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Improved Antioxidant Properties and Vitamin C and B12 Content from Enrichment of Kombucha with Jujube (*Ziziphus jujuba* Mill.) Powder

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Abstract: (1) Objectives: Traditional kombucha (K) is a fermented beverage obtained from black or green tea infusion. Besides traditional substrates, the possibility of using alternative ingredients resulted in changes in metabolic profile and biological activity. The aim of this work was to study an alternative kombucha (KJ) prepared by the addition of jujube powder to black tea. (2) Materials and Methods: Changes in pH, protein, sugars, phenolic (TPC), flavonoid (TFC), and vitamin C and B12 content were evaluated at different time points over a period of 45 days. The identification of polyphenols by HPLC DAD and the antioxidant capacity by DPPH, ABTS, and FRAP tests of all samples was also carried out. (3) Results: The results showed higher protein, total phenolic content, and antioxidant capacity in KJ samples than in K ones. Vitamin C content increased during fermentation and reached its maximum concentration on day 45 (7.1 ± 0.3 mg/100 mL) for KJ. Caffeine in the supplemented samples was the main biocompound among those identified. Vitamin B12 formed on day 4 in K and after 24 h in KJ samples, remaining constant at the initial value of 2.30 ± 0.01 mg/100 mL up to day 45. (4) Conclusions: The results highlight that the fortification of kombucha with jujubes improved its biological activity and the content of bioactive compounds.

Keywords: kombucha; fermentation; jujube powder; black tea; sugars; phenolic content; flavonoid content; antioxidant activity; vitamin C; vitamin B12

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1. Introduction

Kombucha tea is a low-alcoholic functional beverage traditionally prepared from the fermentation of sweetened black or green tea by a symbiotic culture of bacteria and yeasts (SCOBY). The original drink is of Asian origin, but nowadays it is globally consumed due to its beneficial health effects, such as anti-microbial and antioxidant effects [1], treatment for gastric ulcers [2] and high cholesterol [3], and liver detoxification [4].

Kombucha tea contains many compounds, such as esters and organic acids, which are responsible for its flavor [5]. Moreover, it is a drink rich in bioactive ingredients derived from the microbial activity of yeasts and bacteria living symbiotically within a cellulosic backbone, which appears as a layered film, called "tea fungus" or SCOBY (Symbiotic Culture of Bacteria and Yeast) [6]. It is continuously produced during fermentation by acetic acid bacteria and has numerous applications [7]. Black and green tea extracts sweetened with sucrose from 5% to 10% are the traditional substrates used as the fermentation medium, but the growing popularity of kombucha required the use of new materials in terms of the microbiological composition of SCOBY [8], fermentation conditions (temperature and storage time) [9,10], basic ingredients [11], sugar source [11,12], and functional component [13], leading to changes in the chemical composition of the final beverage and

improving its health benefits [14]. In recent years, the rising demands and increasingly health-conscious population in global beverage consumption has promoted the development of alternative kombucha with different organoleptic and functional properties using various plant and fruits that are excellent sources of various bioactive components. So, they may be used to obtain biocompound-rich kombuchas. To date, a great variety of them, used as fresh ingredients or as juice or extracts, like cherry [15], lemon [16], goji berry [17], kiwi [18], red raspberry [19], and fresh or dried herbs and spices such as mint [20,21], cinnamon [22], cardamom [22], thyme [22,23], and inulin [18], have been added to the starting tea before inoculation with SCOBY [20,22] in order to modulate the concentration of bioactive compounds and thus the functional properties of the final beverage.

Among different food matrices, jujube (*Ziziphus jujuba* Mill.) fruit is much admired for its high nutritional value and has been commonly used as a crude drug in traditional Chinese medicine. It is an excellent source of phytochemicals, especially phenolic compounds, triterpenic acids, and polysaccharides, which exert excellent biological activity, such as antibacterial activity [24] and skin-healing properties [25]. Jujube is also rich in fiber and organic acids, and it is considered a rich source of vitamin C [26], which is why it has been called a natural "vitamin C pill".

To the best of our knowledge, jujube fruit has never been used to fortify traditional kombucha. It is important to emphasize the ability of SCOBY microorganisms to naturally synthesize hydro-soluble vitamins [27], but there are no data available in the literature about the effect of fruit addition to kombucha on vitamin B12 content. In this regard, the aim of this study was to obtain alternative kombucha with improved antioxidant activity and nutritional profile, especially with regard to vitamin C and B12 content, by adding jujube powder to the starting sweetened black tea. To achieve this objective, a jujube-enriched kombucha and a traditional one were separately monitored during the whole fermentation process from 0 to 45 days and then the results compared to verify the changes in content of acetic acid, sugars, ethanol, polyphenols, vitamins C and B12 content, as well as antioxidant activity.

2. Materials and Methods

2.1. Chemicals and Reagents

For the preparation of kombucha, Symbiotic Culture of Bacteria and Yeast (SCOBY) and black tea were used. SCOBY was purchased from Kefiralia (Burumart Commerce S.L, Arrasate, Spain), and the black tea (Despar) was from a local market. The powder from jujube (*Ziziphus jujuba* Mill.) fruits was prepared in the laboratory as described below. Analytical studies were carried out by using Folin-Ciocâlteu and Bradford reagents, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid (ABTS), sodium carbonate (Na_2CO_3), potassium persulfate ($K_2S_2O_8$), aluminum chloride ($AlCl_3$), sulfuric acid (H_2SO_4), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), $FeCl_3$, sodium acetate, $FeSO_4$, and butylhydroxytoluene (BHT). They were from Sigma-Aldrich s.r.l. (Milan, Italy), as were the standards used for the quantitative HPLC analyses: sucrose, glucose, fructose, 99.7% purity ascorbic acid, caffeine, epigallocatechin gallate (EGCG), ferulic and chlorogenic acids, quercetin, and $\geq$97.0% purity 5′-Deoxyadenosylcobalamine. All the solvents were provided by Carlo Erba (Milan, Italy).

2.2. Preparation of Jujube Powder

Jujube fruits, yellow-green in color with mahogany spots, were picked in the area of Rombiolo (latitude: 38°35′34″08 N, longitude: 16°0′9″00 E, Calabria, Southern Italy), in October 2017. After collection, the fruits were cleaned up, frozen at −20 °C, and then freeze-dried (Telstar freeze-dryer, model Cryodos, Terrassa, Spain). After that, the dried fruits were deseeded and ground to a powder, which was sieved into 250-micron particles using a 60-mesh screen. The resulting powder was used as a supplement for the preparation of an enriched fermented tea.

2.3. Preparation of Kombucha

Black tea (28 g, 0.7% *w*/*v*) was added to boiling water (4 L) and left to infuse for 10 min in a sterile glass jar. Then, commercial sugar (400 g, 10% *w*/*v*) was added, and the mixture was stirred until it was completely dissolved. After cooling down to room temperature, the entire volume was partitioned into four equal portions (1 L each), and each partition was transferred to a sterile amber container. One portion was used as a control (T), the second one had jujube powder added (7 g, 0.7% *w*/*v*) (TJ), the third portion was inoculated with the SCOBY culture (K), and the fourth one was enriched with 0.7% *w*/*v* of jujube powder and inoculated with the SCOBY (KJ). Glass jars were covered by a clean cloth and the fermentation was allowed to proceed at room temperature up to 45 days. During fermentation, a 100 mL portion was taken from each jar with a periodicity of 1, 4, 8, 14, 21, and 45 days. All the collected samples were kept in the freezer until the analyses. Nine analyses were conducted, each of them in triplicate, and the data were expressed as means ± standard deviation (SD). The results for T, TJ, K, and e KJ were compared.

2.4. pH

An electronic pH meter (Hanna Instruments, George Washington Hwy, Smithfield, RI, USA) was used for pH measurement of all samples. It was previously calibrated at pH 4.0 and 7.0.

2.5. Protein Content

Protein content was determined by Bradford assay with some modifications [28]. Briefly, 20 µL of sample (1 mg/mL) and 200 µL of Coomassie Brilliant blue reagent were added to 800 µL of distilled water. The solution was incubated for 5 min at room temperature in the dark, and the resulting color was measured at 595 nm against a blank (water) by the spectrophotometer (Ultrospec 2100 Pro, Amersham Biosciences/GE Healthcare, Marseille, France). The results were obtained by setting the calibration curve of bovine serum albumin (BSA) in the range of 1 to 0.001 mg/mL.

