

Optimization of kombucha production using organic green tea (*Camellia sinensis*) and specialty coffee (*Coffea arabica*): Production optimization, physicochemical composition, microbial viability, and sensory evaluation

Radharani Melo Serafim Ferreira

Federal University of Bahia (UFBA)

Ana Camile Assis Jesus

Federal University of Bahia (UFBA)

Jaqueline dos Santos de Jesus

Federal University of Bahia (UFBA)

Beatriz Fernandes Vieira

Bahia State Center for Professional Education – Empreende Bahia

Wallysson Wagner Vilela Santos

Federal University of Bahia (UFBA)

Manuela Barreto Nascimento

Agricultural Technology Center of the State of Bahia (CETAB)

Paulo Roberto Ribeiro Mesquita

Agricultural Technology Center of the State of Bahia (CETAB)

Bruno Nicolau Paulino

Federal University of Bahia (UFBA)

Cosme Damião Barbosa

Federal University of Bahia (UFBA)

Maria Eugênia Oliveira Mamede

mmamede@ufba.br

Federal University of Bahia (UFBA)

Research Article

Keywords: Central Composite Rotatable Design (CCRD), Colorimetry, Fermented beverage, Sensory analysis, Total phenolic compounds, Volatile compounds

Posted Date: September 9th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10753055/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

This study evaluated the effects of ingredient combinations on kombucha fermentation using a Central Composite Rotatable Design (CCRD), with tea, coffee, and sugar concentrations as process variables and pH, volatile acidity, and fixed acidity as response variables. Of the 17 formulations, only six met the kombucha identity and quality standards. These six formulations were further analyzed for nutritional composition, color, volatile compounds, total phenolic content, acceptance, and sensory preference. The CCRD models were statistically adequate and showed no lack of fit. Results indicated that tea had the greatest influence on the fermentation system, exhibiting a significant positive linear effect on volatile and total acidity. The pH was influenced by coffee and sucrose, contributing to the beverage's acid-base balance. Multiple Factor Analysis (MFA) demonstrated that initial substrate variation affected the microbiological dynamics of acetic acid bacteria and yeasts, thereby altering the development of secondary metabolites. Formulations with higher residual sucrose content (K7, K8, and K14) exhibited the greatest market potential, with purchase intention exceeding 60%, likely due to higher scores for overall acceptance and flavor. High tea concentration, as observed in K12, increased total phenolic content and acidity, which may have resulted in lower sensory acceptance. Volatile compounds derived from coffee, present in K3 and K12, did not enhance acceptance, even with low volatile acidity. Formulations characterized by high residual sugar, intermediate acidity, and a shared aromatic profile achieved high acceptance scores. These findings suggest that a balanced interaction among the ingredients demonstrates the fermented beverage's technological viability.

1. Introduction

Kombucha is a fermented beverage obtained through the fermentation of a sweetened *Camellia sinensis* infusion by a symbiotic culture of acetic acid bacteria and yeasts, known as Symbiotic Culture of Bacteria and Yeast (SCOBY). During fermentation, sucrose is hydrolyzed into glucose and fructose, which are metabolized by the microorganisms, resulting in the production of ethanol, organic acids, and other metabolites. Consequently, a mildly acidic, naturally carbonated beverage rich in bioactive compounds is formed (Tran et al., 2020; Bishop et al., 2022a).

Growing interest in foods and beverages with health-related claims has driven the global expansion of the kombucha market, making it a widely produced and consumed beverage in both artisanal and industrial settings (Kim et al., 2022; Saito et al., 2024b). The kombucha production sector has experienced significant growth in recent years, particularly following the adoption of regulations that enhanced the safety and standardization of its production and commercialization. This expansion in production and consumption is directly linked to the potential benefits associated with its intake. Various studies highlight the functional properties of kombucha, primarily attributed to the presence of polyphenols, organic acids, vitamins, and compounds resulting from microbial metabolism. Potential effects attributed to kombucha include antioxidant activity (Cardoso et al., 2021; Tong et al., 2026), hypoglycemic and hypolipidemic effects (Tong et al., 2026), anti-inflammatory activity (Cardoso et al., 2021; Cheepchirasuk et al., 2025), and antimicrobial activity (Cheepchirasuk et al., 2025). The final

composition of the beverage is influenced by variables such as the type of substrate, fermentation time and temperature, and the microbiological characteristics of the SCOBY, which determine the profile of organic acids, phenolic compounds, and other metabolites (Barbosa et al., 2021; De Filippis et al., 2018).

Traditionally, kombucha is produced from *Camellia sinensis* tea. However, alternative substrates have been used to diversify the beverage's chemical and functional composition as well as its sensory characteristics; these include fruits (Morales et al., 2023; Silva Júnior et al., 2021), spices (Ikeda et al., 2024), and coffee and coffee-derived substrates (Miranda et al., 2023; Sales et al., 2023; Saito et al., 2024a).

Among the notable matrices, coffee (*Coffea arabica* L.) presents a complex composition—including caffeine, chlorogenic acids, diterpenes, and volatile compounds—that imparts characteristic aroma and flavor (Sales et al., 2023; Córdoba et al., 2021). In this context, coffee stands out as a promising matrix for diversifying kombucha's sensory profile. Incorporating coffee as a partial or total substrate in kombucha has shown potential to modify microbial metabolism and sensory characteristics, resulting in beverages with higher antioxidant content, more intense color, and more complex sensory notes (Saito et al., 2024a; Miranda et al., 2023; Bueno et al., 2021). In addition to contributing to the chemical and aromatic profile, coffee can affect the microbial dynamics of the consortium, as its phenolic compounds exert a mild, selective antimicrobial effect, modulating the relative abundance of yeasts and acetic acid bacteria (Miranda et al., 2023). Recently, Delorme et al. (2025) demonstrated that *Arabica* or *Canephora* coffee by-products are efficient substrates for kombucha production, as they enhance the beverage's antimicrobial activity. The authors also highlighted the value of the addition of coffee farming by-products.

Ingredient concentrations and fermentation conditions directly influence the chemical composition, functional properties, and sensory characteristics of kombucha, making standardization a challenge for large-scale production. Thus, process optimization strategies are essential for identifying combinations of factors that yield higher-quality beverages with greater consumer acceptance. Among these strategies, the statistical Design of Experiments (DoE) stands out by allowing the simultaneous evaluation of effects and interactions among multiple process variables, enabling the determination of optimal production conditions that maximize the beverage's functional properties and sensory acceptance (Sola et al., 2024; Alves et al., 2025).

Although various alternative substrates have demonstrated potential for kombucha production, most studies evaluate them in isolation, limiting the understanding of the combined effects of ingredients on the fermentation process and beverage quality. In this context, the application of statistical optimization tools enables the simultaneous investigation of interactions between traditional substrates (*Camellia sinensis* and sucrose) and alternative substrates, such as coffee. It is hypothesized that the controlled combination of these ingredients promotes a fermentation environment that modulates the beverage's chemical profile without compromising the viability and balance of the microbial consortium. Thus, this study aimed to evaluate, through an experimental design, the effects of ingredient combinations on the

production of coffee-supplemented kombucha from the first fermentation onward, and to characterize the resulting formulations in terms of their physicochemical, chemical, microbiological, and sensory properties.

2. Materials and Methods

2.1 Experimental design and preparation of kombucha formulations.

A Central Composite Rotatable Design (CCRD) was employed with three independent variables: coffee (x_1), green tea (x_2), and sucrose (x_3), resulting in 17 experimental runs. Each variable was assessed at five coded levels (-1.68 , -1 , 0 , $+1$, $+1.68$), as shown in Table 1. The dependent variables measured included pH (Y_1), volatile acidity (mEq/L) (Y_2), and total acidity (mEq/L) (Y_3). The experimental levels reflected the concentrations or proportions of coffee (x_1), green tea (x_2), and sucrose (x_3). The selection of independent variable levels was informed by Saito et al. (2024a).

Table 1
Independent variables and their respective levels and values in Central Composite Rotatable Design (CCDR)

Independent variable		Unit	Levels				
			-1.68	-1	0	+1	+1.68
x_1	Coffee	mL	1.8	3.2	5.2	7.2	8.6
x_2	Tea	g	0.3	1	2	3	3.7
x_3	Sucrose	g	5	7	10	13	15

A green tea (*Camellia sinensis*) infusion was prepared from organic dried leaves (Chá Dō, Yamamotoyama, São Paulo, SP, Brazil). The leaves were infused in potable water heated to 75°C at a concentration of 10% (w/v) and steeped for 15 minutes according to the manufacturer's instructions. The resulting infusion was then filtered and stored in glass containers.

Specialty coffee beans (Catuaí variety) from the Chapada Diamantina region (municipality of Mucugê, Bahia, Brazil) were ground using an electric grinder (Hamilton Beach, model 80393-BZ127, Glen Allen, Virginia, USA) and extracted by cold brew at a ratio of 10 g of ground coffee per 100 mL of potable water (24–26°C). The extraction was performed under refrigeration at 4°C for 24 hours. The beverage was then filtered using a No. 102 paper filter (Melitta, Avaré, São Paulo, Brazil) (Córdoba et al., 2021).

The tea and coffee infusions were combined with granulated sugar and potable water according to the proportions specified for each experimental trial (Table 1). The mixture was homogenized and cooled to

room temperature ($25 \pm 2^\circ\text{C}$). Subsequently, 12.5% (v/v) of previously fermented kombucha (starter) and 12.5% (w/v) of SCOBY were incorporated.

The initial (aerobic) fermentation was performed in 900 mL glass jars covered with paper towels at room temperature ($25 \pm 2^\circ\text{C}$) without agitation for 7 days. Following this period, the SCOBYs were removed, and the fermented beverage was transferred to airtight glass bottles for a second (anaerobic) fermentation under identical temperature conditions for 5 days to promote natural carbonation. All fermentation procedures were conducted in triplicate.

