JING ZHANG

A COMPREHENSIVE STUDY ON KOMBUCHA AND ITS ANALOGUES

LAVRAS-MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

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ABSTRACT

Kombucha is a tea-based, non-alcoholic fermented beverage known for its refreshing scent, anti-microbial and anti-oxidant activity. Traditionally it is produced by fermenting black tea that is sweetened with sucrose and pitched with a SCOBY (a polysaccharide matrix containing yeasts and bacteria) that contains yeast and bacteria. This study aimed to firstly identify the microbial profile of a black tea kombucha produced in Brazil and secondly compare traditional black tea kombucha with analogue kombuchas produced with five alternative substrates, including green tea, white tea, chrysanthemum, honeysuckle and mint infusions. A combination of chemical tests, MALDI-TOF MS and 16s rRNA gene sequencing was used for identification of the microbiota of black tea kombucha. *Debaryomyces hansenii* and *Pichia occidentalis* were the most abundant yeasts, while *Brettanomyces anomalus*, *Rhodotorula mucilaginosa* and *Meyerozyma guilliermondii* were also found. *Acetobacter tropicalis* was the predominate acetic acid bacteria in this culture. Scanning electron microscopy was conducted to examine the ultrastructure of the SCOBY. Both yeast and bacteria were observed and an ultrafine cellulose structure was recorded as well. Throughout the analogue kombucha fermentations, sugars, ethanol and organic acids were monitored by HPLC and volatile compounds were analyzed by GC-MS. The results showed that sugar consumption was substrate-dependent. A total of 46 volatile organic compounds were detected across all kombuchas, including alcohols, esters, acids, aldehydes, ketones and other compounds. The final kombuchas contained compounds that were substrate-specific but also had compounds in common, that were produced during the fermentation. A sensory analysis was carried out by an untrained panel with 58 participants. Mint and green tea kombucha obtained the highest and lowest scores for overall approval, respectively.

Key-words: kombucha, kombucha analogues, scanning electron microscopy, MALDI-TOF, 16s rRNA gene sequencing, Brazilian kombucha microbiology, gas chromatography – mass spectrometry, volatiles of kombucha and its analogues

RESUMO

O kombucha é uma bebida fermentada não alcoólica à base de chá, conhecida por seu aroma refrescante, atividade antimicrobiana e antioxidante. Tradicionalmente, a bebida é produzida pela fermentação do chá preto adoçado com sacarose e preparado com SCOBY (matrix polissacaridica de bacterias e leveduras). Este trabalho teve como objetivo de, primeiramente, identificar o perfil microbiano de um kombucha de chá preto produzido no Brasil e, segundamente, comparar o tradicional kombucha de chá preto com análogos de kombucha produzidos com cinco substratos alternativos, incluindo infusões de chá verde, chá branco, crisântemo, madressilva e hortelã. Uma combinação de testes químicos, MALDI-TOF MS e sequenciamento genético foi utilizada para a identificação da microbiota do chá preto de kombucha. *Debaryomyces hansenii* e *Pichia occidentalis* foram as leveduras mais abundantes, enquanto *Brettanomyces anomalus*, *Rhodotorula mucilaginosa* e *Meyerozyma guilliermondii* também foram encontradas. *Acetobacter tropicalis* foi a bactéria do acido acético predominante nesta cultura. Microscopia eletrônica de varredura foi conduzida para examinar a ultraestrutura do SCOBY. Leveduras e bactérias foram observadas e uma estrutura de celulose ultrafina foi registrada também. Ao longo da fermentação dos analógos de kombucha, açúcares, etanol e ácidos orgânicos foram monitorados por HPLC e os compostos voláteis foram analisados por GC-MS. Os resultados mostraram que o consumo de açúcar foi dependente do substrato. Um total de 46 compostos orgânicos voláteis foram detectados em todos os kombuchas, incluindo álcoois, ésteres, ácidos, aldeídos, cetonas e outros compostos. Os kombuchas finais continham compostos que eram específicos do substrato, mas também tinham compostos em comum, que foram produzidos durante a fermentação. Uma análise sensorial foi realizada por um painel não treinado com 58 participantes. O kombucha de hortelã e chá verde obtiveram as pontuações mais altas e mais baixas na aprovação geral, respectivamente.

Palavras-chave: kombucha, análogos do kombucha, microscopia eletrônica de varredura, MALDI-TOF, sequenciamento genético, microbiologia do kombucha, cromatografia gasosa - espectrometria de massas, voláteis do kombucha e seus análogos

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Chapter 1
1 INTRODUCTION

Kombucha is a fermented beverage that is usually inoculated by back-slopping with a tea fungus that contains bacteria and yeast. It is traditionally fermented with black tea (sometimes green tea) and white sugar. The beverage has a long history of consumption and while its precise origins are unknown, it is clear is that the beverage has been favored by consumers from different countries and in different times, from ancient China to modern America, due to its refreshing sensation. Although kombucha is still a novelty in Brazil, its popularity has risen in recent years as it is being commercialized by many small-scale producers.

Many studies have investigated the microbial consortia present in kombucha cultures. Yeasts and bacteria were isolated and identified and fermentation dynamics were shown to vary depending on a variety of factors, including the origin of the culture, the fermentation substrate and initial sugar concentration that were used as well as fermentation conditions such as temperature and fermentation time.

Also the chemical composition of the kombucha beverage has drawn much attention from researchers, partly because of its presumed health benefits. Most of the studies have focused on its antioxidant activity and on organic acids that may have health promoting effects. Much less attention has been paid to the formation of aroma compounds during the fermentation process and how they relate to the sensory perception of the beverage.

In this comprehensive study of kombucha and its analogues, kombucha is approached first as a microbial ecosystem and second as a functional beverage with potential for innovations.

In the chapter two article 1, the microbial profile of the kombucha beverage was investigated. There is still much to be explored regarding the composition of kombucha cultures, in particular in relation to its geographic origin, and also regarding the potential roles that different groups of micro-organisms fulfill during the fermentation process. To complement this research, scanning electron microscopy was conducted to visualize the ultrastructure of the kombucha tea fungus.

In the chapter two article 2, the chemical composition and sensory characteristics of kombuchas are examined. This part of the study investigated both traditional black tea

kombucha and 5 kombucha analogues, using green tea, white tea, chrysanthemum, honeysuckle and mint infusions as substrates. These substrates were chosen based on their high antioxidant activity, attractive aroma and international popularity. Changes in the composition of sugars, alcohols, organic acids and volatile organic compounds were evaluated. Finally, a sensory analysis was carried out to see how the chemical profiles that were obtained relate to consumer perception.

2 THEORETICAL BACKGROUND

2.1 Kombucha and its history

Kombucha is a fermented beverage that is commonly produced from sweetened black tea. It has a characteristic sour taste and its flavor has been described as cider-like, turning vinegary after prolonged fermentation (Dufresne and Farnworth, 2000; Sievers et al., 1995). The fermentation process is carried out by a consortium of yeast, acetic acid bacteria (AAB) and sometimes lactic acid bacteria (LAB) and results in the production of a cellulose-containing biofilm (Marsh et al., 2014) that floats on top of the beverage, as portrayed in Figure 1. This biofilm is often referred to as 'tea-fungus' or SCOBY (symbiotic culture of bacteria and yeasts).

Figure 1. A jar of fermenting black-tea kombucha containing a cellulose biofilm.

General aspects of the kombucha beverage, including its microbial and chemical composition, its history and its health effects have been reviewed by Greenwalt et al. (2000), Jayabalan et al. (2014) and more recently by Villarreal-Soto et al. (2018), Sinir et al. (2019) and Chakravorty et al. (2019).

There are several origin myths surrounding kombucha, some of which have made their way into the scientific literature, where they are often reported as factual (e.g. Jayabalan et al., 2014). According to these legends, kombucha may have been consumed as early as 220 B.C. by Emperor Qin Shi Huang of China, administered as medicine to an ailing Japanese Emperor by a certain dr. Kombu, or consumed during war-time by the troops of Genghis Khan. However, Crum and LaGory (2016) report that they were unable to substantiate any of these

stories after extensive research. A recurring issue is that it is unknown if certain drinks that are referred to in ancient texts truly correspond to the beverage that is now known as kombucha.

According to the same authors, it is not improbable that kombucha does in fact originate from China, as this is the country where tea originates from. From there, it may have spread to Russia and Germany where the first medical experiments using kombucha occurred around the turn of the twentieth century. Kombucha soon gained a reputation of being able to cure numerous diseases (Hermann, 1929) and was commonly produced in households in Germany and Central-Europe (Mayser et al., 1995).

In the 1960's, kombucha was embraced by the hippie counterculture in the United States but remained relatively unknown to the general public. It is not until 1995 that the first commercial kombucha brewery was founded which, after a relatively slow start, was followed by hundreds of other producers worldwide in what became a billion-dollar industry (Christina Troitino, 2017).

2.2 Kombucha preparation

Kombucha can be prepared according to a variety of recipes that follow similar steps, including tea preparation, sweetening, inoculation and fermentation. A typical home-production process is depicted in Figure 2, based on preparation methods described by Greenwalt et al. (2000) and Sinir et al. (2019).

Black tea is the most commonly used substrate to prepare kombucha, but green tea and alternative substrates may also be used (Battikh et al., 2012; Gaggìa et al., 2018). Tea may be prepared by a variety of methods, with some authors preferring a short steeping time of 10-15 minutes (Sievers et al., 1995; Sun et al., 2015) while others allow the tea to cool down for 30-60 minutes before removing the tea leaves (Aloulou et al., 2012; Greenwalt et al., 1998).

Sweetening is commonly performed with sucrose in concentrations between 50-150 g L^{-1} (Greenwalt et al., 2000). A number of studies explored alternative sweetening agents, such as honey, brown sugar and molasses (Malbaša et al., 2008; Watawana et al., 2015). It should be noted that addition of honey may influence the microbial composition of the SCOBY, in particular the yeast fraction (Reva et al., 2015). High sucrose concentrations (in excess of 90 g L^{-1}) may hinder growth of micro-organisms during the fermentation and have been shown to result in lower cellulose yields and a less pronounced pH decrease compared to moderate sucrose concentrations, likely due to osmotic pressure (Goh et al., 2012).

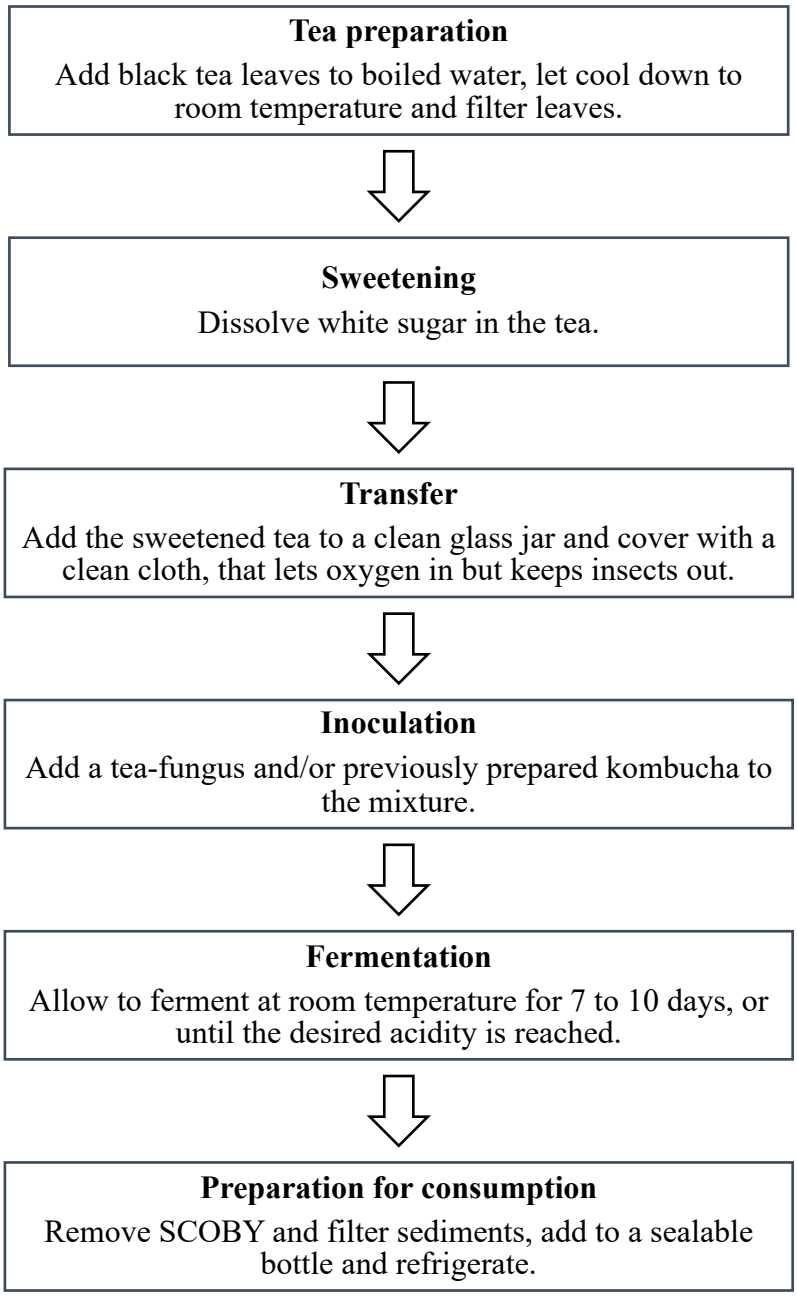

Figure 2. Instructions to prepare a batch of black tea kombucha.

The sweetened tea needs to be cooled down to room temperature before the kombucha culture is added, to avoid heat-shock to the micro-organisms. The tea is typically added to a glass jar that is covered with a clean cloth (Jayabalan et al., 2010; Sievers et al., 1995) which ensures aerobic conditions during the fermentation process and keeps insects out. Nonetheless,

fermentation occurs under non-aseptic conditions and the culture is at risk of being contaminated by pathogenic bacteria, yeasts or molds (Nguyen et al., 2015).

Inoculation usually occurs with both the SCOBY ('tea-fungus') and 10-20% v/v of liquid from a previously made batch of kombucha (Greenwalt et al., 2000; Sinir et al., 2019). In this way, micro-organisms are transferred from an old batch to a new batch, a technique that is known as back-slopping. This method is frequently used in fermentations that involve undefined starter cultures and ensures a high initial load of micro-organisms that are well-adapted to the substrate, accelerating the production process and improving reproducibility (Holzapfel, 1997). Addition of liquid from a previous batch also establishes an acidic environment that limits risk of pathogen growth, especially if the pH drops below 4.2 (Nummer, 2013). It is possible to inoculate kombucha with liquid culture only, which will lead to the formation of a new tea-fungus (May et al., 2017).

Kombucha is normally fermented for 7-10 days (Greenwalt et al., 2000) but in research contexts, fermentation has been monitored for much longer periods (up to 60 days) to gain more insight into the process (Sievers et al., 1995). Prolonged fermentation leads to kombucha with a vinegary aroma and acidity levels that are potentially dangerous to human health (Nummer, 2013; Sievers et al., 1995). For this reason, kombucha fermentation is usually ended at an arbitrary time point where taste and flavor are considered optimal and some residual sugar is still present (Ebersole, 2016; Sinir et al., 2019).

Temperatures for kombucha fermentation typically range from 22 to 30 °C. The temperature may selectively stimulate or inhibit growth of specific groups of micro-organisms in the culture (Villarreal-Soto et al., 2018). The optimal temperature for kombucha fermentation may therefore depend on the composition of the culture and the desired sensory characteristics. Higher fermentation temperatures are associated with faster sucrose consumption and higher production rates of acids and other fermentation metabolites (Lancar et al., 2006).

After fermentation, the liquid is typically filtered, poured into a sealable bottle and refrigerated until consumption. Both homebrewers and commercial brewers may add fruit juice for extra flavoring and submit the beverage to a second fermentation that is alcoholic under anaerobic conditions, which favors yeast growth and leads to a carbonated beverage. (Crum and LaGory, 2016). However, research articles usually omit this second fermentation, very few studies have focused or researched regarding this issue

2.3 Kombucha microbiology

2.3.1 General composition and safety

Kombucha is produced by a symbiosis of bacteria and yeasts that is distributed over two phases: the fermenting tea (liquid phase) and the cellulose bio-film that floats on top of it (solid phase) (Chen and Liu, 2000). The yeasts in kombucha are primarily responsible for breaking down sucrose (or alternative carbon source), producing ethanol and carbon dioxide. Acetic acid bacteria, the main group of bacteria present in kombucha, convert ethanol into acetic acid. Specific strains of AAB are capable of producing cellulose, which leads to the formation of the biofilm ('tea-fungus' or SCOBY) that is characteristic of kombucha and vinegar fermentations. Additionally, a population of LAB is occasionally encountered in kombucha cultures (Chakravorty et al., 2019; Sinir et al., 2019).

The microbial composition of kombucha cultures may differ depending on the geographic origin of the culture, the substrate used and fermentation conditions (Gaggìa et al., 2018; Marsh et al., 2014; Reva et al., 2015). However, the simultaneous production of ethanol and acetic acid combined with the presence of tea tannins and proteins form a barrier against external micro-organisms (Mo et al., 2008). According to Sreeramulu et al. (2000), black tea kombucha inhibits growth of a range of common foodborne pathogens, including *Enterobacter cloacae*, *Pseudomonas aeruginosa, Escherichia coli, Salmonella enteritidis, Campylobacter jejuni* and *Bacillus cereus.*

From a food safety point of view, pH levels below 4.2 are considered to effectively inhibit growth of the majority of foodborne pathogens (Lund et al., 2000). In most cases, addition of 10% of a previously made kombucha to inoculate a new batch of kombucha lowers the pH below this threshold (Nummer, 2013; Sievers et al., 1995).

It should be noted that weak organic acids, such as lactic acid and acetic acid, are considered important stressors to both bacteria and yeasts that are toxic beyond simply lowering the pH (Bauer et al., 2003; Hirshfield et al., 2003). In an acidic external environment, weak organic acids occur in their neutral, protonated form; they can permeate the membrane into the cell, where the pH is higher and they dissociate into their anionic form. This anionic acid can no longer leave the cell, leading to an accumulation of weak organic acids which

disrupts metabolism (Mollapour et al., 2001). For this reason, weak organic acids are effective food preservatives (Hirshfield et al., 2003).

2.3.2 Yeasts

A wide spectrum of yeasts has been isolated from kombucha cultures, comprising species of many different genera. In general, yeast diversity is considered to be greater than bacterial diversity in kombucha cultures (Teoh et al., 2004). The most commonly reported yeasts belong to the genera *Zygosaccharomyces* spp., *Brettanomyces* spp. and *Saccharomyces* spp. (Marsh et al., 2014; Mayser et al., 1995; Teoh et al., 2004). Also yeasts from the genera *Candida* spp., *Pichia* spp. and *Schizosaccharomyces* spp. have been isolated repeatedly (Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Yeasts are found in the tea-fungus as well as the liquid, with the same genera present in both phases and in similar proportions (Marsh et al., 2014).

Common characteristics of yeasts that are found in kombucha is that they are fermentative, osmotolerant and acid tolerant (Teoh et al., 2004). For this reason, species of *Zygosaccharomyces* and *Brettanomyces* are often persistent spoilage yeasts in industrial fermentation processes where domination by *S. cerevisiae* is desired (Kurtzman, 2011).