2.6. Sugars, Ethanol, and Acetic Acid Content by High-Performance Liquid Chromatography–Refractive Index Detectors (HPLC-RI)

Before analyses, samples were centrifugated at 10,000 rpm for 5 min. Then, the supernatants were filtered through a nylon filter with a porosity of 0.45 µm, (diam. 25 mm, Sigma-Aldrich, Milan, Italy) using gentle pressure to transfer. Fructose, glucose, sucrose, acetic acid (AA), and ethanol (EtOH) were quantified by HPLCV (Jasco Technologies, Santa Clara, CA, USA), equipped with a solvent degasser, and coupled to a RI-4030 refractive index detector. The compounds were separated on a Rezex ROA-Organic Acid H + (8%) column (Phenomenex, Torrance, CA, USA) using a 5 mM H_2SO_4 mobile phase at pH 2.25 with a flow rate of 0.5 mL/min. The column thermostat (Jasco co-2060 Plus) was set to 60 °C during the analyses that were completed after 30 min [29]. The external standard method was used to quantify fructose and glucose in the range of 0.1–1 mg/mL, and ethanol and acetic acid in the range of 0.1 to 0.001% (*v*/*v*).

2.7. Vitamin C and B12 Content by High Performance Liquid Chromatography–Diode Array Detector (HPLC-DAD)

The amount of ascorbic acid and vitamin B12 was evaluated by analyzing the samples by HPLC (HPLC, Shimadzu, Kyoto, Japan) equipped with an auto sampler (SIL 20A), two pumps (LC 20AD), a diode array-detector (SPD M20A), and a system controller CMB-20A. Samples were eluted on a Mediterranea SEA (Terrassa, Spain) C18 column (4.6 mm × 25 cm, 5 µm) with a flow rate of 0.6 mL/min and at a wavelength of 254 nm. The mobile phase consisted of (A) water containing 0.1% of formic acid and (B) ACN. All the analyses were performed in gradient elution mode as follows: 0.01 min, 10% B; 20 min, 22% B; 40 min, 40% B; 45 min, 10% B; and 50 min, 10% B. The individual vitamins were quantified by the external standard method using the respective calibration curves in the

range of 0.1–0.5 mM for vitamin C and 0.1–0.01 mM for vitamin B12. The retention times of vitamin C and B12 were 4.5 and 21.4 min, respectively.

2.8. Total Phenolic Content (TPC)

Total phenolic content was determined by using Folin–Ciocâlteu assay [30], with some modifications. Briefly, 0.1 mL of sample (1 mg/mL) was dissolved in 2 mL of distilled water and 0.5 mL of Folin-Ciocâlteu reagent was added, previously diluted with distilled water (Folin-Ciocâlteu: H_2O = 1:10 *v*/*v*). After stirring for 3 min, 0.4 mL of an Na_2CO_3 solution (7.5%) was added. The resulting mixture was incubated for 30 min at room temperature in the dark, before measuring the absorbance at 765 nm by a UV–vis spectrophotometer (model V-550, Jasco, Milano, ItalyEurope) against a blank consisting of 2.1 mL of water, 0.5 mL of reagent, and 0.4 mL of Na_2CO_3 solution. The phenolic content was determined by a five-point gallic acid curve. It was constructed by preparing stock solutions of 0.01, 0.1, 0.25, 0.5, and 1 mg/mL (y = 0.0874x + 0.0207), with a good correlation exhibited between the data and the coefficient R^2 of the linear regression being equal to 0.998. The results were expressed as μg GAE (micrograms of gallic acid equivalents) per 100 mL of sample.

2.9. Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric assay [31]. In total, 0.3 mL of each sample (1 mg/mL) were dissolved in 0.9 mL of MeOH, and 0.06 mL of 10% $AlCl_3$ solution, 0.06 mL of 1 M solution of potassium acetate, and 1.68 mL of distilled water were added. After magnetic stirring for 30 min, the absorbance was measured at 420 nm using a UV-Vis spectrophotometer against a blank (0.3 mL of EtOH, 0.9 mL of MeOH, 0.06 mL of $AlCl_3$, 0.06 mL of 1 M solution of CH_3CO_2K, and 1.68 mL of distilled water). The flavonoid content was evaluated by a quercetin calibration curve (y = 0.0559x + 0.0084; R^2 = 0.999) that was obtained by preparing five stock solutions ranging from 0.1 to 25 μg/mL. The results were expressed as μg QE (micrograms of quercetin equivalents) per 100 mL of sample.

2.10. Identification and Quantification of Biocompounds by HPLC-DAD

The qualitative and quantitative analyses of the major biocompounds in all samples were performed by HPLC coupled to a binary rapid separation pump (LC-20A, Shimadzu, Kyoto, Japan), an auto sampler (SIL-20A, Shimadzu, Kyoto, Japan), and a diode array detector (SPD-M20A DAD, Shimadzu, Kyoto, Japan) [9]. Phenolic compounds were separated on a Mediterranea SEA C18 column (4.6 mm i.d. × 25 cm; 5 μm). Data acquisition, peak integration, and calibrations were performed with a Shimadzu LabSolution Software Platform.

The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B). The system was run with the following gradient elution program: 0 min, 10% B; 20 min, 22% B; 40 min, 40% B; 45 min, 10% B; and 51 min, 10% B. The flow rate was kept constant throughout the analysis at 0.6 mL/min at room temperature, and the injection volume was 10 μL. Before analysis, each sample (1 mg/mL_{DMSO}) was filtered through a membrane filter (0.45 μm) and transferred to a vial. The compounds were identified by comparing the retention time of each peak with that of standard one under the same chromatographic conditions and also by running the samples after the addition of pure standards. The detection of the standards was carried out at their respective maximum absorbance wavelengths as follows: 273 nm for caffeine, 280 nm for epigallocatechin gallate (EGCG), 325 nm for ferulic acid and chlorogenic acid, and 365 nm for quercetin.

Quantification was performed by creating calibration curves for each compound, preparing serial dilution of the stock standards (four set of standard dilutions 0.1, 0.25, 0.5, and 1.0 mg/mL) with DMSO. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard, with R^2 exceeding 0.93: caffeine (y = 956.19x + 64,525; R^2 = 0.9775), EGCG (y = 5992.8x − 106,294; R^2 = 0.9992), ferulic acid (y = 2656.9x − 477.5; R^2 = 0.9643), chlorogenic acid (y = 19,720.19x − 569,622; R^2 = 0.9325),

and quercetin (y = 6895x − 158,694; R^2 = 0.9876). Data were reported as means ± standard deviations of triplicate independent analyses.

2.11. Antioxidant Activity

The antioxidant activities of all samples were investigated by ABTS, DPPH, and FRAP assays. The results for ABTS and DPPH are expressed as µg of Trolox equivalents per mL of samples (µg TE/mL). The ferric reducing antioxidant power (FRAP) values are reported as µM of $FeSO_4$ per mL of sample.

2.11.1. ABTS Assay

The radical scavenging activity of kombucha samples against the azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) was evaluated according to a known protocol [32]. The assay involved the direct production of the blue/green $ABTS^{\bullet+}$ radical cation chromophore, prepared by mixing 2.1 mM ABTS solution and 70 mM potassium persulfate ($K_2S_2O_8$), and allowing the solution to stand for 16 h in the dark at room temperature. It showed absorption maxima at 734 nm. The addition of antioxidants to the preformed radical cation caused discoloration of the chromophore based on the antioxidant activity and its concentration. Different concentrations (5, 10, 20, and 30 µg/mL) of each sample (T, TJ, K, and KJ) at the planned fermentation time were mixed with ABTS solution (3 mL) and then were left to react under magnetic stirring in the dark for 5 min before measuring the absorbance at 734 nm against a blank and a control. Inhibition percentages of kombucha samples against the cationic radical $ABTS^{\bullet+}$ ($\%I_{ABTS}$) were calculated using the following formula:

$$\%I_{ABTS} = [(\text{absorbance of the control} - \text{absorbance of the sample})/\text{absorbance of the control})] \times 100 \quad (1)$$

The results were expressed as µg of Trolox equivalents per mL of samples (µg TE/mL). Trolox (0.1–5.0 mM) was used as a positive control (y = −0.1077x + 0.7305, R^2 = 0.999)

EC_{50} values were calculated from the inhibition percentages $\%I_{ABTS}$ using GraphPad Prism 8 software (GraphPad Inc., San Diego, CA, USA).

2.11.2. DPPH Assay

The DPPH assay was performed according to a previously reported protocol [33]. Different concentrations (10, 25, 50, and 100 µg/mL) of each sample (T, TJ, K, and KJ) at the planned fermentation time were mixed with 0.1 mL of DPPH (1 mM) solution. The scavenging activity was quantified at 517 nm against a blank (3 mL of MeOH) and a control (3 mL solution obtained by diluting 0.1 mL of DPPH with MeOH).

