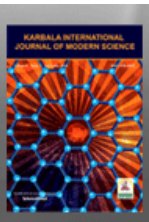

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Antioxidant Properties of Kombucha Beverage Infused with Ganoderma lucidum and Green Tea from Camellia sinensis (L.) Kuntze with Several Fermentation Times

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Abstract

Kombucha is a functional beverage produced through the fermentation of infused tea or *Camelia sinensis* (L.) Kuntze (CS) by a symbiotic culture of bacteria and yeast. Apparently, the substrate of kombucha can be substituted to enhance its functional properties. *Ganoderma lucidum* (GL) is a potential substrate reported to have health benefits. This study aims to evaluate antioxidant properties by comparing different formulations and fermentation times. The formulations prepared in the present study varied in the compositions of GL and CS substrates, namely F1 (100% GL), F2 (75% GL: 25% CS), F3 (50% GL: 50% CS), F4 (25% GL: 75% CS), and F5 (100% CS). The kombucha beverages were fermented for several fermentation times, including 8, 11, 14 and 17 days. The antioxidant activity was evaluated using the free radical scavenging activity of DPPH and the FRP assay. Total phenolics, total flavonoids, and total acids were also determined to support the antioxidant properties, employing the Folin Ciocalteu method, Dowd method, and titration, respectively. Antioxidant activity, total phenolics, and total flavonoids were observed to decrease with the addition of GL substrate, with F5 being identified as the optimum formula based on antioxidant properties. In contrast, total acids were found to increase with an increasing proportion of GL substrate. The use of GL substrate did not prove to enhance the antioxidant properties of kombucha, although a well-produced kombucha beverages continued to be yielded by the fermentation process. Further research should be conducted to evaluate other functional properties of kombucha beverage using GL substrate.

Keywords

Antioxidant; Camellia sinensis (L.) Kuntze; Ganoderma lucidum; Kombucha

Cover Page Footnote

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RESEARCH PAPER

Antioxidant Properties of Kombucha Beverage Infused with *Ganoderma lucidum* and Green Tea from *Camellia sinensis* (L.) Kuntze With Several Fermentation Times

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Abstract

Kombucha is a functional beverage produced through the fermentation of infused tea or *Camelia sinensis* (L.) Kuntze (CS) by a symbiotic culture of bacteria and yeast. Apparently, the substrate of kombucha can be substituted to enhance its functional properties. *Ganoderma lucidum* (GL) is a potential substrate reported to have health benefits. This study aims to evaluate antioxidant properties by comparing different formulations and fermentation times. The formulations prepared in the present study varied in the compositions of GL and CS substrates, namely F1 (100% GL), F2 (75% GL: 25% CS), F3 (50% GL: 50% CS), F4 (25% GL: 75% CS), and F5 (100% CS). The kombucha beverages were fermented for several fermentation times, including 8, 11, 14 and 17 days. The antioxidant activity was evaluated using the free radical scavenging activity of DPPH and the FRP assay. Total phenolics, total flavonoids, and total acids were also determined to support the antioxidant properties, employing the Folin Ciocalteu method, Dowd method, and titration, respectively. Antioxidant activity, total phenolics, and total flavonoids were observed to decrease with the addition of GL substrate, with F5 being identified as the optimum formula based on antioxidant properties. In contrast, total acids were found to increase with an increasing proportion of GL substrate. The use of GL substrate did not prove to enhance the antioxidant properties of kombucha, although a well-produced kombucha beverages continued to be yielded by the fermentation process. Further research should be conducted to evaluate other functional properties of kombucha beverage using GL substrate.

Keywords: Antioxidant, *Camellia sinensis* (L.) Kuntze, *Ganoderma lucidum*, Kombucha

1. Introduction

The current market offers a variety of beverages, such as boba-drinks, coffee, and tea, each featuring an array of flavors and toppings. It is crucial to acknowledge that these beverages may have adverse effects on health, potentially hightening the risk of obesity and diabetes. Boba drinks, in particular, ulitize sweeteners that contribute additional calories to the beverage. The study conducted by Min et al., (2017) revealed that a 16-ounce (473 mL) boba drink with milk tea and tapioca "boba" balls contains 299 calories and 38 g of sugar [1]. Notably, the calorie content of boba drinks surpasses that of carbonated sugary beverages, which typically typically contain about 126 calories per 330 mL [2]. The consumption of excessive sugars from the sweeteners present in boba beverage can

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elevate the risk of weight gain, type 2 diabetes, and various obesity-related comorbidities [1,3]. Consequently, there arises a need for more healthful functional beverages that not only cater to the saturated beverage market, but also provide added value, specifically in terms of health benefits.