A major role that is ascribed to yeasts during kombucha fermentation is the hydrolysis of sucrose to produce glucose and fructose (Villarreal-Soto et al., 2018). As shown in Figure 3, sucrose can be hydrolyzed by the action of extracellular invertases that are attached to the cell wall. The resulting glucose and fructose can diffuse away from the cell or enter the cell via hexose transporters in the membrane (Koschwanez et al., 2011). Both glucose and fructose are detected as metabolites in kombuchas that are sweetened with only sucrose (Sievers et al., 1995). However, not all yeasts produce extracellular invertases; production has been reported for species including *S. cerevisiae* and *Z. bailii* (Arez et al., 2014). Others, such as *Brettanomyces* spp., produce intercellular invertase (Reis et al., 2014) and therefore do not contribute to the kombucha symbiosis in this way.

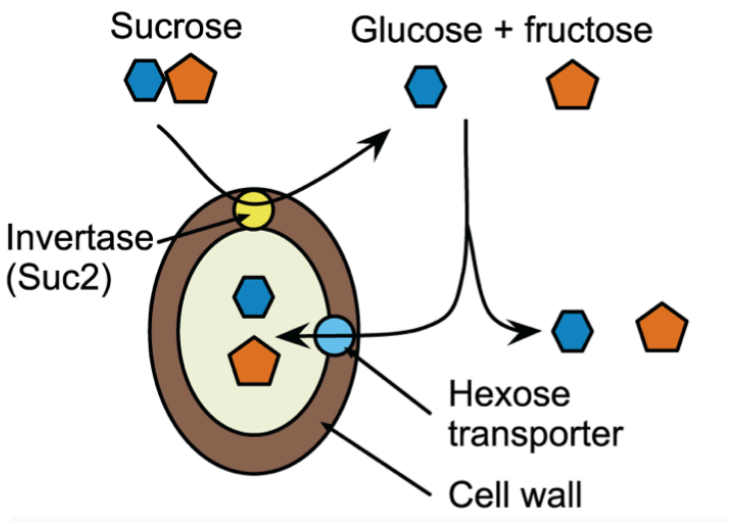

Figure 3. Action of invertase on sucrose in *S. cerevisiae*. From: Koschwanez et al. (2011)

The most important yeasts in kombucha (*Zygosaccharomyces* spp., *Brettanomyces* spp. and *Saccharomyces* spp.) are all fermentative, meaning that they can grow anaerobically and produce ethanol (Freer, 2002; Martín et al., 2002). As discussed below, ethanol can serve as a carbon and energy source for AAB. Some yeasts, such as *Brettanomyces* spp. and specific strains of *S. cerevisiae,* are also known to produce significant amounts of acetic acid themselves (Freer, 2002; Paraggio and Fiore, 2004). It is currently not known to which extent yeasts contribute to the overall acetic acid content of kombucha.

Some authors discuss the possibility that yeasts contribute to aroma development in kombucha by their production of aroma compounds such as esters (Mayser et al., 1995; Teoh et al., 2004). Nonetheless, actual research on aroma compounds in kombucha is scarce. A study by Zhao et al. (2018) described flavor compounds produced during fermentation of pu-erh tea kombucha and claims that a number of esters increases in concentration during fermentation. However, these esters do not correspond to the flavor-active esters that are commonly attributed to yeasts, such as ethyl acetate, ethyl hexanoate, ethyl octanoate, isoamyl acetate and phenethyl acetate (Pires et al., 2014; Steensels et al., 2014), warranting further investigations into this topic.

2.3.3 Acetic acid bacteria

The most frequently isolated AAB in kombucha belong to the genera *Komagataeibacter*, *Gluconacetobacter* and *Acetobacter* (Jayabalan et al., 2014). The precise composition appears to vary significantly between kombucha cultures. A study by Marsh et al. (2014) that compared multiple cultures from different geographic origins did not find any isolates of *Acetobacter* spp. in most cultures. To the contrary, several others identified strains of *A. aceti, A. pasteurianus* or *A. liquefaciens* to be the dominant strains in cultures that they investigated (Abd El-Salam, 2012; Chen and Liu, 2000; Zhang et al., 2011). According to Marsh et al. (2014), AAB are abundant in both the tea-fungus and liquid.

The main role that is ascribed to AAB in kombucha fermentation is the oxidation of ethanol to acetic acid. Oxidation of ethanol happens in two steps, as shown in Figure 4. In the periplasmic space, membrane-bound enzymes can oxidize ethanol to acetaldehyde and then further to acetic acid. In these enzymes, pyrroloquinoline (PQQ) serves as a redox co-factor. It sets in motion an electron transport chain that requires oxygen as final electron acceptor, explaining why acetic acid bacteria are obligate aerobes. The electron transport chain generates a proton motive force which is leveraged by membrane-bound ATPases. Alternatively, acetic acid can enter the cell via passive diffusion and be oxidized there, allowing the cell to obtain acetyl-CoA which is needed to drive anabolic reactions (Vidra and Németh, 2017).

In absence of ethanol, *Acetobacter* spp. are capable of further oxidizing acetic acid to water and carbon dioxide (Raspor and Goranovič, 2008). However, since kombucha that is prepared for human consumption generally does not reach the stage where all sugars and ethanol are consumed (Sievers et al., 1995), it does not seem likely that this would occur during kombucha fermentations for commercial production.

Besides acetic acid, AAB can oxidize sugars such as glucose and fructose. Via the pentose phosphate pathway, glucose can be oxidized to gluconic acid, which occurs in kombucha (Chen and Liu, 2000). Furthermore, *Acetobacter* spp. possess a fully functional Kreb's cycle and can utilize glucose as a carbon and energy source (Mamlouk and Gullo, 2013).

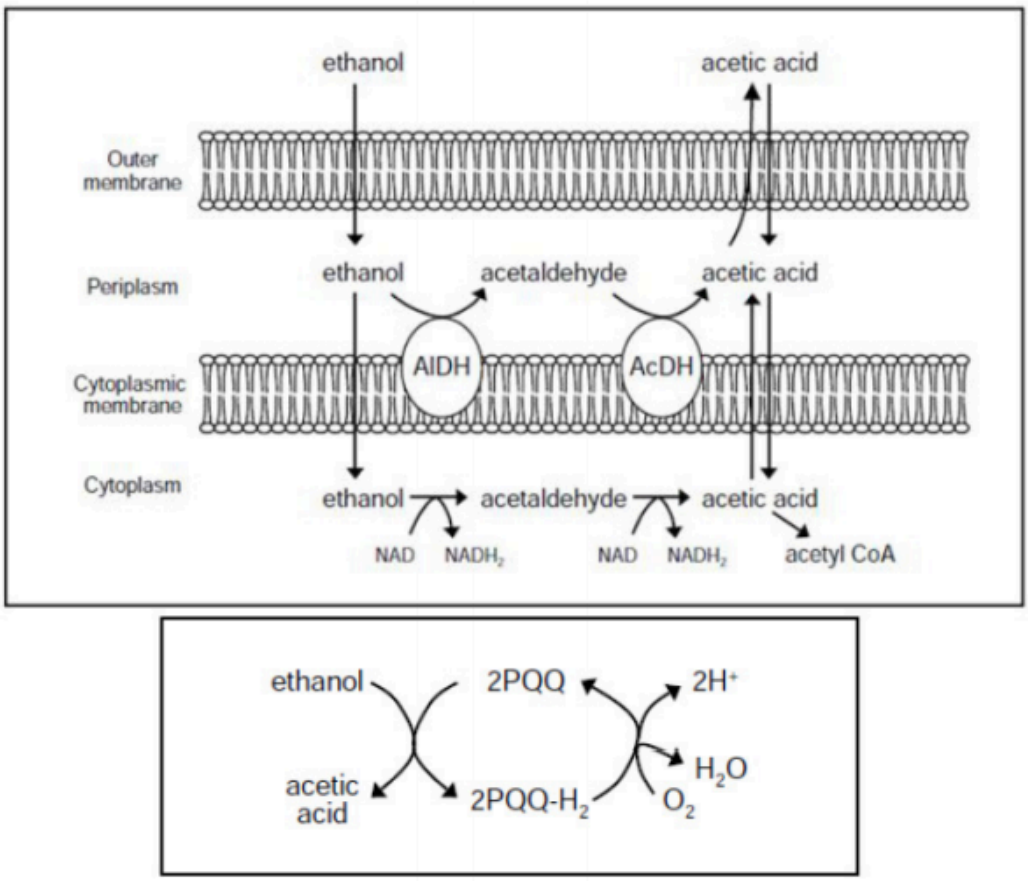

Figure 4. Oxidation of ethanol by AAB. PQQ = pyrroloquinoline; AlDH = alcohol dehydrogenase; AcDH = acetaldehyde dehydrogenase. From: Vidra and Németh (2017)

A number of AAB species is capable of producing bio-cellulose. This cellulose forms a bio-film on the surface of the liquid which enables the bacteria to maintain contact with oxygen (Valera et al., 2015). Bacterial cellulose has received attention from researchers and engineers as its structure and composition can be controlled more easily than cellulose from plant sources. This makes it a promising resource for the development of novel nano-materials (Moon et al., 2011).

Production of bio-cellulose is mediated by genes of the *bcsABZC* operon which is distributed among strains of *Komagataeibacter* spp., *Gluconacetobacter* spp. and *Acetobacter* spp. in a strain-dependent fashion. For example, a number of strains of *A. aceti* and *A. pasteurianus* have been shown to produce bio-cellulose while other strains of the same species did not (Valera et al., 2015).

Another noteworthy species is *K. xylinus* (formerly *G. xylinus* and before that *A. xylinum*), which is the most commonly studied species in the context of bacterial cellulose production (Moon et al., 2011) and became the type-species for the *Komagataeibacter* genus (Yamada et al., 2012). It is among the most commonly isolated species from kombucha (Jayabalan et al., 2014) along with other cellulose producing species, such as *K. kombuchae* and *K. rafaeli* (dos Santos et al., 2014; Dutta and Gachhui, 2007).

2.3.4 Lactic acid bacteria

Although LAB are generally considered less important than AAB and yeasts, they are occasionally isolated from kombucha, mostly belonging to the genera *Lactobacillus* spp., *Lactococcus* spp. and *Oenococcus* spp. (Coton et al., 2017; Marsh et al., 2014). It has been reported that towards the end of fermentation LAB occurred in both SCOBY and liquid, in similar concentrations (Coton et al., 2017).

A testimony to their relatively minor importance is the observation that lactic acid concentrations in kombucha are generally much lower than the concentration of acetic acid (Jayabalan et al., 2014, 2007) or even absent (Sievers et al., 1995).

2.4 Chemical composition of the kombucha beverage

The most common compounds detected in kombucha include sugars (sucrose, fructose and glucose), organic acids (acetic acid, gluconic acid, glucuronic acid, citric acid, malic acid, succinic acid, tartaric acid, oxalic acid and others), ethanol, glycerol, polyphenols ((-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) and theaflavin (TF)) and vitamins (V_{B1}, V_{B6}, V_{B12}, V_C) (Bauer-Petrovska and Petrushevska-Tozi, 2000; Greenwalt et al., 2000; Jayabalan et al., 2014, 2007; Malbaša et al., 2011; Sinir et al., 2019).

Although acetic acid and tea polyphenols are in great part responsible for kombucha's antimicrobial and antioxidant activity, several additional compounds are thought to contribute to kombucha's functional characteristics. Glucuronic has drawn academic attention due to its presumed detoxifying effect. It was first studied by Blanc (1996), who detected glucuronic acid concentrations of more than 30 g l^{-1} in kombucha after 20 days of fermentation. Nguyen et al. (2015) conducted a kombucha fermentation with an artificial starter culture to optimize production of glucuronic acid, and reported that a ratio of 4:6 between cells of *Dekkera*

bruxellensis and *Gluconacetobacter intermedius* resulted in the highest production of glucuronic acid (175.8 mg l^{-1}) after 7 days of fermentation.

Kombucha is considered a functional beverage and is associated with many health claims (Watawana et al., 2015). Positive health effects of long-term kombucha consumption are usually attributed to its presumed pro-biotic nature, to detoxification mechanisms that are associated with the presence of glucuronic acid or to its anti-oxidant activity caused by vitamins and polyphenols (Vīna et al., 2014).Positive health effects are also ascribed to D-saccharic acid -1,4 - lactone (DSL). This compound is a competitive inhibitor of ß-glucuronidase, which is an enzyme that generates toxic and carcinogenic effects in the human body (Wang et al., 2010). Several studies have detected DSL in kombucha. One study found that the strain *Gluconacetobacter sp*. A4 is a major producer of DSL, suggesting this strain would be a potential starter culture candidate to strengthen the health benefits of kombucha (Yang et al., 2009).

An in-vitro study using human prostate cancer cell lines demonstrated reduced survival of cancer cells after treatment with kombucha (Srihari et al., 2013a). While direct evidence of positive health effects in humans is still lacking, several studies using animal models such as mice did obtain promising results (Kapp and Sumner, 2019). These include prevention and treatment of diabetes (Aloulou et al., 2012; Srihari et al., 2013b; Yang et al., 2009), curing of gastric ulcers (Banerjee et al., 2010), reduced physical disturbance upon electromagnetic field exposure (Gharib, 2014), improvement of cardiovascular symptoms (Lobo and Shenoy, 2015), reduced weight gain, increased physical activity and even longevity (Hartmann et al., 2000).

2.5 Tea and alternative substrates for kombucha preparation

Tea plants occur in two main varieties: *Camellia sinensis* var. sinensis and *Camellia sinensis* var. assamica (Dufresne and Farnworth, 2000). White tea, green tea and black tea are all made from the same plant but are subject to different levels of processing. The different levels of processing give rise to the distinct flavor and color of the tea infusions, with white tea having the lightest color and mildest flavor, while black tea has the darkest color and most intense flavor (Rusak et al., 2008). Due to its milder processing, white tea often retains the highest amount of polyphenols such as flavonoids, of which catechins (EC, ECG, EGC, EGCG, GC) are the predominate ones, followed by green tea and black tea (Hara et al., 1995).

While kombucha is traditionally produced with black tea and white sugar, a range of potential alternative substrates has been studied, including green tea, oolong tea, jasmine tea, lemon balm tea, mulberry tea, peppermint, wheatgrass juice, coffee, coffee berry extract, traditional Tunisian plants, fresh, acid and reconstituted sweet whey, coconut water, pear juice, banana peel, nettle leave, oak, Coca-Cola, wine, vinegar, Jerusalem artichoke, Echinacea, milk and others (Sinir et al., 2019).

The outcomes of previous studies on kombucha analogues showed that in some cases these performed superior to black tea kombuchas in terms of sensory characteristics or functional properties (Sinir et al., 2019). For example, kombucha analogues using traditional Tunisian plants such as *Thymus vulgaris* L., *Lippia citriodora*, *Rosmarinus officinalis*, *Foeniculum vulgare* and *mentha piperita* proved to be more effective at inhibiting growth of a wide range of food pathogens (Battikh et al., 2012). Furthermore, kombucha brewed with banana peel and nettle leaf infusions showed a higher antioxidant activity than traditional black tea kombuchas (Pure and Pure, 2016).

3 FINAL CONSIDERATIONS

Kombucha has a long history of consumption but only recently became a popular and economically important beverage, including in Brazil where more and more people start to appreciate this fermented tea.

While kombucha has a long history of consumption, the background research for this thesis project revealed several gaps in the academic understanding of the microbiology of kombucha cultures and of the characteristics of kombucha as a beverage, that this study aims to fill.

Previous studies on the microbial composition of kombucha cultures obtained diverging results that varied significantly by geographic origin, even if most kombucha studies until now have been carried out in Europe and China and none in Brazil. Since kombucha is commonly consumed in its raw, unpasteurized form, the unknown composition of its starter culture may pose a food safety risk.

The kombucha culture is a complex microbial ecosystem in which the occurrence and role of different groups of micro-organisms such as yeasts, acetic acid bacteria and lactic acid bacteria have not yet been properly clarified. Moreover, although a tea fungus is commonly used to inoculate kombucha fermentations, its cellulose structure has not yet been observed by scanning electron microscopy in association with its micro-organisms.

Furthermore, while the chemical composition of kombucha has often been studied in relation to organic acids and anti-oxidants, knowledge of the volatile organic compounds in kombucha is still scarce.

In addition to the fermentation process, the flavor of the kombucha beverage is related to the substrate. While various substrates have been used for kombucha fermentation, some common tea and herbal infusions that are popular worldwide have not yet been studied, in particular white tea, chrysanthemum and honeysuckle. An evaluation of kombucha analogues produced with these substrates, including changes in composition during its fermentation, production of volatile compounds and a sensory analysis, could help to understand the impact of both the substrate and fermentation process on aroma development in kombucha and its influence on consumer perceptions.

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Chapter 2

Article 1: MICROBIAL ECOLOGY OF A BRAZILIAN BLACK TEA KOMBUCHA BEVERAGE AND OBSERVATION OF THE ULTRA-STRUCTURE OF THE TEA-FUNGUS

Abstract

Kombucha is a black tea or green tea-based beverage fermented with white sugar and a “tea fungus” composed of yeast and bacteria. The microbial community of kombucha varies from one culture to another, depending among other factors on the region of origin. This study investigated the microbial profile of a black tea kombucha beverage produced in Brazil. Upon inoculation by back-slopping with a tea-fungus and a previously produced kombucha, pH decreased from neutral to 3.51 and further to 3.09 after 120 h of fermentation. A combination of chemical tests, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and 16s rRNA gene sequencing was used for the identification. Both yeast and bacteria were found, including 9 different species, all from different genera. The most abundant yeasts were *Debaryomyces hansenii* and *Pichia occidentalis*, while *Brettanomyces anomalus*, *Rhodotorula mucilaginosa* and *Meyerozyma guilliermondii* were also found. Among bacteria, *Acetobacter tropicalis* was the predominate acetic acid bacteria in this culture and a significant presence of *Pseudoalteromonas* sp. was also detected. The predominate species that were found in this kombucha culture differed from those reported in previous studies. To complement this research, scanning electron microscopy was conducted to examine the ultrastructure of the kombucha tea-fungus and revealed both yeasts and clusters of bacteria embedded in a fine network of cellulose threads.

Key-words: kombucha, scanning electron microscopy, MALDI-TOF MS, 16s rRNA gene sequencing, *Acetobacter tropicalis*, *Debaryomyces hansenii*

1 Introduction

Kombucha is a vinegary fermented beverage with a long history of production. Traditional kombucha is produced with black or green tea (*Camellia sinensis*) that is sweetened with sucrose and fermented by an undefined starter culture which is transferred by back-slopping. This starter culture is a undefined symbiotic culture of bacteria and yeasts (abbreviated as SCOBY) and it produces a mildly acidic, carbonated beverage with a taste reminding of apple cider (Jarrell et al., 2000).

Acetic acid bacteria and yeasts are the main groups of micro-organisms that are present in the fermentation culture (Greenwalt et al., 2000; Marsh et al., 2014). The basis of this symbiosis is that yeasts break down sucrose into glucose and fructose by action of extracellular invertase and then further into ethanol and carbon dioxide. Acetic acid bacteria produce acetic acid by oxidation of ethanol and gluconic acid by oxidation of glucose. During the fermentation process, extracellular polysaccharides produced by acetic acid bacteria form a biofilm (called 'tea-fungus') that floats on top of the beverage (Jayabalan et al., 2014).

Kombucha tea is an inhospitable environment for micro-organisms to grow in. The black tea substrate is rich in catechins, theaflavins and tannins that inhibit growth of a range of bacteria and yeasts (Almajano et al., 2007; Bansal et al., 2013). Furthermore, fermentation metabolites such as acetic acid and ethanol contribute significantly to the anti-microbial activity that is reported for the kombucha beverage (Dufresne and Farnworth, 2000; Greenwalt et al., 1998). Assuring the microbiological safety of the product is especially important as the beverage is often consumed in unpasteurized form, containing living micro-organisms.

