International Journal of Gastronomy and Food Science 34 (2023) 100844

Contents lists available at ScienceDirect

International Journal of Gastronomy and Food Science

journal homepage: www.elsevier.com/locate/ijgfs

Revamping kombucha production: Achieving consistency and probiotic potential through a tailor-made microbial consortium

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ARTICLE INFO

Keywords:
Fermentation
Tailor-made starter culture
Probiotic beverage
Microbial consortium

ABSTRACT

Revising the production of kombucha, this investigation focused on the utilization of a custom-designed starter culture, aiming to establish a consistent, probiotic-rich beverage. A diverse selection of three acetic acid bacteria and two yeast strains was examined to determine the optimal microbial combination. A meticulous examination of the fermentation timeline was undertaken, juxtaposing forced and natural carbonation techniques. A comprehensive analysis encompassing fermentation metabolites, sensory acceptance, volatile compound profiling, and shelf-life testing was executed to ensure the beverage's superior quality and stability. The resultant probiotic kombucha was produced successfully after 48 h of fermentation with a symbiotic assembly of *Komagataeibacter saccharivorans*, *Brettanomyces anomala*, and *Kluyveromyces marxianus*. Forced carbonated kombucha exhibited acceptance levels rivaling commercial brands, maintaining an alcohol content consistently beneath the 0.5% (v/v) regulatory standard for a 60-day storage period. Specific esters, namely ethyl 3-methyl butanoate, phenethyl acetate, ethyl hexanoate, and 2-methyl-1-propyl acetate, were identified as key determinants of kombucha flavor profiles. The 90-day shelf-life study indicated a consistent presence of viable probiotic *K. marxianus* cells in the kombucha. These findings contribute to understanding probiotic Kombucha fermentation and demonstrate the potential for producing a high-quality beverage with desirable sensory characteristics through a custom-designed microbial consortium.

1. Introduction

Functional foods have gained significant interest from researchers, industries, and consumers due to their potential positive effects on health and wellness, particularly in disease prevention (Kapsak et al., 2011; Pimentel et al., 2021). Among the various functional beverages, kombucha has emerged as a popular fermented beverage in the food industry and is often produced at home. Despite limited scientific evidence supporting its health-promoting effects, kombucha has garnered attention and generated numerous health claims through popular media (Martini, 2018; Vargas et al., 2021; Batista et al., 2022).

Kombucha is a fermented beverage produced by the fermentation of sweetened green and/or black tea decocts using a symbiotic microbiome present in a cellulosic pellicle in the beverage known as SCOBY (Symbiotic Culture of Bacteria and Yeast) (Jayabalan et al., 2014). Acetic acid bacteria (AAB) in this mixed microbial culture are primarily responsible for the production of organic acids, notably acetic and glucuronic acids (Tran et al., 2020). The microbial composition of SCOBY varies depending on its origin, with *Komagataeibacter* (some species reclassified as *Novacetimonas*, Acetobacter, and Gluconacetobacter) being the predominant bacteria reported in the literature and *Brettanomyces/Dekkera* and *Zygosaccharomyces* being the most common yeast strains (Chakravorty et al., 2016; Arıkan et al., 2020; Brandão et al., 2022; Fabricio et al., 2022). Notably, the microbial composition of kombucha can differ

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https://doi.org/10.1016/j.ijgfs.2023.100844
Received 26 May 2023; Received in revised form 30 October 2023; Accepted 14 November 2023
Available online 21 November 2023

significantly among producers, including commercial sources of the beverage (Chakravorty et al., 2016; Suhre et al., 2021; Fabricio et al., 2022).

The microbial diversity within kombucha is closely associated with its functional characteristics, particularly its potential probiotic properties. Probiotic products require an adequate quantity of specific strains capable of positively influencing the intestinal microbiota and conferring benefits to the host (FAO/WHO, 2006). Kombucha contains live microorganisms in its final composition, leading to frequent labeling or marketing of the beverage as a probiotic drink. However, such claims may be inaccurate and inconsistent due to limited knowledge regarding the cellular concentrations of individual species and their probiotic effects (Vargas et al., 2021). Microbial diversity also impacts the biochemical composition and sensory attributes of the final product. Kombucha, often marketed as a probiotic beverage, hosts a diverse array of microorganisms that significantly influence its biochemical composition and sensory attributes. The variations in microbial diversity can result in changes in beneficial organic acids, enzymes, vitamins, flavor profile, aroma, and even the viscosity of the drink. For example, specific strains like *Gluconacetobacter* and *Acetobacter xylinum* can increase the concentrations of detoxifying acids and enhance the drink's viscosity, respectively. Likewise, different yeasts can alter the ethanol content, impacting the flavor and nutritional value. Despite these intriguing factors, the traditional backslopping fermentation method poses challenges for industrial production due to its uncontrolled nature, highlighting the need for more research into microbial strain selection to standardize kombucha production (De Filippis et al., 2018). While the traditional fermentation process involving the backslopping method is suitable for household production of kombucha, it is unsuitable for industrial applications due to the uncontrolled and heterogeneous nature of the process (Vargas et al., 2021). Furthermore, natural microbial consortia may produce inhibitory and/or toxic metabolites that interfere with the natural microbiota of the SCOBY (Che and Men, 2019). Process variables such as temperature, pH, substrate concentration, vessel geometry, and time play a role in controlling the fermentation process but are insufficient to ensure standardized kombucha production (Abaci et al., 2022). The alcohol content of kombucha is a particular concern for industries, as maintaining an alcohol content below 0.5% (v/v) is required by regulations in several countries (Talebi et al., 2017; Jang et al., 2021; Suhre et al., 2021). In light of these technical challenges, the use of a tailor-made starter culture could be a promising approach to ensure the composition, safety, quality, and functional properties. It has been reported that it is possible to produce kombucha using isolated strains and it impacts the antioxidant properties and acid concentration (Malbaša et al., 2011).

Addressing these technical concerns, the present study endeavored to develop a tailor-made starter culture of bacteria and yeasts suitable for probiotic kombucha production, serving as an alternative to the traditional inoculation process (cellulose pellicle + fermented liquid from previous fermentations). The suitability of the microbial consortia was evaluated based on several parameters: metabolite production, cell viability, sugar consumption, and shelf life. The profile of volatile compounds was also analyzed as a part of the study. A sensory analysis was conducted to determine the impact of the fermentation process on the final product's attributes.

By combining scientific rigor with a tailored approach, this study aimed to contribute to the advancement of probiotic kombucha production, paving the way for a more controlled and standardized manufacturing process. The results obtained from this research will serve as a foundation for developing high-quality kombucha products with consistent microbial composition, desired functional properties, and sensory attributes that align with consumer preferences.

Acetobacter aceti, *Novacetimonas hansenii*, *Komagataeibacter saccharivorans*, *Brettanomyces anomala*, and *Kluyveromyces marxianus* were specifically selected for their unique characteristics and contributions to the kombucha fermentation process. *A. aceti* is renowned for its ability to oxidize ethanol to acetic acid, contributing to the beverage's tangy taste. *N. hansenii*, commonly found in kombucha, is thought to enhance the overall flavor profile, although its exact role remains underexplored. *K. saccharivorans* plays a key part in cellulose production, which is integral to the SCOBY formation. *B. anomala*, a yeast species, contributes to the synthesis of aromatic compounds that define kombucha's characteristic aroma. Lastly, *K. marxianus* is known for its high ethanol production capability, which is later converted into acetic acid and other organic acids by acetic acid bacteria. These unique attributes of the selected microorganisms aim to achieve a controlled fermentation process that yields a final product with the desirable taste, aroma, and biochemical properties that define traditional kombucha.

2. Material and methods

2.1. Development of starter culture for kombucha fermentation

2.1.1. Strains, cell maintenance, and pre-inocula

Co-cultures of three acetic acid bacteria (AAB) and two yeasts were tested in this work. The AAB *Acetobacter aceti* (ATCC 15973) and *Novacetimonas hansenii* (formerly *Gluconacetobacter hansenii*) (ATCC 23769) were obtained from André Tosello Research and Technology Foundation (FAT, Campinas, Brazil). *Komagataeibacter saccharivorans* was kindly supplied by the University of Madeira (UMa, Funchal, Portugal). The yeast *Brettanomyces anomala* (UFMG-CM-Y4734) was kindly provided by the Collection of Microorganisms and Cells of the Federal University of Minas Gerais (UFMG, Minas Gerais, Brazil) and the *Kluyveromyces marxianus* (formerly *Kluyveromyces fragilis*) (BO399) was kindly supplied by Turval Company (Udine, Italy). Cells were maintained frozen at −80 °C, in a 40 % glycerol solution-cell suspension. For immediate use, cells of *A. aceti* and *N. hansenii* were kept on plates containing Mannitol agar (25 g/L mannitol, 3 g/L peptone, 5 g/L yeast extract, 15 g/L agar). *K. saccharivorans* was kept on MRS agar (De Man et al., 1960), and yeasts were cultivated on YM agar (10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract, 20 g/L agar). The same culture medium, without agar, was used as a pre-inoculum. The yeast *K. marxianus* B0399 was selected based on its functional and technological properties. This strain presents stability for application in fermented products and probiotic properties, such as immune system stimulation, gut colonization, and the ability to survive in the gastrointestinal tract (Maccaferri et al., 2012; Tabanelli et al., 2016). This yeast strain is included in the list of qualified presumptions as safe by the European Food Safety Authority (EFSA) (Maccaferri et al., 2012).

Pre-inocula were prepared by transferring 0.5 mL of glycerol-solution cell suspension of each microorganism to 250 mL conical flasks containing 50 mL of each respective cultivation medium. Flasks were incubated in a rotary shaker at 37 °C and 180 rpm for *K. marxianus* and 30 °C and 120 rpm for the other microorganisms, until reaching desired cell concentration. The cell pellets were washed twice using sterile distilled water, then centrifuged at 3000 *g* for 15 min, and resuspended in a sweetened green tea infusion to be used as the starter culture.

2.1.2. Preparation of tea infusion and fermentation conditions

All formulations of kombucha were fermented on the same culture medium, consisting of distilled water, 8 g/L of organic green tea (Vemat, SC, Brazil), and organic demerara sugar (Native, SP, Brazil), with varying concentrations of sugar, depending on the experiment, either 60 g/L or 50 g/L (results section). Fermentations were performed in 250 mL glass beakers filled with 250 mL of sweetened green tea, and the flasks were covered with sterile gauze and cheesecloth to create aerobic conditions. Different batches of kombucha were fermented with each starter culture at 28 °C.