Kombucha is a functional beverage produced by acid-tolerant species of the symbiotic culture of bacteria and yeast (SCOBY), commonly known as the "tea fungus" [4]. The fermentation proccess of kombucha involves two stages: initial alcoholic fermentation mediated by yeasts, followed by acetic acid fermentation by acetic acid bacteria (AAB) [5]. Yeast hydrolyzes sucrose into glucose and fructose, a process catalyzed by invertase. Additionally, yeast produce ethanol, primarily utilizing fructose as the preferred substrate through glycolysis [6,7]. *Acetobacter* and *Gluconobacter*, both members of AAB family, convert glucose into gluconic acid and utilize ethanol for the production of acetic acid, with *Acetobacter* playing a crucial role in cellulose pellicle biosynthesis [8–10]. The organic acid present in kombucha collectively lower the pH. This pH reduction serves as a protective mechanism, guarding against potential contamination by pathogenic bacteria [11,12]. Kombucha contains various compounds that are beneficial for health, including amino acids, acetic acids, glucuronic acid, lactic acid, various vitamins, and antioxidants [13,14]. The metabolic activity of kombucha results in a refreshing beverage with the sour taste and many health-beneficial compounds [15]. Kombucha can be utilized to alleviate various conditions such as headaches, gastric illness, diabetes, nervousness, aging-related issues (antioxidant), lowering blood pressure and cholesterol levels, promoting weight-loss, acting as immunomodulator, reducing kidney calcification, and exhibiting anti-cancer and anti-proliferative effects [6,16–19]. The study conducted by Zubaidah et al., (2018) revealed that the fasting plasma glucose of the diabetic rats treated with the kombucha (110.3–189.3 mg/dL) was significantly lower than the diabetic control group (413.3 mg/dL) [20].

Traditionally, kombucha utilizes black tea as its primary substrate. However, several studies have delved into alternative raw materials as substrates to augment the functional value of kombucha. Fermentation of kombucha using alternative substrates such as dried fruits, fruit juice, herbal infusions and other ingredients with potentical health benefits has been explored, yielding satisfactory results concerning the bioological properties of kombucha [21–25]. Kaewkod et al., (2022) demonstrated that antioxidant activity, as well as the total phenolic and flavonoid content, increased in kombucha containing *Terminalia catappa* leaf extract and *Aegle marmelos* fruit compared to kombucha prepared using a black tea substrate. Additionally, this study reported an elevation in the content of organic acid compounds, including glucuronic, gluconic, acetic, ascorbic, and succinic acids, in kombucha with *T. catappa* leaf extract [26].

Ganoderma lucidum (GL) has been utilized for medicinal purposes for thousands of years. Previous studies have revealed that GL comprises more than 400 bioactive compounds, endowing it with various medicinal effects, including antitumor, antimicrobial, anti-atherosclerosis, anti-inflammatory, antioxidant, anti-aging, and immune system enhancing properties [27–29]. These health benefits inherent in GL may enhance the functional properties of kombucha beverage if used as a substrate in the kombucha fermentation process. However, there have been limited studies investigating the use of GL as a substrate for kombucha beverages. In the present study, 5 formulas were prepared by combining GL and CS, including F1 (100% GL), F2 (75% GL: 25% CS), F3 (50% GL: 50% CS), F4 (25% GL: 75% CS), and F5 (100% CS) with the fermentation times of 0, 8, 11, 14, and 17 days. The objective of this study is to evaluate the antioxidant properties by comparing the formula and fermentation time as independent variables, based on DPPH-free radical scavenging activity, ferric reducing power, total phenolic content, total flavonoid content, and total acid.

2. Materials and methods

All chemicals used was pro-analytical grade. DPPH and HCl were purchaed from Sigma–Aldrich® (Germany), while Folin Ciocalteu reagent, Na_2CO_3, gallic acid, $AlCl_3$, methanol, quercetin, CH_3COOK, trichloroacetic acid, NaOH, phelolphthalein indicator, Na_2HPO_4, and NaH_2PO_4 were purchased from Merck® (Germany). $K_3Fe(CN)_6$ were purchased from Himedia® (India). The UV-Vis spectrophotometer used in present study was purchased from Thermo Fisher Scientific®, model Genesys 10-S (USA).

2.1. Preparation of substrate and culture

Kombucha was prepared by fermenting substrate with SCOBY culture. Two substrates were used in the present study, including GL-fruiting body powder and CS-leaf powder. The GL was obtained from a mushroom farming located in Cianjur, West Java, Indonesia. The CS leaf was obtained from tea plantation located in Cisarua, West Java, Indonesia and morphologically identified as *Camelia sinensis* (L.) Kuntze by Herbarium Bogorienses, National

Research and Innovation Agency of Indonesia. Both substrates were partially dried in 40 °C and powdered. The SCOBY culture was purchased in commercial online shop and was recultured in laboratory before used in the experiment.

2.2. Preparation of kombucha tea beverage specifications

The preparation of the kombucha beverage was based on Silva et al., with some modification [25]. Kombucha beverage was prepared using five formulas (F1-F5) with different compositions of GL and CS (Table 1). The substrate (0.5% w/v) and sugar (5% w/v) were placed into a glass jar, which was then immersed in boiling water for 10 minutes, and subsequently cooled to room temperature (25–30 °C). A SCOBY (2.5% w/v) was inoculated and combined with a kombucha liquid starter liquid (20% v/v). The jar was covered with gauze and each kombucha formula was fermented for 8, 11, 14, and 17 days, with day 0 as the control.