Multiple studies have investigated the microbial composition of kombucha cultures (Coton et al., 2017; Marsh et al., 2014; Sievers et al., 1995; Teoh et al., 2004). Yeasts belonging to a wide range of genera have been isolated from kombucha cultures, principally belonging to *Zygossacharomyces* spp., *Brettanomyces* spp. and *Saccharomyces* spp., while also yeasts of *Pichia* spp., *Candida* spp. and *Schizosaccharomyces* spp. are reported frequently (Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Common characteristics of yeasts that are found in kombucha is that they are fermentative, osmotolerant and acidotolerant (Teoh et al., 2004).

Acetic acid bacteria that are isolated from kombucha most frequently belong to the genera *Komagataeibacter*, *Gluconacetobacter* and *Acetobacter* (Jayabalan et al., 2014). The precise composition appears to vary significantly between kombucha cultures. A study by Marsh et al. (2014) that compared cultures from multiple geographic origins found that

Acetobacter spp. were absent in most cultures. However, several others identified strains of *A. aceti, A. pasteurianus* or *A. liquefaciens* to be the dominant strains in cultures that they investigated (Salam, 2012; Chen and Liu, 2000; Zhang and Zhang, 2011).

Previous studies also reported varying results regarding populations of lactic acid bacteria in kombucha cultures. While some reported substantial populations of *Lactobacillus* spp, *Lactococcus* spp. and *Oenococcus* spp. (Coton et al., 2017; Marsh et al., 2014), others did not find any lactic acid bacteria or lactic acid production (Neffe-Skocińska et al., 2017; Sievers et al., 1995). The presence of lactic acid bacteria in kombucha is potentially beneficial as it may enhance anti-oxidant activity and increase the concentration of glucuronic acid (Nguyen et al., 2015).

Kombucha has recently gained popularity in several regions of the world where it has not traditionally been consumed, including in Brazil. The currently available data on the microbiological composition of kombucha cultures suggest significant geographical variation in the microbial composition of kombucha.

This study assesses the microbial composition of a kombucha culture that is produced in Brazil. It assesses development of pH during the fermentation process, species diversity in the beverage and also includes images of the tea-fungus obtained with scanning electron microscopy. Elucidating the microbial composition of kombucha cultures of different origins contributes to a more complete assessment of the safety of its consumption in raw form. Furthermore, it may contribute to answering still outstanding questions regarding the role of different genera of yeasts, acetic acid bacteria and lactic acid bacteria in its fermentation process.

2 Materials and methods

2.1 Preparation of kombucha

Kombucha was prepared as described by Greenwalt et al. (2000) with mild modifications. To prepare the tea, 1 l of mineral water was boiled, 50 g of white sugar was added and left to infuse with 10 grams of black tea until it was cooled down to room temperature.

A SCOBY and 100 ml of a previous batch of kombucha (prepared in the same way) were added to inoculate the fermentation. The kombucha was left to ferment in a 2 l glass jar

covered with a clean cloth at 25 °C. Samples (10 ml) were taken periodically at 24 h intervals over a period of 168 h and frozen at -18 °C until further analysis. All fermentations were carried out in duplicate.

The SCOBY (originally obtained from the Netherlands) was donated by a local household in Lavras, Minas Gerais, Brazil. Mineral water, sugar and black tea were bought in a supermarket in Lavras, Minas Gerais, Brazil.

2.2 Media

Yeast extract peptone dextrose (YEPD) agar was used to isolate yeast from kombucha. It is prepared with 1% yeast extract, 2% peptone, 2% glucose/dextrose, 2% agar with pH adjusted to 3.5. Plates were incubated at 25 °C for 48h. Acetic acid bacteria were isolated on GYC agar, prepared with 10% yeast extract, 50% d-glucose, 5% calcium carbonate and 20% agar. The plates were incubated at 25 °C for 48h. Lactic acid bacteria were isolated on MRS agar, prepared with 1.0 % peptone, 1.0 % beef extract, 0.4 % yeast extract, 2.0 % glucose, 0.5 % sodium acetate trihydrate, 0.1 % polysorbate 80 (Tween 80), 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.02 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate, 1.0 % agar with pH adjusted to 6.2. Plates were incubated at 25°C for 48h. *Lactococcus* spp. were isolated on M17 agar (Merck, Germany). Plates were incubated at 37 °C for 48h. After isolation and purification, single colonies were transferred to cryovials containing 20% glycerol and 80% culture medium. Isolates were stored at -20 °C until identification.

2.3 Enumeration and isolation of micro-organisms

Samples were taken after 0, 24, 72, 120 and 168 hours from the liquid phase of the kombucha for pH measurement (by a pH meter), cell counting and primary cultivation for isolation. Freshly acquired samples at appropriate dilution were immediately plated on four different culture media to determine the amount of colony forming units (CFU).

2.4 Determination of morphology and phenotype

The morphological characteristics of the colonies, including cell size, cell shape, edge, color, and brightness, were observed using a light microscope. The phenotypes of the bacteria

were examined by Gram staining, catalase, oxidase activities, and motility tests (Krieg and Holt, 1984).

2.5 MALDI-TOF MS sample preparation and analysis

After reactivation, strains were grown aerobically on different media at 28°C for 24 h and checked for purity. Prior to MALDI-TOF MS analysis, the strains were sub-cultured at least twice, or until a homogeneous cell culture was observed. An *Escherichia coli* K12 colony was used as calibration. A total of 170 purified isolates were streaked on new agar plates for incubation. YEPG, GYC and MRS was incubated at 25 °C for 24 hours; M17 was incubated at 37 °C for 48 h. Of each colony, 20 mg was freshly transferred to microtubes for protein extraction. Then 6 µL of freshly prepared solution (47.5% acetonitrile and 2.5% trifluoroacetic acid (v/v) for bacteria or a solution of 70% formic acid in water (v/v) for yeasts) was added to the cells and proceeded mixture until homogenous. 0.7 µL of each cell suspension was transferred to the MALDI flex plate, followed by adding 1 µL of matrix solution (α-Cyano-4-hydroxycinnamic acid), a gentle mixture with pipette tips was then conducted. Plates were air dried at room temperature before analysis. Each sample was prepared in triplicate. The methodology of protein extraction, equipment calibration, and data analysis was referenced by Carvalho et al. (2017). A MALDI-TOF microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) was used for the analysis.

2.6 16S rRNA and ITS rDNA gene sequencing analysis

A total of 89 isolates (representative strains obtained by grouping performed by MALDI-TOF MS analysis) were sent for sequencing. DNA was extracted by Instagene (Bio-Rad, Germany). The bacteria DNA extracts were amplified by primers 27F and 1512R for 16S rRNA analysis and yeast DNA extracts were amplified by primer ITS1 and ITS4 for ITS rDNA analysis. (Devereux and Willis, 1995). PCR was performed on a thermal cycler, using the components of the Top Taq Master Mix Kit (QUIAGEN®). The PCR product was gel-loaded with 1.5% agarose (1.5% agarose diluted in 50X TAE buffer), followed by 70 V electrophoresis for 30 min with 1X TAE running buffer. SYBR Green dye was added to each sample to visualize the bands by emission of fluorescence in ultraviolet light. The amplified PCR products were sent to Macrogen USA – Humanizing Genomics (MD, USA) for sequencing.

The obtained sequences were compared for similarity with sequences from the same regions from NCBI, using the 4peaks sequence editor (nucleobytes.com).

2.7 Scanning electron microscopy

Three cubes with sides of 5 mm were cut from the outside of a kombucha tea-fungus fermented for 2 weeks, and prepared for observation. Samples were transferred into microcentrifuge tubes with modified Karnovsky solution at pH 7.2 and kept for 24 h in a refrigerator. They were then transferred to 30% glycerol for 30 minutes and cross sectioned in liquid nitrogen in about 2 mm fragments. These were rinsed in distilled water and followed by a post fix in 1% osmium tetroxide aqueous solution in water for 1- 2 h at room temperature. Dehydration was conducted by washing them with different concentrations of acetone solutions (25, 50, 70 and 90% for 10 minutes, and three times for 10 minutes at 100%). Afterwards, the samples were transferred to a Balzers CPD 030 critical point dryer (Balzers, Liechtenstein, Germany) to complete the drying process with carbon dioxide as a transition fluid. The specimens were mounted on aluminum stubs with double-stick carbon tape on aluminum foil coated with gold using a Balzers SCD 050 sputter and kept in a desiccator with silica gel until observation.

SEM analyses were conducted using a Leo EVO 40 microscope (Leo Electron Microscopy, Cambridge, UK), and the images generated at various magnifications were digitally recorded. The software used was X Ray microanalysis Quantax EDS system (Bruker, Berlin, Germany) (Alves et al., 2013).

3 Results and discussion

3.1 Change in pH during fermentation

In this study, kombucha was inoculated with both the tea-fungus and 10% of a previously produced kombucha to start the fermentation process. Back-slopping with liquid culture does not only transfer the micro-organisms but also imposes an acidic environment that favors the growth of acidophilic micro-organisms and reduces fermentation time. (Greenwalt et al., 2000; Sievers et al., 1995; Sreeramulu et al., 2000; Jayabalan et al., 2014).

As can be seen in Figure 1, the initial tea infusion had a close to neutral pH while addition of the fermentation culture instantly lowered the pH to 3.51. After 168 h of fermentation, pH decreased to 3.06.

The low pH at the beginning of fermentations helps to ensure a safe product, as pH levels below 4.2 are considered to effectively inhibit growth of most food-borne pathogens (Lund et al., 2000; Nummer, 2013).

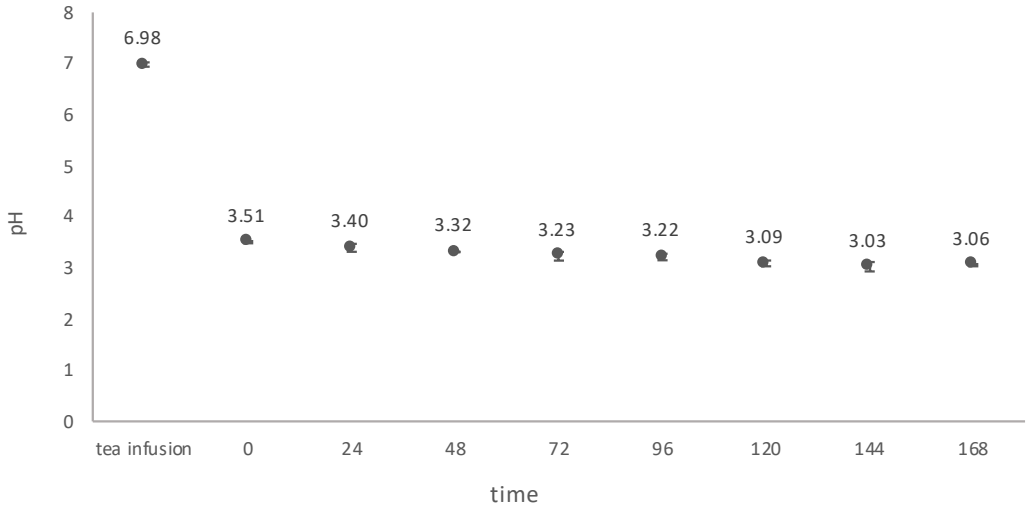

Figure 1. pH changes over course of 168 h fermentation. Tea infusion indicates the cooled down tea without any kombucha culture.

3.2 Microbial growth in kombucha

As the focus of this study is on the kombucha beverage, samples for microbiological analysis were taken over a period of seven days, after which kombucha is considered most drinkable. Prolonged fermentation leads to the development of an unpleasant vinegary flavour and acidity may reach levels that are unfit for human consumption (Greenwalt et al., 2000; Kumar et al., 2016; Sievers et al., 1995).

During the fermentation, CFU counts were obtained on different media which are shown in Figure 2. In the cooled down tea infusion, concentrations of micro-organisms were low (below detection limit and 1.9 log CFU ML^{-1} on the different media). Analogue to what is observed for the pH, inoculation with 10% of a previously produced kombucha increases microbial populations to values near those observed towards the end of fermentation.

Yeast populations (isolated from YEPG) steadily increased until the sixth day of fermentation, reaching a count of 6.15 log CFU ML^{-1}. This number is comparatively lower, as Chen and Liu (2000) analyzed multiple different kombucha cultures in which yeast cell counts all reached values above 7 log CFU ML^{-1} after the same time period.

The results indicate that in the first 48 h yeasts were outperformed by bacteria (isolated from MRS and M17). The CFU counts obtained on GYC (presumed acetic acid bacteria) were lower than that of other groups. It should also be noted that acetic acid bacteria from fermented foods are notoriously difficult to isolate on artificial media and may be present in a viable but non-culturable (VBNC) state (Gomes et al., 2018).

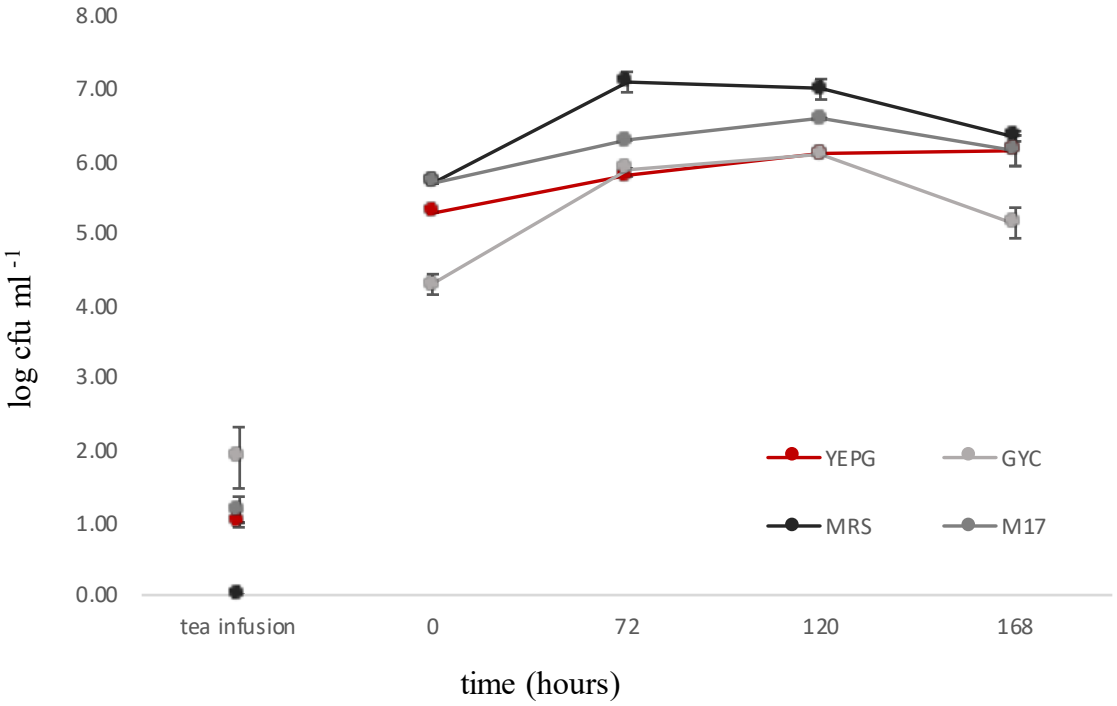

Figure 2. Growth of micro-organisms in kombucha liquid during 168 hours of fermentation.

3.3 Isolates identification

A total of 170 strains were isolated from the kombucha beverage after six days of fermentation. Isolated strains were clustered using MALDI-TOF MS and representative strains for each cluster were sent for sequencing. This resulted in the identification of nine species, all belonging to different genera (Table 1).

The dominating bacterial species in the kombucha beverage was *Acetobacter tropicalis* (59% of identified bacterial isolates) (Figure 3a). *A. tropicalis* was previously identified as one of the main acetic acid bacteria in industrially produced kombucha by Coton et al. (2017) although not as the predominate species. It has been reported that a well-studied strain of this species (*Acetobacter tropicalis* SKU1100) produces a thick pellicle, containing a cellulose-like material that is composed of galactose and rhamnose in addition to glucose (Ali et al., 2011; Deeraksa et al., 2005).

The kombucha culture in this study also had a significant presence of *Pseudoalteromonas* sp. (31%), which has not been found in kombucha cultures before. Although the genus is commonly associated with marine environments, it has also been isolated from Korean fermented foods (Jung et al., 2011; Morya et al., 2014). An interesting property of certain marine strains of this genus is their ability to produce exopolysaccharides that are mainly composed of glucose (Al-Nahas et al., 2011; Finore et al., 2014; Qin et al., 2007).

Since the most commonly identified cellulose producers in kombucha cultures such as *Komagataeibacter xylinum* were not found in this study, it is conceivable that *Acetobacter tropicalis* and potentially also *Pseudoalteromonas sp.* were responsible for the production of polysaccharides in the tea-fungus.

The majority of yeast isolates were identified as *Debaryomyces hansenii* (51%) and *Pichia occidentalis* (29%) while *Meyerozyma guillermondii* (14%), *Rhodotorula mucilaginosa* (4%) and *Bretannomyces anomalus* (2%) were also identified (Figure 3b).

Most previous studies indicate that yeast populations in kombucha are dominated by *Zygosaccharomyces* spp., *Brettanomyces* spp. or *Saccharomyces* spp. (Marsh et al., 2014; Mayser et al., 1995; Teoh et al., 2004). There is one previous report of *Debaryomyces* spp. dominating a kombucha fermentation (Ahmed and Dirar, 2005).

D. hansenii has been found in kombucha before, but in a minor role (Chakravorty et al., 2016). The species is known for its production of killer toxins with activity against other yeasts, as well as its osmotolerance and ability to grow at low pH (≥2.5) (Kurtzman, 2011). Moreover, it has been associated with fermentation of tea leaves, is reportedly able to hydrolyze tea tannins (Kanpiengjai et al., 2016) and has been proposed as a starter culture for tea beverages to improve their nutritional value (Pasha and Reddy, 2005).

Presence of *Pichia* spp. is common in kombucha and both *P. occidentalis* and *M. guilliermondii* (formerly known as *Pichia guilliermondii*) were previously isolated

(Chakravorty et al., 2016; Jayabalan et al., 2014; Reva et al., 2015). They may contribute to aroma development during kombucha fermentation as species of the *Pichia* genus are associated with high production of aroma compounds such as acetate esters (Viana et al., 2008).

In two commercial kombucha cultures, *R. mucilaginosa* was identified as the predominate yeast (Teoh et al., 2004). Both *B. anomalus* and *R. mucilaginosa* have been documented as high producers of extracellular ß-glucosidases (Hu et al., 2016; Wu et al., 2014). These enzymes may be able to hydrolyze cellulose produced by the *Acetobacter* spp. in the tea-fungus which would enable the species to utilize the released glucose for continued fermentation.

Kombucha fermentation is initiated by an undefined starter culture and usually takes place under non-aseptical conditions, hence the interchange of microbial species between tea fungus and local environment is inevitable. To place this study's results in context, Table 2 shows a summary of eight previous studies which focused on kombucha microbiology profiling, among which five were carried out in Europe, one in Australia, one in Sudan and one in India. All studies find the presence of acetic acid bacteria, belonging to the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter*. Lactic acid bacteria (*Lactobacillus* and *Lactococcus*) were found in three European studies (Coton et al., 2017; Marsh et al., 2014; Reva et al., 2015), but not as the major microbiota. There was not a big variation between bacteria profiling and research locations, potentially due to the difficulty of cultivation of acetic acid bacteria, and these genera were often found from back slopped fermentation such as Lambic beer and kefir as well (De Roos and De Vuyst, 2018). *Zygosaccharomyces* and *Dekkera* were dominant in several cultures and were commonly isolated from cultures of European origin (Switzerland, Ireland, Italy and France) (Coton et al., 2017; Gaggìa et al., 2018; Marsh et al., 2014; Sievers et al., 1995). Samples from other continents however showed divergent results, with *Brettanomyces*, *Pichia*, *Candida*, *Schizosacchromyces*, *Tolurospora*, *Rhodotorula*, *Debaryomyces*, *Lachanca*, *Kluyveromyces* and *Meyerozyma* being identified from Australian, Sudanese, Indian and Brazilian cultures, while Zygosaccharomyces was not found from these studies (Ahmed and Dirar, 2005; Chakravorty et al., 2016; Teoh et al., 2004).

