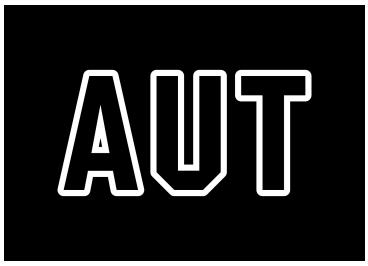

SCHOOL OF SCIENCE

Production of kombucha with new flavour using Chinese oolong tea (*Tie Guan Yin*)

By

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Abstract

Kombucha is a slightly carbonated, sweet and acidic refreshing beverage obtained from the fermentation of tea and sugar with a symbiotic interaction between bacteria and yeasts. Due to its inherent health benefits, it gained strong global market interest over the past decade. From previous studies, oolong tea has many health benefits such as anti-obesity effects and antioxidant capacity. However, oolong tea was rarely used as substrates in kombucha. In this study, kombucha was made with Chinese oolong tea (OT) - *Tie Guan Yin* (TGY), and sucrose. pH, titratable acidity, colour, ethanol, acetic acid, gluconic acid, amino acids, minor organic acids, sugars, total phenolic content, antioxidant activities and volatile organic compounds (VOCs) in kombucha were analysed. As the fermentation time increased, the pH of kombucha declined from 4.69 to 2.80 in 21 days which was mirrored by a significant increase in titratable acidity. In general, the colour of kombucha became lighter as fermentation progressed. The concentration of ethanol and gluconic acid reached the highest values after 21 days of fermentation. However, acetic acid tends to increase significantly and reached a maximum value of about 7.753 g/L at day 14 of fermentation, followed by a decrease to about 4.675 g/L at the end of the 21-day fermentation period. There were nine free amino acids detected in kombucha in this study which included alanine, valine, leucine, isoleucine, proline, threonine, aspartic acid, glutamic acid and phenylalanine. However, all of them decreased significantly (below 0.0025 μmol/mL) after 10 days of fermentation. In terms of minor organic acids after 21 days of fermentation, succinic acid, malic acid, citric acid, maleic acid and malonic acid were detected. The content of sucrose plummeted from 54.59 g/L to 11.95 g/L in the first 10 days of fermentation, and there was little change as fermentation progressed. Besides, glucose and fructose were detected on day 10 of fermentation, but the content of both decreased to 13.15 g/L and 17.48 g/L after 21 days of fermentation, respectively. This is due to the conversion of monosaccharide into other compounds, such as ethanol, acetic acid, and cellulose during fermentation. Notably, the consumption rate of glucose was faster than that of fructose after 10 days, which indicated that glucose was selected preferentially as

the carbon source rather than fructose by kombucha microflora. The antioxidant activity of kombucha were analyzed by three common assays, CUPRAC, FRAP and phosphomolybdenum. All these three methods showed an increasing trend during fermentation, which corresponds to the concentration of polyphenols, and the antioxidant capacity increased more rapidly in the first 10 days. SPME assay indicated that some characteristic aroma compounds of OT were retained in kombucha made with TGY in this study, such as (E)-hex-2-enal and 3-methylbutan-1-ol. Meanwhile, alcohols, acids and esters were generated during fermentation, such as nonanoic acid, ethyl ester, octanoic acid and decanoic acid, formed the unique aroma of kombucha.

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Attestation of Authorship

"*I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning"*

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Signature:

Date: 10 / 1 / 2022

1. Introduction

1.1 Introduction to kombucha

Kombucha is a slightly carbonated, sweet and acidic refreshing beverage obtained from the fermentation of tea and sugar with a symbiotic interaction between bacteria and yeasts. This interaction forms a biofilm called tea fungus or "SCOBY" – symbiotic culture of bacteria and yeasts. Kombucha fermentation is an aerobic brewing process because the oxygen is required for the growth of some microorganisms in the biofilm. Kombucha could be divided into two portions: the sour liquid media and the floating cellulosic pellicle layer (Figure 1).

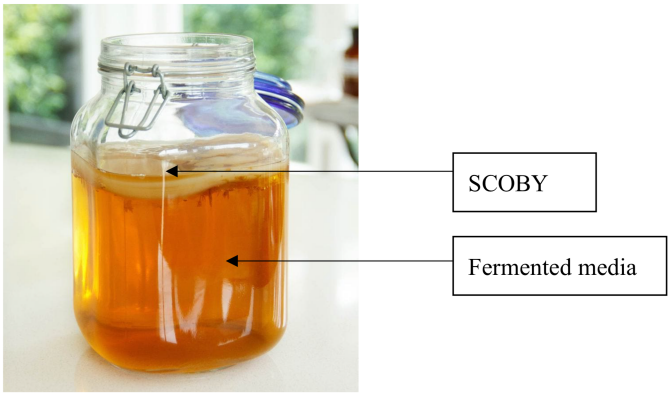


Figure 1： A kombucha beverage with the fermented media and SCOBY.

Kombucha has been consumed in Asia for thousands of years. It is reported that kombucha originated in north-east of China around 220 B.C. and spread to Japan as a medicine to cure the digestive problems by a doctor called Kombu in 414 A.D (Kapp & Sumner, 2019). By the 1900s, it disseminated to Russia and some other European countries, such as France, Italy and Germany, through the Silk Road from Asia (Millin, 2016). For centuries, kombucha was only a local homemade product without commercial intention. However, due to the increasing demand for healthy products, kombucha gained strong global market interest over the past decade (Gaggìa et al., 2019). Many media in the United States have emphasized kombucha for its health benefits, such as *Miami Herald* and the *New York Times*, which

showed that the consumption of kombucha can enhance immunity, relieve arthritis, reduce blood pressure, and treat cancer (Jacobs, 1995; O'Neill, 1994). Therefore, in the United States, the sales of kombucha and other fermented beverages showed a 37.4% growth in 2017, and kombucha alone increased by 49% in 2018 (Kapp & Sumner, 2019). In New Zealand, kombucha has been on a steady increase in popularity during the past five years and now it has a huge market presence with local supermarkets like Countdown and New World reporting high sales (Heard, 2020). Nowadays, kombucha has become one of the most popular low-alcoholic functional fermented beverages in the world. Various flavoured kombucha have been developed to create a novel taste experience for consumers. So, a motivation to develop a new flavour of kombucha made by different types of tea or substrates is highly desirable.

Notably, according to Food Standards Australia New Zealand (FSANZ) Section 2.6.2 (2017), kombucha belongs to the category of brewed soft drinks, so the content of alcohol must be less than 0.5% alcohol by volume (ABV). However, alcohol production is inevitable due to the nature of kombucha as a fermented beverage. Therefore, many kombucha companies control the content of alcohol in their products to less than 0.5% ABV by following methods: heat pasteurization, diluting the drinks or microfiltration of a specific yeast strain producing alcohol (Juyoung & Koushik, 2020). However, these modifications of kombucha have also raised questions among consumers and kombucha industry about whether it can still be regarded as kombucha. A kombucha company in Texas uses a patent pending fermenter that controls the alcohol content in kombucha below 0.5% ABV by reducing the yeast content and increasing the content of bacteria (Juyoung & Koushik, 2020). In addition, some kombucha companies have developed a new alcoholic beverage category recently, called "kombucha beer" or "hard kombucha", which contains 3-11% ABV (Laureys, Britton, & De Clippeleer, 2020).

In addition to its health-promoting effects, another reason for the extensive consumption of kombucha is that it is easy to produce at home. The following materials are used to make the kombucha: sugar, tea leaves, a starter culture (also known as "SCOBY" or tea fungus) and water. Firstly, the tea is brewed by steeping tea leaves in boiling water for at least 5 minutes. The type of tea leaves used will lead to different concentrations of organic acids and polyphenols in kombucha,

which determines the flavour of final product (R Jayabalan, Marimuthu, & Swaminathan, 2007). Although oolong tea (OT) and some other types of tea can be used as substrates, black tea and green tea are the most common choice because they contain high purine derivatives, such as caffeine and theophylline, which provide the nitrogen sources for the development of fungal cells (Yıkmış, 2019). According to Mutukumira, Rutherfurd-Markwick, Wang, Wang, and Archer (2020), green tea contains the highest caffeine content (5%) among all types of tea, and black tea contains 2% caffeine. It is worth noting that tea containing oil, such as Earl tea, is probably not suitable for kombucha production, because oil may interfere with the growth of culture and damage its integrity (Millin, 2016). After steeping of the tea leaves, sugar is added into tea base while the tea is still hot. Although sucrose is usually utilised as a carbon source for the growth of culture, other sugar sources have been applied in some studies to improve nutritional value and change sensory properties of kombucha, such as honey, juice and molasses (Malbaša, Lončar, & Djurić, 2008; Ulusoy & Tamer, 2019; Mindani I. Watawana, Jayawardena, Ranasinghe, & Waisundara, 2017). Before adding the starter culture, the sweetened tea should be cooled down to room temperature (about 20℃) and transferred to the jar. After the inoculation of the starter culture, the top of the jar is covered with a clean cotton cloth by using elastic band to keep out dust, mold and insects and allow the kombucha to ferment under aerobic conditions (Laureys et al., 2020). This also prevents the formation of pressure in the container due to the generation of carbon dioxide and allows oxygen to enter the jar for the growth of some microorganisms. The fermentation of kombucha is carried out at room temperature between 18 to 28℃, and normally last from 7 to 30 days (Kapp, 2019; Lacorn & Hektor, 2018; Leal, Suárez, Jayabalan, Oros, & Escalante-Aburto, 2018; Yıkmış, 2019). It is important to make sure that all equipment used in the production of kombucha are sanitized to prevent undesirable contamination.

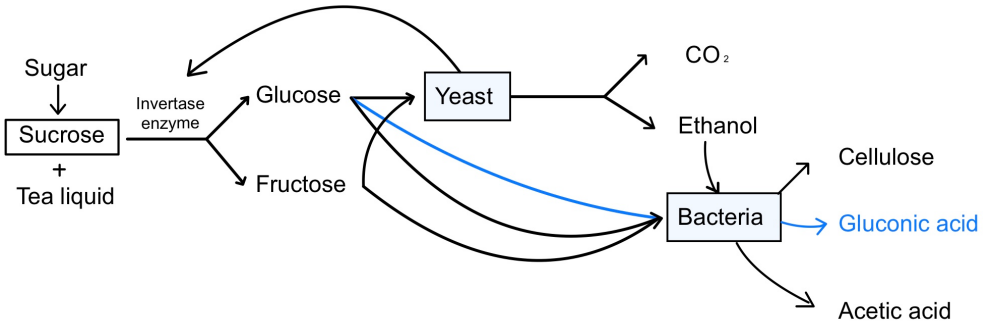


Figure 2: The main mechanism of kombucha metabolism during fermentation.

During kombucha fermentation, invertase enzyme produced by yeasts such as *Brettanomyces*, *Zygosaccharomyces*, *Pichia* and *Saccharomyces* hydrolyse disaccharides (sucrose) into monosaccharides glucose and fructose (May, Narayanan, Alcock, Varsani, & Aktipis, 2019; Neffe-Skocińska, Sionek, Ścibisz, & Kołożyn-Krajewska, 2017). Refer to Figure 2 for an illustration of the main fermentation reaction in kombucha. Afterwards, the yeasts convert these monomers into ethanol and carbon dioxide, and the acetic acid bacteria (AAB) generate acetic acid by oxidizing ethanol. Simultaneously, AAB creates new cellulose floating matrices (biofilm) where the microorganisms are embedded in the cellulose layers and convert most glucose into gluconic acid and glucuronic acid. In addition, vitamins and other nutrients are provided by the autolysis of yeast cells to support the growth of bacteria.

1.2 Introduction to oolong tea

Tea is one of the most consumed drinks in the world. All the different kinds of tea are made from the same species of leaf, *Camellia sinensis* (Ng et al., 2018). Tea can be divided into six categories depending on processing methods and degree of oxidation: green tea (no fermentation), white tea (slight fermentation), yellow tea (mild fermentation), oolong tea (semi fermentation), black tea (full fermentation) and dark tea (post fermentation).

The fermentation of tea refers to the process of enzymatic oxidation of polyphenolic compounds in tea leaves. In the cells of tea leaf, polyphenols exist in the vacuoles, which cannot react directly with oxidase because oxidase is mainly present in the cell

wall. However, when tea leaves are freshly picked, they are very tender. It is easy to damage the vacuole membrane after withering by shaking or rolling in the production process. This causes polyphenols in the tea leaf to react with enzymes and oxygen. The reaction induces a series of oxidation, polymerization and condensation of substances contained in fresh leaves to form coloured substances, such as theaflavins, thearubigins and theabromine (Leal et al., 2018). Therefore, fermentation changes the colour of tea. The higher the oxidation degree in tea polyphenol, the darker in colour of the tea leaves. As shown in Table 1, the colour of six different types of tea changes from green to yellow green, yellow, cyan brown and reddish brown. Polyphenols are also the main components in tea that cause astringency. Thus, fermentation also change the taste of tea. With the reduction of polyphenols in fermentation, the astringency of tea decreases. In addition to black tea, the fermentation of tea does not rely on external microorganisms, such as *Aspergillus* species, but on their own enzymes as catalyst (Tran et al., 2020). High-temperature processing is often used to inhibit the activity of oxidase in tea leaves to control the degree of tea fermentation. For example, in the production of green tea, steaming (200-220℃) is carried out immediately after picking to prevent fermentation. (Lee, Bonn, & Cho, 2018).

OT is unique among all types of tea because research showed that it has weight-reducing effects (Mo, Li, & Zhao, 2007) and can decrease the levels of blood glucose (Hayashino, Fukuhara, Okamura, Tanaka, & Ueshima, 2011). The production process of OT can generally be divided into seven steps: picking, solar or indoor withering (dependent on weather), shaking, panning, rolling, firing and packaging (Ng et al., 2018). After picking, the tea leaves are spread on a bamboo tray and put directly under soft sunshine (from 3 p.m. to 5 p.m.) for 15 to 60 mins to reduce the moisture content and soften the leaves. In the process of withering, the loss of water in tea leaves lead to the change of cell fluid concentration and the enhancement of cell permeability, which make polyphenols contact with oxidase to produce enzymatic oxidation. This procedure is also to break down proteins in tea leaves into free amino acids and caffeine, which could change the taste of the tea. Shaking causes friction and collision between leaves and leaves with appliances resulting in the damage of cells at the edge of the leaves. As mentioned above, the damage of cells turn polyphenols into thearubigins, which make the edges of leaves turn red. This is the reason for the characteristics of oolong tea leaves - the leaves with red border (Figure

Table 1： The introduction of six types of tea.