2.3. Determination of total phenolic content (TPC)

The determination of TPC in kombucha was conducted using the Folin Ciocalteu method, as described by Maeng et al., [30]. A total of 0.2 mL of kombucha sample was mixed with 1.8 mL of distilled water and 0.2 mL of Folin Ciocalteu reagent. The mixture was allowed to stand for 6 minutes before adding 2 mL of sodium carbonate 7% (w/v). After a 90 minutes incubation period, the absorbance was measured at 750 nm. Gallic acid was used as the positive control. The TPC was expressed in milligrams of gallic acid equivalent (GAE) per L of kombucha.

2.4. Determination of total flavonoid content (TFC)

The determination of TFC of kombucha followed the Dowd method described by Aryal et al., [31]. To perform the test, 1 mL of kombucha solution was mixed with 0.2 mL of 10% (w/v) $AlCl_3$ solution in methanol, 0.2 mL of potassium acetate 1 M, and 5.6 mL of distilled water. The mixture was incubated at room temperature for 30 minutes, followed by measuring the absorbance at 415 nm. Quercetin was used as a positive control. TFC content was expressed as a milligram of quercetin equivalent (QE) per L of kombucha.

Table 1. The formulas of kombucha beverage substrate in 100 mL liquid.

Composition	F1	F2	F3	F4	F5
CS substrate (g)	0	0.125	0.25	0.375	0.5
GL substrate (g)	0.5	0.375	0.25	0.125	0
SCOBY (g)	2.5	2.5	2.5	2.5	2.5
Kombucha liquid starter (mL)	20	20	20	20	20
Sucrose (g)	5	5	5	5	5
Water (mL)	ad 100	ad 100	ad 100	ad 100	ad 100

2.5. Antioxidant activity: DPPH scavenging activity assay

DPPH free radical scavenging activity of the kombucha beverage was evaluated following the method established by Boonsong et al., [32]. Each kombucha sample was prepared in six dilution series with 5% (v/v) for the highest concentration. Subsequently, 1 mL of each diluted kombucha was mixed with 3 mL of 0.1 mM DPPH solution in methanol. The mixture was then vigorously shaken and allowed to incubate in the dark at room temperature for 30 minutes then measured at 517 nm.

2.6. Antioxidant activity: Ferric reducing power (FRP) assay

The FRP measurement of the kombucha beverage was carried out following Fu et al., with some modification [33]. A total of 0.3 mL of each kombucha was mixed with 0.6 mL of phosphate buffer (0.2 M; pH 6.6) and 0.6 mL of $K_3Fe(CN)_6$ 1% (w/v). The mixture was then incubated at 50 °C for 30 minutes. Subsequently, 0.6 mL of 10% trichloroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 minutes. A total of 0.5 mL of supernatant obtained was then mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ 0.1% (w/v). The absorbance was measured at 700 nm. The observed increase in absorbance was directly proportional to the concentration of the sample, thereby indicating its reducing power.

2.7. Determination of titrable acidity (TA)

The measurement of total acidity or titrable acidity was conducted following the AOAC method [34,35]. A total of 10 mL of kombucha was diluted with distilled water. Phenolphthalein 1% (w/v) was added as an indicator, and the solution was titrated with NaOH 0.1 N. The end point of the titration was determined by observing the color change in the sample. The concentration of acetic acid was calculated using the following equation:

$$TA\ (\%) = (V._{titran} \times N_{NaOH} \times Eq._{WT.\ of\ Acid}) / (V._{Sample} \times 10)$$

Note: Equivalent weight of acetic acid = 60.05 g/mol.

2.8. Data analysis

All experiments were performed in triplicate and using completely randomized design with formula and fermentation time as dependent variables. Data analysis was using confident level of 95%. Data was analyzed using Minitab-19 with two-way Analysis of Variance (ANOVA) and continue with Tukey's post hoc test. Pearson's correlation analysis was conducted for all dependent variables.

3. Results

3.1. Total phenolic content (TPC)

The TPC in kombucha beverages was determined using the gallic acid equivalent per liter of the sample (mgGAE/L). The linear regression equation for the gallic acid standard curve was $Y = 0.0059x + 0.0006$ and the R^2 value was 99.66%. The TPC is graphically represented in Fig. 1, where it scores constantly increased with the decrease of GL substrate (F1 < F2 < F3 < F4 < F5). The optimum total phenolics was observed on day 8 and day 17 of fermentation for all formulas. Both fermentation times show no significantly different ($\alpha = 0.05$) according to Tukey's post hoc test. The two-way ANOVA analysis indicated that both dependent variables, formulation and fermentation time, along with their interaction, significantly influenced the TPC ($P < 0.05$).

On day 0 of fermentation, serving as a control, it was observed that the TPC consistenly increased with the decrease of GL substrate and the incrase of CS substrate. This suggests that the CS substrate contributes higher phenolics than the GL substrate before starting the fermentation process.

Regarding the increase in TPC after first 8 days of fermentation, identified as one of the optimum TPC levels, all formulas demonstrated an increase in TPC. However, the percentage increases varied in proportion to the decrease of GL substrate (F1 = 7.01%; F2 = 11.39%; F3 = 24.37%; F4 = 26.84%; F5 = 35.33%). This suggests that GL substrate is less effective than CS substrate in increasing TPC during the fermentation.