Table 1. Identification of isolates from kombucha beverage by sequencing of the 16S rRNA gene.

Isolate	possible matches (% similarity)	GenBank reference sequence
YEPG 40	*Brettanomyces anomalus (97%)*	KY103300.1
YEPG 31	*Rhodotorula mucilaginosa (100%)*	MK453052.1
YEPG 5	*Meyerozyma guilliermondii (100%)*	MK044079.1
YEPG 6	*Meyerozyma guilliermondii (100%)*	MK044079.1
YEPG 2	*Debaryomyces hansenii (99%)*	MK352094.1
YEPG 3	*Debaryomyces hansenii (100%)*	MK352094.1
YEPG 16	*Debaryomyces hansenii (99%)*	MK352094.1
YEPG 23	*Debaryomyces hansenii (100%)*	MK352094.1
YEPG 26	*Debaryomyces hansenii (100%)*	MK352094.1
YEPG 53	*Debaryomyces hansenii (100%)*	MK352094.1
YEPG 71	*Debaryomyces hansenii (100%)*	MK352094.1
YEPG 73	*Debaryomyces hansenii (99%)*	MK352094.1
YEPG 35	*Pichia occidentalis (100%)*	KY849376.1
YEPG 59	*Pichia occidentalis (99%)*	KY849376.1
YEPG 68	*Pichia occidentalis (100%)*	KY849376.1
YEPG 69	*Pichia occidentalis (99%)*	KY849376.1
YEPG 70	*Pichia occidentalis (100%)*	KY849376.1
YEPG 75	*Pichia occidentalis (100%)*	KY849376.1
M17 76	*Halomonas sp. GX5A6 (98%)*	KF201593.1
M17 3	*Acetobacter tropicalis (99%)*	KY287775.1
M17 11	*Acetobacter tropicalis (99%)*	CP022699.1
M17 28	*Acetobacter tropicalis (99%)*	NR036881.1
M17 34	*Acetobacter tropicalis (99%)*	NR036881.1
M17 36	*Acetobacter tropicalis (99%)*	NR036881.1
MRS 64	*Pseudoalteromonas sp. (94%)*	FJ025762.1
MRS 92	*Pseudoalteromonas sp. (97%)*	FJ237008.1
MRS 93	*Pseudoalteromonas sp. (96%)*	FJ025764.1
MRS 94	*Vibrio sp. 6-4 (99%)*	FJ237008.1

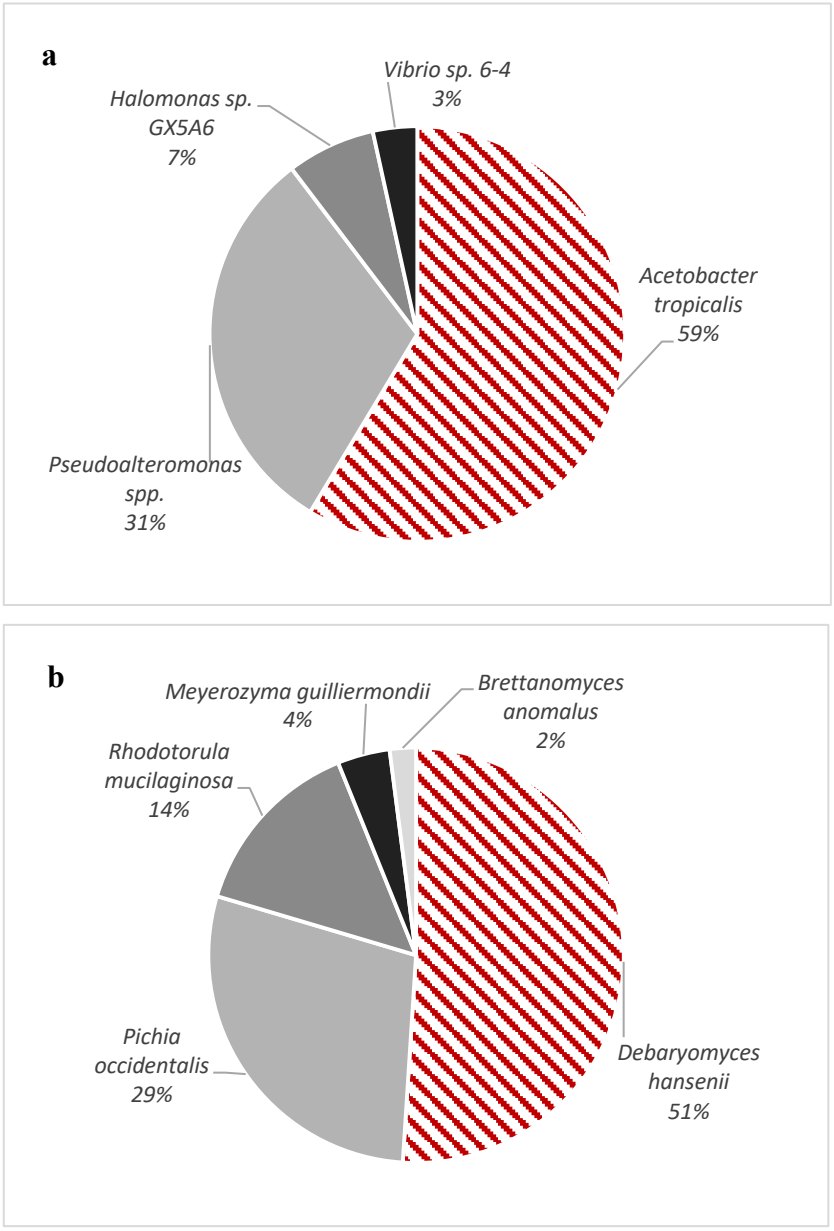

Figure 3. Species diversity of bacteria (a) and yeasts (b) in kombucha expressed as percentage of identified isolates.

Table 2. Summary of previous studies on kombucha microbiology composition research.

Research location	Kombucha sample	Yeast	Bacteria	Reference
Switzerland	Local pharmacy	*Zygosaccharomyces*	*Acetobacter xylinum* *Acetobacter aceti* *Acetobacter pasteurianus*	(Sievers et al., 1995)
Ireland	Commercial brands Canada 1	*Zygosaccharomyces* *Dekkera*	*Gluconacetobacter* *Lactobacillus* *Thermus*	(Marsh et al., 2014)
	Commercial brands Canada 2	*Zygosaccharomyces* *Dekkera*	*Gluconacetobacter* *Lactobacillus* *Lactococcus*	
	Commercial brands United Kindom	*not mentioned*	*Gluconacetobacter* *Thermus* *Lactobacillus*	
	Commercial brands United states	*not mentioned*	*Gluconacetobacter* *Lactobacillus* *Lactococcus*	
	Commercial brands Ireland	*Zygosaccharomyces* *Dekkera*	*Gluconacetobacter* *Lactobacillus* *Thermus* *Lactococcus*	
Italy	British commercial brand	*Zygosaccharomyces parabailii* *Brettanomyces bruxellensis*	*Komagataeibacter spp.* *Komagataeibacter intermedius* *Komagataeibacter rhaeticus* *Gluconacetobacter entanii*	(Gaggìa et al., 2018)
Fance	Local industrial production	*Dekkera bruxellensis* *Hanseniaspora vouyensis* *Dekkera anomala* *Zygotoralaspora florentina* *Zygosaccharomyces bailli* *Pichia membranifaciens* *Picha anomala* *Candida boidinii* *Saccharomyces uvarum* *Torulaspora microellopsopdes*	*Gluconobacter oxydans* *Gluconacetobacter europaeus* *Gluconacetobacter hansenii* *Acetobacter tropicalis* *Acetobacter okinawensis* *Gluconobacter cerinus* *Acetobacter covaniensis* *Oenococcus oeni* *Lactobacillus nagelii* *Lactobacillus satsumensis*	(Coton et al., 2017)
To be continued	Local collection	*Brettanomyces/Dekkera* *Pichia*	*Komagataeibacter* *Gluconobacter*	(Reva et al., 2015)

Ukraine			*Lactobacilli* *Halomonas spp.* *Herbaspirillum spp.*	
Australia	Commercial brand 1, New South Wales	*Candida stellata* *Schizosaccharomyces pombe* *Torulospora delbreuckii* *Rhodotorula mucilaginosa* *Brettanomyces bruxellensis*	*not examined*	(Teoh et al., 2004)
	Commercial brand 2, New South Wales	*Torulospora delbreuckii* *Rhodotorula mucilaginosa*		
	Commercial brand 3, New South Wales	*Schizosaccharomyces pombe*		
	Commercial brand 4, Queensland			
Sudan	Local culture	*Debaryomyces*	*Acetobacter xytinum*	(Ahmed and Dirar, 2005)
India	Local culture	*Candida* *Lachanca* *Kluyveromyces* *Debaryomyces* *Picha*	*Acetobacteraceae* *Komagataeibacter* *Gluconobacter*	(Chakravorty et al., 2016)
Brazil	Local household	*Debaryomyces hansenii* *Picha oxidentalis* *Rhodotorula mucilaginosa* *Meyerozyma guillierondii* *Brettanomyces anomalus*	*Acetobacter tropicalis* *Pseudoalteromonas sp.* *Halomonas sp. GX5A6* *Vibrio sp. 6-4*	This study

3.4 Ultra-structure of the tea-fungus

A scanning electron microscope was used to observe micro-organisms present in the tea-fungus (Figure 4). Pictures are shown in order of increased magnification. Both yeasts and bacteria were observed, but bacteria significantly outnumbered yeasts in the observed sections of the tea-fungus.

According to Tabuchi et al. (2007) bacterial cellulose that is produced by acetic acid bacteria consists of glucan-chains that form micro-fibrils with a width of 50-80 nm and a thickness of 3-8 nm. These micro-fibrils entangle to form a three-dimensional network. In Figure 4a, a net-like structure is shown in a location where the entangled three-dimensional structure appears to be stretched apart (which conceivably occurred as an artefact of sample preparation), showing the individual micro-fibrils. This fine net-like network of fibers is similar to what is observed by Goh et al. (2012).

Several previous studies observed the cellulose structure of the kombucha tea-fungus with scanning electron microscopy, but did not reveal the presence of micro-organisms due to different study objectives or sample preparation methods (Dima et al., 2017; Goh et al., 2012; Neera et al., 2015; Zhu et al., 2014). The micrographs obtained in this study show the presence of budding yeasts as well as clusters of rod- and cocci-shaped bacteria in the outer layer of the tea-fungus. (Figure 4b and 4c). At the highest magnification, it is visible that bacteria are embedded in the cellulose threads (Figure 4d). This may enable the cells, in particular aerobic acetic acid bacteria, to stay in closer contact with oxygen (Valera et al., 2015).

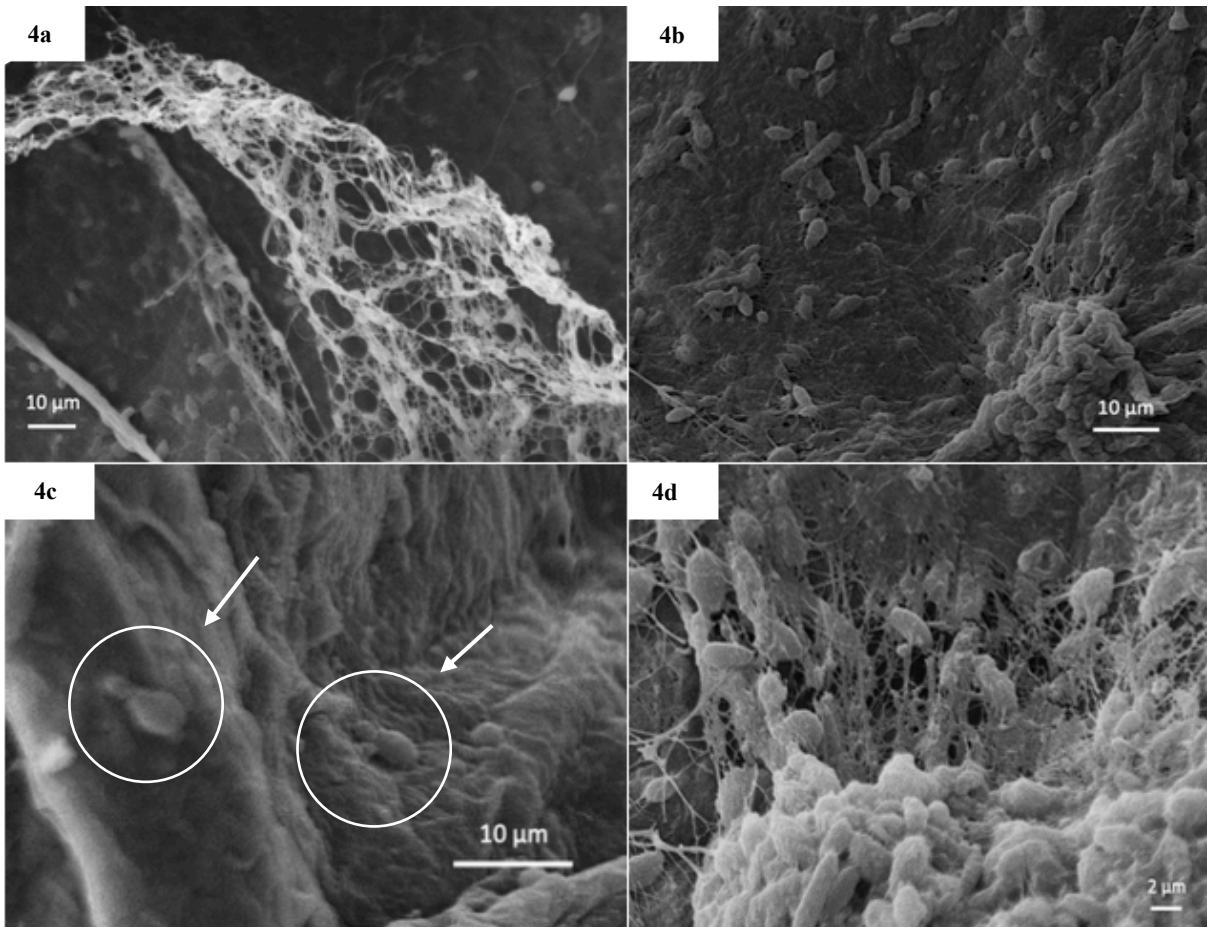

Figure 4. Scanning electron microscope micrographs of a kombucha tea-fungus, featuring a stretched net-like cellulose structure (4a); rod and cocci shaped bacteria (4b); a budding yeast (4c); and rod and cocci shaped bacteria embedded in the cellulose structure (4d).

4 Conclusion

Based on the data, it can be concluded that the composition of the microbial community of the kombucha beverage analyzed in this study differed from that reported in previous studies, in line with the hypothesis that the microbial composition of kombucha cultures strongly depends on the geographic location. Predominate bacterial species that were found included *A. tropicalis* and *Pseudoalteromonas sp.* which may contribute to the production of polysaccharides in the tea-fungus. The major yeast species were *D. hansenii* and *P. occidentalis*, while *M. guilliermondii*, *R. mucilaginosa* and *B. anomalus* were also identified. These species could contribute to the functionality of the kombucha culture in ways that warrant further research, such as the hydrolysis of tea tannis, production of ß-glucosidases that can hydrolyze cellulose in the tea-fungus and aroma production.

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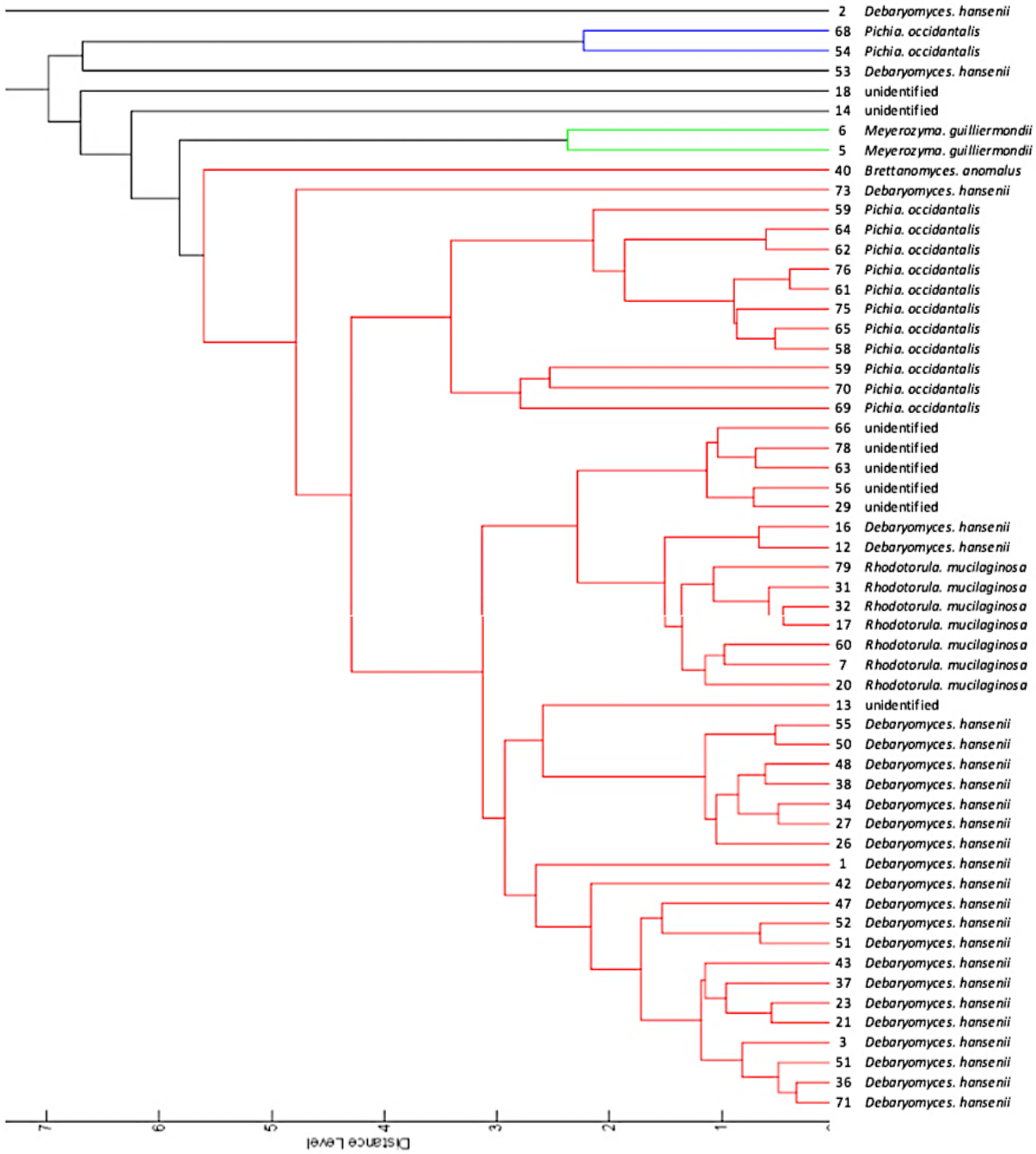
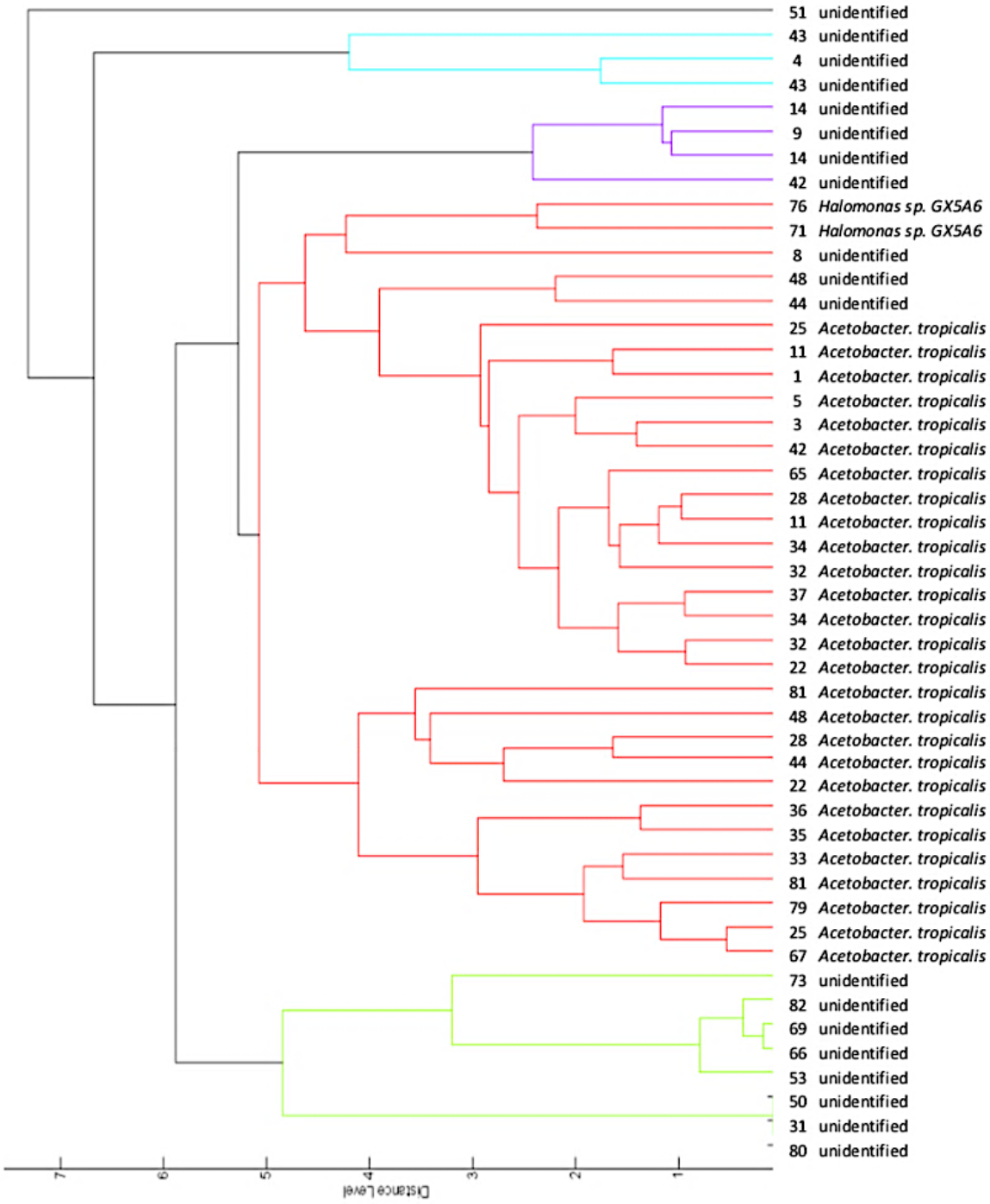
Appendix 1



