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ORIGINAL PAPER

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PHYTOCHEMICAL AND ANTIOXIDANT EVALUATION OF *MORINGA OLEIFERA* AND *ALOE VERA* IN THE FERMENTATION OF KOMBUCHA

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ABSTRACT

Background. Kombucha is a fermented beverage with a very low alcohol concentration. It can be brewed with different substrates, which influence its functional properties.
Methods. The present study elaborated and characterized kombucha based on *Moringa oleifera* and *Aloe vera*. Various combinations of *A. vera* (C: 100:0; T1: 50:50; T2: 75:25; T3: 25:75% W: W) and dextrose were mixed with a single concentration of *M. oleifera*, evaluating the antioxidant capacity, total phenolic compounds, pH, acidity and total sugars of the fermented product at 3, 6, 9, 12 and 15 days.
Results. The percentage acidity and pH ranged between 1.0–1.9% and 2.2–3.2, respectively. At the end of fermentation, no statistical differences were found between treatments for total sugar concentrations (1893–2434 mg/L), total phenols, and antioxidant activity by ABTS, DPPH, and FRAP. However, an increase in DPPH radical activity (734–959 μM TE/mL), Fe oxidation in FRAP (36–83 μM TE/mL), and total phenolic concentration (7.8–9.3 mg GAE/ml) was observed. In contrast, a decrease in ABTS radical activity (786–562 mg TE/mL) is observed.
Conclusion. The results suggest that using *A. vera* and *M. oleifera* is a good alternative for elaborating kombucha.

Keywords: *Moringa oleifera*, *Aloe vera*, fermentation, kombucha, antioxidants

INTRODUCTION

Kombucha is a traditional Asian beverage obtained from the fermentation usually of green or black teas and in recent years it has gained tremendous popularity because it is rich in various bioactive compounds, such as acetic and gluconic acid, B-complex vitamins, minerals, amino acids and polyphenols. These provide health benefits, such as anti-inflammatory, antioxidant, antidiabetic, antimicrobial activity and beneficial effects for the treatment of gastric ulcer (Bortolomedi et al., 2022). Kombucha is a beverage that is fermented by the action of microorganisms that feed on a carbon source.

It has been shown that the type and amount of these bioactive compounds in kombucha are associated not

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only with the properties of the black or green tea used but also with the fermentation conditions, such as fermentation time, pH, carbon source, temperature, and microorganisms of the genus *Acetobacter*, *Saccharomyces*, *Candida*, *and Pichia* (Ashrafi et al., 2018; Majid et al., 2023).

In recent years, the use of teas other than *Camellia sinensis*, such as leaves, fruits, herbs, spices, milk, cereals, mushrooms, and by-products from the food industry, have been tested as viable substrates for preparing kombucha beverages (Freitas et al., 2022).

There are plant species that have been studied for their excellent health benefits, such as the case of *Moringa oleifera*, which has been shown to contain bioactive compounds such as glucosinolates, flavonoids, phenolic compounds, carotenoids, tocopherols, fatty acids, minerals, vitamins and proteins that provide it with antimicrobial, antioxidant and nutrient-rich characteristics, making it a promising plant species with nutritional applications (Saucedo-Pompa et al., 2018).

Another plant with many applications is *Aloe vera*, the most used mucilage, which consists of approximately 99.5% water and 0.5% solid material composed of polysaccharides, vitamins, minerals, and phenols. Studies report its antimicrobial and antioxidant potential that favours maintaining the postharvest quality of fruits, preventing loss of moisture and firmness, as well as controlling the rate of respiration to prolong the shelf life of the products (Shakil et al., 2023).