3.2. Total flavonoid content (TFC)

The TFC in the present study was determined using the standard curve of quercetin, with unit expressed as milligram quercetin equivalent per litter of the sample (mgQE/L). The linear regression equation for quercetin was derived as $Y = 0.005x + 0.0096$, and the R^2 value was 0.9987. TFC in summerizes graphically in Fig. 2. The two-way ANOVA analysis revealed that formulation, fermentation time, and the interaction between formulation and fermentation time significantly contribute to the TFC ($P < 0.05$). Notably, the optimum spike in TFC occured on day 8, as the TFC on day 0 significantly different from the day 8, while the TFC on day 8 was not significantly different from the day 11, day 14, and day 17.

The TFC exhibited a similar pattern to TPC, increasing linearly with the decrease of GL substrate used (F1 < F2 < F3 < F4 < F5). Additionally, the CS substrate contributed more flavonoids than the GL substrate, as indicated by higher TFC levels on the

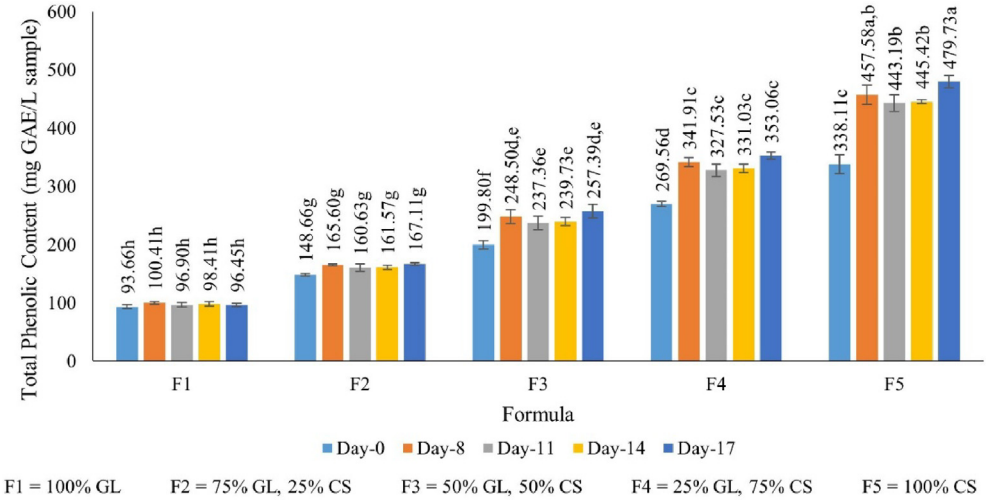

Fig. 1. Total phenolic content of kombucha beverages on F1–F5 with several fermentation times. Values with different letters indicate significant differences (P < 0.05) by Tukey's post hoc test.

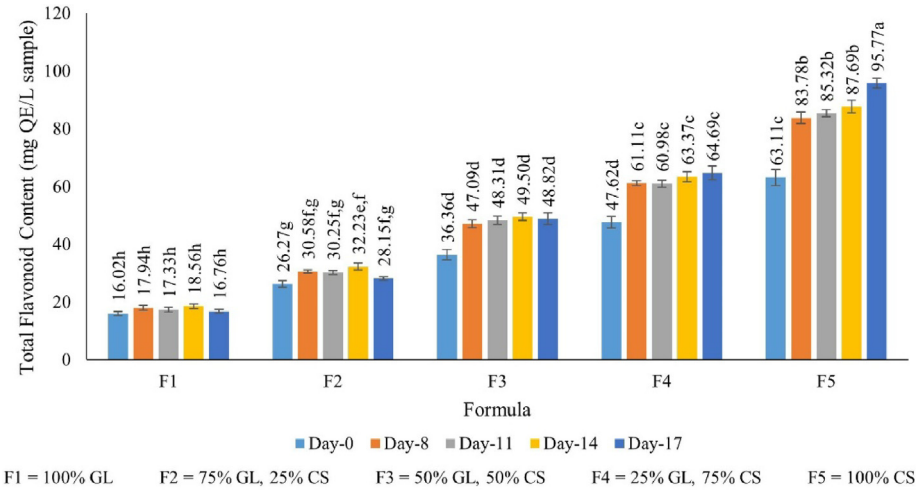

Fig. 2. Total flavonoid content of kombucha beverages on F1–F5 with several fermentation times. Values with different letters indicate significant differences (P < 0.05) by Tukey's post hoc test.

day 0 or before fermentation process. The use of CS substrate also showed its ability to stimulate flavonoid production throughout the fermentation process, showing a significant increase in flavonoids after fermentation with an increased proportion of CS substrate. In contrast, the use of GL substrate only lead to a slight increase in total flavonoids, particulary evident when comparing F1 (100% GL) and F5 (100% CS substrate).