2.3 Analyses of the experimental design

The pH was measured using a calibrated digital pH meter in accordance with the manufacturer's instructions (Hanna Instruments, HI700601, Woonsocket, RI, USA). Total titratable acidity was determined by titrating the formulation with a standardized 0.1 N sodium hydroxide solution, with results expressed as a percentage of acetic acid. Volatile acidity was assessed by distilling the formulation (Gibertini, Super D.E.E, Novate Milanese, MI, Italy), followed by titration of the distillate with a standardized 0.1 N sodium hydroxide solution, and results were reported in mEq/L.

Experimental data were subjected to multiple regression analysis to estimate the coefficients of the linear, quadratic, and interaction terms between the independent variables. The experimental responses were fitted to a second-order polynomial model, as per Eq. 1, aiming to describe the relationship between the independent variables—concentration of coffee (x_1), green tea (x_2), and sucrose (x_3)—and the dependent variables: pH (Y_1), volatile acidity (Y_2), and total acidity (Y_3). The generalized form of the second-order polynomial equation is given as follows.

$$Y_n = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \sum_i \sum_{<j=2}^k \beta_{ij} x_i x_j + e_i$$

1

In the equation, Y denotes the response variable, β_0 is the model intercept, β_i are the linear effect coefficients, β_{ii} are the quadratic effect coefficients, β_{ij} are the interaction effect coefficients between variables, and k is the number of independent variables ($k = 3$).

The significance of the effects was assessed at a 5% probability level ($p < 0.05$), and results were presented using standardized Pareto charts. The quadratic models were subsequently re-parameterized to include only statistically significant terms. Model adequacy was evaluated through Analysis of variance (ANOVA) using the F-test and the coefficient of determination (R^2). All statistical analyses were conducted using Statistica® 10 software (StatSoft Inc., Tulsa, OK, USA). Formulations that met Identity and Quality criteria, as defined by the physicochemical parameters of Normative Instruction No. 41/2019 (Brazil, 2019), were selected based on the CCRD results. Six formulations were subjected to physicochemical and chemical characterization, microbiological viability assessment, and sensory analysis, as detailed in the following sections.

2.4 Physicochemical characterization

Alcohol content was determined by distillation (Super D.E.E., Gibertini, Novate Milanese, MI, Italy) followed by measurement using a hydrostatic balance (Super D.E.E., Gibertini, Novate Milanese, MI, Italy), with results expressed as % v/v. Total Soluble Solids (TSS) were measured using a previously calibrated digital refractometer (Hanna Instruments, model HI96801, Woonsocket, RI, USA) according to the manufacturer's instructions, with results expressed in °Brix. Moisture, ash, lipids, carbohydrates, total acidity (%), and reducing, non-reducing, and total sugars (g/100 mL) were quantified using reference methods (IAL, 2008). Caloric value was calculated from ethanol, lipid, and carbohydrate contents using conversion factors of 7 kcal/g, 9 kcal/g, and 4 kcal/g, respectively (OIV, 2025).

Colorimetric parameters were evaluated using a colorimeter (Konica Minolta, Chroma Meter CR-5) following the *Commission Internationale de l'Éclairage* (CIE L* a* b* / CIE L* C* h) system. The following values were determined: lightness (L*), ranging from 0 (black) to 100 (white); the chromaticity coordinate a*, where negative values indicate green and positive values indicate red; and the coordinate b*, where negative values represent blue and positive values represent yellow. Additionally, chroma/saturation (C*) and hue angle (h) were evaluated. Colorimetric parameters were monitored at the start of fermentation (T0), at the end of the first fermentation (T7), and in the final beverage after the second fermentation (T12).

2.5 Total phenolic compounds

Total phenolic content was determined following the methodology of Alves et al. (2025), with modifications for microplate application. The assay comprised 100 µL of Folin-Ciocalteu reagent (diluted 1:10), 20 µL of kombucha sample (diluted 1:20), and 80 µL of sodium carbonate solution (75 g/L). Absorbance was recorded at 765 nm using a microplate reader (Multiskan 60, Thermo Fisher Scientific, USA). Quantification was performed using a gallic acid standard curve ($y = 0.0063x - 0.0227$; $R^2 = 0.9983$), and results were reported as gallic acid equivalents (mg GAE/100 mL).

2.6 Volatile organic compounds

The volatile organic compound (VOC) profile was determined using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME/GC-MS) and a PAL RSI 85 autosampler. For each analysis, 10 mL of kombucha was transferred to headspace flasks and incubated at 85°C for 20 minutes. An 80 µm DVB/Car/PDMS fiber (Agilent Technologies, California, USA, 5191–5874) was then exposed to the headspace for 78 minutes under constant temperature and agitation at 171 rpm, following the protocol described by Nascimento et al. (2024). The fiber was subsequently introduced into the gas chromatograph injector for 1 minute. Each treatment was extracted in triplicate.

Separation and analysis of volatile organic compounds were performed using a G3540A gas chromatograph (Agilent Technologies, California, USA) coupled with a 5977B mass spectrometer (Agilent Technologies) in splitless injection mode. Helium served as the carrier gas at a flow rate of 0.6

mL/min, and separation was achieved on an HP-5MS column (5% phenylmethylpolysiloxane; 30 m × 0.25 mm internal diameter × 0.25 µm film thickness; Restek®, Bellefonte, USA). The oven temperature program began at 30°C for 3 minutes, increased to 50°C at 1.5°C/min (held for 3 minutes), then to 90°C at 1.0°C/min (held for 2 minutes), followed by 200°C at 2.0°C/min (held for 2 minutes), and finally to 300°C at 15.0°C/min (held for 5 minutes), resulting in a total run time of 130 minutes. The injector and transfer line temperatures were set at 250°C. Data acquisition was conducted in scan mode at 70 eV, with an m/z range of 35 to 350. All analyses were performed in triplicate.

Identification of volatile organic compounds was accomplished by comparing mass spectra with the system data library (NIST 147). The linear retention index (LRI) was calculated using a homologous series of n-alkanes (C7–C30). Relative quantification was performed by normalizing peak areas without applying correction factors.

2.7 Assessment of microbiological viability

Microbial viability of kombucha inocula was determined by quantifying total yeast and acetic acid bacteria (AAB) counts, according to the methodology described by Barbosa et al. (2021; 2023). Yeast enumeration was performed using Sabouraud Agar (ASD) medium supplemented with 0.05% chloramphenicol in a single-layer format. Acetic acid bacteria were enumerated using Mannitol, Yeast Extract, and Peptone (MYP) medium in a double-layer agar setup (0.9% and 1.5%) supplemented with 0.05% cycloheximide.

2.8 Sensory evaluation

Sensory analysis was conducted at the Sensory Analysis Laboratory of the Faculty of Pharmacy at the Federal University of Bahia (UFBA), following approval by the Research Ethics Committee (CEP/FAR/UFBA) under Certificate of Presentation for Ethical Consideration (CAAE) number 57013122.4.0000.8035. Before testing, microbiological safety analyses confirmed the absence of *Escherichia coli* and *Salmonella* spp. (Brazil, 2022). Acceptance, purchase intention, and preference ranking tests were administered to 60 untrained panelists aged 18 years or older, selected from the UFBA academic community, including faculty, technical staff, administrative staff, students, and consumers of fermented beverages.

The formulations were presented monadically, accompanied by mineral water and water crackers to cleanse the palate. For the acceptance test, overall acceptance, aroma, flavor, and appearance were evaluated using a structured 9-point hedonic scale, ranging from “dislike extremely” to “like extremely.” The purchase intention test employed a structured 5-point scale, ranging from “certainly would not buy” to “certainly would buy.” The preference ranking test was conducted on the three formulations with the highest overall acceptance scores. Data were analyzed using the Friedman test and the table of critical values of differences proposed by Christensen et al. (2006), with a significance level set at 5% ($p < 0.05$) (Dutcosky, 2019).

2.9 Statistical Analyses

The fermentations were conducted in a CCRD, with three replicates per formulation, and the analyses were performed in triplicate. The data obtained were subjected to one-way ANOVA, and the ANOVA assumptions were verified using the Shapiro-Wilk, Bartlett, and Durbin-Watson tests to assess normality, homoscedasticity, and independence of errors at the 5% probability level. The means of the physicochemical, volatile, and sensory composition parameters were compared using Tukey's test ($p < 0.05$).

Additionally, Multiple Factor Analysis (MFA) was used to understand the interactions among the active groups of quantitative variables in the dataset of samples classified as kombucha. The active groups used were categorized into: Microbiological viability (yeasts and acetic acid bacteria), Physicochemical and chemical (pH, total acidity, volatile acidity, alcoholic content, total soluble solids, moisture, ash, lipids, protein, carbohydrates, reducing sugar, non-reducing sugar, total sugars, total phenolic compounds, caloric value, L, a, b, c, and h), Sensory (global acceptance, appearance, flavor, aroma and purchase intent), and Volatile organic compound profile (acids, alcohols, aldehydes, esters, ketones, monoterpenes, sesquiterpenes, aromatic hydrocarbons, nitrogen-containing heterocyclic and others), grouped based on the sum of the relative average composition of the identified chemical classes (Morais et al., 2022).

For dimension retention, the eigenvalue criterion was used to select the first two components, which together explain the largest total variance of the data. In addition, cosine squared (Cos^2) values were calculated to represent the quality of each group's representation in the evaluated dimensions; values close to 1 indicate a better projection of the element onto the respective axis (Tassew et al., 2021). All statistical analyzes mentioned were performed using R software (version 4.6.0) and RStudio (version 2026.04.0-526).

