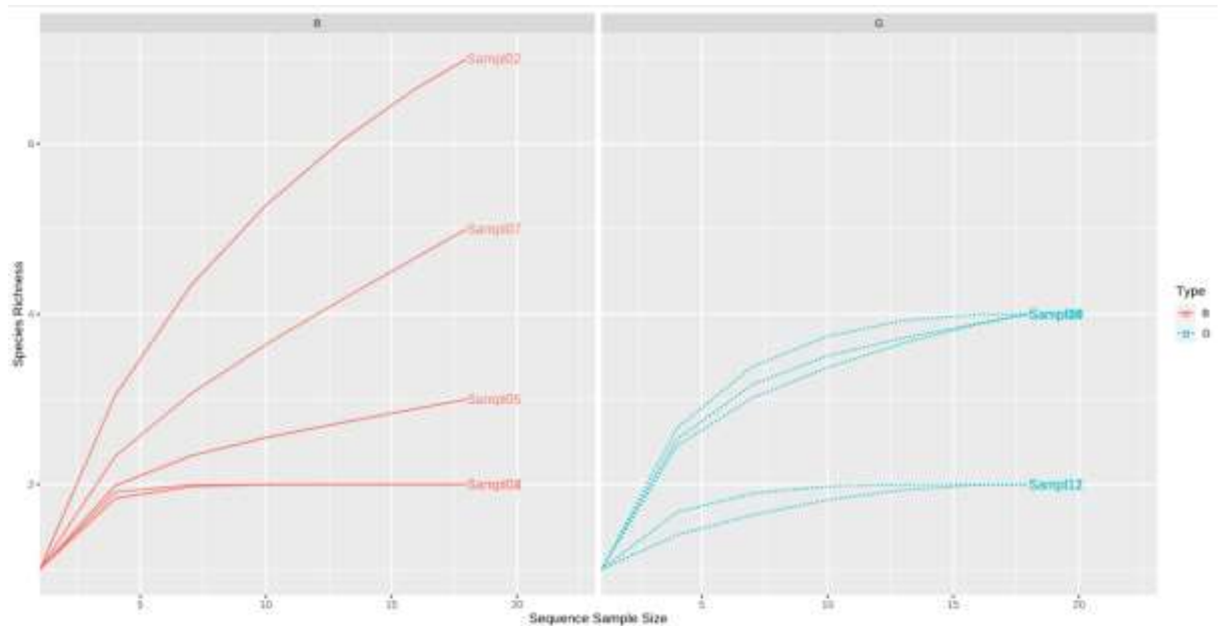
*Selected compounds with at least 50% of identification (Rigobello et al., 2015).

Supplementary Material Table 06 – Compounds differently detected in kombucha produced with green tea (GK) and black tea (BK) and inoculum by GC-MS

Compound	Inoc	GK					BK				
		T0	T3	T7	T10	T15	T0	T3	T7	T10	T15
Acetal	*	–	–	–	–	–	*	*	*	*	*
Glycolaldehyde	–	–	–	–	–	–	*	*	*	*	*
Hydroquinone	–	–	–	–	–	–	*	*	*	*	–
Paromomycin	–	–	–	–	–	–	–	*	–	*	*
Furyl hydroxymethyl ketone	–	–	–	–	–	–	–	*	*	*	*
D-Melezitose	–	*	*	*	*	*	–	–	–	–	–
5-Acetoxymethyl-2-furaldehyde	–	*	*	*	*	*	*	*	*	*	*
Propanol	*	*	*	*	*	*	–	–	–	–	–

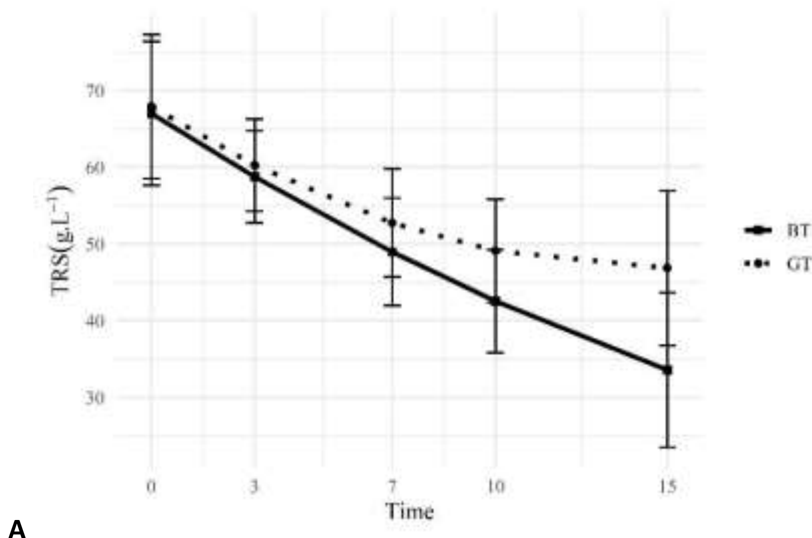
Legenda: Inoc: inoculum; *: detected; - : not detected.

Supplementary Material Figure 01 - Rarefaction curves of amplicons in black (BK) and green (GK) kombucha.



Legend: Sampl02, 03, 04, 05, 07 and Sampl08, 09, 10, 11, 12 correspond to samples collected in T0, T3, T7, T10 and T15 days from BK and GK, respectively. (COLOR SHOULD BE USED)

Supplementary Material Figure 02 – Second-order linear regression of variables. (a) total reducing sugars, (b) pH, (c) ethanol, (d) total tiltable acids, (e) gallic acid, (f) Catechin, (g) Caffein, (h) chlorogenic acids, (i) rutin, and (j) quercetin during fermentation time points for two types of kombucha green (GK) and black kombucha tea (BK). The bars are the 95% confidence intervals.



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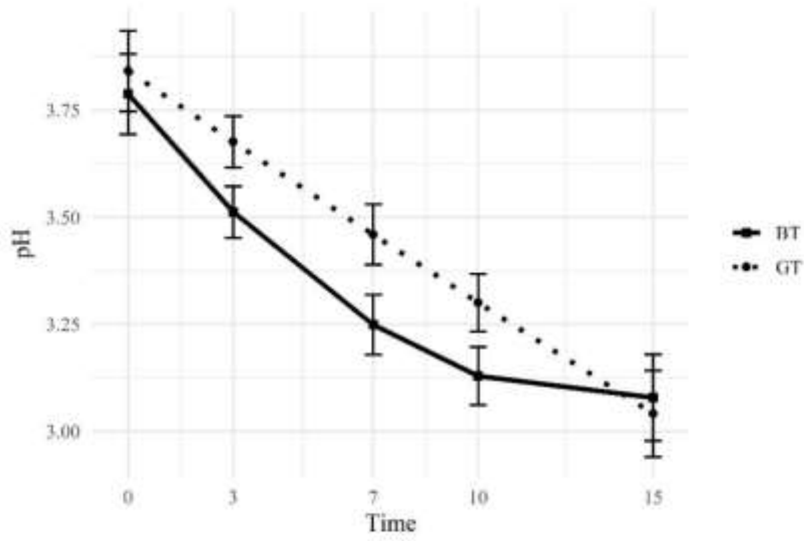

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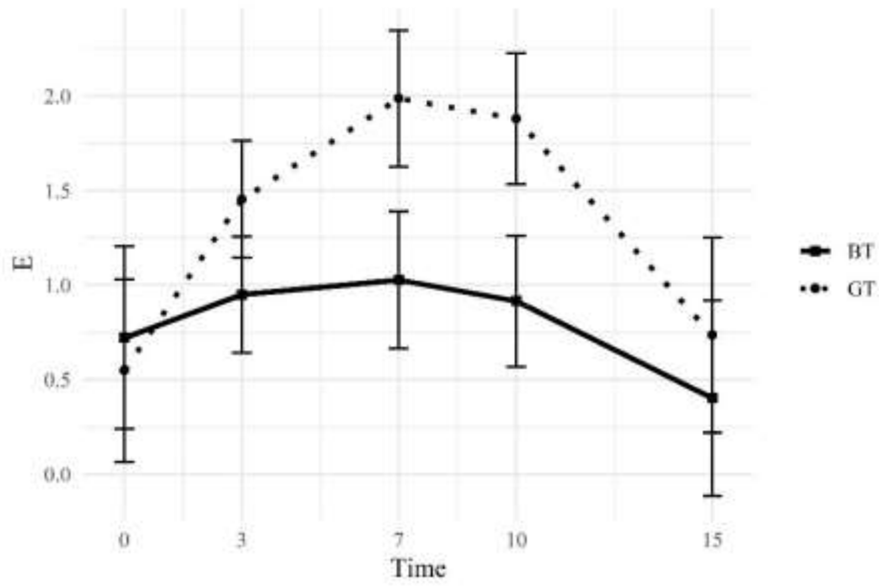

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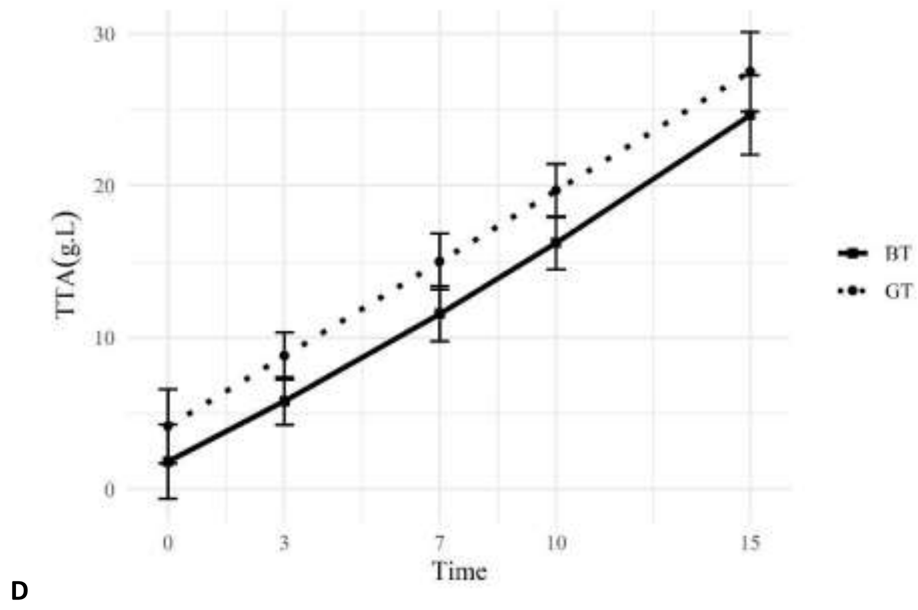

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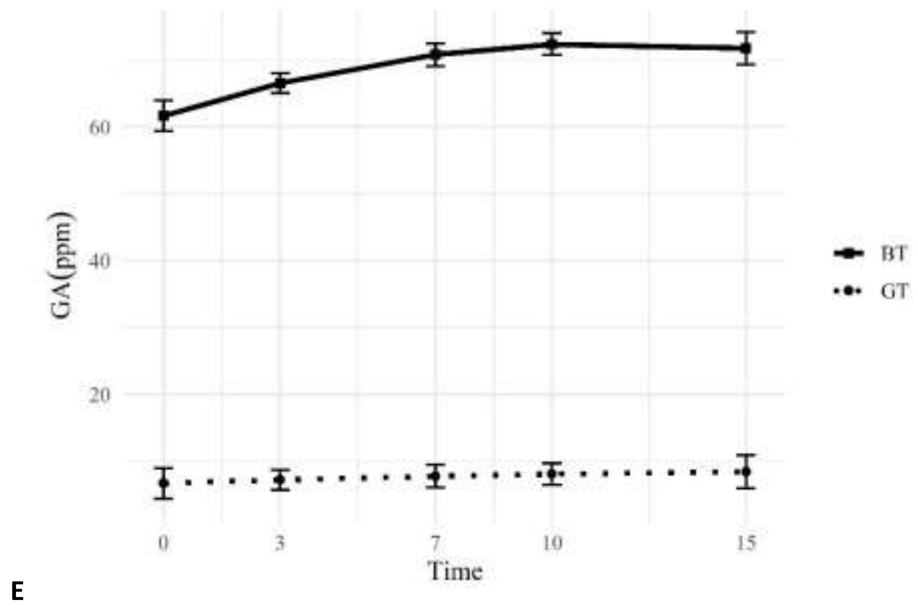