Although *M. oleifera* and *A. vera* have various bioactive compounds and their health benefits have been demonstrated, their behaviour in the formulation and elaboration of fermented kombucha beverages has not been described. Therefore, the objective of the present study is to evaluate the chemical parameters of fermentation and antioxidant activity of kombucha based on *M. oleifera* with different concentrations of *A. vera.*

MATERIALS AND METHODS

Biological material

The leaves of *M. oleifera* were collected, dried in a forced convection oven at 40°C to constant weight, and stored to prepare infusions. The leaves of *A. vera* were collected, washed, and peeled to extract the mucilage, which was liquefied for 5 minutes until a homogeneous mixture was obtained. This procedure was performed every time mucilage was required to avoid browning.

The working bacterial consortium used was taken from a SCOBY composed of bacteria from the genera *Gluconoacentobacter*, *Lactobacillus*, *Lactococcus*, *Ruminococaceae*, *Propionibacterium*, and as unidentified yeasts. The inoculum was maintained with periodic replenishments of green tea infusion with dextrose at room temperature.

Fermentation

3.6 g/L of *M. oleifera* were placed for 15 minutes in distilled water at 85ºC, and different concentrations of *A. vera* mucilage and dextrose (T1 50:50, T2 75:25, T3 25:75%, w/w); only dextrose (50 g/L) was considered as the control. The infusions were filtered and cooled to be placed in the amount of 80 mL in glass bottles, which were inoculated with 3% (V/V) of the working consortium. The containers were covered with gauze and protected from light. All treatments (n = 3) were kept for 15 days at 25°C, and samples were taken on days 3, 6, 9, 12, and 15 for characterizing fermentation.

Characterization of the fermented product

Determination of acidity

The acidity of the fermented product was evaluated by volumetric titration with NaOH (0.1 N), taking 10 mL of sample (n = 5) and adding phenolphthalein (0.1%). The percentage of acidity was determined from the volume spent and the NaOH concentration. The results are expressed as percent acetic acid according to the following formula: % acetic acid = [(mL NaOH)(N NaOH)(0.06)]/(mL sample) × 100.

Determination of pH

The pH value was quantified with a multiparameter meter (n = 5) (Thermo Scientific™ Orion Star™ A211 benchtop pH meter, Massachusetts, USA).

Determination of total sugars

The phenol and sulfuric acid method quantified the concentration of total sugars (López-Legarda et al., 2017). To 1 mL of sample (n = 5), 5 mL of H_2SO_4 and 1 mL of phenol (5%) were added, allowed to stand for 10 min at room temperature, followed by a cold bath, and then analysed using spectrophotometry

(Evolution 201/220 UV Vis, ThermoFisher ScientificTM, Waltham, MA, USA) at 490 nm finally. Glucose at different concentrations was used as a standard for the calibration curve; the results are expressed in mg/mL.

Total phenols

Using the modified Folin-Ciocalteu method, the phenolic components were quantified (Singleton and Rossi, 1965). Na_2CO_3 at 7.5% and Folin reagent (1 N) were used. Inside a tube, 250 µL of the sample (n = 5), 800 µL of distilled water, 50 µL of Folin, and 800 µL of Na_2CO_3 were placed; it was kept for 30 minutes in the dark, and at the end of this time, the reading was done by spectrophotometry at 750 nm. The results were calculated using a calibration curve with different concentrations of gallic acid (GAE) and are expressed in µg GAE/mL.

2,2-diphenyl-1-picrylhydrazyl

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) technique was employed with the preparation method described by Vohra et al. (2019), with some modifications. The DPPH reagent (1 mM) was mixed with methanol (85%), and the absorbance was checked by spectrophotometry in a range from 0.7 and 1 at a wavelength of 517 nm. For the analysis, 50 µL of sample (n = 5) and 1 mL of DPPH reagent were taken, shaken, and kept in the dark for 30 minutes. The results were calculated with a Trolox (TE) calibration curve at different concentrations and expressed in mM TE/mL.

