Article

Development of Potentially Probiotic Mead from Co-Fermentation by *Saccharomyces cerevisiae* var. *boulardii* and Kombucha Microorganisms

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Abstract: Mead is a fermented alcoholic beverage produced from a diluted solution of honey and yeast activity. The objectives of this study were to produce a potentially probiotic mead through mixed fermentation by *Saccharomyces cerevisiae* var. *boulardii* and kombucha microorganisms and to evaluate fermentation kinetics, microbial cell survival and their in vitro resistance to simulated gastrointestinal transit, color parameters and the phenolic and antioxidant potential of the product. The main results of this study show that in order to develop a potentially probiotic mead utilizing the mixed fermentation of *S. boulardii* and kombucha microorganisms, the best condition was a concentration of 25 mL/L (*v*/*v*) of kombucha and 0.75 g/L (*w*/*v*) of *S. boulardii* with fermentation for 9 days at a temperature of 25 °C. In addition, at the end of fermentation, mead with kombucha and *S. boulardii* presented physicochemical characteristics with a pH of 3.48, 0.67% total acidity, 18.76 °Brix soluble solids and 4.77% alcohol content. The *S. boulardii* and lactic acid bacteria (LAB) present in the mead survived conditions reproducing those of the gastrointestinal tract, with counts of more than 6 Log_{10} CFU/mL for both microorganisms after the intestinal phase. In the color analysis, the mead with kombucha and *S. boulardii* had a yellow color with the b* parameter corresponding to 35.93, luminosity (L*) equal to 76.09 and 1.82 for a*. In addition, the mead we produced contains quantities of phenolics and antioxidants. In conclusion, kombucha and *S. boulardii* are presented as alternative microbial sources for obtaining potentially probiotic mead.

Keywords: honey; yeast; in vitro simulated gastrointestinal digestion; phenolics; antioxidants

1. Introduction

Mead is a traditional beverage that is obtained by the alcoholic fermentation of diluted honey and appropriate yeast activity [1–5], which according to Brazilian legislation must have an alcohol content of 4% to 14% by volume of ethanol at 20 °C [6]. In terms of composition, mead is known for its rich amount of nutrients, containing important compounds

such as proteins, carbohydrates (e.g., fructose, glucose and others), minerals, organic acids, vitamins and phenolics, the latter being natural antioxidants that are essential for the maintenance of the human organism because of their potential to inhibit free radicals and thus prevent cell damage [2,4,5,7,8].

For the production of mead, the most commonly used species is *Saccharomyces cerevisiae* (*S. cerevisiae*), which is widely used in the fermented alcoholic beverage industry due to its high fermentative performance in converting sugars into alcohol, a characteristic that is suitable and necessary to obtain other alcoholic beverages that are also fermented, such as beer and wine [9]. Some specific strains of *S. cerevisiae* have also been reported as suitable for mead fermentation, such as BRL-7 [10], C11–3 [11], Premier cru [12], UCD522 [13] and ENSIS-LE5 [14].

According to the scientific literature, some unconventional microorganisms, such as *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii*), associated microorganisms that are found in water kefir and kombucha, have already been reported as suitable for obtaining alcoholic beverages that are also fermented, such as craft beers and rosé wine [15–21], and taking into account the similarity of the production processes, these microorganisms could have potential for the production of mead [8,22,23]. In view of this, current research has highlighted the use of the yeast *S. boulardii* as a promising alternative for obtaining potentially probiotic mead that has shown acceptable sensory characteristics [8,22]. Furthermore, a recent study evaluated the potential of incorporating probiotic bacteria in the development of mead; in this case, an association of *S. cereviseae* with *Lactobacillus paracasei* was used to produce the beverage [5]. Another study demonstrated the potential of water kefir in mixed fermentation with *S. boulardii* to produce a new probiotic mead [23].

Bearing in mind that the search for new sources of microorganisms that are suitable for mead production conditions is still an important open field of research that needs to be investigated, in this study, we initially propose an innovation that consists of obtaining a potentially probiotic mead through the mixed fermentation of *S. boulardii* and kombucha.

S. boulardii is a probiotic yeast that has been used as a therapeutic and preventive agent in the treatment of gastrointestinal diseases such as diarrhea [19,24–26]. Additionally, it is already being incorporated into various types of food and beverages to add functional and probiotic properties [8,19,22,27–31]. Among the products obtained through the use of *S. boulardii* are fermented alcoholic beverages such as beer and wine [16,17,20,28].

Kombucha is a beverage of Asian origin made from black tea and/or green tea (*Camellia sinensis*) that is obtained through fermentation by the proto-cooperation of yeasts and bacteria and in which a cellulose film called a "symbiotic culture of bacteria and yeast" (scoby) is formed [32]. The proto-cooperation of these microorganisms makes kombucha rich in metabolite compounds such as organic acids, proteins, ethanol, polyphenols, antioxidants and anti-inflammatories, thus adding functional characteristics [33–35]. Due to its functional characteristics and beneficial health properties, kombucha has been associated with various effects such as antimicrobials, antihypertensives, antitumor agents and the prevention and treatment of cardiovascular diseases, as well as properties that enable the prevention of neurodegenerative diseases and even diabetes [36,37].

In this context, the aim of this study was to produce a potentially probiotic mead from co-fermentation by *S. boulardii* and kombucha microorganisms, as well as to evaluate the physicochemical characteristics during fermentation, the survival and resistance of the microbial cells after in vitro simulated gastrointestinal digestion, the color parameters and the total phenolic content and antioxidant capacity of the beverage.