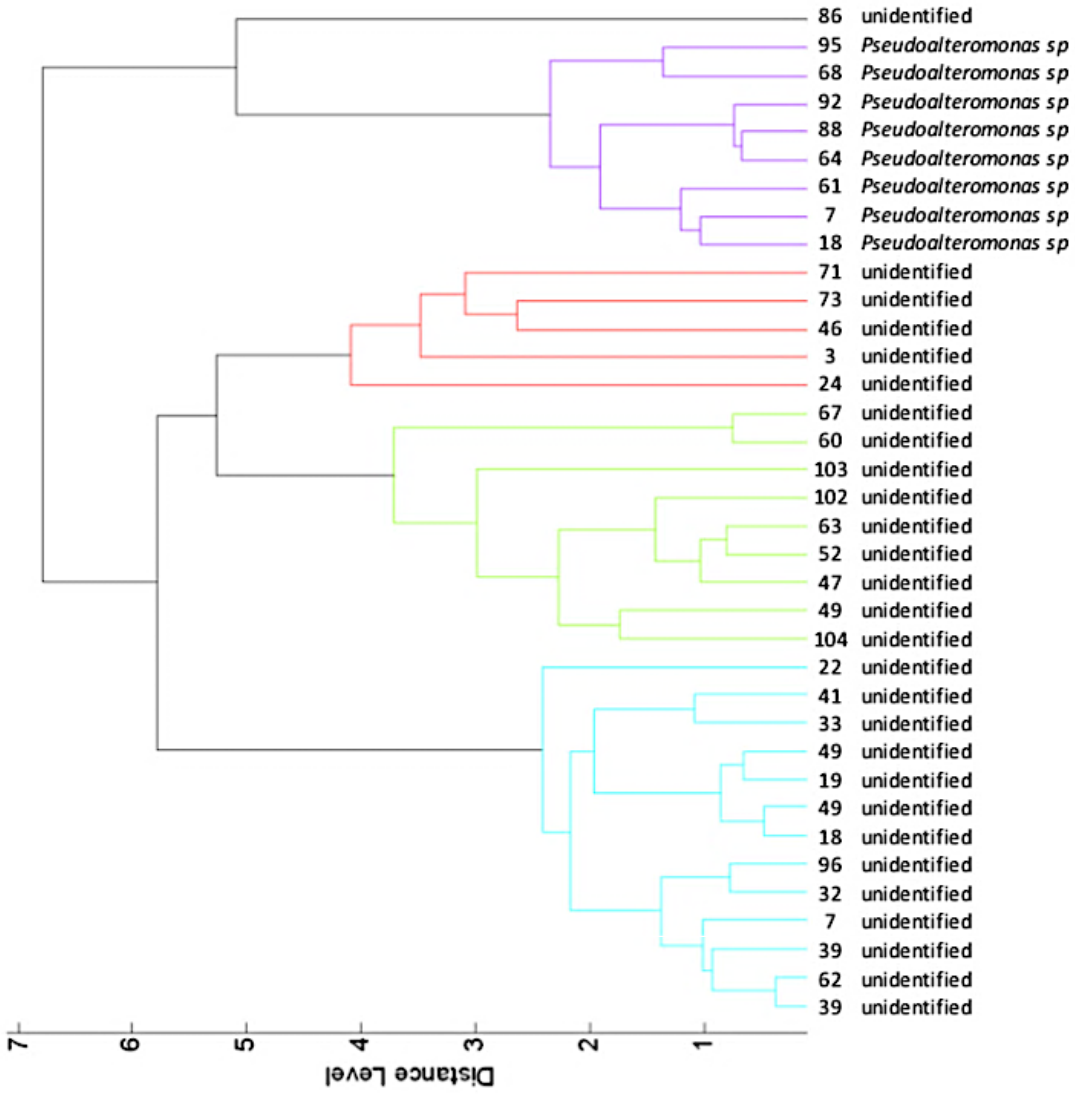
MADI-TOF MS dendogram of microorganism isolated from YEPG.

Appendix 2



MADI-TOF MS dendogram of micro-organisms isolated from M17.

Appendix 3



MADI-TOF MS dendogram of micro-organisms isolated from MRS.

ARTICLE 2: CHEMICAL AND SENSORY ANALYSIS OF KOMBUCHAS FERMENTED ON DIFFERENT SUBSTRATES

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Abstract

Kombucha is a tea-based, non-alcoholic fermented beverage known for its cider-like aroma and refreshing scent. Traditionally it is produced by fermenting black tea that is sweetened with sucrose and pitched with a "tea fungus" that contains yeast and bacteria. This study compared traditional kombucha with kombuchas produced on five alternative substrates, including green tea, white tea, chrysanthemum, honeysuckle and mint infusions. Throughout fermentation, sugars, ethanol and organic acids were monitored by HPLC and volatile compounds were analyzed by GC-MS. The results showed that sugar consumption was substrate-dependent, with mint kombuchas having the highest amount of residual sugar and honeysuckle the lowest. A total of 46 volatile organic compounds were detected across all kombuchas, including alcohols, esters, acids, aldehydes, ketones and other compounds. Among these, 22 compounds were produced during the fermentation process and detected in all kombuchas, some of which represent fruity and floral aromas. Another 24 compounds were substrate-specific. Notably, herbal-based kombuchas (chrysanthemum, honeysuckle, mint) contained a number of compounds that were absent in tea-based kombuchas and are associated with minty, cooling and refreshing aromas. A sensory analysis was carried out by an untrained panel with 58 participants. Mint and green tea kombucha obtained the highest and lowest scores for overall approval, respectively. This study demonstrates the suitability of herbal substrates for the preparation of hedonically acceptable kombuchas with flavor profiles that are distinct from tea-based kombuchas.

Key-words: kombucha, kombucha analogues, green tea kombucha, white tea kombucha, honeysuckle kombucha, chrysanthemum kombucha, mint kombucha, HPLC, GC-MS, kombucha volatiles, kombucha analogues sensory analysis

1 **Introduction**

Kombucha is a non-alcoholic fermented beverage produced from tea that is sweetened with sugar. Fermentation is carried out by a symbiotic culture of bacteria and yeasts that is commonly referred to as SCOBY. During the fermentation process, the culture produces a cellulose-containing biofilm called 'tea-fungus' that floats on top of the beverage and entraps the micro-organisms (Greenwalt et al., 2000). Kombucha has a pleasantly acidic, fruity flavor which may turn vinegary after prolonged fermentation as a result of the accumulation of acetic acid (Sinir et al., 2019).

The symbiosis of yeasts and acetic acid bacteria is responsible for the vinegary character of kombucha. Yeasts break down the carbon source, usually sucrose, into glucose and fructose, and further into ethanol and carbon dioxide. Acetic acid bacteria utilize the ethanol and produce organic acids such as acetic acid (Dufresne and Farnworth, 2000).

While kombucha is traditionally produced with black tea, a range of novel flavors can be developed by using alternative substrates. Previous studies showed that in some cases, these kombucha analogues outperformed black tea kombucha in terms of functional properties such as anti-oxidant activity and antimicrobial activity (Sinir et al., 2019; Battikh et al., 2012; Pure and Pure, 2016).

In this study, black tea kombucha is compared to analogues prepared with green tea and white tea. Teas are produced from the plant *Camellia sinensis* var. sinensis but can be subjected to different levels of post-harvest processing leading to distinct color, flavor and polyphenol contents (Dufresne and Farnworth, 2000; Rusak et al., 2008). Due to its milder processing, white tea often retains the highest amount of polyphenols such as flavonoids, followed by green tea and black tea (Hara et al. 1995a). Likewise, kombucha brewed with green tea trumps traditional black tea kombucha by having a higher amount of polyphenols (Jayabalan et al., 2007). While black and green tea kombuchas have been studied before in relation to its microbial composition and functional properties (Jayabalan et al., 2007; Gaggia et al., 2019), these studies did not focus on its aroma profile and sensory characteristics, knowledge of which is still lacking.

Additionally, three kombuchas are prepared from popular herbal infusions that each have distinct aroma profiles and well documented functional benefits. Chrysanthemum (*Chrysanthemum morifolium*) infusions have a unique aroma that is described as floral and

sweet, with a bitter and sweet aftertaste (Kaneko et al., 2017); it has a high anti-oxidant activity (Duh et al., 1999) as well as anti-inflammatory activity (Li et al., 2014). Honeysuckle infusions *(Lonerica japonica)* possess a mild and sweet aroma and have a high content of anthocyanins (Frejnagel, 2007). Both of these infusions have a long history of consumption, in particular in Asia where they are also applied as traditional medicine. Mint (*Mentha spicata*) is known for its refreshing and herbaceous aroma (Díaz-Maroto et al., 2003) and its extracts are broadly applied in the food, cosmetics and perfume industry while its constituents such as menthol, menthone and carvone also possess anti-oxidant activity (Sivropoulou et al., 1995). Of these herbal substrates, only mint kombucha has been studied before (Velicanski et al. 2013) but again in relation to its functional properties and not its aroma profile.

This study aims to provide inside in aroma development during kombucha fermentations by following the composition of sugars, acids and volatile organic compounds over the course of fermentation of kombucha and kombucha analogues. The final beverages were submitted to a sensory analysis to evaluate their taste and flavor characteristics as well as consumer approval. As kombucha has gained popularity in recent years, understanding of the aroma profile of black tea kombucha and analogue kombuchas may increase control of its sensory characteristics and stimulate the development of novel kombucha flavors with functional benefits.

2 Materials and Methods

2.1 Preparation of kombucha

Two different SCOBY cultures were obtained from the Netherlands (SCOBY 1, used for microbial identification in article 1) and Italy (SCOBY 2). SCOBY cultures were preserved in black tea kombucha at room temperature. Before each experiment, SCOBY cultures were conditioned on the intended substrate for 2 fermentation cycles.

To prepare the teas, 1 l of mineral water was boiled, 50 g of white sugar was added and left to infuse with one of the following substrates: a) 10 g of black tea, b) 10 g of green tea, c) 10 g of white tea, d) 10 g of dried chrysanthemum, e) 10 g of dried honeysuckle, f) 10 g of fresh mint, until they were cooled down to room temperature.
A SCOBY and 100 ml of a previous batch of kombucha (prepared in the same way, using the same substrate) were added. The kombucha was left to ferment in a 2 l glass jar covered with

a clean cloth at 25 °C. Samples (10 ML) were taken periodically at 24-48 h intervals over a period of 13 days and frozen at -18 °C until further analysis. All fermentations were carried out in duplicate. The comparison of SCOBY 1 and 2 was conducted by using black tea only.

Mineral water, sugar, fresh mint, green, black and white tea were bought in a supermarket in Lavras, Minas Gerais, Brazil. Dried chrysanthemum and honeysuckle were purchased on a market in Xining, Qinghai, China.

2.2 Analysis of acids, carbohydrates and ethanol

High-performance liquid chromatography (HPLC) was used to determine the concentration of alcohols, acids and carbohydrates, as described by Puerari et al. (2012). A Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV-Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai) was used. A Shimadzu ion exclusion columns Shim-pack SCR-101 H (7.9 mm x 30 cm) was operated at a temperature of 50 ºC for acids and alcohols and at 30 ºC for carbohydrates. A perchloric acid solution with pH 2.11 was used as the eluent at a flow rate of 0.6 ML/min. Acids were detected via UV absorbance (210 nm), while alcohols and carbohydrates were detected via RID. The concentration of carbohydrates (glucose, fructose and, sucrose), acids (acetic, lactic, citric, malic, tartaric, succinic and oxalic acid) and ethanol were quantified based on standard curves of the pure compounds.

2.3 Extraction of volatile compounds

The volatile compounds of kombucha were determined after extraction and concentration of the headspace compounds with solid phase micro extraction (SPME). Extraction was performed with a 50/30 μm DVB/CAR/PDMS fiber and manual holder (Supelco, Bellefonte, PA, USA). Aliquots of 2 ml of the kombucha samples were transferred to 10 ml vials that were hermetically closed with a teflon septum and screw cap. The SPME was operated at 60 °C, using an equilibrium time of 30 min and an extraction time of 15 min.

2.4 Analysis of volatile compounds

Analysis of volatile compounds was carried out as described by Ribeiro et al. (2017) with minor modifications. After extraction, the thermal desorption in the GC injector was performed at 240 °C for 5 min. The analyses were performed using a GC–MSQP2010 SE

system (Shimadzu) equipped with a DB-WAX column (30 m × 0.25 mm i.d. × 0.25 μm) operated at 50 °C for 5 min, increased at a rate of 3 °C/min up to 190 °C and maintained at 190 °C for 10 min. High-purity helium was used as carrier gas with a constant flow of 1.2 ml min^{-1}. The injections were performed using splitless mode with an injection time of 2 min. After 3 min, the mass spectra were acquired continuously from 45 to 1000 m/z. The temperature of the ion source was 230 °C and the quadrupole 150 °C. Mass spectra were compared against standard spectra from the NIST library version 2011 and further confirmation of the compound's identity is obtained by comparing their Kovat's retention index (using alkane standards as a reference) against references in the NIST RI database.

2.5 Sensory analysis

Kombuchas were prepared for each substrate, using SCOBY 1, and fermented for 6 days. Untrained participants (n = 58) were served a 15 ML sample of each kombucha. Samples were presented in random order and identified with random 3-digit codes. Participants ranked the intensity of attributes related to appearance (turbidity, darkness), aroma (floral, fruity, medicinal, herbal, balanced), taste (intensity, refreshing, acidity, sweetness, balance) and overall preference. All attributes were evaluated on a 9-grade scale. The questionnaire can be found in Appendix 1. The final results were interpreted based on the average score of each parameter (Nollet and Toldra, 2005).

2.6 Data analysis

Statistical analyses (ANOVA and Tukey's HSD post-hoc test) were performed using Python with Statsmodels. A principal component analysis and heat map were produced with Clustvis (https://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015).

3 Results and Discussion

3.1 Dynamics of fermentation

In this study, kombuchas were first produced using two different starter cultures in black tea and then subjected to six different substrates by SCOBY 1. The consumption of sucrose and production of fermentation metabolites were monitored throughout fermentation.

Concentrations of sugars, alcohols and organic acids that were detected after 144 h and 288 h of fermentation are shown in Table 1. The changes in the concentration of the main compounds of interest (sucrose, glucose, fructose, ethanol and acetic acid) over time are shown in Figure 1.

Using different starter cultures (SCOBY 1 and SCOBY 2) to produce black tea kombucha did not result in significant differences in the concentration of metabolites after 288 h of fermentation. However, higher intermediate concentrations of ethanol were observed for SCOBY 2. Keeping ethanol concentrations low is a quality concern during kombucha production as ethanol concentrations need to remain below 0.5% v/v to be sold as a non-alcoholic beverage on the US market or below 1.2% v/v on the EU market (Nummer, 2013). It was chosen to carry out kombucha fermentations on different substrates with SCOBY 1 as black tea kombucha produced with this starter culture did not cross these legal thresholds throughout the observed fermentation period.

A similar trend was observed among all samples: sucrose was consumed while fructose, glucose, ethanol and acetic acid were produced over the course of fermentation. At the final time point, differences in residual sucrose concentrations among tea-based kombuchas (27.3–35.8 g l^{-1}) were not significantly different according to ANOVA with Tukey's HSD post-hoc test. By contrast, residual sugar was lower in herbal-based kombuchas produced with honeysuckle (2.4 g l^{-1}) and chrysanthemum (11.4 g l^{-1}) but higher in kombucha produced with mint (36.6 g l^{-1}). Total residual sugar concentrations between 19.87 and 50.89 g l^{-1} were reported in an analysis of commercial kombuchas from different manufacturers (Ebersole, 2016).

Concentrations of acetic acid were not significantly different after 144 h and 288 h of fermentation. Nonetheless, higher sucrose consumption corresponded to increased accumulation of fermentation metabolites; honeysuckle kombucha had the highest concentration of acetic acid (3.4 g l^{-1} after 144 h and 3.8 g l^{-1} after 288 h) and other fermentation metabolites, while mint kombucha had lowest concentration of acetic acid (1.29 g l^{-1} after 144 h and 1.9 g l^{-1} after 288 h).