Inhibition percentages of kombucha samples against the radical DPPH ($\%I_{DPPH}$) were calculated using the following formula:

$$\%I_{DPPH} = [(\text{absorbance of the control} - \text{absorbance of the sample})/\text{absorbance of the control})] \times 100 \quad (2)$$

The results are expressed as µg of Trolox equivalents per mL of samples (µg TE/mL). Trolox (0.1–5.0 mM) was used as a positive control (y = −0.01x + 0.3431, R^2 = 0.999).

EC_{50} values were calculated from inhibition percentages $\%I_{DPPH}$ using GraphPad Prism 8 software (GraphPad Inc., San Diego, CA, USA).

2.11.3. FRAP Assay

The ferric-reducing antioxidant power (FRAP) method measures the change in absorbance that occurs when the ferric-2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) form in the presence of antioxidant compounds in an acidic medium. The assay was carried out according to a previously reported protocol [33]. Briefly, the FRAP reagent contained 10 mM tripyridyl-triazine (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3$, and 0.25 M sodium acetate buffer (pH 3.6) in a ratio of 1:1:10. Samples were dissolved in DMSO at a concentration of 1 mg/mL, and

0.1 mL of solution was mixed with 2 mL of FRAP reagent and 0.9 mL of H_2O. After 30 min of magnetic agitation in the dark, the absorbance of colored complexes was spectrophotometrically measured at 593 nm. Different concentrations of an $FeSO_4$ solution ranging from 10 to 0.001 g/mL were used to construct the calibration curve. BHT was used as a positive control, and the results are expressed as µmol/mL $FeSO_4$.

2.12. Statistical Analysis

The analyses were carried out in triplicate on three different preparations for each sample. The results are expressed as mean ± standard deviation (SD) of three repetitions. A two-way ANOVA method with the Tukey post hoc test was used for statistical analysis of pH, protein, biocompounds, and vitamins C and B12; the Sidak test was used for statistical analysis of EC_{50} values and FRAP assay. Probability values $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) indicated significant differences.

3. Results

3.1. pH

The pH values of all samples are shown in Figure 1. Black tea (T) was used as the control for unsupplemented samples (K), whereas jujube supplemented tea (TJ) was used as the control for jujube-enriched kombucha samples (KJ). No significant differences in pH were found between the two controls (6.3 ± 0.1 for T and 6.1 ± 0.1 for TJ). The results show that pH values of both the KJ and the K samples significantly decreased by 2.8 and 2.6 units, respectively, during the first day of fermentation (pH 3.5 ± 0.2 for both K1 and KJ1). In the following days, the drop in pH was less than in the first day: On day 4, the pH reduction in both the K and the KJ samples was 0.2 units compared to the pH value of K1 and KJ1. After eight days, the decrease was 0.2 units, and after 14 days it dropped another 0.2 units to a value of 2.9, which remained unchanged for all samples until the end of fermentation (45 days). These values fall within the pH range of 2.5 to 4.2, which is considered safe for human consumption [34].

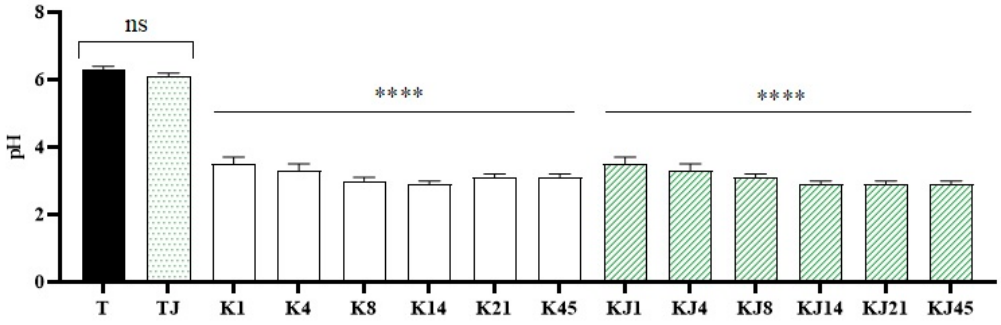

Figure 1. pH values of black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (KJ) at different fermentation times. The numbers in the sample symbols indicate the days of fermentation. The black bar represents the starting tea (T), the bar with green dots represents jujube-supplemented tea (TJ), the white bars represent traditional kombucha (K) samples, and the bars with green lines represent jujube-supplemented kombucha (KJ). Statistically significant differences between the main values of K and KJ compared to the controls T and TJ, respectively, are indicated by asterisks on the bars (**** $p < 0.0001$); ns: not significant value.

3.2. Protein Content

The protein concentration of all samples was determined using the method developed by Bradford [28] and is shown in Figure 2. The results show that the protein content of K1 significantly increased (0.39 ± 0.02 mg/100 mL, * $p < 0.05$) during the first day of fermentation compared to the control T (0.25 ± 0.08 mg/100 mL). Similarly, the protein content of KJ1 (0.50 ± 0.02 mg/100 mL) almost doubled compared to the control TJ (0.27 ± 0.06 mg/100 mL, **** $p < 0.0001$). Then, the protein content of the K samples

decreased to 0.30 ± 0.06 mg/100 mL, remaining approximately unchanged at this value until day 21 and then dropping to 0.17 ± 0.01 mg/100 mL after 45 days of fermentation. The protein content of the KJ samples followed the same fluctuating trend, decreasing to 0.29 ± 0.01 mg/100 mL by day 4 and reaching values of 0.37 ± 0.05 mg/100 mL by day 14 and 0.40 ± 0.03 mg/100 mL by day 21, which was significantly different from the control (* $p < 0.05$). Finally, it dropped to 0.18 ± 0.01 mg/100 mL by day 45, probably due to the acidic pH, which, over a long period of time, can cause the protein denaturation [35].

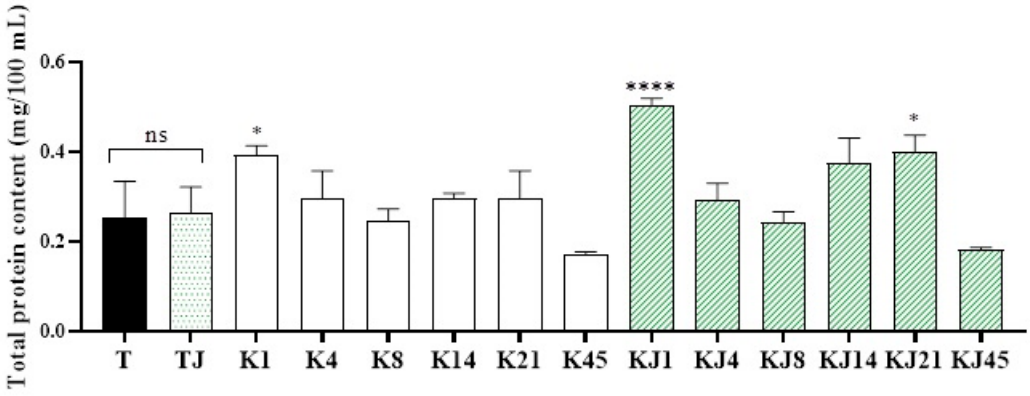

Figure 2. Protein content of black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (KJ) samples at different fermentation times. The numbers in the sample symbols indicate the days of fermentation. The black bar represents the starting tea (T), the bar with green dots represents jujube-supplemented tea (TJ), the white bars represent traditional kombucha (K) samples, and the bars with green lines represent jujube-supplemented kombucha (KJ). Statistically significant differences between the main values of K and KJ compared to the controls T and TJ, respectively, are indicated by asterisks on the bars (* $p < 0.05$, **** $p < 0.0001$, ns: not significant value).