2. Materials and Methods

2.1. Materials

Saccharomyces cerevisiae var. *boulardii* CCT 4308 (UFPEDA 1176) was purchased from a collection of microbial cultures at the Fundação Andre Tosello (FAT, research and technology, Campinas, Brazil). The kombucha scoby was obtained from a microbial cell collection bank at the Laboratório de Engenharia de Bioprocessos (LEB) at FZEA/USP (São Paulo,

Brazil). The honey (Organic–LAMBERTUCCI, KOSHER certification, eucalyptus flowers) was obtained from a local business in Pirassununga/SP. The culture media included the following: yeast extract powder (HIMEDIA, Maharashtra, India), bacteriological peptone (KASVI, Spain), D(+) dextrose monohydrate P.A. (ÊXODO Científica, São Paulo, Brazil) and agar MRS Lactobacilos (Micro MED, Rio de Janeiro, Brazil).

The chemical standards and reagents used in the experiment include the following: sodium chloride P.A. (NaCl), hydrochloric acid P.A. (HCl) (LS Chemicals, São Paulo, Brazil), pepsin from porcine gastric mucosa, 2,4,6-Tris(2-pyridyl)-s-triazine, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 2,2-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammoninum salt (Sigma-Aldrich, São Paulo, Brazil), pancreatin P.A., sodium hydroxide P.A. (NaOH), sodium carbonate P.A. (Na_2CO_3), Folin–Ciocalteau phenol solution (2 M), ferric chloride P.A. ($FeCl_3 \cdot 6H_2O$), potassium persulfate P.A. ($K_2O_8S_2$) (ÊXODO Científica, São Paulo, Brazil), gallic acid P.A. ($C_7H_6O_5 \cdot H_2O$) (Dinâmica, Brazil), and oxgall or bile salts (Progresso, São Paulo, Brazil). Other reagents are all of analytical purity.

2.2. Methods

2.2.1. Obtaining and Cultivating Microorganisms

The *S. boulardii* cells were obtained by Souza et al. [8], with adaptations. The yeast was cultivated in 200 mL of yeast extract–peptone–dextrose (YPD) broth (media concentrations calculated as 2% for peptone, 1% for yeast extract and 2% for dextrose and expressed as *w*/*v*, respectively) in a 500 mL Erlenmeyer flask. The cells were grown at a temperature of 25 °C at 160 rpm for 72 h in a shaker-type incubator (TECNAL, model TE-424). The yeast cell biomass was then recovered by centrifugation (Excelsa II, FANEM, centrifuge model 206 BL) at 5000 revolutions per minute (rpm) for a time of 5 min at a temperature of 30 °C, washed three times with phosphate buffer pH 7.2 and immediately used to produce mead.

For the production of kombucha, pure organic green tea (*Camellia sinensis*) was used (GUNPOWDER, herbal flavor), imported from China (Hunan province). The tea was brewed using drinking water heated to 80 °C and 6 g/L of green tea buds and leaves. The container was then covered and infused for 10 min. After infusing the tea, the liquid was filtered three times using a traditional coffee filter. In addition, 50 g/L of commercial crystal sugar was added to the solution. To ferment the tea, 300 mL of the infusion solution was added to previously sanitized 500 mL glass containers. The solution was cooled to a room temperature of 25 °C. Next, 10% scoby (*m*/*v*) (equivalent to a mass of 30 g) and 10% (*v*/*v*) (equivalent to a volume of 30 mL) of previously fermented kombucha were added, and the glass containers were kept half-open (lightly covered with flannel lids) to remove the gas produced. Finally, fermentation and kombucha production took place for 20 days at a temperature of 25 °C. Figure 1 shows the kombucha that was obtained at the end of fermentation.

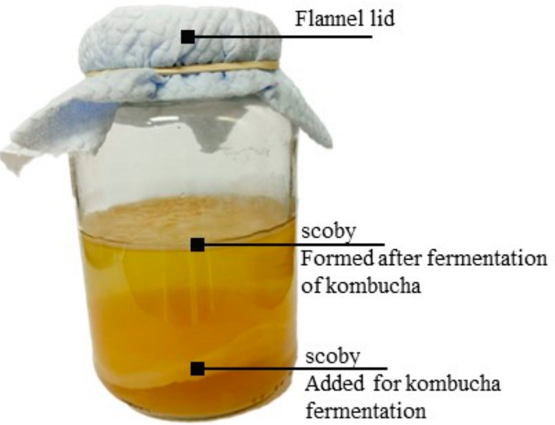

Figure 1. Kombucha obtained from green tea (*Camellia sinensis*), after 20 days of fermentation, at a temperature of 25 °C. scoby = symbiotic culture of bacteria and yeast.

2.2.2. Central Composite Design (CCD): Preliminary Tests

According to the method presented by Souza et al. [8], with adaptations, a Central Composite Design (CCD) for two variables was applied, with 2^n (n = 2 independent variables) + 3 central points (Cp) to evaluate the variables *S. boulardii* concentration (g/L) (w/v) and kombucha concentration (mL/L) (v/v), as shown in Table 1. Given this, the preliminary tests were designed to determine the best conditions for obtaining mead. The response (dependent) variables evaluated in the preliminary tests were pH, soluble solids (°Brix), total acidity (%), alcohol content (%) and the viable cell counts of yeasts and lactic acid bacteria (LAB), expressed in Log_{10} (CFU/mL).

Table 1. Central Composite Design (CCD) with 2^n (n = 2) + 3 central points (Cp) for obtaining mead.