For the preparation of sweetened tea, a solution of distilled water added with sucrose was sterilized at 121 °C for 15 min. Green tea leaves were infused for 10 min in boiling water and then filtered using a

membrane pore size of 0.22 μm. After cooling, tea infusion was added to the sugar solution, and 250 mL of sweetened tea was placed in each beaker. The initial pH was set to 4.5, using a sterile phosphoric acid solution (1 M) to avoid mold growth.

2.1.3. Starter culture design

A Plackett-Burmann (PB) design was performed on four microbial strains (variables): *A. aceti*, *N. hansenii*, *K. saccharivorans*, and *B. anomala* for determining suitable microbial combinations producing Kombucha. The yeast *K. marxianus* was used as a fifth, fixed ingredient because of the probiotic properties and its ability to hydrolyze sucrose, as observed in previous experiments in our laboratory (results not shown). The microbial concentrations, as determined based on data from previous experiments (data not shown), were 1×10^7 CFU/mL for bacteria and 1×10^5 CFU/mL for yeasts. An 8-run PB design (Table 1) was used to evaluate the survival of each strain and production of acetic acid and ethanol (the two key products in kombucha) after 10 days of fermentation, using 60 g/L of sugar.

For the PB experiments, the culture media were inoculated with respective starter cultures followed by incubation at 28 °C for 10 days. At the end of fermentation, samples of 20 mL were collected from beakers for analysis of microbial growth by surface inoculation on agar, pH, sugar concentrations, and fermentation metabolites (see 2.2).

2.1.4. Growth kinetics of kombuchas fermented with the tailor-made starter culture

The most suitable mixture of microorganisms obtained in the PB design was further studied. Kinetics of fermentation were performed in the same condition to observe the evolution of metabolite production and sugar consumption. Then, the same starter culture, with a higher concentration of the yeast *K. marxianus* (1×10^6 CFU/mL), was fermented to improve the hydrolysis of sucrose, probiotic activity in the final product, and a shorter fermentation time. After obtaining a shorter fermentation time of only 3 days, the concentration of 50 g/L of sucrose was tested to find whether a complete fermentation in a shorter time and residual sugar concentrations similar to commercial brands were possible to obtain. Fermentations were performed as described by the PB design (described in sections 2.1.2 and 2.1.3). Samples were collected along fermentations to evaluate pH, microbial growth, sugar consumption, acids, and alcohol production.

The fermented kombuchas were bottled in sterile hermetic glass bottles, and two different processes of carbonation were carried out. A natural carbonation process was performed, in which the yeasts were responsible for converting sugars into CO_2 and consisted of bottling and fermenting at 28 °C for 48 h (Fabricio et al., 2022). The second process was forced carbonation, consisting of infusing CO_2 into the liquid from a 2 kg-gas cylinder connected to a CO_2-regulator manometer operating at 1 bar and a hose-fitting, similar to the process performed in industries. In this case, the kombucha was refrigerated at 4 ± 0.5 °C, and then CO_2 was injected at constant agitation until the bottle pressure stabilized at 1 bar. This stage aimed to evaluate the most suitable process for kombucha carbonation, as well as to compare the influence of the carbonation method on the final product characteristics and shelf-life. The natural and forced carbonated kombuchas' sensory acceptance and volatile profile were assessed and compared with two commercial brands. Volatile compounds of non-fermented tea were also evaluated.

Table 1
Process variables and experimental results of the 8-run Plackett-Burmann design to study the impact of co-cultured strains on kombucha fermentation. Results are expressed in g/L.

Run	AA	NH	BA	KS	Acetic acid	Ethanol
1	−1	−1	−1	1	0.00 ± 0.00	0.00 ± 0.00
2	1	−1	−1	−1	0.31 ± 0.01	9.08 ± 0.15
3	−1	1	−1	−1	0.29 ± 0.06	8.60 ± 0.17
4	1	1	−1	−1	0.33 ± 0.02	9.08 ± 0.21
5	−1	−1	1	1	3.39 ± 0.67	0.27 ± 0.02
6	1	−1	1	−1	0.32 ± 0.02	6.22 ± 0.48
7	−1	1	1	−1	0.31 ± 0.04	6.74 ± 0.12
8	1	1	1	1	5.03 ± 0.34	0.28 ± 0.07

AA: *A. aceti;* NH: *N. hansenii;* BA: *B. anomala;* KS; *K. saccharivorans.*
(-1) absence of variable; (1) presence of variable.

2.1.5. Shelf-life study design

A shelf-life study of the natural and forced carbonated kombuchas was performed to evaluate the stability of the final product, especially in terms of the cell viability of the probiotic yeast and alcohol content. Kombucha samples (at 4 °C) after carbonation were analyzed for pH, cell viability, sugars, and metabolite concentrations after 10, 20, 30, 45, 60, and 90 days of storage.

2.2. Analytical methods

2.2.1. Enumeration of microorganisms

Microorganisms were enumerated (CFU counting) by surface inoculation on agar, using specific enumeration media adapted according to literature (Morace et al., 1991; Pereira et al., 2012, 2015) and incubated at 30 °C. To inhibit yeast growth, *A. aceti* and *N. hansenii* were plated on Mannitol agar containing 128 μg/mL of fluconazole. *K. saccharivorans* was cultivated on MRS agar with 128 μg/mL of fluconazole and 0.1 % (v/v) of cysteine-HCl to inhibit the growth of *A. aceti* and *N. hansenii*. To inhibit bacterial growth, yeast enumeration was performed on YM agar plates containing 34 μg/mL chloramphenicol (Sigma-Aldrich, Germany).

2.2.2. Determination of substrate consumption and fermentation metabolites

Collected samples were centrifuged (3000×*g*, 15 min), and the supernatant was filtered through 0.22 μm membrane pore size. The concentration of glucose, sucrose, fructose, glycerol, ethanol, acetic, lactic, and succinic acids were calculated using calibration curves obtained by a standard of each compound. Analyses were performed in HPLC using Bio-Rad Aminex 87C for sugars and Bio-Rad Aminex 87H for organic acids and alcohols. HPLC assay conditions used for each column were run according to a previous publication (Fabricio et al., 2022).

2.3. Sensory analysis

The kombuchas produced in this work (natural and forced carbonated) and two Brazilian commercial brands were subjected to an acceptance test using a 9-point hedonic scale (1- dislike extremely; 9-like extremely). Panelists evaluated the attributes of appearance, aroma, taste, acidity, and overall acceptance. The Acceptance Index (AI) was calculated by:

$$\text{AI}\ (\%) = (\text{Attribute media} \times 1/9) \times 100 \quad (1)$$

One hundred untrained panelists, aged between 18 and 60 years old, were served with randomized samples of 30 mL coded with a three-digit random number. The panelists were asked if they have tried kombucha before and the results were analyzed separately for all participants and for only the participants that have tried kombucha before.

The research was approved by the University Ethical Committee (UFRGS, Protocol nº 18613419.8.0000.5347).

2.4. Volatile compound profile

Volatile compounds (VCs) were extracted by headspace solid-phase microextraction (HS-SPME) using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) 2 cm–50/30 μm covering fiber (SupelcoTM, Darmstadt, Germany), according to Bernardi et al. (2014). For analysis, 5 ± 0.1 mL of kombucha were transferred into 20 mL glass

vials (hermetically closed with a silicone/PTFE cap), added with 1.6 ± 0.1 g of NaCl and 10 μL of 3-octanol (internal standard (IS), 8.5 μg/mL). The vials were then immersed in a thermostatic water bath at 35 °C for 10 min, followed by a 45 min exposure for adsorption of the compounds. Each treatment was done in triplicate. The VCs analysis was run in a gas chromatograph coupled to a mass spectrometer (GC/MS) (Shimadzu GC/MS-QP, 2010 Plus, Kyoto, Japan). The fiber was thermally desorbed into the injector at 250 °C for 10 min in splitless mode (1 min split-off). Helium was the carrier gas at a constant flow of 1.3 mL/min. The VCs were separated using a polar phase fused silica capillary column (ZB-Wax, Phenomenex, USA; 60 m × 0.25 mm; 0.25 μm of thickness film). The initial oven temperature was set at 35 °C for 1 min, followed by a 3 °C/min temperature ramp to 180 °C and then, increasing 5 °C/min up to 230 °C, remaining for 2 min. The GC/MS interface and the ionization source were kept at 250 °C and 230 °C, respectively. The MS data were collected in the electron impact ionization mode at +70 eV, using mass range scanning of 35–350 m/z. The identification of VCs was performed by comparing the mass spectrum available in the National Institute of Standards and Technology (NIST) library, the linear retention index (LRI) from literature, with those experimentally obtained data. The experimental LRI was obtained through a series of n-alkanes at the same GC conditions. The VCs concentration was determined by internal standardization using the equivalent of a 3-octanol IS solution. The response factor between IS and each analyte was assumed as one. All analyses were carried out in triplicate and the results were expressed as mg/L.

2.5. Statistical analysis

The results obtained from fermentations were submitted to analysis of variance (ANOVA), and the means were compared using Tukey's test ($p < 0.05$). The significant results of volatile compounds analysis and sensory evaluation were subjected to principal component analysis (PCA) using the Statistica 12.5 software (StatSoft Inc., USA). For this, each variable was auto-scaled to obtain the same weight for all variables (mean = 0 and variance = 1) before PCA analysis.

3. Results and discussion

3.1. Selection of the microorganisms for kombucha fermentation

Table 1 displays the results of the Plackett-Burman experiments designed to elucidate the cross-feeding interactions between strains, which are critical to fermentation. The aim was to determine the optimal consortium of microorganisms for converting sweetened green tea into kombucha, similar to traditional SCOBY. Regulatory limits on alcohol and acetic acid levels, 0.5% (v/v) and between 1.8 and 7.8 g/L, respectively, served as benchmarks for the fermentation process. The lower limit of acetic acid was the target to avoid imparting an unpleasant aroma to the product (BRAZIL, 2019).