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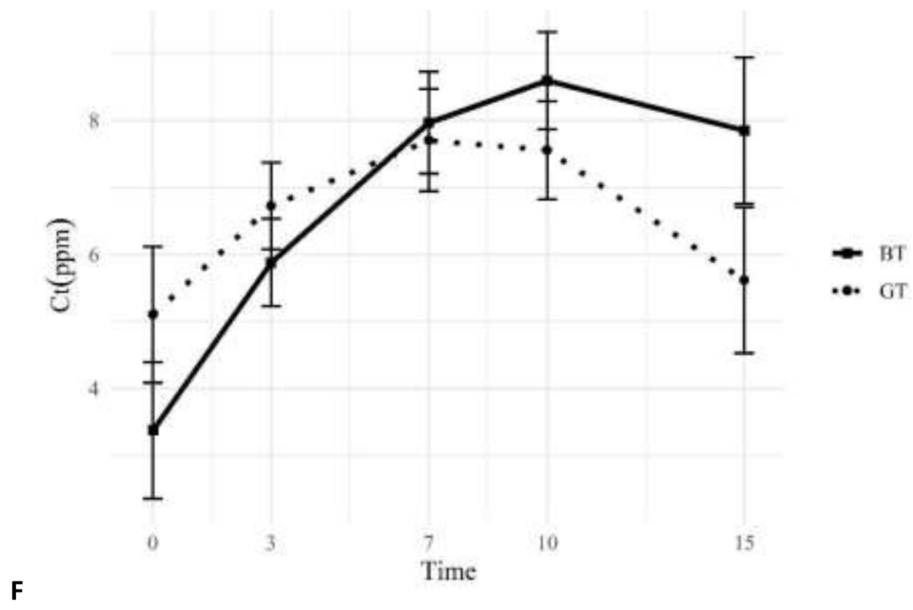

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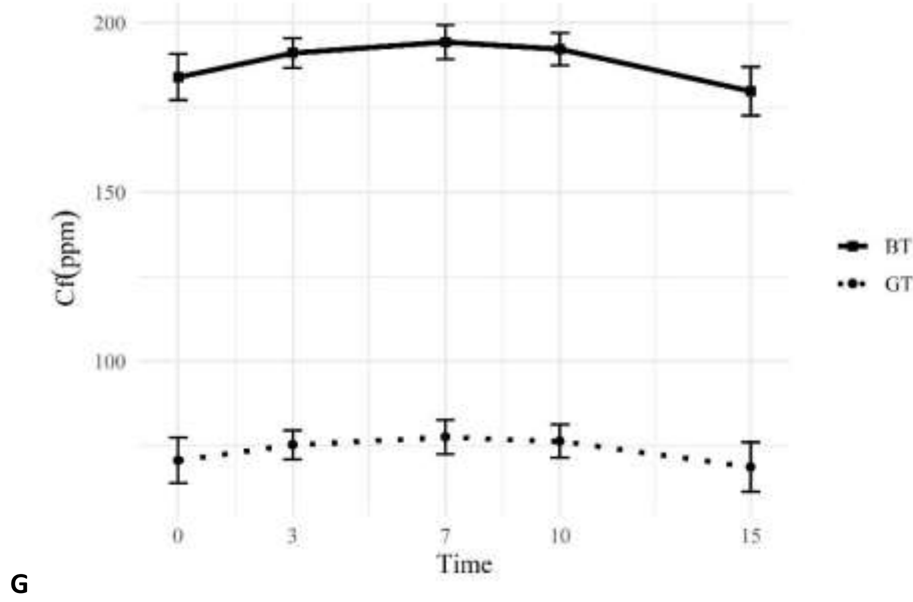

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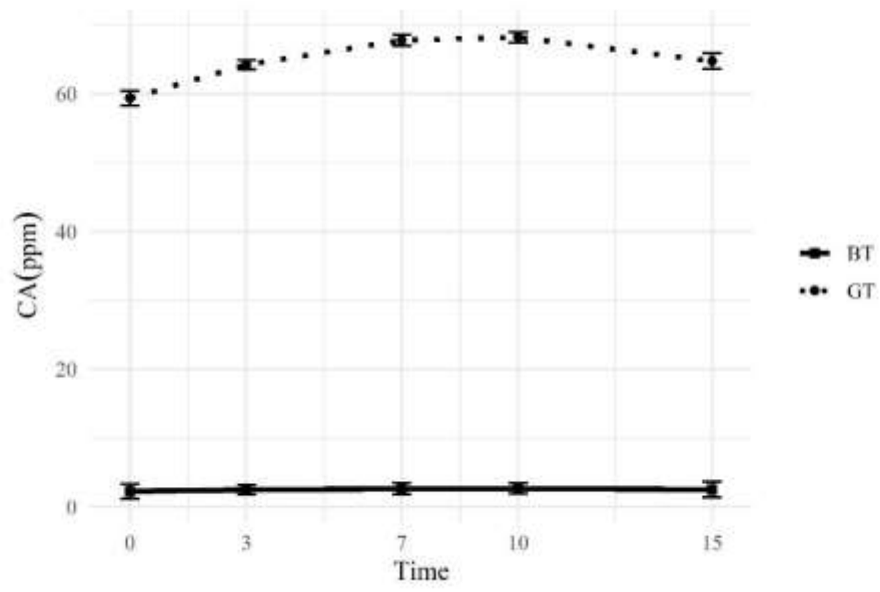

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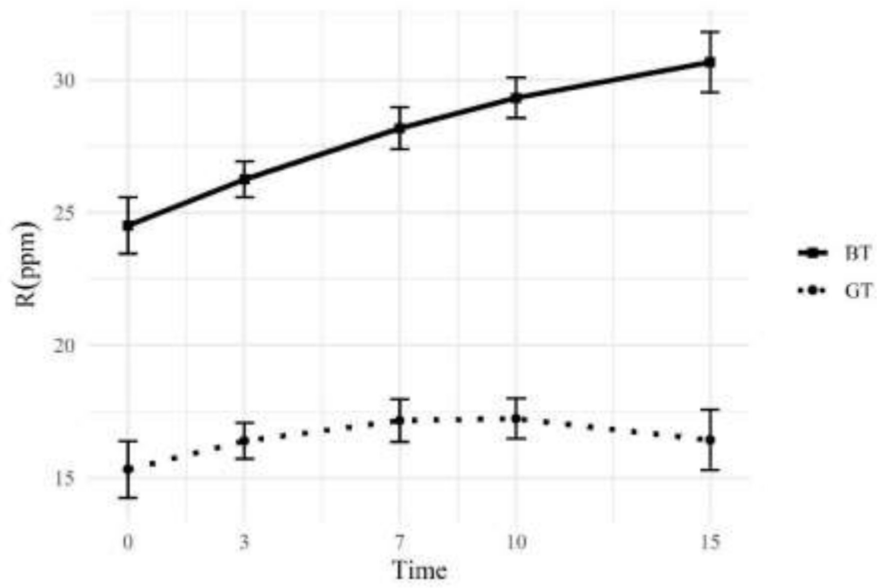

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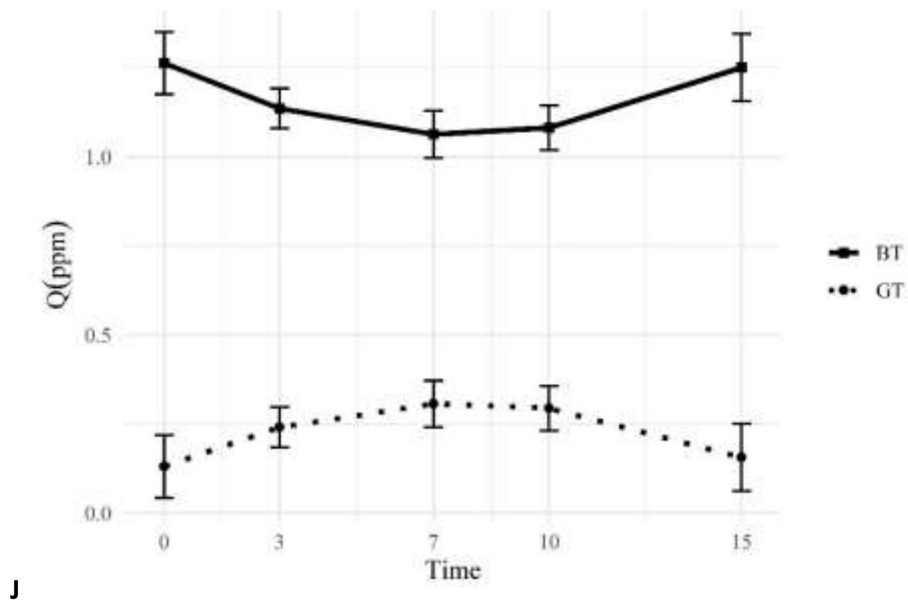
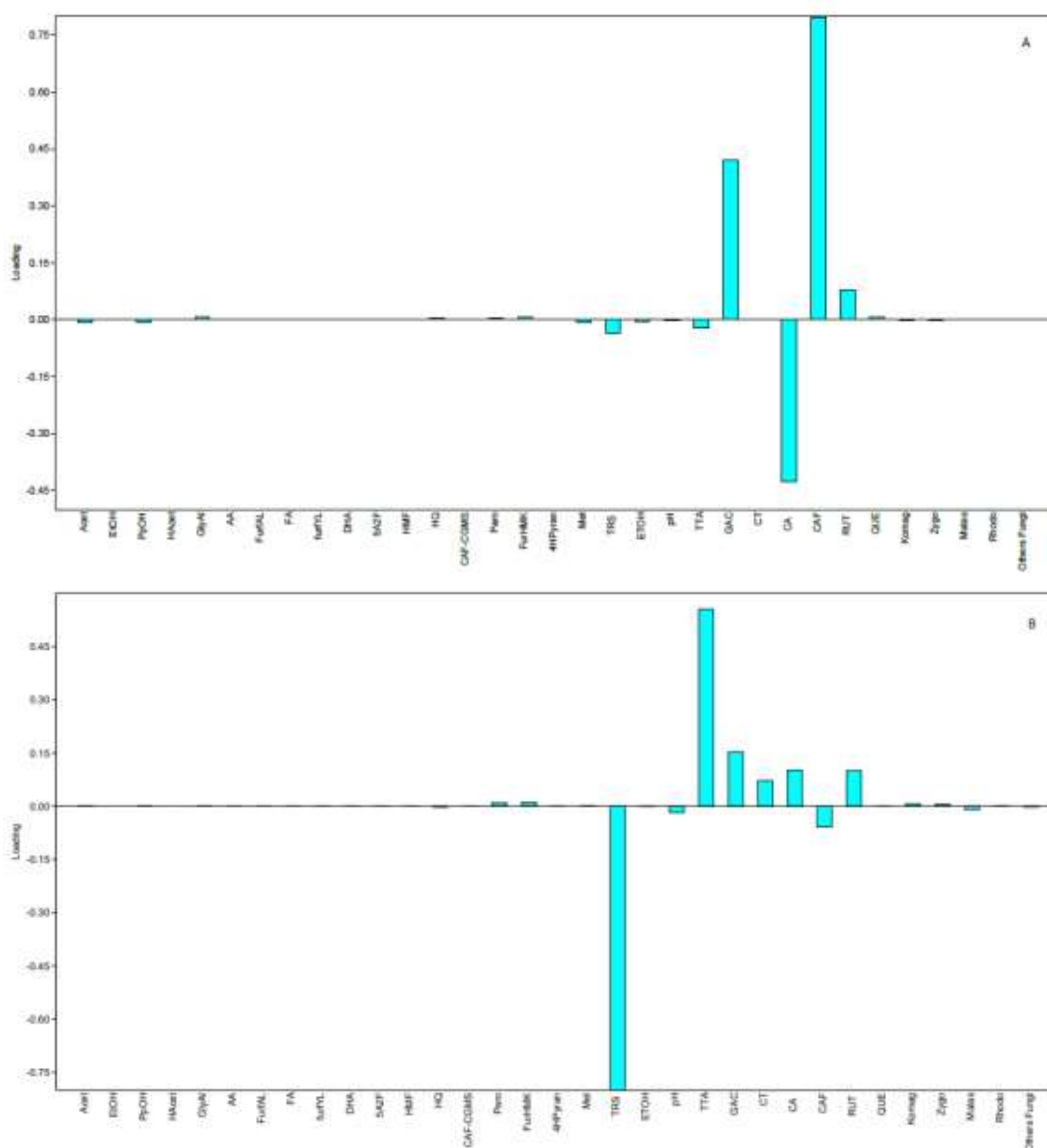