Significant differences were observed in the concentration of isovaleric acid, which was higher in tea-based kombuchas (1.22-4.01 g l^{-1}) than in herbal-based kombuchas (0.08-0.30 g l^{-1}). In most tea-based samples its concentration exceeded that of acetic acid, making it a major fermentation metabolite.

The difference in fermentation performance in tea- and herbal-based kombuchas may be attributed to antimicrobial activity of the substrates. Previous research indicated essential oil of mint was able to inhibit the growth of micro-organisms, including *Escherichia coli* and *Staphylococcus aureus*; its volatiles like piperitone, carvone, carveol, dihydrocarveol, menthone and pluegone possess antimicrobial activity (Sivropoulou et al., 1995). Furthermore, tea is known to inhibit growth of a range of yeasts and bacteria due to the presence of catechins, theaflavins and tannins (Almajano et al., 2007; Bansal et al., 2013).

Based on this data, the time point of 144 h was chosen to carry out the sensory analysis as at this time point, all samples were still compliant with EU and US legislation for non-alcoholic beverages. Kombucha fermentations are typically carried out for 7-10 days (Greenwalt et al., 2000). Prolonged fermentation leads to further increases in the concentration of both ethanol and acetic acid which imparts a vinegary character on the beverage (Sievers et al., 1995) and may lead to adverse health effects in susceptible consumers (Greenwalt et al., 2000).

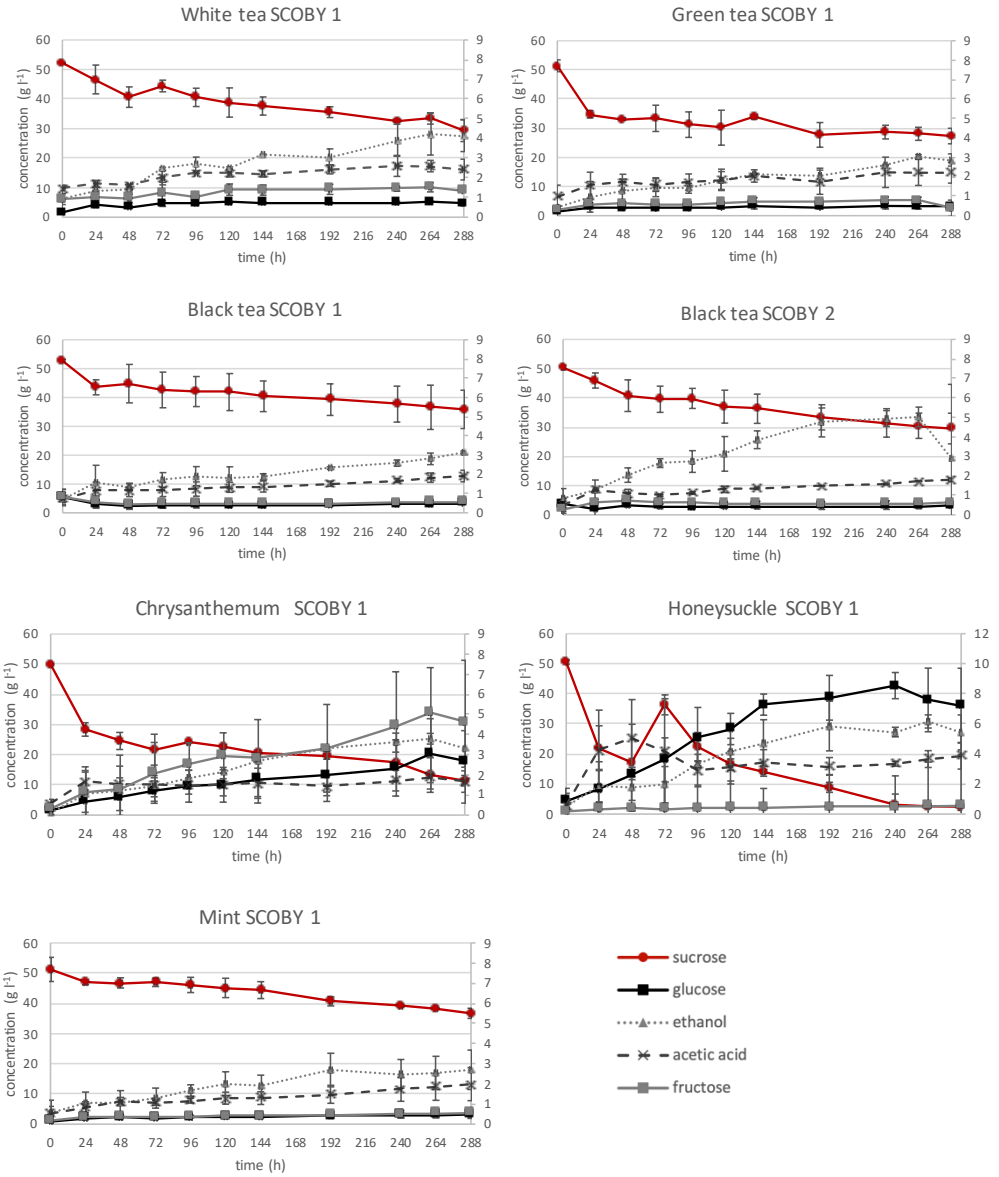

Figure 1. Evolution of the concentration of sucrose (g l^{-1}; primary axis) and glucose, fructose, ethanol and acetic acid (g l^{-1}; secondary axis) over the course of fermentation for seven different kombuchas produced with white tea, green tea, black tea, chrysanthemum, honeysuckle and mint (SCOBY 1) and black tea (SCOBY 2).

Table 1. Sugars, alcohols and organic acids detected by HPLC in kombuchas produced with different substrates, at 0 h, 144 h and 288 h of fermentation (g l^{-1}, mean ± sd).[1]

	White tea SCOBY 1	Green tea SCOBY 1	Black tea SCOBY 1	Black tea SCOBY 2	Chrysan-themum SCOBY 1	Honeysuckle SCOBY 1	Mint SCOBY 1	ANOVA
Sucrose								
0 h	52.2±0.06	51.4±2.02	52.6±0.45	50.3±0.81	49.9±0.23	50.5±0.70	51.2±3.99	NS
144 h	37.6±3.07^{ab}	33.8±0.76^{abc}	40.4±5.31^{a}	36.2±5.00^{ab}	20.4±1.11^{bc}	14.0±0.60^{c}	44.3±2.79^{ab}	**
288 h	29.3±3.72^{a}	27.3±2.65^{a}	35.8±6.64^{a}	29.5±5.24^{a}	11.4±1.73^{b}	2.44±1.23^{b}	36.6±1.67^{a}	***
Glucose								
0 h	0.24±0.00	0.20±0.15	0.81±0.38	0.56±0.78	0.23±0.00	0.85±0.85	0.12±0.09	NS
144 h	0.75±0.01^{b}	0.49±0.05^{b}	0.37±0.03^{b}	0.42±0.12^{b}	1.76±1.16^{b}	7.28±0.70^{a}	0.36±0.04^{b}	***
288 h	0.68±0.11	0.47±0.01	0.48±0.05	0.46±0.10	2.68±2.08	7.23±2.46	0.48±0.19	*
Fructose								
0 h	0.92±0.28	0.30±0.18	0.83±0.40	0.26±0.08	0.31±0.05	1.66±0.23	0.17±0.01	NS
144 h	1.37±0.25^{b}	0.75±0.17^{b}	0.46±0.05^{b}	0.59±0.09^{b}	2.83±1.91^{b}	7.14±1.28^{a}	0.42±0.06^{b}	***
288 h	1.33±0.00^{b}	0.38±0.41^{b}	0.57±0.04^{b}	0.61±0.10^{b}	4.61±3.07^{ab}	6.89±3.40^{a}	0.54±0.23^{b}	**
Glycerol								
0 h	0.01±0.00	0.03±0.05	0.02±0.02	0.06±0.08	0.02±0.01	0.07±0.02	0.06±0.09	NS
144 h	0.15±0.12	0.11±0.09	0.21±0.05	0.24±0.08	0.15±0.12	0.26±0.07	0.13±0.16	NS
288 h	0.19±0.13	0.11±0.15	0.32±0.06	0.38±0.07	0.31±0.03	0.52±0.06	0.21±0.27	NS
Ethanol								
0 h	0.93±0.68	0.43±0.52	0.68±0.15	0.84±0.01	0.10±0.08	0.50±0.13	0.55±0.32	NS
144 h	3.18±0.01^{ab}	2.13±0.01^{b}	1.87±0.16^{b}	3.85±0.45^{ab}	2.68±0.04^{ab}	4.73±1.54^{a}	1.88±0.54^{b}	*
288 h	4.13±0.80	2.86±0.31	3.14±0.07	2.89±3.79	3.29±0.90	5.43±1.16	2.69±0.97	NS
Acetic acid								
0 h	1.48±0.04	0.96±0.61	0.71±0.34	0.49±0.41	0.59±0.20	0.60±0.78	0.52±0.67	NS
144 h	2.19±0.15	2.04±0.31	1.36±0.28	1.37±0.04	1.58±0.75	3.40±0.88	1.29±0.31	NS
288 h	2.41±0.52	2.21±0.53	1.91±0.30	1.80±0.08	1.66±0.53	3.85±0.86	1.98±0.81	NS
Isovaleric acid								
0 h	1.78±0.17	1.15±0.76	3.73±0.56	3.99±0.72	0.05±0.05	0.00±0.00	0.04±0.04	NS
144 h	3.37±0.58^{a}	1.26±0.59^{ab}	3.96±0.74^{a}	3.80±0.34^{a}	0.24±0.18^{b}	0.13±0.00^{b}	0.10±0.01^{b}	***
288 h	3.72±0.56^{a}	1.21±0.39^{b}	3.99±0.68^{a}	4.01±0.51^{a}	0.30±0.24^{b}	0.11±0.00^{b}	0.08±0.05^{b}	***
Citric acid								
0 h	0.24±0.03	0.24±0.17	0.40±0.09	0.39±0.06	0.02±0.02	0.09±0.02	0.23±0.06	NS
144 h	0.33±0.13	0.25±0.05	0.06±0.09	0.44±0.08	0.26±0.00	0.78±0.17	0.17±0.24	NS
288 h	0.30±0.07	0.27±0.05	0.14±0.20	0.55±0.05	0.23±0.05	0.79±0.18	0.14±0.20	NS

(Continued)

	White tea SCOBY 1	Green tea SCOBY 1	Black tea SCOBY 1	Black tea SCOBY 2	Chrysan-themum SCOBY 1	Honeysuckle SCOBY 1	Mint SCOBY 1	ANOVA
Succinic acid								
0 h	0.05±0.03	0.02±0.01	ND	ND	1.40±0.99	0.03±0.02	0.20±0.14	NS
144 h	0.26±0.03^{ab}	0.24±0.14^{ab}	0.38±0.05^{a}	0.40±0.01^{a}	0.09±0.01^{b}	0.11±0.01^{b}	0.21±0.02^{ab}	**
288 h	0.23±0.00^{ab}	0.24±0.11^{ab}	0.40±0.06^{a}	0.42±0.06^{a}	0.11±0.02^{b}	0.12±0.00^{b}	0.22±0.01^{ab}	**
Lactic acid								
0 h	0.16±0.22	0.09±0.09	ND	ND	0.04±0.02	0.14±0.19	0.09±0.13	NS
144 h	0.14±0.10	0.06±0.04	ND	ND	1.39±1.20	0.19±0.13	0.16±0.11	NS
288 h	0.17±0.12	0.10±0.07	ND	ND	0.99±0.79	0.10±0.07	0.10±0.07	NS
Malic acid								
0 h	ND	ND	ND	0.11±0.00	0.09±0.08	0.04±0.03	0.04±0.02	NS
144 h	0.14±0.19	0.10±0.10	ND	ND	0.24±0.11	0.12±0.00	0.08±0.12	NS
288 h	0.13±0.17	0.12±0.13	ND	ND	0.25±0.07	0.11±0.04	0.08±0.11	NS
Propionic acid								
0 h	ND	ND	0.41±0.06	0.40±0.03	ND	ND	ND	NS
144 h	ND	ND	0.14±0.05	0.11±0.05	0.07±0.06	0.03±0.02	0.04±0.03	*
288 h	ND	ND	0.15±0.02^{ab}	0.13±0.02^{ab}	0.05±0.05^{bc}	0.03±0.02^{c}	0.02±0.02^{c}	**
Oxalic acid								
0 h	ND	ND	ND	ND	ND	0.08±0.05	ND	NS
144 h	ND	ND	0.37±0.02^{a}	0.34±0.01^{a}	ND	ND	ND	***
288 h	0.05±0.07^{b}	ND	0.36±0.02^{a}	0.36±0.05^{a}	ND	ND	ND	***
Tartaric acid								
0 h	ND	ND	ND	ND	ND	0.08±0.05	ND	NS
144 h	ND	ND	ND	ND	ND	ND	0.10±0.07	NS
288 h	ND	ND	ND	ND	ND	ND	0.11±0.08	NS

*[1]nd = not detected; ANOVA: NS = not significant. * = P < 0.05. ** = P < 0.01. *** = P < 0.001; different letters in same row mean samples are significantly different according to Tukey's HSD post- hoc test (P<0.05)*

3.2 Analysis of volatile compounds

The volatile composition of kombuchas produced using each of the different substrates was evaluated after 144 h of fermentation (Table 2), which was the time point used for the sensory analysis. In total, 46 volatiles compounds were detected across all kombuchas, including 15 alcohols, 9 esters, 7 fatty acids, 5, aldehydes, 5 ketones and 5 other compounds. The volatile composition after 0 h and 288 h of fermentation was also analyzed and can be found in Appendix 2, along with a table made to describe the change of volatiles over the course of fermentation and their scent (Appendix 2).

A total of 22 compounds were detected in all kombuchas, independent of the substrate; mostly alcohols, acids and esters. These include aroma-active compounds that are known microbial metabolites, such as ethyl octanoate (apricot aroma), p-ethylguiacol (spice) and

phenyl ethyl alcohol (lilac). All these compounds increase in concentration during the fermentation process. In honeysuckle kombucha, the sample with the largest extent of fermentation, a significantly higher amount of fermentation metabolite phenyl ethyl alcohol was found than in mint kombuchas, which had the lowest extent of fermentation.

Few significant differences were observed in the volatile profiles of white, green and black tea kombuchas; no significant differences were found between the volatile composition of black tea kombuchas produced with SCOBY 1 and SCOBY 2. White tea kombuchas contained the volatile compounds neodihydrocarveol and p-methylanisate, which were absent in green and black tea kombuchas; such differences may be attributed to different levels of tea leaves processing. (Hara et al., 1995a; Hara et al., 1995b).

Another 17 compounds were present in one or more of the herbal kombuchas, but not in tea-based kombuchas. Eugenol, having a sweet, woody scent, was only found in chrysanthemum and mint kombuchas; several carveol alcohols (trans-carveol, sis-carveol, pinocarveol) and p-cymene were found in honeysuckle and mint; volatiles like dihydro-carveol, 3-octanol, alpha-cadinol, (-)-trans-dihydrocarvyl acetate, dihydro-carvone, piperitenone and jasmone were exclusively found in mint kombucha, and are reported in the literature as common volatiles in mint, contributing to its characteristic, refreshing scent (Kaneko et al., 2017; Padmini et al., 2010). This suggests that mint kombuchas were able to retain signature mint aroma compounds during the fermentation process.

Combined, these results suggest that the kombuchas in this study were able to retain characteristic aroma compounds from their substrate. Simultaneously, acids, alcohols and esters were produced during the fermentation process that may contribute to a recognizable kombucha aroma.

To analyze the variety among the volatile profiles of the kombuchas after 144 h of fermentation, a principal component analysis (PCA) was carried out. Along PC1 (which explains 33.5% of variance) mint kombucha is separated from the other samples, while honeysuckle kombucha is separated along PC2 (31.9% of variance). Tea-based kombuchas had relatively similar compositions and are therefore in the same cluster, together with chrysanthemum kombuchas. For the latter sample, fewer characteristic aroma compounds were detected than in mint and honeysuckle kombuchas.

Table 2. Volatiles detected by HS-SPME/GC-MS in kombucha after 144 h of fermentation (logarithmic peak areas, mean ± sd).

144 h	White tea SCOBY1	Green tea SCOBY1	Black tea SCOBY1	Black tea SCOBY2	Chrysanthemum SCOBY1	Honeysuckle SCOBY1	Mint SCOBY1	ANOVA
Alcohols								
phenyl ethyl alcohol	5.57±0.02^{ab}	5.50±0.12^{b}	5.43±0.10^{b}	5.82±0.08^{ab}	5.84±0.04^{ab}	5.89±0.12^{a}	5.50±0.05^{b}	**
benzyl alcohol	4.50±0.05^{abcd}	4.57±0.16^{abc}	4.51±0.12^{abcd}	4.64±0.02^{ab}	3.95±0.13^{acd}	3.57±0.03^{cd}	4.36±0.25^{abcd}	*
1-decanol	4.20±0.12	2.14±3.03	4.36±0.00	4.39±0.35	4.36±0.01	3.92±0.03	4.03±0.39	NS
linalool	4.33±0.08^{b}	4.24±0.22^{b}	4.09±0.09^{b}	4.17±0.27^{b}	4.73±0.00^{a}	3.80±0.01^{b}	4.06±0.25^{b}	**
alpha-terpineol	3.96±0.12	4.14±0.04	3.75±0.27	3.87±0.16	4.41±0.07	4.64±0.12	2.43±2.42	NS
Neodihydro-carveol	1.77±1.77^{b}	ND	ND	ND	1.57±1.57^{b}	3.70±0.33^{b}	5.84±0.25^{a}	*
Dihydro-eugenol	ND	ND	4.27±0.03^{ab}	4.31±0.35^{ab}	4.64±0.18^{a}	ND	ND	*
trans-carveol	ND	ND	ND	ND	ND	4.95±0.10^{b}	5.99±0.17^{a}	**
carveol	ND	ND	ND	ND	ND	4.16±0.10^{b}	5.24±0.21^{a}	**
1-hexadecanol	ND	ND	ND	ND	ND	4.66±0.23^{a}	ND	**
pinocarveol	ND	ND	ND	ND	ND	4.62±0.07a	ND	***
dihydro-carveol	ND	ND	ND	ND	ND	ND	4.91±0.62	NS
3-octanol	ND	ND	ND	ND	ND	ND	5.23±0.02^{a}	***
alpha-cadinol	ND	ND	ND	ND	ND	ND	4.09±0.19^{a}	**
1-butanol, 3-methyl-	6.35±0.10	6.18±0.16	6.44±0.07	6.54±0.17	6.43±0.09	6.44±0.03	6.33±0.16	NS
Esters								
nonanoic acid, ethyl ester	4.23±0.00	2.37±3.37	4.42±0.69	4.21±0.41	4.77±0.14	4.59±0.34	4.59±0.97	NS
octanoic acid, ethyl ester	4.17±0.10	2.37±2.37	4.40±0.72	4.15±0.44	4.76±0.10	2.17±2.17	4.54±0.96	NS
decanoic acid, ethyl ester	3.99±0.06	2.18±2.18	3.93±0.61	2.12±2.12	4.09±0.31	1.93±1.93	2.44±2.44	NS
dodecanoic acid, ethyl ester	1.86±1.86	ND	1.82±1.82	1.99±1.99	1.66±1.66	3.91±0.29	2.58±2.58	NS
Hexadeca-noic acid, ethyl ester	2.26±2.26	2.04±2. 04	4.71±0.86	5.08±0.81	2.20±2.20	2.69±2.69	2.54±2.54	NS
9,12-octadecenoic acid, methyl ester	ND	ND	1.80±1.80	3.62±0.07	3.9±0.14	2.30±2.30	2.24±2.24	NS
.gamma.-palmitolactone	2.10±2.10	1.81±1.81	3.82±0.13	3.78±0.32	2.03±2.03	1.97±1.97	3.58±0.03	NS
methyl p-anisate	1.81±1.81^{b}	ND	ND	ND	ND	ND	5.08±0.20^{a}	**

