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MICROBIOLOGY

Development of flavored kombuchas with Amazonian fruits: bioactive compounds evaluation and antioxidant capacity

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Abstract: Using Amazonian fruits to flavor kombuchas is a promising proposal, as it adds nutritional value to the drink. This work sought to develop kombucha flavored with Amazonian fruits and evaluate the bioactive compounds and antioxidant capacity of the formulations. Three kombucha formulations were prepared using green tea (*Camellia sinensis*) and three Amazonian fruits: cupuassu (*Theobroma grandiflorum*), tapereba (*Spondias lutea* L.) and bacuri (*Platonia insignis*). Kombucha fermentations were evaluated before and after the insertion of nectars through the analysis of phenolic compounds, vitamin C and antioxidant capacity. Analyzes of pH, total sugars, acetic acid, ethanol, and microbiological characterization of final formulations were also carried out. For the first fermentation, were found values of phenolic compounds and antioxidant capacity of 30.60 ± 0.93 mg EAG/L and 295.02 ± 5.59 µmol ET/mL, and the formulation with tapereba showed the highest values for total phenolic compounds (34.92 ± 12.25 mg EAG/L), antioxidant capacity (320.57 ± 9.53 µmol ET/mL) and vitamin C (198.25 mg/100g). Thus, the formulations developed had a crucial nutritional appeal to stimulate consumption by the population, in addition to enabling the valorization and addition of commercial value to the Amazonian fruits used.

Key words: Cupuassu, Bacuri, Flavoring,Fermentation,Vitamin C, Symbiotic Colony.

INTRODUCTION

Kombucha is a fermented food originated in Asia, based on black tea or sweetened green tea (5-10% sugar) and a biofilm of bacteria and yeast called *SCOBY (Symbiotic Colony of Bacteria and Yeast)* (Marco et al. 2021). The final product is obtained after at least 14 days of the mixture fermentation. It is important not to confuse this product with tisanes (infusion with other herbs), produced by infusing dry or fresh leaves, flowers, or roots of other plants (Freitas et al. 2022). Large-scale consumption of this beverage is recommended due to the high antioxidant capacity due to the bioactive compounds present in tea after fermentation, which makes it beneficial to health (Dutta & Paul 2019), in addition to other benefits, such as reduction and control of cholesterol and diabetes levels (Bellassoued et al. 2015, Hosseini et al. 2015).

Kombucha is made using various subtypes of *Camellia sinensis*, such as black tea and green tea. However, other inputs are part of its production, both in the first fermentation and the second fermentation (tasting stage), such as fruit pulp, plant extracts, other teas, and spices (Zubaidah et al. 2022). Thus, flavouring kombucha with different types of fruit, such as those of Amazonian origin, is a promising proposal, considering that some fruits from the region have a high amount of bioactive compounds with high antioxidant activity, which

can add nutritional value to the food drink (Santos et al. 2018).

In addition, to obtain a quality fermented beverage, the proportion of its components (tea, sugar, and culture) must be well adjusted. Therefore, it is noteworthy that the amount of sugar (sucrose) used directly interferes with the acidity and pH of the product since the variable acts as a substrate for the generation of carbon dioxide, ethanol, and organic acids by microorganisms (Nascimento & Lima 2019). The organic compounds produced by kombucha give the drink a functional character, as many studies show the relationship between a healthy diet and the consumption of its compounds (such as flavonoids), in addition to attesting to its role in combating chronic diseases such as obesity, acting as an inflammation modulator (Dário & Weschenfelder 2020). The long history of consumption of this beverage has global appeal and has demonstrated through the scientific literature antimicrobial effects and benefits to the general health of the consumer (Mohsin et al. 2022).

Several Amazonian fruits have great commercial appeal for consumption due to their flavor, color, and intrinsic health benefits that are associated with nutritional and antioxidant properties, such as cupuaçu (Alvarez et al. 2017), bacuri (Yamaguchi et al. 2021) and taperebá (Aniceto et al. 2021). Combining these characteristics with the already recognized properties of kombucha allows the development of commercially attractive biotechnological products with high nutritional potential.

Although there are already many commercial kombuchas, there are practically no products flavored with Amazonian fruits. For that reason, this work demonstrates innovation by developing three different kombucha formulations, with the inclusion of recognized Amazonian fruits, with the premise of obtaining new products with a differentiated taste, high antioxidant and nutritional capacity.

