Sains Malaysiana 51(11)(2022): 3715-3729
http://doi.org/10.17576/jsm-2022-5111-16

Effects of Starter Culture and Sweetener on Biochemical Compounds and Microbial Diversity of Kombucha Tea

(Kesan Kultur Pemula dan Pemanis pada Sebatian Biokimia dan Kepelbagaian Mikrob Teh Kombucha)

Ahmad Azfaralariff[1,6], Batul Vohra[2], Shazrul Fazry[1,2,3], Douglas Law[5], Fareed Sairi[4] & Babul Airianah Othman[1,2,3,*]

[1]*Department of Food Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*
[2]*Tasik Chini Research Center, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*
[3]*Innovative Centre for Confectionery Technology (MANIS), Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*
[4]*Department of Biology Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*
[5]*Faculty of Health and Life Sciences, Inti International University, Persiaran Perdana BBN Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia*
[6]*Bioresource Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang, Malaysia*

Received: 22 March 2022/Accepted: 28 June 2022

ABSTRACT

Kombucha tea has been claimed to have several health benefits. Many factors influence the properties of kombucha tea produced. This study focused on the effects of starter cultures (kombucha liquid broth (KLB) and cellulosic pellicle (KCP)) and sweetener (white sugar (S), honey (H) and jaggery (J)) used in the production of kombucha tea. The results showed that all kombucha teas prepared using KLB had a lower pH and a higher concentration of acetic acid during fermentation. The ethanol content for samples prepared using KLB increased (0.7 ± 0.26 mg/L to 1.73 ± 0.58 mg/L) during the fermentation period, compared to KCP which was the maximum after 72 h fermentation, and continued to decrease (2.97 ± 1.24 mg/L to 0.90 ± 0.44 mg/L). Although not too much differences in pH and ethanol content were observed when different sweetener sources were used, they did have significant differences in antioxidant properties and antimicrobial activity. Samples prepared using jaggery had the lowest antioxidant activity while kombucha tea prepared using KLB and white sugar (KLB-S) had the highest antioxidant and antibacterial activity and was mostly colonized by *Acetobacteracea* and *Aspergillus fumigatus*. Fermentation significantly increases the number of active compounds present in KLB-S from 11 to 25 compounds. New compounds such as docosanedioic acid, muramic acid and thiolactomycin were formed. Thiolactomycin, a natural antibiotic is suggested to contribute to the high antimicrobial activity of KLB-S. In conclusion, KLB and white sugar are better suited in preparing kombucha tea as more benefits and consistent results were observed.

Keywords: Antimicrobial; antioxidant; cellulose; kombucha; pellicle; starter cultures

ABSTRAK

Teh kombucha dilaporkan mempunyai pelbagai manfaat kesihatan. Terdapat banyak faktor yang mempengaruhi ciri-ciri teh kombucha yang dihasilkan. Kajian ini memberi tumpuan kepada kesan penggunaan kultur pemula (air teh kombucha (KLB) dan lapisan selulosa (KCP)) serta pemanis (gula putih (S), madu (H) dan gula palma (J)) yang berbeza dalam penghasilan teh kombucha. Hasil kajian menunjukkan bahawa semua teh kombucha yang disediakan menggunakan KLB mempunyai pH yang lebih rendah dan kepekatan asid asetik yang lebih tinggi sepanjang tempoh fermentasi. Kandungan etanol untuk sampel yang disediakan menggunakan KLB juga semakin meningkat (0.7 ± 0.26

mg/L kepada 1.73 ± 0.58 mg/L) sepanjang tempoh fermentasi, berbanding KCP yang mempunyai kepekatan maksimum selepas 72 jam dan terus menurun (2.97 ± 1.24 mg/L hingga 0.90 ± 0.44 mg/L). Walaupun tiada perbezaan yang ketara pada pH dan kandungan etanol direkodkan apabila sumber pemanis yang berbeza digunakan, keputusan analisis menunjukkan perbezaan yang ketara dilihat pada sifat antioksidan dan aktiviti antimikrob. Sampel yang disediakan menggunakan gula palma mempunyai aktiviti antioksida yang paling rendah manakala teh kombucha yang disediakan menggunakan KLB dan gula putih (KLB-S) mempunyai aktiviti antioksida dan antibakteria yang paling tinggi dan dikoloni oleh *Acetobacteracea* dan *Aspergillus fumigatus*. Fermentasi didapati telah mengubah komposisi sebatian aktif yang terdapat dalam KLB-S daripada 11 sebatian kepada 25 sebatian. Sebatian baru seperti asid dokosanedioik, asid muramik dan tiolaktomisin telah terhasil. Tiolaktomisin yang merupakan antibiotik semula jadi dalam sampel KLB-S dipercayai menjadi punca aktiviti antimikrob yang tinggi. Sebagai kesimpulan, KLB dan gula putih lebih sesuai digunakan dalam penyediaan teh kombucha kerana menghasilkan teh kombucha yang lebih banyak faedah dengan hasil dan keputusan yang lebih konsisten.

Kata kunci: Antimikrob; antioksida; kombucha; kultur pemula; pelikel; selulosa

Introduction

Kombucha is a fermented tea beverage, which is said to have originated in China (Chakravorty et al. 2016; Soares et al. 2021). Kombucha is now consumed worldwide not only as health supporting drink but also as an alternative to soda drinks due to its effervescent and fizzy taste. The most common way to prepare kombucha is by adding a starter culture that contains a symbiotic culture of bacteria and yeast or also known as SCOBY into a sweetened black tea. The proportion of the microbial composition in SCOBY was reported to be diverse and difficult to elucidate as it differs from sample to sample (Laavanya et al. 2021) but mainly consists of acetic acid bacteria (AAB) namely, *Acetobacter, Gluconobacter* sp. (De Roos & De Vuyst 2018; De Filippis et al. 2018), lactic acid bacteria (LAB) namely, *Lactobacillus, Lactococcus* (Marsh et al. 2014) and yeasts such as *Saccharomyces cerevisiae, Zygosaccharomyces bailii*, *Saccharomyces lugwigii, Kloeckera apiculata*, and *Brettanomyces bruxellensis* (Coton et al. 2017). Under aerobic conditions, the microbes in the starter culture are responsible for the metabolite diversity in kombucha. The yeasts cut down the sucrose to glucose and fructose then ethanol. The bacteria then use ethanol and glucose to produce acetic acid and gluconic acid, respectively (Sievers et al. 1995; Vitas et al. 2019). This overall process converts the sweetened tea into a slightly carbonated and sour, refreshing beverage.

Kombucha consumption is linked with health benefits such as immunity enhancement, cancer prevention, diabetes control, toxin excretion prevention, and anti-ageing (Ahmed et al. 2020; Dufresne & Farnworth 2000) as well as a potential antimicrobial agent (Ivanišová et al. 2020; Sreeramulu et al. 2001; Vohra et al. 2019b). The acid content and other biochemical compounds restrict the growth of harmful bacteria and therefore only selected species are associated with the kombucha fermentation. Many have reported on the chemical composition of kombucha which showed the presence of various metabolites including various kinds of sugars; vitamins B complex and vitamin C; various organic acids such as acetic, glucuronic and gluconic acids; various amino acids and proteins, lipids, phenols and polyphenols, minerals, and ethanol (Ahmed et al. 2020; Amarasinghe et al. 2018; Sreeramulu et al. 2001).