J

Supplementary Material Figure 03 - Correlation of PC1 (A) and PC2 (B) of physical-chemical and microbial results of fermentation of green (GK) and black (BK) kombucha tea



Legend: A: PC1; B: PC2; TRS: Total Reducing Sugars; ETOH: ethanol detected quantitatively; pH: hydrogen potential; TTA: Total Titratable Acidity; GAC: gallic acid; CT: catechin detected quantitatively; CA: chlorogenic acid; CAF: caffein; RUT: rutin; QUE: quercetin; Komag: *Komagataeibacter* spp.; Zygo: *Zygosaccharomyces* spp.; Malas: *Malassezia* spp.; Rhodo: *Rhodotorula* spp.; Others Fungi: others fungi; Acet: Acetal; EtOH: Ethanol detected by GC-MS; PpO: propanol; HQ: Hydroxyacetone; GlyAl: glycolaldehyde; AA: Acetic acid; FurfAL: Furfural; FA: formic acid; furfYL: furfuryl alcohol; DHA: Dihydroxyacetone; 5A2F: 5-Acetoxymethyl-2-furaldehyde; HMF (5-hydroxymethylfurfural); HQ: hydroquinone; CAF-CGMS: caffeine detected by GC-MS; Paro: Paromomycin; FurHMK: Furyl hydroxymethyl ketone; 4HPyran: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; Mel: D-Melezitose.

CHAPTER II

Data fusion of UPLC data, NIR spectra and physicochemical parameters with chemometrics as an alternative to evaluating kombucha fermentation

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Data fusion of UPLC data, NIR spectra and physicochemical parameters with chemometrics as an alternative to evaluating kombucha fermentation

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Abstract

Kombucha consumption has become popular due to health benefits and sensorial properties. Different analytical techniques were associated with unsupervised chemometrics to evaluate kombucha fermentation using a low-level data fusion strategy. Kombucha was prepared from green and black teas (*Camellia sinensis*). The samples were analyzed by ultra-performance liquid chromatography (UPLC), portable near-infrared spectroscopy (NIR), and physicochemical analyses (ethanol, pH, total reducing sugars, and total titratable acidity). Also, the bioactive phenolic compounds were evaluated in the fermented samples. The merged results were analyzed by principal component analysis (PCA). The PCA was applied to discriminate substrate for kombucha production and the fermentation time. Although the fermentation behavior was similar for both substrates, the kombucha showed quantitative differences in physicochemical parameters. They also showed differences in bioactive compounds for each kombucha. Data fusion strategy was more effective to differentiate the two types of kombucha than single analysis. The portable NIR can be a reliable and robust analysis for fermentation monitoring. Moreover, the results can be used as a base to further studies and mainly to help in which substrate composition is better to be produced on an industrial scale.

Keywords: Fermented beverage; Bioactive compounds; PCA analysis; acetic acid bacteria.

1. Introduction

Kombucha is an Asiatic beverage obtained through the fermentation of sugared tea infusion, usually prepared with *Camellia sinensis*. The characteristics flavor and aroma of kombucha are sweet, slightly acidic, and naturally sparkling (Jayabalan et al., 2011; Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014). The substrate composition, e.g., black or green tea, can provide some biologically active substances, including polyphenols, flavonoids, and phenolic acids. These substances are known to be antioxidant, antimicrobial, anti-inflammatory, and anti-carcinogenic activities. Therefore, health benefits may be associated with kombucha consumption (Sreeramulu, Zhun, & Knol, 2000; Banerjee et al., 2010; Jayabalan et al., 2011; Jayabalan et al., 2014; Shahidi, & Ambigaipalan, 2015; Bhattacharya et al., 2016).

Several factors affect kombucha composition and sensorial characteristics such as the substrate, source, inoculum viability, temperature, and cell growth (Sreeramulu et al., 2000; Jayabalan et al., 2008; Jayabalan et al., 2011; Jayabalan et al., 2014; De Filippis, Troise, Vitaglione, & Ercolini, 2018). During the fermentation, the combined effects of microbial growth and substrate are among the main factors to influence the production of different metabolite profiles. Variation in substrate composition may lead to different pathways during fermentation. To several biochemical reactions occur in formation ethanol, bioactive substances, and volatile organic compounds (Battikh, Bakhrouf, & Ammar, 2012; Neffe-Skocińska, Sionek, Ścibisz, & Kołożyn-Krajewska, 2017).

The chemical composition encompasses organic acids, sugars, vitamins, biogenic amines, pigments, lipids, proteins, some hydrolytic enzymes, ethanol, antimicrobial

compounds, carbon dioxide, and some tea polyphenols – in green tea: catechin and epicatechin; in black tea: quercetin (Jayabalan et al., 2014, Malbaša, Lončar, & Kolarov, 2004).

The kombucha is a complex food matrix in which the determination of its quality requires a holistic characterization. Thus, a single parameter or analytical technique may not be sufficient for a comprehensive evaluation of fermentation. In this case, a combination of different analytical methods may be interesting to study the relationship of distinct chemical information on the same samples, aiming to evaluate the stages of kombucha fermentation objectively. In the field of food and beverage analysis, ultra-performance liquid chromatography (UPLC) is a well-established technique widely applied for dealing with the determination of specific bioactive substances in a sample (Jayabalan et al., 2007; Essawet et al., 2015). On the other hand, other analytical approaches such as vibrational spectroscopy have been proposed for a simpler, faster and relatively less expensive analysis compared with chromatographic techniques (Correia et al., 2018; Baqueta, Coqueiro, Março, & Valderrama, 2019; Lobato, Alamar, Caramês, & Pallone, 2018). Near-infrared spectroscopy (NIR) has emerged as a non-target technology for direct food analysis. The advantage of NIR spectroscopy over chromatographic techniques, *e.g.,* UPLC, is that it performs non-destructive analysis and does not require extensive sample preparation (Monteiro et al., 2018).

Moreover, in this study, a portable NIR spectrometer (microNIR) was used. It has the same applications as conventional NIR, with the advantages that it is compact, low-cost, quick, and sensitive (Correia et al., 2018). In particular, the UPLC complements the molecular information from NIR with more specific composition information, and for this reason, these techniques were used in this study.

The use of data fusion may be considered as a strategy to combine data from distinct instrumental techniques (Assis, Pereira, Amador, Augusti, de Oliveira, & Sena, 2019; Li, Xie,

Ning, Chen, & Zhang, 2019; Ríos-Reina, Callejón, Savorani, Amigo, & Cocchi, 2019). The advantage of data fusion is merging relevant chemical information from various equipment or experiments, resulting in more valuable information extraction from a single sample set (Banerjee, Roy, Tudu, Bandyopadhyay, & Bjhattacharyya, 2018). There are three principal approaches in data fusion: low, mid, and high-level. More information about data fusion strategies can be found in the literature (Ríos-Reina et al., 2019; Cuevas, Pereira-Caro, Moreno-Rojas, Muñoz-Redondo, & Ruiz-Moreno, 2017; Godinho et al., 2014). Here, low-level approach fusion was used because it is the only approach that fully exploits the multivariate advantage. In this approach, the data from different analytical sources are concatenated into a common data matrix after suitable pre-processing and weighting (Schwolow, Gerhardt, Rohn, & Weller, 2019). Then, some chemometric tools are applied in the data fusion matrix to obtain relevant information about the sample set analyzed under different techniques. In particular, principal component analysis (PCA) has been proposed with encouraging results as an unsupervised method for exploratory purposes (Gonçalves et al., 2020; Schwolowet al., 2019). So far up to the knowledge of the authors, no studies have been carried out using the data fusion approach associated with chemometrics for kombucha fermentation.

This study aims to assess differences in kombucha composition using UPLC, NIR, and physicochemical analyses merged in a low-level data fusion approach. Despite the limitations, this approach can provide qualitative differentiation of complex food matrices such as kombucha. Moreover, it allows the description of biochemical changes in kombucha fermentation (Biancolillo, Boqué, Cocchi, & Marini, 2019).

2. Material and Methods

2.1 Reagent, substrate and culture

All reagents and the UPLC standards (chlorogenic acid, gallic acid, caffeine, catechin, quercetin, and rutin) used in this study were of analytical grade and were obtained from Sigma–Aldrich. Three batches of black and green tea samples (*Camellia sinensis*) were purchased in the local market at Belo Horizonte, Brazil. After that, the samples were brought to the laboratory where they were randomly sorted before the experiments were carried. An artisanal producer provided the kombucha starter culture (fermented broth and cellulosic pellicle). The located of an artisanal producer was Uberlândia, Minas Gerais-Brazil with the following geographical coordinates: 18º 55' 07" S; 48º 16' 38" W.

2.2 Inoculum preparation

The beverages were individually prepared by infusion of 22.5 g of green or black tea leaves in 1.5 L of boiling mineral water for 15 min; then, the infusions were filtered through a filter membrane followed by sucrose addition (120 g). After cooling (at 25 °C) of both teas, the kombuchas were produced by inoculating of the starter culture (150 mL of fermented broth and 75 g cellulosic pellicle) from our supplier and incubating for 15 days at 28 ± 2 °C in a bioreactor.

2.3 Sampling

The fermentation process was carried out for 15 days, and samples were collected at different times: 0, 3, 7, 10, and 15 days. A total of 6 fermentations were performed for green tea (n=3) and black tea (n=3). All fermentation processes were done in triplicate.

All samples were analyzed to determine bioactive substances (chlorogenic acid, gallic acid, caffeine, catechin, quercetin, and rutin), physicochemical parameters (pH, total reducing sugars, total titratable acidity, and ethanol) and NIR absorbance from kombucha beverages. The analyses are described below.

2.4 Identification of bioactive compounds by UPLC

Identification of bioactive compounds was carried out according to previous works (Eça, Machado, Hubinger, & Menegalli, 2015; Chisté, Freitas, Mercadante, & Fernandes, 2012). It was used as a UPLC system (Acquity ™, Waters) with a diode array detector. An Aquacity UPLC BEH C18 column (100 mm x 2.1 mm) with a 1.7 µm particle size was used for chromatographic separations. The samples were filtered through a membrane filter (KASVI, 0.45 µm) before injection. The mobile phase components were acetonitrile (A) and ultrapure water containing 0.5% formic acid (B) at a flow rate of 0.3 $mL.min^{-1}$. Aliquots of 10 µL of samples were injected under the linear gradient starting at 5% A (0-3 min); 8% A (3-10 min); 19% A (10-14 min); 27% A (14-20 min). Bioactive compounds monitored were detected at 271 nm (gallic acid, catechin, and caffeine), 320 nm (chlorogenic acids), 354nm (rutin), and 371 nm (quercetin).