3.3. Sugar, Ethanol, and Acetic Acid Content

After the addition of sucrose (100 g/L) to the T and the TJ samples, before inoculation, the total sugar content ranged from 100.2 ± 1.7 mg/mL in T and 102.5 ± 2.3 mg/mL in TJ (Table 1). The jujube solution (J), prepared by dissolving 0.7% w/v jujube powder in water, naturally contained glucose (2.3 ± 0.1 mg/mL), fructose (1.5 ± 0.1 mg/mL), and sucrose (2.2 ± 0.3 mg/mL). During fermentation, sucrose was hydrolyzed into glucose and fructose, and in the following days all the sugars were consumed without accumulating in the fermented medium. Since the yeasts in the consortium of microorganisms hydrolyzed sucrose into its monomeric constituent units, namely, glucose and fructose, the sucrose concentration in the K samples decreased by 32% (68.5 ± 4.5 mg/mL) after the first 24 h of fermentation and by 64% (37.3 ± 1.4 mg/mL) after 21 days compared with the sucrose amount initially added. By day 45, the residual amount of sucrose was very low (2.9 ± 0.3 mg/mL). The sucrose content decrease in the KJ samples was greater than that in the K samples: The reduction was 39% on day 1, 44% on day 8, and up to 76% on day 21. In the K samples, glucose deriving from sucrose hydrolysis by yeast increased from a value of 17.6 ± 0.2 mg/mL on day 1 to 27.3 ± 0.3 mg/mL by day 8 and then dropped to a value of 1.9 ± 0.1 mg/mL by day 45. Fructose decreased over 45 days from the initial value of 8.1 ± 0.5 mg/mL to 0.4 ± 0.01 mg/mL, showing an anomalous higher value on the eighth day of fermentation (11.8 ± 0.2 mg/mL), as well as for glucose.

Table 1. Content of sugars (mg/mL), ethanol, and acetic acid (%) of jujube (J), black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (KJ) at the planned fermentation times.

Unsupplemented	Glucose (mg/mL)	Fructose (mg/mL)	Sucrose (mg/mL)	EtOH (%)	Acetic Acid (%)
T	0 ^{d,B}	0 ^{d,B}	100.2 ± 1.7 ^{a,A}	0 ^{a,A}	0 ^{a,A}
K1	17.6 ± 0.2 ^{b,A}	8.1 ± 0.5 ^{b,A}	68.5 ± 4.5 ^{b,A}	0 ^{a,A}	0 ^{a,A}
K4	18.8 ± 0.8 ^{b,A}	8.4 ± 0.3 ^{b,A}	64.3 ± 3.6 ^{b,A}	0.40 ± 0.01 ^{a,A}	0.40 ± 0.01 ^{a,A}
K8	27.3 ± 0.3 ^{a}	11.8 ± 0.4 ^{a,B}	68.2 ± 2.8 ^{b,A}	0.80 ± 0.03 ^{a,A}	0.90 ± 0.03 ^{a,A}
K14	21.6 ± 0. 1 ^{b}	6.8 ± 0.1 ^{b,A}	66.2 ± 3.3 ^{b,A}	1.00 ± 0.01 ^{a,A}	1.8 ± 0.2 ^{a,A}
K21	14.1 ± 2.3 ^{c}	2.6 ± 0.4 ^{c,A}	37.3 ± 1.4 ^{c,A}	1.00 ± 0.02 ^{a,A}	3.0 ± 0.1 ^{a,A}
K45	1.9 ± 0.1 ^{d}	0.40 ± 0.1 ^{d,A}	2.9 ± 0.3 ^{d,A}	0.30 ± 0.01 ^{a,A}	1.0 ± 0.1 ^{a,A}
J-Supplemented	**Glucose (mg/mL)**	**Fructose (mg/mL)**	**Sucrose (mg/mL)**	**EtOH (%)**	**Acetic Acid (%)**
J	2.3 ± 0.1 ^{a,b}	1.5 ± 0.1 ^{b,B}	2.2 ± 0.3 ^{g}	0 ^{a,A}	0 ^{b}
TJ	2.7 ± 0.5 ^{a,b,A}	1.4 ± 0.3 ^{b,A}	102.5 ± 2.3 ^{a,A}	0 ^{a,A}	0 ^{b,A}
KJ1	15.5 ± 0.4 ^{a,A}	7.3 ± 0.9 ^{a,A}	62. 2 ± 2.6 ^{b,B}	0 ^{a,A}	0 ^{b,A}
KJ4	13.2 ± 0.1 ^{a,B}	6.1 ± 0. 5 ^{a,A}	56.9 ± 1.2 ^{c,B}	0.40 ± 0.01 ^{a,A}	0.30 ± 0.03 ^{b,A}
KJ8	13.2 ± 0.1 ^{a}	6.6 ± 0.5 ^{a,B}	55.8 ± 2.9 ^{d,B}	0.50 ± 0.03 ^{a,A}	0.80 ± 0.02 ^{b,A}
KJ14	13.8 ± 0.1 ^{a}	6.3 ± 0.1 ^{a,A}	54.2 ± 0.4 ^{e,B}	1.10 ± 0.05 ^{a,A}	1.9 ± 0.1 ^{b,A}
KJ21	12.6 ± 0.1 ^{a}	2.9 ± 0. 1 ^{b,A}	24.9 ± 0.3 ^{f,B}	1.40 ± 0.03 ^{a,A}	3.1 ± 0.1 ^{a,A}
KJ45	1.8 ± 0.2 ^{b}	0.30 ± 0.1 ^{b,A}	1.2 ± 0.3 ^{h,B}	0.60 ± 0.04 ^{a,A}	0.8 ± 0.1 ^{b,A}

Letters indicate the significance of the data. Values with the same letter are not significantly different; values with different letters are significantly different. Small letters indicate significance in the same sample (K or KJ) but with different fermentation times, while capital letters indicate significance between the K and KJ samples, with the same fermentation times

Similar to the K samples, in the KJ ones, glucose diminished during the 45-day fermentation period, ranging from 15.5 ± 0.4 mg/mL to 1.8 ± 0.2 mg/mL, and fructose from 7.3 ± 0.9 mg/mL to 0.3 ± 0.01 mg/mL, without exhibiting atypical increases by day 8. The alcohol concentration was zero before inoculation and remained so even after the first day of fermentation in both the K and the KJ samples; it increased slightly as fermentation progressed, reaching its maximum value on the 21st day of fermentation, (1.0 ± 0.01%, *v*/*v*, for K21 and 1.4 ± 0.03% for KJ21).

After day 21, the alcohol content decreased in both samples. Acetic acid, a metabolite of the fermentation process, was absent in the T and TJ samples and in K1 and KJ1. During the following days of fermentation, the content increased, reaching the maximum value on day 21 (3.0% for K21 and 3.1% for KJ21). Thereafter, the acetic acid content of K21 and KJ21 decreased to 1.0% and 0.8%, respectively.

3.4. Vitamin C and B12 Content by HPLC-DAD

L-Ascorbic acid and vitamin B12 were quantified by HPLC-DAD analysis (Table 2). The results highlight that vitamin C was present in the control (T) in small amounts (0.25 ± 0.07 mg/100 mL). Since jujube fruit is a source of vitamin C (69 mg/100 g) [36], the addition of jujube powder to tea justified the significantly higher vitamin C content of TJ (2.9 ± 0.4 mg/100 mL) compared to T (**** $p < 0.0001$). Vitamin C concentration significantly increased on the first day of fermentation (K1, 1.32 ± 0.03 mg/100 mL, **** $p < 0.0001$), decreasing from this amount to 0.74 ± 0.14 mg/100 mL by day 45.

Table 2. The content of vitamins C and B12, expressed as mg/100 mL, in black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (KJ) at different fermentation times.

	mg/100 mL	
Unsupplemented	**Vitamin C**	**Vitamin B12**
T	$0.25 \pm 0.07^{b,B}$	$0^{b,A}$
K1	$1.32 \pm 0.03^{a,B}$	$0^{b,A}$
K4	$0.24 \pm 0.01^{b,B}$	$2.26 \pm 0.02^{a,A}$
K8	$0.65 \pm 0.11^{a,b,B}$	$2.30 \pm 0.05^{a,A}$
K14	$0.69 \pm 0.19^{a,b,B}$	$0^{b,A}$
K21	$1.22 \pm 0.02^{a,b,B}$	$0^{b,A}$
K45	$0.74 \pm 0.32^{a,b,B}$	$0^{b,A}$
Supplemented	**Vitamin C**	**Vitamin B12**
J	2.9 ± 0.1^{c}	$0^{b,A}$
TJ	$2.87 \pm 0.35^{c,A}$	$0^{b,A}$
KJ1	$2.04 \pm 0.05^{c,A}$	$2.35 \pm 0.07^{a,A}$
KJ4	$4.15 \pm 0.01^{b,A}$	$2.40 \pm 0.01^{a,A}$
KJ8	$4.38 \pm 0.01^{b,A}$	$2.27 \pm 0.01^{a,A}$
KJ14	$6.72 \pm 0.70^{a,A}$	$2.30 \pm 0.01^{a,A}$
KJ21	$6.51 \pm 1.14^{a,A}$	$2.36 \pm 0.03^{a,A}$
KJ45	$7.12 \pm 0.31^{a,A}$	$2.29 \pm 0.01^{a,A}$

Letters indicate the significance of the data. Values with the same letter are not significantly different; values with different letters are significantly different. Small letters indicate significance in the same sample (K or KJ), but with different fermentation times, while capital letters indicate significance between K and KJ samples with the same fermentation times.