These metabolites composition and concentration of kombucha are dependent on the source of starter culture (geographical and various microbial combinations) (Ahmed et al. 2020; Malbasa et al. 2011), sugar and tea concentration, temperature, as well as fermentation time (Chen & Liu 2000; Jayabalan et al. 2008; Villarreal-Soto et al. 2018; Watawana et al. 2017). Nowadays, kombucha is being brewed with various substrates, not limited to black tea, and is fermented with a different form of starter culture. The common starter culture used is normally the floating cellulosic pellicle, however, some would also use the liquid broth portion of matured kombucha as well as a combination of both. There is no definite standard nor control on the form of starter cultures used. As both forms of starter cultures have different microorganism's consortium (Chakravorty et al. 2016; Coton et al. 2017; Marsh et al. 2014), therefore, it can be hypothesized that they will behave differently with the substrates, thus, will lead to a different product, with different properties. These will include the concentration of ethanol as well as other metabolites of kombucha tea.

This study aims to explore the influence of two kombucha starter cultures, kombucha liquid broth (KLB) and cellulosic pellicle (KCP), on the ethanol and acetic acid content as well as antioxidant activity, antibacterial

activity and chemical composition of the kombucha within several variations of sweeteners as substrates. The sweeteners chose based on the commonly used by brewers. The microbial identification on the starter cultures has also been performed to build up a better understanding. The findings of this study will help in designing better kombucha beverages with optimum efficacy levels.

Materials and Methods

Materials

The black tea (Lipton, Malaysia), white sugar (CSR brand, Malaysia) and jaggery (Bintang brand, Malaysia) were obtained from the local market. Kelulut honey (Stingless bee, *Heterotrigona itama* sp.) was obtained from Malaysian Genome Institute (MGI), Selangor, Malaysia. Liquid broth (KLB) and cellulosic pellicle (KCP) were generously provided by local kombucha manufacturing company namely SMS Wellness Beverages Sdn. Bhd. Malaysia, located in Rawang, Selangor, Malaysia.

Preparation of Kombucha Samples

Teas at 0.2% (w/v) were boiled together with 10% (w/v) of sweetener: white sugar (S), honey (H), and jaggery (J), respectively, for 10 min. This mixture was then allowed to cool down to room temperature and a portion of 20 mL was transferred into 50 mL-Falcon tubes. Starter culture, in which either the KLB or KCP, at 7 days old, were added by 10% (v/v for KLB and w/v for KCP), respectively, to each sample. The samples were covered with cheesecloth and kept at room temperature for fermentation. Prior to further examination, all samples were collected and filtered using a 0.2 millipore-syringe filter to ensure that no debris remains.

pH Measurement and Acetic Acid Assay

A 10 mL aliquot of the samples was taken in a test tube and the pH was measured by using an electronic pH meter (Sartorius PB-10, Göttingen, Germany). Megazyme kit (K-ACET, Wicklow, Ireland) was used to measure the acetic acid content as by procedure given in the assay manual.

Ethanol Content Assay

Ethanol content was measured by a dichromate assay (Williams & Darwin Reese 1950) with modification as in Sumbhate et al. (2012). The ethanol standard graph of 1.6 mg/mL to 8 mg/mL was prepared. To a 1 mL aliquot of ethanol stock solution and the sample, 5 mL of 40 mg/mL sodium dichromate solution, 5 mL of pH 4.3 acetate buffer and 25 mL of 1 M sulfuric acid were added in a volumetric flask and gently shaken for 1 min. This mixture was incubated at room temperature for 2 h prior to a 578 nm wavelength reading using a microplate reader (BioTek Epoch, Santa Clara, USA).

Antioxidant Activity

Antioxidant activity of kombucha samples was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Ascorbic acid was used as a standard and distilled water was used as the blank. The DPPH reagent was prepared by dissolving 4 mg of reagent powder in 100 mL methanol. A total of 100 µL of each sample and reagent, respectively, were pipetted into a 96 well microplate and incubated in a dark environment for 30 min at room temperature. The plates were read at 517 nm using a microplate reader (BioTek Epoch, Santa Clara, USA). DPPH inhibition was calculated using the formula:

$$\frac{A_0 - A_i}{A_o} \; X \; 100$$

where A_0 is the absorbance of blank; A_i is the absorbance of sample.

Antimicrobial Activity

Antibacterial activity was measured via the disk diffusion method according to the procedure followed by Greenwalt et al. (2000) with few modifications against common human pathogens, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacilus subtilis*, *Staphylococcus aureus,* and *Serratia marcescens*. A single colony of each bacterium was inoculated in 5 mL of nutrient broth media and incubated for 24 h at 30 °C. A total of 0.1 mL of culture broth of each pathogen was spread and plated evenly on nutrient agar (Sigma Aldrich, Germany, now Merck). Whatman cellulose filter paper was used to prepare 0.5 mm disks. Using sterile forceps, the discs were then saturated with 14, 28 and 60 -day -old kombucha samples and placed on a spread plate. The plates were incubated for 24 h at 30 ºC. The antimicrobial activity is displayed by the inhibition zone of the specimen around the paper disc.

Microbial Analysis of Kombucha Samples

To determine the microbial type in KLB and KCP, a metagenomics study was done using three duplicates.

Samples prepared by using sugar as substrate and 7 days old were used. The PureLink Microbiome DNA Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) was utilised to extract high-quality DNA from the kombucha samples, which was then used as a template for Hypervariable regions of 16s V3-V4 for the bacterial identification and ITS-2 region for sequencing fungal DNA on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Ribosomal Database Project (RDP) classifier Bayesian algorithm at 97% similarity level was used to obtain the species classification information which is corresponding to Operational Taxonomic Units (OTUs).

COMPOSITION ANALYSIS

The 14 day old sample of fresh black tea without sugar and KLB-S was selected for chemical compound identification through UPLC-MS. The sample was filtered and injected (2 uL) into Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer (LC-MS) with dual ionization source electrospray (ESI) source (Agilent, USA). For chromatographic separation, Agilent Zorbax Eclipse XDB-C18 (150 mm × 2.1 mm, 3.5 um) column maintained at 30 °C and flow rate of 0.5 mL/min of the mobile phases was used: ultra-pure water containing 0.1% formic acid and 5 mM ammonium formate (mobile phase A); and LC-MS-grade acetonitrile containing 0.1% formic acid (mobile phase B), according to the following gradient: 0 min - 97% A; 11.80 min - 50% A; 12.38 min - 15% A; 14.23 m -15% A; 14.70 min - 97% A. Data were acquired in MSE mode using argon as collision gas, applying low and high collision energy with a ramp from 25 to 55 V. Acquisitions were performed in positive and centroid mode between m/z 100 and 3200. The ionization conditions were applied: cone voltage 30 V, capillary voltage 3.0 kV; desolation gas (N2) 1,200 L/h at 600 °C; cone gas 50 L/h and source temperature at 150 °C. Data processing was performed with the software HunterLab (Agilent, USA) and the identification by the comparison with standards library based on isotope distribution of neutral mass, the retention time and the MS/MS fragments spectra.

STATISTICAL ANALYSIS

Experiments were performed at least in triplicate. The IBM SPSS 18.0 (SPSS Inc., Chicago, USA) was used to analyze the variance (ANOVA) in order to determine the differences. Means were considered significantly different at $P<0.05$ using pair-wise multiple comparison procedures (Tukey's Test).

RESULTS AND DISCUSSION

pH and acetic acid content are considered the basic measures for the acidity of the beverage and hence changes in pH and acetic acid concentration of the drink was recorded with variation in media, and starter cultures as well for different fermentation time. Figure 1 shows the pH changes and acetic acid produced during 60 days of fermentation. Drastic pH changes were recorded on the first day for samples prepared by using honey and jaggery.