MATERIALS AND METHODS

Materials

The green tea used was obtained from the company CHÁ DÕ® (São Paulo, Brazil) and the SCOBY colony was donated from the city of Belém. Sugar (União, São Paulo, Brazil) and fruit pulp from Petruz® (Belém, Brazil) were purchased from a supermarket chain in the city of Belém.

Methods

Tea preparation

To prepare the tea, 2 liters of water were boiled at 100°C, leaving it in the boiling phase for 3 minutes so that all the chlorine present was removed (Silva et al 2021). 14 g of *Camellia Sinensis* herb (green tea) was added and left to infuse for 15 minutes, with the addition of 40 g of sugar. The preparation was filtered and the substrate (mixture of tea + sugar) was left to cool at room temperature.

First fermentation

The first fermentation was carried out by adding the SCOBY to the previously prepared and sweetened tea. The SCOBY used already contained a small amount of green tea, in which it is normally stored. The mixture was added to a 2 L beaker at 25 °C and the container was closed and stored for 7 days (Figure 1).

Characterization of the first fermentation process

The following analyzes were performed: determination of pH, using a calibrated benchtop pHmeter (Onda Digital®), titratable total acidity (using a standardized 0.1 N NaOH solution),

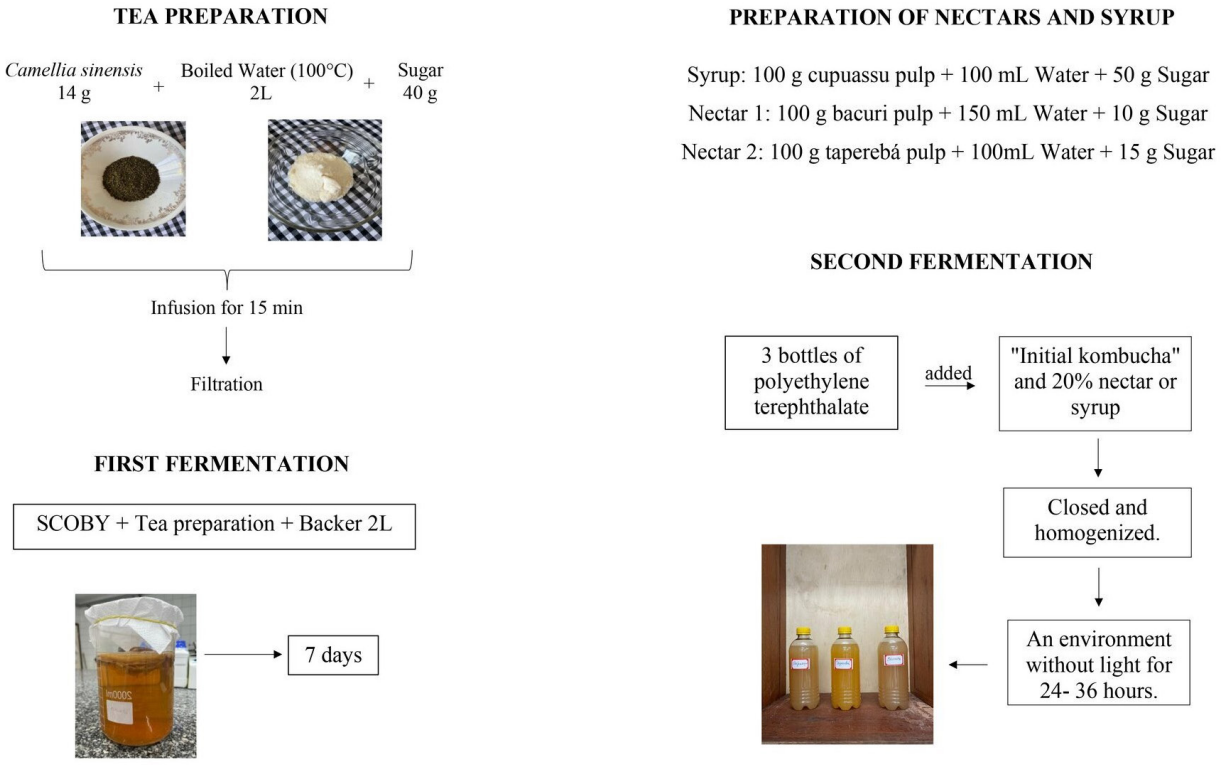

Figure 1. The production stages of kombucha fermentations.

total soluble solids with a digital refractometer (Kasvi®), all analyzes followed AOAC (2019).