3.3. Antioxidant activity: DPPH scavenging activity

The antioxidant activity expressed as IC_{50} score, based on DPPH scavenging activity is presented in Fig. 3. The two way-ANOVA analysis indicated that the IC_{50} score was significantly influenced by both formula and fermentation time. The IC_{50} score was calculated throuh linear regression between DPPH scavenging activity and sample concentration, reflecting the concentration of the sample required to neutralize 50% of free radicals of DPPH. Consequently, the IC_{50} score is inversely proportional to antioxidant activity, where a lower IC_{50} score corresponds to the higher antioxidant activity. The two-way ANOVA analysis futher demonstrated that both formulation and fermentation time, as well as the interaction between formulation and fermentation time, significantly influenced the DPPH-IC_{50} score ($P < 0.05$).

The data reveals aconsistent decrease in the IC_{50} score along with the reduction of GL substrate (F1 > F2 > F3 > F4 > F5), indicating that a decrease in the GL substrate elevating antioxidant activity (F1 < F2 < F3 < F4 < F5). This suggest that the GL substrate contributes less to antioxidant activity compared to the CS substrate. Futhermore, when comparing the patterns of F1 (100% GL substrate) and F5 (100% CS substrate), it is evident that the use of GL substrate results in higher IC_{50} than CS substrate. This signifies that the CS substrate significantly contributes to high antioxidant activity when compared to the GL substrate.

3.4. Antioxidant activity: Ferric reducing power

The antioxidant activity, assessed through ferric reducing power (FRP), involves the reduction of Fe^{3+} to Fe^{2+} which can be measured at 700 nm. The occurrence of this reaction is indicative of the presence of antioxidant compound. A contrasting pattern was observed between ferric reducing power and DPPH-IC_{50} score, revealing that FRP is directly proportional to DPPH scavenging activity (Fig. 4). The two-way ANOVA analysis demonstrated that formulation, fermentation time, and interaction between formulation and fermentation time significantly influence the ferric reducing power ($P < 0.05$). FRP is proportionally similar to total phenolic and total flavonoid content, as well as inversely proportional to DPPH-IC_{50} score. The optimum ferric reducing power score was consistently observed at day 8 fermentation for all formulas, indicates the optimal fermentation time based on ferric reducing power. Futhermore, the graph in Fig. 4 illustrates that the use of GL substrate diminishes ferric reducing power, particularly evident when comparing F1 (100% GL substrate) and F5 (100% CS substrate). It implies that the antioxidant activity by the FRP assay wincreases

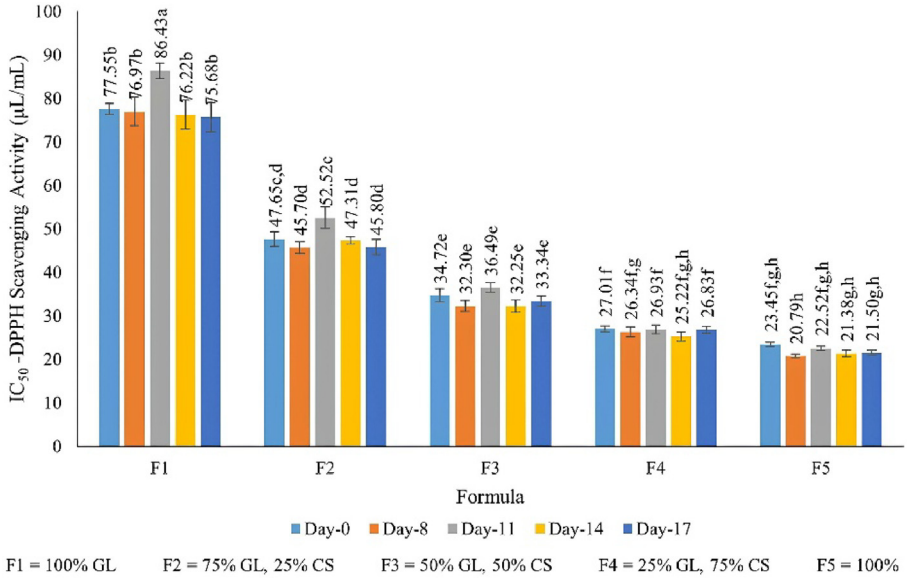

Fig. 3. IC_{50}-DPPH scavenging activity of kombucha beverages on F1–F5 with several fermentation times. Values with different letters indicate significant differences ($P < 0.05$) by Tukey's post hoc test.

with the decrease in GL substrate, mirroring the pattern observed in DPPH scavenging activity.

3.5. Titrable acidity (TA)

The determination of TA in kombucha beverages was conducted using the acid-base titration method and expressed as the percentage of acetic acid, as acetic acid constitutes the predominant component of the final product in kombucha fermentation. The result of TA is presented as total acids and illustrated in Fig. 5.

