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The effect of fermentation time on the development and antioxidant activity of kombucha from different starter cultures in Malaysia

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Abstract Popularity of kombucha has increased significantly in recent years due to growing interest in its beneficial health effects. Result revealed that across the six kombucha starter cultures, pH decreased value throughout the fermentation, reaching 2.49 to 3.00 by day 30. The beverages became increasingly turbid, while their surface pellicles thickened over time and differed in colour and texture between starter cultures. Total phenolic content was generally higher than total flavonoid content. A positive correlation was noted between total phenolic, total flavonoid and antioxidant activity of each kombucha sample; however, no definite trend could be deduced from the bioactive compound analyses. Both measures fluctuated during fermentation, as did ferric reducing antioxidant power, with the patterns varying among cultures. These findings are indicated that starter culture affects pellicle characteristics and bioactive profiles, while fermentation duration alone does not reliably predict antioxidant activity. They provide a basis for selecting starter cultures and fermentation conditions to achieve desired kombucha characteristics.

Keywords: Kombucha, Fermentation time, Antioxidant, Development

Introduction

Natural sources, such as fruit and tea are rich in antioxidant compounds, and these compounds may help prevent or treat diseases by protecting against ROS-mediated damage (Halliwell, 2023). Phenolic compounds are secondary metabolites found in plants and plant-based products, contributing to their antioxidant activity (Rajashekar and Rajashekar, 2023). Both phenolic and non-phenolic compounds contribute to antioxidant strength, as they reverse free radical activity by decomposing peroxides and unpaired oxygen molecules, adsorbing and neutralizing the free radicals to render them harmless to the body (Hassanpour and Doroudi, 2023; Gebicki *et al.*, 2021).

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Research interest has focused on various foods containing natural substances, with fermented products being increasingly utilized due to their health benefits and preference for causing fewer side effects. For many years, fermentation has played a significant role in food preservation, flavor enhancement, and nutritional enrichment (Yuan *et al.*, 2024; Abbaspour, 2024). Fermentation involves the conversion of complex organic compounds into simpler ones through chemical transformation which involves the action of enzymes and organic catalysts produced by microorganisms such as yeast and bacteria, which contribute to the unique flavor and texture of the fermented foods (Abbaspour, 2024). These compounds or metabolites produced during the fermentation process provide nutrients and energy for body maintenance, growth and reproduction (Rusu *et al.*, 2023).

Fermented beverages, such as kombucha, provide health benefits through their antioxidant activity, immune-boosting effects, and modulation of gut microbiota (Shah *et al.*, 2023). Kombucha, a spontaneously fermented tea beverage, is a homemade natural product consisting of a microbial consortium, including acetic acid bacteria (AAB) and yeasts (de Miranda *et al.*, 2022). Recently, the consumption of this fermented tea beverage has been gaining popularity in many countries, including Malaysia, due to its probiotic nature and purported health benefits. Kombucha consumption has been associated with various health benefits, including antioxidative, anti-inflammatory, antimicrobial, and anticancer properties (Batista *et al.*, 2022; de Oliveira *et al.*, 2023) . The microflora in kombucha depends on the origin of the symbiotic culture of bacteria and yeast (SCOBY), the sugar source (sucrose), and the substrate used, all of which can influence the biochemical composition and flavor of kombucha. During the fermentation process, AAB contributes to the formation of the pellicle (a floating thin biofilm) and the production of various acids, such as acetic, gluconic, and malic acids, which are responsible for its sour taste and other bioactive properties (Bishop *et al.*, 2022). Simultaneously, yeast fermentation produces ethanol, which is often present in the final beverage.

The popularity of kombucha has grown, like many other traditional beverages, due to its beneficial effects on human health. Exploring and understanding the nature of kombucha starter cultures can aid in its development and enhance its potential health-promoting properties. While the production of metabolites and health benefits of kombucha can vary based on the source of the starter culture, fermentation time, and substrate used. The research finding was investigated the effect of fermentation time of different kombucha starter cultures in Malaysia on viscosity, pellicle formation, pellicle morphology, pH, total phenolic content, total flavonoid content, and antioxidant activity.

Materials and methods

Preparation of sabah tea kombucha

Six starter cultures of kombucha were purchased online from local seller in Malaysia. Kombucha was then prepared following the method by Zubaidah *et al. (*2020). In brief, eight grams of black tea was added to 1 liter of sterile water containing 10% sucrose (w/v) and brought to a boil for 15 minutes. The tea was then poured into a sterile container and allowed to cool to room temperature before adding 10% of the starter culture (v/v). The container was covered with a sterile cloth and kept in the dark at room temperature for 30 days to allow fermentation. Sampling was performed aseptically at the beginning of fermentation and on days 7, 14, 21, and 30 to measure the pH and antioxidant activity of the kombucha. The formation of the pellicle throughout the fermentation process was also observed and recorded.