(Continued)

144 h	White tea SCOBY1	Green tea SCOBY1	Black tea SCOBY1	Black tea SCOBY2	Chrysanthemum SCOBY1	Honeysuckle SCOBY1	Mint SCOBY1	ANOVA
(-)-trans-dihydrocarvyl acetate	ND	ND	ND	ND	ND	ND	2.36±3.34	NS
Acid								
octanoic acid	6.07±0.03	6.07±0.21	6.27±0.12	6.14±0.23	6.07±0.02	6.08±0.13	6.20±0.18	NS
benzeneaceticacid	4.94±0.04	4.85±0.08	4.97±0.12	5.06±0.22	5.07±0.07	5.20±0.02	4.91±0.16	NS
decanoic acid	6.12±0.10	6.31±0.21	6.29±0.21	6.28±0.23	6.13±0.06	6.20±0.33	6.28±0.34	NS
dodecanoic acid	4.45±0.08	4.84±0.32	4.83±0.09	4.91±0.16	4.68±0.00	5.13±0.36	5.07±0.17	NS
isovaleric acid	5.49±0.05^{b}	5.53±0.18^{b}	5.43±0.22^{b}	5.67±0.02^{ab}	5.82±0.09^{ab}	5.89±0.00^{a}	5.43±0.15^{b}	**
hexanoic acid	5.14±0.01	5.20±0.12	5.44±0.09	5.44±0.16	5.52±0.10	5.49±0.07	5.28±0.10	NS
isobutyric acid	4.95±0.04^{b}	5.08±0.24^{b}	4.89±0.05^{b}	5.24±0.04^{b}	5.29±0.06^{b}	5.52±0.03^{a}	4.97±0.08^{b}	***
Aldehydes								
2-decenal	4.17±0.18	1.89±1.89	3.85±0.04	2.36±2.36	ND	1.91±1.91	1.98±1.98	NS
2,4-dimethylbenzaldehyde	4.62±0.24	4.53±0.19	4.50±0.01	4.78±0.28	4.64±0.59	4.88±0.60	4.46±0.47	NS
benzaldehyde	3.53±0.01	3.54±0.23	3.71±0.09	3.86±0.14	3.36±0.13	1.97±1.797	ND	NS
nonanal	ND	ND	1.99±1.99	2.10±2.10	1.81±1.81	ND	ND	NS
Ketones								
d-carvone	2.40±2.40^{b}	2.05±2.05^{b}	1.82±1.82^{b}	4.12±0.23^{b}	ND	4.13±0.29^{b}	6.19±0.19^{a}	**
dihydro-carvone	ND	ND	ND	ND	ND	ND	5.43±0.19^{a}	**
piperitenone	ND	ND	ND	ND	ND	ND	4.98±0.24^{a}	*
jasmone	ND	ND	ND	ND	ND	ND	4.45±0.35	NS
procymidone	ND	ND	ND	ND	ND	4.14±0.13	ND	NS
Others								
p-ethylguai-col	5.04±0.00	5.12±0.22	5.80±0.09	5.92±0.20	5.71±0.09	4.93±0.02	5.94±0.33	NS
indole	1.70±1.70^{b}	1.83±1.83^{b}	1.83±1.83^{b}	1.74±1.74^{b}	1.75±1.75^{b}	5.33±0.07^{a}	1.74±1.74^{b}	***
eugenol	ND	ND	ND	ND	4.54±0.17^{b}	ND	5.46±0.06^{a}	***
phenol, 2,4,6-trichloro-	ND	ND	ND	ND	ND	4.53±0.10^{a}	ND	***
p-cymenene	ND	ND	ND	ND	ND	4.51±0.00^{a}	3.67±0.02^{b}	***
4-pyridone	ND	ND	ND	ND	ND	6.05±0.02^{a}	ND	***

*ND = not detected; ANOVA: NS = not significant. * = P < 0.05. ** = P < 0.01. *** = P < 0.001; different letters in same row mean samples are significantly different according to Tukey's HSD post- hoc test (P<0.05)*

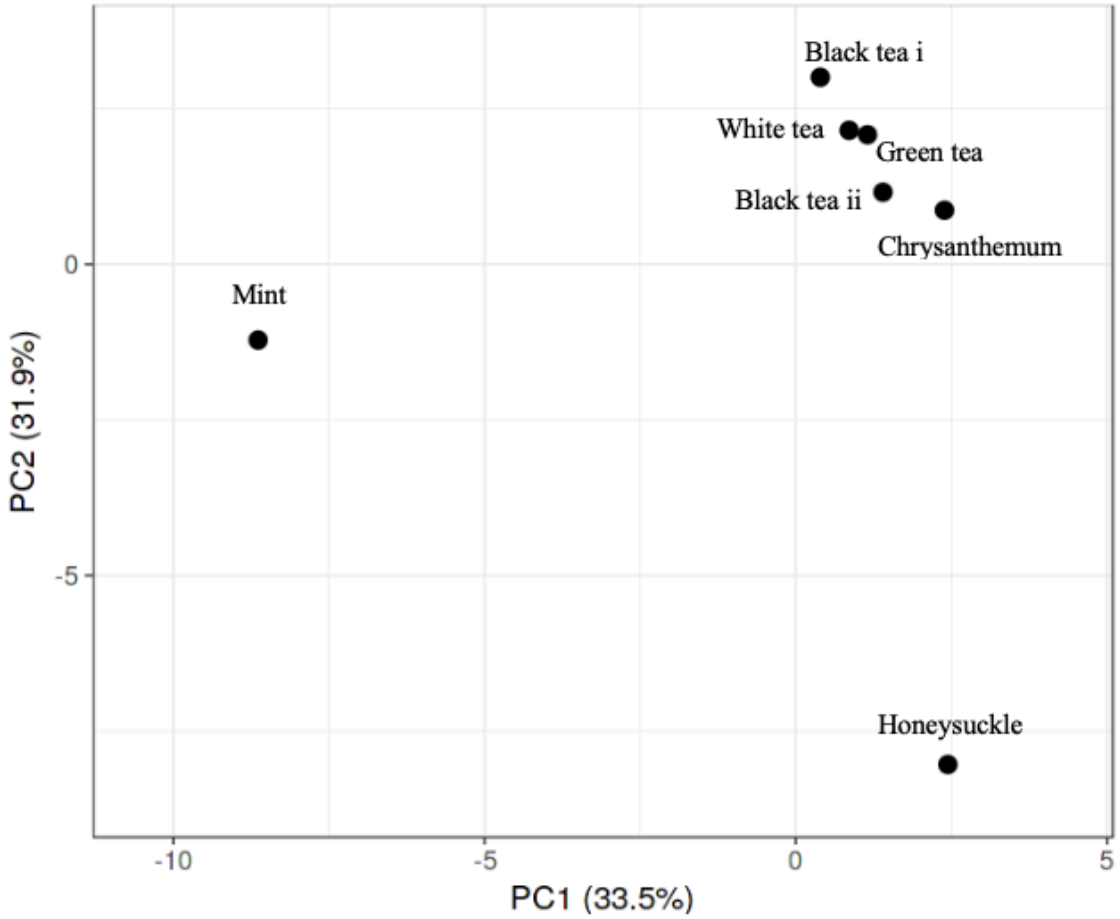

Figure 2. Principal component analysis for volatile compounds found in kombuchas fermented using six different substrates after 144 h of fermentation, based on log-normalized peak areas, after centering and unit variance scaling of the data. Black tea i = black tea kombucha fermented with SCOBY 1, Black tea ii = black tea kombucha fermented with SCOBY 2.

3.3 Sensory analysis

To analyze the appearance, taste and odor attributes as well as the overall approval of the different kombuchas, a sensory analysis was carried out with an untrained panel (n=58). Results are shown in Figure 3. An ANOVA analysis revealed that panelists were able to discriminate between samples ($P<0.05$) on most attributes.

All herbal-based kombuchas obtained higher approval scores than any of the tea-based kombuchas. The reason for the worse performance of tea-based samples in this study appears to be related to their taste (rather than their flavor), which was considered less balanced than that of herbal-based samples. Specifically, the acidity of tea-based kombuchas was considered

less pleasant than that of herbal-based kombuchas, while differences in sweetness were not statistically different.

As discussed previously, the concentrations of acetic acid and lactic acid did not differ significantly between tea and herbal based samples. The unpleasant acidity of tea-based kombuchas may therefore be related to isovaleric acid, which is the only acid that occurred in much higher concentrations in tea-based kombuchas (range 1.2 – 3.9 g l^{-1}) than herbal based kombuchas (0.1 – 0.3 g l^{-1}). Isovaleric acid is associated with a sweaty aroma and naturally occurs in tea (Schieberle and Schuh, 2006). In the context of fermented foods it is a main contributor to the undesired *Brettanomyces* off-flavor (Romano et al., 2009).

The sample that obtained the highest approval, mint kombuchas, had the most pleasant acidity and was also evaluated as the most refreshing and sweet. Participants were able to give comments to mint kombucha such as minty, cooling or refreshing without knowing the substrate (data not shown). This corresponds to the GC-MS results, where various volatiles that are described as minty and cooling were found exclusively in mint kombucha and persisted throughout the fermentation process. The high score for sweetness corresponds to its higher sugar content and may have contributed to its high approval. Sweetness can mask acidity in beverages and higher ratio of sugars to acidic constituents, sometimes referred to as °Brix/acid ratio, is closely related to the sensory acceptability of beverages (Jayasena and Cameron, 2008).

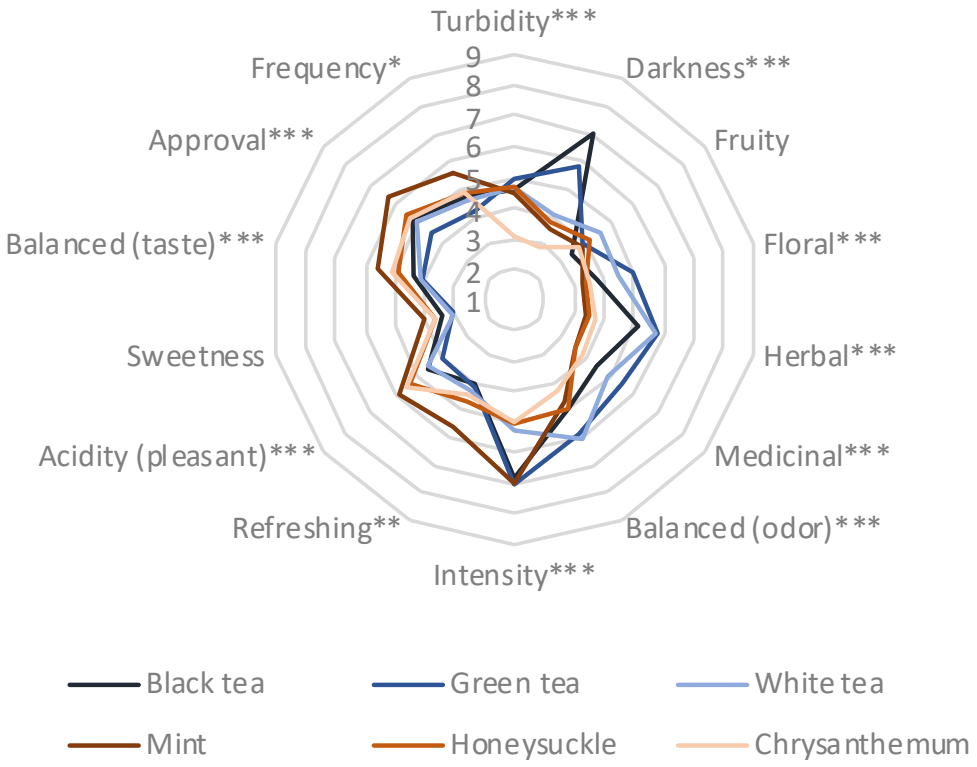

Figure 3. Results of sensory analysis including appearance (darkness, turbidity), odor (fruity, floral, herbal, medicinal, balanced), taste (intensity, refreshing, pleasant acidity, sweetness, balanced), overall appreciation and frequency of consumption (hedonic scale 1-9) as judged by an untrained panel (n=58), comparing kombucha produced from six different substrates. Stars next to attribute labels refer to significance level according to ANOVA (*: P <0.05, **: P<0.01，***：P<0.001).

Scores for overall approval by individual panelists are recorded in Figure 4. This reveals that each sample obtained a wide range of evaluations, both positive and negative. Some participants either liked (red cells) or disliked (blue cells) all samples, while the majority of panelists showed an intermediate behavior, liking some of the kombuchas. Herbal-based kombuchas were clearly favored by this group.

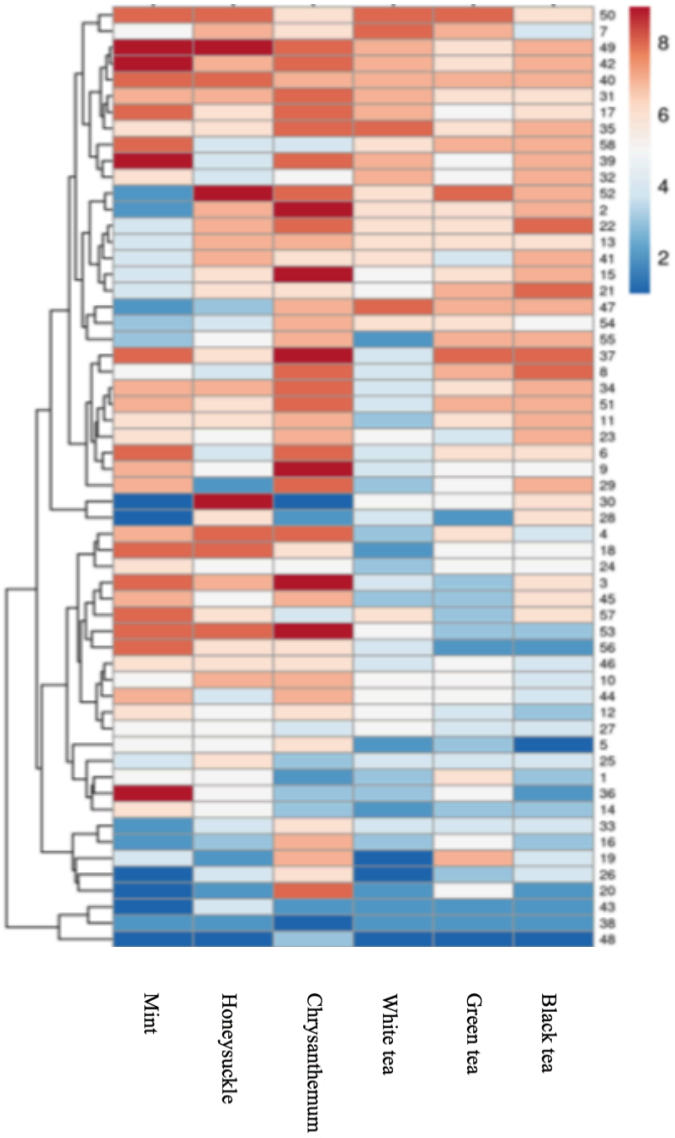

Figure 4. Heatmap recording overall approval (hedonic scale, 1-9) for kombucha samples expressed during the sensory analysis by individual panelists (numbered 1-58). Rows are clustered using Canberra distance and complete linkage.

4 Conclusion

The tea- and herbal based substrates used in this study were each suitable for the production of kombucha with characteristic accumulation of metabolites such as acetic acid and ethanol. Compared with tea-based substrates, fermentation occurred faster in chrysanthemum and honeysuckle infusions and slower in mint infusions. The volatile profile

of the final kombuchas had a number compounds in common, that may contribute to a signature kombucha aroma, but also maintained characteristic aroma compounds from their original substrates. The sensory analysis revealed a preference for herbal-based kombuchas over tea-based kombuchas. The low scores obtained by tea-based kombuchas may be related to their high content of isovaleric acid, which naturally occurs in tea but also accumulated during fermentation and is associated with off-flavor in fermented beverages. Mint kombucha obtained the highest approval score and was identified as both the sweetest and most refreshing sample. The higher residual sugar content in mint kombuchas may have aided in masking its acidity. The results reveal the potential for herbal preparations, especially mint infusions, to be applied as an alternative to the more conventional tea-based kombuchas, yielding beverages with a distinct aroma profile and pleasant sensory characteristics.

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Appendix 1

Figure 1. Questionnaire used for the sensory analysis (translated from Portuguese)

age _____ sample number _____
gender _____

1. The appearance (Questions in this section are **only** related to the **appearance** of the beverage)

How would you describe the colour of the beverage?

very light				neutral				very dark
1	2	3	4	5	6	7	8	9

How would you describe the clearness of the beverage?

very clear				neutral				very turbid
1	2	3	4	5	6	7	8	9

2. The smell (every question in this section is **only** related to the **smell** of the beverage)

Do you think the smell is fruity or not

not fruity at all				neutral				very fruity
1	2	3	4	5	6	7	8	9

Do you think the smell is floral or not?

not floral at all				neutral				very floral
1	2	3	4	5	6	7	8	9

Do you think the smell is herbal or not?

not herbal at all				neutral				very herbal
1	2	3	4	5	6	7	8	9

Do you think the smell is medicinal or not?

not medicinal at all				neutral				very medicinal
1	2	3	4	5	6	7	8	9

Do you think the smell is well balanced or not?

not balanced at all				neutral				very well balanced
1	2	3	4	5	6	7	8	9

Are there any other words you would use to describe the smell of this beverage?

_____

3. The taste (every question in this section is **only** related to the **taste** of the beverage)

Do you think the taste is intense or not?

not intense at all				neutral				very intense
1	2	3	4	5	6	7	8	9

Do you think the taste is refreshing or not

not refreshing at all				neutral				very refreshing
1	2	3	4	5	6	7	8	9

Do you think the taste is acid or not?

not acid at all				neutral				very acid
1	2	3	4	5	6	7	8	9

Do you think the taste is sweet or not?

not sweet at all				neutral				very sweet
1	2	3	4	5	6	7	8	9

Do you think the taste is well balanced or not?

not balanced at all				neutral				very well balanced
1	2	3	4	5	6	7	8	9

Are there any other words you would use to describe the taste of this beverage?

_____

4. overall opinion (the questions in this section are **only** related to **overall opinions**)

Do you like this beverage or not?	Would you drink it often?
like extremely	I would drink this every opportuity I had
like very much	I would drink this very often
like moderately	I would regularly drink this
like slightly	I like this and would drink this now and then
neither like nor dislike	I would drink this if available but would not go out of my way
dislike slightly	I do not like it but would drink it on an occasion
dislike moderately	I would hardly ever drink this
dislike very much	I would only this if there were no other drink choices
dislike extremely	I would drink this only if I were forced to

How much money you would like to spend on a bottle (500ml) of this beverage?

- 1-5 reals
- 5-10 reals
- 10-15 reals
- 15-20 reals
- more than 20 reals

Are there any comments you would like to add?

_____

Appendix 2

Table 1. Volatiles detected by HS-SPME/GC-MS in kombucha after 0 h of fermentation (logarithmic peak areas, mean ± sd).