Data indicated that *A. aceti* and *N. hansenii* bacteria were not suitable for kombucha fermentation in co-culture because both strains lost viability (Fig. 1). The co-cultures involving *K. marxianus* with *A. aceti* and/or *N. hansenii* (runs 2, 3, 4, 6, and 7) produced high concentrations of ethanol and succinic acid, a scenario absent in consortia utilizing *K. saccharivorans* (runs 1, 5, and 8) (Table 1). Also, the final alcohol concentrations in runs 2, 3, 4, 6, and 7 surpassed legal limits. The pH values were dependent on bacterial growth and acetic acid production. Consortia incorporating *A. aceti* and *N. hansenii* resulted in a final pH of 3.3 with acetic acid levels below 0.5 g/L, whereas those using *K. saccharivorans* led to a pH of 2.5 with acetic acid production between 3.4 and 5 g/L (Fig. 1, Table 1). This evidence points to the low pH tolerance of *K. marxianus* and *B. anomala*, indicative of their robustness for kombucha fermentation.

Run 5, which included *K. saccharivorans*, *B. anomala*, and *K. marxianus*, demonstrated the most promising combination of

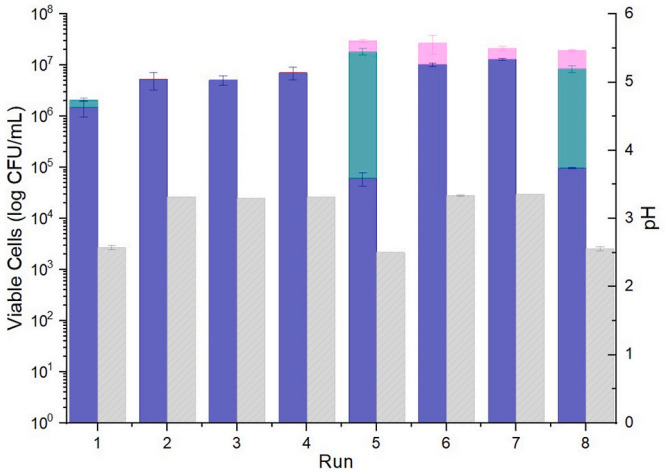

Fig. 1. pH and viable cells of bacteria and yeast populations after 10 days of fermentation with different starter culture designed by the 8-Run PB experiments presented in Table 1. Blue: *K. marxianus*; Green: *K. saccharivorans*; Pink: *B. anomala*; Gray: *N. hansenii* and *A. aceti*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

microorganisms for kombucha fermentation. *K. saccharivorans* maintained viability and produced acetic acid within legal limits in this configuration. While run 8 also exhibited favorable values for ethanol and acetic acid, it involved two acetic acid bacteria (*A. aceti* and *N. hansenii*) that did not survive beyond 10 days of fermentation. Using this combination would impact the economic feasibility without improving the kombucha composition.

Therefore, the microbial consortium from run 5 was selected for subsequent experiments. This choice was based on the mutualistic relationship observed. The chosen consortium exemplifies mutualism, a symbiotic relationship that ensures the survival and growth of all microorganisms involved (Che and Men, 2019). In this case, the yeasts not only hydrolyzed sucrose into glucose and fructose for bacterial sustenance but also produced ethanol. The AAB in the consortium could then oxidize this ethanol into acetic acid. Moreover, the AAB also have the capability to oxidize glucose into gluconic acid, further diversifying the product of fermentation. As yeast cells proliferate, the nutrients they generate create an environment conducive to the growth of AAB. Over time, the accumulation of organic acids metabolized by the AAB slows down yeast growth, avoiding excessive alcohol production during fermentation. Thus, these complex metabolic interactions contribute to the production of a balanced, legal kombucha product.

3.2. Kinetics of kombucha fermented using the tailor-made starter culture

To understand the metabolism of the chosen starter culture, 10-day kinetics was performed under the same conditions of previous experiments (Table 1, run 5), with results presented in Fig. 2. The pH decreased from 4.50 to 2.85 in 3 days, further dropping to 2.44 at the 7th day of fermentation, which is related to microbial growth and acid production. *K. marxianus* population increased until the 3rd day, remaining constant until the 10th day. The population of this yeast is responsible for ethanol production because it has a faster growth rate as compared to *B. anomala*. Most of the ethanol produced was oxidized to acetic acid by *K. saccharivorans*, which remained stable through the 10 days of fermentation. The amount of *B. anomala* increased to 7.14 log CFU/mL after 10 days of fermentation, suggesting that this strain is tolerant to low pH environments. After the 8th day of fermentation, the pH value was below legal limits (pH 2.5), suggesting the fermentation should be interrupted at the 8th day, at most. The sugar consumption was low, with 53.96 g/L of the total sugar remaining on the 10th day.

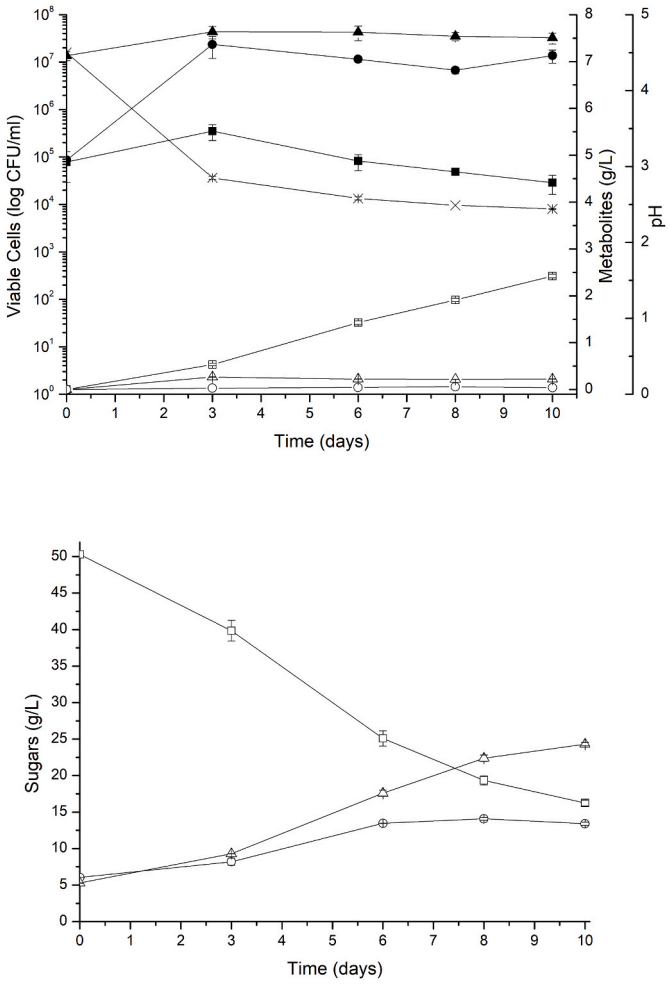

Fig. 2. 10-days kinetics of kombucha fermentation with the tailor-made consortia (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B. anomala,* and 10^5 CFU/mL of *K. marxianus*) from run 5 of PB experiments. **A)** Metabolites: pH (×), ethanol (Δ), glycerol (○), acetic acid (□); and viable counts: *K. saccharivorans* (▲), *K. marxianus* (●), and *B. anomala* (■). **B)** Sugars: sucrose (□), glucose (○) and fructose (Δ). Experiments are mean of duplicate.

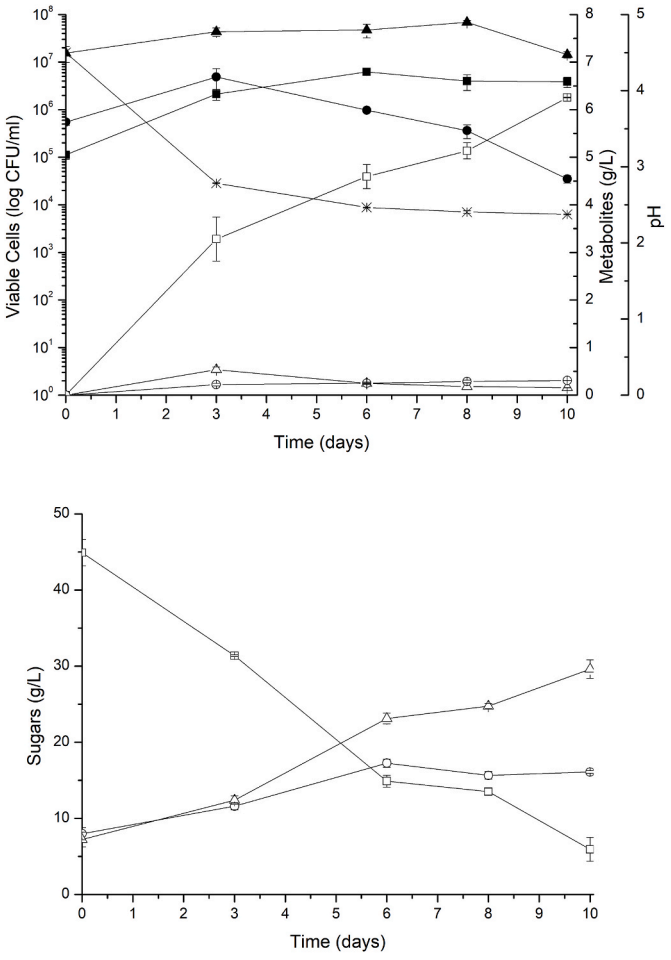

Fig. 3. 10-days kinetics of kombucha fermentation with the tailor-made consortia higher concentration of the probiotic yeast (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B.* anomala, and 106 CFU/mL of K. marxianus). A) Metabolites: pH (×), ethanol (Δ), glycerol (○), acetic acid (□), viable counts of K. saccharivorans (▲), K. marxianus (●), and B. *anomala* (■). **B)** Sugars: sucrose (□), glucose (○) and fructose (Δ). Experiments are mean of duplicate.