2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

Antioxidant activity by 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was performed as proposed by Tanticharakunsiri et al. (2020), with some modifications. A solution of ABTS (7 mM) and potassium persulfate (140 mM) was prepared. For the preparation of ABTS reagent, 88 µL of potassium persulfate was mixed in 5 mL of ABTS reagent and mixed with pure methanol. The absorbance was checked spectrophotometrically in the range between 0.7 and 1 at a wavelength of 734 nm. The samples were quantified by mixing 25 µL of the fermentate (n = 5) and 1 mL of ABTS reagent, kept in darkness for 45 minutes, and finally analysed by means of spectrophotometry (734 nm). The results were calculated with a Trolox (TE) calibration curve at different concentrations and expressed as mM TE/mL.

Ferric reducing antioxidant power

The antioxidant activity quantified by the ferric reducing antioxidant power (FRAP) technique was performed as reported by Aung and Eun (2021). The solutions used were hydrochloric acid at 40 mM, sodium acetate buffer (pH 3.6), ferric chloride (0.54 g in 10 mL), and 2, 4, 6-Tris-(2-pyridyl)-s-triazine (TPTZ) (0.156 g in 5 mL of 40 mM HCl). In individual vessels, 10 mL of buffer, 10 mL of ferric chloride, and 5 mL of TPTZ reagent were placed in a dry bath until 37°C was reached. A beaker covered in light was used to prepare the FRAP reagent, and 10 mL of buffer, 1 mL of ferric chloride, and 1 mL of TPTZ were mixed. Quantification was performed by mixing 25 µL of the fermentate (n = 5) and 1 mL of the FRAP reagent. The samples were kept in the dark for 90 minutes, and the reading was performed spectrophotometrically at 595 nm. A calibration curve was prepared at different concentrations of Trolox (TE), and the results are expressed in µM TE/mL.

Statistical analysis

The data were statistically processed using the MiniTab 17 programme, performing the analysis of variance (ANOVA), where comparisons of means were made, and Tukey's method was used when there was a significant difference between treatments ($p < 0.05$). Graphs were constructed in OriginPro 8.0.

RESULTS AND DISCUSSION

Determination of acidity

The acidity of the control and T2 increased considerably as the days of fermentation passed, obtaining the highest concentrations at day 12, at 3.2 ±0.98 and 2.96 ±0.96%, respectively (Fig. 1). At the same time, T1 and T3 obtained the highest acidity percentage on day 6, with 1.6 ±0.57 and 1.06 ±0.31, respectively. Statistical differences ($p < 0.05$) were found between treatments, T3, control and T2 with the rest of the treatments. Treatment T3 had the lowest concentration of *A. vera* and T2 the highest, suggesting a relationship between its concentration and the percentage of acidity.

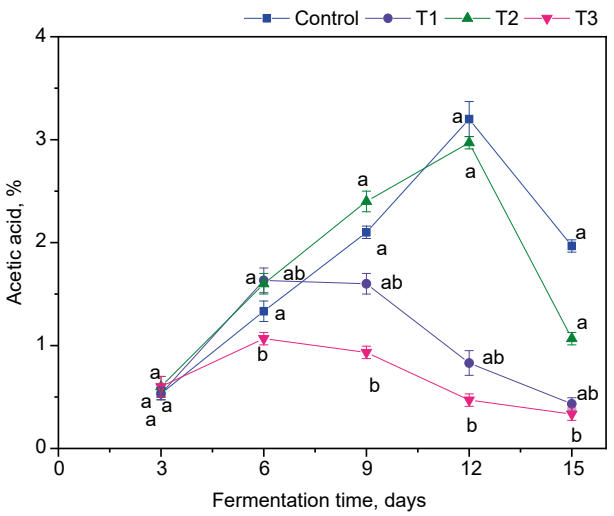

Fig. 1. The percentage of acidity values of kombucha beverages during the fermentation process ($p < 0.05$). Control: dextrose (100%); T1: *A. vera* (50%) + dextrose (50%); T2: *A. vera* (75%) + dextrose (25%); T3: *A. vera* (25%) + dextrose (75%). Different letters indicate significant differences ($p \leq 0.05$)