3. Results and discussion

3.1 Optimization of kombucha fermentation using Central Composite Rotatable Design (CCRD)

The CCRD response surface methodology is a widely utilized statistical tool for evaluating the simultaneous effects of multiple independent variables on responses of interest in food and beverage products. In addition to facilitating the optimization of formulations and processes, CCRD can be employed to investigate the individual and combined effects of experimental variables on physicochemical, technological, and quality parameters, even when the primary objective is not to determine optimal conditions. CCRD supports the construction of predictive mathematical models that describe response behavior within the experimental region, thereby enhancing understanding of the system under evaluation. In this study, the responses analyzed included pH, volatile acidity, and total acidity, which are critical parameters for the identity and quality of kombucha (Brazil, 2019). The results obtained from CCRD varied according to the combinations of coffee, tea, and sucrose added to the kombucha (Table S1). Preliminary analysis indicated that pH values ranged narrowly from 3.18 to 3.29,

demonstrating that all formulations remained within the acidic range characteristic of kombucha-type fermented beverages. Furthermore, the observed values complied with the standards established by Normative Instruction No. 41/2019, which defines a pH range of 2.5 to 4.2 for the product (Brazil, 2019).

Table 2

Central Composite Rotatable Design (CCRD) matrix with real values of independent variables (tea, coffee, sugar) and response variables (pH, volatile acidity, total acidity) for coffee kombucha

Kombuchas	Independent variables			Response variables		
	Tea (g)	Coffee (mL)	Sucrose (g)	pH	VA (mEq/L)	TA (%)
K1	1	3.2	7	3.21 ± 0.01 ^a	22.25 ± 0.53 ^e	53.60 ± 1.01 ^e
K2	1	7.2	7	3.29 ± 0.01 ^a	18.46 ± 0.25 ^f	54.61 ± 1.01 ^e
K3	3	3.2	7	3.21 ± 0.01 ^a	38.68 ± 0.15 ^c	88.99 ± 2.34 ^c
K4	3	7.2	7	3.23 ± 0.02 ^a	37.17 ± 1.75 ^c	88.99 ± 1.54 ^c
K5	1	3.2	3	3.25 ± 0.01 ^a	17.70 ± 0.51 ^f	41.46 ± 1.01 ^f
K6	1	7.2	13	3.23 ± 0.02 ^a	24.52 ± 0.25 ^e	51.58 ± 1.01 ^e
K7	3	3.2	13	3.19 ± 0.03 ^a	48.04 ± 0.39 ^b	106.19 ± 1.5 ^{bd}
K8	3	7.2	13	3.21 ± 0.02 ^a	36.15 ± 0.64 ^c	102.14 ± 1.5 ^{bd}
K9	2	1.8	10	3.19 ± 0.02 ^a	30.09 ± 1.48 ^d	76.86 ± 2.55 ^d
K10	2	8.6	10	3.24 ± 0.04 ^a	28.31 ± 0.00 ^d	83.94 ± 2.11 ^c
K11	0.3	5.2	10	3.26 ± 0.01 ^a	12.89 ± 0.00 ^g	46.52 ± 4.63 ^f
K12	3.7	5.2	10	3.25 ± 0.00 ^a	51.83 ± 0.16 ^a	116.30 ± 0.5 ^{ae}
K13	2	5.2	5	3.25 ± 0.01 ^a	29.07 ± 1.02 ^c	73.82 ± 1.17 ^d
K14	2	5.2	15	3.18 ± 0.01 ^a	33.88 ± 0.67 ^c	74.84 ± 0.58 ^d
K15	2	5.2	10	3.23 ± 0.01 ^a	28.32 ± 0.88 ^d	66.75 ± 0.00 ^d

Kombuchas	Independent variables			Response variables		
	Tea (g)	Coffee (mL)	Sucrose (g)	pH	VA (mEq/L)	TA (%)
K16	2	5.2	10	3.22 ± 0.01 ^a	29.58 ± 0.00 ^d	71.80 ± 0.58 ^d
K17	2	5.2	10	3.21 ± 0.01 ^a	34.64 ± 0.76 ^c	85.96 ± 0.58 ^c

Values are expressed as mean ± standard deviation of three experimental replicates. Different letters subscripts in the same column indicate significant differences by Tukey test. TA – Total acidity; VA – volatile acidity.

The homogeneous and symmetrical distribution, concentrated within narrow ranges, demonstrates kinetic pH stability during fermentation. The absence of outliers across all experimental conditions indicates that, despite inherent variability among formulations, the system exhibited a buffering effect. This effect results from the presence of organic acids generated during fermentation, which help stabilize pH throughout the process. Additionally, CO₂ produced by yeast dissolves in the matrix, forming carbonic acid (H₂CO₃), which contributes to the system's acid-base equilibrium (Villarreal-Soto et al., 2018). This mechanism accounts for the minor residual pH variations observed.

Volatile acidity values ranged from 12.89 to 51.83 mEq/L (Table 2), indicating that independent variables substantially influenced volatile acid production during fermentation. The highest volatile acidity occurred in the trial with the greatest tea concentration (+ 1.68), whereas the lowest value was recorded under the lowest tea concentration (– 1.68). According to Brazilian regulations established by Normative Instruction No. 41/2019 (Brazil, 2019), the acceptable range is 30 to 130 mEq/L. This parameter is essential for evaluating the conformity and stability of the fermented product. Notably, service values below the minimum recommended limit suggest that specific variable combinations can markedly reduce the formation of volatile acidic compounds.

Although coffee is a source of caffeine and polyphenols, it contains a high concentration of chlorogenic acids—compounds widely known for their antimicrobial and bacteriostatic activity against various cell types (Lin et al., 2022). However, increasing the proportion of tea optimized the supply of macro- and micronutrients characteristic of *Camellia sinensis*, a trend also reported by Alves et al. (2025). Thus, the components of both substrates act synergistically, mitigating potential inhibitory effects caused by major coffee compounds while serving as essential metabolic stimulants for acetic acid bacteria. Consequently, these bacteria achieve a higher rate of ethanol conversion to acetic acid, the primary component of volatile acidity in kombucha fermentation.

Total acidity exhibited the greatest variation among the analyzed responses, with values ranging from 41.46 to 116.30 mEq/L (Table 2). The highest acidity levels occurred in treatments with elevated tea concentrations, indicating increased organic acid production due to the metabolic activity of fermenting

microorganisms. In contrast, formulations with reduced tea content and higher sucrose levels demonstrated lower total acidity.

The acidity of kombucha involves the presence of non-volatile organic acids—such as glucuronic and gluconic acids—in addition to acetic acid; these acids can originate from either the tea or microbial metabolism (Bishop et al., 2022a). Total acidity therefore refers to the quantification of all acidic species present in both the volatile and fixed fractions of the beverage, which accounts for the substantially higher values observed compared to volatile acidity levels. In this study, higher tea concentrations were found to promote the production of both fixed and volatile acids by the microbial consortium, a trend also reported by Miranda et al. (2023).

The trials conducted at the center point produced closely aligned values across all evaluated responses, demonstrating strong experimental repeatability and effective implementation of the experimental design. Specifically, pH values at the center point ranged from 3.21 to 3.23, volatile acidity from 28.32 to 34.64 mEq/L, and total acidity from 66.75 to 85.96 mEq/L (Table 2). These findings indicate stability in the fermentation process and minimal experimental variability under the central conditions of the design.

Following the execution of the rotatable central composite design, the experimental data underwent statistical analysis, including assessment of estimated effects, regression coefficients, ANOVA, and Pareto charts of standardized effects (Fig. 1). These procedures enabled the evaluation of the individual influence of the independent variables, as well as their quadratic effects and interactions, on the responses under study.

Regarding pH, the analysis of estimated effects showed that the linear effects of coffee and sucrose were significant ($p < 0.05$), with positive and negative coefficients, respectively (Table S2). Coffee exhibited a significant positive linear effect (coefficient = 0.013480; $p = 0.038016$), indicating that increasing the coffee volume led to a slight rise in kombucha pH (Tables S2a and S3a). Conversely, sucrose showed a significant negative linear effect (coefficient = -0.013014; $p = 0.040620$), indicating a trend toward lower pH with increasing sucrose concentration. The linear effect of tea was borderline statistically significant (coefficient = -0.011483; $p = 0.051298$), suggesting a possible trend toward decreasing pH with increasing tea concentration.

Furthermore, the quadratic terms and most interactions were not statistically significant ($p > 0.05$), suggesting a lack of pronounced curvature in the pH response within the experimental range. ANOVA indicated that the model produced a coefficient of determination (R^2) of 0.78745 and an adjusted R^2 of 0.51416, reflecting a moderate capacity to explain experimental variability (Table 3a). The lack of significant lack-of-fit ($p = 0.171393$) further demonstrated that the fitted model adequately represented the experimental data (Table 3). The Pareto chart confirmed that the linear effects of coffee and sucrose were the primary factors influencing pH changes, as these were the only variables to exceed the statistical significance threshold at the 95% confidence level (Fig. 1a).