Modifying the starter culture composition individually is essential to make the tailor-made consortia cost-effective, stable, and robust (Che and Men, 2019). A shorter time of fermentation would be of economic advantage to turn the process more industrially efficient. To accelerate kombucha fermentation, kinetics using a higher initial population of *K. marxianus* to 1 × 10^6 CFU/mL was performed. This step aimed to obtain a higher concentration of the probiotic yeast in the final beverage, in addition to reducing the fermentation time. The results showed a higher production of ethanol and glycerol and, consequently, acetic acid production (Fig. 3). In this experiment, although the synthesis of ethanol was higher, the acetic acid bacteria were able to maintain the final amount of alcohol very low, as in the previous experiment (less than 0.4 g/L). After 10 days of fermentation, acetic acid concentration was high (6.26 ± 0.01 g/L), and the pH was low (<2.5), suggesting that this condition has a positive impact on fermentation time, and it would result in the same product as the previous experiment in only 3 days. On day 3, the highest viable counts of the probiotic yeast *K. marxianus* were observed. The cell concentration had a considerable effect on sugar hydrolysis, thereby reducing sucrose and increasing glucose and fructose in the final product. The total sugar consumption on the 10th day was around 11 % of the initial sugar. Thus, the following experiments were performed for 3 days using 50 g/L of sucrose instead of 60 g/L.

The 3-day kinetics results (Fig. 4) showed that the consortia had a remarkable ability to ferment kombucha, and it would be possible to interrupt fermentation as early as 48 h to obtain a product with low amounts of alcohol and acetic acid within the legislation in place.

The next step was to test the carbonation of the kombucha after 48 h of fermentation and to analyze its sensory acceptance and composition of volatile compounds. The results of organic acids, pH, and alcohols of the final products made by natural carbonation (NC), forced carbonation (FC), and two commercial brands of kombucha (CB1 and CB2) are shown in Table 2. The brands were chosen regarding the production process because CB1 is produced by natural fermentation, whereas CB2 is produced using the forced carbonation method.

The secondary fermentation of kombucha is carried out to obtain a sparkling beverage through the production of ethanol and carbon dioxide in the anaerobic environment of the bottle. Under this condition, the metabolism of acetic acid bacteria is inhibited, and the yeasts carry out alcoholic fermentation by converting residual sugars. Natural carbonation is a common procedure used by industries. However, the control of the fermentation is influenced by many variables, such as the addition of fruits or juices as flavoring agents, which adds more sugar and other microorganisms to the beverage (Kim and Adhikari, 2020; Tran et al., 2020). This may interfere with the fermentation, risking spoiling an entire batch of kombucha production. The production of

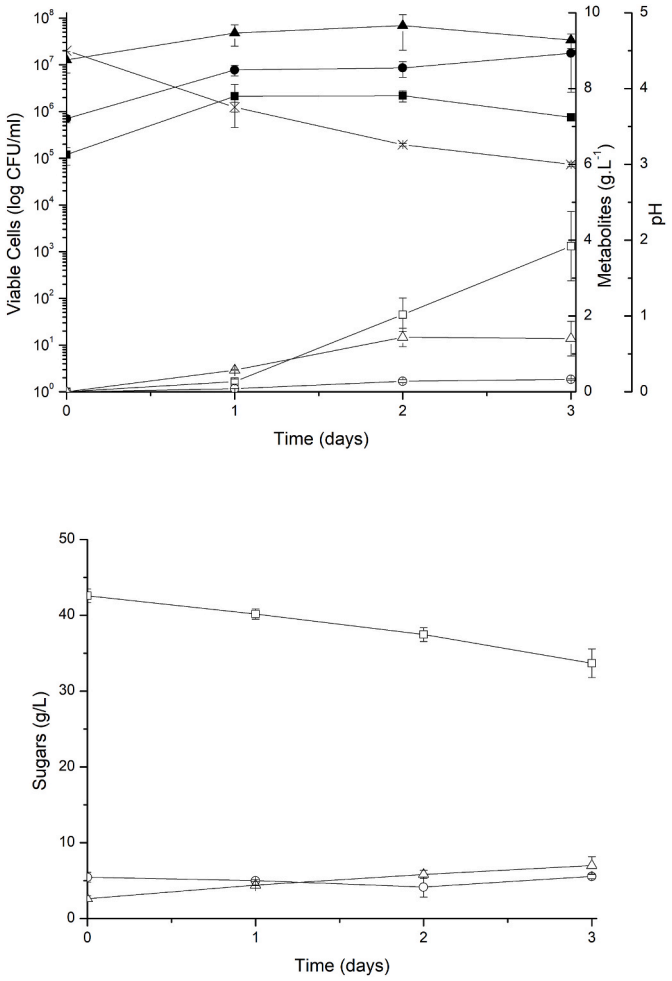

Fig. 4. 3-days kinetics of kombucha fermentation with the tailor-made consortia with lower sugar concentration (50 g/L) (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B. anomala,* and 10^6 CFU/mL of *K. marxianus*. **A**) Metabolites: pH (×), ethanol (Δ), glycerol (○), acetic acid (□), viable counts of *K. saccharivorans* (▲), *K. marxianus* (●), and *B. anomala* (■). **B**) Sugars: sucrose (□), glucose (○) and fructose (Δ). Experiments are mean of triplicate.

Table 2
Concentrations of sugars, organic acids, and pH of kombuchas made by NC and FC and two commercial brands (CB1 and CB2). Results are expressed in g/L.

Sample	FC	NC	CB1	CB2
Acetic acid	1.74 ± 0.05^{b}	1.88 ± 0.07^{b}	5.74 ± 1.10^{a}	3.03 ± 0.34^{b}
Glycerol	0.28 ± 0.14^{b}	0.45 ± 0.05^{ab}	0.53 ± 0.13^{a}	0.06 ± 0.02^{c}
Ethanol	1.96 ± 0.21^{b}	4.83 ± 1.32^{b}	18.24 ± 3.35^{a}	2.01 ± 0.10^{b}
Lactic acid	0	0	1.05 ± 0.25^{a}	0
Succinic acid	0	0	0.14 ± 0.01^{a}	0
pH	3.20 ± 0.01^{b}	3.20 ± 0.01^{b}	3.39 ± 0.06^{a}	3.18 ± 0.06^{b}
Sucrose	37.46 ± 0.05^{a}	23.37 ± 1.08^{b}	2.05 ± 0.85^{c}	32.77 ± 1.86^{a}
Glucose	3.39 ± 0.01^{d}	6.06 ± 0.29^{c}	13.52 ± 0.26^{a}	10.17 ± 0.66^{b}
Fructose	3.78 ± 0.01^{c}	9.99 ± 1.40^{b}	22.63 ± 1.50^{a}	9.27 ± 1.07^{b}

FC: forced carbonation; NC: natural carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same row are significantly different as determined by the Tukey test ($p \leq 0.05$).
FC and NC kombuchas were fermented with tailor-made microbial starter culture (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B. anomala,* and 10^6 CFU/mL of *K. marxianus*) and 50 g/L of sucrose.

carbon dioxide also increases bottle pressures, risking their ruptures (Kim and Adhikari, 2020). Another critical issue about natural carbonation is the amount of ethanol produced because the acetic acid bacteria are unable to oxidize ethanol into acetic acid under the anaerobic environment of the bottle. In this work, it was possible to observe that natural carbonation resulted in a 3-fold increase in ethanol content compared to forced carbonation (Table 2), exceeding the legislation limits. Given these problems, forced carbonation is a useful way to avoid excess ethanol production and fluctuations between batches. Although second fermentation is widely used, few studies have focused on this topic (Tran et al., 2020).

The label on kombucha CB1 states alcohol content as 0.9 % (v/v), contrasting with analysis obtained in this work showing concentrations twice as high (2.31 %, v/v). This increased alcohol content might be due to the effects of the storage period, which is a lacking topic of investigation in the literature. NC kombucha slightly exceeded the legal limits (0.61 %, v/v), reinforcing that natural carbonation is the most critical step in controlling ethanol during kombucha fermentation. FC kombucha suggested that forced carbonation is, indeed, a very useful alternative for controlling the process and ensuring the quality and safety levels of kombucha.

3.3. Sensory analysis of kombuchas

Presently, there are no standards regarding the sensory characteristics of kombucha, except for its vinegary sour taste (Tran et al., 2020). In this work, a sensory test was performed to evaluate the consumers' acceptance of kombuchas produced using tailor-made consortia. The scores for all attributes, given by the 100 panelists, varied from 4.61 to 7.21, with samples of kombuchas showing significant statistical differences ($p < 0.05$) (Table 3). The NC kombucha had the lowest scores for all attributes, except appearance. The CB1 and CB2 kombuchas had higher scores and differed statistically ($p < 0.05$) from FC and NC concerning aroma and overall acceptance.

Kombucha is not widely consumed in Brazil, sold mainly in specialty stores or health food stores at unaffordable prices. For this reason, the panelists were asked if they had ever tried kombucha and if they considered themselves consumers of such beverages. Of 100 panelists, 53 had tried and enjoyed kombucha before. Analyzing the data from those 53 panelists, the average acceptance ranged from 4.72 to 7.51, with samples differing statistically regarding aroma and overall acceptance attributes ($p < 0.05$) (Table 4).

The commercial samples presented a higher average ($p < 0.05$) compared to FC and NC regarding the aroma. The differences observed may be explained by the volatile profile of kombuchas (discussed below), as CB1 and CB2 had higher amounts and diversity of esters (Table 5), which are responsible for fruity notes. Although the CB1

Table 3
Acceptance of kombuchas sensory attributes evaluated by 100 participants.

	FC	NC	CB1	CB2
Appearance	7.19 ± 1.54^{a}	6.93 ± 1.54^{a}	6.63 ± 1.72^{ab}	7.22 ± 1.82^{a}
Aroma	5.15 ± 1.89^{b}	$4.64 \pm 1.97b^{b}$	6.37 ± 2.02^{a}	6.22 ± 1.63^{a}
Flavor	6.00 ± 2.03^{bc}	5.56 ± 1.97^{c}	6.47 ± 1.96^{ab}	6.68 ± 1.76^{a}
Acid flavor	6.20 ± 1.83^{b}	6.12 ± 1.91^{b}	6.47 ± 1.90^{ab}	6.81 ± 1.80^{a}
Overall acceptance	6.06 ± 1.97^{b}	5.63 ± 1.78^{b}	6.62 ± 1.69^{a}	6.90 ± 1.57^{a}

FC: forced carbonation; NC: natural carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same row are significantly different as determined by the Tukey test ($p \leq 0.05$).
FC and NC kombuchas were fermented with tailor-made microbial starter culture (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B. anomala,* and 10^6 CFU/mL of *K. marxianus*) and 50 g/L of sucrose.