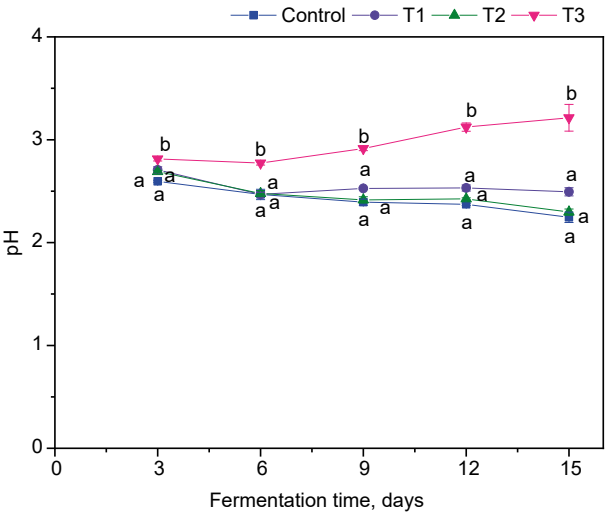

Fig. 2. The pH values of kombucha beverages during the fermentation process ($p < 0.05$). Control: dextrose (100%); T1: *A. vera* (50%) + dextrose (50%); T2: *A. vera* (75%) + dextrose (25%); T3: *A. vera* (25%) + dextrose (75%). Different letters indicate significant differences ($p \leq 0.05$)

This behaviour may be due to the activity of lactic and acetic acid bacteria that consume glucomannan polysaccharide, glucose, galactose, arabinose, fructose, and other hydrolyzable sugars present in the mucilage and convert them to organic acids (Jayabalan et al., 2014).

Determination of pH

The pH of the control, T1, and T2 decreased as fermentation time passed without obtaining a significant difference ($p < 0.05$), obtaining values of 2.24, 2.49, and 2.29 at the end of fermentation. Meanwhile, T3 reached a value of 3.21 on day 15, statistically different from the rest of the treatments (Fig. 2). Low pH values have been reported in fermentations of laver kombucha (*Porphyra dentata*) and black tea-based kombucha, which is indicative of a successful fermentation due to the production of organic acids, mainly acetic acid (Aung and Eun, 2022). The pH values obtained are characteristic of microbial activity of Gluconobacter, Saccharomyces, and Brettanomyces genera, primarily found in a kombucha fermentate (Sanwal et al., 2023). Soluble compounds (vitamins, lipids, carbohydrates) in *A. vera* can modify the pH, which changes microbial activity in the fermentate.

Determination of total sugar

The control and T3 treatments showed significant differences ($p < 0.05$) during the entire fermentation period, except for 12 days. At the end of fermentation, no differences were found between treatments (Fig. 3). The treatments with the highest concentration of total sugars were the control (6640 ±294.22 mg/mL) on day 12 and T2 (3340 ±50.91mg/mL) on day 6. Because *A. vera* contains mannose, glucose, galactose, xylose, and arabinose, a drastic drop in concentration was not observed, as the microorganisms have different carbon sources that they can utilize during fermentation (Neffe-Skocińska et al., 2017). It was observed that by varying concentrations of *A. vera*, there are statistical differences in the contribution of sugars during fermentation, which may be associated with the complexity of the substrate and the consumption rate of the microorganisms. However, on day 15 of fermentation, no differences in sugar concentration are shown. This indicates that the fermentation process is similar between substrates. Replacing dextrose with *A. vera* adds vitamins, minerals, anthroquinones, amino acids and enzymes to the fermented product (Domínguez-Fernández et al., 2012), generating a better functional beverage.