The type of tea		Green Tea	White Tea	Yellow Tea	Oolong Tea	Black Tea	Dark Tea
The degree of fermentation		no fermentation	slight fermentation	mild fermentation	semi fermentation	full fermentation	post fermentation
The degree of oxidation in tea polyphenols		weak → strong					
The colour of tea							
Pigments in tea	chlorophyll	✓			✓		
	lutein	✓	✓	✓			
	theaflavins		✓	✓	✓	✓	
	thearubigins				✓	✓	✓
	theabromine					✓	✓
Astringency of tea		strong → weak					
Typical type		Long Jing	Baihao Yinzhen	Mengding yellow tea	Tie Guan Yin	Qimen black tea	Pu’er

3). Panning is accomplished by moderately heating the tea leaves (can be up to 193°C), and the enzyme is decomposed at high temperature to stop further oxidation. After that, rolling is used for shaping and breaking down the cell wall to make the juice come out and then stick back to the surface of the tea leaves, which will increase the scent while brewing tea. Due to the semi fermentation process of OT, the different number of repetitions or operation time of withering and shaking makes the range of oxidation degree from 10 to 70% (Leal et al., 2018), which leads to a variety of types of OT, such as *Tie Guan Yin*, *Da Hong Pao* and *Dong Ding* oolong. *Tie Guan Yin* is one of the most common of all oolong teas.

Figure 3: The characteristics of OT leaves - the leaves with red border.

Several different chemical components that benefits human health have been found in OT, including alkaloids (theobromine and caffeine), polyphenols, organic acids, amino acids, polysaccharides, proteins, vitamins and minerals (Ng et al., 2018). Polyphenols in OT can be divided into four subgroups: tannins, catechins, flavonols and flavonol glycosides. Tea polysaccharides isolated from OT is water-soluble which are mainly composed of D-glucose, D-galactose, D-rhamnose and L-arabinose (Huixian Jiang & Xiao, 2013). In addition, OT is rich in minerals with potassium (K) being the most abundant, followed by phosphorus (P), sodium (Na), calcium (Ca), magnesium (Mg), manganese (Mn), and essential trace elements such as copper (Cu), zinc (Zn), iron (Fe) and so on (Shen & Chen, 2008).

Since OT is a semi-oxidized tea, it gives a taste between green tea (no fermentation) and black tea (full fermentation). Compared to green tea and black tea, the contents of flavonoids, catechin and tea polysaccharides are highest in oolong tea, but the concentration of caffeine and amino acid are lowest (Table 2)

Table 2: The difference of concentration of compounds in oolong tea, green tea and black tea.

		Oolong Tea	Green Tea	Black Tea	Reference
Tea Polyphenols		Medium (not reported)	Highest (not reported)	Lowest (not reported)	H. Zhang, Qi, and Mine (2019)
Flavonoids		Highest (not reported)	Lowest (not reported)	Medium (not reported)	
Catechin		Medium (201.88 μg/g)	Highest (300.09 μg/g)	Lowest (7.97 μg/g)	Vu and Alvarez (2021)
Tea Saccharides	Sucrose	Highest (3.70 mg/g)	Medium (1.08 mg/g)	Lowest (0.16 mg/g)	
	Glucose	Highest (22.04 mg/g)	Medium (16.07 mg/g)	Lowest (not detected)	
	Fructose	Highest (3.24 mg/g)	Lowest (2.84 mg/g)	Medium (2.94 mg/g)	
Caffeine		Lowest (not reported)	Medium (not reported)	Highest (not reported)	Chaisricharoen, Srimaharaj, and Manurkar (2020)
Amino Acids		Lowest (6.17 mg/g)	Highest (15.62 mg/g)	Medium (25.89 mg/g)	Hao Jiang et al. (2020)

In addition, OT has many health benefits (Ng et al., 2018). Scientific reports showed that OT has anti-obesity effects because catechins, caffeine and polymerized polyphenols in OT can enhance lipid metabolism (Cheng, Jenner, Low, & Lee, 2006; Okuda, 1999). A study in Taiwan found that habitual OT consumption (i.e. drinking 120 mL oolong tea per day for more than a year) has a protective effect on hypertension (Yi-Ching et al., 2004). This might be because (i)-epicatechin gallate and (i)-epigallocatechin gallate in OT can reduce cholesterol (Yang & Koo, 1997). OT is also a good antioxidant drink. Its antioxidant capacity mainly comes from polyphenols because it can effectively scavenge free radicals and metals (Leal et al., 2018). When metals such as copper and iron are in free state or not combined with proteins, they can promote oxidation, which will damage lipids and nucleic acids. The chelating property of antioxidants in tea make them bound to free metals to reduce the possibility of destruction of important molecules involved in physiological processes. Polyphenols could modulate the activity of several cell receptors and enzymes to inhibit oxidative stress resulted from reactive oxygen species (Tsao, 2010). Therefore, they play an important part in preventing various diseases through a defence against oxidative stress, like cardiovascular diseases (CVDs), neurodegenerative diseases and cancers (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004).

2. Literature review

2.1 Microorganisms in kombucha

The exact microbial components of kombucha during fermentation are hard to disseminate due to its variety. The composition of various microorganisms present in kombucha depends on the origin of SCOBY and its preparation method (Reiss, 1994). However, it has been found that bacteria and yeast remain in most kombucha cultures, such as *Komagataeibacter xylinum* and *Zygosaccharomyces parabailii* (Villarreal-Soto, Beaufort, Bouajila, Souchard, & Taillandier, 2018). Figure 4 shows the electron micrograph of yeast and acetic acid bacteria present in the symbiotic culture.

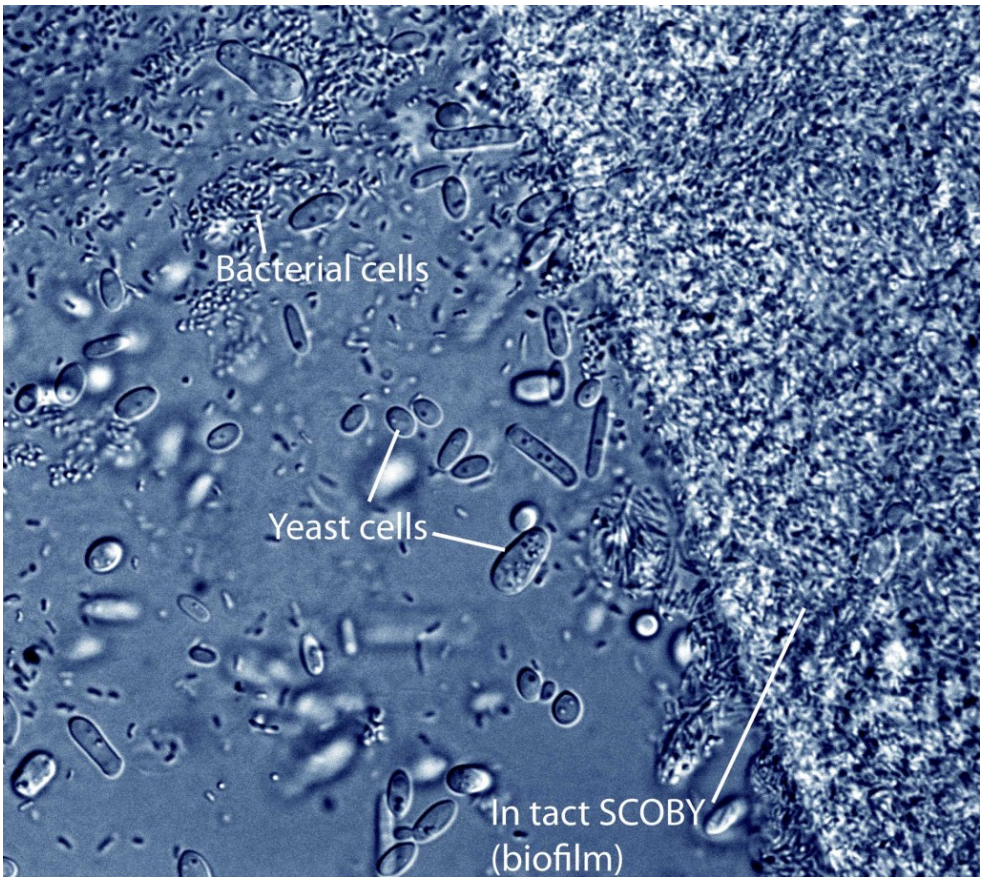


Figure 4: Electron micrograph of kombucha colony present in SCOBY (Wolfe, 2014)

Yeasts are single-celled small eukaryotic microorganisms, which belongs to fungi of the phylum Ascomycota (Neiman, 2005). They are non-motile and facultatively anaerobic, which can survive under aerobic and anaerobic conditions. Most yeasts present in kombucha are facultative fermentative, such as *Saccharomyces cerevisia*e and *Dekkera bruxellensis*, thus they can produce energy through respiration or fermentation. As shown in Figure 4, yeasts are oval or spherical, and have a diameter of about 8 μm. The most suitable temperature for their growth is about 20-30 ℃ and pH value is around 4.5-7.0, however some yeasts can still survive at pH 2.5 (Laureys et al., 2020).

As shown in Table 3, it has been reported that there are many yeasts species in kombucha culture, such as *Saccharomyces sp., Saccharomycodes sp., Schizosaccharomyces sp., Zygosaccharomyces sp., Brettanomyces Dekkera, Candia sp., Torulospora, Koleckera, Pichia, Macotorula, and Mycoderma* (Chakravorty et al., 2016; Alan J Marsh, Hill, Ross, & Cotter, 2014). Moreover, Mindani I Watawana,

Jayawardena, Gunawardhana, and Waisundara (2016) suggested that *Zygosaccharomyces sp.* are the main yeast (84.1%), and *Dekkera* and *Pichia* species occupy 6% and 5%, separately. Another study by Gaggìa et al. (2019) also showed that although the yeast population in kombucha display heterogeneity, *Zygosaccharomyces* sp. and *Brettanomyces* sp. are two typical yeast genera found in kombucha. In addition, *Brettanomyces* sp. can adapt in the harsh environment with low pH (Gaggìa et al., 2019) and high concentration of ethanol (Liberal, Basílio, Resende, Brasileiro, & Morais, 2010).

Alcohol and carbon dioxide are usually produced by yeast from carbohydrates. Under high osmotic pressure, yeasts can also produce glycerol to maintain the internal redox balance (Pigeau, Bozza, Kaiser, & Inglis, 2007). Another substance generated by yeast to maintain the balance of internal redox is acetic acid (Uscanga, Délia, & Strehaiano, 2003). Higher alcohols and esters are also produced in this process, which are responsible for the aroma of kombucha. Higher alcohols are formed by pyruvate anabolism or by the degradation of amino acids through Ehrlich pathway (Hazelwood, Daran, Maris, Pronk, & Dickinson, 2008). Although biogenic amines may be generated by some yeasts as well, and excessive intake of biogenic amines can cause headache, allergic response or gastrointestinal ulcer (Hanaa & El-Agizy, 2005), yeasts usually do not have a negative effect on health. Some yeasts even have health benefits. For example, *Saccharomyces boulardii* has been proved that it is helpful to the prevention of antibiotic associated diarrhea and the treatment of acute gastroenteritis in pediatric population (Vandenplas, Brunser, & Szajewska, 2009).

Sugar can be fermented to ethanol by most of the yeasts, and *Saccharomyces cerevisiae* is commonly used in alcoholic fermentation because of its high efficiency. However, in order to enhance the flavors and improve the complexity and kinetics of

Table 3: Microorganism found in kombucha with corresponding references.

Microorganism name			**Type of kombucha**	**Carbohydrate source**	**Fermentation time and temperature**	**Reference**
Bacteria	*Komagataeibacter* (formerly *Gluconacetobacter*, *Acetobacter*)	*K. xylinus*	Green or black tea	sucrose	For 21 days at 20 °C	De Filippis, Troise, Vitaglione, and Ercolini (2018)
			Black tea	sucrose	For 14 days at 28°C	Reva et al. (2015)
				honey		
		K. intermedius	Black tea	sucrose	For 14 days at 28°C	Reva et al. (2015)
				honey		
		K. rhaeticus	Rooibos tea	sucrose	For 14 days at 27±1°C	Gaggìa et al. (2019)
		K. saccharivorans	Green or black tea	sucrose	For 21 days at 30 °C	De Filippis et al. (2018)
	Gluconobacter	*G. oxydans*	Black tea	sucrose	For 10 days at 23 °C	Alan J. Marsh, O'Sullivan, Hill, Ross, and Cotter (2014)
	Lactobacillus	*L. kefirgranum*				
Yeast	*Zygosaccharomyces*	*Z. parabailii*	Black tea	sucrose	For 14 days at 27±1°C	Gaggìa et al. (2019)
	Candida	*C. stellata*	Black tea	sucrose	For 14 days at 20–22 °C	Teoh, Cox, and Heard (2004)
	Torulaspora	*T. delbreuckii*				
	Pichia		Black tea	sucrose	For 14 days at 28°C	Reva et al. (2015)
	Brettanomyces/Dekkera	*B. bruxellensis*	Black or Rooibos tea	sucrose	For 14 days at 27±1°C	Gaggìa et al. (2019)
	Schizosaccharomyces	*S. pombe*	Black tea	sucrose	For 14 days at 20–22 °C	Teoh et al. (2004)
	Rhodotorula	*R. mucilaginosa*				

the fermented product, non-*Saccharomyces* yeasts are also introduced into the concoction (Lopez, Beaufort, Brandam, & Taillandier, 2014). This microbial interaction between *Saccharomyces* and non-*Saccharomyces* yeast are used to allow the modification and flavour enhancement of kombucha.