Nectar and syrup preparation

The nectars were standardized based on legislation (Brazil 2013). 100 g of pulp were used for all the preparations, while the amounts of sugar and water were defined by preliminary tests, generating the final formulations (Table I). For the bacuri nectar, 150 mL of water and 10 g of sugar were used, and for the taperebá nectar, 100 mL of water and 15 g of sugar. The cupuaçu pulp was made into syrup, to which 100 mL of water and 50 g of sugar were added. All the mixtures were homogenized, filtered and stored at room temperature.

Second fermentation

For the second fermentation, 3 bottles of polyethylene terephthalate with a capacity of 500 mL were used, in which the product of the first fermentation was added: "initial kombucha" and 20% nectar or syrup, values shown below in Table I (Kombucha Brewers International, 2021). The container was closed, homogenized, and placed in an environment without contact with light for a period of 24-36 hours.

Fermented products characterization

Initial kombucha and flavored kombuchas pH, acidity, soluble solids and reducing sugars

The pH, total titratable acidity and soluble solids were determined using the same parameters explained in the previous topic. Furthermore, analyses of reducing sugars were performed by DNS in a spectrophotometer (Cirrus 80 PR, Femto®) at 540 nm. For such analysis, a 1.0 g/L glucose solution was used for the calibration curve. A 2 N NaOH solution and a DNS reagent solution were prepared, containing 1 g of 3,5-dinitro salicylic acid, 30 g of double sodium

Table I. Composition of Formulations.

Formulation	KC	KT	KB
Initial Kombucha	400mL	400mL	400mL
Pulp of fruit	100g (40%v/v)	100g (46%v/v)	100g (40%v/v)
Water	100mL	100mL	150mL
Sugar	50g	15g	10g

KC (Kombucha with Cupuassu); KT (Kombucha with Tapereba); and KB (Kombucha with Bacuri).

potassium tartrate, and 20 mL of NaOH 2N solution following the methodology described by Miller (1959).

Total phenolic compounds and antioxidant capacity

The analysis of total phenolic compounds followed the Folin-Ciocalteu method described by Singleton & Rossi (1965), and it took place in a microplate reader (NovoStar, BMG LabTech®) with a filter of 725 nm. A calibration curve was prepared with gallic acid (Sigma-Aldrich ®) at a concentration ranging from 25-200 µg/mL. The results were expressed in mg of gallic acid equivalents (GAE)/mL or mg of extract.

The DPPH method (stable free radical 2,2-diphenyl-1-picrylhydrazyl) followed the method described by Macedo et al. (2011) to assess the antioxidant capacity of kombuchas. The analyses and reactions took place in a 96-well microplate reader (NovoStar, BMG LabTech®) at an absorbance of 520 nm. The reaction mixture followed the sequence: 50 µL of the sample under study was mixed with 150 µL of DPPH reagent (Sigma-Aldrich®) prepared at 0.2 mM in methanol. After the reaction period (90 min), DPPH decolorization was measured. The results were expressed as µmol Trolox equivalent (TE)/mL of kombucha.

Vitamin C content

The test was carried out according to Strohecker (1967), who based it on Tillmans' titrimetric method, in which the ascorbic acid content is measured by reducing the reagent 2,6-dichlorophenolindophenol (DFI). Solutions of 1% oxalic acid, 0.2% sodium 2,6-dichlorophenolindophenol and 50 mg/mL ascorbic acid were used. After standardizing the DFI solution, 4 mL of the sample extracts were collected and added to a 125 mL erlenmeyer flask with 46 mL of deionized water, which was titrated with the standardized DCFI solution until a persistent pink color appeared for 15 seconds. The results were expressed in mg of vitamin C/100g of extract.