On the day 0 of fermentation, all formulas exhabited no significant difference in TA (F1=F2=F3=F4=F5), indicating that both substrates, GL, and CS, had no or trace contribution to TA. The TA on the day 0 in all formulas was expected to originate from the liquid of the kombucha liquid stater. The graph in Fig. 5 showed a consistent pattern on 8, 11, 14, and 17 day fermentation, where in TA decreased along with the reduction of GL substrate (F1 > F2 > F3 > F4 > F5). Additionally, the graph indicated an increase in TA along the fermentation time for all formulas. The GL substrate was anticipated to contribute more to the production of acids during the fermentation compared to the CS substrate. The data on TA, along with two-way ANOVA analysis, revealed that the formulation,

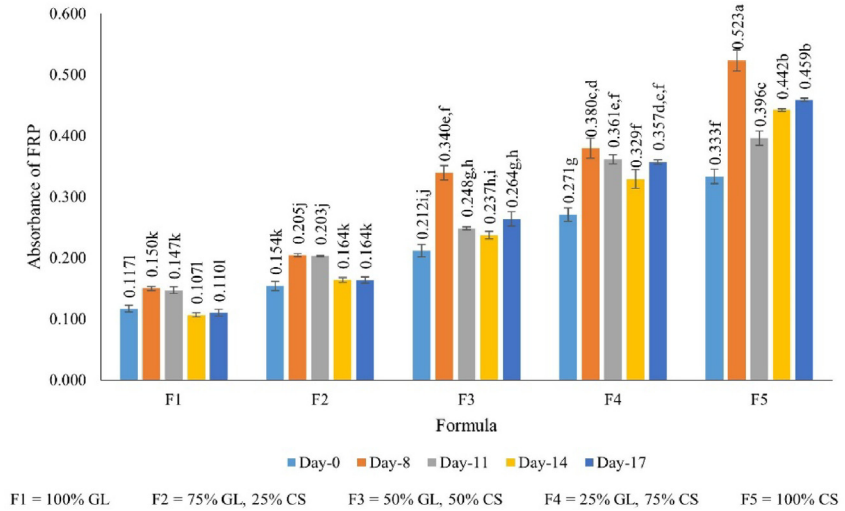

Fig. 4. Ferric reducing power of kombucha beverages on F1–F5 with several fermentation times. Values with different letters indicate significant differences ($P < 0.05$) by Tukey's post hoc test.

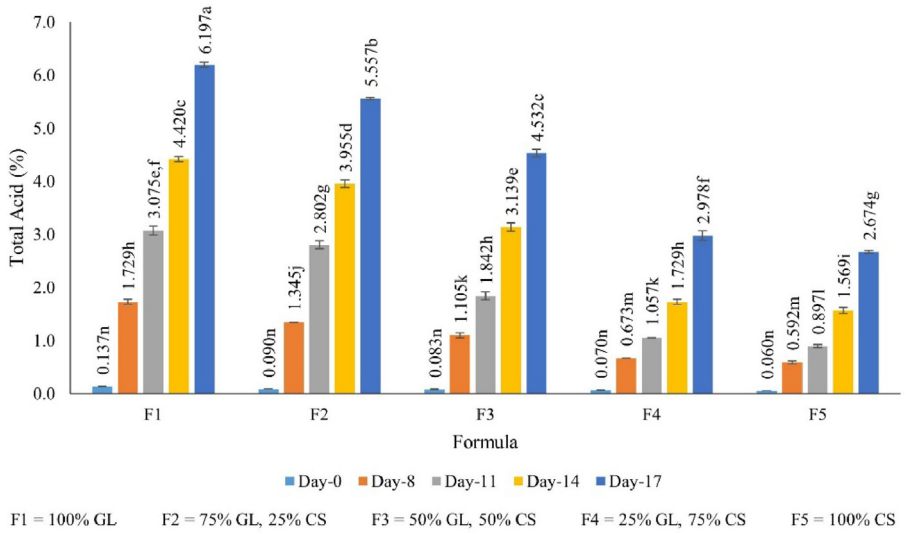

Fig. 5. Total acids of kombucha beverages on F1–F5 with several fermentation times. Values with different letters indicate significant differences ($P < 0.05$) by Tukey's post hoc test.

fermentation time, and interaction between formulation and fermentation time significantly influence the TA ($P < 0.05$).

3.6. The Pearson's correlation analysis

The Pearson's correlation bivariate analysis was conducted to examine the relationships between all dependent variables in the present study, including TPC, TFC, IC_{50}-DPPH, FRP, and TA. The Pearson's analysis results are presented in Table 2, where the positive score indicate proportional correlation, and negative score indicate inversely proportional correlation. Strong collerations were observed between antioxidant activity represented by IC_{50}-DPPH and FRP with TPC and TFC, as indicated by absolute values of correlation coefficient higher than 0.70. Conversely, total acids exhabited weak correlations with all dependent variables (TPC, TFC, IC_{50}-DPPH, and FRP), with absolute values of correlation coefficient less than 0.40. In the present study, the Person's correlation coefficient showed that the TPC and TFC significantly contribute to the antioxidant activity represented by both IC_{50}-DPPH and FRP. However, the TA has a comparatively lesser contribution to the antioxidant activity of kombucha beverages in present study.

Table 2. The Pearson's correlation analysis of TPC, TFC, IC_{50}-DPPH, FRP, and TA.