The pH of all prepared kombucha tea was decreased as fermentation time was prolonged (Figure 1(a)). The decrease in pH is due to the production of acetic acid as a fermentation product. As the fermentation prolonged, the concentration of acetic acid in all samples was increased (Figure 1(b)). During the fermentation process, yeasts and bacteria metabolize the sugar as a carbon source and produce a number of by-products such as cellulose, alcohol and organic acids including acetic acids, lactic acids, citric acid and malic acids (Jayabalan, Marimuthu & Swaminathan 2007; Sreeramulu et al. 2000). The sugar used influenced the formation of these products. Sucrose gave the lowest pH followed by honey and jaggery. This difference in acidity may be due to different compositions of the sweeteners which affect the microbial ability to grow and utilize the carbon sources (Keshk & Sameshima 2005). White sugar is consist of pure sucrose that can be easily breakdown into glucose and fructose, thus it provides a simple carbon source compared to honey and jaggery that contain mixed sugar together with other complex ingredients (Jagannadha Rao et al. 2010; Veena et al. 2018). Kelulut honey has fructose, glucose as well as maltose present in it, while jaggery is constituted of sucrose, protein and insoluble fibre (Jagannadha Rao et al. 2010). Conversion of the sugar present to the organic acid will reduce the pH of kombucha tea during the fermentations process (Amarasinghe et al. 2018; Sreeramulu et al. 2000).

Other factors that influenced the rate of fermentation was the starter culture used. In this study, kombucha tea prepared using KLB has a higher fermentation rate compared to KCP. This difference is caused by the different microbial compositions between both starter cultures used (Watawana et al. 2017) where KLB produced more acetic acid thus lowering the pH. Different types of tea also have been reported to influence the

acidity of the fermented kombucha tea (Coton et al. 2017). All cultures show saturation points after 30 days of fermentation where the pHs were in the range of 2.8 to 3.8 where such values are within the range considered safe for human consumption, pH 2.5 to 4.2 (Nummer 2013). Values below pH 2.5 pose a health risk to the consumers since it has a high concentration of acetic acid, while, pH values greater than 4.2 can compromise the beverage's microbiological safety (Cardoso et al. 2020).

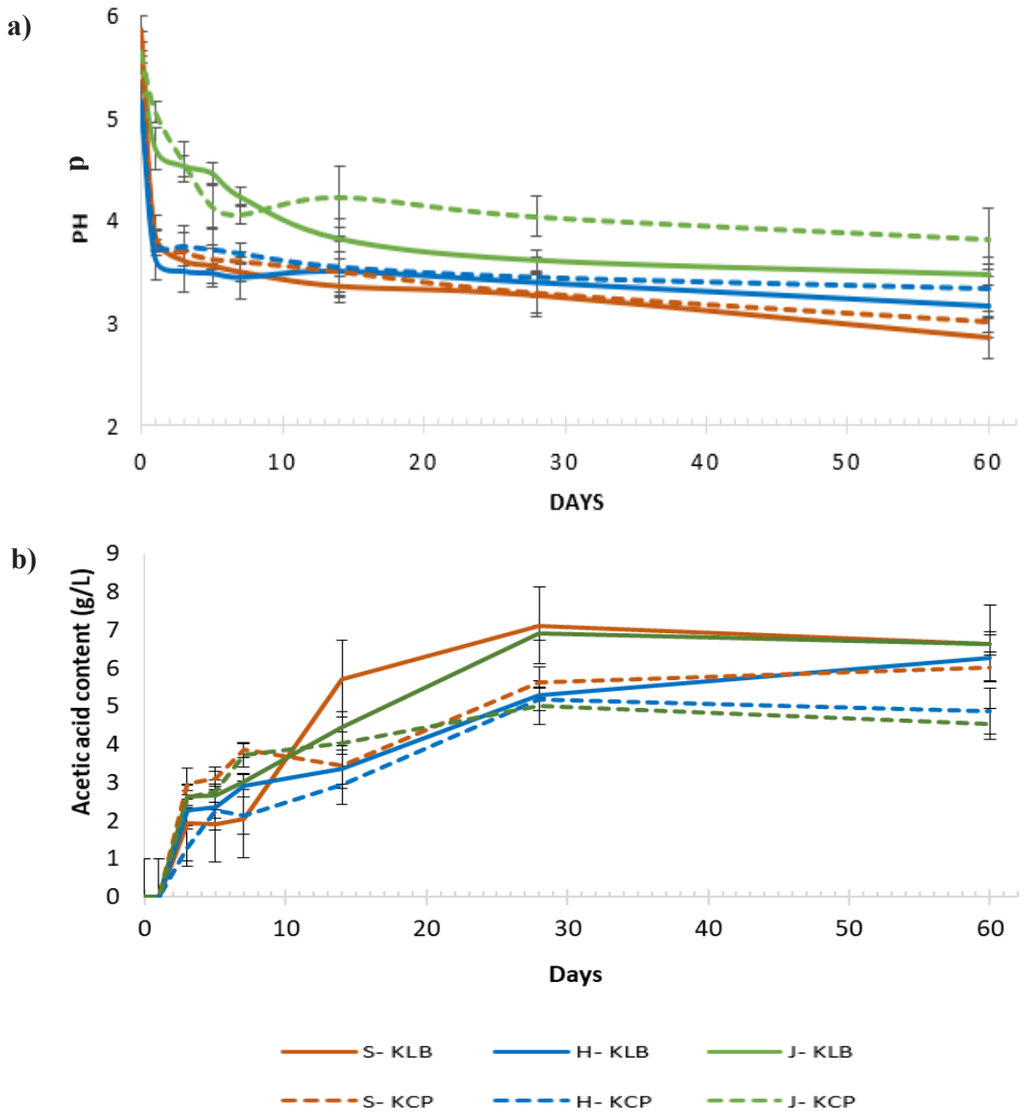

FIGURE 1. Changes occur to Kombucha tea prepared by using different sweeteners (S: white sugar, H: honey, J: jaggery) and starter cultures (KLB or KCP) during fermentation: a) pH changes; and b) acetic acid content

ETHANOL CONTENT

During the fermentation process, three products were produced: ethanol; organic acid such as acetic acid or gluconic acid; and cellulose mat (Greenwalt et al. 2000). The ethanol content of kombucha was measured to better understand how the difference in starter cultures and sweeteners will affect kombucha tea composition. Ethanol is one of the by-products in the fermentation process that is very important to monitor as it is seen as a health hazard. We hypothesized that the production of ethanol was dependent on the starter culture used. Figure 2 shows the changes in ethanol contents during the fermentation process of the kombucha tea.

The starter culture used, significantly affects the production of the ethanol during the fermentation process. Kombucha tea prepared using KCP had a higher ethanol content at the beginning of fermentation and decreased with prolonged fermentation time (Figure 2(a)), while samples prepared using KLB had a lower ethanol content at the beginning and increased over time. The ethanol content of kombucha prepared by using KLB generally start to increase significantly on the 14th day while the sample prepared by using KCB decreased on the 14th day.