Ethanol content by HPLC

Ethanol was quantified in the samples using the methodology described by Oliveira et al. (2020). A 300 mm × 7.8 mm column (HPX-87H,Aminex®) contained in a liquid chromatograph (1260 Infinity, Agilent®) was used. This equipment had an IR refractive index detector and UV-vis DAD. The mobile phase used was 0.05 M H2SO4 with an eluent flow rate of 0.6 ml/min at 30°C. 15 µl of the sample were injected and the total analysis time was 30 min.

Microbiological analyzes

Aerobic mesophiles, molds, yeasts and thermotolerant coliforms were analyzed. 25 ml of sample was diluted in 225 ml of distilled water for all counts. The mesophile count was carried out according to Apha (1992), by taking 1 ml from three or more dilutions and placing it in a Petri dish with Plate Count Agar (PCA) culture medium (Kasvi ®), followed by incubation in an

oven at 35°C for 48 hours. Counting was carried out on a Quebec counter and plates containing 30 to 300 colonies were selected.

For molds and yeasts analyses, the DRBC agar base medium (Kasvi®) was used. 0.01 µL of sample was inoculated onto plates with solidified medium and spread with a Drigalski loop. The plates were incubated in an oven at 37°C for 72 hours (Apha 1992). Finally, thermotolerant coliforms were analyzed using Lauryl Sulphate broth (Kasvi®). The presumptive test was carried out by immersing the inverted Duran tube inside a test tube with the sample and incubating it in an oven at 37°C for 24 to 48 hours (Silva et al 2017).

Statistical Analysis

For statistical evaluation, all the samples were tested in triplicate and the means and standard deviations were calculated. A comparison was performed between the samples using the Tukey test ($p \leq 0.05$), using the Minitab 16.1.1 statistical program.

RESULTS AND DISCUSSION

Characterization of the fermentation process of initial kombucha

Figure 2 shows the parameters evaluated over the 14 days of the first fermentation. According to the figure, it was possible to verify a standard behavior of the kombucha fermentation processes, in which there is, over time, a reduction in the values of soluble solids (°Brix), a reduction in the pH values with a consequent increase in acidity, caused mainly by the increase in acetic acid levels (Zubaidah et al. 2018).

Figure 2. Variation of values of Brix° (a), pH (b) and Acidity (c) during the first fermentation.