During the fermentation process, the amount of yeast cells reached the highest value, and then declines gradually (Mutukumira et al., 2020). There are two reasons for the decrease of the number of yeast cells in the later stage of fermentation: 1) the increasingly acidic environment; 2) with the progress of fermentation, the carbon dioxide produced by sugar and yeast probably accumulate between the cellulose membrane and liquid media. Thus, the transfer of nutrients from the liquid broth to the culture is prevented, which makes yeast and bacteria hard to survive. The autolysis of yeast cells begins when yeasts die, whereby the compounds present in the cells are degraded by endogenous enzymes, such as lipids, proteins, polysaccharides, and nucleic acids. This self-degradation process generates a variety of compounds, which contribute to the overall flavour of final product (Alexandre & Guilloux-Benatier, 2006). In addition, the released vitamins and other nutrients can be used for the growth of bacteria (such as AAB or LAB).

The predominant bacteria in kombucha culture are acetic acid bacteria (AAB), which are used to produce acetic acid from alcohol by the invertase enzyme acetaldehyde dehydrogenase (R. Jayabalan, Swaminathan, & Marimuthu, 2007). AAB are gram-negative bacteria, which belong to the family of Acetobacteraceae (Kersters, Lisdiyanti, Komagata, & Swings, 2006). As shown in Figure 4, they are oval to rod-shaped, and about 1-4 μm long and 0.5 μm wide. In contrast to yeast, AAB are aerobic bacteria, which need large amount of oxygen to grow and maintain their activity. The most suitable temperature for their growth is about 25-30 ℃ at pH around 5.0-6.5, but many of them can still grow at pH 3.0 or even lower. AAB are not considered to generate toxic substances, so it is usually not pathogenic to humans. The most typical feature of AAB is that under aerobic conditions, carbon sources (sugars, aldehydes or alcohols) are oxidized by dehydrogenase on the outer surface of cell plasma membrane (Kersters et al., 2006; Rodrigo et al., 2018). For instance, ethanol is oxidized to acetic acid, and glucose can be converted into glucuronic acid, gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid and 2,5-diketogluconic acid.

According to Chen and Liu (2000), *Komagataeibacter* spp. (previously known as *Gluconacetobacter* and *Acetobacter* spp.) is the major bacterial genus in kombucha, including *K. xylinus*, *K. intermedius*, *K. rhaeticus* and *K. saccharivorans*, and other bacteria species (such as *Gluconobacter* and *Lactobacillus spp.*) are also found in the system (Table 3) (Reva et al., 2015). For example, a study conducted by Alan J. Marsh et al. (2014) showed that the relative abundance of *Komagataeibacter* in liquid medium and cellulose membrane was between 86% and 99% during fermentation by rRNA sequence analysis. Generally, *Komagataeibacter* species are present in alcohol rich environments and prefer to oxidize ethanol than glucose (Rodrigo et al., 2018). However, *Gluconobacter* species is related to sugar rich environment and prefer the oxidation of glucose, gluconic acid and glycerol.

The existence of lactic acid bacteria (LAB) in kombucha is inconsistent. They are usually absent or low in content (Gaggìa et al., 2019; Alan J. Marsh et al., 2014). LAB are gram-positive bacteria, which belong to the phylum of the Firmicutes (Laureys et al., 2020). They are non-motile and facultatively anaerobic, with spherical or rod-shaped cells. The most suitable temperature for their growth is about 25-40 ℃ at pH around 4.0-6.0. According to Pascal, Denis, Bernard, and Aline (2005), LAB are related to the production of lactic acid during kombucha fermentation, and some other metabolites (such as carbon dioxide, ethanol, acetic acid and mannitol)can also generated by LAB.

The most characteristic microorganism during the whole process of kombucha fermentation is *K. xylinus*, and it is considered to be the main reason for the formation of cellulose membrane (Rasu et al., 2014). The bacterial cellulose production is based on the transport of carbon source. During kombucha fermentation, glucose enters the cell of *Komagataeibacter* bacterium from the liquid media, and then cellulose precursor uridine diphosphoglucose (UDPGlc) is synthesized by glucose 1-phosphate and glucose 6-phosphate (Cacicedo et al., 2016; Villarreal‐Soto et al., 2018). After that, up to 200,000 glucose residues are polymerized into β-1,4-glucan chains through cellulose CS synthase in each *Komagataeibacter* bacterium cell per second, and these newly formed chains are extruded out of the cell membrane. As shown in Figure 5, each *Komagataeibacter* bacterium cell has around 50 to 80 complex terminals (CTs) or pores with a diameter of 3.5 nm for extruding polymer chains. The polymer chains passing through the pores combine with each other to form subfibrils with a width of

1.5 nm, and then subfibrils are automatically assembled into nanofibril (2-4 nm thick), followed by a formation of cellulose nanoribbon with a thickness of 3-8 nm and a width of 40-60 nm (Shi, Zhang, Phillips, & Yang, 2014). These fibrils form a special three-dimensional network structure, which is full of hydrogen bonds and can easily interact with water, nanoparticles and other molecules (Cacicedo et al., 2016). Thus, it can hold 200 times more water of its dry mass and has great elasticity. According to Villarreal‐Soto et al. (2018), this form of cellulose production has two advantages: 1) cellulose can be produced from various carbon sources, such as glucose, ethanol, sucrose and glycerol. 2) bacteria grow rapidly under controlled conditions. The production rate of cellulose is directly proportional to the growth rate of *K. xylinus*, and the final yield of cellulose depends on the carbon source, the specific strain, and other factors (Mamlouk & Gullo, 2013). In addition, the existence of caffeine and other xanthines found in tea seems to stimulate the production of cellulose as well.

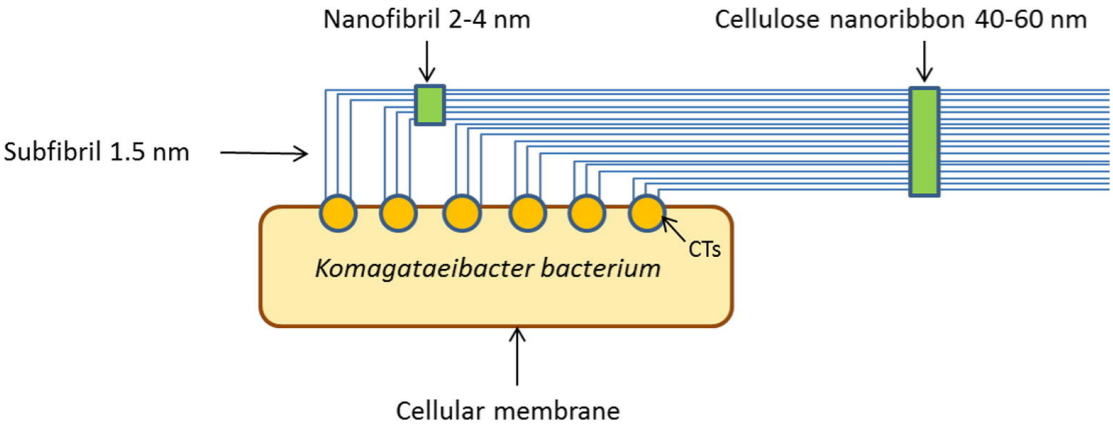


Figure 5: Schematic image of cellulose microfibrils by *Komagataeibacter* bacterium (Cacicedo et al., 2016)

It is difficult to understand the fermentation kinetics of kombucha because of the diversity of microorganisms present in kombucha and their interactions. Most microorganisms generate metabolites that can inhibit or stimulate the specific growth rate of other species and establish symbiotic or non-symbiotic interactions (Villarreal‐Soto et al., 2018). Therefore, they must be widely analyzed to understand this complex coexistence phenomenon. Although the role of some bacterial groups and yeast species (such as AAB, LAB and *Saccharomyces cerevisiae*) in the

fermentation process has been understood, the roles of many other microorganisms and their interactions have not been widely described even till now.

2.2 Chemical composition of kombucha

The presence and amount of chemical components in kombucha are variable, which mainly depends on the following factors such as type of tea, origin of the SCOBY for kombucha production, fermentation temperature, fermenting time and type of sugar used. However, many studies reported that the general chemical components present in all kombucha samples includes sugars, ethanol, carbon dioxide, organic acid, polyphenols, water soluble vitamins, amino acids and minerals (Leal et al., 2018; Rasu et al., 2014).

Normally, sucrose is used as sugar source in kombucha production. It is hydrolyzed into fructose and glucose via invertase from yeasts within the starter culture. Although the yeasts could convert both sugars into ethanol and carbon dioxide, the consumption rate of these two sugars are not same, which indicates that their usage by acetic acid bacteria during the fermentation process are slightly different (Chen & Liu, 2000). The main use of fructose is to produce acetic acid, but glucose is used to generate gluconic and glucuronic acid and synthesising cellulose biofilms by acetic acid bacteria. In addition, ethanol present in kombucha is formed by yeast. Yeasts convert glucose into pyruvate through glycolysis, which is then metabolized to acetaldehyde, and then generated to ethanol through fermentation. With the decrease of sucrose content, ethanol tends to increase to the highest value, followed by a slow decline (Chen & Liu, 2000). The decrease is mainly due to the lack of sugar to maintain the ethanol production in the later stage of fermentation, and ethanol is used as a carbon source by AAB to produce acetic acid in a nutrient-depleted environment (Chakravorty et al., 2016).

The metabolism and growth of AAB, LAB and yeasts lead to the accumulation of organic acids in kombucha (Rasu et al., 2014). Acetic acid, glucuronic and gluconic acids are the main organic acids present in kombucha, and lactic, tartaric, citric, folic, succinic, malic, malonic, pyruvic and oxalic acids may also be found in small quantities (Neffe-Skocińska, Sionek, Ścibisz, & Kołożyn-Krajewska, 2017). Acetic acid is a weak acid, and it provides the acidic taste and smell of vinegar. The anti-

pathogenic bacterial effect of kombucha is mainly due to acetic acid. Acetic acid present in kombucha is produced by AAB from ethanol (Leal et al., 2018), and the trend of its content is almost the same as ethanol. However, the decrease is attributed to the reduction of ethanol metabolism by yeast in the later stage of fermentation and acetic acid is used as a carbon source for the growth of culture when sugar substrate is depleted. (Chen & Liu, 2000). In addition, the amount of acetic acid also depends on the type of sugar used in the kombucha production. For example, according to Rasu et al. (2014), when molasses is used as a carbon source, the concentration of acetic acid is much lower. As mentioned above, glucose is converted into gluconic acid by microorganisms embedded in the cellulose layers via the pentose phosphate pathway, which is further transformed into glucuronic acid by the acetic acid bacteria (dominated by *K. xylinum*) (Martínez-Leal, Ponce-García, & Escalante-Aburto, 2020). Gluconic acid is a non-volatile acid sugar, and glucuronic acid is a precursor of ascorbic acid (Vitamin C) (Sastry & Sarma, 1957). During the fermentation process, the pH value of kombucha drops because these organic acids are produced (Dufresne & Farnworth, 2000).

Polyphenols are also found in kombucha, such as epigallocatechin (EGC) and epicatechin (EC), and the total content of them is dependent on many factors, such as the type of tea, age of tea leaves, microorganism in kombucha and temperature and time of fermentation (De Filippis et al., 2018; Yıkmış, 2019). As mentioned in the previous section, the degree of oxidation of polyphenols in each type of tea is different, which leads to the different initial polyphenol content in kombucha. According to Gaggìa et al. (2019), compared with kombucha made from black tea, green tea kombucha has extremely high content of catechins after fermentation for 14 days at 27 ± 1°C. Because green tea is non-fermented tea, few polyphenols are oxidized during the production process. EGCG, EGC, ECG and EC are four main polyphenol classes in green tea. However, these compounds in black tea are oxidized and dimerized due to the production methods. Therefore, thearubigins and theaflavin are two major phenolic compounds in black tea. During kombucha fermentation, the increasingly acidic environment leads to the degradation of complex tea polyphenols such as thearubin into simple molecules with high antioxidant capacity. Bacteria and yeasts present in kombucha such as *Candida tropicalis* may produce some unknown enzymes that can catalyze the

biodegradation of catechins, theaflavin and thearubigins resulting in colourless phenolic acids (Ettayebi et al., 2003; R. Jayabalan et al., 2007). This is thought to be the reason for the increase of total polyphenol content and the lighter color of kombucha during fermentation (Chu & Chen, 2006; Rasu et al., 2014).

According to Bauer-Petrovska and Petrushevska-Tozi (2000) and Malbasa, Loncar, Vitas, and Canadanovic-Brunet (2011), five water soluble vitamins have been found in kombucha, including vitamin B_1, B_2, B_6, B_{12} and C, and the concentration of them increased during fermentation. In addition to vitamins, trace amount of minerals are also detected (iron, copper, zinc, nickel and manganese) (Leal et al., 2018). It is worth noting that the contents of toxic elements (chromium and lead) present in kombucha were very small, and even decreased during fermentation compared to unfermented sweet tea infusion (Bauer-Petrovska & Petrushevska-Tozi, 2000). The decrease proved that kombucha has detoxification property, possibly due to the presence of glucuronic acid. In addition, D-saccharic acid-1,4-lactone (DSL), a bioactive compound derived from D-glucaric acid is generated by *Gluconacetobacter* spp. The compound has antioxidant activity and the concentration can range from 57.99 to 132.72 μg/mL (Leal et al., 2018).

All the chemical components (including sugar, organic acids and CO_2) are commonly found in kombucha and are important compounds in determining the final flavour of the product. For example, higher content of acetic acid will produce stronger acidity and astringency, while more gluconic acid will create a milder taste.

2.3 Effects of fermentation condition on kombucha

The chemical components in kombucha varies with fermentation conditions, such as the type of tea used, the type and content of sugar, temperature, duration of fermentation and oxygen transfer rate (Greenwalt, Steinkraus, & Ledford, 2000; Rasu et al., 2014).