Table 4
Acceptance of kombuchas' sensory attributes evaluated by the 53 participants that had already tried kombucha before.

	FC	NC	CB1	CB2
Appearance	7.30 ± 1.54[a]	7.02 ± 1.68[a]	6.75 ± 1.80[a]	7.51 ± 1.81[a]
Aroma	5.17 ± 1.88[b]	4.72 ± 2.00[b]	6.68 ± 2.04[a]	6.09 ± 1.75[a]
Flavor	6.42 ± 1.88[a]	5.94 ± 1.78[a]	6.58 ± 1.91[a]	6.66 ± 1.70[a]
Acid flavor	6.49 ± 1.79[a]	6.13 ± 1.92[a]	6.34 ± 2.02[a]	6.66 ± 1.91[a]
Overall acceptance	6.38 ± 1.94[ab]	5.85 ± 1.74[b]	6.79 ± 1.72[a]	6.85 ± 1.51[a]

FC: forced carbonation; NC: natural carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Different letters in the same row are significantly different as determined by the Tukey test ($p \leq 0.05$).
FC and NC kombuchas were fermented with tailor-made microbial starter culture (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B. anomala,* and 10^6 CFU/mL of *K. marxianus*) and 50 g/L of sucrose.

sample had high concentrations of acetic acid (Table 2), the acceptance for aroma and overall quality was higher than for the kombuchas fermented with the tailor-made consortia. This is probably because of the presence of esters in CB1, as noted by the comments of panelists such as "apple aroma" and "green grape aroma".

Despite the differences between the FC formulation (this work) and commercial brands (CB1 and CB2), the Acceptance Index for all of them was above 70 %, which indicates that FC has the potential for commercialization. The microbial consortium used in FC kombucha would be suitable for the industrial production of this beverage, since FC kombucha had adequate acceptance, presented total control of the fermentation process, and produced low concentrations of alcohol.

3.4. Volatile profile of kombuchas

The aromatic profile of kombucha originates both from the tea base and the volatile metabolites generated during the fermentation process. These metabolic by-products impart a distinct "vinegary" and "cidery" character to the beverage, with the former attribute connected to the production of acetic acid and the latter linked to yeast activity, particularly in the synthesis of superior alcohols and esters (Tran et al., 2020). In this work, 102 volatile compounds were detected in the sweetened tea and kombuchas by HS-SPME-GC/MS (Table 5). These volatile metabolites encompass 12 distinct chemical classes: acids, alkanes, alcohols, aldehydes, amines, ketones, esters, ethers, lactones, phenols, sulfurs, and terpenes.

The non-fermented sweetened tea was analyzed to detect which volatile compounds were consumed and produced by the tailor-made consortia of microorganisms. Among the 56 volatile compounds found in non-fermented tea, the most abundant group was acids (n = 15), followed by alcohols (n = 12). The most abundant compounds were 2-methylbutanal, 2-butanone, benzoic acid, and β-damascenone. The aldehyde 2-methylbutanal is derived from amino acid degradation (Pripis-Nicolau et al., 2000) and is responsible for almond, cocoa, fermented, hazelnut, and malt notes (Kim et al., 2016). This compound was fully consumed in FC kombucha and increased in NC kombucha, the most abundant compound in this sample. The terpenic ketone β-damascenone has a low odor threshold and is considered one of the most potent flavor constituents in teas (Yang et al., 2013). The β-damascenone was fully consumed during fermentation in both FC and NC kombuchas. Other tea volatile compounds such as 1-nonanol, 2-heptenoic acid, benzoic acid, (2E)-2-Octen-1-ol, ethyl 2-hydroxy-3-methylbutanoate, and β-cyclocitral were consumed during fermentation. The metabolization of some volatile compounds and the production of others explain why kombucha flavors remarkably differ from tea (Tran et al., 2020).

A comparison between non-fermented tea and fermented kombucha illuminates the dramatic biochemical transformations that occur during fermentation. The non-fermented sweetened tea primarily contains a mixture of acids and alcohols among its 56 detected volatile compounds, such as 2-methylbutanal, 2-butanone, benzoic acid, and β-damascenone. However, these compounds are substantially consumed during fermentation. Conversely, the process of fermentation introduces a plethora of new volatile compounds, a product of microbial activity, significantly shaping kombucha's unique flavor profile. Additionally, the carbonation method employed can further modulate this volatile landscape. Hence, this juxtaposition underscores the transformative nature of fermentation from tea to kombucha, driven by intricate biochemical processes.

Even with the same starter culture, the carbonation method used resulted in some differences between kombuchas. The anaerobic in-bottle fermentation in NC kombucha produced more ethanol and different volatile compounds, such as (2E)-dec-2-enoic acid, 2-methylbutanal, 3-methylbutanal, ethyl 3-methyl butanoate, methyl dihydrojasmonate, and 3-methylsulfanylpropan-1-ol. The latter, a volatile compound also found in CB1, is a sulfur flavor found in wine and soy sauce, which imparts off-flavor cauliflower-like and potato-like and has a low odor threshold of 1–3 ppm (Lwa et al., 2015).

Esters and acids were the main chemical classes found in the fermented kombuchas and both classes have great importance in the volatile profile and the acceptance of the beverage (Savary et al., 2021). The aldehydes hexanal, nonanal, and benzaldehyde were detected in all fermented samples, and those compounds are related to off flavors, such as rancid, fat, and green odor descriptors (Kim et al., 2016). However, due to synergistic effects, the perception of some off flavors may be masked or enhanced by the presence of other volatile compounds (Savary et al., 2021). Many of those aldehydes have been detected in kombucha before (Tran et al., 2022).

All fermented kombuchas also presented the esters 2-methyl-1-propyl acetate (fruit, apple, and banana notes), ethyl butyrate (fruit and pineapple notes), 3-methyl butyl acetate (banana notes), and phenethyl acetate (rose, honey, and tobacco notes), and the terpenes menthol (peppermint notes), α-Terpinol (anise, mint notes), geranylacetol (magnolia and green notes), and nerolidol (wood and flower notes), compounds which are expected to contribute positively to the aroma. CB1 and CB2 presented a different profile of esters and higher diversity of those compounds compared to FC and NC, which may have contributed to masking their off flavors, thus the higher scores for aroma on sensory analysis.

A principal component analysis (PCA) was performed to explore and visualize groupings and discrimination in the volatile profile and sensory analysis. For that, the results of the volatile compounds that differed significantly ($p < 0.05$) in the fermented samples (NC, FC, CB1, and CB2) and the sensory attributes (overall acceptance, aroma, acid flavor, and flavor) were analyzed. Principal components PC 1 and PC 2 explained 50.25 and 40.29 % of the variation in the data, respectively (Fig. 5a). PC 1 separated CB2 from the other 3 samples, while PC 2 separated CB1 and CB2 from the other samples (FC and NC). The right-hand upper quadrant suggests a similar volatile composition between NC and FC kombuchas, since they differ by the carbonation process only, and discriminated those samples from the commercial kombuchas. The loadings plot (Fig. 5b) showed that CB2 was characterized by having higher concentrations of 2-methyl-1-propyl acetate, an ester with apple and banana sensory notes, which agree with the comments of the participants in the sensory analysis. The volatile profile of this sample was also related to ethyl hexanoate, phenethyl acetate, and eucalyptol. The attributes of the sensory evaluation are more correlated to commercial brands because of the higher scores of those samples and their higher concentrations of fruity esters and terpenes. Regarding terpenes, (*E*)-Ethyl cinnamate (honey, cinnamon) was found only in CB1, eucalyptol (mint, sweet) exclusively in CB2, and FC and NC presented β-Ionone epoxide (fruit, wood). The FC and NC kombuchas were mainly

Table 5
Volatile compounds of non-fermented sweetened tea and kombuchas. Results are expressed in mg/L.