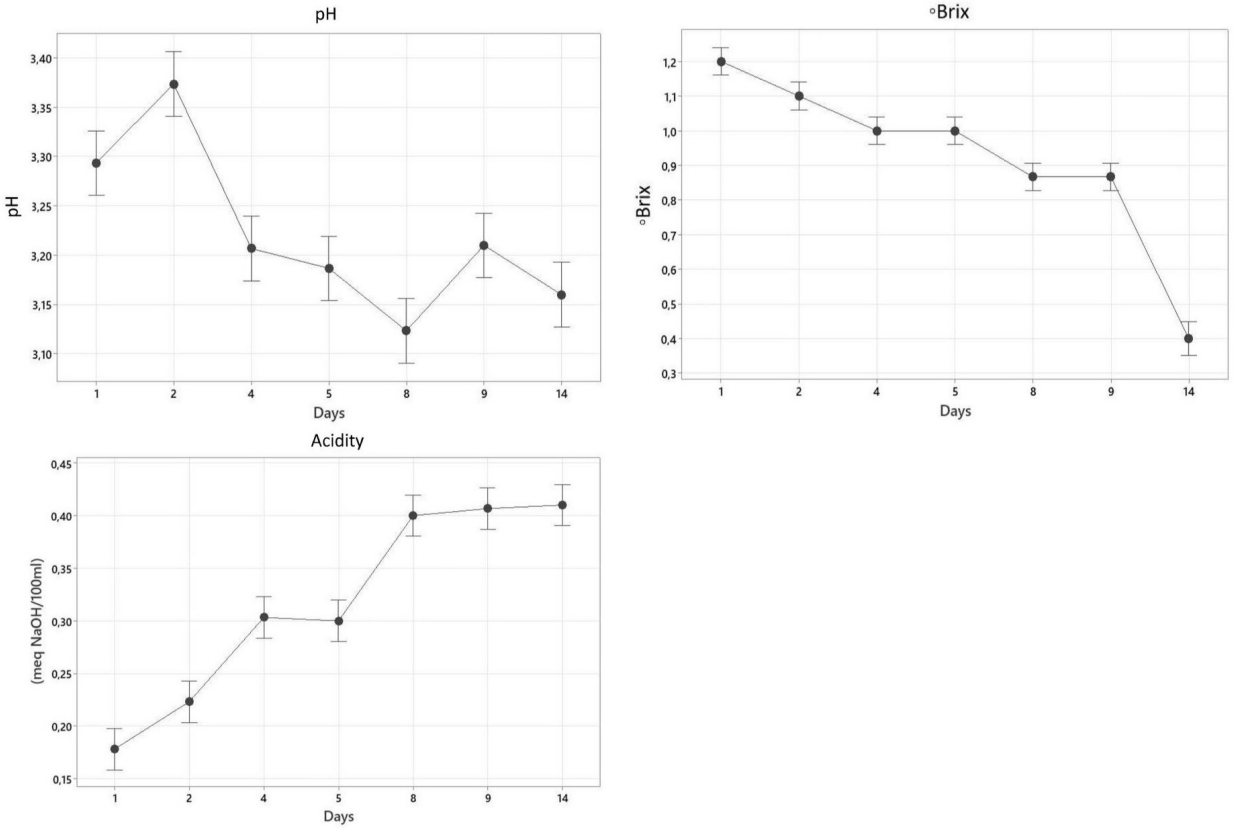

Figure 2. Variation of values of Brix° (a), pH (b) and Acidity (c) during the first.

The average values of soluble solids along the first ranged from 1.2±0.00-0.4±0.00 °Brix; for the pH values, the range of 3.28 ± 0.005 – 3.13 ± 0.025 was found and finally, the increase in acidity during the analyzed period (0.18 ± 0.01-0.41 ± 0.00 g.100 mL^{-1}), the decrease in soluble solids, and the increase in acidity occurred due to the consumption of sucrose during the fermentation process and the production of organic acids, such as acetic acid, by aceto-acid bacteria present in the symbiotic colony (Muzaifa et al. 2022).

Physicochemical characterization of initial kombucha and flavored kombuchas

The values quantified in the characterization of traditional and flavored kombuchas, shown in table II, are in accordance with the safety and quality standards for the type of fermented beverage produced, provided for in Normative Instruction No. 41 (Brazil 2019) and international legislation, according to Kombucha Brewers International – Code of Practice (Kombucha Brewers International 2021), about pH range, titratable acidity and amount of sugars.

Table II. Physicochemical characterization of initial kombucha and flavored kombuchas.

The pH values directly linked to acidity, present in Table II, maintain an inversely proportional relationship with the fermentation time so that more extended fermentation periods promote acidification of the medium and a consequent decrease in pH. Therefore, the data obtained for kombucha in the first fermentation (KF1) (pH = 3.2) and flavored drinks with pH values of 3.18; 3.10 and 2.98 for KB, KC and KT, respectively, have a significant decrease ($p \leq 0.05$), which is coherent with the longer fermentation time and the addition of sugars from the nectars.

Acetic acid is one of the most prevalent organic compounds in kombucha, a result of the fermentation process of bacteria present in the drink, and responsible for the vinegary flavor (Amarasinghe 2018, Khosravi et al. 2019). In the present work, the acetic acid calculated for kombucha KF1 e KT were similar (0.39 ± 0.00 and 0.39 ± 0.11 g acetic acid.mL^{-1}), and statistically different when compared to the other flavored kombucha (KC= 0.49 ± 0.03 and KB=0.41 ± 0.01), which demonstrated higher acidity. The data obtained were higher than those determined by Abuduaibifu and Tamer (2019), in their flavored kombuchas with goji berry and black tea substrate and those observed by Neffe-Skocińska et al. (2017) (1.42 to 1.52 g. L^{-1}).

The soluble solids analisis (°Brix) for the initial kombucha (1.0 ± 0.00) demonstrated lower amount than what is found in the literature, in studies with green tea-based kombucha at the end of the fermentation

Table II. Physical-chemical characterization of initial kombucha and flavorings.