The KJ samples were significantly richer in vitamin C than the K samples and the T and TJ controls. After an initial decrease on day 1 (KJ1, 2.04 ± 0.05 mg/100 g), the quantity increased as the fermentation progressed, doubling by days 4 and 8 (4.15 ± 0.01 mg/100 mL and 4.38 ± 0.01 mg/100 mL, respectively), tripling by days 14 and 21 (6.72 ± 0.70 mg/100 mL and 6.51 ± 1.15 mg/100 mL, respectively), and reaching the maximum value of 7.11 ± 0.30 mg/100 mL on day 45, which was significantly higher than vitamin C content of KJ1 (**** $p < 0.0001$).

Vitamin B12 was absent in both control T and TJ, indicating that tea and jujube did not contain it but was biosynthesized by acetic acid bacteria during fermentation, as previously observed [37].

As shown in Table 2, with regard to the K samples, vitamin B12 was absent during the first three days of fermentation, and it reached the significant values of 2.26 ± 0.02 mg/100 mL on day 4 (**** $p < 0.0001$) and 2.30 ± 0.05 mg/100 mL on days 8, but disappeared in the following days of fermentation. With regard to the KJ samples, it formed on day 1 (KJ1, 2.35 ± 0.07 mg/100 mL) and remained unchanged till day 45.

3.5. Total Phenolic (TPC) and Flavonoid Content (TFC)

The analysis of TPC and TFC exhibited a fluctuating trend during the fermentation process in both the K and the KJ samples (Figures 3A and 3B, respectively). The control (T) showed good initial phenolic content of 588.4 ± 1.2 μg GAE/100 mL, but the addition of jujube powder to the starting tea led to a significant increase (**** $p < 0.0001$) in phenolic concentration by 1.7-fold (977.5 ± 2.0 μg GAE/100 mL). The results highlight that the phenolic content of KJ samples was higher than that of the corresponding K samples until day 45, at which point the phenolic content was almost the same for both samples (857.7 ± 0.9 μg GAE/100 mL for K45 and 872.9 ± 0.4 μg GAE/100 mL for KJ45). After 24 h of fermentation, the phenolic content of the K and KJ samples increased by 32% and 20%, respectively. However, the phenolic content of the K samples reached its lowest value on day 8 (719 ± 2 μg GAE/100 mL) and showed an increased on day 21. In contrast, the TPC value of the KJ1 sample decreased by only about 7% over the next 13 days and then

returned to the content of the KJ1 sample by day 21. The most significant values for samples K and KJ compared with T and TJ, respectively (**** $p < 0.0001$), were found after 1 day (864.2 ± 1.9 μg GAE/100 mL for K1 and 1215.8 ± 4.0 μg GAE/100 mL for KJ1) and 21 days of fermentation (970.6 ± 2.4 μg GAE/100 mL for K21 and 1210.5 ± 1.2 μg GAE/100 mL for KJ21).

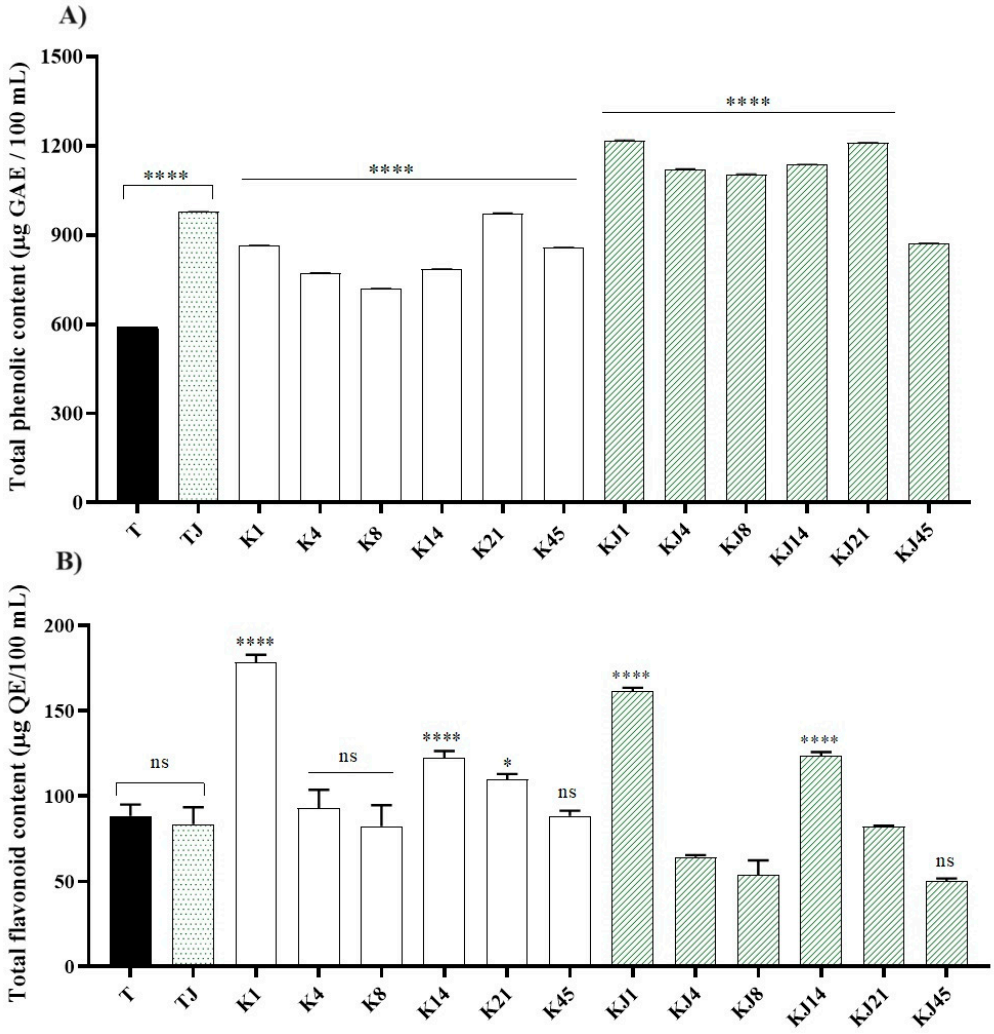

Figure 3. (**A**) Total phenolic content (TPC) is expressed as μg of gallic acid equivalents (GAE) per 100 mL of sample. (**B**) Total flavonoid content (TFC) is given as μg of quercetin equivalents (QE) per 100 mL of sample. The black bar represents the starting tea (T), the bar with green dots represents jujube-supplemented tea (TJ), the white bars represent traditional kombucha (K) samples, and the bars with green lines represent jujube-supplemented kombucha (KJ). The asterisks on the bars indicate the significance values of K (kombucha) and KJ (kombucha supplemented with jujube) versus the respective controls T (black tea) and TJ (black tea supplemented with jujube) (**** $p < 0.0001$, * $p < 0.05$; ns: not significant value).

The flavonoid concentration highlighted a floating trend during fermentation. In contrast to the results obtained for total phenolic content, it was evident that jujube supplementation did not improve the flavonoid content. However, the data collected show that the flavonoid content of the K and KJ samples compared to the T and TJ values, respectively, increased on the first day of fermentation by 50.5% and 48.3%. The flavonoid content of K1 and KJ1 dropped by 54% and 67%, respectively, after 8 days, increasing again on day 14.

The highest significant concentrations of flavonoids (**** $p < 0.0001$) were found after the first day of fermentation for both K1 (178.30 ± 4.60 μg QE/100 mL) and KJ1

(161.42 ± 2.10 μg QE/100 mL), with these values being twice as high as those of the samples before inoculation (88.3 ± 6.7 μg QE/100 mL for T and 82.5 ± 10 μg QE/100 mL for TJ). Significant flavonoid concentrations (**** $p < 0.0001$), compared with the corresponding controls for T and TJ were also found after 14 days of fermentation for both K14 (122.2 ± 4.2 μg QE/100 mL) and KJ14 (123.42 ± 2.40 μg QE/100 mL).