Compounds	Non-fermented tea	FC	NC	CB1	CB2
Acids					
Propionic acid	nd	184.46 ± 9.22^{a}	181.57 ± 61.9^{a}	93.08 ± 3.82^{ab}	154.73 ± 28.3^{a}
2-Methylpropanoic acid	8.87 ± 2.43^{ab}	3.15 ± 0.33^{c}	3.52 ± 0.26^{c}	7.49 ± 0.38^{b}	11.92 ± 0.56^{a}
Butanoic acid	nd	115.45 ± 30.8^{a}	145.28 ± 11.88^{a}	111.17 ± 18.09^{a}	nd
3-Methylbutanoic acid	7.23 ± 1.47^{a}	0.79 ± 0.08^{c}	0.87 ± 0.03^{c}	1.36 ± 0.06^{bc}	2.75 ± 0.37^{b}
Hexanoic acid	3.56 ± 0.73^{c}	15.5 ± 1.14^{b}	14.09 ± 0.61^{b}	3.54 ± 0.10^{c}	28.08 ± 3.83^{a}
Ethyl hexanoic acid	12.20 ± 3.38^{d}	53.3 ± 1.84^{ab}	46.07 ± 1.94^{b}	30.68 ± 4.73^{c}	63.13 ± 10.25^{a}
Heptanoic acid	8.81 ± 1.15^{b}	26.41 ± 1.82^{b}	31.29 ± 3.7^{b}	25.21 ± 1.87^{b}	93.02 ± 27.07^{a}
Octanoic acid	2.29 ± 0.51^{bc}	4.24 ± 0.20^{b}	4.15 ± 0.59^{b}	0.34 ± 0.01^{c}	8.44 ± 2.00^{a}
2-Heptenoic acid	13.73 ± 3.98^{a}	nd	nd	nd	nd
Nonanoic acid	2.15 ± 0.30^{b}	5.77 ± 0.64^{b}	7.97 ± 2.37^{b}	6.81 ± 2.82^{b}	16.13 ± 4.76^{a}
2-Octenoic acid	37.49 ± 20.25^{a}	nd	nd	nd	nd
Decanoic acid	3.86 ± 0.87^{b}	6.29 ± 0.98^{ab}	4.05 ± 1.12^{b}	0.45 ± 0.06^{c}	7.32 ± 1.63^{a}
9-Decenoic acid	nd	338.22 ± 84.83^{a}	162.16 ± 65.74^{b}	13.43 ± 1.99^{bc}	nd
Geranic acid	nd	327.84 ± 18.59^{a}	253.62 ± 85.49^{a}	42.55 ± 7.1^{b}	302.46 ± 83.82^{a}
3-Decenoic acid	nd	nd	nd	21.36 ± 1.82^{b}	189.02 ± 20.97^{a}
(2*E*)-dec-2-enoic acid	20.27 ± 4.22^{c}	nd	52.36 ± 6.12^{b}	114.15 ± 12.58^{a}	nd
Benzoic acid	72.47 ± 20.54^{a}	nd	nd	nd	nd
Dodecanoic acid	9.69 ± 1.83^{c}	29.05 ± 6.32^{ab}	27.5 ± 9.52^{ab}	10.07 ± 2.74^{c}	36.52 ± 7.22^{a}
Tetradecanoic acid	11.57 ± 2.23^{b}	41.44 ± 6.05^{a}	49.44 ± 17.43^{a}	23.98 ± 6.99^{ab}	52.81 ± 10.24^{a}
Pentadecanoic acid	3.34 ± 0.88^{c}	10.99 ± 1.45^{ab}	18.51 ± 10.34^{ab}	7.12 ± 2.78^{ab}	20.53 ± 9.34^{a}
Alkanes					
Cyclohexane	nd	nd	nd	1.83 ± 0.31^{a}	1.97 ± 0.28^{a}
m-xylene	21.22 ± 0.98^{b}	110.56 ± 14.83^{ab}	185.62 ± 90.09^{a}	203.78 ± 45.29^{a}	146.8 ± 24.56 ^{ab}
Alcohols					
2-Methyl-propan-1-ol	39.73 ± 21.11^{a}	2.88 ± 0.35^{b}	2.34 ± 0.24^{b}	6.1 ± 0.27^{b}	26.03 ± 2.88^{ab}
2-Methyl-1-butanol	17.28 ± 2.22^{a}	2.29 ± 0.24^{b}	2.06 ± 0.24^{b}	2.31 ± 0.14^{b}	22.03 ± 5.28^{a}
3-Methyl-1-butanol	8.48 ± 2.23^{a}	0.46 ± 0.05^{b}	0.37 ± 0.02^{b}	0.26 ± 0.01^{b}	2.31 ± 0.08^{b}
Hexan-1-ol	30.60 ± 12.45^{c}	147.42 ± 20.86^{b}	299.09 ± 33.82^{a}	75.61 ± 14.65^{c}	83.66 ± 2.10^{c}
Cis-3-hexen-1-ol	nd	nd	nd	9.26 ± 0.43^{b}	320.44 ± 26.16^{a}
2-Ethylhexan-1-ol	3.17 ± 0.47^{b}	5.5 ± 0.93^{b}	5.02 ± 1.49^{b}	4.67 ± 0.37^{b}	15.4 ± 1.12^{a}
1,11-Undecanediol	3.37 ± 1.91^{a}	53.97 ± 43.22^{a}	nd	25.27 ± 8.39^{a}	52.23 ± 50.65^{a}
1,3-Butanediol	nd	nd	nd	48.34 ± 14.95^{a}	nd
(2*E*)-2-Octen-1-ol	5.17 ± 1.32^{a}	nd	nd	nd	nd
1-Nonanol	7.75 ± 1.15^{b}	nd	nd	nd	25.01 ± 5.68^{a}
1-Decanol	22.79 ± 3.68^{b}	177.18 ± 9.78^{a}	196.91 ± 57.89^{a}	62.86 ± 5.2^{b}	nd
1-Undecanol	nd	nd	nd	nd	198.9 ± 66.94^{a}
2-Phenylethan-1-ol	28.62 ± 11.06^{a}	1.54 ± 0.16^{b}	0.67 ± 0.02^{b}	0.46 ± 0.01^{b}	2.15 ± 0.33^{b}
1-Dodecanol	13.42 ± 5.48^{b}	56.62 ± 28.8^{ab}	54.35 ± 15.16^{ab}	47.15 ± 22.18^{b}	106.34 ± 14.51^{a}
Hexadecanol	52.21 ± 13.20^{c}	269.72 ± 38.51^{a}	235.52 ± 14.99^{ab}	167.17 ± 44.33^{b}	258.37 ± 36.68^{a}
Aldehydes					
Acetaldehyde	8.04 ± 2.01^{ab}	24.64 ± 17.73^{ab}	21.19 ± 7.92^{a}	nd	nd
Isobutyraldehyde	nd	nd	nd	nd	60.51 ± 54.73
2-Methylbutanal	144.46 ± 4.94^{a}	nd	2514.18 ± 2262.39^{a}	nd	nd
3-Methylbutanal	nd	nd	429.39 ± 192.27^{a}	nd	nd
Hexanal	5.33 ± 0.30^{c}	82.00 ± 22.30^{bc}	128.01 ± 65.46^{ab}	192.08 ± 20.98^{a}	80.05 ± 6.65^{bc}
Octanal	9.74 ± 2.95^{ab}	40.76 ± 20.39^{ab}	80.31 ± 54.09^{a}	nd	nd
(2*Z*)Hept-2-enal	4.43 ± 1.13^{c}	61.42 ± 14.72^{b}	nd	127.68 ± 32.99^{a}	86.11 ± 22.85^{ab}
Nonanal	2.97 ± 1.71^{a}	34.57 ± 31.80^{a}	28.50 ± 22.15^{a}	16.34 ± 1.35^{a}	48.93 ± 29.87^{a}
Benzaldehyde	nd	95.64 ± 6.53^{b}	99.00 ± 55.58^{b}	56.95 ± 10.69^{b}	262.19 ± 47.58^{a}
2-Dodecenal	nd	218.70 ± 18.08^{a}	195.27 ± 124.06^{a}	132.36 ± 12.32^{ab}	nd
2,6-bis(1,1-dimethylethyl)-4-methylphenol	nd	nd	nd	147.17 ± 66.63^{b}	376.17 ± 74.12^{a}
Amines					
Ethylacetamide	nd	nd	nd	109.27 ± 17.24^{a}	nd
Ketones					
2-Butanone	105.34 ± 68.65^{a}	nd	nd	nd	nd
(3*E*)-3-penten-2-one	18.63 ± 2.42^{b}	177.62 ± 31.57^{ab}	333.14 ± 189.24^{a}	120.62 ± 22.23^{ab}	23.49 ± 1.75^{b}
(5*S*)-5-Methyl-3-heptanone	2.05 ± 0.23^{b}	84.01 ± 31.07^{a}	110.32 ± 14.45^{a}	34.92 ± 5.11^{b}	83.85 ± 2.63^{a}
(3*S*)-3-Hydroxy-2-butanone	nd	nd	nd	11.91 ± 0.12^{b}	17.77 ± 4.34^{a}
1-Octen-3-one	4.60 ± 0.68^{b}	66.17 ± 10.60^{ab}	211.01 ± 63.94^{a}	nd	116.71 ± 28.67^{b}
5-Octen-2-one	nd	97.76 ± 23.73^{a}	77.92 ± 14.06^{a}	30.14 ± 1.34^{b}	nd
6-Methyl-5-heptene-2-one	6.15 ± 1.06^{c}	98.72 ± 5.56^{a}	51.44 ± 18.32^{b}	54.93 ± 1.96^{b}	80.77 ± 3.69^{a}
3,5-Octadien-2-one	10.51 ± 2.34^{d}	263.90 ± 17.09^{a}	nd	82.80 ± 4.14^{c}	180.01 ± 21.94^{b}
Esters					
Ethyl acetate	1.22 ± 0.18^{a}	1.20 ± 0.18^{a}	0.87 ± 0.04^{b}	nd	0.70 ± 0.06^{b}
2-Methylpropyl ethanoate	15.52 ± 2.62^{c}	40.90 ± 7.91^{b}	29.67 ± 5.98^{bc}	19.54 ± 1.69^{c}	90.17 ± 3.19^{a}
Ethyl butyrate	2.31 ± 0.20^{c}	31.83 ± 6.62^{ab}	41.27 ± 15.47^{a}	42.35 ± 4.61^{a}	13.02 ± 0.27^{bc}
Ethyl 3-methyl butanoate	nd	nd	225.89 ± 19.79^{a}	61.90 ± 8.66^{b}	247.95 ± 29.65^{a}
3-Methyl butyl acetate	0.71 ± 0.10^{c}	6.47 ± 1.18^{a}	4.76 ± 2.17^{ab}	2.37 ± 0.34^{bc}	5.61 ± 0.04^{ab}
Ethyl 3-methyl-2-butenoate	nd	nd	nd	nd	1095.32 ± 209.69^{a}
Ethyl hexanoate	nd	nd	nd	16.52 ± 2.46^{b}	128.32 ± 15.14^{a}
3-Methylbutyl butyrate	nd	nd	nd	nd	183.16 ± 35.21^{a}
3-Methylbutyl isovalerate	nd	nd	nd	nd	153.38 ± 41.67^{a}
Ethyl 2-hydroxypropanoate	nd	nd	nd	8.30 ± 0.61^{b}	205.10 ± 13.41^{a}

(*continued on next page*)

Table 5 (*continued*)