The different kinds of sugar have distinct effects on the formation of ethanol, lactic, acetic, gluconic and glucuronic acids (Blanc, 1996). Reiss (1994) suggested that the types of sugar could have influence on both yeasts and acetic acid bacteria. Sucrose is hydrolyzed by invertase enzyme from yeasts into fructose and glucose. Firstly, the yeast could convert fructose, glucose and maltose into ethanol. Glucose

and fructose are mainly metabolized to gluconic acid and acetic acid by AAB, respectively. However, only a small amount of acetic acid could be produced from maltose by AAB. Therefore, sucrose is still the traditional carbon source for kombucha fermentation.

During fermentation, the optimum temperature provides better fermentation condition for the microbial growth and enzyme activity. Temperature also influences the formation of phenolic compounds, which affects the antioxidant activity of kombucha (Hur, Lee, Kim, Choi, & Kim, 2014). Kombucha fermented at higher temperature contains higher contents of acids, metabolites and vitamin C (Chakravorty et al., 2016). A study from Lončar, Djurić, Malbaša, Kolarov, and Klašnja (2006) showed that the concentration of vitamin C in kombucha fermented at 30°C (0.175 mg/L) was much higher than that fermented at 20°C (around 0.0025 mg/L). The fermentation time is also an important factor for the production of kombucha. Chakravorty et al. (2016) claimed that the level of polyphenols and antioxidant activity increased sharply after 7 days of fermentation, and they increased continuously with the fermentation time (Chu & Chen, 2006; R Jayabalan et al., 2007). Although better antioxidant activity can be obtained by prolonged fermentation time at higher temperature, it is still not recommended because of excessive accumulation of organic acids. This will lead to a negative influence on the consumer acceptance because the kombucha becomes too sour. Generally, during 10 to 20 days of fermentation at 20 to 30°C, kombucha contains high levels of organic acids, polyphenols and vitamins and has low pH value to avoid contamination from pathogenic bacteria (Rasu et al., 2014).

Oxygen transfer rate is the ratio between the surface exposed to air and the whole kombucha liquid volume. It also influences the final kombucha product. There are two different simultaneous reaction routes during kombucha fermentation (Villarreal-Soto et al., 2018). One is the aerobic reaction which converts oxygen and ethanol into acetic acid. The second is anaerobic reaction for formation of alcohols and lactic acid. Therefore, higher oxygen exposure in kombucha will elevate the content of acetic acid. However, if the oxygen level is too high, the anaerobic fermentation would be limited, which would affect the balance between microorganisms.

2.4 Flavour compounds in kombucha

During the fermentation process, the component and concentration of flavour compounds are plentiful. According to Zhao et al. (2018), Pu-erh tea was used to make kombucha in their study. Generally, these volatile flavour compounds identified in the kombucha broth during the whole fermentation process could be divided into seven different categories: alcohols, aldehydes, ketones, acids, esters, benzenoids and others.

21 different volatile compounds were identified on day 0, and most of them derived from the Pu-erh tea itself. As shown in Table 4, the largest group in the broth was aldehydes, accounting for 18.16% of the total volatile compounds. The second portion was alcohols, which contained 9 different kinds of flavour components, occupying 13.59%.

Table 4: The major volatile flavour compounds in the kombucha during fermentation.

Fermentation time	Category	Volatile compounds
Before fermentation (Day 0)	Aldehydes	benzeneacetaldehyde, heptaldehyde, 2,4-dimethyl benzaldehyde, nonanal, furfural and Z-7-hexadecenal
	Alcohols	linalool, phenethyl alcohol, terpineol, 2-methyl decanol, 2,6-dimethyl-4-ethyl-4-heptanol, 5-methyl-2-isopropyl hexanol and. 2-hexyl-1-decanol
After fermentation (Day 10)	Acids	acetic acid, butanoic acid, phenylacetic acid, citric acid, 2-methyl succinic acid, L-lactate, succinic acid, oxalic acid and 2-hydroxy-3-methbutyric acid
After fermentation (Day 14)	Acids	acetic acid, citric acid, butanoic acid, succinic acid, and L-lactate, 2-methyl succinic acid, 2-oxo-malonic acid and glucuronic acid
	Esters	2,6-hexadecyl-1(+)-ascorbic ester, methoxyacetic acid-4-hexadecane ester, octadecyl acetate, 3-methyl propionic acid-2-acrylic ester and isobornyl thiocyanoacetate

There were 56 volatile flavour compounds in the kombucha broth after 10 days fermentation. During this fermentation period, the content of alcohols and aldehydes reduced dramatically, but the acidic compounds increased continuously. Acids group (including 22 different kinds of acid compounds) was abundant, accounting for 57.21%. The top five components in the acid group were acetic acid, butanoic acid, phenylacetic acid, citric acid and 2-methyl succinic acid, occupying 21.64%, 3.41%, 3.35%, 3.21% and 2.78%, separately. In addition, 53 volatile flavor components were found from the kombucha broth after 14 days fermentation, mainly composed of acids and esters. However, the largest group in the broth was still acid, sharing 57.62% of the total volatile compounds.

2.5 Benefits of kombucha and potential toxicity

Kombucha is considered a functional food because of its inherent health benefits from the tea as mentioned in the previous section and the added benefits from metabolites after the fermentation process.

As mentioned in section 2.2, acetic acid, glucuronic and gluconic acids are the main organic acids present in kombucha. According to Murooka and Yamshita (2008), acetic acid has many health-promoting effects, such as antioxidant capacity, anti-diabetes, lowering blood pressure and inhibiting cancer cell growth. Gluconic acid helps to improve intestinal health by promoting the growth of probiotics in the large intestine. Glucuronic acid is one of the very important and valuable component in kombucha (Neffe-Skocińska et al., 2017). Glucuronic acid is a precursor of ascorbic acid (Vitamin C) (Sastry & Sarma, 1957) and can be converted into glucosamine in the human body, which are used to treat osteoarthritis (Clegg et al., 2006).

Although tea is known to be a good antioxidant drink, kombucha has higher free radical scavenging activity (Rasu et al., 2014). The reason might be when the complex phenolic compounds are subjected into an acidic environment, the phenolic compound degrade into smaller molecules by enzymatic activity from microorganisms in kombucha culture (R Jayabalan et al., 2007). For example, (-)-epigallocatechin gallate (EGCG) degraded into (-)-epigallocatechin (EGC) and (-)-epicatechin gallate (ECG) converted into (-)-epicatechin (EC), which increases the

total phenolic compounds in kombucha. D-saccharic acid-1,4-lactone (DSL) found in kombucha is a product of the glucuronic acid pathway, which also has antioxidant properties (Leal et al., 2018) and inhibits the activity of glucuronidase, which is indirectly related to cancer (Mindani, Nilakshi, Chaminie, & Viduranga, 2015). In addition, vitamin C is also one of the better known antioxidant, and it can strengthen the immune system, promote tooth and bone health, maintain healthy collagen in the skin, and helps to prevent some illnesses related to oxidation, such as heart disease, arthritis, and cancer (Malbasa et al., 2011).

Even though most bioassays in kombucha were performed in vitro, several studies have been conducted in vivo by using rats and have shown some interesting results. A study by Bhattacharya, Gachhui, and Sil (2013) showed that kombucha had a protective effect on different organs of diabetic rat models (including heart, kidney, pancreas and liver) and significant antidiabetic potential by restoring pathophysiological changes. Srihari, Karthikesan, Ashokkumar, and Satyanarayana (2013) assessed the anti-hyperglycemic effect of kombucha on diabetic rats by using different contents of kombucha extract within 45 days. They detected that taking 6 mg / kg body weight per day reduced glycosylated hemoglobin and increased plasma insulin. In addition, Bellassoued, Ghrab, Makni-Ayadi, Jos Pelt, and Elfeki (2015) found that the concentration of thiobarbituric acid reactive substances (TBARS) in liver and kidney decreased significantly after treatment with kombucha by testing rats fed with high levels of TBARS and a high cholesterol diet. This showed that kombucha could be thought as an agent for the treatment of liver and kidney toxicity. In order to confirm the claimed health benefits of kombucha, clinical research and more in vivo evaluation are still needed.

However, many kombucha production is homemade, so it is important to be cautious about the pathogenic microorganism contamination during the preparation. Some individuals cases of headache, dizziness, allergy and nausea have been reported (Rasu et al., 2014; Villarreal‐Soto et al., 2018). The U.S. Food and Drug Administration has also conducted the analysis and reported that it is safe for the consumption of kombucha, but due to the microbial complexity of this beverage, it needs to be produced according to FDA Food Standards (Nummer, 2013).

2.6 Project aims and objectives

The aim of this study is to evaluate the efficacy of using TGY and sucrose to produce kombucha. In addition, the study seeks to understand the effect of fermentation conditions on kombucha properties such as:

- Colour
- pH and total acidity
- Ethanol content
- Acetic acid, gluconic acid, amino acids and minor organic acids
- Total phenolic content and antioxidant activity and sugars content
- Volatile compound profiling

2.7 Novelty of this project

Most kombucha are made with black tea, and this is the first scientific study on developing kombucha using Chinese oolong tea (TGY). For the first time, free amino acids in kombucha were detected by using methyl chloroformate (MCF) derivatization and GC-MS, and solid phase microextraction (SPME) was used to analyse the change of volatile compounds in kombucha during fermentation.

3. Experimental methods

3.1 Materials

A kombucha starter culture (SCOBY) was purchased from Get Cultured Limited, New Zealand (NZ), and maintained in the Food Laboratory at Auckland University of Technology, New Zealand. *Tie Guan Yin* (TGY) tea samples were purchased from Hiland Tea Ltd, NZ. Copper (II) chloride, citric acid, malic acid, tartaric acid, galactose, sodium chloride and sucrose were provided by VWR International, US. 1,2 Dichlorobenzene, 3-Methyl-1-phenyl-2-pyrazoline-5-one (PMP), amino acid

standard, C7-C40 Saturated alkanes standards, Folin-Ciocalteu reagent, gallic acid, gluconic acid, L-amino acid kit, malonic acid, methanol, neocuproine, pyridine, trolox, erlose and raffinose were obtained from Sigma-Aldrich, US. Iron (III) chloride, anhydrous sodium sulphate and succinic acid were sourced from Scharlau, Spain. Caffeine, glucuronic acid and mannose were acquired from Acros Organics, US. Ammonium heptamolybdate, hydrochloric acid, sodium carbonate, sulfuric acid, lactose and maleic acid were purchased from Ajax Fine Chemical, Australia. Ammonium acetate, acetonitrile, ethanol, formic acid, glacial acetic acid, toluene, chloroform, acetone and butanol were obtained from Fisher Chemical, US. Milli-Q water was purchased from Suez Water Technologies & Solutions, US. Sodium bicarbonate, methyl chloroformate, sodium acetate trihydrate and monopotassium phosphate were purchased from Panreac (Spain), Merck Millipore (US), LabServ (UK) and Interchem (NZ), respectively.

3.2 The preparation of kombucha

All the glass jars and appliances for the preparation of the fermentation were sanitized in iodine Star Sanitizer solution for 10 min. 16 g of Chinese oolong tea (TGY) was added into 1 L boiling water and stir for 5 min. After that, 120 g sucrose was mixed with the tea. The tea was poured into a glass jar, which contained 1 L distilled water by using a mesh strainer to remove the tea leaves. After the solution cooled down to 25 ℃, 50 g of SCOBY was added. The fermentation was carried out for 10 and 21 days at room temperature, and three independent fermentation batches were incubated for each fermentation time. Before the chemical and microbiological analyses, all kombucha samples were stored at 4 ℃. All analyses were carried out on the same batch of samples in triplicate. When the SCOBYs were not in use, they were stored in a “SCOBY Hotel” for the growth of new cellulose membrane. The “SCOBY Hotel” was made by the same method as above, except that the content of materials increased (40 g TGY, 500 g sucrose, 2.5 L distilled water and all SCOBYs not in use). The “SCOBY Hotel” was remade once a month to ensure that the SCOBYs are healthy.

3.3 pH and total acidity determination

The pH value of kombucha fermented tea was determined using an electronic pH meter (Eutech Instruments, model pH 700) on fermented day 0, 3, 7, 10, 14, 17 and 21. The total acidity of kombucha was assessed according to Nielsen (2010). Kombucha was titrated with 0.05 M NaOH until pH value reached to 7. Results were expressed as grams of acetic acid per liter of sample.

The total acidity is determined from the equation below:

$$\%\ acid\ \left(\frac{wt}{vol}\right) = \frac{\mathrm{N} \times V_1 \times Eq.wt}{V_2 \times 10}$$

Where N = normality of titrant, which is NaOH in this study (mEq/mL), V_1 = volume of titrant (mL), Eq. wt = Equivalent weight of predominant acid, which is acetic acid in this study (60.05 mg/mEq), V_2 = volume of sample (mL)

3.4 Ethanol, acetic acid and gluconic acid analysis using GC-FID

For ethanol, acetic acid and gluconic acid were analysed using gas chromatography and flame ionization detector (GC-FID), 1 ml kombucha samples (in triplicates) were pipetted into 2 ml GC vials, spiked with 5 µL of butanol (internal standard) and capped with a screw septum cap. The samples were performed on Shimadzu GC-2010 gas chromatograph with an FID detector equipped with a DB-FATWAX UI column (30 m × 0.25 mm ID × 0.25 µm). The initial temperature of GC column was 40°C, and then it was increased at a gradient of 10°C min−1 until it reaches 240°C.

3.5 Total phenolic content

Total phenolic concentration of fermented kombucha was quantified by using Folin-Ciocalteu's (FC) assay (Singleton, Orthofer, & Lamuela-Raventós, 1999). One mL kombucha sample solution was mixed with 0.5 mL FC reagent in a 10 mL glass vial. After 5 minutes of incubation, 1.5 mL 20% sodium carbonate was added. The glass vial was covered by foil to incubate for two hours in the dark. The absorbance of mixture was measured at 765 nm using a UV spectrophotometer (a

Ultrospec 2100 Pro) as with all the other antioxidant analysis. Gallic acid (2.5 mg/L to 40 mg/L) were used as an external standard as well and a standard curve was generated with an R^2 value of 0.9991 (refer to Appendix D). The results were expressed as mg of gallic acid equivalent (GAE) per liter of sample.