Tests	*S. boulardii* (g/L)	Kombucha (mL/L)
1	−1 (0.5)	−1 (20.0)
2	+1 (1.0)	−1 (20.0)
3	−1 (0.5)	+1 (30.0)
4	+1 (1.0)	+1 (30.0)
5 *Cp	0 (0.75)	0 (25.0)
6 *Cp	0 (0.75)	0 (25.0)
7 *Cp	0 (0.75)	0 (25.0)

*Cp = Center points.

2.2.3. Mead Production

For the production of mead, the methodology of Souza et al. [8] was used, with adaptations. Initially, the quantities of honey and drinking water were calculated, and the water was heated to a temperature of 65 °C. The honey was then dissolved in drinking water at 65 °C, and the concentration of soluble solids in the must was standardized at 25 °Brix for all treatments. The mixture was then pasteurized. The must was then cooled to 25 °C. Next, *S. boulardii* and kombucha were added to the must, according to Table 1, and then it was gently homogenized. For the fermentation, 900 mL of must was prepared and added to polypropylene buckets (with a total volume of 1 L for each bucket). The buckets were placed in a BOD-type oven (model 347 CD, São Paulo, Brazil, MERSE) with a controlled temperature of 25 °C. Fermentation took place over a period of 9 days, and the system was kept in anaerobiosis (by airlocks). A scoby was observed forming on the surface of the mead inside the fermentation bucket. Finally, the mead was filled into transparent glass bottles (previously cleaned and sanitized) and stored at 7 °C ± 1 °C for analysis.

2.2.4. Experimental Validation of the Pilot Scale and Fermentation Kinetics

For experimental validation, a pilot scale was carried out considering the improved condition for obtaining mead, according to the preliminary tests of the Central Composite Design (CCD) with 2^n (n = 2) + 3 central points (Cp), shown in Table 1. For this, 3.0 L of mead was produced in polypropylene buckets (5 L volume) for the best condition. Fermentation took place in accordance with the methodology described above in "Section 2.2.3 Mead production". In order to compare the mead we obtained with the *S. boulardii* yeast, a standard mead was made using *S. cerevisiae* Mangrove Jack's M05, which was considered the best condition according to preliminary tests. For fermentation kinetics, the fermentation process was evaluated over 9 days of fermentation, monitoring the parameters pH (using a bench pH meter, model PG 1800 FARMA), soluble solids (°Brix) (refractometer, model RSG-100ATC), total acidity (%) [38] and alcohol content (%) (using an ebulliometer Kit–0700, CIENLAB). The alcohol content measured by the ebulliometer determines the decimal alcohol content by boiling the sample. This equipment is effective for extremely precise measurements of the sample's boiling temperature, which, in comparison with the calibration boiling temperature of water, determines the alcohol concentrations (%) by the equivalence ratio on a millimeter ruler that relates boiling temperature to alcohol content.

2.2.5. Viable Cell Count of Lactic Acid Bacteria (LAB) and Yeast

Yeast cell counts were determined according to the method presented by Zamora-Vega et al. [39] and Souza et al. [8], with modifications. Initially, about 1 mL of mead was added to 9 mL of saline solution (0.1%) that was previously sterilized and then serially diluted. Subsequently, aliquots of 100 µL were spread (by surface spreading) on plates containing yeast extract–peptone–dextrose agar medium (media concentrations calculated as 2% for peptone, 1% for yeast extract and 2% for dextrose and expressed as *w*/*v*, respectively) and incubated in a BOD incubator (BOD Incubator, MA 425, MARCONI) for 48 h at a controlled temperature of 35 °C. The yeast count was obtained by direct determination on plates and expressed as Log_{10} of colony-forming units (CFU) per mL of mead.

The viable cells of the LAB were evaluated according to the methodology presented by Silva et al. [40]. Samples of 25 mL of mead were added to 225 mL of previously sterilized peptone water (0.1%), and serial dilutions were made. Next, 1 mL of the dilution was added to a pour plate on plates overlaid with De Man, Rogosa and Sharpe (MRS) agar medium. The plates were then incubated inverted at 37 °C for 48 h in a BOD incubator (MA 425, MARCONI). Viable LAB cells were determined directly on plates and expressed as Log_{10} CFU per mL of mead.

2.2.6. Survival of Microorganisms after Simulated Gastrointestinal Digestion

The survival of microorganisms after simulated gastrointestinal digestion in vitro at 37 °C was analyzed according to the method presented by Souza et al. [23], based on Mathara et al. [41] and Fonseca et al. [42], with modifications. To simulate the gastric phase, about 1 mL of mead was added to 9 mL of sterile saline solution (concentration of 0.85% NaCl, *w*/*v*), and the pH of the mixture was adjusted to 2.0 using a solution of hydrochloric acid (HCl, 1 M), containing pepsin at a concentration of 0.5% (*w*/*v*). Subsequently, this first mixture was incubated at a temperature of 37 °C for 90 min with constant stirring at 130 rpm in order to prevent the sedimentation of the microorganisms. To simulate the intestinal phase, 9 mL of the gastric phase was mixed with intestinal juice. The intestinal juice was prepared by adding a solution of oxgall (4 mL, concentration 0.3%, *w*/*v*) and pancreatin (17 mL, concentration 0.1%, *w*/*v*), and the pH was adjusted to 7.0 by adding a solution of sodium hydroxide (NaOH, 1 M). The mixture was then incubated at a temperature of 37 °C for 150 min and stirred at 130 rpm. Viable yeast and LAB cell counts were obtained by plate counting at the end of the gastric and intestinal phases. In addition, the survival rate was obtained using Equation (1) and expressed as a percentage (%).

$$\text{Survival rate } (\%) = \frac{\text{Log}_{10}\ \text{CFU/mL (final)}}{\text{Log}_{10}\ \text{CFU/mL (initial)}} \times 100 \quad (1)$$

2.2.7. Color Analysis

The color of the mead was determined according to the method presented by Souza et al. [23]. The parameters L* (luminosity), b* (yellow color for positive values and blue for negative values) and a* (red color for positive values and green for negative values) were measured.