2.5 Near-infrared analysis and spectra preprocessing

A NIR spectrum was obtained from all kombucha samples, where each replicate was considered as one sample (in total 90 samples). The absorbance was recorded by a portable microNIR (MicroNIR^{TM} 1700, JDSU Uniphase Corporation) at room temperature (22 ± 2 °C) using a glass cuvette. The spectral profile of each sample was acquired as the mean of 32 scans and in the spectral range of 900 to 1.650 nm. The blank was measured using a NIR reflectance standard (Spectralon™) with a diffuse reflection coefficient of 99%, while a dark reference (zero - to simulate non-reflection) was obtained with the lamp off. No sample preparation was made. In the present study, pre-treatments on microNIR spectrum were: multiplicative scatter correction (Geladi, MacDougall, & Martens, 1985) and Savitzky–Golay smoothing with a

window size of five points and first derivative with first-order polynomial (Savitzky, & Golay, 1964) using the Matlab software version R2019a.

2.6 Determination of physicochemical parameters

Ethanol, pH, total reducing sugars, and total titratable acidity were analyzed for all samples. The ethanol was determined using a Thermo Plate TP Reader (600nm) spectrophotometry based on the method described by Salik & Povoh (1993). The pH was measured using a pH meter with a combined electrode (MS Tecnopon MPA 210), according to AOAC (2007). For the evaluation of total reducing sugars, a spectrophotometry technique (Thermo Plate TP Reader) with 3,5-dinitrosalisylic acid (DNS) at 540nm was used (Miller, 1959). Finally, total titratable acidity was determined by titration with 0.1 N NaOH, according to Instituto Adolfo Lutz (2008).

2.7 Data fusion approach

In this study, a low-level data fusion approach was implemented, in which a previous preprocessing was independently performed over each dataset. To applied data fusion, all variables studied were organized in matrices, where the lines represented the samples and the columns the variables.

Here, each replicate was considered as one sample, resulting in 90 samples studied. Samples were numbered from 1 to 90, comprising kombucha prepared from black (1–45) and green (46–90) teas. Low-level data fusion was applied to a matrix composed of 90 samples and 131 variables). The variables that form the matrix were bioactive compounds chlorogenic acid, gallic acid, caffeine, catechin, quercetin, and rutin - 06 variables), physicochemical parameters total reducing sugars, ethanol, pH, total titratable acidity - 04 variables) and NIR absorbance

(121 variables). In the development of our data fusion, each dataset was previously pre-processed before combining the fusion matrix. Bioactive substances and physicochemical parameters were autoscaled, while NIR spectra were pre-treated by multiplicative scatter correction and Savitzky–Golay smoothing as previously described. After individual preprocessing, both datasets were individually normalized and joined in a fusion matrix with all variables (Gonçalves et al., 2020).

2.8 Multivariate analysis

The results obtained in this study were merged in a data fusion approach with a subsequent evaluation by classical PCA. Several multivariate tools have been used to collect valuable information regarding data fusion matrices (Gonçalves et al., 2019; Schwolow et al., 2019). Here, however, we are specifically concerned with the potential of PCA for exploring kombucha fermentation. PCA was performed according to established recommendations and more information on the unsupervised pattern recognition technique can be found in the literature (da Silva et al., 2019; Penttilä, Martikainen, Gritsevich, & Muinonen, 2018; Santos et al., 2018). The model was built with a mean center preprocess.

3. Results and Discussion

3.1 Statistics on chemical values

Due to the dimensionality of the table, the results for each sample are presented in **Table S1** as supplementary material. The kombucha samples were analyzed by UPLC, NIR, and physicochemical analysis. Regarding UPLC analysis, the bioactive compounds chlorogenic acids, gallic acid, caffeine, catechin, quercetin, and rutin were identified in both kombucha (**Figure 1**). The concentration of gallic acid (71.04 ± 2.59 mg L^{-1}), caffeine (177.37 ± 1.09 mg

L^{-1}), rutin (30.19 ± 0.31 mg L^{-1}), quercetin (1.22 ± 0.01 mg L^{-1}) and catechin (8.00 ± 1.40 mg L^{-1}) in black tea kombucha (KB) is higher green tea kombucha (KG). While KG showed higher chlorogenic acid concentration (65.42 ± 0.63 mg L^{-1}).

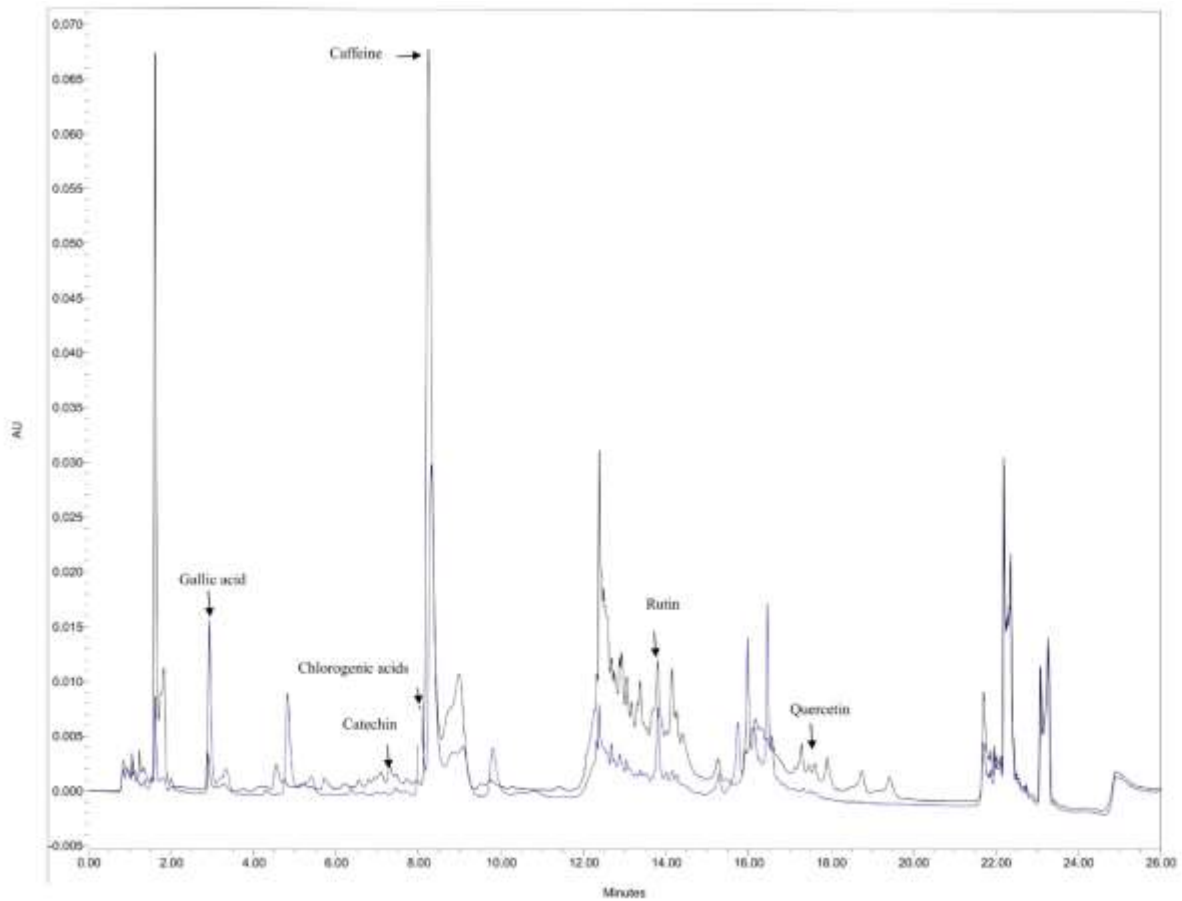

Figure 01. Bioactive compounds identification of the kombucha prepared with green (blue line) and black tea (black line).

Many of the protective effects of bioactive substances present in green and black teas are attributed to phenolic compounds such as phenolic acids, except rutin and quercetin, which are flavonoids. Phenolic acids occur in the form of hydroxybenzoic acids and hydroxycinnamic acids in vegetal fonts as in tea (Shahidi, & Ambigaipalan, 2015). In general, the results obtained in this work indicated the presence of these biologically active compounds in both kombuchas produced.

Concerning to physicochemical parameters, at the end of fermentation, the pH was 3.05 ± 0.03 and 3.09 ± 0.01 for green tea and black tea kombucha, respectively, the total titratable acidity was of 27.44 ± 0.01 g L^{-1} for green tea and 24.17 ± 6.05 g L^{-1} for black tea. The final content of ethanol was less than 1 g L^{-1} (1%) for both kombuchas, which characterizes the kombuchas as non-alcoholic beverages. The total reducing sugars decreased during the fermentation process. This fact may be inferred from the good viability of the inoculum. The parameters showed that both kombuchas fermentation carried off a satisfactory way until 15 days. In this process, the sucrose was converted to ethanol for yeast, followed by acetylation to acetic acid. The pH and titratable acidity showed changes during the fermentation process due to the formation of organic acids.

3.2 NIR analysis

The raw microNIR spectra during the fermentation time of all samples are shown in **Figure 1S** as supplementary material. The microNIR preprocessed spectra for the kombucha samples exhibited absorption bands in the regions of 906–1050 nm, 1150–1250 nm, and 1300–1500 nm, corresponding to the third, second, and first overtones, respectively (**Figure 2**) (Metrohm, 2013; Osborne, 2000). Absorptions in the microNIR spectrum refer to the presented functional groups, such as OH, CH, and NH (Correia et al., 2018). However, how NIR spectroscopy is not selective, it is difficult to conclude only by observing the spectra of both kombuchas. There is no evident pattern in the spectroscopic profile of kombucha prepared from black tea and green tea. For this reason, a chemometric treatment is necessary to extract valuable information on this data.

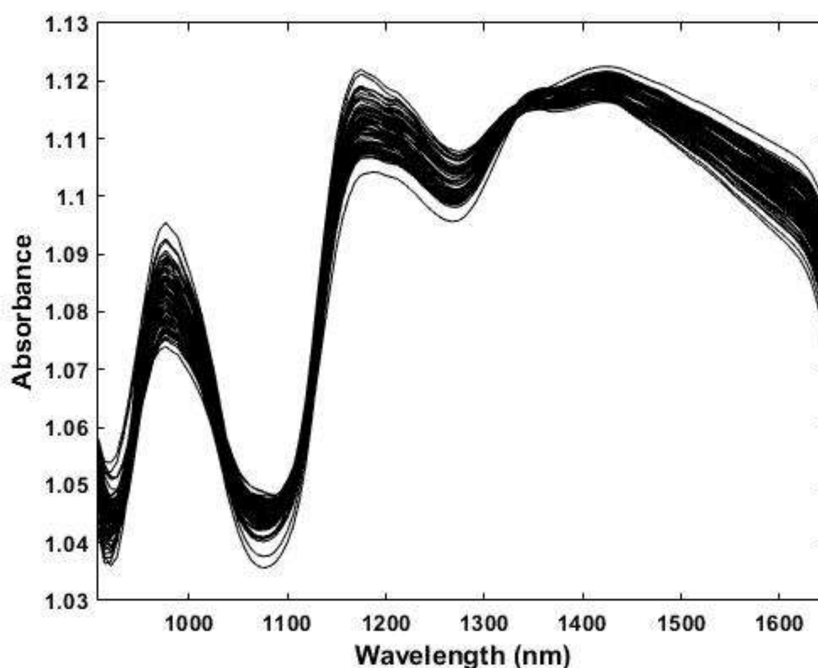

Figure 02. Preprocessed NIR spectra from all kombucha samples.

3.3 Data fusion approach with chemometrics

For the data fusion approach applied in this study, all results from UPLC, NIR, and physicochemical analyses were used. After low-level data fusion applications on our datasets, classical PCA was used to provide informative plots through scores and loadings. Also, the data fusion combined with PCA proposed allows the assessment of which portion of each variable or analytical technique has a greater influence under the dispersion presented in the scores.

The first two PCA dimensions were sufficient to explain 80.01 % of the variance in the data fusion matrix (56.62% in principal component (PC) 1 and 23.39 % in PC2). Both dimensions present valuable information regarding the chemical composition and fermentation process of both kombuchas. The first PCA dimension mainly differentiated between the kombucha produced with different substrates (black or green tea) (**Figure 3A**). The kombucha prepared with black tea presented positive scores, while kombucha made with green tea

presented a negative score. It was possible to verify that the samples are separated along PC1 into two clusters that show a score profile of each kombucha.