Compounds	Non-fermented tea	FC	NC	CB1	CB2
Ethyl 2-hydroxy-3-methylbutanoate	8.60 ± 2.16^{b}	nd	nd	347.65 ± 87.36^{a}	nd
Diethyl butanedioate	nd	nd	nd	82.37 ± 11.43^{a}	nd
Ethyl (*Z*)-dec-4-enoate	nd	nd	nd	69.32 ± 19.36^{a}	nd
Decyl methacrylate	nd	nd	nd	nd	36.53 ± 3.38^{a}
Benzyl ethanoate	nd	nd	nd	155.38 ± 27.34^{a}	nd
Ethyl phenylacetate	nd	nd	nd	22.43 ± 0.90^{a}	nd
Phenethyl acetate	nd	13.49 ± 2.49^{b}	0.46 ± 0.06^{c}	8.93 ± 0.74^{b}	30.55 ± 4.97^{a}
Ethyl dodecanoate	nd	nd	nd	47.79 ± 5.26^{a}	nd
Methyl ethyl tetradecanoate	36.75 ± 14.25^{a}	84.13 ± 33.66^{a}	nd	88.43 ± 63.89^{a}	nd
Hexyl salicylate	nd	158.5 ± 63.59^{b}	nd	nd	666.40 ± 295.18^{a}
Methyl Dihydrojasmonate	24.11 ± 12.33^{bc}	nd	121.68 ± 40.02^{ab}	94.2 ± 69.90^{bc}	207.82 ± 21.68^{a}
Diisooctyl adipate	nd	nd	nd	75.19 ± 12.23^{b}	141.11 ± 5.99^{a}
Diethyl Phthalate	14.27 ± 4.18^{d}	62.27 ± 10.29^{bc}	66.74 ± 4.55^{b}	47.55 ± 10.33^{c}	101.96 ± 1.93^{a}
Ether					
1.1-Diethoxyethane	2.39 ± 0.37^{a}	nd	nd	nd	nd
Phenols					
Phenol	nd	94.15 ± 14.21^{a}	45.60 ± 16.74^{b}	nd	nd
2-ethylphenol	nd	42.55 ± 3.60^{b}	14.56 ± 2.26^{c}	18.44 ± 3.31^{bc}	178.24 ± 19.36^{a}
2,6-di*tert*-butylphenol	3.56 ± 1.31^{b}	16.52 ± 5.09^{ab}	14.56 ± 7.89^{ab}	8.08 ± 2.04^{b}	28.05 ± 7.39^{a}
Lactones					
Butyrolactone	nd	62.95 ± 9.69^{bc}	80.14 ± 11.10^{ab}	5.57 ± 0.88^{c}	144.17 ± 56.91^{a}
Sulfurs					
Dimethyl sulfide	nd	nd	nd	7.81 ± 1.46^{a}	nd
3-methylthiolane 1,1-dioxide	nd	nd	nd	nd	227.04 ± 11.28^{a}
3-methylsulfanylpropan-1-ol	nd	nd	26.56 ± 6.09^{b}	82.06 ± 20.39^{a}	nd
Terpenes					
Eucalyptol	nd	nd	nd	nd	226.76 ± 18.20^{a}
Cardene	nd	nd	nd	20.51 ± 2.27^{b}	577.37 ± 94.24^{a}
(+)-4-Carene	nd	nd	774.81 ± 113.86^{a}	nd	nd
β-Cyclocitral	29.93 ± 5.26^{a}	nd	nd	nd	nd
Menthol (isomer)	21.31 ± 5.44^{b}	131.04 ± 29.9^{a}	102.45 ± 46.03^{ab}	52.06 ± 0.75^{ab}	108.63 ± 32.89^{ab}
α-Terpinol	36.49 ± 12.80^{a}	30.58 ± 4.97^{ab}	16.59 ± 0.93^{bc}	7.83 ± 0.19^{c}	13.43 ± 1.50^{c}
Citronellol (isomer)	nd	nd	nd	10.32 ± 0.39^{ab}	348.2 ± 271.69^{a}
β-Damascenone	53.84 ± 3.35^{a}	nd	nd	nd	nd
β-Ionone	11.00 ± 2.69^{c}	78.89 ± 11.92^{b}	53.61 ± 8.39^{bc}	28.96 ± 1.45^{bc}	169.29 ± 42.2^{a}
Geranylacetol	nd	52.17 ± 9.21^{b}	36.52 ± 3.19^{b}	95.3 ± 10.13^{b}	303.99 ± 95.25^{a}
β-Ionone epoxide	9.37 ± 3.47^{b}	85.63 ± 6.57^{a}	82.58 ± 25.44^{a}	nd	nd
Ethylguaiacol	nd	45.1 ± 6.10^{b}	11.46 ± 1.25^{c}	110.69 ± 12.45^{a}	nd
Nerolidol	nd	211.39 ± 58.21^{a}	73.03 ± 29.12^{ab}	65.58 ± 16.57^{ab}	206.08 ± 96.51^{a}
(*E*)-Ethyl cinnamate	nd	nd	nd	193.44 ± 12.35^{a}	nd

FC: forced carbonation; NC: natural carbonation; CB1: commercial brand 1; CB2: commercial brand 2. Results are expressed as mean peak areas (× 10^{5}) ± standard deviation; (nd = not detected). The values followed by different letters in the same row differed significantly in the Tukey test ($p \leq 0.05$). The results are expressed by the mean of the triplicates.

FC and NC kombuchas were fermented with tailor-made microbial starter culture (10^{7} CFU/mL of *K. saccharivorans*, 10^{5} CFU/mL of *B. anomala,* and 10^{6} CFU/mL of *K. marxianus*) and 50 g/L of sucrose.

correlated with aldehydes, such as acetaldehyde (originated from sugars) and octanal (originated from lipid oxidative process), and this may explain the lower aroma scores on the sensory analysis since those compounds are associated with rancid, pungent, and fat aroma sensory descriptors (Ribeiro et al., 2020). At the same time, the concentration of the esters that help to mask those off-flavors was lower compared to commercial brands (Villavicencio et al., 2021).

Since there is no expected profile of volatile compounds for kombuchas and only a few studies were performed on this subject, further research is needed to define which volatile compounds are essential in kombucha and what are their origins (Savary et al., 2021; Zhang et al., 2021; Leali et al., 2022; Tran et al., 2022). Thus, the use of tailor-made starter cultures would help to control the production of volatile and non-volatile metabolites, allowing the production of high-quality and standardized kombucha.

3.5. Shelf-life evaluation

The shelf-life of kombucha has been studied in literature to evaluate the changes in the antioxidant properties and metabolites in kombuchas made from different teas, such as black tea, green tea mixed with aromatic herbs and black tea mixed with soursop tea (Jayabalan et al., 2008; Tan et al., 2020; La Torre et al., 2021; Grassi et al., 2022). In this work, a shelf-life study of 90 days was carried out to evaluate the stability of kombuchas produced in this work, and the results are presented in Table 6. The viable cells of *K. marxianus* remained above 6 logs CFU/mL (1 million CFU/mL) for 90 days, meaning that the ingestion of 30 mL of kombucha a day would offer the dosage that delivers beneficial effects defined in previous studies (at least 30 million CFU/day) (Maccaferri et al., 2012; Lisotti et al., 2013). This result shows that it was possible to produce a probiotic kombucha and brings a new alternative to plant-based products, as most probiotic sources commercially available are dairy products. This fermentation technology innovates the industrial production and research on kombucha, as the probiotic strain is present in the inoculum, and it is responsible for the fermentation of the product. This avoids pasteurization or filtration, which is needed when the probiotic strain is added at the end of the process in traditional kombucha production. The viable counts of *K. saccharivorans* severely dropped on the 90th day in both FC and NC kombuchas. Despite being viable for 60 days, the bacteria were not able to oxidize the ethanol produced by yeasts into acetic acid because of the absence of oxygen in the bottle. For this reason, an increase in ethanol in NC and FC kombuchas during storage was observed. The pH also slightly increased in both samples. The ethanol in NC kombucha exceeded the legal limits (0.5 %, v/v), reaching 1.2 % (v/v) on the 45th day of storage. FC kombucha, which showed the potential for industrial production of a probiotic beverage, remained stable as the parameters were within legal limits for 60 days under refrigeration.

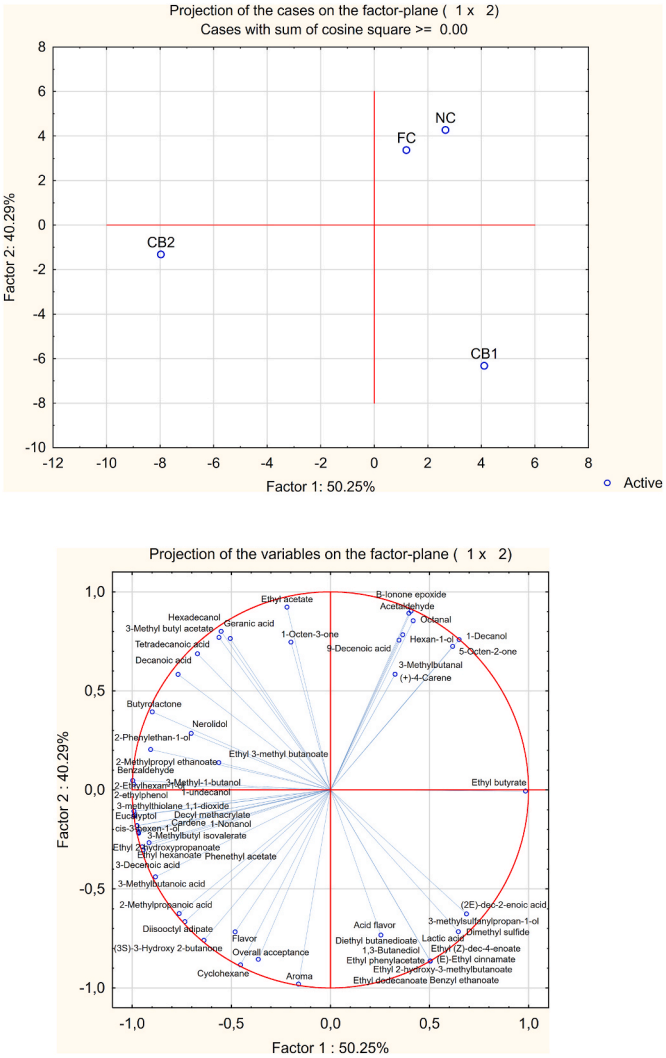

Fig. 5. Principal Component Analysis plots of volatile compounds and sensory attributes found in fermented kombuchas (FC, NC, CB1, and CB2). **A**) Score plot. **B**) Loadings plot (volatile compounds, sensory attributes, and organic acids).

Results showed that the fermentation process continued at a low pace when stored at 4 °C. The yeasts continued to hydrolyze the sucrose, which decreased during the 90 days of storage, with the glucose and fructose that were released being little consumed. Tan et al. (2020) observed the hydrolysis of sucrose and decreased ethanol and acetic acid content in soursop kombucha stored for 21 days. To the best of our knowledge, this is the first study that evaluates the storage time of kombuchas fermented by a tailor-made microbial consortium.

3.6. Limitations and call for research

This research has introduced an innovative approach to kombucha production employing a tailor-made microbial consortium, thereby contributing meaningful insights toward the establishment of a standardized probiotic kombucha beverage. Yet, it is critical to acknowledge certain limitations while simultaneously charting potential domains for future investigations.