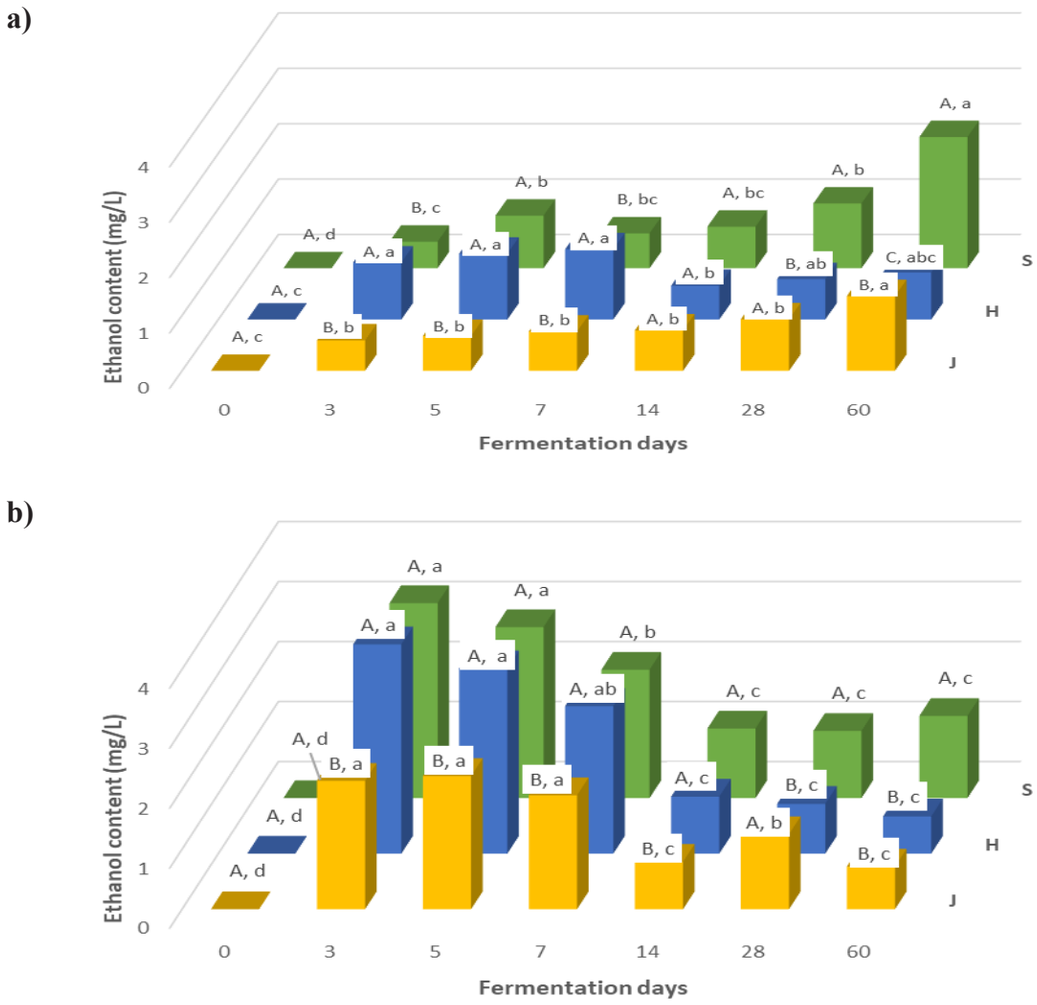

FIGURE 2. Changes in the ethanol content of the kombucha tea prepared by using a) KLB; and b) KCP, respectively, in different sweeteners: white sugar (S), honey (H) and jaggery (J). The different letter indicates significant different ($P<0.05$): capital letters for the differences between S, H and J; small letters for the differencess between the fermentation days

During the fermentation process, sugar is broken down to alcohol by the yeasts which the produced alcohol is converted to acetic acid by the bacteria. This process occurs simultaneously and the amount of product produced is highly dependent on the microbial concentration. As the distribution of the microorganism in the KCP is very complex and dense through the cellulosic network, it has more microorganisms especially various yeast species that work symbiotically with bacteria during fermentation (Greenwalt et al. 2000) compared to the KLB which only has free-floating microbes. Glucose is primarily used up by the yeasts to yield ethanol and carbon dioxide. The ethanol is then oxidized by the bacteria to acetaldehyde and then to acetic acid. As a result, the alcohol content of kombucha is reduced and the acetic acid concentrations may rise to higher levels as the fermentation is prolonged as demonstrated in Figure 1(b) previously.

Between the three sweeteners used, the highest ethanol content was produced when honey (3.49 ± 0.2 mg/L) was used, followed by sucrose (3.25 ± 0.3 mg/L) and jaggery (2.14 ± 0.2 mg/L). These difference is due to the difference in the composition of the sweetener used. Sugar plays important role in microbial growth as it provide the carbon source needed (Quiao-Won & Teves 2018). Compared to white sugar, Kelulut honey has high maltose content besides other simple sugars such as glucose and sucrose. When all the simple sugar was converted into alcohol in the first 7 days, the yeast has difficulties in hydrolysing the remaining maltose, thus producing less ethanol for the rest of the fermentation period.

ANTIOXIDANT ACTIVITY

One of the reported beneficial properties of kombucha tea is antioxidant activity (Chu & Chen 2006; Fu et al. 2014; Lobo, Dias & Shenoy 2017). Figure 3 shows the antioxidant activity of kombucha tea prepared by using different starter cultures and media. Results show that

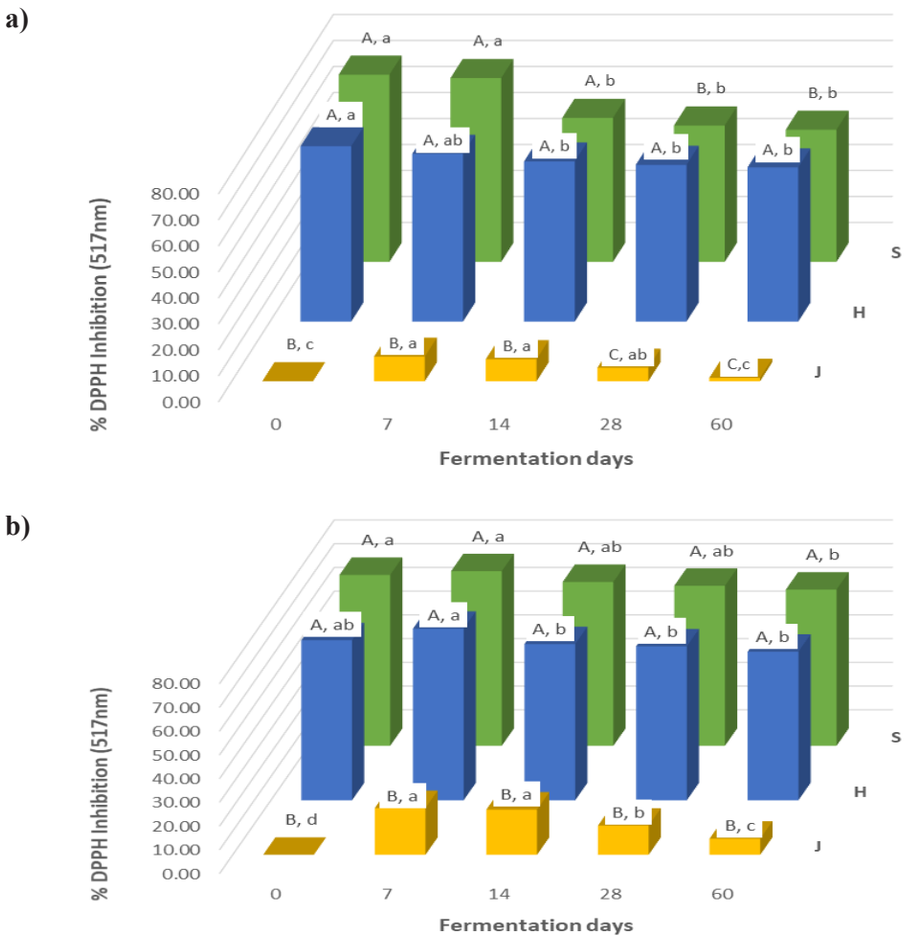

FIGURE 3. The % of DPPH inhibition of kombucha tea prepared by using a) KLB; or b) KCP, respectively, in different sweeteners: white sugar (S), honey (H) and jaggery (J). The different letter indicates significant different ($P<0.05$): capital letters for the differences between S, H and J; small letters for the differences between the fermentation days

the antioxidant activity of kombucha tea decreased as the fermentation was prolonged. There are no significant differences in antioxidant activity when either white sugar or honey is used, but kombucha tea prepared by using jaggery show the lowest antioxidant activity. A similar result has been reported by Watawana et al. (2017), who found that kombucha tea prepared using jaggery showed the lowest antioxidant activity (12500 umol TE/mL) compared to other sweeteners (>12500 umol TE/mL, respectively, for bees' honey, brown sugar, glucose , Caryota urens honey, sucrose and white sugar). The samples prepared by using KCP have higher antioxidant activity (62% - 73% of DPPH Inhibition) compared to KLB (50% - 72% of DPPH Inhibition). It is well known that the antioxidant properties of kombucha were contributed by the black tea used which contains natural antioxidants mainly polyphenols (Malbaša et al. 2011). One of them is catechins which belong to the flavanols group that could donate hydroxyl hydrogen due to stabilisation and it is effective in removing free radicals by playing a preventive role in the oxidative changes of the substrate (Gramza-Michalowska et al. 2016).