Assessing NIR spectroscopy contributions for sample distribution (**Figure 3B**), all spectral region (906–1650 nm) has importance for clusters observed in scores. The NIR spectroscopy influences can be assigned to a shift in the absorption bands in the regions of the first, second, and third overtones, probably overdue to the differences in the amount of the compounds that present these bonds in both kombuchas. Thus, it can be suggested that the kombucha prepared using different substrates had differences in chemical composition that the NIR spectroscopy detected and illustrated by the first PCA dimension.

The kombucha samples prepared with black tea were discriminated by the first model dimension, which mostly described variation in the gallic acid, catechin, caffeine, rutin, and quercetin bioactive compounds (**Figure 3C**). Kombucha produced from black tea shows higher differences of these compounds compared to kombucha prepared from green tea. In opposite, kombucha prepared with green tea were better discriminated by the variations if total reducing sugars, ethanol, pH, total titratable acidity, and mainly chlorogenic acids, which varies largely in comparison to kombucha produced with green tea as can see in the Table S1.

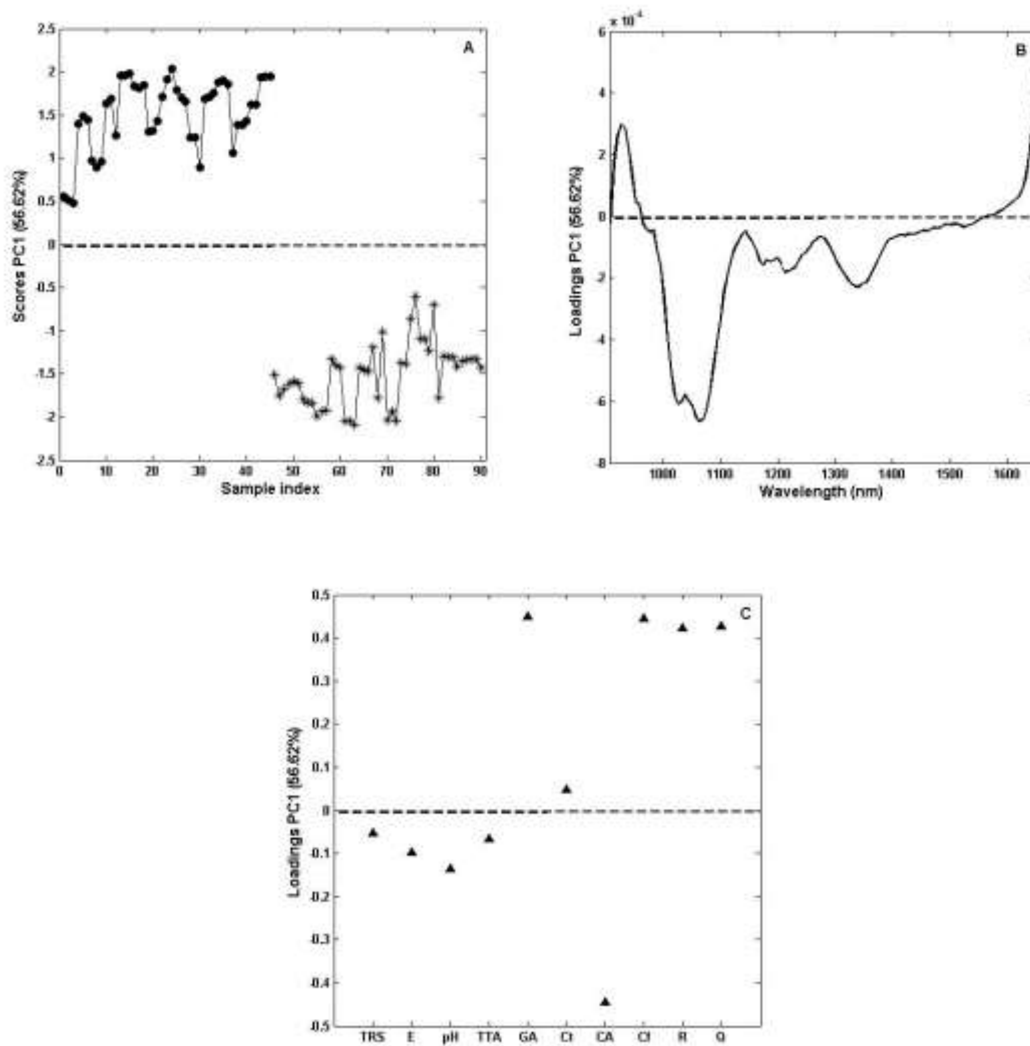

Figure 03. PCA maps obtained from PC1. (A) Scores, (B) NIR loadings, and (C) loadings of the physicochemical parameters.

The results showed similar behavior for kombucha fermentation. In PCA second dimension (**Figure 4A**) could be observed in the sample dispersion by fermentation time. The scores profiles in PC2 showed fermentation evolution along the time. The total period of fermentation (15 days) was divided into three main phases: start, middle, and end. Thus, a trend visual separation of clusters can be noted, where the behavior of both kombuchas samples (prepared with green or black tea) along fermentation seem similar. The samples at the starting stage of fermentation (zero-day) are distributed on the superior side (most positive scores) of PC2. The samples at the end of fermentation (fifteen days) are dispersed in a lower area (from

negative scores), and the samples at the middle of fermentation (three, seven and ten days) are distributed in an intermediate zone, showing positive and negative scores along second PCA dimension. These results indicate that kombucha fermentation occurs in a similar way using different substrates. This finding can be useful in both quality control of the food industry and future applications.

Regarding the NIR analysis, all spectral region again has importance for clusters observed in second PCA dimension scores (**Figure 4B**). However, this dimension could verify the differences and similarities of both kombuchas focusing on the fermentative process. Therefore, it is possible to deduce that despite qualitative differences in the chemical composition of the kombucha, the NIR spectroscopy associated with the PCA also can extract information beyond that already obtained in lower projections.

Considering the distribution from the samples in the second dimension, the total reducing sugars, pH, and total titratable acidity were the main variables associated with the observations found (**Figure 4C**). The total reducing sugars and the pH were the most critical variables for discriminating the fermentation starts (at day zero). In contrast, the total titratable acidity was more associated with the fermentation end (at fifteen days). These differences in acidity could be attributed to the decrease in total reducing sugars along the fermentative process, which decreases the pH and consequently increase the total titratable acidity as expected. In the middle stage of the fermentation (at three, five, and ten days), the samples were better discriminated by the variations in catechin, chlorogenic acids, caffeine, rutin, and quercetin bioactive compounds. Ethanol and gallic acid differences were not statistically significant in this dimension.

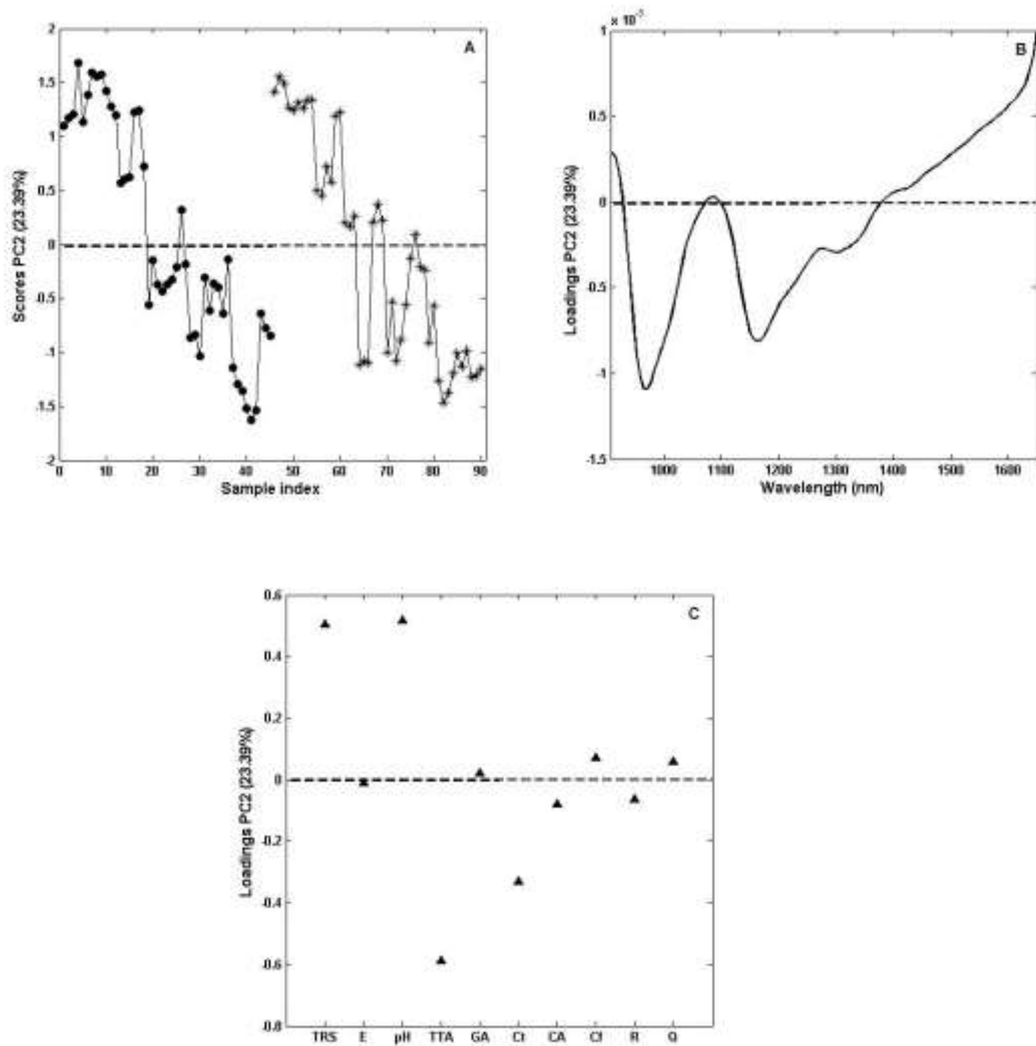

Figure 04. PCA maps obtained from PC2. (A) Scores, (B) NIR loadings, and (C) loadings of the physicochemical parameters.

Additionally, the results gather evidence to show the significant differences between green and black kombucha – the green kombucha has higher concentrations of chlorogenic acids, and black has higher concentrations of caffeine, rutin, and quercetin. The key factors involved in these differences may be useful information to be used in the industrial fermentation to produce the highest quality and quantity kombucha. Furthermore, the results suggest that the use of a portable NIR is a reliable tool for on-line monitoring fermentation, especially when different substrates are compared.

4. Conclusions

In this study, kombucha fermentation was investigated by different analytical techniques, including UPLC, portable NIR spectroscopy, and physicochemical analyses. The results were merged in a low-level data fusion approach coupled with unsupervised chemometrics, which showed informative plots and an understanding of kombucha fermentation, mainly when different substrates are used in its production. Despite their quantitative differences, concerning bioactive compounds and physicochemical properties. The results show that both kombuchas, prepared with green or black tea, behaved similarly during fermentation. The achieved results allowed to conclude that the data fusion of UPLC, NIR, and physicochemical parameters at low-level combined with unsupervised chemometrics can be used as an alternative method to evaluate kombucha fermentation. Moreover, the results can be used as a base to further studies and mainly to help in which substrate composition is better to be produced in an industrial scale. Furthermore, portable NIR can be a reliable and robust analysis for fermentation monitoring.

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Supplementary Material

Figure 1S. Raw microNIR spectrum of all samples during the kombucha fermentation.