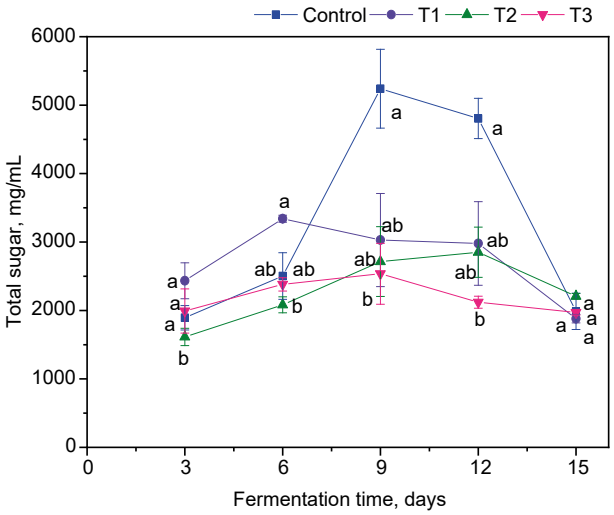

Fig. 3. Total sugar concentration of kombucha beverages the during the fermentation processes ($p < 0.05$). Control: dextrose (100%); T1: *A. vera* (50%) + dextrose (50%); T2: *A. vera* (75%) + dextrose (25%); T3: *A. vera* (25%) + dextrose (75%). Different letters indicate significant differences ($p \leq 0.05$)

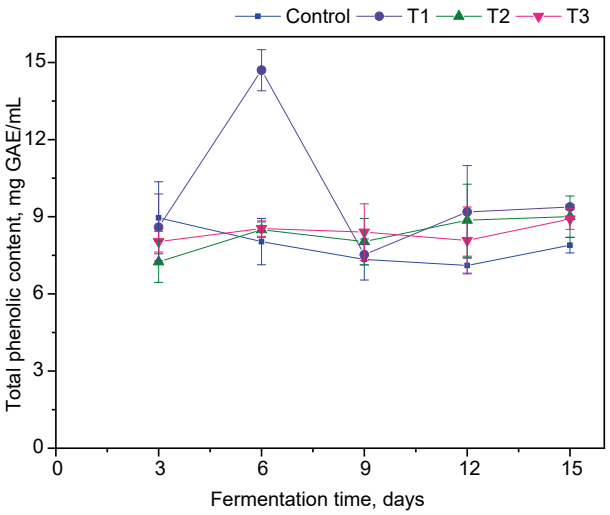

Fig. 4. Concentration of phenolic compounds in kombucha beverages during the fermentation processes ($p > 0.05$). Control: dextrose (100%); T1: *A. vera* (50%) + dextrose (50%); T2: *A. vera* (75%) + dextrose (25%); T3: *A. vera* (25%) + dextrose (75%)

Phenolic compounds

The content of total phenols increased as the days of fermentation passed. However, no significant differences were obtained between treatments ($p < 0.05$). T1 had the highest concentration (1.80 ±1.96 mg GAE/mL) on day 6 (Fig. 4). The control, T2, and T3 treatments obtained a total phenol content of 8.26 ±0.39, 8.87 ±1.40, and 8.91 ±0.42 mg GAE/mL at 3, 12 and 15 days, respectively. Cardoso et al. (2020) report in green tea and black tea kombucha 0.70 ±0.09 and 1.09 ±0.07 mg GAE/mL, respectively, values well below those reported in the present work. The concentration of total phenols in the fermentates is related to compounds present in *A. vera* and *M. oleifera*, such as barbaloìna, gallic acid, chlorogenic acid, coumaric acid, flavonoids, tannins, coumarins, quinones, and anthocyanin (Amaya et al., 2022; Rodríguez-González et al., 2023).