3.6 Antioxidant analysis

To eliminate the influence of false positive and false negative on the results, the antioxidant capacity of the kombucha produced in this study was measured by three antioxidant assays: Cupric ion reducing antioxidant capacity (CUPRAC) assay, ferric reducing antioxidant power (FRAP) assay and phosphomolybdenum (PM) assay.

3.6.1 Cupric ion reducing antioxidant capacity (CUPRAC) Assay

Cupric ion reducing antioxidant capacity (CUPRAC) Assay was performed as following procedures described by Özyürek, Güçlü, and Apak (2011) with slight modification. One mL 1.0 M ammonium acetate buffer at pH 7.0, 1 mL of 0.01 M CuCl2, 1 mL 0.0075 M Neocuporine and 100 µL deionized water were added into 1mL kombucha solution diluted by a factor of 50. The mixture was kept for 5 minutes at room temperature to allow the reaction to take place, and absorbance value was measured at 450 nm. Concentrations of 5 mg/L through to 160 mg trolox /L were used as an external standard and a standard curve was produced with an R^2 value of 0.9965 (refer to Appendix A).

3.6.2 Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain (1996). 100 µL kombucha was mixed with 900 µL deionized water and 2 mL FRAP reagent, which is a mixed solution of 0.3 M acetate buffer, 10 mM TPTZ solution in 40 mM HCl, and 20 mM ferric chloride at the ratio of 10:1:1 in volume. After keeping it for 4 minutes at room temperature, absorbance value was determined at 593 nm. Trolox (5 mg/L to 160 mg/L) were used as an external standard as well and a standard curve was generated with an R^2 value of 0.9996 (refer to Appendix B).

3.6.3 Phosphomolybdenum (PM) assay

Phosphomolybdenum (PM) assay with some modifications was carried out as described by Prieto, Pineda, and Aguilar (1999). One mL kombucha sample was combined with 2.8 mL monopotassium phosphate, 6 mL sulphuric acid, 0.4 mL ammonium heptamolybdate and 0.8 mL distilled water in a 10 mL glass vial. The vials were capped and incubated in oven at 90 ℃ for 120 minutes. After the samples cooled down to the room temperature, absorbance value was determined at 700. Concentrations of 12.5 mg/L through to 400 mg/L Trolox were used as an external standard as well and a standard curve was made with an R^2 value of 0.9931 (refer to Appendix C).

3.7 Colour

According to European Brewery Convention (1998), Colour of kombucha was determined. The absorbance of kombucha solution was measured at 430 nm by using a UV spectrophotometer.

The colour in EBC Units is determined from the equation below:

$$C = A_{430} \times f \times 25$$

Where A430 = Absorbance at 430 nm, f = dilution factor which is 5 in this study. The colour corresponding to EBC units are shown in Figure 6.

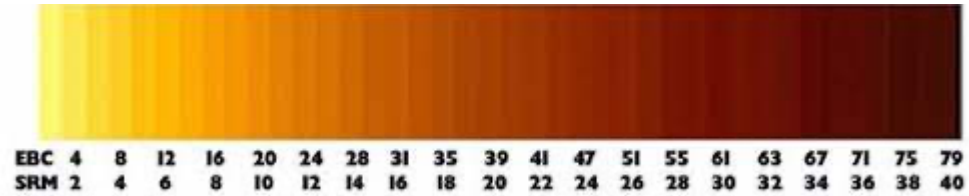


Figure 6: The colouring chart of EBC unit.

3.8 Amino acids and organic acids analysis using methyl chloroformate (MCF) derivatization and GC-MS

3.8.1 MCF derivatisation

Amino acids and organic acids in the kombucha was identified and quantified using the methyl chloroformate (MCF) alkylation developed by Smart, Aggio, Van Houtte, and Villas-Bôas (2010). 20 µL of 10 mM d4-alanine was mixed with 1 mL kombucha solution in a 4 mL silanised glass vial, and it was kept in the freeze dryer overnight. Freeze-dried sample was resuspended in 200 µL of 1 M sodium hydroxide, 34 µL of pyridine and 167 µL of methanol. After 30 seconds vortex, 20 µL of MCF was mixed with the mixture and it was mixed vigorously for another 30 seconds. Again, 20 µL of MCF was added and vortex for 30 seconds. To separate the MCF derivatives from the mixture, 400 µL of chloroform and 400 µL of 50 mM Sodium hydrogen carbonate were added, and 10 seconds vortex was performed after the addition of both these two reagents. Thereafter, the upper aqueous layer was removed using a glass pipette. Anhydrous sodium sulphate (0.30 g) was added into the chloroform layer, and 100 µL of chloroform mixture was transferred into a GC glass micro-insert, which was placed inside a 2 mL GC vial with screw septum cap.

3.8.2 GC-MS Parameters

Gas chromatography-mass spectrometry detection (GC–MS) analysis was carried out with an Agilent 5977B GC/MSD with a 7890B GC system fitted with a fused silica capillary DB-1701 column (30 m x 0.25 mm, film thickness 0.25 µm). The temperature of GC oven was initially held at 45 °C for 2 min. Thereafter, the temperature was increased at a gradient of 9 °C /min until it reaches 180 °C. This temperature (180 °C) is held for 5 min. A second gradient was applied to reach 220 °C at 40 °C /min and held for 5 min again. The temperature was then raised with a gradient of 40 °C /min until 240 °C, where it was held for 11.5 min. The final gradient was used to reach 280 °C at 40 °C/min and held for 2 min. Gas flow through the column is held constant at 1.1 ml of He per min. In addition, 1 µl sample was injected into the GC-MS. The temperature of the inlet is 290 °C and the interface temperature is 250 °C.

3.9 Sugars analysis using HPLC

3.9.1 Sample Extraction

Three mL of sample and 60 µL of 0.05 g/mL xylitol (internal standard) were mixed into a 15 mL falcon tube. After that, 3 mL of chloroform was added for the extraction. After 30 seconds vortex, the mixture was centrifuged at 4000RPM at 10°C for 10 minutes. The bottom chloroform phase was removed into a halogenated solvent waste bottle by using a glass Pasteur pipette. This extraction procedure was repeated twice to ensure all non-polar organic compounds have been removed from the aqueous layer. 1.2 mL mixture from aqueous layer was transferred into a micro-centrifuge tube, and the tube was centrifuged at 10000 RCF for 15 minutes. Finally, 1 mL of centrifuged liquid was placed into a 2 mL GC/LC vial for HPLC-ELSD analysis.

3.9.2 HPLC parameters

The parameters of high-performance liquid chromatography (HPLC) with some modifications were determined by Puerari, Magalhães, and Schwan (2012). HPLC analysis was carried out with a Shimadzu LC-10ATvp series chromatograph system equipped with an Agilent 385-ELSD detector. Samples were separated by a Luna Omega Sugar 100Å column (250 x 4.6 mm, 3 µm) with Isocratic Elution. Acetonitrile: MilliQ water (80:20, v/v) was used as a mobile phase. The pump flow rate was 0.5mL/min, and the total analysis time was 75 minutes. The analysis was operated at 25°C, and the injection volume was 10 µL. Results were expressed as concentration in g/L.

3.10 Solid phase microextraction (SPME) for volatile compound analysis

3.10.1 Sample preparation

Solid phase microextraction (SPME) was accomplished by using a Supelco 50/30 µm, DVB/CAR/PDMS, Stableflex, 24GA Fiber. 2 mL of sample, 0.5 g of NaCl and 10 µL of 89 µM 1,2 Dichlorobenzene were added into a vial. The mixture was vortexed to make NaCl fully dissolve into the sample liquid. The SPME incubation temperature was 50°C, with an incubation time of 15 minutes and an extraction time of 25 minutes. Then the samples were run on the GC-MS for SPME analysis, and

the C7-C40 Alkane standard was run under identical conditions to obtain the retention index of the compounds identified.

3.10.2 GC-MS parameter

After extraction, the analysis was performed by using an Agilent 7980B Gas Chromatograph System coupled with an Agilent 5977B Mass Spectrometer (Agilent, USA) equipped with an Extractor Electron Ionization (EI) source. The column was Agilent DB-FATWAX-UI (30 m × ID 0.25 mm × 0.25 μm). Helium (He) was used as a carrier gas with a constant flow of 1.1 mL/min. The inlet temperature was 250°C. In addition, the ion source temperature was 250°C, and quad temperature was 150°C. Compounds were identified by comparing the mass spectra and retention index to the National Institute of Standards and Technology (NIST) Mass Spectral Database 2014.

3.12 Data analysis

In terms of the results of SPME, principal component analysis (PCA) of GC-MS data was performed. In addition, one-way ANOVA (analysis of variance) was carried out using XL-STAT to determine if significant difference existed between the samples for the rated attributes. According to Graham and Edwards (2001), one-way ANOVA was only useful when one factor was varied; in this case, the factor varied was the kombucha fermentation time. Where significant differences were identified, a post-hoc test (in this case, Tukey's test) was carried out to determine which of the samples were significantly different and which samples they were significantly different to.

4. Results and discussion

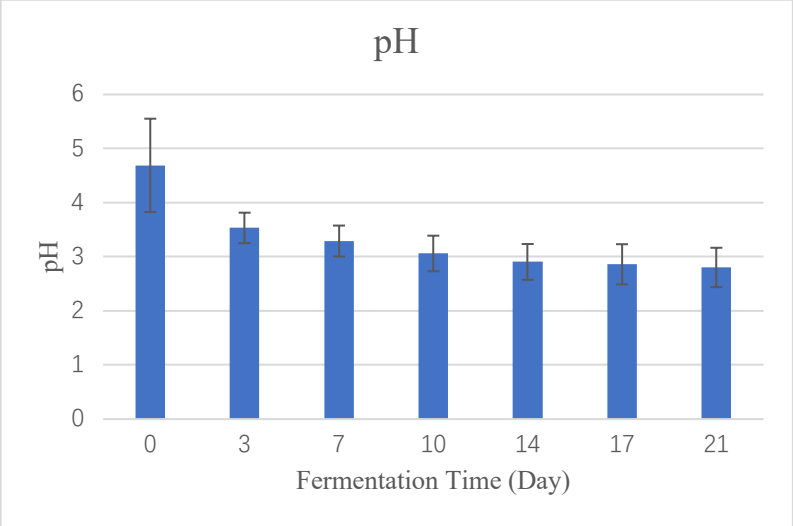
4.1 pH and total acidity determination

Low pH can lead to an unacceptable level of sensory quality of kombucha, so the measurement of pH value is necessary. The measurement is used to monitor the

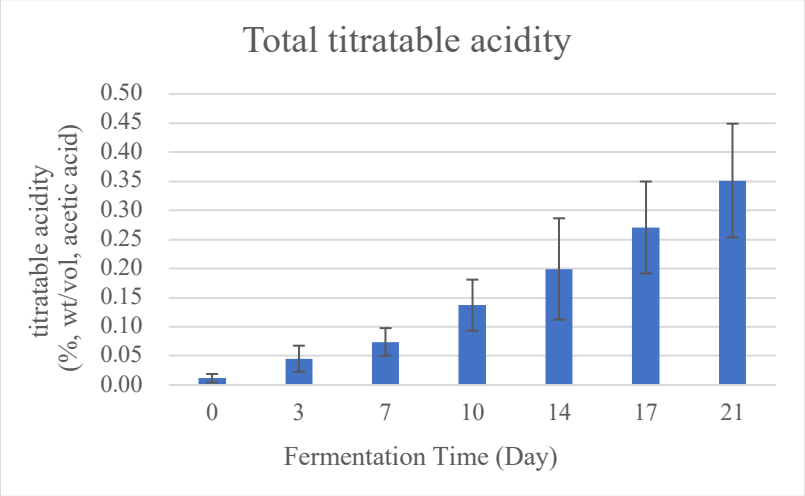
fermentation and is a factor that determines the end of the fermentation process (Malbaša, 2008; Neffe-Skocińska et al., 2017).

The change in kombucha pH at different fermentation times are presented in Figure 7a. The pH results showed that as the fermentation time increased, the pH declined from 4.69 to 2.8 in 21 days. In the first 3 days, the pH value dipped rapidly to 3.53, but the value remained stable at around 2.9 after 10 days. The findings are consistent with Yıkmış (2019)'s work who also reported that the pH of all kombucha samples decreased with time. This is caused by the organic acids formed during the fermentation process and evidence will be provided in Section 4.6. Acetic acid bacteria converted ethanol into acetic acid and glucose into gluconic acids (Rasu et al., 2014). Glucuronic acid is formed by glucose transformation when glucose is oxidized at the C-6 position (R Jayabalan et al., 2007). Acetic acid bacteria also utilize ethanol and acetic acid to produce lactic acid.

Although the pH value reached a plateau after 10 days of fermentation, a significant increase in titratable acidity occurs during the whole fermentation, indicating an increased concentration of organic acids (Figure 7b). This might be caused by carbon dioxide produced in the fermentation process, which resulted in the buffer effect (Kallel, Desseaux, Hamdi, Stocker, & Ajandouz, 2012). This buffering capacity is caused by the dissociation of carbon dioxide in water, which produces amphoteric bicarbonate anions (HCO_3^-). This anion can react with hydrogen ions (H^+) from organic acid produced by fermentation to inhibit further changes in the concentration of hydrogen ion in kombucha (Mindani et al., 2015). According to Figure 7b, the total titratable acidity of kombucha at the end of 10 days and 21 days was 0.137%, wt/vol, acetic acid and 0.351%, wt/vol, acetic acid, respectively.



(a)



(b)

Figure 7: (a) The average pH values of kombucha (n= 3) (b) Titratable acidity of kombucha with a strong base sodium hydroxide on day 0, 3, 7, 10, 14, 17 and 21 of fermentation (n=3).

4.2 Ethanol, acetic acid and gluconic acid analysis using GC-FID

Figure 8 shows the concentration of ethanol and acetic acid and Figure 9 shows gluconic acid by using gas chromatography and flame ionization detector (GC-FID) at different fermented time. The concentration of ethanol showed an upward trend during the fermentation process. After 21 days of fermentation, it showed the highest value (3.338 g/L), which was twice as much as that of the kombucha sample at 10 days of fermentation (1.6 g/L). The increase of ethanol concentration was

because yeast convert sucrose into glucose and fructose, and then generate ethanol by glycolysis (Neffe-Skocińska et al., 2017).