2.2.8. Total Phenolics and Antioxidant Potential of Mead

Total phenolic compounds were analyzed according to the method presented by Everette et al. [43] and adopted for mead analysis by Souza et al. [23]. The absorbance of each sample was measured in a spectrophotometer (JENWAY, model 7305, London, UK) at an optical density of 700 nm. A standard curve of gallic acid (0.01–0.05 mg/mL) was drawn up, and the results obtained were expressed in units of mg of gallic acid equivalent (GAE) per 100 mL of mead.

The antioxidant potential of the mead was measured using the 2.2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical assay (ABTS), according to the method pre-

sented by Re et al. [44]. The absorbance of the samples was read at 734 nm on a spectrophotometer. A standard curve was drawn up using Trolox equivalents (100–2000 μmol/L). Finally, the results obtained were expressed in units of μmol of Trolox equivalent per 100 mL (μmol TE/100 mL) of mead.

The antioxidant potential was also assessed using ferric reducing antioxidant power (FRAP) [45]. The absorbance of the samples was read at 593 nm on a spectrophotometer. A standard curve was drawn up using Trolox equivalents (5.0–25 μmol/L). Finally, the results obtained were expressed in units of μmol of Trolox equivalent per 100 mL (μmol TE/100 mL) of mead.

2.2.9. Statistical Analysis

A Completely Randomized Design (CRD) was adopted, and it took into account the homogeneity of the experimental unit (environment) as well as the basic principles of statistical experimentation. For the results of the analyses of fermentation kinetics, viable cell counts of microorganisms, survival after simulated gastrointestinal digestion in vitro, color analysis, phenolic compounds and the antioxidant capacity of the beverage, all the data were tabulated and expressed as mean ± standard deviation. Analysis of variance (ANOVA) and Tukey's test for differences in means were then carried out, considering a 95% confidence level ($p \leq 0.05$), using R Software version 4.3.1 for Windows. All the determinations were carried out with three repetitions and triplicates for the analyses.

3. Results and Discussion

3.1. Characteristics of Kombucha

After the fermentation period (20 days) at a temperature of 25 °C, the kombucha had the following aspects: pH 2.73, soluble solids of 4.63 °Brix, a total acidity of 0.75% (% lactic acid) and a LAB count of 3.4 Log_{10} (CFU/mL).

3.2. Central Composite Design (CCD): Preliminary Tests

The results obtained in the preliminary tests for the Central Composite Design (CCD) with 2^n (n = 2) + 3 central points (Cp) are shown in Table 2. Table 2 shows that the soluble solids content is reduced at the end of fermentation. Consequently, there is an increase in the final alcohol content (%), demonstrating the action of microorganisms, especially yeasts, in converting fermentable sugars into ethanol [8,46].

The counts of viable yeast cells and LAB both exceeded 7 Log_{10} CFU/mL. These counts are above the recommended minimum therapeutic amount (6 Log_{10} CFU/mL) for probiotic products, which is generally accepted to promote beneficial health effects [8,16,22,23,47,48]; however, high levels of alcohol can contribute to the stress of probiotic cells, since ethanol in high concentrations can decrease the vitality and increase the death of microorganisms [8,23,49–51]. For this reason, it was clearly established that the central points (0.75 g/L of *S. boulardii* and 25 mL/L of kombucha), shown in Table 2, are the best conditions for the development of a potentially probiotic mead, since lower levels of ethanol (average of 6.1%) were observed at the end of fermentation.

Table 2. Results of the preliminary tests for the Central Composite Design (CCD) with 2^n (n = 2) + 3 central points (Cp) showing the impact of *S. boulardii* and Kombucha concentrations on the physicochemical properties and yeast as well as LAB counts of the mead during 9 days of fermentation at 25 °C.

Tests	*S. boulardii* (g/L) (Initial)	Kombucha (mL/L) (Initial)	pH (Final)	Total Acidity (% Lactic Acid) (Final)	Soluble Solids (°Brix) (Final)	Alcohol Content (%) (Final)	Log_{10} Yeast Count (CFU/mL) (Final)	Log_{10} Lactic Acid Bacteria Count (CFU/mL) (Final)
1	−1 (0.5)	−1 (20.0)	3.17	0.60	17.90	6.45	7.36	8.23
2	1 (1.0)	−1 (20.0)	3.19	0.53	16.90	6.80	7.25	7.65
3	−1 (0.5)	1 (30.0)	3.20	0.54	17.90	7.10	7.80	8.08

Table 2. *Cont.*

Tests	*S. boulardii* (g/L) (Initial)	Kombucha (mL/L) (Initial)	pH (Final)	Total Acidity (% Lactic Acid) (Final)	Soluble Solids (°Brix) (Final)	Alcohol Content (%) (Final)	Log_{10} Yeast Count (CFU/mL) (Final)	Log_{10} Lactic Acid Bacteria Count (CFU/mL) (Final)
4	1 (1.0)	1 (30.0)	3.17	0.64	17.15	8.05	7.22	7.87
5 *Cp	0 (0.75)	0 (25.0)	3.17	0.55	18.25	6.18	7.34	7.54
6 *Cp	0 (0.75)	0 (25.0)	3.33	0.59	18.45	6.05	7.51	7.20
7 *Cp	0 (0.75)	0 (25.0)	3.17	0.59	18.85	6.06	7.21	7.59

*Cp = Center points.