DPPH

In the antioxidant activity by DPPH, a trend of increasing activity was observed as the days passed (Fig. 5). However, no statistical differences ($p < 0.05$) were found between treatments. Treatments T2 and

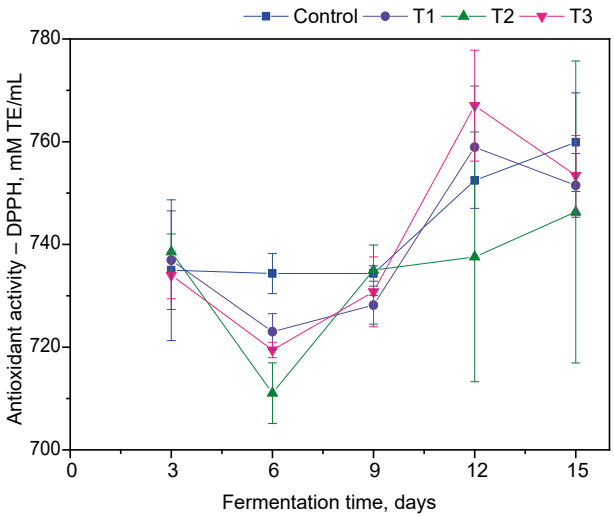

Fig. 5. Antioxidant activity of the DPPH radical of kombucha beverages during the fermentation process ($p > 0.05$). Control: dextrose (100%); T1: *A. vera* (50%) + dextrose (50%); T2: *A. vera* (75%) + dextrose (25%); T3: *A. vera* (25%) + dextrose (75%)

the control showed a higher capacity to eliminate the radical on day 15 (763.30. ±0.69 mM TE/mL and 759.90 ±9.56 mM TE/mL), while T3 and T1 on day 12 (767.02.93 ±10.83 mM TE/mL and 758.93 ±11.93 mM TE/mL). This behaviour may be due to the concentration of *A. vera* used, which is reported to have antioxidant activity due to anthraquinones (Franco-Quin et al., 2016). The values obtained in the present investigation are higher than those reported by Kaewkod et al. (2019); in green tea-based fermentates (0.0155 mM GAE/ml) and those reported by Braham et al., (2019); in moringa leaf extracts (530 µmol ET/g DM).

ABTS

The ABTS method determines the radical reduction activity by transferring electrons or hydrogen atoms, which is determined by decolorization. The antioxidant activity of the ABTS radical of the fermented decreases over time, and no statistical differences ($p < 0.05$) were found between treatments (Fig. 6). The highest concentrations of the radical were quantified on days 3 and 6, ranging from 771 to 786 mM TE/mL. In green tea fermentations, concentrations of 8.22 ±0.86 µM TE/mL have been reported (Cardoso et al., 2020), values lower than those obtained in this research. This behaviour may be due to the presence of flavonoids and anthroquinones that inhibit the generation of reactive oxygen species and are present in *A. vera* and vitamins A, C, E, isothiocyanates, polyphenols and quercetin present in *M. oleifera* that act as an electron transfer mechanism preventing oxidation (Franco-Quino et al., 2016; Cohen et al., 2017; Wang et al., 2020).

FRAP

The FRAP method consists of the redox reaction between the antioxidant substance and Fe^{3+} ions through electron transfer, in which the ion is reduced to Fe^{2+} and determined according to the colour change. The results show a trend of increasing concentration as the days of fermentation pass, with no significant difference ($p < 0.05$) between treatments (Fig. 7). The control treatment and T1 had the highest concentrations, 83.69 ±2.19 mM TE/mL and 78.94 ±2.82 Mm TE/mL, respectively, on days 15 and 12. While T2 and T3 on day 12 presented the highest concentrations, 55.10 ±7.31 mM TE/mL and 65.20 ±8.46 mM TE/mL, respectively. The concentrations in the present study are higher than