Analysis	KF1	KC	KT	KB	IN. N 41/2019
pH	3.20 ± 0.03 ^{d}	3.10 ± 0.02^{a}	2.98 ± 0.05^{b}	3.18 ± 0.02^{c}	2,5- 4,2
Total soluble solids (°Brix)	1.0 ± 0.00 ^{d}	4.01 ± 0.00^{a}	2 ± 0.00^{b}	2.11 ± 0.00^{b}	-
Total Titratable Acidity (meq NaOH.100mL-1)	65.3 ± 0.60 ^{d}	81.01 ± 1.73^{a}	65.03 ± 1,00^{c}	70.04 ± 1.00^{b}	30 - 130
Total Titratable Acidity (g acetic acid.mL^{-1})	0.39 ± 0.00 ^{d}	0.49 ± 0.03^{a}	0.39 ± 0.11^{b}	0.41 ± 0.01^{c}	-
Reducing sugars (g.L^{-1})	0.09 ± 0.00^{d}	0.23 ± 0.03^{a}	0.39 ± 0.10^{b}	0.16 ± 0.00^{c}	-
Ethanol (g.L^{-1})	0.54±0.021^{a}	5.07±0.33^{b}	3.51±0.32^{c}	2.27±0.21^{d}	-

KF1 – Kombucha first Fermentation (inicial Kombucha); KC (kombucha with Cupuassu); KT (Kombucha with Tapereba); and KB (Kombucha with Bacuri); IN.N 41/2019- Normative Instruction N 41 September, 17 of 2019.

process (Treviño-Garza et al. 2020). For Akarca (2021), the significant reduction in soluble solids is associated with the hydrolysis of sucrose into the monosaccharides glucose and fructose, consumed by microorganisms for the production of different metabolites. However, there was a significant increase ($p \leq 0.05$) in preparations with the Amazonian fruits (KC=4.01 ± 0.00; KT=2,00 ± 0.00; KB=2.00 ± 0.00), which was expected from the flavoring process.

Sugar is an essential element in the preparation of kombucha and its quality and quantity may or may not boost the fermentation process (Martínez et al. 2018). Therefore, the reducing sugars estimated in the flavorings were significantly increased ($p \leq 0.05$) compared to the initial kombucha (Table II).

The present work demonstrated lower values of reducing sugars than those found in the literature, such as Laver's kombucha (*Porphyra dentata*), which presented 0.45 $g.L^{-1}$ (Aung & Eun 2021). In this view, to Neffe-Skocinska et al. (2017), fructose and glucose development through yeasts fermentation, the chosen substrate and the amount of sugar added during the process can increase the amount of reducing sugars.

The lowest value of ethanol we observed in KF1, which refers to kombucha without flavoring. The insertion of fruit pulps allowed the addition of sugars capable of promoting the chemical transformation characteristics. Thus, it is possible to verify the ethanol concentation varied between 0.54 - 5.07 $g.L^{-1}$, with KC being the most alcoholic, probably due to the higher content of added sugars in the form of commercial sucrose, and transformed by the action of scoby yeast, in ethanol. Nhan et al. (2020) in their fermentation with the addition of typical fruit of Vietnam, found an ethanol value within the range obtained for developed formulations (0,88 $g.L^{-1}$). Generally, Kombucha's ethanol content generally ranges from 3.6 to 10 g/L^{-1}, including values observed for developed kombuchas (Jayabalan et al. 2014, Greenwalt et al. 2000).

Besides, the highest levels of ethanol in the amazonian fruits flavored kombuchas is significant ($p \leq 0.05$), when compared to the initial kombucha. Table III shows the results found for the analysis of phenolic compounds and antioxidant capacity.

The amount of phenolic compounds in kombucha is related to several factors, such as the type of tea used, the amount of sugar, fermentation time, and temperature (Antolak et al. 2021). The values found for the phenolic compounds in the present work did not show any statistical difference between them at 95 % statistical confidence. It's important to notice that there was an increase in total phenolic compounds in flavored formulations compared to KF1, which is explained by the addition of fruit nectars and the phenolic compounds biotransformation. Besides, the values estimated were smaller than those observed in the literature for other fruits (Dada et al. 2021, Gramza-Michaiowska et al. 2016, Kayisoglu & Coskun 2021).

The bacuri flavored kombucha (KB) showed the highest value of total phenolic

Table III. Analysis of Phenolic Compounds and Antioxidant Activity.