Kombucha fermentation significantly reduced the antioxidant properties of the teas due to the biodegradation of tea catechins by the enzymes secreted by the SCOBY consortium (Jayabalan, Marimuthu & Swaminathan 2007). The previous study has proven that tea fermented without the presence of sugar has higher antioxidants even though fermented at the same period due to low microbial metabolism even using the same starter culture (Vohra et al. 2019b). In addition, unwanted metabolites might be formed as the fermentation was prolonged (Watawana et al. 2017).

Although the antioxidant activity of Kombucha is linked to many of its claimed health benefits, including cancer prevention, immunity enhancement, and alleviation of inflammation and arthritis (Ahmed et al. 2020; Malbaša et al. 2011; Vohra et al. 2019b), these results indicated that the source of sweetener is a very important element that influence the variation in the antioxidant capacity of the beverage. While jaggery has been reported to have higher antioxidants, this study shows kombucha tea prepared by using jaggery has the lowest antioxidant activity. These different results may be due to the quality of the jaggery used. Several factors have been reported to influent the antioxidant properties of jaggery such as refining process, processing temperature and storage condition (Chand et al. 2011; Singh 2013).

ANTIBACTERIAL ACTIVITY

Generally, kombucha is known to exhibit antimicrobial activity against a variety of common pathogenic bacteria either Gram-positive/negative bacteria or yeasts (Chakravorty et al. 2016; Deghrigue et al. 2013; Quiao-Won & Teves 2018; Sreeramulu et al. 2000). The antibacterial activities of kombucha tea studied against some pathogenic microorganisms are shown in Table 1. The results showed that the use of starter cultures and different sweeteners would significantly affect the antibacterial activity of the kombucha tea produced. All samples show no antimicrobial activities after 7 days of fermentation (data not shown) but prolonged fermentation increase the antibacterial activity. This suggests that the active antibacterial components are very likely metabolites produced by the bacteria and/or yeasts responsible for the fermentation of kombucha.

The results showed that kombucha tea prepared using white sugar as a carbon source has a higher antimicrobial activity compared to the kombucha tea prepared by using honey and jaggery. It has high susceptibility to almost all pathogenic bacteria tested. The use of KLB as a starter culture produced a broth that had antimicrobial activity as earlier as 14 days compared to the KCP which mostly showed no antibacterial activity at 14 days of fermentation. Compared to samples prepared using KLB showed antibacterial activity against all microbes tested, samples prepared using KCP did not show antibacterial activity against *S. marecens* even after 60 days of fermentation. However, it showed higher antibacterial activity against *S. aureus* and *P. aeruginosa* (19 mm and 29 mm inhibition zones at 28 days, respectively). Although honey naturally has antibacterial properties (da Silva et al. 2013), kombucha tea prepared using honey has a low susceptibility to *B. subtilis*. Similar to honey, samples prepared using jaggery showed generally low antimicrobial activity.

This study shows that the antibacterial properties of kombucha tea are the result of microbial metabolic activity. The significant differences in microbial inhibition are possibly due to the difference in acidity when the different sweeteners and starter cultures are used. Both factors will determine the available microbial bioactivity. Sweeteners contain different composition of carbohydrates to be utilized as the carbon sources by various microbes in the starter cultures. For example, *Acetobacteraceae* bacteria are known for their ability to partially oxidise a variety of carbohydrates and to release the corresponding metabolites such as aldehydes, ketones and many organic acids including acetic acids

TABLE 1. Effect of fermentation period on antibacterial activity of kombucha tea broth inoculate by using KLB and KCP respectively in different media

			Inhibition zone (mm)*				
Sample		Days	*B. Subtilis*	*E.Coli*	*S.aureus*	*S.marecen*	*P.aeruginosa*
Sugar							
	KLB	14	0^{b}	9 ± 2^{b}	11 ± 2^{b}	15 ± 2^{b}	0^{c}
		28	7 ± 2^{a}	13 ± 2^{b}	11 ± 1^{b}	19 ± 2^{a}	11 ± 2^{b}
		60	9 ± 1^{a}	21 ± 3^{a}	15 ± 2^{a}	21 ± 1^{a}	17 ± 2^{a}
	KCP	14	0^{b}	0^{b}	0^{c}	0^{a}	0^{b}
		28	7 ± 2^{a}	0^{b}	19 ± 3^{b}	0^{a}	29 ± 3^{a}
		60	7 ± 1^{a}	7 ± 2^{a}	25 ± 2^{a}	0^{a}	33 ± 2^{a}
Honey							
	KLB	14	0^{a}	0^{b}	0^{b}	9 ± 1^{b}	0^{b}
		28	0^{a}	9 ± 1^{a}	9 ± 1^{a}	11 ± 2^{ab}	9 ± 1^{a}
		60	0^{a}	11 ± 1^{a}	9 ± 2^{a}	13 ± 1^{a}	11 ± 1^{a}
	KCP	14	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}
		28	0^{b}	0^{b}	9 ± 1^{a}	33 ± 3^{a}	17 ± 2^{a}
		60	7 ± 2^{a}	15 ± 2^{a}	9 ± 2^{a}	1 ± 1^{b}	19 ± 1^{a}
Jaggery							
	KLB	14	0^{c}	0^{b}	0^{b}	0^{b}	13 ± 1^{b}
		28	9 ± 1^{b}	0^{b}	21 ± 1^{a}	0^{b}	17 ± 1^{a}
		60	11 ± 1^{a}	7 ± 2^{a}	21 ± 1^{a}	7 ± 1^{a}	17 ± 2^{a}
	KCP	14	0^{b}	0^{b}	0^{b}	0^{b}	0^{c}
		28	7 ± 1^{a}	0^{b}	7 ± 2^{a}	21 ± 2^{a}	33 ± 1^{b}
		60	7 ± 2^{a}	7 ± 2^{a}	9 ± 2^{a}	18 ± 1^{a}	37 ± 2^{a}

*Data are the mean of three repetitions ± SD (The value was rounded to the true number). Different letters indicate significant differences between samples according to Tukey test ($p \leq 0.05$)

into the media in turn lowering the pH of kombucha tea (Mamlouk & Gullo 2013). It can be observed in the previous discussion (Figure 1(b)) sample prepared by using sugar have higher acetic acid content compared to the sample prepared by using jaggery. The increase in acetic acid concentration along the fermentation period also increased antibacterial activity. Acetic acid is considered to be responsible for the inhibitory effect on a number of microbes tested (Cardoso et al. 2020; Sreeramulu et al. 2000), thus, it is not surprising that the sample prepared by using sugar inhibited a higher number of pathogenic bacteria. An undissociated molecule of the acid could enter the bacterial cell and the dissociation would take place in the cytoplasm and inhibit the cell

growth. However, the increase in acidity might alter the activity of the biologically available compound in honey such as protein or enzyme that is very sensitive and require specific pH to be active (Battikh et al. 2012). This may explain the low antibacterial properties even though honey has its own antibacterial active compound. Finally, the simultaneous and synergic effect of ethanol and acetic acid production by yeast and bacteria, respectively, prevents the competition of other microorganisms. This relationship illustrates the defined level of symbiosis and compatibility between the organisms in the tea colony (Greenwalt et al. 2000).