Table 3
Analysis of variance (ANOVA) for pH (a), volatile acidity (b) e total titratable acidity (c)

Source	Sum of squares	df	Mean square	F-value	p-value
(a) Y_1					
Model					
x_1	0.002481	1	0.002481	24.81464	0.038016*
x_2	0.001801	1	0.001801	18.00697	0.051298
x_3	0.002313	1	0.002313	23.12857	0.040620*
$x_1 x_2$	0.000050	1	0.000050	0.50000	0.552786
$x_1 x_3$	0.001250	1	0.001250	12.5000	0.071523
$x_2 x_3$	0.000050	1	0.000050	0.50000	0.552786
x_1^2	0.000048	1	0.000048	0.48072	0.559792
x_2^2	0.001644	1	0.001644	16.44310	0.055776
x_3^2	0.000048	1	0.000048	0.48072	0.559792
Lack of fit	0.002561	5	0.000512	5.12139	0.171393
Pure error	0.000200	2	0.000100		
Total	0.012988	16			
R ²	0.78745				
Adj-R ²	0.51416				
(b) Y_2					
Model					
x_1	13.077	1	13.077	1.1687	0.392688

Source	Sum of squares	df	Mean square	F-value	p-value
x_2	1488.958	1	1488.958	133.0741	0.007431*
x_3	23.565	1	23.565	2.1061	0.283816
$x_1 x_2$	33.743	1	33.743	3.0158	0.224593
$x_1 x_3$	0.007	1	0.007	0.0006	0.982813
$x_2 x_3$	5.831	1	5.831	0.5211	0.545345
x_2 x_1	5.951	1	5.951	0.5319	0.541653
x_2 x_2	1.720	1	1.720	0.1538	0.732811
x_2 x_3	0.068	1	0.068	0.0061	0.944898
Lack of fit	70.950	5	14.190	1.2682	0.496092
Pure error	22.378	2	11.189		
Total	1669.295	16			
R ²	0.94409				
Adj-R ²	0.87221				
(c) Y_3					
Model					
x_1	26.398	1	26.398	0.26618	0.657279
x_2	6696.647	1	6696.647	67.52556	0.014488*
x_3	20.902	1	20.902	0.21077	0.691235
$x_1 x_2$	28.804	1	28.804	0.29045	0.643900

Source	Sum of squares	df	Mean square	F-value	p-value
$x_1 x_3$	3.200	1	3.200	0.03227	0.873986
$x_2 x_3$	259.009	1	259.009	2.61171	0.247457
x_2 x_1	6.259	1	6.259	0.06312	0.825094
x_2 x_2	13.696	1	13.696	0.13810	0.745851
x_2 x_3	22.125	1	22.125	0.22310	0.683210
Lack of fit	150.423	5	30.085	0.30336	0.877835
Pure error	198.344	2	99.172		
Total	7442,859	16			
R ²	0.95314				
Adj-R ²	0.89289				

x_1 = Coffee; x_2 = Tea; x_3 = Sucrose. *Statistically significant at the $p < 0.05$ level by ANOVA.

Figure 1 Pareto chart showing the effect of the variables on the pH (a), volatile acidity (mEq/L) (b), and total acidity (%) (c)

Regarding volatile acidity, statistical analysis revealed that only the linear effect of the tea was significant (coefficient = 10.44157; $p = 0.007431$), demonstrating a strong positive influence of this variable on the formation of volatile acidic compounds in kombucha (Tables S2b and S3b). Increasing the tea concentration led to a marked rise in volatile acidity, possibly due to a greater availability of substrates and phenolic compounds that influence the fermentative metabolism of the microorganisms (Alves et al., 2025).

Other factors, including coffee, sucrose, quadratic effects, and interactions, did not demonstrate statistical significance ($p > 0.05$). The ANOVA indicated no significant lack of fit ($p = 0.496092$), confirming that the fitted model adequately describes the response behavior (Table 3b). A decreasing trend in volatile acidity was observed in formulation 5% (se (29.07 m, /L) and formulation K14 with 15% sucrose (33.88 mEq/L). This finding suggests that the availability of the carbon source was not a limiting factor for volatile acidity synthesis under the tested conditions. Similarly, varying only the coffee concentration, as seen in the comparison between formulation K9 at 1.8% (30.09 mEq/L) and K10 at

8.6% (28.31 mEq/L), did not result in significant changes (Table 2). Therefore, fluctuations in these two independent variables, coffee and sucrose, within the evaluated ranges, play a secondary role in regulating volatile acidity compared to the dominant effect of the tea substrate.

The model exhibited strong predictive power, with $R^2 = 0.94409$ and adjusted $R^2 = 0.87221$, indicating that approximately 94.4% of the experimental variability was explained by the fitted mathematical model (Table 3b). The Pareto chart indicated that the linear effect of tea was the most influential factor, substantially surpassing the statistical significance threshold, while other variables had minimal impact on volatile acidity (Fig. 1b).

For total acidity, similar to volatile acidity, the linear effect of tea concentration was the only significant factor influencing the response (coefficient = 22.14386; $p = 0.014488$), indicating that higher tea concentrations favored an increase in the kombucha's total acidity in kombucha (Tables S2c and S3c). This behavior suggests greater organic acid production during fermentation when higher proportions of tea are used. The production of acids characteristic of kombucha, such as acetic acid—the primary component of the volatile fraction—occurs partly through the oxidation of ethanol (derived from sugar) by acetic acid bacteria (Villarreal-Soto et al., 2018). The pronounced effect of tea may be attributed to its provision of essential nutrients, as tea supplies nitrogenous compounds, vitamins, and minerals that stimulate the metabolic activity and growth of the microbial consortium (SCOBY).

Furthermore, according to Sola et al. (2025), kombucha acidity involves the presence of acids beyond acetic acid, including glucuronic and gluconic acids, which either originate from tea leaves or are synthesized by acetic acid bacteria from tea-derived compounds. The availability of these substrates, as well as bioactive compounds from tea and coffee, modulates microbial metabolic dynamics and directly influences the rate and intensity of acidification during fermentation (Barros et al., 2024).

The effects of coffee, sucrose, quadratic terms, and interactions were not statistically significant ($p > 0.05$), indicating that these variables had limited influence on total acidity within the evaluated experimental range. ANOVA indicated no significant lack of fit ($p = 0.877835$), confirming the adequacy of the proposed model (Table 3c). Additionally, the model demonstrated a high goodness of fit, with $R^2 = 0.95314$ and adjusted $R^2 = 0.89289$, indicating excellent agreement between experimental and predicted values (Table 3c). The Pareto chart corroborated the statistical results, highlighting that the linear effect of tea was the primary factor responsible for the observed changes in the total acidity of the coffee-flavored kombucha (Fig. 1c).

The results indicate that the independent variables exert distinct influences on the physicochemical parameters of coffee-supplemented kombucha. Tea was the variable with the greatest impact on acidity parameters, with a significant positive linear effect on both volatile and total acidity. In contrast, pH was primarily affected by the linear effects of coffee and sucrose, suggesting that specific formulation components distinctly influence the acid-base characteristics of the fermented beverage (Miranda et al., 2022).

Although the CCRD was initially structured to facilitate quadratic modeling and optimization, the results demonstrate that the design was more effective for evaluating the effects of independent variables on kombucha characteristics when coffee was used as an alternative substrate, rather than for defining an optimal processing condition. The absence of statistical significance for most quadratic terms and interactions suggests that the responses were predominantly linear within the evaluated experimental region. Consequently, constructing classical response surfaces is less applicable in this context, as such surfaces are most informative when the system exhibits significant curvature and variable interactions.

Furthermore, in the present study, only one variable had a significant effect on volatile and total acidity, which precludes robust interpretation of three-dimensional surfaces involving factor interactions. However, the models did not show significant lack of fit and exhibited high coefficients of determination for volatile and total acidity, indicating adequate descriptive capability within the experimental range. This experimental design enabled the identification of variables that most strongly influenced the beverage's identity and quality parameters, thereby contributing to understanding of this novel fermentation system.

Based on the observed behavior and screening results from the experimental design, formulations K3, K4, K7, K8, K12, and K14 were selected for further physicochemical and chemical characterization, microbiological viability assessment, volatile compound profiling, and sensory analysis to support the technological consolidation of coffee kombucha.

3.3 Characterization of the selected beverages

The univariate characterization of each group is presented, including microbiological viability (Table S4), physicochemical and chemical properties (Table 4), volatile compounds (Fig. S1), sensory attributes assessed through acceptance tests (Fig. S2), purchase intention (Fig. S3), preference ranking (Table S5), Pearson's Correlation Coefficient (r) (Fig. S4). Means, standard deviations, and results of mean comparison tests are reported. To integrate these findings and elucidate the interactions among the different variable groups in the fermented beverages, data from formulations K3, K4, K7, K8, K12, and K14 (Fig. S5) were analyzed using MFA (Fig. 2).

Table 4
Physicochemical, chemical and colorimetric characterization of Coffee Kombuchas