Antioxidant activities of sabah tea kombucha

The kombucha samples taken on day 0, 7, 14, 21 and 30 days were tested for their total phenolic (TPC), total flavonoid (TFC) and ferric reduction antioxidant power (FRAP) activities using colorimetric assays according to Mohamad *et al.* (2018).

Total phenolic content (TPC)

The total phenolic content of the samples was prepared by adding 1 mL of diluted Folin-Ciocalteu's phenol reagent to 0.2 mL of kombucha solution or gallic acid standard, followed by incubation for 5 minutes. Next, 0.8 mL of 7.5% Na_2CO_3 solution was added to the mixture and incubated for 2 hours at room temperature in the dark. Distilled water was used as a blank. The absorbance was measured at 765 nm using an ELISA plate reader, and the experiment was performed in triplicate.

Total flavonoid content (TFC)

The total flavonoid content was measured by adding 150 µL of kombucha solution or catechin standard to 9 µL of 5% NaNO3, followed by incubation for 6 minutes in the dark. Then, 9 µL of 10% $AlCl_3$ was added, and the mixture was incubated for another 5 minutes. Next, 60 µL of 1M NaOH and 72 µL of distilled water were added to the solution and mixed well by briefly vortexing. The absorbance was measured at 430 nm using an ELISA plate reader, and the experiment was performed in triplicate.

Ferric reduction antioxidant power (FRAP)

For FRAP assay, the working solution was prepared by adding 4mL of TPTZ and 4mL of $FeCl_3.6H20$ in 40mL of acetate buffer. The solution was warmed to 37°C in dark before use. Then, 20µl of the sample was added into 150µl of FRAP working solution and incubated for 10 minutes. Absorbance was measured using ELISA plate reader at 593nm. Results were calculated from the standard $FeSO^4$calibration curve and expressed in µM of Fe^{2+}.

Statistical analysis

The experiments were conducted in triplicate and analyzed using Prism 9.5.1 for Windows (GraphPad Software Inc., San Diego, CA, USA). Quantitative data were expressed as the mean and standard deviation values and were analyzed using one-way ANOVA and Tukey's multiple comparison test. Statistical significance was defined as a *p*-value of less than 0.05 ($p<0.05$), while a *p*-value of less than 0.001 ($p<0.001$) was considered highly significant.

Results

The effect of fermentation time on viscosity, pellicle formation and morphological appearance

The effect of fermentation time on kombucha's viscosity and pellicle formation is shown in Figure 1. On day 0, the mixture of a sugared black tea and the starter culture gave a clear solution, with no pellicle formation observed. As fermentation progressed, the solution becomes slightly turbid, and a very thin layer of the pellicle started to form on the surface. By day 14, the solution became more turbid with a visibly thicker pellicle was observed on the surface. More layers of pellicle formed on day 21 and 30, during which the solution became completely turbid, releasing a more pungent smell.

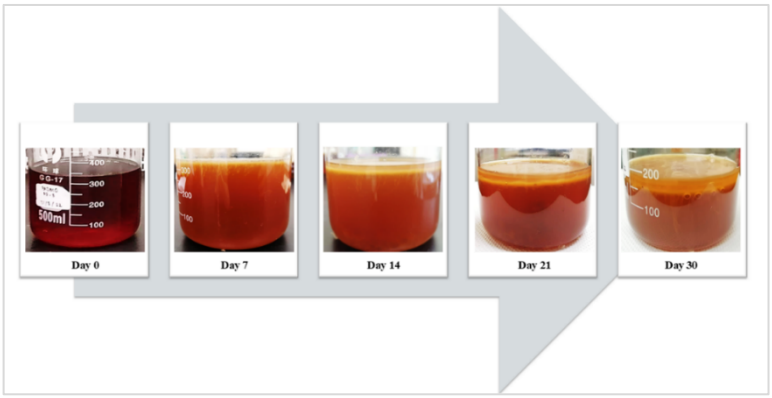


Figure 1. Pellicle formation after 30 days of fermentation

The morphology of the pellicle formed from different kombucha on day 14 is illustratyed in Figure 2. Based on the observations, the pellicle formed from different starter culture of kombucha exhibited variations in color and texture.