Component	K F1	KC	KT	KB
Total Phenolic Compounds (mg EAG.L^{-1})	30,60 ± 0,93^{a}	33,40 ± 1,60^{a}	34,92 ± 12,25^{a}	38,3 ± 0,93^{a}
Antioxidant capacity (µmol ET.mL^{-1})	320,57 ± 9,53^{a}	295,02 ± 5,59^{b}	295,39 ± 3,89^{b}	307,98 ± 2,51^{ab}

KF1 – Kombucha first Fermentation (inicial Kombucha); KC (kombucha with Cupuassu); KT (Kombucha with Tapereba); and KB (Kombucha with Bacuri).

compounds (38.3 ± 0.93 mg GAE.L^{-1}) compared to the formulation with tapereba (KT) (34.92 ±12.25 mg GAE.L^{-1}) and cupuassu (KC) (33.40 ± 1.60 mg GAE.L^{-1}). This fact may be related to the different phenolic profiles of each fruit and how these phenols behave in the face of a biotransformation process during fermentation (Wang et al. 2020).

Regarding antioxidant activity, when comparing the result obtained by kombucha from the first fermentation (295.02 ± 5.59 µmolET. mL^{-1}) to some literature, our result was lower than that of Zou et al. (2021). Antioxidants are sensitive to some environmental factors, such as pH; temperature and oxidation time, and are usually related to the amount of phenolic molecules in the studied sample (Degirmencioglu et al. 2021 and Tang et al. 2022). The KB formulation showed the highest antioxidant activity value (307.98 ± 2.51 µmolET.mL-1), followed by the KT (295.39 ± 3.89 µmolET.mL-1) and KC (295,02 ± 5,59 µmolET.mL-1), similar to what was observed for total phenolic compounds.

Figure 3 shows the vitamin C estimated at the initial and final stage of the first fermentation, the fruit nectars and the same stages of the flavored kombucha formulations.

Figure 3. Quantification of vitamin C during kombucha production.

Regarding the quantification of vitamin C (Figure 3), after adding fruit nectars, the vitamin C levels of all formulations increased significantly ($p \leq 0.05$). At the end of second fermentation, it was observed increased levels of vitamin C in all developed formulations, similarly when *Annona muricata* was used by Candra et al. (2021). The flavor with the highest concentration was kombucha with tapereba (398.38 ± 2.22 mg.100 mL^{-1}), followed by bacuri flavoring (375.25 ± 2.22 mg. 100 mL^{-1}) and finally the fermented drink with cupuassu (360.57 ± 13.12 mg.100 mL^{-1}).

The value obtained for flavored kombucha with cupuassu, the sensitivity to sudden changes