Parameters	Kombucha samples					
	K3	K4	K7	K8	K12	K14
Alcoholic content (% v/v)	0.62 ± 0.02 ^b	0.91 ± 0.02 ^{ab}	1.12 ± 0.25 ^a	1.10 ± 0.03 ^a	0.82 ± 0.13 ^{ab}	0.82 ± 0.04 ^{ab}
Moisture (%)	95.03 ± 0.14 ^a	94.81 ± 0.04 ^b	90.72 ± 0.10 ^c	91.17 ± 0.05 ^d	92.93 ± 0.02 ^e	89.26 ± 0.04 ^f
Ash (%)	0.08 ± 0.00 ^{bc}	0.10 ± 0.01 ^{ab}	0.07 ± 0.01 ^c	0.10 ± 0.01 ^{ab}	0.12 ± 0.01 ^a	0.06 ± 0.01 ^{ab}
Lipids (%)	0.51 ± 0.00 ^c	0.32 ± 0.00 ^e	0.58 ± 0.00 ^b	0.33 ± 0.00 ^d	0.62 ± 0.00 ^a	0.24 ± 0.00 ^f
Carbohydrates (%)	3.75 ± 0.12 ^d	3.86 ± 0.05 ^d	7.50 ± 0.27 ^b	7.30 ± 0.04 ^b	5.51 ± 0.11 ^c	9.63 ± 0.03 ^a
Reducing sugar (%)	0.15 ± 0.02 ^c	0.23 ± 0.02 ^a	0.22 ± 0.00 ^a	0.34 ± 0.02 ^{ab}	0.28 ± 0.01 ^b	0.30 ± 0.05 ^b
Non-reducing sugar (g/100 mL)	0.13 ± 0.03 ^d	0.13 ± 0.03 ^d	5.45 ± 0.22 ^c	5.94 ± 0.36 ^b	4.02 ± 0.26 ^c	8.69 ± 0.14 ^a
Total sugar (g/100 mL)	0.27 ± 0.05 ^d	0.35 ± 0.04 ^d	5.67 ± 0.26 ^b	6.27 ± 0.43 ^b	4.29 ± 0.30 ^c	8.98 ± 0.23 ^a
Total soluble solids (°Brix)	5.83 ± 0.11 ^e	5.66 ± 0.11 ^e	9.76 ± 0.15 ^c	10.66 ± 0.05 ^b	8.23 ± 0.05 ^d	11.86 ± 0.05 ^a
pH	3.24 ± 0.00 ^b	3.23 ± 0.00 ^c	3.16 ± 0.01 ^d	3.26 ± 0.00 ^{ab}	3.27 ± 0.00 ^a	3.27 ± 0.00 ^a
Volatile acidity (mEq/L)	38.76 ± 0.14 ^c	38.17 ± 1.75 ^{cd}	48.12 ± 0.38 ^b	36.23 ± 0.63 ^d	51.89 ± 0.16 ^a	33.62 ± 0.66 ^e
Total acidity (%)	90.34 ± 2.33 ^c	89.33 ± 1.54 ^c	105.84 ± 1.54 ^b	102.47 ± 1.54 ^b	115.96 ± 0.58 ^a	74.49 ± 0.58 ^d
TPC (mg GAE/100 mL)	151.25 ± 3.28 ^b	101.42 ± 0.94 ^d	91.45 ± 1.25 ^e	146.43 ± 3.72 ^b	224.39 ± 7.88 ^a	129.18 ± 4.74 ^c
L*	97.39 ± 0.01 ^a	85.91 ± 0.16 ^c	94.51 ± 0.0 ^b	97.39 ± 0.01 ^a	85.91 ± 0.16 ^c	94.51 ± 0.06 ^b
a*	90.63 ± 0.01 ^a	84.48 ± 0.02 ^b	83.52 ± 0.09 ^c	90.63 ± 0.01 ^a	84.48 ± 0.02 ^b	83.52 ± 0.09 ^c

Parameters	Kombucha samples					
	K3	K4	K7	K8	K12	K14
b*	94.57 ± 0.01 ^a	86.91 ± 0.42 ^b	82.97 ± 0.05 ^c	94.57 ± 0.01 ^a	86.91 ± 0.42 ^b	82.97 ± 0.05 ^c
C*	91.48 ± 0.01 ^a	90.00 ± 0.09 ^b	87.56 ± 0.28 ^c	91.48 ± 0.01 ^a	90.00 ± 0.09 ^b	87.56 ± 0.28 ^c
H	92.20 ± 0.01 ^a	84.56 ± 0.06 ^c	89.42 ± 0.22 ^b	92.20 ± 0.01 ^a	84.56 ± 0.06 ^c	89.42 ± 0.22 ^b
Caloric value (kcal/100 mL)	29.00 ± 0.69 ^e	29.81 ± 0.23 ^e	50.00 ± 0.46 ^b	43.74 ± 0.22 ^c	38.36 ± 0.07 ^d	54.23 ± 0.21 ^a

Values are expressed as mean ± standard deviation of three experimental replicates. Different letters subscripts in the same line indicate significant differences by the Tukey test. TPC – Total phenolic compounds.

Figure 2 Multiple Factor Analysis (MFA) applied to kombucha samples. a) Correlation plot of the active variables grouped by category: Microbiological viability (MV) in red, Sensory (SEN) in green, Physicochemical and chemical (PCC) in blue, and Volatile organic compound profile (VOC) in pink. b) Factor map of individual samples in the first two dimensions (Dim 1 and Dim 2)

AAB – acetic acid bacteria; GA – Global acceptance; PI – Purchase intent; MOI – Moisture; LIP – Lipids; CARB – Carbohydrates; RS – Reducing sugar; NRS – Non-reducing sugar; TS – Total sugar; CV – Caloric value; TSS – Total soluble solids; VA – Volatile acidity; TA – Total acidity; AC – Alcoholic content; TPC – Total phenolic compounds; NCH – Nitrogen-containing heterocyclic; AH – Aromatic hydrocarbons.

MFA accounted for 55.20% of the total data variability across the first two components (Dim1 and Dim2), which explained 30.38% and 24.82% of the variance, respectively. The first component (Dim1) was primarily influenced by the physicochemical and chemical (PCC; 39.28%) and sensory (SEN; 37.58%) groups. In contrast, microbiological viability (MV; 48.08%) contributed most substantially to the second component (Dim2). The VOC group contributed moderately to both components, representing 13.85% of Dim1 and 26.84% of Dim2 (Table S6).

The lower-right quadrant demonstrated an association between sensory attributes (overall acceptance, aroma, flavor, appearance, and purchase intention) and variables related to yeast fermentative activity, including ester, acid, and alcohol groups. The strong positive correlation between yeasts and esters ($r = 0.92$) (Fig. S4) is attributable to the role of yeasts in ester biosynthesis during alcoholic fermentation, primarily via the enzymatic esterification of higher alcohols and acyl-CoA catalyzed by alcohol acetyltransferases (Zhang et al., 2021). These findings suggest that these metabolites are fundamental to the sensory perception of the beverage and flavor (Zhang et al., 2021). In addition to esters, certain higher alcohols, when present at moderate concentrations, also enhance the beverage's aromatic

complexity. Notable examples include 2-methyl-1-butanol and 3-methyl-1-butanol, which are frequently associated with fruity and malty odors (Duan et al., 2026). Formulations K7 and K14 were prominent in this quadrant, both characterized by the highest sucrose concentrations in the experimental design. These formulations received the highest scores for the sensory attributes evaluated: overall acceptance (6.31 and 6.55), aroma (5.60 and 5.85), flavor (6.60 and 6.63), and appearance (6.35 and 6.52), respectively (Fig. S2). A positive correlation was also observed between overall acceptance and carbohydrates ($r = 0.90$), non-reducing sugars ($r = 0.87$), total sugars ($r = 0.87$), and total soluble solids ($r = 0.89$) (Fig. S4).

Conversely, the opposing orientation of these vectors indicates that formulations with elevated yeast activity are less strongly associated with high concentrations of phenolic compounds and acidity, as demonstrated by the separation of these variables along the first two dimensions of the MFA. This pattern is further supported by Pearson correlation analysis, which reveals negative correlations between yeast activity and total phenolic compounds ($r = -0.77$), volatile acidity ($r = -0.67$), and total acidity ($r = -0.66$) (Fig. S4).

The purchase intention test, assessed using a diverging Likert scale, demonstrates consumer behavior toward the evaluated formulations (Fig. S3). Formulations K7, K8, and K14 possess the greatest commercial potential, with over 60% of consumers indicating a positive purchase intention ("probably would buy" or "certainly would buy"). In contrast, formulation K12, positioned in the opposite quadrant, exhibits the highest rejection rates, corresponding to its low scores for flavor and overall acceptance in the hedonic evaluation (Fig. S2). Purchase intention exhibited positive correlations with carbohydrate content ($r = 0.62$), non-reducing sugars ($r = 0.65$), and total sugars ($r = 0.65$). Strong correlations were also identified with sensory attributes, including aroma ($r = 0.91$), flavor ($r = 0.73$), appearance ($r = 0.72$), and overall acceptance ($r = 0.82$), indicating that sensory perception substantially influenced purchase decisions. In contrast, purchase intention demonstrated a moderate negative correlation with total phenolics ($r = -0.52$) and the color parameters b^* ($r = -0.56$) and c ($r = -0.55$) (Fig. S4). Although differences in chemical composition and associations identified by MFA were observed, the formulations K7, K8, and K14 did not differ significantly in the preference-ranking test (Table S5), suggesting that these samples were equally preferred by consumers. Fig. S5 displays the six coffee kombucha formulations produced, along with the response percentages for each formulation corresponding to each scale term.

In the upper-right quadrant, formulation K8 is associated with variables indicative of acetic acid bacteria activity and the availability of fermentable carbohydrates, including total soluble solids, carbohydrates, reducing sugars, non-reducing sugars, total sugars, and caloric value. The caloric value demonstrated a similar pattern, reflecting its direct dependence on the beverage's carbohydrate content (Fig. 2). This association is supported by the characterization results, which indicate that K8 contains a high level of fermentable sugars (10.66 °Brix and 6.27 g/100 mL total sugars), an intermediate caloric value (43.74 ± 0.22 kcal/100 mL) (Table 4), and a high acetic acid bacteria count (6.18 CFU/mL) (Table S4). The metabolic succession dynamics characteristic of kombucha fermentation explain this association.

Yeasts, including the genera *Saccharomyces spp.* and *Zygosaccharomyces spp.*, initiate sucrose hydrolysis through the enzyme invertase and convert the resulting monosaccharides into ethanol and carbon dioxide. Acetic acid bacteria, such as *Komagataeibacter spp.* and *Gluconacetobacter spp.*, then utilize the produced ethanol as the primary substrate for synthesizing acetic, gluconic, and glucuronic acids (Li et al., 2025; Bishop et al., 2022b). These acids contribute to the beverage's perceptible acidity and antimicrobial properties (Chandran et al., 2026).

The AAB count results (Table S4) indicate the presence of an active and viable consortium across all evaluated formulations, which is essential for initiating fermentation and producing acetic acid, a defining characteristic of the beverage. The culture media employed produced favorable AAB counts, thereby confirming the suitability of RAE and MYP for kombucha analysis. As noted by Barbosa et al. (2023), these media support AAB colony enumeration because their compositions promote the growth of microorganisms that metabolize carbohydrates (MYP), ethanol, and acetic acid (RAE).