3.6. Identification and Quantification of Biocompounds by HPLC-DAD

HPLC analyses confirmed the presence in all samples of caffeine and four polyphenols, which were EGCG, ferulic and chlorogenic acids, and quercetin (Table 3). The data highlighted that the concentration of polyphenols during the 45 days changed in accordance with the variation in total phenols, with a higher content for each polyphenol in the KJ samples than in their traditional K counterparts, with the absolute highest concentration for KJ samples after 1 and 21 days of fermentation. The highest ECGC content was for KJ1 (3.12 ± 0.01 mg/100 mL), followed by KJ21 (2.97 ± 1.07 mg/100 mL), whereas in the K samples, the ECGC content reached values above 2.0 mg/100 mL only on day 14 and the maximum value in the K21 sample (2.56 ± 0.21 mg/100 mL). Chlorogenic acid was found to be significantly high (**** $p < 0.0001$) in jujube-enriched tea TJ (4.94 ± 1.06 mg/100 mL) before the fermentation process and in KJ1 (5.89 ± 0.01 mg/100 mL), the content of which was reduced by 50% after 4 days (2.91 ± 0.01 mg/100 mL) and remained unchanged until day 21 (3.01 ± 0.03 mg/100 mL). In the K samples, the content of chlorogenic acid did not change compared to that of the starting tea (2.89 ± 0.01 mg/100 mL) for the whole fermentation process.

Table 3. Content of biocompounds identified in the K an KJ samples, expressed as mg/100 mL.

Unsupplemented	**EGCG λ 280 nm**	**Chlorogenic Acid λ 327 nm**	**Caffeine λ 273 nm**	**Ferulic Acid λ 325 nm**	**Quercetin λ 365 nm**
T	1.89 ± 0.05 ^{a,A}	2.89 ± 0.01 ^{a,B}	280.18 ± 0.84 ^{a,B}	1.12 ± 0.03 ^{d,B}	2.31 ± 0.01 ^{a,A}
K1	1.88 ± 0.07 ^{a,B}	2.89 ± 0.01 ^{a,A}	160.20 ± 0.97 ^{c,B}	0.22 ± 0.02 ^{g,B}	2.73 ± 0.01 ^{a,A}
K4	1.80 ± 0.0 ^{a,A}	2.88 ± 0.38 ^{a,A}	178.10 ± 0.55 ^{b,B}	0.97 ± 0.04 ^{e,B}	2.30 ± 0.02 ^{a,A}
K8	1.98 ± 0.06 ^{a,A}	2.89 ± 0.02 ^{a,A}	97.30 ± 1.10 ^{f,B}	0.60 ± 0.03 ^{f,B}	2.35 ± 0.01 ^{a,A}
K14	2.17 ± 1.21 ^{a,A}	2.88 ± 0.01 ^{a,A}	127.48 ± 0.43 ^{d,B}	1.87 ± 0.07 ^{c,A}	2.46 ± 0.08 ^{a,A}
K21	2.56 ± 0.21 ^{a,A}	2.87 ± 0.01 ^{a,A}	146.14 ± 0.49 ^{d,A}	2.18 ± 0.07 ^{b,B}	2.37 ± 0.01 ^{a,A}
K45	2.17 ± 0.05 ^{a,A}	2.89 ± 0.01 ^{a,A}	66.94 ± 0.04 ^{g,A}	2.64 ± 0.04 ^{a,B}	2.32 ± 0.02 ^{a,A}
J-Supplemented	**EGCG λ 280 nm**	**Chlorogenic Acid λ 327 nm**	**Caffeine λ 273 nm**	**Ferulic Acid λ 325 nm**	**Quercetin λ 365 nm**
J	n.d. ^{b}	2.90 ± 0.01 ^{b}	270.97 ± 0.56 ^{c}	0.22 ± 0.01 ^{d}	n.d. ^{b}
TJ	2.36 ± 0.12 ^{a,A}	4.94 ± 1.06 ^{a,A}	338.27 ± 0.15 ^{a,A}	1.26 ± 0.02 ^{c,B}	2.31 ± 0.01 ^{a,A}
KJ1	3.12 ± 0.01 ^{a,A}	5.89 ± 0.01 ^{a,B}	300.02 ± 0.71 ^{b,A}	3.75 ± 0.07 ^{a,A}	2.74 ± 0.02 ^{a,A}
KJ4	2.26 ± 0.07 ^{a,A}	2.91 ± 0.0 ^{b,A}	171.92 ± 0.08 ^{d,A}	1.23 ± 0.01 ^{c,A}	2.04 ± 0.01 ^{a,A}
KJ8	2.87 ± 0.9 ^{a,A}	2.90 ± 0.06 ^{b,A}	139.43 ± 0.26 ^{f,A}	2.26 ± 0.02 ^{b,A}	2.02 ± 0.08 ^{a,A}
KJ14	2.82 ± 1.39 ^{a,A}	2.90 ± 0.05 ^{b,A}	140.60 ± 0.61 ^{e,A}	1.52 ± 0.06 ^{b,B}	2.59 ± 0.01 ^{a,A}
KJ21	2.97 ± 1.07 ^{a,A}	3.01 ± 0.03 ^{b,A}	101.97 ± 0.02 ^{g,B}	3.91 ± 0.01 ^{a,A}	2.39 ± 0.01 ^{a,A}
KJ45	2.67 ± 0.14 ^{a,A}	2.91 ± 0.02 ^{b,A}	29.3 ± 0.40 ^{h,B}	3.81 ± 0.01 ^{a,A}	2.09 ± 0.01 ^{a,A}

Letters indicate the significance of the data. Values with the same letter are not significantly different; values with different letters are significantly different. Small letters indicate significance in the same sample (K or KJ), but with different fermentation times, while capital letters indicate significance between K and KJ samples with the same fermentation times.

Ferulic acid, despite its low content in jujubes (0.22 ± 0.01 mg/100 mL), reached a value of 3.75 ± 0.07 mg/100 mL after just 1 day of fermentation in KJ1, which decreased to 1.23 ± 0.01 mg/100 mL after 4 days and increased to the highest value (3.91 ± 0.01 mg/100 mL, **** $p < 0.0001$) on day 21. In the K samples, the initial amount of 0.22 ± 0.02 mg/100 mL increased by 74.3% after 4 days (0.97 ± 0.04 mg/100 mL) and then doubled compared to the latter in K14 (1.87 ± 0.07 mg/100 mL, * $p < 0.05$), reaching the maximum value after 45 days (2.64 ± 0.04 mg/100 mL). The concentration of quercetin showed a decreasing trend in both

the K and the KJ sample after the first day of fermentation, accordingly to the TFC values: The highest values were obtained for samples K1 and KJ1 (2.74 ± 0.02 mg/100 mL). The concentration of caffeine which, was high in the starting tea (280.18 ± 0.84 mg/100 mL), was degraded by kombucha microorganisms, leading to a 43% reduction in K1 after the first day of fermentation (160.20 ± 0.97 mg/100 mL, **** $p < 0.0001$). Then, its content decreased by 58% by day 45 (66.94 ± 0.04 mg/100 mL), with a fluctuating trend. In the KJ samples, the initial caffeine content (TJ, 338.27 ± 0.15 mg/100 mL) was found to have degraded by 11.2% on day 1 (300.02 ± 0.71 mg/100 mL), by 49% by day 4 (171.92 ± 0.08 mg/100 mL), and finally by 91% by day 45 (29.3 ± 0.40 mg/100 mL).

3.7. Antioxidant Activity

The antioxidant activity of all samples was evaluated by performing three in vitro assays: ABTS, DPPH, and FRAP tests.

3.7.1. ABTS Assay

TEAC values against ABTS were reported in Table 4. The supplemented samples exhibited higher antioxidant activity than the unsupplemented ones, especially at the higher concentrations (30 and 20 µg/mL). At the concentrations of 30 and 20 µg/mL, the TEAC values of TJ (62.69 ± 0.94 µg TE/mL and 45.35 ± 5.14 µg TE/mL, respectively) were 1.74-and 1.55-fold higher than those of T (36.02 ± 1.69 µg TE/mL and 29.10 ± 4.05 µg TE/mL, respectively). The highest TEAC values were found for the K14 and KJ14 samples at all tested concentrations, with the KJ samples showing higher values than K ones. In order to determine which of the samples was most effective as an antioxidant against the cation radical ABTS$^{\bullet+}$, the EC_{50} values of samples required to scavenge 50% of the initial cation radical concentration were calculated, and the results are expressed as µg/mL (Table 5).

Table 4. Trolox equivalent antioxidant capacity (TEAC) values, expressed as µg TE/mL, against the cation radical ABTS$^{\bullet+}$ of black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (KJ) at different fermentation times.