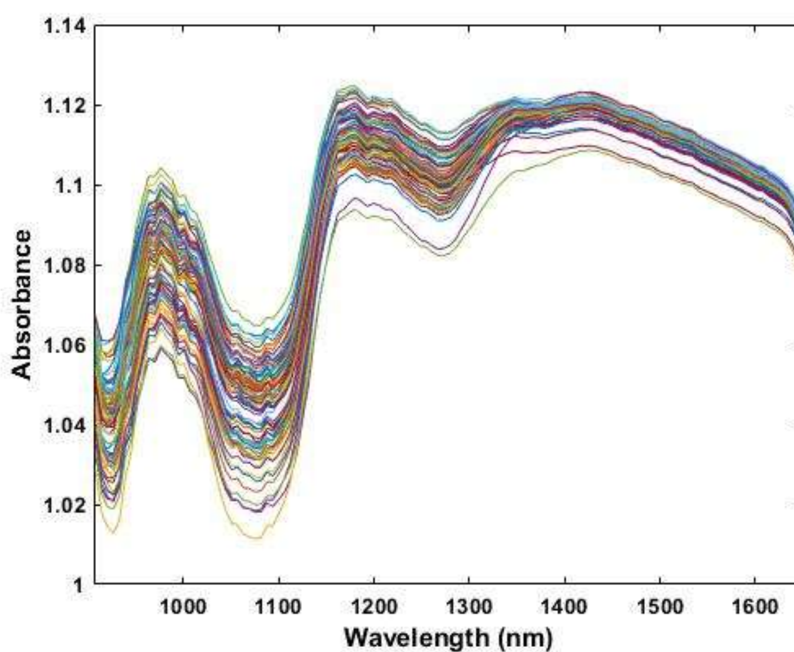

Table S1. Descriptive measurements on samples. TRS= Total Reducing Sugars; E= Ethanol; pH; TTA= Total titratable acidity; GA= gallic acid Ct= Catechin; Cf= caffeine; R= rutine and Q= Quercetin.

Sample	TRS ($g.L^{-1}$)	E	pH	TTA (g.L)	GA (ppm)	Ct (ppm)	CA (ppm)	Cf (ppm)	R (ppm)	Q (ppm)
					Green tea kombucha					
KGT0R1.1	77.0968	0.1129	3.7510	3.9200	7.0325	4.3129	56.8088	64.9257	14.8190	0.1568
KGT0R1.2	74.2294	0.5968	3.7700	3.9200	7.0325	4.3129	56.8088	64.9257	14.8190	0.1568
KGT0R1.3	75.6631	0.9194	3.7790	3.9200	7.0325	4.3129	56.8088	64.9257	14.8190	0.1568
KGT0R2.1	76.7384	1.3226	3.8440	3.9200	5.9541	6.5647	59.3642	73.1704	15.4317	0.0955
KGT0R2.2	72.7957	0.5161	3.8660	3.9200	5.9541	6.5647	59.3642	73.1704	15.4317	0.0955
KGT0R2.3	76.3799	0.9194	3.8700	3.9200	5.9541	6.5647	59.3642	73.1704	15.4317	0.0955
KGT0R3.1	66.7025	0.1935	3.8040	5.8800	6.4933	5.4388	58.0865	69.0480	15.1254	0.1261
KGT0R3.2	69.9283	0.5161	3.8040	5.8800	6.4933	5.4388	58.0865	69.0480	15.1254	0.1261
KGT0R3.3	70.6452	0.6774	3.8100	5.8800	6.4933	5.4388	58.0865	69.0480	15.1254	0.1261
KGT3R1.1	49.1398	1.5645	3.6530	7.8400	6.8124	5.7548	63.9116	73.7378	16.0386	0.1019
KGT3R1.2	49.4982	1.1613	3.6130	7.8400	6.8124	5.7548	63.9116	73.7378	16.0386	0.1019
KGT3R1.3	57.0251	0.7581	3.6530	7.8400	6.8124	5.7548	63.9116	73.7378	16.0386	0.1019
KGT3R2.1	44.4803	1.6452	3.8660	5.8800	8.3532	6.2737	68.9682	82.7015	17.3482	0.4827
KGT3R2.2	73.1541	1.8065	3.8700	5.8800	8.3532	6.2737	68.9682	82.7015	17.3482	0.4827
KGT3R2.3	72.4373	1.2419	3.8440	5.8800	8.3532	6.2737	68.9682	82.7015	17.3482	0.4827
KGT3R3.1	38.7455	1.1613	3.7090	9.8000	7.5828	6.0142	66.4399	78.2196	16.6934	0.2923
KGT3R3.2	38.0287	1.2419	3.7000	9.8000	7.5828	6.0142	66.4399	78.2196	16.6934	0.2923
KGT3R3.3	40.8961	1.4839	3.7050	9.8000	7.5828	6.0142	66.4399	78.2196	16.6934	0.2923
KGT7R1.1	35.8781	1.4032	3.3020	15.6800	7.2255	9.4683	66.3228	75.3737	16.9730	0.1992
KGT7R1.2	35.8781	1.5645	3.3340	15.6800	7.2255	9.4683	66.3228	75.3737	16.9730	0.1992
KGT7R1.3	35.1613	1.8065	3.3341	15.6800	7.2255	9.4683	66.3228	75.3737	16.9730	0.1992
KGT7R2.1	65.2688	2.6935	3.5120	11.7600	8.2661	6.6235	69.6834	79.4561	17.3843	0.1201
KGT7R2.2	67.0609	2.1290	3.5060	11.7600	8.2661	6.6235	69.6834	79.4561	17.3843	0.1201
KGT7R2.3	69.5699	3.0161	3.5070	11.7600	8.2661	6.6235	69.6834	79.4561	17.3843	0.1201

KGT7R3.1	40.5376	1.5645	3.5100	19.6000	7.7458	8.0459	68.0031	77.4149	17.1787	0.1596
KGT7R3.2	56.3082	0.2742	3.5000	19.6000	7.7458	8.0459	68.0031	77.4149	17.1787	0.1596
KGT7R3.3	38.3871	1.7258	3.5000	19.6000	7.7458	8.0459	68.0031	77.4149	17.1787	0.1596
KGT10R1.1	55.5914	1.2419	3.2590	19.6000	7.2206	9.4964	64.7662	74.0233	16.9343	0.3164
KGT10R1.2	69.2115	0.9194	3.2650	19.6000	7.2206	9.4964	64.7662	74.0233	16.9343	0.3164

Continued.

KGT10R1.3	87.1326	0.3548	3.2330	19.6000	7.2206	9.4964	64.7662	74.0233	16.9343	0.3164
KGT10R2.1	78.1720	4.1452	3.3316	15.6800	8.3732	5.8531	68.0776	74.9853	16.9878	0.5265
KGT10R2.2	53.0824	2.5323	3.3120	15.6800	8.3732	5.8531	68.0776	74.9853	16.9878	0.5265
KGT10R2.3	51.6487	2.5323	3.3090	15.6800	8.3732	5.8531	68.0776	74.9853	16.9878	0.5265
KGT10R3.1	48.7814	2.3710	3.2300	23.5200	7.7969	7.6748	66.4219	74.5043	16.9611	0.4214
KGT10R3.2	43.0466	3.5000	3.2300	23.5200	7.7969	7.6748	66.4219	74.5043	16.9611	0.4214
KGT10R3.3	46.2724	1.9677	3.3000	23.5200	7.7969	7.6748	66.4219	74.5043	16.9611	0.4214
KGT15R1.1	30.5018	0.4355	3.0490	27.4400	8.4631	5.4633	66.2283	70.0405	16.8352	0.1186
KGT15R1.2	36.2366	0.5161	3.0250	27.4400	8.4631	5.4633	66.2283	70.0405	16.8352	0.1186
KGT15R1.3	43.7634	0.2742	3.0390	27.4400	8.4631	5.4633	66.2283	70.0405	16.8352	0.1186
KGT15R2.1	50.2151	1.1613	3.1050	27.4400	8.5682	5.5547	64.7004	69.0314	16.2245	0.1396
KGT15R2.2	47.3477	0.6774	3.0400	27.4400	8.5682	5.5547	64.7004	69.0314	16.2245	0.1396
KGT15R2.3	52.3656	0.1935	3.0760	27.4400	8.5682	5.5547	64.7004	69.0314	16.2245	0.1396
KGT15R3.1	41.6129	0.5161	3.0500	27.4400	8.5156	5.5090	65.4643	69.5359	16.5298	0.1291
KGT15R3.2	40.1792	0.3548	3.1000	27.4400	8.5244	5.5166	65.3370	69.4519	16.4790	0.1308
KGT15R3.3	48.7814	1.7258	3.0000	27.4400	8.5346	5.5255	65.1885	69.3537	16.4196	0.1329
					Black tea kombucha					
KBT0R1.1	53.0824	0.1935	3.9010	1.9600	54.6125	2.0629	1.9756	166.6482	22.6311	1.1547
KBT0R1.2	58.8172	1.0000	3.8990	1.9600	54.6125	2.0629	1.9756	166.6482	22.6311	1.1547
KBT0R1.3	60.9677	1.4839	3.9000	1.9600	54.6125	2.0629	1.9756	166.6482	22.6311	1.1547
KBT0R2.1	75.6631	1.0000	3.7190	3.9200	67.5373	3.9246	2.4841	198.0260	26.2570	1.4314
KBT0R2.2	49.8566	0.6774	3.7310	3.9200	67.5373	3.9246	2.4841	198.0260	26.2570	1.4314
KBT0R2.3	61.6846	0.8387	3.7240	3.9200	67.5373	3.9246	2.4841	198.0260	26.2570	1.4314
KBT0R3.1	78.8889	0.1935	3.7020	1.9600	61.0749	2.9937	2.2298	182.3371	24.4441	1.2931

KBT0R3.2	82.4731	0.3548	3.7030	1.9600	61.0749	2.9937	2.2298	182.3371	24.4441	1.2931
KBT0R3.3	77.4552	0.2742	3.7100	1.9600	61.0749	2.9937	2.2298	182.3371	24.4441	1.2931
KBT3R1.1	68.1362	1.5435	3.5950	3.9200	66.0270	6.9036	2.3034	192.8099	25.8470	1.0457
KBT3R1.2	62.0430	1.1613	3.6160	3.9200	66.0270	6.9036	2.3034	192.8099	25.8470	1.0457
KBT3R1.3	68.4946	2.1290	3.6300	3.9200	66.0270	6.9036	2.3034	192.8099	25.8470	1.0457
KBT3R2.1	50.2151	0.4355	3.4620	5.8800	71.9739	6.3176	2.8489	204.3298	28.3764	1.1662
KBT3R2.2	52.0072	0.3548	3.4520	5.8800	71.9739	6.3176	2.8489	204.3298	28.3764	1.1662
KBT3R2.3	54.1577	0.1935	3.4280	5.8800	71.9739	6.3176	2.8489	204.3298	28.3764	1.1662
KBT3R3.1	67.7778	0.9194	3.4900	3.9200	69.0005	6.6106	2.5761	198.5698	27.1117	1.1060

Continued.