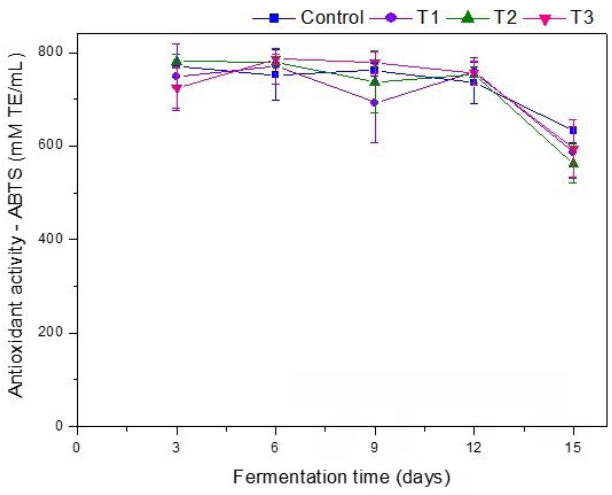

Fig. 6. Antioxidant activity of the ABTS radical of kombucha beverages during the fermentation process. Control: dextrose (100%); T1: *A. vera* (50%) + *dextrosa* (50%); T2: *A. vera* (75%) + *dextrosa* (25%); T3: *A. vera* (25%) + *dextrosa* (75%). Bars with the same letter mean that there are no significant differences ($p \leq 0.05$)

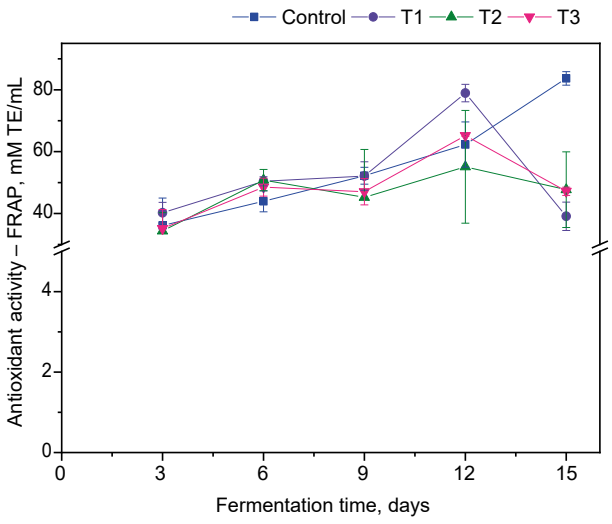

Fig. 7. The FRAP values of kombucha beverages during the fermentation process ($p > 0.05$). Control: dextrose (100%); T1: *A. vera* (50%) + dextrose (50%); T2: *A. vera* (75%) + dextrose (25%); T3: *A. vera* (25%) + dextrose (75%)

those reported by Aung and Eun (2021), who reported 5.26 mM TE/mL in black tea, 5.67 mM TE/mL in green tea, and 0.86 mM TE/mL in an algal infusion. The above indicates that the presence of *A. vera* and *M. oleifera* increases the antioxidant activity capable of reducing Fe ions by the presence of phytochemical compounds (Zubaidah et al., 2019; Barragán et al., 2023). This increase in antioxidant activity may be due to a higher affinity for iron ions and radicals structurally similar to the ABTS cation (Wang et al., 2020).

In the fermentation process, kombucha microorganisms can change the bioactive compounds present in the fermentation medium through enzymatic action and substrate type, favouring the increase of antioxidant compounds (Ashrafi et al., 2018; Jakubczyk et al., 2020).

CONCLUSION

In this research, we found that using *M. oleifera* and *A. vera* to elaborate on kombucha is a good alternative due to the contribution of bioactive compounds. During fermentation, an increase in the antioxidant activity of the DPPH radical, total phenols, and iron reduction in FRAP was observed. Sugar consumption was similar among treatments, which indicates that dextrose can be replaced by *A. vera* mucilage, reducing the concentration of added sugars. The kombucha obtained is an alternative beverage containing antioxidants, phenolic compounds and no added sugars. Future studies should focus on consumer perception and acceptance and the evaluation of fermentation parameters to increase antioxidant activity and phenol content.

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DECLARATIONS

Data statement
All data supporting this study has been included in this manuscript.

Ethical Approval
Not applicable.

Competing Interests
The authors declare that they have no conflicts of interest.

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