In the upper-left quadrant, the positioning of formulation K12 is noteworthy and can be attributed to its higher proportion of tea (3.7 g) combined with intermediate concentrations of coffee (5.2 mL) and sucrose (10 g) (Table 2). *Camellia sinensis* tea serves as a significant source of phenolic compounds, including catechins and flavonoids (Wang et al., 2022), while coffee primarily contributes chlorogenic acids as its main phenolic constituents (Yeager et al., 2021). The combination of these substrates appears to have enhanced the chemical profile of the formulation, as evidenced by its association with elevated values for total phenolic compounds (224.39 ± 7.88 mg GAE/100 mL), total acidity ($115.96 \pm 0.58\%$), volatile acidity (51.89 ± 0.16 mEq/L) (Table 4), and other physicochemical parameters in this quadrant (Fig. 2a). In contrast, the orientation of these vectors opposite to the sensory attributes indicates an inverse relationship between physicochemical composition and sensory perception along the first dimension of the MFA. This interpretation is further supported by the negative correlation between flavor and total phenolic compounds ($r = -0.52$) (Fig. S4), suggesting that the concentration of these compounds affects the sensory perception of this attribute. The K12 beverage demonstrated reduced volatile profile complexity, with a higher relative proportion of ketones and a lower contribution of esters (Fig. S1). As esters are key compounds responsible for fruity and floral aromas in fermented beverages (Wang et al., 2024), the distribution of these volatiles likely contributed to the inferior sensory performance observed in this formulation. Variations in the volatile compounds of kombucha are attributable to factors such as the fermentation process, the initial microbial composition of the SCOBY, and the substrates employed (Venegas et al., 2023).

The lower-left quadrant corresponded to the positioning of formulations K3 and K4, which were primarily associated with volatile compounds including aldehydes, monoterpenes, heterocyclic nitrogen compounds, and aromatic hydrocarbons (Fig. 2). In contrast to the other quadrants analyzed, this region was distinguished by compounds linked to the aromatic composition of the coffee matrix. This finding suggests a greater relative contribution of markers originating from the raw material rather than metabolites typically produced during kombucha fermentation. Among these, heterocyclic nitrogen compounds (Fig. S1), mainly pyrazines and pyrroles, were notable. These compounds are generated

through the Maillard reaction during the roasting of coffee beans and are associated with roasted, cocoa, caramel, and nutty aromas and odors (Silva et al., 2025). The lowest counts of acetic acid bacteria were observed in these formulations, specifically for K3 (5.77 Log₁₀ CFU/mL) and K4 (5.49 Log₁₀ CFU/mL) (Table S4). Although both formulations exhibited high yeast counts (K3: 6.89 and K4: 6.39 Log₁₀ CFU/mL) (Table S4), the reduced acetic acid bacteria population indicates a lower intensity of the oxidative fermentation stage (Wang et al., 2024). This condition favors the preservation of aromatic compounds derived from the coffee matrix over the formation of metabolites typically associated with kombucha fermentation.

Collectively, MFA demonstrated that the proportions of initial substrates (tea, coffee, and sucrose) determined the microbial ecology of the SCOBY and, consequently, the secondary metabolite synthesis pathway. Modulation of volatile, chemical, and physicochemical profiles substantially influenced sensory acceptance, indicating that interactions between chemical and biological components are the primary determinants of the beverages' distinctive characteristics.

4. Conclusions

The results demonstrate the feasibility of producing kombucha by adding coffee (*Coffea arabica*) at the initial fermentation stage, supporting the introduction of a substrate intended to enhance the beverage's chemical and sensory properties. Although the experimental design data did not yield statistically significant quadratic and linear terms, which prevented the construction of classical response surfaces, the application of the CCRD effectively identified the factors that most influenced the response variables. This approach provided robust statistical data, contributing to a clearer understanding of the parameters that may affect kombucha fermentation.

MFA revealed that the concentrations of key beverage components, including sucrose, tea, and coffee, drive metabolic pathways during fermentation. These metabolic pathways shape the beverage's physicochemical, chemical, and volatile profiles, ultimately influencing consumer acceptability. Consequently, precise control of these raw materials is essential for standardizing fermentation processes and maintaining kombucha quality. Formulations K7, K8, and K14 demonstrated the greatest market potential, characterized by higher residual sugar concentrations and enhanced flavor and aromatic complexity attributed to the biosynthesis of esters and higher alcohols. Conversely, combining high levels of tea and coffee increased the concentrations of total phenolic compounds and acidity. However, this combination did not favor K12 acceptance, indicating that an optimal balance between tea and coffee levels is necessary.

Regarding prospects, *scale up* the experimental conditions is recommended to evaluate the reproducibility of the process optimization, the beverage's stability during storage, and the confirmation of its biological properties *in vitro*.

Declarations

CRedit authorship contribution statement

Radharani de Melo Serafim Ferreira: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. Ana Camile Assis de Jesus: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. Jaqueline dos Santos de Jesus: Formal analysis, Methodology, Writing – original draft. Beatriz Fernandes Vieira: Formal analysis, Writing – original draft. Wallysson Wagner Vilela Santos: Methodology, Software, Writing – review & editing. Manuela Barreto Nascimento: Formal analysis, Methodology, Writing – original draft. Paulo Roberto Ribeiro de Mesquita: Methodology, Resources. Bruno Nicolau Paulino: Methodology, Writing – original draft. Cosme Damião Barbosa: Formal analysis, Methodology, Resources. Maria Eugênia de Oliveira Mamede: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, writing – review & editing.

Ethics approval and consent to participate

This study was approved by the Pharmacy Ethics Committee of the Federal University of Bahia (57013122.4.0000.8035) and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants and, where applicable, from their legal guardian(s).

Consent for publication

The informed consent forms signed by all participants stated that their data would be used for publication.

Competing interests

The authors declare no competing interests.

Funding

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) [grant numbers: 88887.915671/2023-00; 88887.214297/2025-00], Universidade Federal da Bahia (UFBA) [with Scientific Initiation scholarships from students Jaqueline Jesus and Beatriz Vieira], and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) [grant numbers: 304377/2025-2 - research; 402596/2025-0 - Universal Project].

Acknowledgements

The authors are grateful to the Laboratório de Bromatologia and to the Laboratório de Análise Sensorial, both of the Faculdade de Farmácia da Universidade Federal da Bahia; to the Centro Tecnológico

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

References

1. Alves, R.O., Oliveira, R.L., Moraes, M.M., Santos, W.W.V., Câmara, C.A.G., Silva, S.P., Porto, C.S., Porto, T.S. (2025). Evaluation of the impact of fermentation conditions, scale-up, and stirring on physicochemical parameters, antioxidant capacity, and volatile compounds of green tea kombucha. *Fermentation*, 11, 201. <https://doi.org/10.3390/fermentation11040201>
2. Barbosa, C.D., Uetanabaro, A.P.T., Santos, W.C.R., Caetano, R.G., Albano, H., Kato, R., Cosenza, G.P., Azeredo, A., Góes-Neto, A., Rosa, C.A., Teixeira, P., Alvarenga, V.O., Lacerda, I.C.A. (2021). Microbial–physicochemical integrated analysis of kombucha fermentation. *LWT*, 148, 111788. <https://doi.org/10.1016/j.lwt.2021.111788>
3. Barbosa, C.D., Costa, I.M., Santos, W.C.R., Alvarenga, V.O., Lacerda, I.C.A., Simiqueli, G.F. (2023). Acetic acid bacteria analysis using non-conventional culture media in kombucha fermentation. *Ensaio e Ciência: Ciências Biológicas, Agrárias e da Saúde*, 27, 370–375. <https://doi.org/10.17921/1415-6938.2023v27n3p370-375>
4. Barros, V.C., Botelho, V.A., Chisté, R.C. (2024). Alternative Substrates for the Development of Fermented Beverages Analogous to Kombucha: An Integrative Review. *Foods*, 13, 1768. <https://doi.org/10.3390/foods13111768>
5. Bishop, P., Pitts, E.R., Budner, D., Thompson-Witrick, K.A. (2022a). Chemical composition of kombucha. *Beverages*, 8, 45. <https://doi.org/10.3390/beverages8030045>
6. Bishop, P., Pitts, E.R., Budner, D., Thompson-Witrick, K.A. (2022b). Kombucha: Biochemical and microbiological impacts on the chemical and flavor profile. *Food Chemistry Advances*, 1, 100025. <https://doi.org/10.1016/j.focha.2022.100025>
7. Brazil, 2019. *Instrução Normativa nº 41, de 17 de setembro de 2019: estabelece o Padrão de Identidade e Qualidade da Kombucha em todo o território nacional*. <https://www.in.gov.br/en/web/dou/-/instrucao-normativa-n-41-de-17-de-setembro-de-2019-216803534> (accessed 16 July 2026).
8. Brazil, 2022. *Instrução Normativa nº 161, de 1º de julho de 2022: estabelece as listas oficiais com os padrões microbiológicos dos alimentos*. <https://www.in.gov.br/en/web/dou/-/instrucao-normativa-in-n-161-de-1-de-julho-de-2022> (accessed 16 July 2026).
9. Bueno, F., Chouljenko, A., Sathivel, S. (2021). Development of coffee kombucha containing *Lactobacillus rhamnosus* and *Lactobacillus casei*: Gastrointestinal simulations and DNA microbial analysis. *LWT*, 142, 110980. <https://doi.org/10.1016/j.lwt.2021.110980>