Parameters	TFC	IC_{50}-DPPH	FRP	TA
TPC	0.99	−0.76	0.95	−0.11
TFC	1	−0.75	0.92	−0.10
IC_{50}-DPPH		1	−0.70	0.15
FRP			1	−0.15

4. Discussion

In this study, the variations in TPC in kombucha can be attributed to several factors. One significant factor is the elevated content of insoluble bound phenols within the plant cell wall, which poses a challenge for their release into the kombucha product. Furthermore, the solubility of phenolics is another crucial factor influencing the release of these compounds into kombucha. Moreover, the solubility of phenolics itself can be influenced by other metabolites generated by the microbial community during kombucha fermentation [36] Additionally, a reduction in phenolic content within kombucha can occur due to the degradation of polyphenols into other metabolites by hydrolytic enzymes produced by SCOBY. These metabolites are subsequently utilized by kombucha microbes as a source of nutrients for their metabolic activities, resulting in a decrease in TPC within kombucha products [13,37,38]. The TFC in *G. lucidum* kombucha (F1) in this study exhibited a low value compared to other formulas. This is was not homogeneosly mixes, leading to insufficient dissolution of active compounds from GL into kombucha filtrate [39,40].

The evaluation of TFC in kombucha revealed an increase following fermentation on day 8. The optimum TFC was observed in F5, and this increase persisted until day 17 of fermentation. Additionaly, the TPC of kombucha in this study increased during fermentation on day 8. Subsequently, the value

exhibited consistent fluctuations throughout the fermentation, persisting until the day 17 of fermentation. Throughout the fermentation process, various bioactive compounds inherent to tea, such as polyphenols and amino acids, are released into kombucha broth [36,41]. The fermentation process contributes to an augmentation in both TPC and TFC [42,43]. Kombucha involves the generation of numerous enzymes, including cellulase, glucanase, xylanase, pectinase phytase, α-galactosidase, tannase, and glucosidase. These enzymes may reduce large molecular compounds into smaller polyphenol monomers, thereby increasing the total polyphenol and flavonoid contents of kombucha [36,44,45]. Meanwhile, Hsieh et al., reported that the TFC of green tea kombucha exhibited consistent fluctuations throughout the fermentation period (4–20 days), reaching approximately 200 mgQE/L kombucha. Notably, in addition to F5 kombucha, the TFC also demonstrated a tendency for consistent fluctuations during the fermentation process [46].

Jakubczyk et al., and Hsieh et al., reported that the fluctuation and degradation observed in TFC in kombucha might be attributed to flavonoid conversion by various microbial species whitin the SCOBY during fermentation [46,47]. Meanwhile, according to Phung et al., changes in TFC during fermentation may be caused by oxidative reactions of polyphenols, as well as other factors such as reduction and esterification reactions that occur during fermentation [37]. In this present study, fluctuations in TFC may be attributed to variations in substrate composition used. These variations can subsequently influence the fermentation process and microbial growth, thereby impacting the activity of SCOBY microbial enzymes involved in flavonoid conversion of flavonoids [46].

TPC and TFC are crucial antioxidants, and based on the above results, the change in their antioxidant activity were consistent with the alteration in flavonoids and polyphenols levels. Jakubczyk et al., reported the highest antioxidant activity was obtained in green tea kombucha with an inhibition value of 91.40% on the day 7 of fermentation [47]. In this present work, the antioxidant activity of F5 kombucha was higher compared to the research conducted by Jacubczyk et al. [47], which reported an inhibition value of 93.626 % for F5 kombucha on day 8 of fermentation. Green tea produced from the fresh leaves of the plant preserves a higher polyphenol content with a greater concentration of catechins and compounds with functional activity compared to other teas produced from *C. sinensis* [48]. Green tea kombucha exhabits the highest antioxidant activity among oolong tea and black tea. Tea is known to contain many compounds with antioxidant properties, such as catechin, which are released during kombucha fermentation, resulting in green tea kombucha with enhanced antioxidant activity [13]. Catechins, along other polyphenol groups, including flavonoids and phenolics, act as antioxidants with the ability to counteract free radicals, reactive oxygen species and prevent metal ion oxidation [13,49–51].

The IC_{50} value represents the concentration of the sample with antioxidant activity needed to reduce 50% of DPPH free radicals, serving as a measure of a substance's effectiveness in inhibiting specific biological or biochemical processes. A lower IC_{50} value signifies a more potent antioxidant capacity in reducing DPPH radicals [52,53]. The IC_{50} value signifies of F5 kombucha was 20.790 μL/mL on day 8 fermentation. Velićanski et al., reported that black tea kombucha had IC_{50} values ranging from 60.2 μL/mL at day 7 of fermentation [54]. In contrast, the IC_{50} value of F1 kombucha was 75.683 μL/mL on day 17 fermentation. Previous studies have indicated that certain complex phenolic compounds in *G.lucidum* can bind to fungal polysaccharides. The phenolic compounds are frequently glycosylated, leading to the binding of free hydroxyl groups. This glycosylation makes the molecule less reactive to radicals, consequently affecting its ability to counteract these radicals [40].