Figure 2. Morphology of different kombucha pellicle at 14 days of fermentation

The effect of fermentation time on pH changes

The pH of kombucha fermented from six different starter cultures over 30 days is presented in Table 1. The pH of the sugared black tea taken at the beginning of the study was 5.52 ± 0.22, which falls within a normal pH range of the black tea. A substantial drop in pH values was observed in all kombucha samples immediately after the starter culture was mixed with the substrate (sugared black tea) and continued to decrease throughout the study.

The effect of fermentation time on total phenolic content, total flavonoid content and antioxidant activity

The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of different kombucha samples over 30 days of fermentation are shown in Figure 3. The correlation data showed a consistent trend in TPC, TFC, and FRAP for all samples throughout the 30-day period, as depicted in Figure 3. Meanwhile The antioxidant activity of kombucha at five time points during fermentation, is presented in Figure 4. No definite trend in antioxidant activity was observed in relation to fermentation time for any of the kombucha samples, which aligns with the data observed for TPC

and TFC values. The data also revealed that fluctuations in TPC, TFC, and antioxidant activity varied among kombucha samples from the beginning to the end of the study.

Table 1. pH of the kombucha

Kombucha	Day 0	Day 7	Day 14	Day 21	Day 30
1	3.25±0.13	3.03±0.31	2.87±0.33	2.76±0.31	2.74±0.18
2	3.69±0.26	3.16±0.50	2.88±0.62	2.80±0.42	2.71±0.12
3	3.69±0.11	3.21±0.28	3.19±0.13	3.10±0.08	3.00±0.31
4	3.49±0.09	3.17±0.33	3.00±0.34	2.95±0.11	2.87±0.18
5	3.19±0.22	3.06±0.24	2.86±0.24	2.72±0.27	2.70±0.12
6	3.25±0.36	3.01±0.12	2.66±0.16	2.61±0.17	2.49±0.17
Sugared black tea	5.52±0.22	-	-	-	-

Discussion

Kombucha is a functional drink that has been consumed since ancient times for its purported health benefits. The gradual drop in pH values during the fermentation process of kombucha results from the symbiotic activity of bacteria and yeast, which metabolize sugar into acetic acid and other organic acids (Coelho *et al.*, 2020). As shown in Table 1, the pH of kombucha from six different starter cultures ranged between 3.69 (highest) and 2.49 (lowest), which falls within the reported pH range of kombucha (de Miranda *et al.*, 2022). While the pH range varied among samples, all samples exhibited a decreasing trend in pH values throughout the study, which is consistent with the data reported by Ahmed *et al.* (2020).

During fermentation of kombucha, the pellicle is produced as a byproduct of sugar metabolism by acetic acid bacteria (Coelho *et al.*, 2020). As fermentation progresses, a new pellicle continuously forms on top of the old pellicle, increasing the overall thickness, as shown in Figure 1. This pellicle, or bacterial cellulose, accommodates both bacteria and yeast within its gelatinous matrix, facilitating oxygen supply to the microbes near the liquid surface while also acting as a protective layer for the kombucha solution (Coelho *et al.*, 2020). On the other hand, the morphology of kombucha pellicle from different starter cultures was also examined in this study. As reported by Bishop *et al.* (2022), the microbial diversity in kombucha is complex and varies depending on the starter culture used, fermentation time, substrate, and overall fermentation process. Consequently, differences in texture and color among pellicles can be observed across kombucha samples, indicating variations in microbial composition within the starter cultures, as shown in Figure 2.