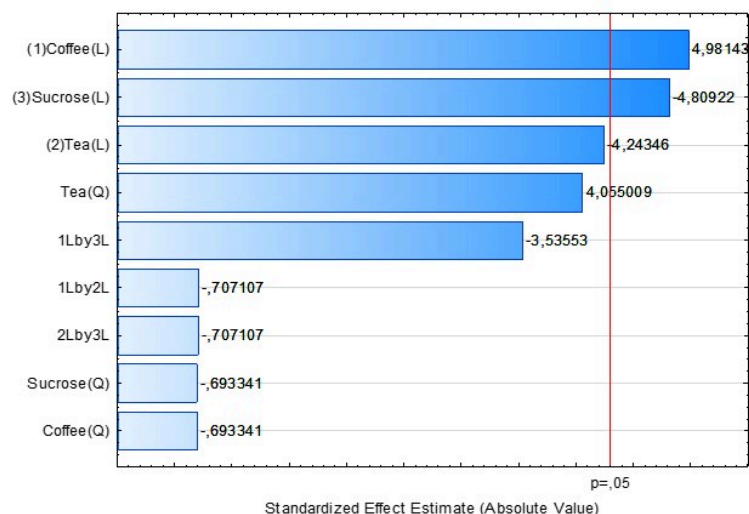
10. Cardoso, R.R., Moreira, L.P.D., Costa, M.A.C., Toledo, R.C.L., Grancieri, M., Nascimento, T.P., Ferreira, M.S.L., Matta, S.L.P., Eller, M.R., Martino, H.S.D., Barros, F.A.R. (2021). Kombuchas from green and black teas reduce oxidative stress, liver steatosis and inflammation, and improve glucose metabolism in Wistar rats fed a high-fat high-fructose diet. *Food Function*, 12, 10813–10827. <https://doi.org/10.1039/D1FO02106K>
11. Chandran, A., Wyka, J., Klein, G.-R., Stefanska, B., Kolniak-Ostek, J. (2026). Shaping the bioactive properties of kombucha drinks by using raw materials alternative to tea. *Molecules*, 31, 1170. <https://doi.org/10.3390/molecules31071170>
12. Cheepchirasuk, N., Kaewkod, T., Suriyaprom, S., Intachaisri, V., Ngamsaard, P., Tragoolpua, Y. (2025). Functional metabolites and inhibitory efficacy of kombucha beverage on pathogenic bacteria, free radicals and inflammation. *Scientific Reports*, 15, 19187. <https://doi.org/10.1038/s41598-025-03545-z>
13. Córdoba, N., Moreno, F.L., Osorio, C., Velásquez, S., Ruiz, Y. (2021). Chemical and sensory evaluation of cold brew coffees using different roasting profiles and brewing methods. *Food Research International*, 141, 110141. <https://doi.org/10.1016/j.foodres.2021.110141>
14. Christensen, Z.T., Ogden, L.V., Dunn, M.L., Eggett, D.L. (2006). Multiple comparison procedures for analysis of ranked data. *Journal of Food Science*, 71, S132–S139. <https://doi.org/10.1111/j.1365-2621.2006.tb08916.x>
15. De Filippis, F., Troise, A.D., Vitaglione, P., Ercolini, D. (2018). Different temperatures select distinctive acetic acid bacteria species and promotes organic acids production during kombucha tea fermentation. *Food Microbiology*, 73, 11–16. <https://doi.org/10.1016/j.fm.2018.01.008>
16. Delorme, M.M., Machado, B.S., dos Santos Ferreira, F., Alves, R.N., Ferreira, D.C., De Lima Moreira, J.P., Castelo Branco, V.N., Uekane, T.M., Fernandes, F.F., Lima, A.R. (2025). Harnessing arabica and canephora coffee cascara as substrates for kombucha: Comparative study of chemical, antioxidant, antibacterial and antifungal properties. *Biocatalysis and Agricultural Biotechnology*, 66, 103570. <https://doi.org/10.1016/j.bcab.2025.103570>
17. Duan, S., Dong, J., Chen, Y., Yu, L., Liu, S., Yu, R., Du, Z., Shen, Y., Lu, X., Fu, J., Yang, R., Fang, C. (2026). Kombucha inoculated fermentation reshapes microbial ecology and flavour metabolism in Yunnan Arabica coffee. *Npj Science of Food*, 10, 194. <https://doi.org/10.1038/s41538-026-00852-1>
18. Dutcosky, S.D. (2019). *Análise sensorial de alimentos*, 5th ed. PUCPRESS, Curitiba.
19. Ikeda, M.E.T.S., Quináia, S.P., Pucholobek, G., Guerrero, M.D.C.L., Cardoso, F.A.R., Perdoncini, M.R.F.G., Rigobello, E.S. (2024). Unveiling the functional potential: Mineral analysis of kombucha enriched with spices as a flavorful health beverage. *South African Journal of Botany*, 171, 497–503. <https://doi.org/10.1016/j.sajb.2024.06.030>
20. Instituto Adolfo Lutz (IAL). (2008). *Métodos físico-químicos para análise de alimentos*, 4th ed. Instituto Adolfo Lutz, São Paulo.
21. International Organisation of Vine and Wine (OIV). (2025). *Update of the OIV International standard for the labelling of spirituous beverages of vitivinicultural origin (resolution OIV-ECO 402-2012)* -

- Information about nutritional declaration. <https://www.oiv.int/node/4095> (accessed 16 July 2026).
22. Kim, J., Bhattarai, U., Adhikari, K. (2022). The healthy eater's idea and related behavior of a healthy diet—A case study with kombucha drinkers. *Beverages*, 8, 25.
<https://doi.org/10.3390/beverages8020025>
 23. Li, S., Chu, K., Guo, R., Wu, J., Wang, X., Zhang, Y., Liu, J. (2025). Tailoring a simplified and steady-state kombucha consortium for newly formulated kiwifruit beverage production: Impacts on untargeted/targeted metabolites, antioxidant capacities and flavor profiles. *Food Research International*, 220, 118217. <https://doi.org/10.1016/j.foodres.2025.118217>
 24. Lin, Y.-H., Huang, H.-W., Wang, C.-Y. (2022). Effects of high pressure-assisted extraction on yield, antioxidant, antimicrobial, and anti-diabetic properties of chlorogenic acid and caffeine extracted from green coffee beans. *Food Bioprocess Technology*, 15, 1529–1538.
<https://doi.org/10.1007/s11947-022-02828-x>
 25. Miranda, J.F., Ruiz, L.F., Silva, C.B., Uekane, T.M., Silva, K.A., Gonzalez, A.G.M., Fernandes, F.F., Lima, A.R. (2022). Kombucha: A review of substrates, regulations, composition, and biological properties. *Journal of Food Science*, 87, 503–527. <https://doi.org/10.1111/1750-3841.16029>
 26. Miranda, J.F., Belo, G.M.P., Lima, L.S., Silva, K.A., Uekane, T.M., Gonzalez, A.G.M., Castelo Branco, V.N., Pitangui, N.S., Fernandes, F.F., Lima, A.R. (2023). Arabic coffee infusion based kombucha: Characterization and biological activity during fermentation, and in vivo toxicity. *Food Chemistry*, 412, 135556. <https://doi.org/10.1016/j.foodchem.2023.135556>
 27. Morais, S.M., Galvão, M.S., Olegario, L.S., Carvalho, L.M., Pereira, G.E., Lima, L.L.A., Silva, F.L.H., Madruga, M.S. (2022). Identification of chemical markers of commercial tropical red wine candidates for the São Francisco Valley Geographical Indication. *Food Analytical Methods*, 15, 1237–1255. <https://doi.org/10.1007/s12161-021-02225-8>
 28. Morales, D., Gutiérrez-Pensado, R., Bravo, F.I., Muguerza, B. (2023). Novel kombucha beverages with antioxidant activity based on fruits as alternative substrates. *LWT*, 189, 115482.
<https://doi.org/10.1016/j.lwt.2023.115482>
 29. Nascimento, M.B., Amorim, L.R., Nonato, M.A.S., Roselino, M.N., Santana, L.R.R., Ferreira, A.C.R., Rodrigues, F.M., Mesquita, P.R.R., Soares, S.E. (2024). Optimization of HS-SPME/GC-MS method for determining volatile organic compounds and sensory profile in cocoa honey from different cocoa varieties (*Theobroma cacao* L.). *Molecules*, 29, 3194. <https://doi.org/10.3390/molecules29133194>
 30. Saito, M.S., Santos, W.A., Mamede, M.E.O. (2024a). Coffee-flavoured kombucha: Development, physicochemical characterisation, and sensory analysis. *Fermentation*, 10, 334.
<https://doi.org/10.3390/fermentation10070334>
 31. Saito, M.S., Ferreira, R.M.S., Jesus, A.C.A., Mamede, M.E.O. (2024b). Analysis of innovations in kombucha production and the protection of intellectual property. *Biotechnology Research and Innovation*, 8, e2024016. <https://doi.org/10.4322/biori.00162024>
 32. Sales, A.L., Iriondo-DeHond, A., DePaula, J., Ribeiro, M., Ferreira, I.M.P.L.V.O., Miguel, M.A.L., del Castillo, M.D., Farah, A. (2023). Intracellular antioxidant and anti-inflammatory effects and bioactive