3.3. Fermentation Kinetics of the Pilot Scale

The fermentation kinetics of the mead are shown in Figure 2, where it can be seen that the parameters of pH, soluble solids, total acidity and alcohol content behave similarly for mead with the probiotic yeast *S. boulardii* and kombucha (T1) and mead with the commercial yeast *S. cerevisiae* and kombucha (T2). The evolution of the fermentation process shows a decrease in pH (Figure 2A) and an increase in total acidity (Figure 2B) for both treatments. This pH and acidity were expected due to the formation of different types of acids during fermentation [5]; furthermore, the acidification of the mead is also related to the co-fermentation of kombucha in T1 and T2. As kombucha is a symbiotic association between different microorganisms, such as yeasts, acetic bacteria and LAB, these microorganisms form a consortium with a complex microbiological composition by acting on the substrates (fermentable sugars) and contributing to the production of organic acids, thus favoring the acidification of the environment [5,33,34].

For soluble solids (Figure 2C), there was a decrease during the fermentation time with values varying from 25 °Brix in the initial must to 18.76 °Brix in T1 and 17.16 °Brix in T2 at the end of fermentation. The must soluble solids are derived from honey [9], which is composed, in particular, of carbohydrates (80%), of which around 75% is fructose and glucose [52]. Other compounds are also present in honey in small quantities, such as proteins, vitamins, minerals, amino acids, enzymes, polyphenols and others [9,52]. Given this, the decrease in soluble solids during mead fermentation was expected and is an important indication of the action of the microorganisms that are acting on the substrates, such as fermentable carbohydrates and allowing the fermentation process to take place [8,23,46]. In the study by Souza et al. [23], a similar behavior was also observed for soluble solids, in which the authors observed a reduction in initial soluble solids from 25 °Brix to a final soluble solid of 17.28 °Brix in mead with water kefir and *S. boulardii*, and 16.40 °Brix for water kefir and commercial *S. cerevisiae*.

Figure 2D shows the increase in alcohol content during fermentation. Note that the highest alcohol content is reached by mead with kombucha and commercial *S. cereviseae* yeast (T2), with a statistically significant difference ($p > 0.05$) in relation to mead with kombucha and probiotic *S. boulardii* yeast (T1). The higher alcohol content observed in T2 is due to the high fermentative performance of the commercial *S. cereviseae* yeast in converting fermentable sugars into alcohol; this yeast has the characteristics required for the production of other fermented alcoholic beverages such as wine and beer [9]. The production of ethanol during the mead fermentation process takes place through the conversion of sugars (glucose and fructose) by the action of yeasts, consequently leading to the generation of ethanol. As such, in the conversion process that takes place inside the cell, the monosaccharides are converted into pyruvic acid (pyruvate) through a sequence of enzymatic reactions known as glycolysis, and then, from pyruvic acid, alcoholic fermentation takes place under anaerobic conditions, giving rise to the final product of the process, ethanol [53]. Recently, in a study that evaluated the development of potentially probiotic mead by *S. boulardii* and a standard treatment by *S. cerevisiae*, the evolution of the increase in ethanol during fermentation was also observed [8], corroborating the results of this study. Fu et al. [5] found values of 17.0% (v/v) of alcohol content evaluating the fermentation of *S. cerevisiae* and *L. paracasei* in the production of mead, and this value was higher than those found in

this study. On the other hand, Souza et al. [23] evaluated the development of probiotic mead from the mixed fermentation of *S. boulardii* and water kefir and found an alcohol content of 7.05%, higher than in this study.