The concentration of acetic acid tended to increase significantly and reached a maximum value of about 7.753 g/L at day 14 of fermentation, followed by a decrease to about 4.675 g/L at the end of the 21-day fermentation period. As mentioned above, the increase in the first two weeks was because the acetic acid bacteria can generate acetic acid by using ethanol. However, when sugars in the samples were used up, acetic acid would be a carbon source for bacteria (Leal et al., 2018). This might be the reason why the decrease in the concentration of acetic acid initiated after 14 days.

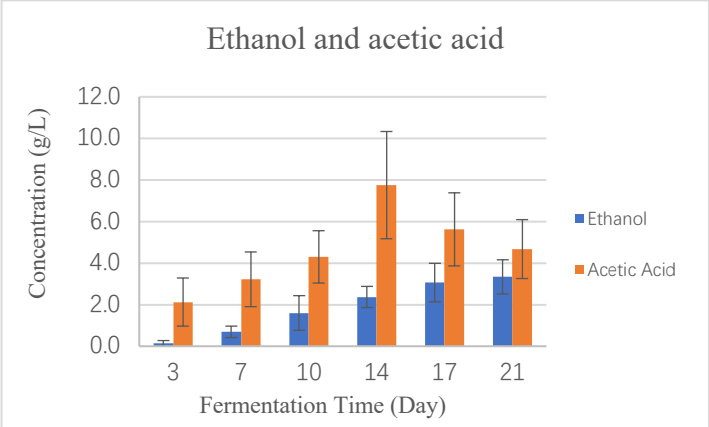


Figure 8: The concentration of ethanol and acetic acid of kombucha on day 0, 3, 7, 10, 14, 17 and 21 of fermentation (n=3).

In addition, according to Figure 9, the gluconic acid content increased slowly from 17 mg/L to 24 mg/L during the first 14 days fermentation period and then the concentration of gluconic acid in kombucha remained stable until day 21. The increase in gluconic acid was because sucrose was hydrolysed into fructose and glucose by invertase enzyme in yeast cells, and then acetic bacteria turned glucose into gluconic acid during the fermentation (Neffe-Skocińska et al., 2017).

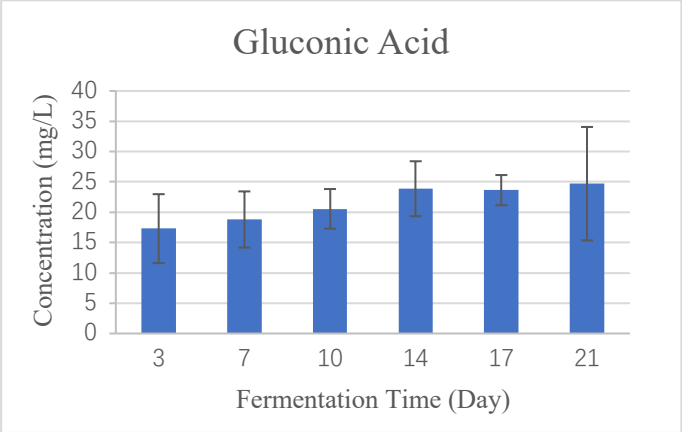


Figure 9: The concentration of gluconic acid of kombucha on day 0, 3, 7, 10, 14, 17 and 21 of fermentation (n=3).

A study by Chen and Liu (2000) showed that the concentration of ethanol in kombucha made by black tea and sucrose reached the highest value of about 0.55% (w/v) at day 20 of fermentation, and then decrease slowly to 0.4% (w/v) after 30 days fermentation. The decrease is because ethanol was used by acetic acid bacteria to produce acetic acid (Chakravorty et al., 2016). However, this expected decrease in ethanol was not observed in this study, possibly because of insufficient fermentation time.

The major organic acid in kombucha was acetic acid. The variation and trend of acetic acid concentration in this study was similar to that previously reported by Leal et al. (2018). However, the content of gluconic acid was much lower than that shown in the study from Chakravorty et al. (2016) (7.36 g/L). This might be due to the higher fermentation temperature (28 ± 2 °C) they used in the research. Higher temperature could promote the growth of *G. saccharivorans*, and the concentration of gluconic acid is directly proportional to its microbial count. This was also proved by De Filippis et al. (2018). In their study, the concentration of gluconic acid was much higher in the kombucha samples fermented at 30°C (2.3 g/L) than that at 20 °C (70 mg/L).

4.3 Total phenolic content

Total phenolic content of kombucha sample was measured by using FC assay, and the results were shown in Figure 10. According to the results, the concentration of

total phenolic content in kombucha sample increased from 437 mg GAE/L to 522 mg GAE/L within 10 days and then kept increasing to 641 mg GAE/L after 21 days of fermentation. The breakdown of complex polyphenol by enzymes from microorganisms in kombucha culture and the release of extra catechins by acid-sensitive cell lysis might be the reason for the increase of the concentration of total polyphenols in kombucha during the fermentation (R. Jayabalan et al., 2007; May et al., 2019).

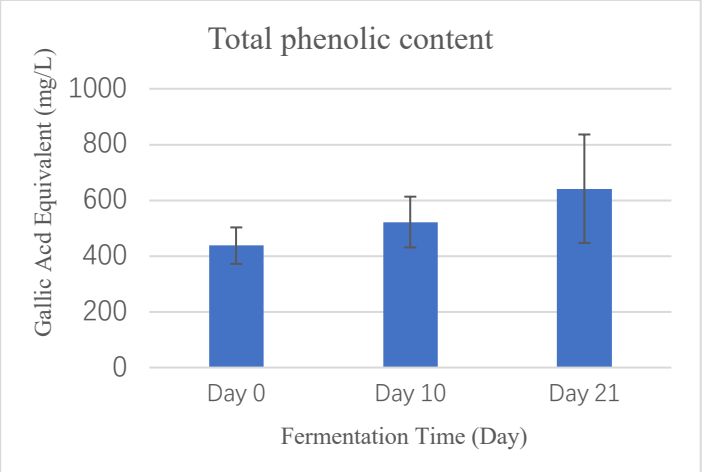


Figure 10: FC assay performed on kombucha on day 0, 10 and 21 of fermentation (n=3).

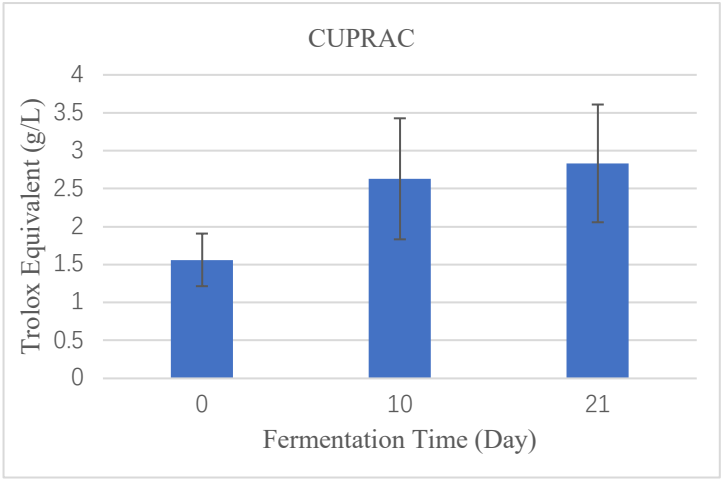
However, Yıkmış (2019) found that the amount of total polyphenol and flavonoid showed a decreasing trend during 30 days of fermentation in all kombucha samples made by black tea. This decrease might be a result of chemical transformation of black tea polyphenols in kombucha or the direct metabolism of polyphenols by microorganisms. Moreover, the study from De Filippis et al. (2018) showed that the types of tea leaves have an effect on the total polyphenol content when the fermentation was carried out at 20°C. Although the content of total polyphenol in kombucha made by green tea was higher than that of black tea at the beginning of fermentation, the concentration of polyphenols in kombucha made by green tea did not change within 21 days, while the content of polyphenols in black tea increased with time. The total polyphenols of both kinds of kombucha at 30°C did not change within 15 days but then showed a downward trend from 15 days to 21 days. This result showed that temperature was also one of the important factors affecting the

concentration of polyphenols in kombucha. From the diversity of results from literature, the total polyphenols content in kombucha is affected by factors such as tea leaf type, production methods, and microorganism in kombucha.

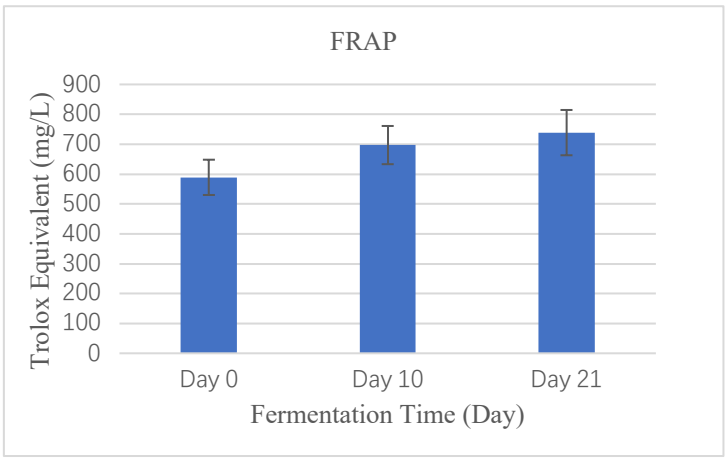
4.4 Antioxidant analysis

Figure 11 presents the antioxidant activity of kombucha made by TGY and sucrose by using three common assays, CUPRAC, FRAP and Phosphomolybdenum on fermented day 0, 10 and 21. All these three methods showed an increasing trend during fermentation time, and the antioxidant capacity increased more rapidly in the first 10 days.

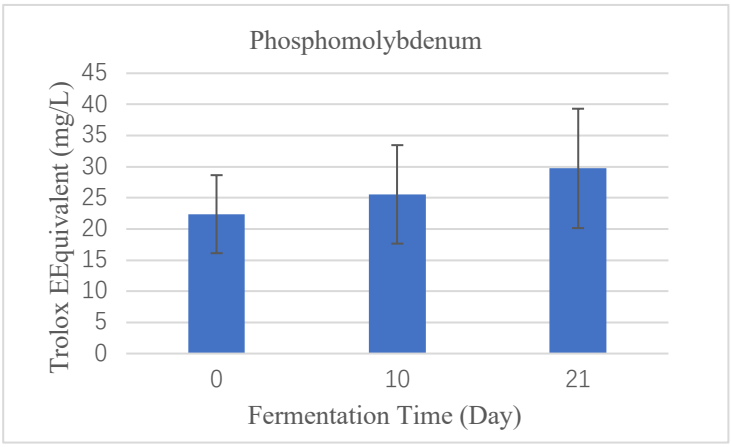
Through CUPRAC assay, the initial antioxidant capacity of kombucha was 1562 mg Trolox equivalent/L kombucha sample. After 21 days of fermentation, the antioxidant activity of kombucha was about twice that of the original (2833 mg Trolox equivalent/L kombucha sample). According to the results from the FRAP assay, the antioxidant capacity of kombucha increased from 589 mg Trolox equivalent/L kombucha sample to 739 mg Trolox equivalent/L kombucha sample during 21 days of fermentation. The results of phosphomolybdenum analysis showed that there was an increase in the antioxidant activity of kombucha, reaching 29729 mg/L at 21 days of fermentation.



(a)



(b)



(c)

Figure 11: (a) CUPRAC (b) FRAP (c) Phosphomolybdenum assay performed on kombucha on day 0, 10 and 21 of fermentation (n=3).

Antioxidant activity plays an important part in fermented beverages because antioxidants have scavenging effects on free radicals, therefore they can delay or prevent oxidative damage. As shown in Section 1.3, the increase of antioxidant capacity in kombucha tea is due to the enzymatic degradation of complex polyphenols obtained from tea leaves during fermentation, such as catechins and anthocyanin, and the production of low molecular weight substances, such as DSL and ascorbic acid, which corresponds to the increase in the concentration of polyphenols.

According to Kaewkod, Bovonsombut, and Tragoolpua (2019), the kombucha prepared from green tea showed the highest antioxidant capacity (2.642 mg GAE/mL kombucha tea) after 15 days of fermentation. Kombucha made by OT

showed the highest antioxidant ability on the 9th day, but it was stable after 12 to 15 days. In the study made by Sheng Che Chu (2006), the results of antioxidant capacity of kombucha samples were still different. During 15 days of fermentation, some samples showed an increasing trend, but the antioxidant ability of some kombucha samples decreased firstly and began to rise after 6 to 9 days of fermentation. Different kombucha samples showed diverse free radical scavenging ability, which might be related to the normal microbiota.

4.5 Colour of kombucha

Colour is an important factor to determine the visual quality of beverage. The colour of kombucha in this study was expressed in the EBC unit. As can be seen from Figure 12, the values decreased as the fermentation time increased, which indicates the colour of kombucha become lighter. The reason for this is that part of brown pigments, such as thearubibin undergo microbial transformation and is converted into theaflavin (C. H. Liu, Hsu, Lee, & Liao, 1996; Sheng Che Chu, 2006), which changes the colour of kombucha into lighter brown.

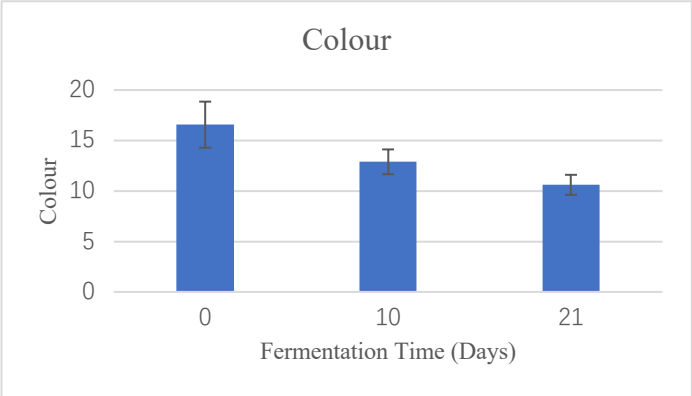


Figure 12: Colour in EBC Units detected on kombucha on day 0, 10 and 21 of fermentation (n=3).