TABLE 2. Taxonomic classification of the predominant bacteria and yeast in 7 days old liquid broth (KLB) and cellulosic pellicle (KCP) growth in black tea prepared by using white sugar

	KLB		KCP	
	Species Name	%	Species Name	%
Bacteria	*Acetobacteracea*	97	*Acetobacteracea*	100
	Gluconobacter	1		
	Bacillus sp. *(unidentified)*	1		
	B. ginsenghihumi	0.50		
	B. flexus	0.20		
	A. chiangmaiensis	0.02		
	B. firmus	0.01		
Yeast	*Aspergillus fumigatus*	23	*Zygosaccharomyces bisporus*	25
	Penicillium	15	*Phlebia setulosa*	7
	Candida etchellsii	7	*Polyporales*_sp.	6
	Auricularia mesenterica	5	*Fomitopsis meliae*	6
	Lenzites warneiri	4	*Ascomycota*_sp.	6
	Kodamaea ohmeri	4	*Eurotiales* sp.	5
	Mallasezia restricta	3	*Sterigmatomyces halophilus*	5
	Zygosaccharmomyces bisporus	3	*Theleporus membranaceus*	5
	Hymenochaete sphaerospora	3	*Mallasezia restricta*	3
	Uwebraunia musea	3	*Bipolaris microstegii*	3
	Marasmius rotula	2	*Dothideomycetes*	2
	Russulates sp.	2	*Hannaela luteola*	2
	A.penicilloides	2	*Coprinopsis_insignis*	2
	Resinicium sachharicola	2	*Cruentomycena_kedrovaya*	2
	Agaricomycetes sp.	2	*Paramicrosporidium_saccamoebae*	2
	Hypochnium punctulatum	2	Others <1%	19
	Trichoderma asperullum	2		
	Rhodotorula calyptogenae	2		
	Others <1%	14		

DIVERSITY OF MICROBIAL POPULATION IN KOMBUCHA

As the antimicrobial properties of samples prepared by using sugar show the highest antioxidant and antimicrobial activity, metagenomic analysis was conducted on the 7 days samples to identify the microbial species diversity of the KLB and KCP where the results are shown in Table 2. For the classification of bacteria, it was found that only *Acetobacteraceae* is present in KCP compared to KLB which also contains 3% of other bacteria. These results are consistent with previous studies reporting the predominance of *Acetobacteraceae* in kombucha tea (Arıkan et al. 2020; Laavanya et al. 2021). *Acetobacteraceae,* especially *Acetobacter xylinum* has a very important function in kombucha tea where it serves to produce cellulose pellicle during fermentation (Al-Kalifawi 2014). Although *Gluconobacter* is also reported to be the bacterium that dominates kombucha tea (Laureys et al. 2020; Quiao-Won & Teves 2018), only 1% is found in KLB while none in KCP.

Yeasts also play an important role during fermentation where it produces alcohol and other volatile compounds that enhance the aroma, the general sensory quality and the functional properties of fermented beverages (Tu et al. 2019). Usually, the yeast component generally includes *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *B. lambicus*, *B. custersii*, Candida, and Pichia species (Quiao-Won & Teves 2018). In this study, 7 days old starter cultures have significantly different predominant yeast compositions where KLB is predominated by *Aspergillus fumigatus* (23%) while KCP is predominated by *Zygosaccharomyces bisporus* (25%). While *Zygaosaccaromyces* sp. was considered a common yeast present in kombucha tea during fermentation, *Aspergillus fumigatus* and *Penicilium* are mostly known as the common opportunistic fungus that presents as a contaminant in food fermentation and the brewing process (Gyllang & Martinson 1976; Nazemi et al. 2019).

Although *Aspergillus* spp. has been known as contaminated during fermentation, it has been reported to give unique characteristics, especially towards the sensory benefits of beverages. It has been reported to produce many metabolic enzymes such as tannase, laccase and vanillyl-alcohol oxidase that can catalyse the conversion and degradation of certain phenolic compounds including (±)-catechin, gallic acid, quercetin and produced unique flavour during tea fermentation (Jin et al. 2020; Ma et al. 2021). This later will increase beneficial bioactive compounds such as theabrownins that contribute to decreasing astringent taste, increasing mellow flavour and promoting the health benefits of the fermented beverages (Ma et al. 2021; Wang et al. 2014). Theabrownin has been shown to boost ileal conjugated bile acids (BAs), which in turn, block the intestinal FXR-FGF15 signalling pathway, leading to increased hepatic BA synthesis and faecal excretion, lower hepatic cholesterol, and reduced lipogenesis (Huang et al. 2019). Since the KLB used for this metagenomic analysis is only 7 days old broth in which cellulosic pellicle layer is still underdeveloped, we speculate that the growth of *Aspergillus fumigatus* will be suppressed and controlled as the fermentation period is prolonged. According to a previous study, kombucha tea can exert an inhibitory effect against the growth of *A. fumigatus* strains after prolong fermentation (Nazemi et al. 2019).

KOMBUCHA BIOCHEMICAL COMPOSITION

To know in more detail about the biochemical changes in the fermented kombucha tea, the 14 days old black tea and S-KLB sample composition were analyzed. The composition of those black teas and kombucha tea were presented in Table 3. Uninoculated black tea that has been fermented for 14 days had a total of 11 identified compounds while for 14 days fermented S-KLB has 25 identified compounds. These compounds are composed of sugar, protein, enzyme, phenolic, flavonoid, and antibiotic. Among 11 compounds identified in black tea broth, only 1 compound namely (S)-2,3-Dihydro-3,5-dihydroxy-2-oxo-3-indoleacetic acid 5-[glucosyl-(1->4)-b-D-glucoside] was not identified in S-KLB. Fermented kombucha 14 days old seems to have various metabolites that have antimicrobial properties as well as acids that cause the pH to decrease (Vohra et al. 2019a). This test proved that the fermentation process produces new compounds as a result of the metabolic activity of the microbial colonies present in kombucha tea. It appears that 15 metabolites such as simple sugars, protein, fatty acids, phenolic, and flavonoids were produced. Interestingly, among these 15 compounds, in addition to phenolic compounds and flavonoids that have been known to contribute to antioxidant and antimicrobial properties, there is a natural antibiotic also present, which is thiolactomycin.

Thiolactomycin is a potent and highly selective inhibitor of type II dissociated fatty acid synthases of plants and bacteria (Brown et al. 2003). It is a natural product that is commonly produced by both *Nocardia* and

Streptomyces spp. (Oishi et al. 1982). The unique mode of action of thiolactomycin and its low toxicity makes it an attractive compound for the development of new antimicrobial agents. The presence of Thiolactomycin is likely to be a major factor contributing to the antimicrobial properties of S-KLB. However, *Nocardia* or *Streptomyces* spp. was not detected by using metagenomic analysis on the KLB and KCP sample. Therefore, the further characterization needs to be done to identify and confirm the formation or presence of these natural antibiotics.