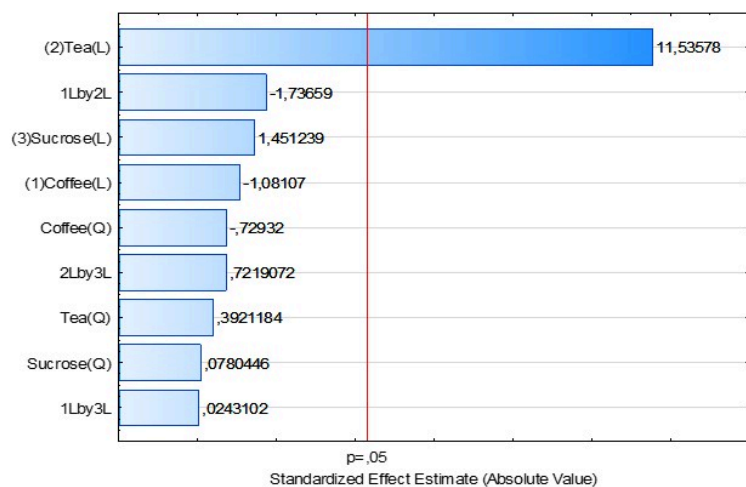
- profiles of coffee cascara and black tea kombucha beverages. *Foods*, 12, 1905.
<https://doi.org/10.3390/foods12091905>
33. Silva Júnior, J.C., Magnani, M., Costa, W.K.A., Madruga, M.S., Olegário, L.S., Borges, G.S.C., Dantas, A.M., Lima, M.S., Lima, L.C., Brito, I.L., Souza, E.L. (2021). Traditional and flavored kombuchas with pitanga and umbu-cajá pulps: Chemical properties, antioxidants, and bioactive compounds. *Food Bioscience*, 44, 101380. <https://doi.org/10.1016/j.fbio.2021.101380>
 34. Silva, M.E., Oliveira, R.L., Moraes, M.M., Câmara, C.A.G., Arruda, L.L.A.L., Silva, S.P., Porto, T.S. (2025). Impact of fermentation time on the bioactive and volatile composition of coffee: Insights for producers and researchers. *Food Chemistry*, 490, 145067.
<https://doi.org/10.1016/j.foodchem.2025.145067>
 35. Sola, I.M.M.S., Marcondes, A., Fischer, T.E., Nunes, G., Nogueira, A., Alberti, A. (2025). Enhanced kombucha fermentation with yerba mate and purple pitanga (*Eugenia uniflora* L.). *Journal of Food Measurement and Characterization*, 19, 1373–1385. <https://doi.org/10.1007/s11694-024-03051-2>
 36. Tassew, A.A., Yadessa, G.B., Bote, A.D., Obso, T.K. (2021). Influence of location, elevation gradients, processing methods, and soil quality on the physical and cup quality of coffee in the Kafa Biosphere Reserve of SW Ethiopia. *Heliyon*, 7, e07790. <https://doi.org/10.1016/j.heliyon.2021.e07790>
 37. Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., Tournet-Maréchal, R. (2020). Microbial dynamics between yeasts and acetic acid bacteria in kombucha: Impacts on the chemical composition of the beverage. *Foods*, 9, 963. <https://doi.org/10.3390/foods9070963>
 38. Tong, K., Liao, Y., Tang, Y., Luo, Y., Liu, X., Yu, D., Zhou, J., Hou, C., Li, Z. (2026). Red yeast rice-driven kombucha fermentation: A novel strategy for developing functional beverages with enhanced hypoglycemic and hypolipidemic properties. *Foods*, 15, 747.
<https://doi.org/10.3390/foods15040747>
 39. Venegas, C., Saona, L.A., Urbina, K., Quintrel, P.A., Peña, T.A., Mardones, W., Cubillos, F.A. (2023). Addition of *Saccharomyces eubayanus* to SCOBY fermentations modulates the chemical and volatile compound profiles in kombucha. *Food Microbiology*, 116, 104357.
<https://doi.org/10.1016/j.fm.2023.104357>
 40. Villarreal-Soto, S.A., Beaufort, S., Bouajila, J., Souchard, J.-P., Taillandier, P. (2018). Understanding kombucha tea fermentation: A review. *Journal of Food Science*, 83, 580–588.
<https://doi.org/10.1111/1750-3841.14068>
 41. Wang, C., Han, J., Pu, Y., Wang, X. (2022). Tea (*Camellia sinensis*): A review of nutritional composition, potential applications, and omics research. *Applied Sciences*, 12, 5874.
<https://doi.org/10.3390/app12125874>
 42. Wang, S., Li, C., Xu, Q., Wang, Y., Zhang, Y., Yuan, L. (2024). Addition of lactic acid bacteria modulates microbial community and promotes the flavor profiles of kombucha. *Food Bioscience*, 60, 104340.
<https://doi.org/10.1016/j.fbio.2024.104340>
 43. Yeager, S.E., Batali, M.E., Guinard, J.-X., Ristenpart, W.D. (2023). Acids in coffee: A review of sensory measurements and meta-analysis of chemical composition. *Critical Reviews in Food Science and*

44. Zhang, J., Van Mullem, J., Dias, D.R., Schwan, R.F. (2021). The chemistry and sensory characteristics of new herbal tea-based kombuchas. *Journal of Food Science*, 86, 740–748. <https://doi.org/10.1111/1750-3841.15613>

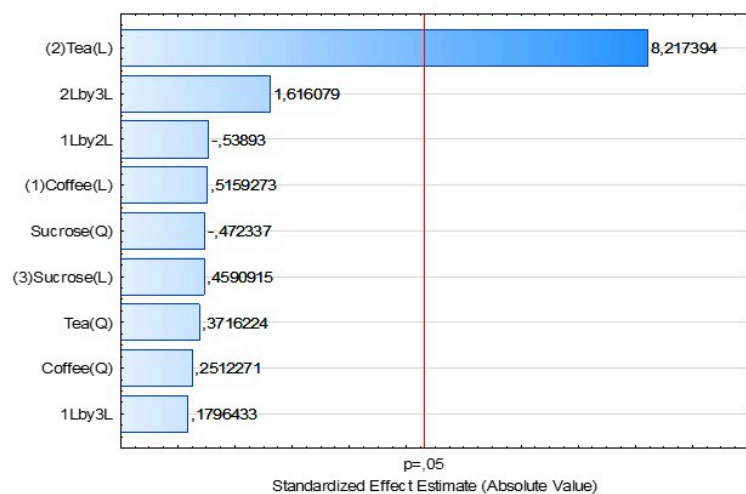
Figures



A



B



C

Figure 1

Pareto chart showing the effect of the variables on the pH (a), volatile acidity (mEq/L) (b), and total acidity (%) (c)

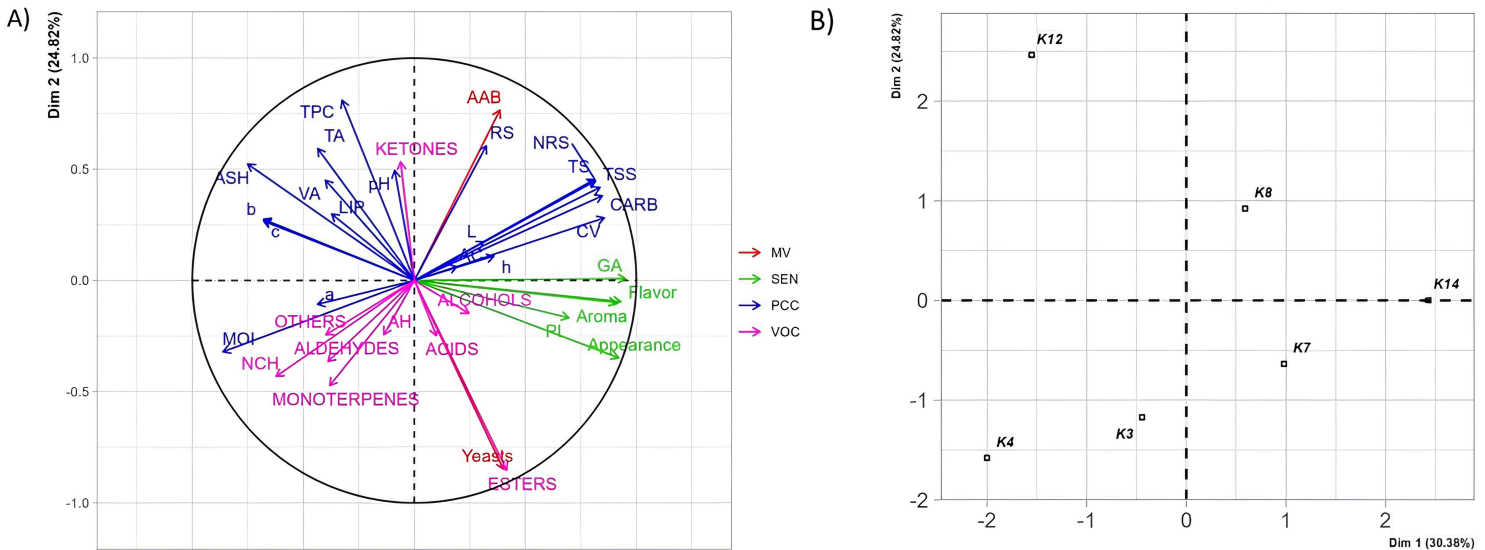


Figure 2

Multiple Factor Analysis (MFA) applied to kombucha samples. a) Correlation plot of the active variables grouped by category: Microbiological viability (MV) in red, Sensory (SEN) in green, Physicochemical and chemical (PCC) in blue, and Volatile organic compound profile (VOC) in pink. b) Factor map of individual samples in the first two dimensions (Dim 1 and Dim 2)

AAB – acetic acid bacteria; GA – Global acceptance; PI – Purchase intent; MOI – Moisture; LIP – Lipids; CARB – Carbohydrates; RS – Reducing sugar; NRS – Non-reducing sugar; TS – Total sugar; CV – Caloric value; TSS – Total soluble solids; VA – Volatile acidity; TA – Total acidity; AC – Alcoholic content; TPC – Total phenolic compounds; NCH – Nitrogen-containing heterocyclic; AH – Aromatic hydrocarbons.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.png](#)
- [SupplementaryMaterials.docx](#)