	µg TE/mL						
µg/mL	**T**	**K1**	**K4**	**K8**	**K14**	**K21**	**K45**
30	36.02 ± 1.69	62.72 ± 3.88	43.15 ± 3.01	58.59 ± 0.53	61.75 ± 1.88	46.74 ± 1.41	54.40 ± 4.60
20	29.10 ± 4.05	47.10 ± 0.97	32.61 ± 2.96	48.72 ± 3.40	58.32 ± 1.21	38.15 ± 1.88	42.67 ± 1.40
10	20.14 ± 0.74	28.32 ± 0.62	15.25 ± 3.90	30.93 ± 0.70	39.88 ± 3.30	17.27 ± 2.33	26.67 ± 3.40
5	11.40 ± 2.40	17.50 ± 2.44	7.44 ± 1.84	12.75 ± 2.10	28.03 ± 0.69	19.26 ± 2.15	17.95 ± 0.76
µg/mL	**TJ**	**KJ1**	**KJ4**	**KJ8**	**KJ14**	**KJ21**	**KJ45**
30	62.69 ± 0.94	66.61 ± 1.08	61.16 ± 1.50	64.46 ± 2.89	66.32 ± 2.01	64.37 ± 1.30	56.02 ± 1.85
20	45.35 ± 5.14	45.72 ± 4.8	44.40 ± 0.18	54.43 ± 1.62	65.82 ± 1.50	59.51 ± 0.13	45.56 ± 0.28
10	25.82 ± 4.72	24.26 ± 4.30	25.14 ± 1.13	29.48 ± 0.74	41.73 ± 0.80	30.85 ± 0.27	33.22 ± 4.40
5	14.94 ± 1.11	7.44 ± 0.73	15.79 ± 0.27	10.34 ± 1.19	33.63 ± 0.05	16.99 ± 2.24	17.04 ± 3.00

EC_{50} values decreased as fermentation proceeded, reaching the minimum value, which corresponded to the maximum antioxidant capacity, on day 14 in both the KJ and the K samples (4.39 ± 0.63 µg/mL and 6.09 ± 0.77 µg/mL, respectively). It is worth pointing out that the EC_{50} value of KJ14 was 0.72-fold lower than that of K14. Moreover, the obtained EC_{50} values confirmed that, with the exception of the KJ1 sample, the KJ samples exerted a higher radical scavenging ability towards ABTS$^{\bullet+}$ than that of the K ones.

Table 5. EC_{50} values for ABTS, expressed as μg/mL, of black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (KJ) at different fermentation times.

	EC_{50} (μg/mL)	
Samples	**Unsupplemented**	**J-Supplemented**
Trolox	1.98 ± 0.25	
T	28.92 ± 1.45 ^{e,B}	11.84 ± 1.06 ^{d,A}
K1	10.64 ± 1.03 ^{b,A}	12.65 ± 1.10 ^{d,B}
K4	26.23 ± 1.42 ^{e,B}	12.27 ± 1.09 ^{e,d,A}
K8	11.25 ± 1.04 ^{b,B}	10.16 ± 1.01 ^{c,A}
K14	6.09 ± 0.77 ^{a,B}	4.39 ± 0.63 ^{a,A}
K21	18.50 ± 1.27 ^{d,B}	8.38 ± 0.91 ^{b,A}
K45	13.15 ± 1.12 ^{c,B}	11.05 ± 1.03 ^{d,A}

Letters indicate the significance of the data. Values with the same letter are not significantly different; values with different letters are significantly different. Small letters indicate significance in the same sample (K or KJ) but with different fermentation times, while capital letters indicate significance between the K and KJ samples, with the same fermentation times.

3.7.2. DPPH Assay

TEAC values against DPPH are reported in Table 6.

Table 6. Trolox equivalent antioxidant capacity (TEAC) values, expressed as μg TE/mL against the radical DPPH of black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (kJ) at different fermentation times.

	μg TE/mL						
μg/mL	**T**	**K1**	**K4**	**K8**	**K14**	**K21**	**K45**
100	21.88 ± 1.47	30.78 ± 1.46	30.57 ± 1.17	32.58 ± 2.17	31.23 ± 3.07	31.73 ± 3.06	28.93 ± 3.16
50	20.43 ± 2.94	27.81 ± 3.33	29.43 ± 2.26	26.53 ± 0.30	22.11 ± 2.01	28.11 ± 6.09	23.18 ± 0.87
25	6.43 ± 0.07	12.91 ± 4.38	20.43 ± 3.11	16.48 ± 4.04	16.88 ± 1.93	14.41 ± 1.94	8.23 ± 1.94
10	0	0	6.14 ± 0.01	0.18 ± 0.01	5.01 ± 1.10	3.38 ± 1.04	0
μg/mL	**TJ**	**KJ1**	**KJ4**	**KJ8**	**KJ14**	**KJ21**	**KJ45**
100	25.81 ± 1.72	31.98 ± 1.25	31.31 ± 1.84	32.48 ± 0.76	35.68 ± 3.79	31.18 ± 1.90	30.23 ± 0.94
50	22.61 ± 2.96	28.28 ± 2.71	30.81 ± 3.02	30.88 ± 4.45	29.73 ± 3.33	31.17 ± 0.65	25.31 ± 3.96
25	14.43 ± 4.06	16.33 ± 1.90	22.01 ± 1.81	15.23 ± 2.02	27.73 ± 1.66	20.73 ± 1.66	13.18 ± 1.65
10	1.01 ± 0.10	0	0	0.04 ± 0.01	13.43 ± 1.71	5.61 ± 0.95	0.23 ± 0.01

Unlike the results obtained for the radical cation $ABTS^{\bullet+}$, the results of the TEAC of the K and KJ samples for DPPH were comparable at concentrations of 100, 50, and 25 μg/mL, with the exception of KJ14, which showed TEAC values higher than those of K14 at all concentrations. The substantial difference of TEAC values between the K and KJ samples was found to be at the lowest concentration (10 μg/mL). The antioxidant effectiveness of the samples was evident from the EC_{50} values (Table 7), which, excluding the value relating to the 4th day of fermentation, appeared to be lower for the KJ samples, reaching the maximum inhibitory concentration on the 14th day of fermentation (8.19 ± 0.90 μg/mL).

Table 7. EC_{50} values for DPPH, expressed as μg/mL, of black tea (T), jujube-supplemented tea (TJ) traditional kombucha (K), and jujube-supplemented kombucha (KJ) at different fermentation times.

	EC_{50} (μg/mL)	
Samples	**Unsupplemented**	**J-Supplemented**
Trolox	22.95 ± 1.36	
T	46.09 ± 1.66 ^{g,B}	23.57 ± 1.36 ^{e,A}
K1	24.75 ± 1.39 ^{e,B}	20.69 ± 1.32 ^{d,A}
K4	14.56 ± 1.15 ^{a,A}	19.31 ± 1.29 ^{d,B}

Table 7. *Cont.*

Samples	EC_{50} (μg/mL)	
	Unsupplemented	J-Supplemented
K8	$23.13 \pm 1.35^{d,B}$	$17.27 \pm 1.24^{c,A}$
K14	$16.62 \pm 1.21^{b,B}$	$8.19 \pm 0.90^{a,A}$
K21	$19.59 \pm 1.28^{c,B}$	$13.91 \pm 1.13^{b,A}$
K45	$34.06 \pm 1.52^{f,B}$	$23.82 \pm 1.38^{e,A}$

Letters indicate the significance of the data. Values with the same letter are not significantly different; values with different letters are significantly different. Small letters indicate the significance in the same sample (K or KJ) but with different fermentation times, while capital letters indicate significance between the K and KJ samples.

3.7.3. FRAP Assay

All samples reduced Fe^{3+} ions to Fe^{2+} ions that were complexed by 2, 4, 6-tripyridyl-s-triazine (TPTZ) at a concentration of 33.3 μg/mL. FRAP values (Table 8), according to the TEAC values for both DPPH and ABTS, confirmed that the supplementation improved the antioxidant properties of the samples, ranging from 45.72 ± 0.40 μmol/mL $FeSO_4$ on day 45 to 87.42 ± 0.67 μmol/mL $FeSO_4$ on day 14. Similarly to the ABTS and DPPH assay fundings, the data show the same trend of reducing power over 45 days for both the K and the KJ samples; the lowest values were found for samples on days 1, 4, and 8, and then they gradually increased until day 14 (62.89 ± 0.67 μmol/mL $_{FeSO4}$ for K and 87.42 ± 0.67 μmol/mL $_{FeSO4}$ for KJ). FRAP values showed a decrease on day 21 and reached the lowest values after 45 days (29.48 ± 0.92 μmol/mL $_{FeSO4}$ for K45 and 45.72 ± 0.40 μmol/mL $_{FeSO4}$ for KJ45).