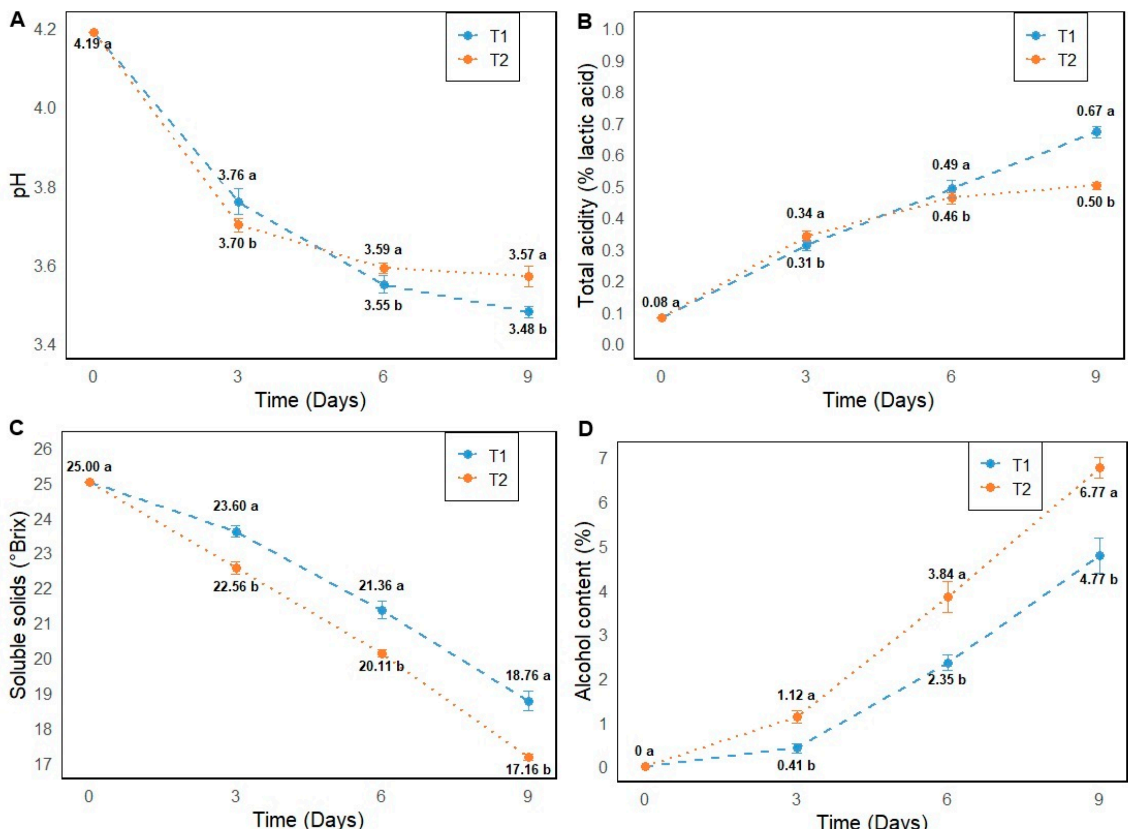

Figure 2. Mead fermentation kinetics over 9 days at a temperature of 25 °C. (**A**) pH, (**B**) Total acidity (% lactic acid), (**C**) Soluble solids (°Brix) and (**D**) Alcohol content (%). T1 = mead with kombucha and *S. boulardii*; T2 = mead with kombucha and commercial *S. cerevisiae*. Different letters indicate that there are significant differences between the treatments when the Tukey test is applied ($p > 0.05$).

3.4. Viable Cell Count and Simulated In Vitro Digestibility at 37 °C

The results relating to the viable cell count and the survival of the microorganisms after simulated in vitro digestion are shown in Figure 3. According to Figure 3A, the viable yeast count in the mead is higher than 8 Log_{10} CFU/mL for both treatments (T1 and T2), being statistically equal ($p < 0.05$). These results clearly show that honey must is an environment with the right conditions and nutrients for the growth and action of *S. boulardii* and *S. cereviseae*. Furthermore, after the intestinal phase of the simulated in vitro digestibility at 37 °C, it was found that the counts of *S. boulardii* in T1 were still high, with values above 6 Log_{10} CFU/mL, despite the various extrinsic factors and stressful conditions that the microorganisms encounter during digestion. In the study by Souza et al. [23], similar results were found, in which it was observed that *S. boulardii* presented quantities of 7.45 Log_{10} CFU/mL after the intestinal phase in probiotic mead obtained through the fermentation of water kefir and *S. boulardii*.