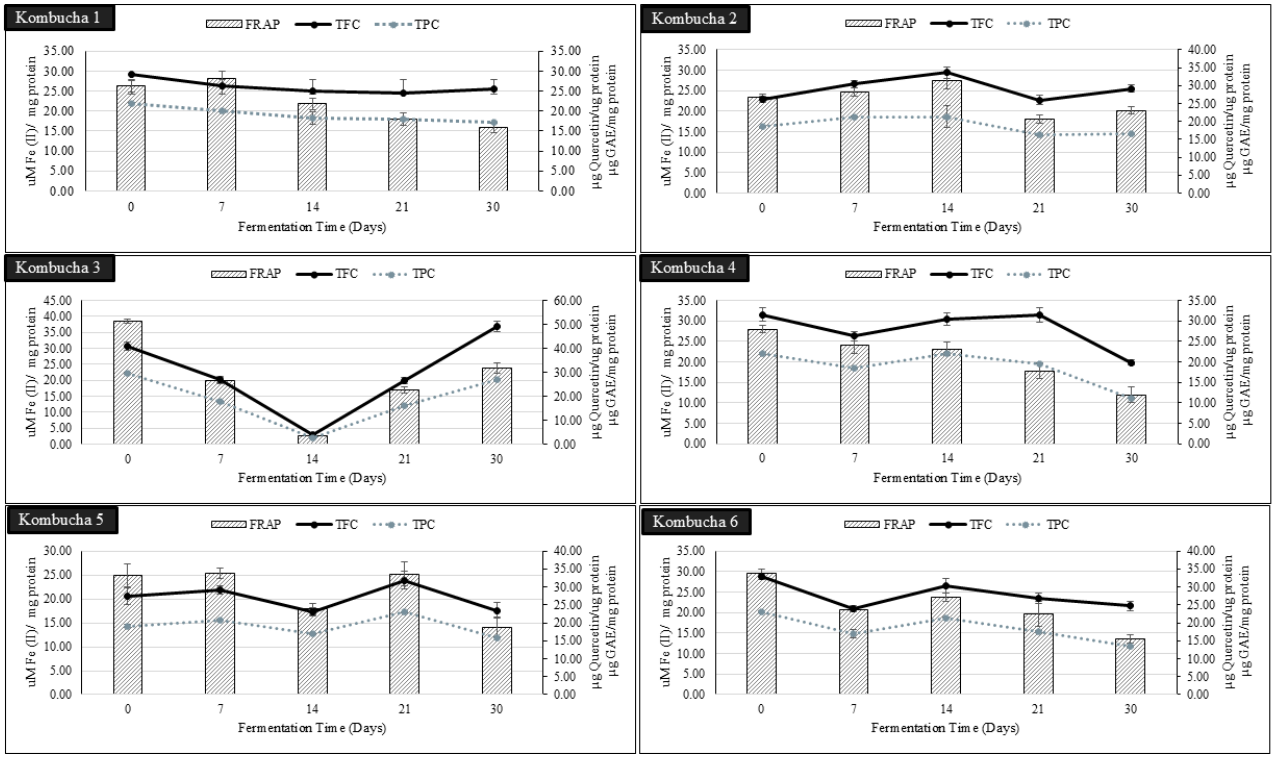


Figure 3. Total phenolic content, total flavonoid content and antioxidant activity of different types of kombucha at 30 days fermentation

Note: Data are shown as mean ± standard deviation and * indicate significant differences among the same kombucha at different time points where p of less than 0.001 was regarded as highly significant (* $p < 0.05$; ** $p < 0.01$. *** $p<0.001$).