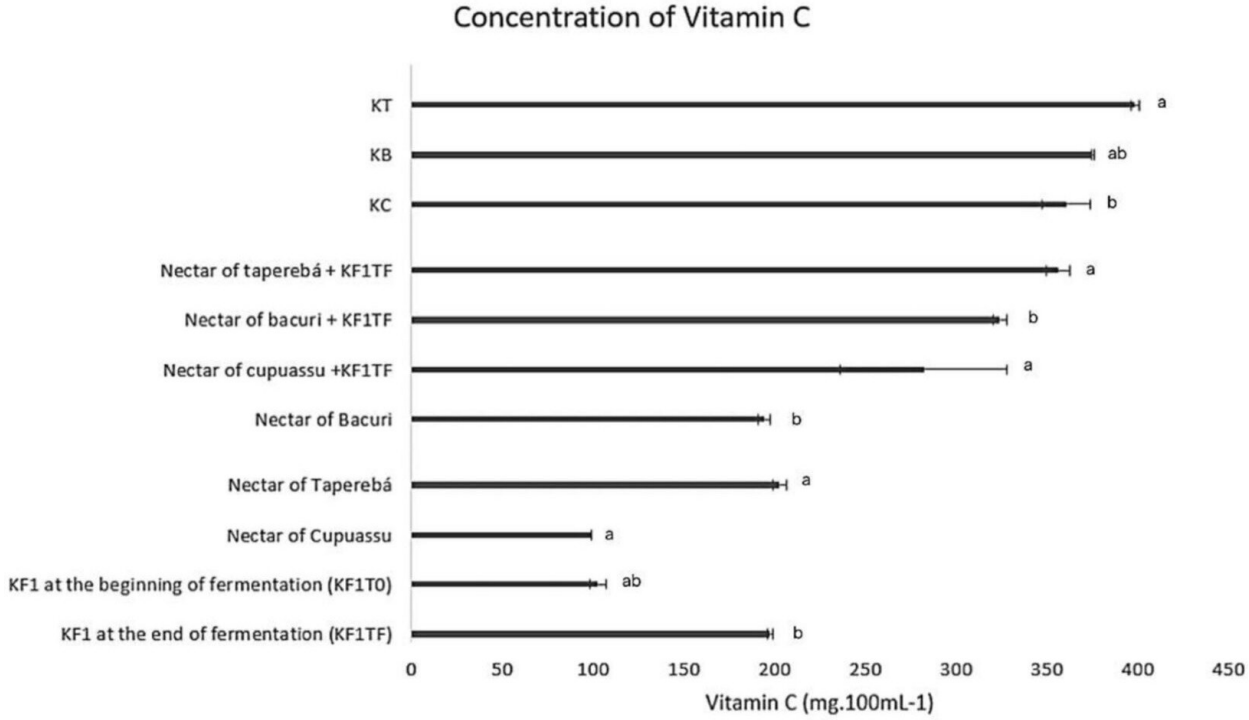

Figure 3. Quantification of vitamin C during kombucha production.

in temperature and light may be responsible for the decreased vitamin C levels in the drink. This fact was demonstrated by Vieira et al. (2000), who evaluated the kinetics of the degradation of ascorbic acid present in cupuassu nectar, which proved to be unstable to various external factors.

After the first fermentation (KF1T0), the vitamin C decreased from 112.98 ± 4.44 to 99.25 ± 1.70 mg.100 mL^{-1} (KF1TF). The values calculated can be explained by the micronutrient instability in an acidic medium, such as the present in kombucha, in which the pH ranges between 3 and 6 (Bishop et al. 2022).

Despite that, the quantified values of vitamin C in flavored kombuchas were promising, since they supply the daily amount that should be ingested by an adult (Brazil 2005). In addition, the total micronutrient found was higher compared to cashew apple juice (NEPA 2011).

Microbiological assessment

With the premise of guaranteeing food safety and the quality of the products developed in this study, the final formulations were evaluated from a microbiological point of view. The results are shown in Table IV.

The fermented beverage kombucha still does not have a specific legislation for the microbiological standard, being used for comparison with the parameters found in other literature. Brunini et al (2019), found a similar count ($1{,}3.10^2$ UFC/100mL) of mesophilic bacteria, molds and yeast found in this present study. In relation to thermotolerant coliforms, there was no colony count, similar to what happened in the study by Yikmis &Tuggum (2019) of kombucha based on purple basil (*Ocimum basilicum L.*).

These findings ensure compliance with the hygiene standards during the kombucha preparation, since the presence of the investigated microorganisms is associated with failures in the cleaning, disinfection, transport, storage, and temperature control during the manufacturing process of the products (Bruini 2019, Silva et al. 2017).

CONCLUSIONS

From this work, it was observed that the flavored kombuchas had high amounts of vitamin C, an important micronutrient in the human diet and necessary for the functioning of the immune system, as well as satisfactory levels of phenolic compounds and antioxidant capacity. In addition, the physicochemical characteristics and microbiological analysis of the fermented products proved that they were developed in accordance with the quality and safety standards laid down in the legislation.

As a result, it can be understood that the flavored formulations added nutritional and functional properties to the original fermented beverage that increased its potential for commercialization. Therefore, the association between the chosen fruits and traditional kombucha proves to be a sustainable and promising alternative for combining a probiotic fermented food with the diversity of bioactives

Table IV. Microbiological Analysis.

Formulation	Mesophilic bacteria	Molds and Yeasts	Thermotolerant Coliforms
KC	$1{,}2.10^{2a}$	6.10^{4b}	Aus.
KT	4.10^{1c}	9.10^{3ab}	Aus.
KB	$1{,}5.10^{1c}$	$6{,}1.10^{4b}$	Aus.

KC (kombucha with Cupuassu); KT (Kombucha with Tapereba); and KB (Kombucha with Bacuri).

found in fruit species from the Amazonian Region.

Finally, it is necessary to carry out sensory and consumption tests to complement the analysis of these new products, but our work is a tool to initiate further studies on kombuchas flavored with Amazonian fruits.

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