KBT3R3.2	68.1362	1.0806	3.4960	3.9200	69.0005	6.6106	2.5761	198.5698	27.1117	1.1060
KBT3R3.3	50.2151	1.2419	3.4900	3.9200	69.0005	6.6106	2.5761	198.5698	27.1117	1.1060
KBT7R1.1	41.9713	1.6452	3.3290	11.7600	65.2384	9.1945	2.5996	176.1951	26.0102	0.8443
KBT7R1.2	57.7419	1.0806	3.3320	11.7600	65.2384	9.1945	2.5996	176.1951	26.0102	0.8443
KBT7R1.3	48.4229	0.2097	3.3350	11.7600	65.2384	9.1945	2.5996	176.1951	26.0102	0.8443
KBT7R2.1	34.8029	0.6774	3.2780	11.7600	66.3005	6.3487	2.5261	180.2328	25.1458	1.1113
KBT7R2.2	38.7455	1.2419	3.2800	11.7600	66.3005	6.3487	2.5261	180.2328	25.1458	1.1113
KBT7R2.3	39.8208	0.2742	3.2830	11.7600	66.3005	6.3487	2.5261	180.2328	25.1458	1.1113
KBT7R3.1	47.7061	1.5645	3.2800	9.8000	65.7695	7.7716	2.5628	178.2139	25.5780	0.9778
KBT7R3.2	64.1935	1.8065	3.2750	9.8000	65.7695	7.7716	2.5628	178.2139	25.5780	0.9778
KBT7R3.3	48.4229	1.8871	3.2800	9.8000	65.7695	7.7716	2.5628	178.2139	25.5780	0.9778
KBT10R1.1	40.1792	0.5968	3.2330	23.5200	76.6419	11.5367	2.6098	209.5405	31.9593	1.2705
KBT10R1.2	41.9713	0.5161	3.2360	23.5200	76.6419	11.5367	2.6098	209.5405	31.9593	1.2705
KBT10R1.3	35.8781	0.8387	2.2370	23.5200	76.6419	11.5367	2.6098	209.5405	31.9593	1.2705
KBT10R2.1	48.7814	1.4032	3.1650	17.6400	75.7682	5.0169	2.7593	200.1087	31.2573	1.1259
KBT10R2.2	34.8029	1.5403	3.1630	17.6400	75.7682	5.0169	2.7593	200.1087	31.2573	1.1259
KBT10R2.3	45.1971	0.6774	3.1820	17.6400	75.7682	5.0169	2.7593	200.1087	31.2573	1.1259
KBT10R3.1	49.1398	0.3145	3.1400	11.7600	76.2051	8.2768	2.6845	204.8246	31.6083	1.1982
KBT10R3.2	37.3118	0.3387	3.1450	11.7600	76.2051	8.2768	2.6845	204.8246	31.6083	1.1982
KBT10R3.3	61.6846	0.3548	3.1400	11.7600	76.2051	8.2768	2.6845	204.8246	31.6083	1.1982

KBT15R1.1	16.8817	0.8387	3.1290	29.4000	67.8646	6.5980	2.2707	176.0312	30.4978	1.2300
KBT15R1.2	27.9928	0.2742	3.0850	29.4000	67.8646	6.5980	2.2707	176.0312	30.4978	1.2300
KBT15R1.3	25.4839	0.3548	3.0700	29.4000	67.8646	6.5980	2.2707	176.0312	30.4978	1.2300
KBT15R2.1	24.7670	0.5968	3.1010	27.4400	74.2200	9.4000	2.7509	178.7144	29.8758	1.2068
KBT15R2.2	30.1434	0.6774	3.0920	27.4400	74.2200	9.4000	2.7509	178.7144	29.8758	1.2068
KBT15R2.3	35.5197	0.5968	3.0740	27.4400	74.2200	9.4000	2.7509	178.7144	29.8758	1.2068
KBT15R3.1	50.9319	0.1935	3.1000	15.6800	71.0423	7.9990	2.5108	177.3728	30.1868	1.2184
KBT15R3.2	45.5556	0.2742	3.0990	15.6800	71.0423	7.9990	2.5108	177.3728	30.1868	1.2184
KBT15R3.3	43.0466	0.2742	3.0960	15.6800	71.0423	7.9990	2.5108	177.3728	30.1868	1.2184

Footnote: Analytical data were described in replications with three repetitions. Each line represents a replication (1.1; 1.2; 1.3 …) of the respective repetition (R1).

CHAPTER III

Evaluation of antibacterial activity of black and green tea kombucha

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Evaluation of antibacterial activity of black and green tea kombucha

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ABSTRACT

Currently, the search for natural antimicrobial agents has increased. Thereat, plant extracts, products of fermentative processes, and microbial growth have been tested. Kombucha is a fermented beverage that resulted from the fermentation of the infusion of *Camellia sinensis*. There are reports about biological activities, such as anti-inflammatory, digestibility, antioxidant, antimicrobial, and others. The study aimed to evaluate the antibacterial activity of black and green tea kombucha against eight pathogenic microorganisms. The antibacterial activity was analyzed using the agar well diffusion method. Each Kombucha consisted of three treatments: natural, neutralized, and filtered. The solutions of acetic acid (4% v/v) and Kanamycin (30µg.L^{-1}) were used as a control. The black and green tea kombucha beverages, not neutralized, showed antibacterial activity against *Escherichia coli* serotypes. Black and Green Tea Kombucha did not show antibacterial activity against *Staphylococcus aureus*, *Salmonella Flexneri*, and *Salmonella Typhimurium*. The inhibition may be attributed for acetic acid production during the fermentation process. Therefore, it appears that Kombucha may have a potential antimicrobial agent.

Keywords: *Camellia sinensis*, acetic acid, fermentation, infusion

1. Introduction

Natural antimicrobials are compounds capable of inhibiting the growth of microorganisms (FOOD INGREDIENTS BRASIL, 2010; CUSHNIE E LAMB, 2011). The characterization and use of these substances can significantly decrease the demand for new drugs, microbial multidrug resistance, and represent lower risks of toxicity to the organism. These characteristics are of interest to the pharmaceutical and cosmetic sectors (PENNA et al., 2001; MICHELIN et al., 2005; LIMA et al., 2006; BARBOSA-FILHO et al., 2007; FOOD INGREDIENTS BRASIL, 2010). Various compounds are formed during the fermentation process, such as organic acids, bacteriocin, and peptides. Kombucha is a fermented beverage of *Camellia sinensis* infusion. In the process, the microorganisms change sucrose to ethanol, cellulose, acetic acid, glucuronic acid, gluconic acid, and others (GREENWALT et al., 1998; JAYABALAN et al., 2010; JAYABALAN et al., 2014; POTHAKOS et al., 2016; VITAS et al., 2018).

The kombucha consumption is related to its functional properties and health-promoting effects: antimicrobial property (GREENWALT et al., 1998; SREERAMULU et al., 2000; BATTIKH et al., 2012; BHATTACHARYA et al., 2016; VITAS et al., 2018), antioxidant (CHU; CHEN, 2006; YANG et al., 2009; BHATTACHARYA et al., 2013; FU et al., 2014; VITAS et al., 2018; KAEWKOD et al., 2019), anticarcinogenic activity (JAYABALAN et al., 2011; VITAS et al., 2018), anti-diabetic (ALOULOU et al., 2012; SRIHARI et al., 2013; ZUBAIDAH et al., 2018), hepatoprotective (BHATTACHARYA et al., 2011; ABSHENAS et al., 2012; WANG et al., 2014), and hypocholesterolemic (YANG et al., 2009).

Polyphenols are present in kombucha composition through tea leaves. These compounds result from the secondary metabolism of plants being are responsible for the protection of the plant body against thermal and water stress, ultraviolet radiation, attacks by herbivores, and others. The polyphenols are found in fruits and vegetables, in addition, to drinks derived from them, such as juice, tea, and wine (DAGLIA et al., 2012). Can be divided into two large groups, such as, flavonoid and non-flavonoid (HERNANDEZ et al. 2009; LIMA et al., 2009; ASHIHARA et al., 2010; DAGLIA et al., 2012; PERES, 2004). The flavonoids group include flavonols, flavones, flavanones, anthocyanins, flavanols, and isoflavones. Non-flavonoid compounds are phenolic acids derived from benzoic acid (gallic acid and protocatechuic acid) and cinnamic acid (coumaric acid, ferulic acid, caffeic acid, and others) (DAGLIA et al., 2012) The antimicrobial activity of polyphenols has been widely investigated. Compounds such as flavanols, flavonols, and tannins have shown a broad spectrum of action and higher

antimicrobial activity when compared to other polyphenols. They have also been able to suppress microbial virulence factors, such as reduction of ligand adhesion in the host, inhibition of biofilm formation, and neutralization of bacterial toxins, in addition to demonstrating synergism with antibiotics (DAGLIA et al., 2012). Due to the antimicrobial properties, there are proposals for the development of food preservatives from polyphenols (DAGLIA et al., 2012).

The composition of *Camellia sinensis* tea is similar to that found in the leaves. The content of phenolic compounds founded in the leaves can reach 30% of the dry weight of the plant (CHAN et al., 2007). The chemical composition of the leaves is affected by several factors, such as edaphoclimatic conditions, preparation, and conditioning of the plant material. The catechin monomers are the most abundant flavonols in green tea. For black tea, the most abundant phenolic compounds are theaflavins and thearubigins (MUKHTAR, 2000; MANACH et al., 2004; RETO et al., 2008; SENGER et al., 2010).

According to DAGLIA et al (2012), catechin, present mainly in green tea (*C. sinensis*), has antibacterial activity. In *in vitro* tests, it was able to inhibit the growth of several bacteria (*Campilobacter jejuni*, *Clostridium perfringes Escherichia coli*, *Streptococcus mutans*, and *Vibrio cholerae*).

The flavonols, tannins, and nonflavonoid showed antimicrobial activity reported by CUSHNIE et al. (2007); ALVESALO et al. (2006); MANACH et al. (2004); COTE et al. (2010); HEINONEN et al. (2007), and SAAVEDRA et al. (2010). The antimicrobial activity of phenolics can be attributable to both direct actions against microorganisms and the suppression of microbial virulence factors.

Studies on the mechanism of action of phenolic compounds against microorganisms suggest that compounds such as flavonol, flavan-3-ol, and flavolan classes damage the cytoplasmic membrane (SIRK et al., 2008; TAMBA et al., 2007; KUSUDA et al., 2006). The flavan-3-ols and isoflavones can inhibit nucleic acid synthesis. This action can occur by inhibition of topoisomerase and/or dihydrofolate reductase (GRADISAR et al., 2007; ULANOWSKA et al., 2006; WANG et al., 2010; NAVARRO- MARTINEZ et al., 2005). Also, demonstrating inhibition of energy metabolism by inhibiting ATP synthase (CHINNAM et al., 2010).

Currently, search for antimicrobial agents from natural sources has increased, such as plant extracts. Thus, we aimed to evaluate the antibacterial activity of black and green tea kombucha for using the agar well diffusion method against foodborne pathogens.

2. Material and Methods

2.1 Kombucha fermentation

The inoculum (fermented broth and cellulosic pellicle), used for experiments, was provided by an artisanal producer of Uberlândia, Minas Gerais, Brasil (18º 55' 07" S; 48º 16' 38" W). Fermentation was performed on infusion made by dissolving 1.5% (w/v) of black or green tea leaves in water for 15 minutes (JAYABALAN ET AL., 2007; JAYABALAN ET AL., 2014; TEOH ET AL; 2004; NEFFE- SKOCINSKA ET AL., 2017; BARBOSA ET AL., 2020). After boiling, 8.0% (w/v) of sugar was added, and the infusion was filtered in a paper filter. The sweetened infusion was cooled at 25° C, and the inoculum was added with 10% (v/v) fermented broth and 5% (w/v) cellulosic pellicle. Processes were carried out in sterile glasses bioreactor (volume 3L and diameter 12 cm). The bioreactors (glass flaskes) were filled up to 50% of full capacity and covered with cotton gauzes. Fermentation was carried out for 15 days at 28°C ± 2°C.

2.2 Microbial enumeration

The microbial population was monitored at the final of the fermentation. The samples were tenfold diluted in 0.1 % peptone saline water (and spread plated in MYP (Manitol Yeast Peptone) for acetic acid bacteria (AAB) and in GYMP (Glucose, Yeast Extract, Malt Extract, and Peptone) with 0.05% chloramphenicol for yeast (Spinosa, 2002). All plates were incubated at 30 °C for five days; the relative humidity was maintained at 90% (SPINOSA, 2002; OLIVEIRA ET AL., 2010).

2.3. pH and Total Titratable Acidity

Total Titratable Acidity (TTA) was performed by titration with NaOH solution (0.1 mol. L^{-1}) according to procedures described by INSTITUTO ADOLFO LUTZ (2008). The method for determining total titratable acidity is to neutralize the total acids present in the sample by using a base. The results were expressed as of NaOH 0.1 mol L1 /100 g kombucha). The pH measurements were done at 25°C using a pHmeter with a combined electrode (MS Tecnopon MPA 210) previously calibrated with pH 4.0 and 7.0 buffer solutions according to procedures described by AOAC (2007).