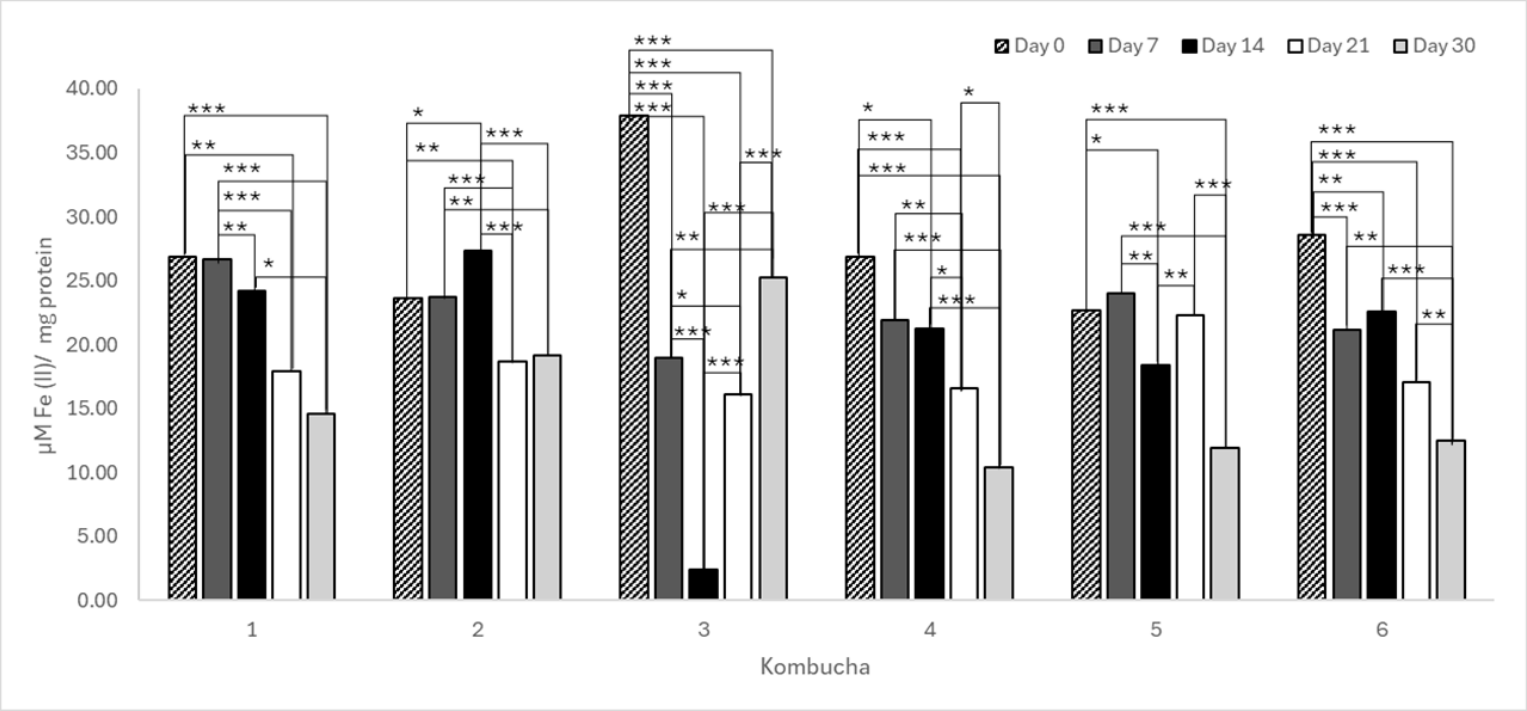


Figure 4. Antioxidant activity of different types of kombucha at 30 days fermentation

Note: Data are shown as mean ± standard deviation and * indicate significant differences among the same kombucha at different time points where p of less than 0.001 was regarded as highly significant (* $p < 0.05$; ** $p < 0.01$. *** $p<0.001$).

Phenolics, such as polyphenols (e.g. flavonoids) are compounds that can be found in plant-based foods and fermented products (Sun and Shahrajabian, 2023). Phenolic compounds have been sought after for their health-promoting effects as potential bioactive agents (Sun and Shahrajabian, 2023; Rana *et al.*, 2022; Di Lorenzo *et al.*, 2021). Traditional kombucha is made by incorporating the starter culture, also known as the symbiotic culture of bacteria and yeast (SCOBY), with sugared black tea (Massoud *et al.*, 2022). Black tea is well-documented to contain bioactive compounds, such as polyphenols (Zhou *et al.*, 2022; Massoud *et al.*, 2022). The polyphenols in black tea and the metabolites produced during the spontaneous fermentation of kombucha have been reported to influence its antioxidant and other bioactive properties (Shi *et al.*, 2023). Figures 3 and 4 demonstrate a positive correlation between total flavonoids, total phenolics, and antioxidant activity in all kombucha samples. Based on the data, total phenolic content in kombucha samples was higher compared to total flavonoid content. Nevertheless, fluctuations in total flavonoid content, total phenolic content, and antioxidant activity were observed throughout the fermentation period and among the samples, as depicted in Figures 3 and 4. This is in agreement with a study reported by Chu and Chen (2006) and Ahmed *et al.* (2020), where these fluctuations may be caused by differences in the diversity of microbes in kombucha and their complex symbiotic relationships, leading to variations in metabolite production and antioxidant activity.

On a related note, Figure 4 shows a comparison of the antioxidant activity of each kombucha sample at different time points over the 30-day period. In this study, the ferric reducing antioxidant power (FRAP) assay was used to measure the antioxidant activity of kombucha. FRAP is one of the standard assays for determining the antioxidant capacity of food by measuring a sample's ability to reduce ferric ions to ferrous ions (Munteanu and Apetrei, 2021). Based on observations of the antioxidant activity of kombucha, no clear deduction can be made regarding its correlation with fermentation time or differences between samples. The data revealed that different kombucha samples exhibited higher antioxidant activity at different time points. Notably, only samples 1 and 4 showed a decrease in antioxidant activity over the fermentation period, while the rest displayed fluctuations.

The data suggest that kombucha produced from different starter cultures contains distinct microbial consortia. This variation is evident through morphological assessments of the formed pellicle and analyses of bioactive compounds. Understanding the properties of kombucha is essential for optimizing its production to maximize health benefits.

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Conflicts of interest

The authors have declared that no conflict of interest exists.

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