These results are in line with those mentioned by Chu and Chen (2006), which said that the colour parameters of kombucha made by black tea decreased gradually during fermentation. In addition, the study from Yıkmış (2019) also showed the colour of kombucha made by the mixture of black tea and purple basil during the 20

day fermentation period became light through L, a, b, c and h values. However, the fermentation process usually has little influence on the colour intensity of kombucha made by fruit tea.

4.6 Free amino acids and minor organic acids in kombucha

The free amino acids and minor organic acids were analyzed by GC-MS assay after MCF derivatization. There were nine free amino acids dertected in kombucha in this study, including alanine, valine, leucine, isoleucine, proline, threonine, aspartic acid, glutamic acid and phenylalanine. The five minor organic acids that were also detected in the kombucha included malonic acid, maleic acid, succinic acid, malic acid and citric acid.

According to Figure 13, the concentration of malonic acid, maleic acid and citric acid in kombucha sample had a slight growth with the increase of fermentation time. The content of malic acid doubled after 10 days of fermentation (26 mg/L) but decreased to 16 mg/L after 21 days. The concentration of succinic acid increased significantly from 8 mg/L to 95 mg/L during the first 10 days and had a slight decrease to 90 mg/L after 21 days. In addition, after 21 days of fermentation, among these five organic acids, the content of succinic acid was highest, followed by malic acid, citric acid, maleic acid and malonic acid.

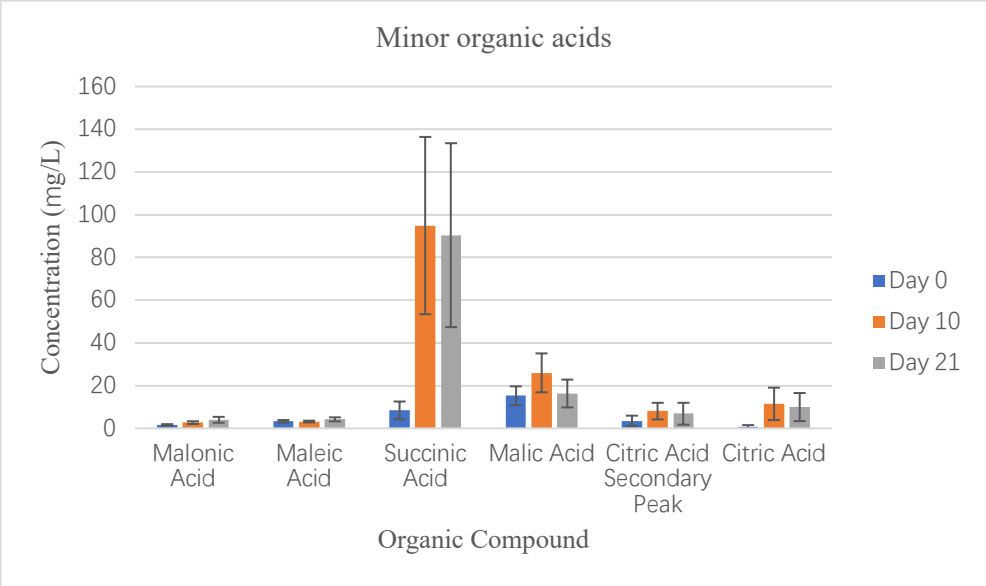


Figure 13: Concentration of minor organic acids of kombucha on fermented day 0, 10 and 21 (n= 3).

As shown in Figure 14, the concentration of all amino acids decreased during the fermentation. Before fermentation, the content of alanine was the highest (0.0352 µmol/mL), followed by glutamic acid (0.0154 µmol/mL), aspartic acid (0.0137 µmol/mL) and valine (0.007 µmol/mL). The concentration of the remaining five amino acids was less than 0.005 µmol/mL before fermentation. However, the concentration of amino acids decreased significantly below 0.0025 µmol/mL after 10 days fermentation.

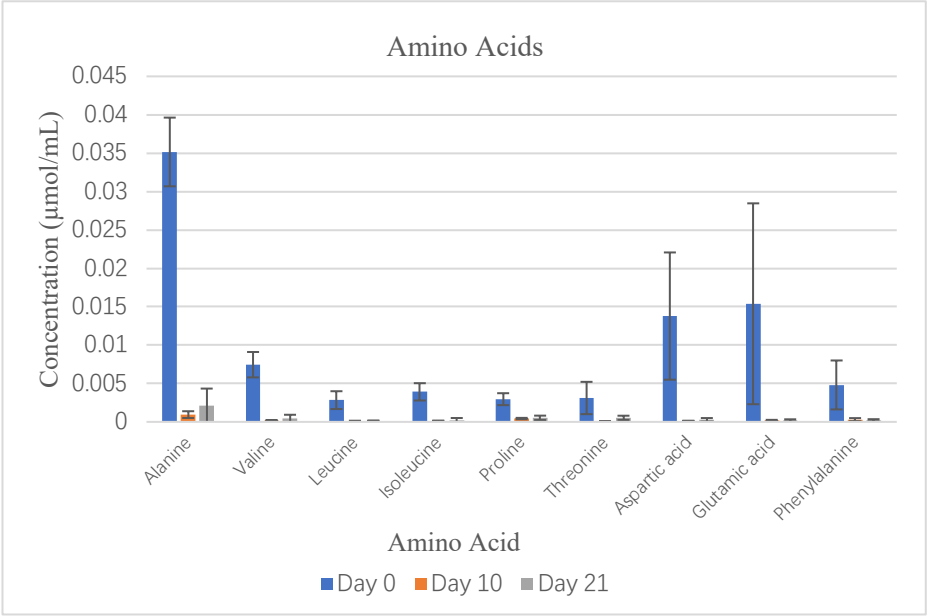


Figure 14: Concentration of amino acids of kombucha on fermented day 0, 10 and 21 (n= 3).

Research by De Filippis et al. (2018) showed that the selection of tea used to make kombucha had no effect on the microbiome and the content of organic acid. But due to the complicated microbial consortium in kombucha, a study by Tran et al. (2020) discussed the interaction between microorganisms and the effects of monocultures on the composition of organic acids in kombucha fermented at 26 ℃ in both open and close incubation condition. They found that the presence of succinic acid was associated with *K. Saccharivorans*, especially under the condition of limited oxygen. Besides, the production of lactic acid was related to the presence of lactic acid bacteria. The existence of lactic acid bacteria (LAB) in kombucha is inconsistent. Generally, they are absent or low in content. In this study, lactic acid was not detected, probably because LAB was not present in the SCOBY used and LAB cannot grow below pH 3.5 (Coton, Pawtowski, Taminiau, Burgaud, & Coton, 2017). While according to Leal et al. (2018), lactic acid was one of minor organic acid of kombucha made by green tea in their study.

In addition, amino acids detected on fermentation Day 0 came from OT itself. A study from Hao Jiang et al. (2020) showed that aspartic acid, glutamic acid and alanine are the most abundant amino acids in OT except theanine. It is difficult to compare the amino acid content of kombucha during fermentation to literature because this is the first time such study was conducted. However, according to

Matsuoka, Tsuchida, Yoshinaga, Matsushita, and Adachi (1996), amino acids are used as nitrogen sources or as stimulators to enhance cellulose yield and improve the cell growth and production during the early stage of fermentation, which is also the reason for the decrease of amino acid content during fermentation.

4.7 Sugars

The concentration of sugars in kombucha detected by HPLC was shown in Figure 15. The sucrose content plummeted from 54.59 g/L to 11.95 g/L in the first 10 days of fermentation, and there was little change until the last day of fermentation. Besides, glucose and fructose were detected on the day 10 of fermentation, and the concentration were 16.92 g/L and 19.11 g/L, respectively. As mentioned above, this is because sucrose was hydrolyzed into glucose and fructose by invertase enzyme during the fermentation. However, the content of both decreased to 13.15 g/L and 17.48 g/L after 21 days of fermentation, respectively. This is due to the conversion of monosaccharide into other compounds, such as ethanol, acetic acid, gluconic acid, carbon dioxide and cellulose during fermentation. Notably, the consumption rate of glucose was faster than that of fructose after 10 days, which indicated that glucose was selected preferentially as the carbon source rather than fructose by kombucha microflora.

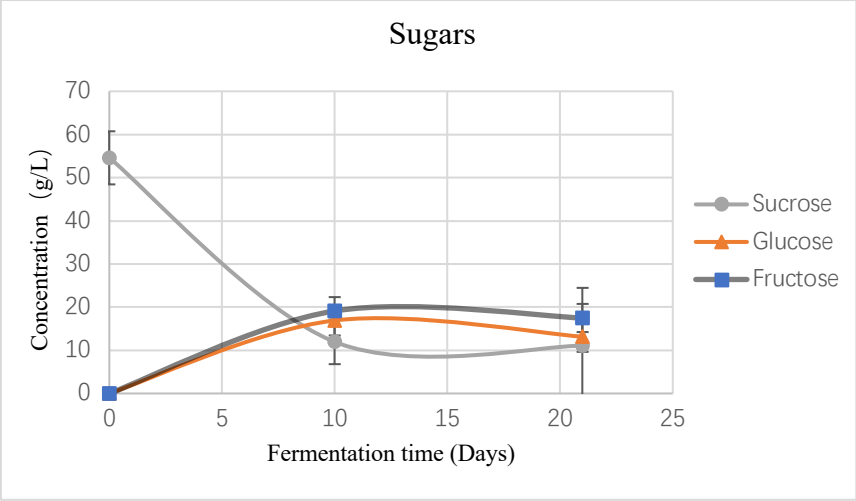


Figure 15: Concentration of sucrose, glucose and fructose of kombucha on fermented day 0, 10 and 21 (n= 3).

A study by Kallel et al. (2012) mentioned that the concentration of sucrose decreased linearly with time within the whole two weeks of green tea kombucha fermentation, but the decrease was only in the first week in black tea kombucha. From previous studies, they showed that the range of sucrose consumption rate was around 2 to 15 $g \cdot L^{-1} \cdot d^{-1}$ (Chen & Liu, 2000; Sreeramulu, Zhu, & Knol, 2010). The consumption rate of sucrose in this study was 4.264 $g \cdot L^{-1} \cdot d^{-1}$. The difference between these values was likely to be related to the property and content of kombucha microflora, which is consistent with their respective invertase activities. The elimination degree of invertase inhibitors in the tea production process may also be one of the factors. This was also the reason why the consumption trends of sucrose in kombucha made with different tea were different. Consistent with the results here, Kallel et al. (2012) and Toyosaki et al. (1995) all have reported that glucose was preferred to fructose as the carbon source for kombucha microflora, especially *Acetobacter* strains.

4.8 Volatile compounds

Volatile organic compounds (VOCs) are very critical in the food industry because they play an important role in determining the sensory characteristics of the final food products (Capozzi et al., 2016). VOCs refer to volatile organic molecules with a molecular weight of up to 300 Dalton at room temperature, and they are primary and secondary metabolites generated by microorganisms (Capozzi et al., 2016; Liszkowska & Berlowska, 2021). In addition to ethanol and carbon dioxide, a small amount of volatile organic compounds are formed (Januszek, Satora, Tarko, & Wajda, 2020).

The volatile compounds in kombucha made by TGY were evaluated at day 0, 10 and 21 of fermentation by SPME technique, and principal component analysis (PCA) was performed to analyze the data. Figure 16 showed PCA scores plot of the volatile compounds found in kombucha on fermented day 0, 10 and 21, and the volatile clustering based on fermentation time. In addition, the kombucha from Day 0 of fermentation was separated from Day 10 and Day 21 along PC1, which explained 79.6% of the variance.

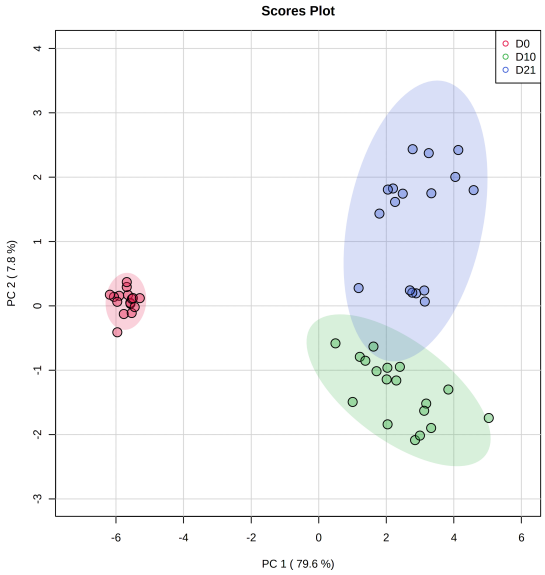


Figure 16: PCA scores plot of the volatile compounds found in kombucha on fermented day 0, 10 and 21.

Overall, there were 92 VOCs detected in kombucha samples during fermentation, and in this study, it was narrowed down to 20 main ones based on literature findings, including 6 fatty acids, 6 alcohols, 3 aldehydes, 2 esters, 1 ketone and 2 other compounds. Figure 17 showed PCA biplot of these main VOCs found in kombucha on fermented day 0, 10 and 21, and Table 5 listed their category and relative content. The relative content is expressed as a ratio between the response of the compound and the response of the internal standard (ISTD ratio). As shown in Figure 17, hexanal, (E)-pent-2-enal, 1-penten-3-ol, sulcatone were detected in kombucha before fermentation. According to H. Liu et al. (2021), all of these four aroma compounds came from oolong tea. Hexanal is a colourless transparent liquid with the smell of raw oil, grass and apple, and 1-penten-3-ol is colourless to light yellow liquid with fruit aroma. Sulcatone, a colourless liquid, has fresh fruit aroma. During fermentation, the concentration of these compounds decreased, and the formation of some other VOCs occurred.