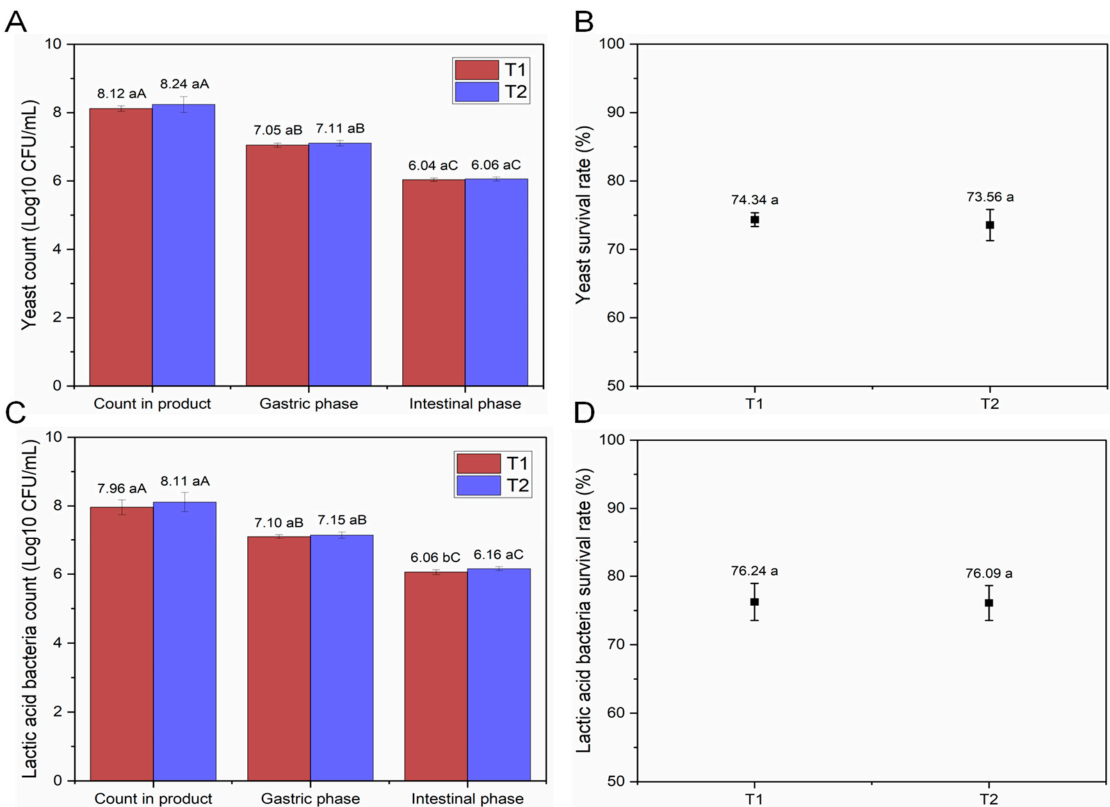

Figure 3. Viable cell count in mead and simulated in vitro digestibility at 37 °C. (**A**) Yeast count (Log_{10} CFU/mL), (**B**) Yeast survival rate (%), (**C**) Lactic acid bacteria count (Log_{10} CFU/mL) and (**D**) Lactic acid bacteria survival rate (%). T1 = mead with kombucha and *S. boulardii*; T2 = mead with kombucha and commercial *S. cerevisiae*. Different lowercase letters indicate that there is a significant difference between the treatments. Different capital letters above the bars indicate that there are significant differences between the different phases, for each treatment. The Tukey test ($p > 0.05$) was applied to the difference in means.

The results obtained after the intestinal phase (Figure 3A) are highly relevant because, according to the scientific literature, it is widely accepted that a viable cell count of 6 log_{10} CFU/mL is the minimum therapeutic dose for probiotic products to have a positive effect on the host [8,16,22,23,47,48,54]. The high counts of *S. boulardii* obtained after the intestinal phase (Figure 3A) support scientific evidence demonstrating the ability and tolerance of this yeast to survive conditions that resemble gastric and intestinal transit [50,51,55–57]. In addition, *S. boulardii* showed a high survival rate after in vitro digestibility, with values higher than 74% in T1, as shown in Figure 3B.

The counts of viable LAB cells in the mead are shown in Figure 3C. The quantities of LAB were 7.96 ± 0.22 and 8.11 ± 0.28 Log_{10} CFU/mL for T1 and T2, respectively, being statistically equal ($p < 0.05$) (Figure 3C). When we compare the LAB counts in the mead with the initial counts in the kombucha (presented in the subsection "Section 3.1 Characteristics of kombucha"), in which LAB was present at 3.4 Log_{10} CFU/mL of kombucha, we can infer that honey must offers good conditions for the development of these microorganisms, highlighting the use of kombucha as a mixed starter with *S. boulardii* and *S. cerevisiae* as a potential strategy to produce the beverage. When simulated in vitro digestibility was carried out at 37 °C, high LAB counts were also observed after the intestinal phase, with quantities of more than 6.0 Log_{10} CFU/mL of mead for both treatments (Figure 3C). In addition, the LAB survival rate was over 76% for both treatments (Figure 3D). Although the LAB are subjected to adverse conditions such as the alcoholic stress of the mead, the acidic and low pH environment, as well as the salt and bile stress of in vitro digestion, these microorganisms are resistant and survive the simulated gastric and intestinal con-

ditions. This may have been due to the presence of probiotic LAB in the kombucha that have developed in the mead, such as possible probiotic strains of *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Bifidobacterium bifidum* and others [58].

The viable cell counts and survival of the microorganisms in the mead we produced could potentially characterize a positive association between the alcohol and the probiotics. As such, the consumption of alcoholic beverages in adequate doses, which cause beneficial effects on health, is recommended by various organizations and countries that stipulate in their legislation the minimum requirements for moderate consumption. In the European Commission, consumption recommendations can be found in the "National low-risk drinking recommendations (or drinking guidelines) and standard units" [59]. In some countries, such as Brazil, there is no specific legislation on moderate alcohol consumption, in which case the dose recommended by the World Health Organization (WHO) is stipulated, which defines 10 g of pure ethanol as the standard dose [60]. In view of this information, our product is within the recommended alcohol content and dose limits, and in this dose level, probiotic amounts above 6 Log_{10} CFU/mL are found, making the beverage an alternative for probiotic consumption, since the microbial cells that are present in the mead survive the conditions that reproduce gastrointestinal transit (Figure 3).

3.5. Color Analysis of the Mead

Figure 4 shows the visual color aspects of mead, revealing that the products tend towards a brownish-yellow color.

Table 3 shows the colorimetric analysis of the meads we produced. It can be seen that the mead with *S. boulardii* probiotic yeast (T1) had a lower luminosity (L*) than the mead with commercial *S. cerevisiae* (T2), which is a significant difference ($p > 0.05$). The higher luminosity of T2 may be associated with the high fermentative activity of commercial yeast [9,61], which allows for greater consumption of fermentable sugars (substrates) and, consequently, soluble solids are reduced (Figure 2C), leading to the higher luminosity of the sample.

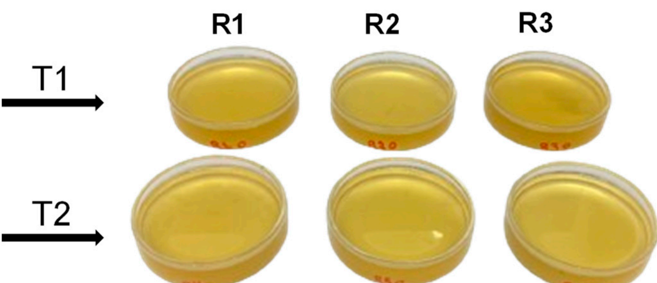

Figure 4. Visual appearance and color of the meads produced. T1 = mead with kombucha and *S. boulardii*; T2 = mead with kombucha and commercial *S. cerevisiae*. R1, R2 and R3 = repetitions 1, 2 and 3, respectively.