TABLE 3. Chemical compounds present in kombucha tea after 7 days of fermentation prepared by using white sugar (S) and liquid broth (KLB)

		*Availability	
	Chemical compounds	**Control	S-KLB
1.	(S)-2,3-Dihydro-3,5-dihydroxy-2-oxo-3-indoleacetic acid 5-[glucosyl-(1->4)-b-D-glucoside]	+	
2.	(2R,3S)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (Catechin)	+	+
3.	Caffeine	+	+
4.	DL-pipecolic acid	+	+
5.	Fructose	+	+
6.	Glucose	+	+
7.	Kaempferol 3-neohesperidoside-7-(2”-p-coumaryllaminaribioside)	+	+
8.	Nigerose (Sakebiose)	+	+
9.	Oleamide	+	+
10.	Sucrose	+	+
11.	Tetradecylamine	+	+
12.	2-Dehydro-3-deoxy-L-rhamnonate		+
13.	4,4’-Biphenyldithiol		+
14.	7-Hydroxy-2-methyl-4-oxo-4H-1-benzopyran-5-carboxylic acid 7- glucoside		+
15.	Aminocaproic acid		+
16.	b-D-Glucopyranosiduronic acid		+
17.	Docosanedioic acid		+
18.	Ethyl glucuronide		+
19.	His Pro		+
20.	L-Galactose		+
21.	Methyl 6-O-galloyl-beta-D-glucopyranoside		+
22.	Muramic acid		+
23.	Mycosporine		+
24.	N-Acetyl-D-mannosaminolactone		+
25.	Piperonal		+
26.	Pro Leu		+
27.	Thiolactomycin		+

* + sign indicate the present of the chemical compounds in the sample. **Control used was 7 days old prepared black tea

Conclusion

The selection of starter cultures and sweeteners to be used in the preparation of kombucha tea is a very important step. It was found that the samples prepared using KLB had a higher acetic acid content and a higher alcohol content after 60 days of fermentation compared to the samples prepared using KCP. Samples prepared using white sugar or honey showed similar antioxidant activity despite using different starting cultures. However, all samples prepared using jaggery showed very low antioxidant activity. All samples have antimicrobial activity but at a different level with KLB-S showing the highest antimicrobial activity. Metagenomic analysis of the starter culture showed that it was both dominated by *Acetobacteracea* bacteria, and yeast: *Aspergillus fumigatus* in KLB; and *Zygosaccharomyces bisporus* in KCP. Fermentation significantly changed the black tea composition. When 14 days of fermented KLB-S broth was subjected to bioactive compound determination, 26 compounds were identified compared to 11 compounds for 14 days old black tea. The presence of thiolactomycin, a natural antibiotic is likely to contribute to the highest antimicrobial activity of KLB-S. How thiolactomycin is produced in this fermentation is interesting to know. Therefore, further characterization is essential for identifying changes in chemical compounds formed during the fermentation process, and for understanding the transformation mechanisms and possible structural formation of new compounds.

Acknowledgements

This study was supported by Universiti Kebangsaan Malaysia, grant no. GUP-2017-053 and DCP-2018-006/1. This article is composed of our original work and contains no material previously published or written by another person except where due reference has been made in the text. The authors declare that they have no competing interests.

References

Ahmed, R.F., Hikal, M.S. & Abou-Taleb, K.A. 2020. Biological, chemical and antioxidant activities of different types Kombucha. *Annals of Agricultural Sciences* 65(1): 35-41.

Al-Kalifawi, E.J. 2014. Produce bacterial cellulose of kombucha (Khubdat Humza) from honey. *Journal of Genetic and Environmental Resources Conservation* 2(1): 39-45.

Amarasinghe, H., Weerakkody, N.S. & Waisundara, V.Y. 2018. Evaluation of physicochemical properties and antioxidant activities of kombucha "Tea Fungus" during extended periods of fermentation. *Food Science and Nutrition* 6(3): 659-665.

Arıkan, M., Mitchell, A.L., Finn, R.D. & Gürel, F. 2020. Microbial composition of Kombucha determined using amplicon sequencing and shotgun metagenomics. *Journal of Food Science* 85(2): 455-464.

Battikh, H., Bakhrouf, A. & Ammar, E. 2012. Antimicrobial effect of Kombucha analogues. *LWT - Food Science and Technology* 47(1): 71-77.

Nummer, B.A. 2013. Kombucha brewing under the food and drug administration model food code: Risk analysis and processing guidance abstract. *Journal of Environmental Health* 76(4): 8-11.

Brown, M.S., Akopiants, K., Resceck, D.M., McArthur, H.A.I., McCormick, E. & Reynolds, K.A. 2003. Biosynthetic origins of the natural product, thiolactomycin: A unique and selective inhibitor of type II dissociated fatty acid synthases. *Journal of the American Chemical Society* 125(34): 10166-10167.

Cardoso, R.R., Neto, R.O., dos Santos D'Almeida, C.T., do Nascimento, T.P., Pressete, C.G., Azevedo, L., Martino, H.S.D., Cameron, L.C., Ferreira, M.S.L. & de Barros, F.A.R. 2020. Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. *Food Research International* 128: 108782.

Chakravorty, S., Bhattacharya, S., Chatzinotas, A., Chakraborty, W., Bhattacharya, D. & Gachhui, R. 2016. Kombucha tea fermentation: Microbial and biochemical dynamics. *International Journal of Food Microbiology* 220: 63-72.

Chand, K., Shahi, N.C., Lohani, U.C. & Garg, S.K. 2011. Effect of storage conditions on keeping qualities of jaggery. *Sugar Tech* 13(1): 81-85.

Chen, C. & Liu, B.Y. 2000. Changes in major components of tea fungus metabolites during prolonged fermentation. *Journal of Applied Microbiology* 89(5): 834-839.

Chu, S.C. & Chen, C. 2006. Effects of origins and fermentation time on the antioxidant activities of kombucha. *Food Chemistry* 98(3): 502-507.

Coton, M., Pawtowski, A., Taminiau, B., Burgaud, G., Deniel, F., Coulloumme-Labarthe, L., Fall, A., Daube, G. & Coton, E. 2017. Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiology Ecology* 93(5): 1-16.

da Silva, I.A.A., da Silva, T.M.S., Camara, C.A., Queiroz, N., Magnani, M., de Novais, J.S., Soledade, L.E.B., de Oliveira Lima, E., de Souza, A.L. & de Souza, A.G. 2013. Phenolic profile, antioxidant activity and palynological analysis of stingless bee honey from Amazonas, Northern Brazil. *Food Chemistry* 141(4): 3552-3558.

De Filippis, F., Troise, A.D., Vitaglione, P. & Ercolini, D. 2018. Different temperatures select distinctive acetic acid bacteria species and promotes organic acids production during Kombucha tea fermentation. *Food Microbiology* 73: 11-16.

De Roos, J. & De Vuyst, L. 2018. Acetic acid bacteria in fermented foods and beverages. *Current Opinion in Biotechnology* 49: 115-119.

Deghrigue, M., Chriaa, J., Battikh, H. & Abid, K. 2013. Antiproliferative and antimicrobial activities of kombucha tea. *African Journal of Microbiology Research* 7(27): 3466-3470.

Dufresne, C. & Farnworth, E. 2000. Tea, Kombucha, and health: A review. *Food Research International* 33: 409-421.

Fu, C., Yan, F., Cao, Z., Xie, F. & Lin, J. 2014. Antioxidant activities of kombucha prepared from three different substrates and changes in content of probiotics during storage. *Food Science and Technology* (Campinas) 34(1): 123-126.

Gramza-Michalowska, A., Kulczynski, B., Xindi, Y. & Gumienna, M. 2016. Research on the effect of culture time on the kombucha tea beverage's antiradical capacity and sensory value. *Acta Scientiarum Polonorum, Technologia Alimentaria* 15(4): 447-457.

Greenwalt, C.J., Steinkraus, K.H. & Ledford, R.A. 2000. Kombucha, the fermented tea: Microbiology, composition, and claimed health effects. *Journal of Food Protection* 63(7): 976-981.

Gyllang, H. & Martinson, E. 1976. *Aspergillus fumigatus* and *Aspergillus amstelodami* as causes of gushing. *Journal of the Institute of Brewing* 82(3): 182-183.

Huang, F., Zheng, X., Ma, X., Jiang, R., Zhou, W., Zhou, S., Zhang, Y., Lei, S., Wang, S., Kuang, J., Han, X., Wei, M., You, Y., Li, M., Li, Y., Liang, D., Liu, J., Chen, T., Yan, C., Wei, R., Rajani, C., Shen, C., Xie, G., Bian, Z., Li, H., Zhao, A. & Jia, W. 2019. Theabrownin from Pu-erh tea attenuates hypercholesterolemia via modulation of gut microbiota and bile acid metabolism. *Nature Communications* 10(1): 4971.