2.4. Preparation of antibacterial extracts

The antibacterial extract used for the test were: kombucha beverages (black and green tea), filtered kombucha beverage, neutralized Kombucha, acetic solution (4% v/v), and Kanamycin solution (30µg. L^{-1}). For preparing filtered antibacterial extract, both kombucha beverages were filtered, separately, in PES milipore membrane pore size 0.22 µm (Kasvi, São José dos Pinhais-PR) according to SREERAMULU et al. (2000). The neutralized extract was prepared by neutralizing kombucha beverages with 1 mol. L^{-1} of NaOH until pH 6.5 (GREENWALT et al., 1998). The acetic acid solution was prepared to dilute 0,4 mL of acetic acid P.A (Labsynth) in 10 mL sterile distilled water. For kanamycin solution, the Kanamycin (Himedia) was dissolved in distilled water and filtered in in PES milipore membrane pore size 0.22 µm (Kasvi) (BHATTACHARYA et al., 2016).

2.5 Antibacterial activity

2.5.1. Bacteria strains

Strains of *Escherichia coli (E. coli)* 25922, enteropathogenic *E. coli*- EPEC (CDC 0126), enterohaemorrhagic *E. coli* - EHEC (CDC EDL-933), enteroinvasive *E. coli* - EIEC, enterotoxigenic *E. coli* - ETEC (H10407), *Salmonella* Typhimurium (ATCC 14028), *Salmonella* Flexneri (ATCC 12002) e *Staphylococcus aureus (*ATCC 33591) were grown in nutrient broth at 35°C. All microorganisms were provided by the Collection of Cultures of Mycology Laboratory, Department of Microbiology - UFMG (Minas Gerais, Brazil). Microorganisms were kept Brain Heart Infusion (BHI) broth with 10% (v/v) glycerol at -20°C until use. All strains were evaluated for sensitivity to kanamycin solution (30µg. L^{-1}), according to BHATTACHARYA et al. (2016). The microorganisms used are standard strains and are commonly involved in processes of food poisoning.

2.5.2 Antibacterial tests

Inoculum preparation followed the methodology proposed by DE CAMARGO et al. (2017). Bacteria cell concentration was standardized by optical density (OD) measurement in 0.5 (1.5 × 10^8 cells. mL^{-1}) of the standard McFARLAND scale. Well Diffusion Method was used to assess the antibacterial activity of Kombucha (SREERAMULU et al., 2000; BATTIKH et al., 2012). Müller Hinton agar were distributed in sterile Petri dishes, separately. After plates

set, a hole of 8 mm bores was punched aseptically with a sterile borer. The wells were filled up 50 µL antibacterial extract (see item 2.4). Before the test, all plates were store at the refrigerator for overnight for complete absorption of the extracts. After storage, the agar plates were inoculated in superficies with 100 µl of bacterial suspension adjusted at 0.5 McFarland scale. The inoculated Petri dishes were incubated at 36°C for 24h. After incubation, the diameter of the inhibition zone was measured. Measurements were carried out, in triplicate, with a digital pachymeter (Mitutoyo, 500-19730B). All experiments were performed in duplicate.

3.Results and Discussion

3.1 Characterization of antibacterial extracts

The pH values for kombucha beverage was pH 3.5 (black tea) and 3.4 (green tea). Total Titratable Acidity was 18 $g.L^{-1}$ for black tea and 15 $g.L^{-1}$. The population of AAB and yeast was 10^6 CFU. mL^{-1} for both Kombucha. Filtered Kombucha showed the absence of microorganisms, pH was ranged from 3.4 (black) to 3.6 (green), and TTA was 16.5 g. L^{-1} for black Kombucha and 13 $g.L^{-1}$ for green Kombucha. The pH for neutralized kombucha beverage varied from 6.7 (black) to 6.8 (green), and TTA was 1.5 g. L^{-1} for black Kombucha and 1.0 g. L^{-1} for green Kombucha. Also, the microbial population was reduced with neutralization; for yeast, the counts ranged from 10^3 $CFU.mL^{-1}$(green) to 10^4 $CFU.mL^{-1}$ (black), and for AAB, the counts varied from 10^4 $CFU.mL^{-1}$(green) to 10^5 $CFU.mL^{-1}$ (black). The pH for Acid Acetic Solution was 2.6. The filtration hasn't modified the pH of Kombucha (IVANISOVA et al., 2020). However, the neutralization increased the pH, as expected. The same behavior was observed for TTA; the addition of NaOH modified the acidity of kombucha beverage, which showed low values.

3.2 Antibacterial activity

The inhibition zone diameter of kombucha extracts against selected bacterial strains is shown in Table 1. Concerning, Gram-Negative bacteria, the highest efficacy of Kombucha was found against enterotoxigenic *E. coli* ETEC (H10407). Whereas, *Salmonella* Typhimurium, *Salmonella* Flexneri, and *Staphylococcus aureus* showed high resistance for kombucha beverages (Table 1).

E. coli was the most inhibited pathogen by fermented beverages. For kombucha beverage, the inhibition zone diameter for *E. c*oli ranged from 14.8 mm to 18.0 mm for black tea and 15.8 mm to 17.0 mm for green tea kombucha. Filtered Kombucha showed similar behavior was observed, the inhibition zone varied from 13.5 mm to 17.0 mm. The antimicrobial activity of Kombucha could be attributed to low pH and high TTA (18 $g.L^{-1}$(black tea) and 15 $g.L^{-1}$ (green tea)). The antimicrobial properties of organic acids have been widely studied scientifically (RICKE, 2003; IN et al., 2013; NIETO-PEÑALVER et al., 2014; OLAIMAT et al., 2018). In fermented Kombucha, the presence of a high concentration of gluconic acid and acetic could explain the higher inhibition of pathogenic microorganisms (NIETO-PEÑALVER et al., 2014). The neutralized beverage wasn't showed antibacterial activity for both kombuchas. Although neutralized Kombucha did not show antibacterial activity, some authors showed that phenolics might act as antimicrobials through phenolic-membrane interaction, DNA gyrase inhibition, and metal sequestering (ANDJELKOVIĆ et al., 2006; WANG et al., 2010; REYGAERT, 2014). On the other hand, some studies (GREENWALT et al., 1998; SREERAMULU et al., 2000; BATTIKH et al., 2012; BHATTACHARYA et al., 2016) present little effect or absence of antibacterial activity for neutralized fermented beverages. These authors suggest that antibacterial effects are due to organic acids, mainly acetic acid. Also, lower pH is useful to control for pathogenic bacteria. Moreover, it's the mechanism of action is not entirely known; non-dissociated acetic acid can increase lipid solubility, allowing an increased accumulation of fatty acids in the cell membrane or other cell wall structures.

The positive controls used in this study showed high inhibition for pathogens, except for *Staphylococcus aureus*. The inhibition zone for acetic acid solution ranged from 12.0 mm to 22.0 mm. The effectiveness of pathogen control is for lower pH and high concentration of acetic acid. Also, kanamycin solution inhibited all pathogens. The higher inhibition zone was 24.1 mm for *E. coli* ETEC (H10407), and the lower inhibition zone was 10 mm for *Staphylococcus aureus*.

Notwithstanding, the neutralized Kombucha did not show an antimicrobial effect. The kombucha beverage showed a high inhibition effect for *E. coli* (18.0 mm) when compared with the acetic acid solution (17.5 mm) and Kanamycin (24.1 mm). The interaction with organic acid and tea catechins work synergistically to inhibit *E. coli* (FANAKI et al., 2008; CUI et al., 2012; PASSAT, 2012). Catechins have synergistic effect with antibiotics such as amoxicillin, azithromycin, chloramphenicol, gentamycin, levofloxacin, sulfamethoxazole, and other (NOORMANDI; DABAGHZADEH, 2015). Catechins and fractions showed efficacy to inhibit of the microorganisms, especially *E. coli*. The inhibition occurs by different mechanisms

such as damage to bacterial membrane (IKIGAI et al., 1993; CUI et al., 2012), damage to the cytoplasmic membrane (HOSHINO et al., 1999), action of hydrogen peroxide by catechin fractions (ARAKAWA et al., 2004).

Table 1: Inhibition zone diameter (mm) for antibacterial extracts

	Positive Control		**Green Kombucha beverage**			**Black Kombucha Beverage**		
Strain	**Kanamycin (30 µg.L^{-1})**	**AAS**	**Kombucha**	**Filtered**	**Neutralized**	**Kombucha**	**Filtered**	**Neutralized**
***E. coli* 25922**	24.0 ± 0.1	18.8 ± 0.2	15.3 ± 0.3	15.5 ± 0.4	ND	15.8 ± 0.2	15.0 ± 0.1	ND
EPEC (CDC 0126)	21.5 ±0.1	19.2 ± 0.3	16.0 ± 0.0	15.8 ± 0.9	ND	16.4 ± 0.4	15.7 ± 0.4	ND
EIEC	22.4 ± 0.0	23.0 ± 0.0	14.9 ± 0.3	13.5 ± 0.5	ND	15.9 ± 0.2	14.9 ± 0.2	ND
EHEC (CDC EDL-933)	20.0 ± 0.0	18.5 ± 0.4	16.0 ± 0.7	15.8 ± 0.6	ND	17.0 ± 0.1	17.1 ± 0.1	ND
ETEC (H10407)	24.1 ± 0.2	17.6 ± 0.4	18.0 ± 0.8	15.9 ± 0.5	ND	16.9 ± 0.1	17.2 ± 0.3	ND
***Salmonella* Typhimurium (ATCC 14028)**	15.0 ± 0.1	12.0 ± 0.1	ND	ND	ND	ND	ND	ND
***Salmonella* Flexneri (ATCC 12002)**	16.2 ± 0.1	15.0 ± 0.5	ND	ND	ND	ND	ND	ND
***Staphylococcus aureus* (ATCC 33591)**	10.0 ± 0.3	ND	ND	ND	ND	ND	ND	ND

ND: not detected inhibition zone AAS: Acetic Acid Solution

Conclusion

Antibacterial activity of kombucha beverage can be related to low pH and organic acid, as acetic acid and gluconic acid. The presence of catechins might work synergistically with other compounds present in Kombucha. Future research can be evaluating the potential of Kombucha as an effective antimicrobial control for emerging multidrug-resistant microorganisms.

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INTEGRATED CONCLUSIONS AND FINAL CONSIDERATIONS

Analysis of the kombucha microbial composition revealed low species diversity in both yeasts and acetic acid bacteria. The *Komagataeibacter* and *Zygosaccharomyces* are the genera of bacteria and yeasts, respectively, dominant in fermentation. *Zygosaccharomyces bailli* is a yeast resistant to high concentrations of sugars, ethanol, and acids. This yeast in the inoculum is fundamental for understanding the composition of the microbiota and its interaction during the fermentation process. The acetic acid bacteria was the group most abundant in the inoculum of kombucha. For establishing a starter culture, the use of osmotolerant yeasts, such as *Z. bailli*, is recommended in association with species of acetic acid bacteria of the genus *Komagataeibacter*.

Both kombuchas showed similar concentrations for pH, TRS, ethanol, and TTA. There was a difference in the concentration of phenolic compounds among the kombuchas analyzed. The black tea kombucha showed high caffeine and gallic acid concentrations, whereas the chlorogenic acid had higher concentrations in green tea kombucha.

The data fusion strategy making it possible to distinguish them in terms of matter press used. In the future, this strategy may be viable for the predilection of the composition of beverages by less invasive and low-cost methods. In the PCA analysis of both the integrative approach and the data fusion approach, phenolic compounds were responsible for separating kombuchas concerning the type of tea.

The kombucha samples analyzed showed antimicrobial activity against serotypes of *E. coli*. These beverages may be a possible source of natural antimicrobials. Improving the kombucha fermentation process and directing the production of certain compounds may, in the future, configure a strategy for obtaining these compounds in a sustainable, safe, and inexpensive way.

Kombucha can be a source of substances with functional properties and of microorganisms with industrial application.