0 h	White tea SCOBY1	Green tea SCOBY1	Black tea SCOBY1	Black tea SCOBY2	Chrysanthemum SCOBY1	Honeysuckle SCOBY1	Mint SCOBY1	ANOVA
Alcohols								
phenyl ethyl alcohol	4.87±0.65	4.72±0.49	5.01±0.46	4.97±0.28	5.27±0.15	5.06±0.64	4.90±0.25	NS
benzyl alcohol	4.01±0.14	3.99±0.06	4.00±0.17	4.14±0.08	3.83±0.24	3.48±0.09	4.06±0.02	NS
1-decanol	ND	1.86±1.86	1.95±1.95	4.03±0.43	2.16±2.16	1.77±1.77	1.80±1.80	NS
linalool	3.58±0.64	4.21±0.36	3.65±0.21	3.97±0.18	4.21±0.44	ND	3.78±0.30	NS
alpha-terpineol	2.33±1.89^{b}	2.00±2.00^{b}	3.42±0.21^{ab}	3.64±0.10^{a}	3.99±0.27^{b}	4.47±0.19^{b}	ND	*
neodihydrocarveol	ND	ND	ND	3.75±0.15^{b}	ND	1.55±1.55^{b}	5.31±0.06^{b}	***
dihydroeugenol	ND	ND	1.84±1.84	2.03±2.03	4.09±0.16	ND	ND	NS
trans-carveol	ND	ND	ND	ND	ND	4.84±0.18^{b}	5.57±0.05^{b}	***
carveol	ND	ND	ND	ND	ND	3.66±0.17^{b}	4.88±0.22^{b}	**
1-hexadecanol	ND	ND	ND	ND	ND	2.05±2.05	ND	NS
pinocarveol	ND	ND	ND	ND	ND	4.62±0.20^{b}	ND	**
dihydro-carveol	ND	ND	ND	ND	ND	ND	4.82±0.57	NS
3-octanol	ND	ND	ND	ND	ND	ND	4.31±0.43	NS
alpha-cadinol	ND	ND	ND	ND	ND	ND	2.03±2.03	NS
1-butanol, 3-methyl-	6.12±0.11	3.20±3.20	6.38±0.00	6.54±0.06	6.27±0.10	6.42±0.03	6.37±0.03	NS
Esters								
nonanoic acid, ethyl ester	ND	ND	ND	1.93±1.93	ND	1.87±1.87	2.19±2.19	NS
octanoic acid, ethyl ester	ND	1.78±1.78	ND	1.94±1.94	ND	1.91±1.91	2.17±2.17	NS
decanoic acid, ethyl ester	ND	ND	ND	1.98±1.98	ND	1.77±1.77	2.05±205	NS
dodecanoic acid, ethyl ester	1.62±1.62	ND	1.85±1.85	2.08±2.08	ND	1.75±1.75	1.94±1.94	NS
hexadecanoic acid, ethyl ester	2.84±2.84	2.10±2.10	4.76±0.21	ND	ND	4.65±0.29	5.13±0.49	NS
9,12-octadecenoic acid, methyl ester	2.10±2.10	ND	1.91±1.91	3.77±0.15	ND	ND	1.87±1.87	NS
.gamma.-palmitolactone	4.02±0.18	3.99±0.23	4.24±0.26	3.81±0.17	1.79±1.79	2.18±2.18	2.05±2.05	NS
methyl p-anisate	1.82±1.82^{b}	ND	ND	ND	ND	ND	5.10±0.23^{b}	**

(Continued)

0 h	White tea SCOBY1	Green tea SCOBY1	Black tea SCOBY1	Black tea SCOBY2	Chrysanthemum SCOBY1	Honeysuckle SCOBY1	Mint SCOBY1	ANOVA
(-)-trans-dihydrocarvyl acetate	ND	ND	ND	ND	ND	ND	2.45±3.47	NS
Acid								
octanoic acid	5.38±0.20	5.30±0.20	5.64±0.09	5.71±0.53	5.50±0.13	5.25±0.39	5.62±0.14	NS
benzeneacetic acid	4.31±0.51	2.00±2.00	4.79±0.25	4.63±0.28	4.63±0.17	4.59±0.22	4.61±0.39	NS
decanoic acid	5.25±0.07	5.31±0.26	5.71±0.03	5.98±0.44	5.68±0.20	5.40±0.18	5.67±0.20	NS
dodecanoic acid	4.34±0.33	2.02±2.02	4.65±0.28	4.96±0.24	4.35±0.32	4.35±0.03	4.76±0.14	NS
isovaleric acid	5.05±0.12	4.86±0.15	5.18±0.05	5.05±0.21	5.28±0.04	5.02±0.68	5.00±0.16	NS
hexanoic acid	4.79±0.11	4.78±0.04	5.12±0.00	5.20±0.23	5.26±0.21	5.17±0.09	4.94±0.15	NS
isobutyric acid	4.49±0.14	4.42±0.12	4.69±0.21	4.59±0.24	4.71±0.01	4.93±0.26	4.58±0.10	NS
Aldehydes								
2-decenal	4.25±0.02	4.06±0.35	ND	ND	1.70±1.70	4.16±0.42	4.19±0.43	NS
2,4-dimethyl benzaldehyde	4.01±0.35	4.53±0.25	4.41±0.07	4.58±0.36	4.48±0.11	4.47±0.39	4.62±0.09	NS
benzaldehyde	3.57±0.09	3.79±0.38	4.40±0.68	4.49±0.70	3.50±0.27	3.78±0.02	1.63±2.31	NS
nonanal	ND	1.93±1.93	2.08±2.08	1.88±1.88	ND	1.89±1.89	ND	NS
Ketones								
d-carvone	2.36±2.36^{b}	4.10±0.19^{b}	1.86±1.86^{b}	4.53±0.00^{b}	1.93±1.93^{a}	4.55±0.58^{b}	6.44±0.15^{b}	***
dihydro-carvone	ND	ND	ND	ND	ND	ND	4.79±0.12^{b}	***
piperitenone	ND	ND	ND	ND	ND	ND	5.00±0.22^{b}	**
jasmone	ND	ND	ND	ND	ND	ND	4.36±0.27^{b}	*
procymidone	ND	ND	ND	ND	ND	4.06±0.57	ND	NS
Others								
p-ethylguaiacol	4.34±0.16	4.31±0.22	5.07±0.19	5.05±0.62	4.86±0.00	4.00±0.40	5.21±0.34	NS
indole	ND	ND	1.86±1.86	3.82±0.06	ND	1.92±1.92	1.74±1.74	NS
eugenol	ND	ND	ND	ND	4.03±0.19^{a}	ND	4.80±0.28^{b}	*
phenol, 2,4,6-trichloro-	ND	ND	ND	ND	ND	4.51±0.30^{b}	ND	*
p-cymenene	ND	ND	ND	ND	ND	3.98±0.56	3.38±0.01	NS
4-pyridone	ND	ND	ND	ND	ND	6.12±0.11^{b}	ND	***

*ND = not detected; ANOVA: NS = not significant. * = P < 0.05. ** = P < 0.01. *** = P < 0.001; different letters in same row mean samples are significantly different according to Tukey's HSD post- hoc test (P<0.05)*

Table 2. Volatiles detected by HS-SPME/GC-MS in kombucha after 288 h of fermentation (logarithmic peak areas, mean ± sd).

288 h	White tea SCOBY1	Green tea SCOBY1	Black tea SCOBY1	Black tea SCOBY2	Chrysanthemum SCOBY1	Honeysuckle SCOBY1	Mint SCOBY1	ANOVA
Alcohols								
phenyl ethyl alcohol	5.79±0.13	5.66±0.11	5.51±0.13	5.95±0.03	5.93±0.05	6.04±0.19	5.71±0.03	NS
benzyl alcohol	4.59±0.00^{abc}	4.56±0.03^{abc}	4.49±0.16^{abcd}	4.65±0.01^{ab}	3.87±0.08^{acd}	3.47±0.44^{cd}	4.46±0.25^{abcd}	**
1-decanol	4.12±0.17	4.28±0.07	4.42±0.04	4.51±0.00	4.43±0.16	4.10±0.14	4.21±0.46	NS
linalool	4.39±0.22^{ab}	4.57±0.31^{ab}	4.09±0.14^{ab}	4.30±0.02^{ab}	4.74±0.02^{a}	1.73±1.73^{b}	4.16±0.11^{ab}	*
alpha-terpineol	4.08±0.23	4.27±0.20	3.73±0.31	4.05±0.01	4.30±0.05	4.38±0.34	4.74±0.24	NS
neodihydrocarveol	1.81±1.81^{b}	ND	ND	ND	1.68±1.68^{b}	3.64±0.49^{b}	6.00±0.18^{a}	**
dihydroeugenol	ND	ND	4.34±0.03^{bc}	4.54±0.09^{abc}	4.56±0.07^{ab}	ND	ND	***
trans-carveol	ND	ND	ND	ND	ND	4.71±0.30^{b}	6.14±0.02^{a}	***
carveol	ND	ND	ND	ND	ND	3.98±0.34^{b}	5.43±0.06^{a}	***
1-hexadecanol	ND	ND	ND	ND	ND	4.60±0.37	ND	NS
pinocarveol	ND	ND	ND	ND	ND	4.31±0.16^{a}	ND	**
dihydro-carveol	ND	ND	ND	ND	ND	ND	4.99±0.46	NS
3-octanol	ND	ND	ND	ND	ND	ND	5.37±0.04^{a}	***
alpha-cadinol	ND	ND	ND	ND	ND	ND	4.04±0.23^{a}	**
1-butanol, 3-methyl-	6.37±0.05	6.40±0.03	6.40±0.03	6.55±0.10	6.33±0.26	6.30±0.11	6.48±0.05	NS
Esters								
nonanoic acid, ethyl ester	4.12±0.22	4.47±0.50	4.28±0.35	4.03±0.11	4.76±0.12	4.47±0.27	4.84±0.74	NS
octanoic acid, ethyl ester	4.07±0.24	4.45±0.49	4.22±0.42	3.90±0.26	4.73±0.13	4.49±0.30	4.77±0.80	NS
decanoic acid, ethyl ester	1.95±1.95	3.88±0.72	3.88±0.31	3.95±0.06	4.26±0.16	4.19±0.22	4.40±0.62	NS
dodecanoic acid, ethyl ester	ND	ND	1.75±1.75	1.76±1.76	1.77±1.77	3.63±0.02	2.45±2.45	NS
hexadecanoic acid, ethyl ester	2.23±2.23	4.09±0.41	2.59±2.59	ND	2.59±2.59	2.30±2.30	2.44±2.44	NS
9,12-octadecenoic acid, methyl ester	ND	ND	ND	ND	1.89±1.89	ND	2.13±2.13	NS
.gamma.-palmitolactone	3.83±0.36	3.50±0.16	3.51±0.14	3.66±0.29	2.02±2.02	1.74±1.74	2.24±2.24	NS
methyl p-anisate	1.79±1.79^{b}	ND	ND	ND	ND	ND	5.08±0.12^{a}	***
(-)-trans-dihydrocarvyl acetate	ND	ND	ND	ND	ND	ND	2.00±2.00	NS

(Continued)

288 h	White tea SCOBY1	Green tea SCOBY1	Black tea SCOBY1	Black tea SCOBY2	Chrysanthemum SCOBY1	Honeysuckle SCOBY1	Mint SCOBY1	ANOVA
Acid								
octanoic acid	6.23±0.14	6.20±0.02	6.35±0.16	6.31±0.07	6.02±0.14	6.01±0.28	6.45±0.10	NS
benzeneacetic acid	5.08±0.03^{b}	4.94±0.05^{b}	5.09±0.12^{b}	5.18±0.12^{b}	5.29±0.06^{ab}	5.44±0.02^{a}	5.05±0.15^{b}	**
decanoic acid	6.10±0.17	6.33±0.02	6.37±0.25	6.45±0.16	6.11±0.27	6.22±0.21	6.45±0.24	NS
dodecanoic acid	4.29±0.06	4.66±0.06	4.76±0.15	4.94±0.21	4.53±0.19	4.89±0.21	5.05±0.40	NS
isovaleric acid	5.68±0.15	5.66±0.05	5.49±0.27	5.82±0.02	5.72±0.02	5.88±0.29	5.67±0.09	NS
hexanoic acid	5.30±0.08	5.30±0.04	5.48±0.15	5.52±0.09	5.43±0.00	5.37±0.24	5.49±0.09	NS
isobutyric acid	5.14±0.14	5.18±0.12	4.97±0.13	5.36±0.05	5.25±0.00	5.54±0.25	5.14±0.04	NS
Aldehydes								
2-decenal	4.34±0.62	1.82±1.82	1.96±1.96	ND	ND	ND	1.88±1.88	NS
2,4-dimethyl benzaldehyde	5.03±0.17	4.81±0.29	4.80±0.48	4.91±0.46	4.70±0.60	2.53±2.53	5.00±0.16	NS
benzaldehyde	3.41±0.20	3.45±0.38	3.73±0.13	3.69±0.30	3.26±0.07	ND	ND	NS
nonanal	2.08±2.08	1.90±1.90	ND	2.10±2.10	ND	ND	ND	NS
Ketones								
d-carvone	2.27±2.27^{b}	3.56±0.29^{b}	1.68±1.68^{b}	3.73±0.32^{b}	ND	ND	6.02±0.00^{a}	***
dihydro-carvone	ND	ND	ND	ND	ND	ND	5.60±0.04^{a}	***
piperitenone	ND	ND	ND	ND	ND	ND	5.03±0.11^{a}	***
jasmone	ND	ND	ND	ND	ND	ND	4.51±0.22^{a}	**
procymidone	ND	ND	ND	ND	ND	4.16±0.10^{a}	ND	***
Others								
p-ethylguaiacol	5.13±0.10^{b}	5.20±0.00^{b}	5.80±0.14^{ab}	6.04±0.03^{a}	5.58±0.01^{ab}	4.75±0.19^{b}	6.02±0.20^{a}	**
indole	ND	ND	ND	ND	ND	4.52±0.30^{a}	ND	*
eugenol	ND	ND	ND	ND	4.45±0.09^{b}	ND	5.69±0.08^{a}	***
phenol, 2,4,6-trichloro-	ND	ND	ND	ND	ND	4.41±0.10^{a}	ND	***
p-cymenene	ND	ND	ND	ND	ND	4.51±0.02^{a}	3.86±0.01^{b}	***
4-pyridone	ND	ND	ND	ND	ND	5.77±0.06^{a}	ND	***

*ND = not detected; ANOVA: NS = not significant. * = P < 0.05. ** = P < 0.01. *** = P < 0.001; different letters in same row mean samples are significantly different according to Tukey's HSD post- hoc test (P<0.05)*

Alcohols	White tea	Green tea	Black tea	Black tea IT	Chrysanthemum	Honeysuckle	Mint	Characteristics
phenyl ethyl alcohol	↑	↑	↑	↑	↑	↑	↑	fermentation product, floral scent, antimicrobial
benzyl alcohol	↑	↑	↑	↑	➡	➡	➡	common in tea and fruit, floral fruity scent
1-decanol	↑	↑	↑	➡	↑	↑	↑	floral, citris scent
linalool	↑	➡	➡	➡	↑	↑	➡	common in plant, floral and touch of spice scent
Alpha-terpineol	↑	↑	➡	➡	➡	➡	↑	abandunt in black tea, lilac odor
Neodihydrocarveol	↑				↑	↑	↑	metabolites of carveol and dihydrocarveol by Mos
Dihydroeugenol			↑	↑	↑			natually found in plant, sharp and spicy scent
Trans-carveol						➡	➡	naturally found in mint, minty scent
carveol						➡	➡	naturally found in mint, minty scent
1-hexadecanol						↑		natually found in honeyckle, floral and clean scent
pinocarveol						➡		naturally found in honeyckle, herbal woody scent
Dihydro-carveol							➡	naturally found in mint, minty scent
3-octanol							↑	naturally found in mint, herbal minty scent
Alpha-cadinol							↑	naturally found in mint, herbal woody scent, anti fungal
1-butanol, 3-methyl-							➡	naturally found in plant ad fruit, fruity scent
Esters								
nonanoic acid, ethyl ester	↑	↑	↑	↑	↑	↑	↑	yeast metabolism, fruity and winey scent
octanoic acid, ethyl ester	↑	↑	↑	↑	↑	↑	↑	yeast metabolism, fruity and flower scent
decanoic acid, ethyl ester	↑	↑	↑	↑	↑	↑	↑	yeast metabolism, fruity and sweet scent
dodecanoic acid, ethyl ester	↓		➡		↑	↑	↑	yeast metabolism, fruity and sweet scent
hexadecanoic acid, ethyl ester	➡	➡	➡	↑	↑	↓	↓	yeast metabolism, fruity and sweet scent
.gamma.-Palmitolactone	↓	↓	↓	➡	➡	➡	➡	natually found in plant
9,12-Octadecenoic acid, methyl ester	↓		↓	↓	↑	↑	➡	
Methyl p-anisate	➡						➡	natually found in plant
(-)-trans-Dihydrocarvyl acetate							➡	naturally found in mint, floral minty cooling scent
Acids								
octanoic acid	↑	↑	↑	↑	↑	↑	↑	yeast metabolism, antimicrobial
Benzeneacetic acid	↑	↑	➡	➡	↑	↑	➡	yeast metabolism, natually found in fruit and plant, honey-like scent, antimicrobial
decanoic acid	↑	↑	↑	➡	➡	↑	↑	yeast metabolism, antimicrobial
dodecanoic acid	➡	↑	➡	➡	➡	➡	➡	yeast metabolism
Isovaleric acid	↑	↑	➡	↑	➡	↑	↑	yeast metabolism (*Brettanomyces),* spicey smoky scent in small amount
hexanoic acid	➡	➡	➡	➡	➡	➡	➡	yeast metabolism
isobutyric acid	➡	➡	➡	➡	➡	➡	➡	MOs metabolism (*Brettanomyces* or *Lactobacillus*)

Aldehydes	White tea	Green tea	Black tea	Black tea IT	Chrysanthemum	Honeysuckle	Mint	Characteristics
2-decenal	➡	↓	↑	↑	↓	↓	↓	fermentation product
2,4-dimethyl benzaldehyde	↑	➡	➡	➡	➡	➡	➡	naturally found in plant, cherry and vanila scent
benzaldehyde	➡	↓	↓		➡	↓	↓	naturally found in plant, almond fruity scent
nonanal	↑	➡	➡	➡	↑	↓		naturally found in plant and fruit,citris scent
2,4-heptadienal, (E,E)-	↓	↓	↓	↓	↓	↓		naturally found in plant and fruity,citris and spice scent
Others								
p-Ethylguaiacol	↑	↑	↑	↑	↑	↑	↑	yeast metabolism (*Brettanomyces*), spicey smoky scent in small amount
indole	↑	↑	➡	↓	↑	↑	➡	MOs metabolism, flowery scent in small amount
p-cymene						➡	➡	natually found in plant, fresh woody scent
eugenol					➡		➡	natually found in plant, sweet woody scent, antimicrobial
Phenol, 2,4,6-trichloro-						➡		pesticide residule, anti fungal
Ketones								
D-carvone	➡	↓	➡	➡	↓	↓	➡	natually found in plant, abandund in mint, minty refreshing scent
Dihydro-carvone							↑	natually found in plant, abandund in mint, minty refreshing scent
Piperitenone							➡	natually found in plant, abandund in mint, minty refreshing scent
jasmone							➡	natually found in plant, woody herba floral scent
Procymidone						➡		pesticide residule, anti fungal

* (Cheryll Williams, 2011; Olaniran et al., 2017; Romeo et al., 2007; Shale et al., 2002)

Figure 1. Volatiles found in kombuchas. Arrows indicate an increase (green), decrease (red) or stability (yellow) through fermentation.