The kombucha beverage with the highest absorbance value was obtained in F5 kombucha with a maximum absorbance of 0.523 at day 8 of fermentation. The measurement of absorbance serves as a crucial indicator of a compound's potential antioxidant activity, as it reflects its capacity to reduce other substances. A higher absorbance value of the sample indicates a greater reducing capacity [33]. The higher absorbance value indicates that the sample has the potential as a strong reductant that can reduce the formation of radical species by using metal ions for oxidation [39,55]. Fu et al., reported that kombucha from green tea has a stronger potential reducing power with an absorbance value of 0.304 at 700 nm [33]. Tea contains 20–30% of polyphenolic group compounds, making tea itself a valuable source of natural antioxidants [49–51]. Polyphenol groups have properties as metal ion chelating agents, which contribute to reducing the production of oxidant species [56].

The reducing power of F1 kombucha exhibited absorbance values ranging from 0.107 to 0.150 at 700 nm. Sknepnek et al., investigated the potential reducing power of *G. lucidum* kombucha was 1.185 ± 0.055 [40]. The chelating ability of iron ions (Fe^{3+}) decreased with increasing concentration of

G.lucidum. This phenomenon is attributed to the elevated presence of polysaccharides within *G. lucidum* [40]. Polysaccharides are recognized as one of the primary constituents responsible for the biological activity of *G. lucidum* [39,57]. The polysaccharide content can result in reduced solubility, leading to incomplete dissolution of *G. lucidum* bioactive compounds in the kombucha solution. Consequently, this occurrence leads to a notable reduction in the potential antioxidant efficacy of F5 kombucha [40]. In this present work, *G. lucidum* powder was heated in boiling water for 10 minutes. However, the bioactive compound in *G.lucidum* could not completely dissolve in the kombucha solution, leading to the assumption that the bioactive compounds of *G. lucidum* present in the kombucha solution were at a low concentration. Resulted in low absorbance value and weakened the potential reducing power of *G. lucidum* kombucha [39,40].

In this study, the titrable acidity of kombucha was found to be increasing linearly along with the fermentation process. The optimum titrable acidity was found in F1 kombucha with fermentation time on the day 17, i.e 6.197 ± 0.048%. During the fermentation process under anaerobic respiration, yeast consumes dissolved oxygen and metabolizes sucrose into glucose and fructose, leading to a gradual increase in ethanol content. Subsequently, this ethanol undergoes oxidation to form acetaldehyde, a process catalyzed by acetic acid bacteria, ultimately leading to the production of acetic acid. Furthermore, the sugars are converted into various organic acids including, acetic, gluconic acid, glucuronic acid, pyruvic acid, succinic acid, lactic acid, malic acid, citric acid, saccharic acid, vitamins, and amino acids [13]. These acids contribute significantly to the acidity level and functional attributes of kombucha, such as its antimicrobial activity [8,58]. Glucuronic acid is considered to be one of the key components found in kombucha due its detoxifying action through conjunction [59,60]. In the late stage of fermentation, the bacteria ultimately result in the formation of synthetic fiber substances. Furthermore, it is important to note that the fermentation process has the capacity to produce organic acids and vitamin C, which influences the variations in the overall antioxidant capacity [6,61]. In comparison to an unfermented beverage, the heightened beneficial activities observed in kombucha suggest that certain alterations are linked to the composition of the microbial community present throughout the fermentation process.

The results of the Pearson's correlation analysis are expressed by the correlation coefficient (r), where an r value of 0.70–0.89 indicates a strong correlation between two variables and an r value 0.90–1.00 indicates a very strong correlation [62,63]. The results of the correlation analysis of TFC with TPC show a very strong correlation with an r value of 0.99, indicating a significant correlation between the TFC and TPC of kombucha. The results reveal that flavonoids are part of the phenolic compound group [64,65]. Based on the results of the Parson's correlation analysis, it has been revealed that TPC and TFC highly contribute to the antioxidant activity represented by both IC_{50}-DPPH and FRP of the kombucha beverages.

5. Conclusion

The antioxidant properties of kombucha beverages were assessed in the present study through the combination use of GL and CS substrate formulated in F1 – F5 with several fermentation times. Both independent variables (formula and fermentation time) and their interaction were found to significantly influence ($P < 0.05$) the score of all dependent variables (TPC, TFC, IC50-DPPH, FRP, and TA). The antioxidant properties were observed through the antioxidant activity represented by DPPH scavenging assay and FRP assay. Both antioxidant activity assays were contributed to TPC and TFC, as indicaed by a strong correlation in the Person's correlation coefficient, but were less affeected TA, which revealed a weak correlation. Comparing the formula and fermentation time, it was concluded that F5 (100% CS substrate) on the day 8 of fermentation exhabited the most optimum antioxidant properties. The CS substrate showed a contribution to the phenolic and flavonoid compounds, which can be utilized to produce new phenolic and flavonoid compounds along fermentation process, resulting the optimum antioxidant activity. The use of GL substrate has not been proven to increase the antioxidant properties of kombucha, although the fermentation process was still running well to produce kombucha beverage. Further research should be conducted to reveal other bioactivities of kombucha beverages using the combination of GL and CS substrate.

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