Ivanišová, E., Meňhartová, K., Terentjeva, M., Harangozo, Ľ., Kántor, A. & Kačániová, M. 2020. The evaluation of chemical, antioxidant, antimicrobial and sensory properties of kombucha tea beverage. *Journal of Food Science and Technology* 57(5): 1840-1846.

Jagannadha Rao, P.V.K., Das, M. & Das, S.K. 2010. Effect of moisture content on glass transition and sticky point temperatures of sugarcane, palmyra-palm and date-palm jaggery granules. *International Journal of Food Science and Technology* 45(1): 94-104.

Jayabalan, R., Marimuthu, S. & Swaminathan, K. 2007. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chemistry* 102(1): 392-398.

Jayabalan, R., Subathradevi, P., Marimuthu, S., Sathishkumar, M. & Swaminathan, K. 2008. Changes in free-radical scavenging ability of kombucha tea during fermentation. *Food Chemistry* 109(1): 227-234.

Jin, X., Song, J. & Liu, G-Q. 2020. Bioethanol production from rice straw through an enzymatic route mediated by enzymes developed in-house from *Aspergillus fumigatus*. *Energy* 190: 116395.

Keshk, S.M.A.S. & Sameshima, K. 2005. Evaluation of different carbon sources for bacterial cellulose production. *African Journal of Biotechnology* 4(6): 478-482.

Laavanya, D., Shirkole, S. & Balasubramanian, P. 2021. Current challenges, applications and future perspectives of SCOBY cellulose of Kombucha fermentation. *Journal of Cleaner Production* 295: 126454.

Laureys, D., Britton, S.J. & De Clippeleer, J. 2020. Kombucha tea fermentation: A review. *Journal of the American Society of Brewing Chemists* 78(3): 165-174.

Lobo, R., Dias, F. & Shenoy, C. 2017. Kombucha for healthy living: Evaluation of antioxidant potential and bioactive compounds. *International Food Research Journal* 24(2): 541-546.

Ma, Y., Ling, T-J., Su, X-Q., Jiang, B., Nian, B., Chen, L-J., Liu, M., Zhang, Z-Y., Wang, D-P., Mu, Y-Y., Jiao, W-W., Liu, Q-T., Pan, Y-H. & Zhao, M. 2021. Integrated proteomics and metabolomics analysis of tea leaves fermented by *Aspergillus niger, Aspergillus tamarii* and *Aspergillus fumigatus*. *Food Chemistry* 334: 127560.

Malbaša, R.V., Lončar, E.S., Vitas, J.S. & Čanadanović-Brunet, J.M. 2011. Influence of starter cultures on the antioxidant activity of kombucha beverage. *Food Chemistry* 127(4): 1727-1731.

Mamlouk, D. & Gullo, M. 2013. Acetic acid bacteria: Physiology and carbon sources oxidation. *Indian Journal of Microbiology* 53(4): 377-384.

Marsh, A.J., O'Sullivan, O., Hill, C., Ross, R.P. & Cotter, P.D. 2014. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. *Food Microbiology* 38: 171-178.

Nazemi, L., Hashemi, S.J., Daie Ghazvini, R., Saeedi, M., Khodavaisy, S., Barac, A., Modiri, M., Akbari Dana, M., Zare Shahrabadi, Z. & Rezaie, S. 2019. Investigation of *cgrA* and *cyp51A* gene alternations in *Aspergillus fumigatus* strains exposed to kombucha fermented tea. *Current Medical Mycology* 5(3): 36-42.

Oishi, H., Noto, T., Sasaki, H., Suzuki, K., Hayashi, T., Okazaki, H., Ando, K. & Sawada, M. 1982. Thiolactomycin, a new antibiotic: I. taxonomy of the producing organism, fermentation and biological properties. *The Journal of Antibiotics* 35(4): 391-395.

Quiao-Won, M.E. & Teves, F.G. 2018. Characteristics of kombucha fermentation from different substrates and cytotoxicity of tea broth. *Sustainable Food Production* 4: 11-19.

Sievers, M., Lanini, C., Weber, A., Schuler-Schmid, U. & Teuber, M. 1995. Microbiology and fermentation balance in a kombucha beverage obtained from a tea fungus fermentation. *Systematic and Applied Microbiology* 18(4): 590-594.

Singh, J. 2013. Manufacturing jaggery, a product of sugarcane, as health food. *Agrotechnology* 01(S11): 10-12.

Soares, M.G., de Lima, M. & Reolon Schmidt, V.C. 2021. Technological aspects of kombucha, its applications and the

symbiotic culture (SCOBY), and extraction of compounds of interest: A literature review. *Trends in Food Science and Technology* 110(May 2020): 539-550.

Sreeramulu, G., Zhu, Y. & Knol, W. 2001. Characterization of antimicrobial activity in kombucha fermentation. *Acta Biotechnologica* 21(1): 49-56.

Sreeramulu, G., Zhu, Y. & Knol, W. 2000. Kombucha fermentation and its antimicrobial activity. *Journal of Agricultural and Food Chemistry* 48(6): 2589-2594.

Sumbhate, S.V., Nayak, S., Goupale, D., Tiwari, A. & Jadon, R.S. 2012. Colorimetric method for the estimation of ethanol in alcoholic drinks. *Journal of Analytical Techniques* 1(1): 1-6.

Tu, C., Azi, F., Huang, J., Xu, X., Xing, G. & Dong, M. 2019. Quality and metagenomic evaluation of a novel functional beverage produced from soy whey using water kefir grains. *Lwt* 113(February): 108258.

Veena, K.S., Sameena, M.T., Padmakumari, A.K.P., Srinivasa, G.T.K., Nishanth, K.S. & Reshma, M.V. 2018. Development and validation of HPLC method for determination of sugars in palm sap, palm syrup, sugarcane jaggery and palm jaggery. *International Food Research Journal* 25(2): 649-654.

Villarreal-Soto, S.A., Beaufort, S., Bouajila, J., Souchard, J.P. & Taillandier, P. 2018. Understanding kombucha tea fermentation: A review. *Journal of Food Science* 83(3): 580-588.

Vitas, J.S., Vukmanović, S.Z., Malbaša, R.V. & Tepić Horecki, A.N. 2019. Influence of process temperature on ethanol content in Kombucha products obtained by fermentation of flotated must effluent. *Acta Periodica Technologica* 50: 311-315.

Vohra, B., Fazry, S., Sairi, F. & Othman, B.A. 2019a. Effects of medium variation and fermentation time towards the pH level and ethanol content of Kombucha. *AIP Conference Proceedings* 2111(1998): 298-302.

Vohra, B., Fazry, S., Sairi, F. & Othman, B.A. 2019b. Effects of medium variation and fermentation time on the antioxidant and antimicrobial properties of Kombucha. *Malaysian Journal of Fundamental and Applied Sciences* 15(2-1): 298-302.

Wang, Q., Gong, J., Chisti, Y. & Sirisansaneeyakul, S. 2014. Bioconversion of tea polyphenols to bioactive theabrownins by *Aspergillus fumigatus*. *Biotechnology Letters* 36(12): 2515-2522.

Watawana, M.I., Jayawardena, N., Ranasinghe, S.J., Waisundara, V.Y., Gunawardhana, C.B., Waisundara, V.Y., Ranasinghe, S.J. & Waisundara, V.Y. 2017. Evaluation of the effect of different sweetening agents on the polyphenol contents and antioxidant and starch hydrolase inhibitory properties of Kombucha. *Journal of Food Processing and Preservation* 41(1): e12752.

Williams, M.B. & Darwin Reese, H. 1950. Colorimetric determination of ethyl alcohol. *Analytical Chemistry* 22(12): 1556-1561.

*Corresponding author; email: airianah@ukm.edu.my