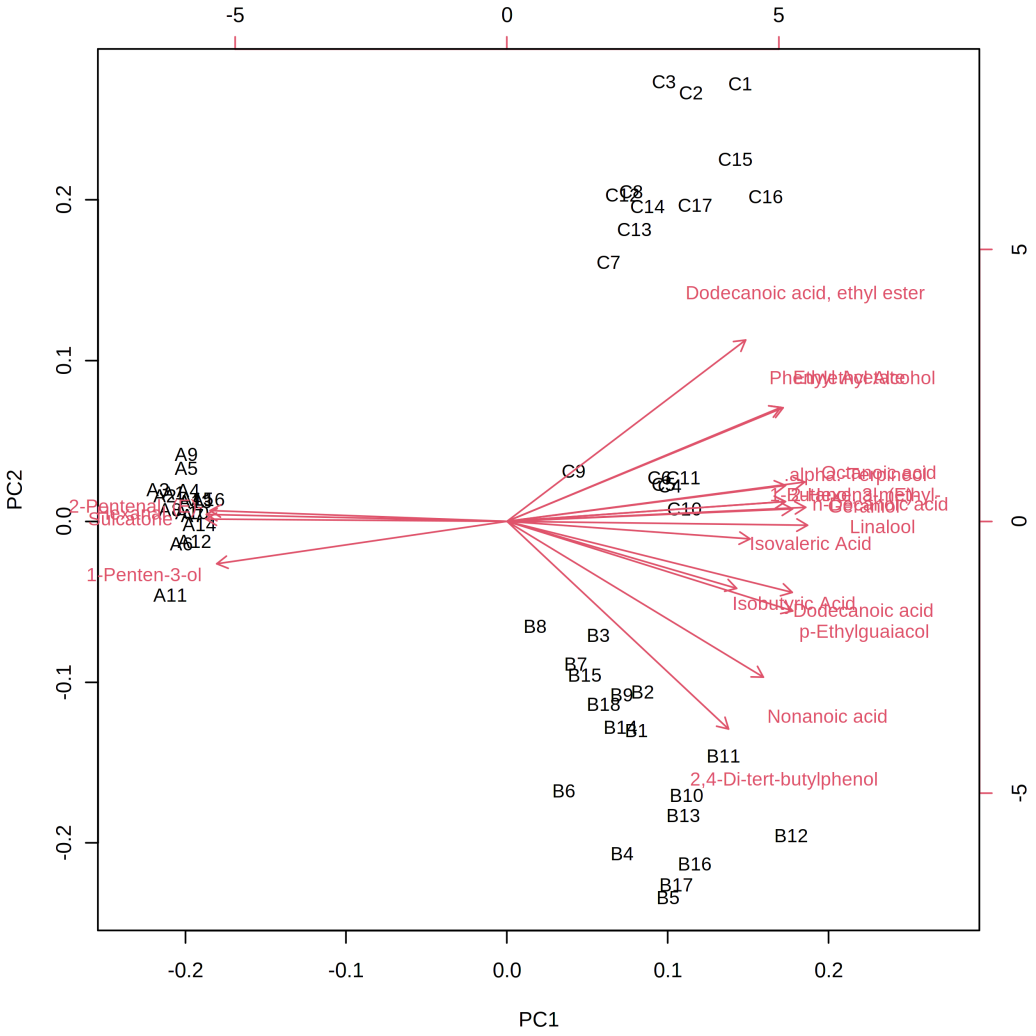


Figure 17: PCA biplot of the main volatile compounds found in kombucha on fermented day 0, 10 and 21.

Table 5: The relative content of main volatile compounds found in kombucha on fermented day 0, 10 and 21.

No.	Category	Compounds	Relative content (ISTD Ratio)		
			D0	D10	D21
1	Aldehydes	hexanal	0.054	0.001	0
2		(E)-pent-2-enal	0.006	0	0
3		(E)-hex-2-enal	0.011	0.182	0.172
4	Alcohols	1-penten-3-ol	0.125	0.029	0

5		3-methylbutan-1-ol	0.011	0.181	0.172
6		linalool	0.021	0.112	0.115
7		alpha-terpineol	0.002	0.032	0.045
8		geraniol	0.009	0.031	0.031
9		2-phenylethanol	0.024	0.185	0.297
10	Ketones	sulcatone	0.041	0.003	0.002
11	Acids	isobutyric acid	0	0.159	0.128
12		isovaleric acid	0	0.288	0.340
13		octanoic acid	0.013	0.867	1.168
14		nonanoic acid	0.062	0.403	0.248
15		n-decanoic acid	0.020	1.253	1.579
16		dodecanoic acid	0.008	0.555	0.510
17	Esters	ethyl acetate	0.005	0.667	1.083
18		ethyl dodecanoate	0	0.017	0.037
19	Others	p-ethylguaiacol	0	0.185	0.140
20		2,4-Di-tert-butylphenol	0.253	0.780	0.456

According to Figure 17, VOCs detected after fermentation were mainly composed of acids and esters compounds. Several volatile acids were detected in kombucha after fermentation, such as isobutyric acid, isovaleric acid, octanoic acid, nonanoic acid, n-decanoic acid and dodecanoic acid. Many volatile acids are only intermediate products in the fermentation process, like acetic acid, isovaleric acid and n-decanoic acid, in which they will be converted into esters. For example, isovaleric acid produced by *Brettanomyces* has unpleasant rancidity and pungent smell (J. Zhang, Van Mullem, Dias, & Schwan, 2021). However, it is an important raw material to produce perfume 2-phenylethyl 3-methylbutanoate, which was also detected during fermentation. 2-phenylethyl 3-methylbutanoate has chrysanthemum, fruit, sweet and rose aroma. Nonanoic acid has a light aroma of fat and coconut. In addition, dodecanoic acid with a slight fragrance of laurel oil and ethyl dodecanoate has a special fruity smell.

It is worth noting that some aroma compounds from oolong tea remained in kombucha after fermentation, such as (E)-hex-2-enal and 3-methylbutan-1-ol

(Pripdeevech, Rothwell, D'Souza, & Panuwet, 2017). Therefore, kombucha made by OT in this study retained some characteristic aroma compounds of oolong tea. At the same time, alcohols, acids and esters were generated during fermentation, such as nonanoic acid, ethyl ester, octanoic acid and decanoic acid which form a distinguishable aroma of kombucha.

In fermented food, a large number of these compounds are synthesized or released from original materials during fermentation. According to J. Zhang et al. (2021), 3-methylbutan-1-ol, octanoic acid, decanoic acid, dodecanoic acid, isovaleric acid, hexanoic acid, isobutyric acid, benzaldehyde and p-ethylguaiacol were also found in kombucha made by both green tea and black tea. However, dihydroeugenol, ethyl dodecanoate and nonanal are the VOCs also found in this study, were only detected in black tea kombucha, and these differences may be attributed to different process of tea leaf production.

5. Conclusion

The study revealed that it is possible to produce kombucha with TGY and sucrose. In particular, during fermentation, the pH of kombucha made by TGY decreased and stabilized after 10 days, while the titratable acid continued to rise until the end of 21 days of fermentation. The colour of kombucha became lighter. While the content of sucrose and free amino acids (alanine, glutamic acid, aspartic acid and valine) decreased during fermentation, the content of fructose, glucose, ethanol, gluconic acid, minor organic acid (malonic acid, maleic acid, succinic acid, malic acid and citric acid) and total phenolic compounds and antioxidant capacity in kombucha increased. SPME assay indicated that some characteristic aroma compounds of OT were retained in kombucha mad by TGY in this study, such as (E)-hex-2-enal and 3-methylbutan-1-ol. Meanwhile, alcohols, acids and esters were generated during fermentation, such as nonanoic acid, ethyl ester, octanoic acid and decanoic acid, forming the unique aroma of kombucha.

6. Recommendation for future work

- The microbial community in the SCOBY used in this study should be analyzed as this can explain the metabolites formed in the kombucha.
- Sensory analysis could be conducted on TGY kombucha from this study as it can determine the acceptability of this product in terms of colour, odour and taste and overall liking.

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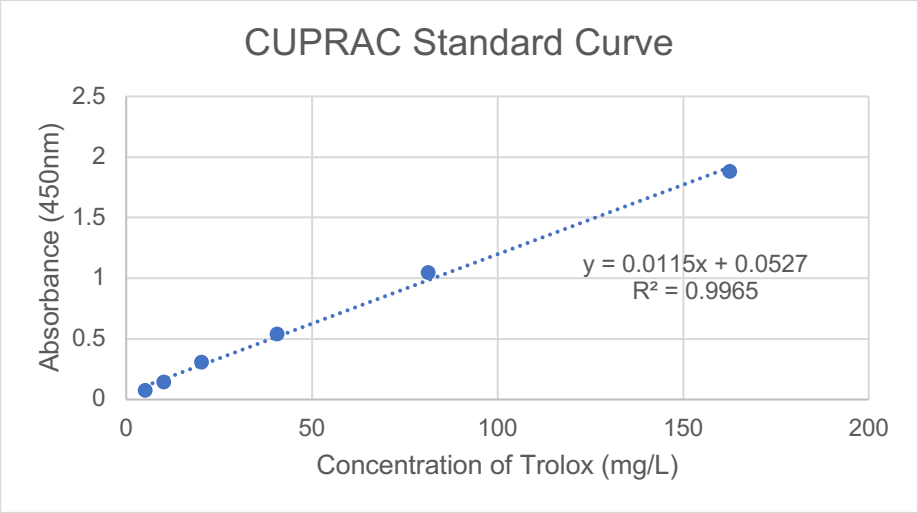
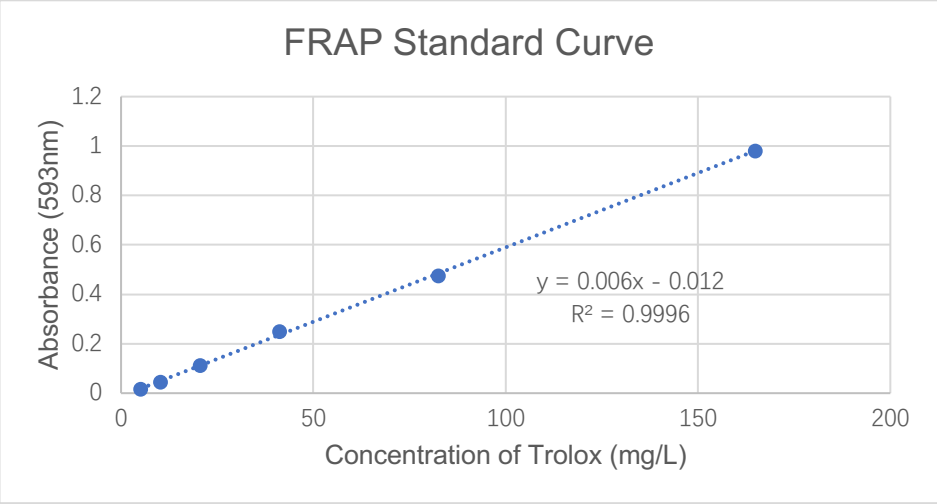
Appendix

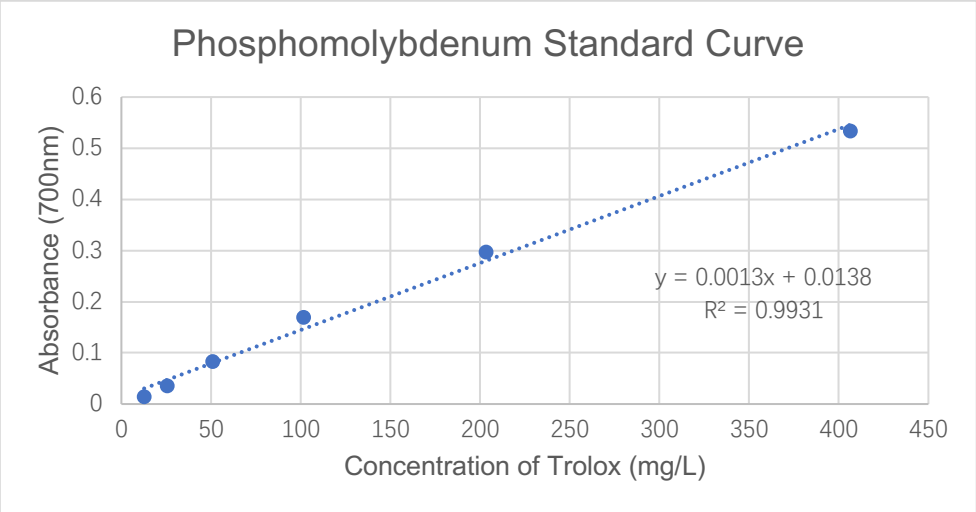
Appendix A: Antioxidant assay calibration Curves



(a)



(b)



(c)

Figure 18: The standard calibration curve for (a) CUPRAC (B) FRAP (c) PM antioxidant test

Appendix B: Total phenolic content assay calibration Curves

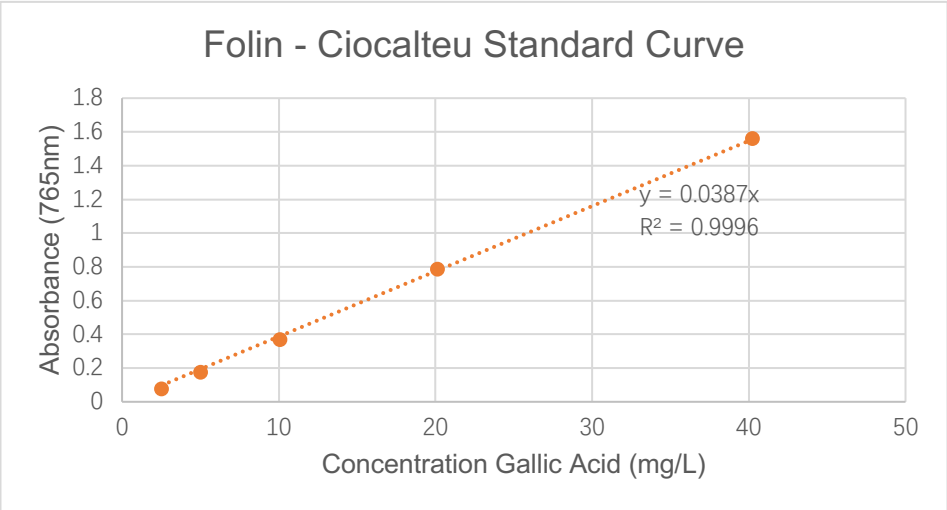


Figure 19: The standard calibration curve for FC assay