The yeast *K. marxianus*, selected for its promising fermentation attributes and possible probiotic properties, demonstrated advantageous outcomes for this study conducted in Brazil, where the utilization of *K. marxianus* for probiotic claims isn't restricted and its role in kombucha production isn't widely recognized. Nevertheless, it has yet to be granted permission for probiotic claims in specific regions, including Europe. Despite these hurdles, the successful incorporation of *K. marxianus* in this study underscores the potential for expanding the microbial spectrum in kombucha production, thus stimulating further exploration into yeasts of varied characteristics, including those globally acknowledged as probiotics.

The fermentation procedure executed in this study resulted in a kombucha variant with a higher concentration of residual sugars compared to certain commercial counterparts. Although this disparity in sugar content might have influenced sensory analysis and comparison with commercial brands, it also presents an opportunity for future research to concentrate on refining the fermentation process to reduce residual sugars, potentially facilitating a more precise comparison with commercially available alternatives.

In addition, the implications of this study and prospective future research may not be confined to scientific exploration, with a potential reach extending into sectors associated with gastronomy and food production. The unique flavor profile of kombucha, combined with its functional benefits, could foster innovative approaches in food pairing, culinary techniques, or even integration into unique recipes. This progress could precipitate the emergence of new product lines, thus

Table 6
The effect of storage time on natural carbonation and forced carbonation kombuchas fermented with a tailor-made microbial starter culture (10^7 CFU/mL of *K. saccharivorans*, 10^5 CFU/mL of *B. anomala,* and 10^6 CFU/mL of *K. marxianus*).

Time (days)	Microbial counts (log CFU/mL)			Metabolites (g/L)			Sugars (g/L)		
	KMF	BA	KS	Acetic acid	Ethanol	Ph	Sucrose	Glucose	Fructose
Natural carbonation									
0	7.24 ^{a}	6.48 ^{a}	6.45 ^{a}	1.88 ± 0.07 ^{a}	4.83 ± 1.32 ^{c}	3.20 ± 0.01 ^{bc}	23.37 ± 1.08 ^{a}	6.06 ± 0.29 ^{g}	9.99 ± 1.40 ^{e}
10	6.60 ^{b}	6.23 ^{b}	6.71 ^{a}	1.99 ± 0.11 ^{a}	5.64 ± 0.84 ^{bc}	3.16 ± 0.01 ^{c}	19.41 ± 0.78 ^{b}	7.95 ± 0.04 ^{f}	11.41 ± 0.09 ^{e}
20	6.83 ^{ab}	6.67 ^{a}	6.64 ^{a}	2.34 ± 0.34 ^{a}	6.19 ± 0.01 ^{ab}	3.24 ± 0.08 ^{abc}	15.84 ± 0.08 ^{c}	9.54 ± 0.12 ^{e}	14.46 ± 0.46 ^{d}
30	6.56 ^{bc}	6.60 ^{ab}	5.24 ^{c}	2.04 ± 0.06 ^{a}	8.01 ± 0.08 ^{a}	3.25 ± 0.13 ^{abc}	10.97 ± 0.11 ^{d}	10.89 ± 0.17 ^{d}	16.25 ± 0.09 ^{cd}
45	6.59 ^{b}	6.30 ^{ab}	5.71 ^{bc}	2.23 ± 0.09 ^{a}	8.19 ± 0.24 ^{a}	3.31 ± 0.07 ^{ab}	8.27 ± 0.31 ^{e}	12.02 ± 0.04 ^{c}	17.99 ± 0.02 ^{bc}
60	6.70 ^{b}	6.41 ^{ab}	6.18 ^{ab}	2.00 ± 0.01 ^{a}	8.58 ± 0.42 ^{a}	3.36 ± 0.01 ^{a}	4.90 ± 0.10 ^{f}	13.42 ± 0.11 ^{b}	19.54 ± 0.06 ^{ab}
90	6.06 ^{c}	6.56 ^{ab}	2.05 ^{d}	1.95 ± 0.02 ^{a}	8.77 ± 0.29 ^{a}	3.37 ± 0.01 ^{a}	3.46 ± 0.11 ^{f}	14.72 ± 0.13 ^{a}	20.35 ± 0.22 ^{a}
Forced carbonation									
0	6.71 ^{abc}	6.40 ^{a}	7.16 ^{a}	1.76 ± 0.01 ^{c}	1.96 ± 0.21 ^{b}	3.20 ± 0.01 ^{b}	37.46 ± 0.05 ^{a}	3.39 ± 0.01 ^{e}	3.78 ± 0.01 ^{e}
10	6.96 ^{ab}	6.01 ^{b}	7.21 ^{a}	1.77 ± 0.04 ^{bc}	2.28 ± 0.04 ^{b}	3.20 ± 0.01 ^{b}	31.99 ± 1.04 ^{b}	5.62 ± 0.38 ^{de}	8.09 ± 1.83 ^{d}
20	7.11 ^{a}	6.44 ^{a}	6.67 ^{b}	2.17 ± 0.02 ^{a}	3.12 ± 0.02 ^{b}	3.15 ± 0.21 ^{b}	28.79 ± 0.45 ^{c}	7.40 ± 0.13 ^{cd}	9.71 ± 0.35 ^{d}
30	7.05 ^{ab}	6.40 ^{a}	6.83 ^{b}	2.16 ± 0.05 ^{a}	4.18 ± 0.05 ^{ab}	3.25 ± 0.08 ^{ab}	22.17 ± 0.07 ^{d}	9.89 ± 0.23 ^{bc}	13.25 ± 0.17 ^{c}
45	6.77 ^{abc}	6.18 ^{ab}	6.54 ^{b}	1.92 ± 0.06 ^{b}	4.22 ± 0.06 ^{ab}	3.32 ± 0.07 ^{ab}	17.73 ± 0.01 ^{e}	12.14 ± 1.09 ^{b}	15.48 ± 0.48 ^{bc}
60	6.46 ^{bc}	6.33 ^{a}	5.25 ^{c}	1.93 ± 0.03 ^{b}	3.83 ± 0.03 ^{ab}	3.33 ± 0.01 ^{ab}	12.10 ± 0.16 ^{f}	15.15 ± 0.07 ^{a}	18.33 ± 0.24 ^{ab}
90	6.43 ^{c}	6.14 ^{ab}	2.65 ^{d}	1.83 ± 0.01 ^{bc}	5.65 ± 0.01 ^{a}	3.42 ± 0.04 ^{a}	9.21 ± 0.98 ^{g}	15.69 ± 1.62 ^{a}	19.95 ± 0.62 ^{a}

KMF: *K. marxianus;* BA: *B. anomala;* KS; *K. saccharivorans.* Different letters in the same column are significantly different as determined by the Tukey test ($p \leq 0.05$).

stimulating novel market opportunities for kombucha manufacturers. It could also enhance the consumer experience, introducing new facets to the appreciation of this fermented beverage. Hence, this research, while contributing to the scientific knowledge base, also holds substantial potential to positively impact both the commercial landscape and the understanding of Gastronomy and Food Science.

This work lays the groundwork for future investigations, broadening the understanding of kombucha production using an innovative microbial consortium. It is envisaged that these endeavors will spearhead the generation of kombucha beverages featuring controlled quality, consistency, and probiotic potential, satisfying a wide array of regulatory standards and consumer preferences.

4. Conclusion

The findings from this study indicate that the tailor-made microbial consortium, developed using the amalgamation of *K. saccharivorans, B. anomala*, and *K. marxianus*, efficiently produced a probiotic-certified kombucha. This consortium presents a viable alternative to traditional starter cultures. The replacement of the conventional, artisanal backslopping process used by producers signifies an innovative approach to control the fermentation process. It offers an assurance of food safety and consistent quality across different batches and over time. Furthermore, the adoption of a known and controlled starter culture, as proposed in this work, opens new avenues for exploring the health benefits of kombucha, as the suggested fermentation process is universally reproducible. Nonetheless, further studies employing other strains of yeasts and bacteria and differing process conditions are necessary to enhance the kombucha's volatile profile and, as a consequence, its sensory acceptance. This research underscores the importance of scientific investigation in driving innovations in kombucha production and substantiating its health claims.

5. Consent to participate

Informed consent was obtained from all individual participants included in the study.

Implications for gastronomy

This study brings implications for gastronomy in the context of probiotic-rich foods and beverages. Kombucha, valued for its distinct taste and potential health benefits, often presents inconsistent outcomes due to the inherent variability and unpredictability of the symbiotic cultures traditionally used in its fermentation. However, the innovative strategy presented here, which uses a controlled, tailor-made microbial consortium, creates new possibilities for the culinary arts and beverage industry.

- Standardization and Consistency: Using a defined microbial consortium provides consistency for an overall sensory profile of kombucha, minimizing variation between batches. This consistency is paramount for culinary professionals and beverage manufacturers who aim to deliver a consistent product to their customers.
- Amplified Probiotic Potential: The confirmed probiotic properties of the kombucha produced using this consortium enhanced its health-promoting attributes and increased its appeal as a functional beverage, important in gastronomy because of growing consumer interest in probiotic-rich foods and beverages.
- Regulatory Compliance: Keeping alcohol content within regulatory limits for non-alcoholic beverages during 60-day storage is crucial for the commercialization of kombucha. This presents a practical benefit for the culinary and beverage industries.
- Flavor Profiling: The identified ester compounds in kombucha contribute to its unique flavor profile, allowing creative food preparation in gastronomy, and allowing chefs and beverage professionals to craft menus and beverage lists that underscore the distinctive qualities of kombucha.
- Sustainable Production: The method proposed here could enhance the sustainability of kombucha production by minimizing waste and maximizing yields, both particularly important in the gastronomy industry.

Authors contributions

MF, BK, and MZA designed the project. MF, BK, BT, RW, SRR, and SF developed the experimental work. MF, BK, BT, RW, SRR, SF, NC, and MZA analyzed the data. MF, BK, and MZA wrote the main text. NC, RW, and MZA revised the main text. NC and MZA provided the funding through projects. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

This work was funded by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnologico (CNPq, Brazil), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, RS), and scholarships from CAPES Code Fund 001 (Brazil). N. Cordeiro thanks the financial support of the Foundation for Science and Technology through CIIMAR - UIDB/04423/2020 and UIDP/04423/2020, and European Territorial Cooperation Programme PCT-MAC 2014–2020 through REBECA-CCT (MAC/1.1. B/269) project.

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