Table 3. Colorimetric analysis of the mead produced.

Treatment	L*	a*	b*
T1	76.09 ± 1.26 b	1.82 ± 0.82 a	35.93 ± 3.05 a
T2	77.56 ± 1.11 a	1.58 ± 0.49 a	34.12 ± 1.23 a

T1 = mead with kombucha and *S. boulardii*; T2 = mead with kombucha and commercial *S. cerevisiae*. Different lowercase letters in the columns show that there are significant differences between the treatments. The Tukey test ($p > 0.05$) was applied to the difference in means.

With regard to the a* coordinate (red/green coordinate, which indicates variations in red for positive values and green for negative values) (Table 3), it can be seen that both treatments tend towards a red color (positive values) and are statistically equal ($p < 0.05$). For the *b coordinate (yellow/blue coordinate, which indicates variations from yellow for

positive values and blue for negative values), with yellow being the most relevant color for mead, it can be seen that T1 and T2 tend towards a yellow color, being statistically equal ($p < 0.05$). The yellow color of mead may be related to the presence of various compounds in this drink, including flavonoids, phenolics and minerals. It can also be affected by compounds produced in the middle of fermentation by reactions that allow oxidation and/or condensation and adsorption by the yeasts that act on the product [8,9,61,62]. The color parameters of T1 found in this study were close to the results reported by Souza et al. [23], who, evaluating water kefir and *S. boulardii* in the production of probiotic mead, observed luminosity values (L*) of 73.55, 2.18 for a* and 35.07 for b*, as well as a trend towards yellow in products. Fu et al. [5], evaluating the fermentation of mead using a mixed fermentation of *S. cerevisiae* and *L. paracasei*, found higher L* and a* values of 83.69 and 4.32, respectively, and lower b* values of 20.97.

3.6. Total Phenolics and Antioxidant Potential of Mead

The total phenolic and antioxidant results of the mead are shown in Table 4, which shows that T1 and T2 have the same amounts of total phenolics and antioxidants according to the ABTS and FRAP methods and are statistically equal ($p < 0.05$). According to studies, honey is rich in phenolics, such as phenolic acids and derivatives as well as flavonoids, and these compounds contribute to bioactive properties such as antioxidant and antimicrobial capacity [7,9]. Mead is therefore a source of bioactive compounds such as phenolics and antioxidants [8,23]. The phenolic compound results of this study are supported by the scientific literature that shows mead as a source of phenolic compounds [5,23,63]. As such, the study by Souza et al. [23] found values of 15.24 and 15.63 mg of GAE/100 mL of phenolic compounds in mead made from water kefir and *S. boulardii* and water kefir and S. cerevisiae, respectively, which are lower than the results of the present study.

Table 4. Bioactive compounds in the mead produced.

Bioactive Compounds	T1	T2
Total phenolic (mg de GAE/100 mL)	17.34 ± 0.22 a	17.16 ± 0.15 a
ABTS (μmol TE/100 mL)	62.92 ± 5.54 a	68.03 ± 5.04 a
FRAP (μmol TE/100 mL)	4.93 ± 0.09 a	4.94 ± 0.04 a

TE = Trolox equivalent; GAE = gallic acid equivalent; T1 = mead with kombucha and *S. boulardii*; T2 = mead with kombucha and commercial *S. cerevisiae*. Different lowercase letters in the rows show that there are significant differences between the treatments. The Tukey test ($p > 0.05$) was applied to the difference in means.

According to the literature, the presence of antioxidant compounds in mead can be influenced by several factors, including the amount of honey and composition of the raw material used, types and methods of processing such as fermentation and aging, and the addition of ingredients such as herbs, fruits and spices [2,3,8,23,64,65]. In the case of this study, it can be seen that, for example, the presence of acetobacteria, normally found in kombucha, may have contributed to the antioxidant activity of the products [66]. According to the scientific literature, the presence of antioxidant compounds has been confirmed in mead obtained by various production methods and processes. In the study by Kawa-Rygieslka et al. [2], the antioxidant activity measured by the ABTS method showed values of 0.29 μmol TE/mL in mead with dandelion syrup and 0.89 μmol TE/mL with grape seeds. For the FRAP method, these same authors reported values of 0.40 and 0.74 μmol TE/mL for the beverages, respectively. The results presented by Kawa-Rygieslka et al. [2] are lower than those found in this study. On the other hand, Souza et al. [23] reported higher antioxidant values for ABTS with 85.23 μmol TE/100 mL and lower values for FRAP with 4.52 μmol TE/100 mL in a study of probiotic mead made by co-fermenting water kefir and *S. boulardii*; however, it is extremely important to note that the presence of antioxidants in mead is highly relevant due to the ability of these compounds to inhibit free radicals that act negatively and cause damage to cells, thus contributing to the improvement of the body and human health [2,7,8,23,67,68].

4. Conclusions

The co-fermentation of kombucha and *S. boulardii* made it possible to develop a beverage typical and characteristic of mead. The probiotic yeast *S. boulardii* and LAB survive the simulated in vitro conditions that reproduce gastrointestinal transit, attesting to the probiotic potential of these microorganisms. The mead produced tends to be yellow in color. In addition, the beverage was found to have phenolic compounds and antioxidants, which could contribute to the functional aspects of the beverage due to the ability of antioxidants to inhibit free radicals that can cause damage to cells. In short, this study has shown that the use of kombucha and *S. boulardii*, when combined in mixed fermentation, is a potential alternative for obtaining a potentially probiotic mead and serves as a basis for future studies.

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