Table 8. FRAP values expressed as μmol/mL of black tea (T), jujube-supplemented tea (TJ), traditional kombucha (K), and jujube-supplemented kombucha (kJ) at different fermentation times.

Samples	$FeSO_4$ (μmol/mL)	
	Unsupplemented	J-Supplemented
BHT	$145.81 \pm 0.03^{a,A}$	
T	$115.43 \pm 3.74^{f,C}$	$125.53 \pm 0.93^{f,B}$
K1	$34.21 \pm 1.47^{d,C}$	$55.06 \pm 0.01^{d,B}$
K4	$35.47 \pm 0.47^{d,C}$	$56.13 \pm 0.32^{d,B}$
K8	$36.74 \pm 0.52^{d,C}$	$57.23 \pm 0.67^{d,B}$
K14	$62.89 \pm 0.67^{b,C}$	$87.42 \pm 0.67^{b,B}$
K21	$54.40 \pm 0.40^{c,C}$	$67.89 \pm 0.27^{c,B}$
K45	$29.48 \pm 0.92^{e,C}$	$45.72 \pm 0.40^{e,B}$

Letters indicate the significance of the data. Values with the same letter are not significantly different; values with different letters are significantly different. Small letters indicate significance in the same sample (K or KJ) but with different fermentation times, while capital letters indicate significance between the K and KJ samples.

4. Discussion

The jujube-enriched kombucha and the traditional kombucha were separately monitored during the whole fermentation process from day 0 to day 45, verifying the changes in content of organic acid, acetic acid, sugars, ethanol, polyphenols, vitamins C and B12, and antioxidant activity. The results obtained for the K and KJ samples with the same fermentation time were compared. Changes in chemical parameters, such as pH values for the KJ and K samples, were similar during all fermentation processes. It drastically dropped to a value of 2.9 ± 0.1 by day 45, ensuring the safety of the resulting beverages, since the pH values for both K and KJ fell within the safe range for human consumption. Kombucha, with pH values below 2.5, has a high concentration of acetic acid, which could be a risk to the health of consumers. Likewise, pH values above 4.2 do not guarantee the microbiological safety of the beverage. The pH reduction was due to the generation of some organic acids, such as acetic and gluconic acids, by the symbiotic microorganisms of the SCOBY during fermentation [38].

The protein content in K samples was highest on day 1 of fermentation, in agreement with the results obtained by Kaashyap et al. [39]. Since the Coomassie Brilliant Blue dye does not usually react with the proteins within microorganism cells, it was suggested that kombucha proteins consisted primarily of extracellular enzymes excreted by the bacteria and yeast into the liquid around them in order to break down larger molecules of different nutrients into smaller ones [40]. Then, they were able to cross the phospholipid membrane barrier to penetrate the cell. It should be noted that the protein content of the KJ samples on days 1, 14, and 21 was higher than of the corresponding K samples. Since the protein contribution was not ascribable to the supplemented jujube powder, as shown by the protein content of TJ, it is likely that the higher protein values in the KJ samples resulted from the presence of endogenous enzymes, including proteases, released by the microorganisms in the liquid, which increased proteolytic activity during the fermentation processes. Reyes-Flores et al. also reported that the addition of plant material such as hempseed improved the protein concentration of the fermented samples [41].

The analyses of sugar content highlight that the maximum concentration of sucrose was found in the T and TJ samples. Sucrose decreased during fermentation, achieving its lowest value on day 45 (2.9 $\pm$ 0.3 mg/100 mL for K45 and 1.2 $\pm$ 0.3 mg/100 mL for KJ45). The rate at which the sucrose concentration in the K and KJ samples decreased over time was different: On the first day of fermentation, the sucrose reduction in K1 was found to be 32%, and in KJ1 it was 39%. No additional disappearance of sucrose was observed in the K sample during the following 14 days, whereas in the KJ ones, a gradual decrease from the KJ1 content of 8.5% on day 4, 10% after another 4 days of fermentation, and 13% on day 14 was observed. In previous studies, sucrose was found to linearly disappear during the first week [42], while Chen and Liu found that sucrose linearly disappeared for up to four weeks of kombucha fermentation [42]., The rate at which the sucrose content of both the K and the KJ samples decreased during the first 24 h of fermentation was very high (32 and 40 g, respectively), whereas in the following 14 days it decreased in the KJ samples at a rate of 1.8 g/d until day 4, then by 1.1 g/d until day 8 and by 0.27 g/d in the next 7 days. From day 14, the rate of disappearance of sucrose in both samples was approximately 4 g/d. Over the next 24 days, the rate of sucrose disappearance decreased to values of 1.4 g/d in the K samples and 1.0 g/d in the KJ samples. The data show that the rate of carbohydrate consumption by the microorganisms became similar as the fermentation time increased until the sugar content was reduced to values below 2%. The same results were obtained by Tejedor-Calvo and Diego Morale [35].

The discrepancy between these velocity values is most likely related to the nature and abundance of kombucha microflora, in conformity with their respective invertase activities. This could be explained by the higher invertase activity in the KJ samples than in the K ones, resulting from partial or total elimination of invertase inhibitors by the jujube powder. Villarreal-Soto et al. [43] worked with green tea kombucha and presented sucrose consumption curves where sucrose content was reduced from 8% on day 0 to almost 0% after 21 days [43]. Once fermentation began, yeast and bacteria of the kombucha consortium utilized the available nutrients in different but complementary ways. At the initial stage of fermentation, yeasts hydrolyzed sucrose into glucose and fructose by invertase and produced ethanol via glycolysis and carbon dioxide. In this metabolic pathway, they preferred fructose as a substrate [44]. Instead, acetic acid bacteria made use of glucose to produce gluconic acid and ethanol to yield acetic acid. The metabolic activity of acetic acid bacteria has been reported to be lower than that of yeasts because the nutrient source of bacteria must initially be utilized and produced by yeasts [45].

In the K samples, glucose content increased to a maximum value of 27.3 $\pm$ 0.3 mg/100 mL on day 8, which halved over the next 14 days and then decreased to small amounts by day 45. In KJ, the starting glucose corresponded to that contained in the jujube powder and increased its content to a maximum value of 15.5 $\pm$ 0.3 mg/100 mL during the first 24 h of fermentation. It slowly decreased by 12% over the next 20 days of fermentation and then

dropped to 1.8 ± 0.2 mg/100 mL during the last 24 days. Fructose consumption exhibited the same trend as glucose in both the K and the KJ samples.

The concentrations of glucose and fructose in the K samples were higher than those in the KJ ones. It also appears that the consumption of glucose and fructose decreased with time, but the fructose content was lower than that of glucose during the whole fermentation process. This suggests that yeast cells preferred fructose as the carbon source [7].

In conjunction with yeast activity, acetic acid bacteria consumed glucose to produce acetic acid, which was one of the main metabolites, as well as being the predominant organic acid of kombucha beverages, representing 62% of the titratable acidity [46]. In addition to acetic acid, other organic acids are usually produced during the fermentation process, such as gluconic acid, glucuronic acid, malic acid, tartaric acid, lactic acid, succinic acid, and citric acid [47,48]. These organic acids exhibited antibacterial properties that inhibited the pathogenic population responsible for contaminating kombucha [49]. Acetic acid, which was absent in the first 24 h of fermentation in both the K and the KJ samples, increased with time to a maximum value of 3.0–3.1% on day 21 and then decreased in the following 24 days to 0.9%. Regarding the rate of production and consumption of acetic acid, no significant differences were found between the K and KJ samples. So, the addition of jujube powder to the starting tea did not result in significant changes in acetic acid production. Cardoso et al. found similar results for the acetic acid content produced in kombucha from black and green tea [4]. Our results are dissimilar to those reported by Chen and Liu [46] in a study where they found that the maximum concentration of acetic acid was 11 g/L after 30 days of fermentation.

The data obtained regarding ethanol production highlight that it was dependent on the availability of sugars and the length of the fermentation process. Since the initial amount of sugar added was the same for both the K and the KJ samples, the natural sugars in jujube led to the production of greater amounts of ethanol. In fact, the ethanol content was zero before inoculation, and increased with fermentation time, with a different trend in the K and KJ samples. In the K samples, the ethanol concentration reached the highest value of 1.0% on the 14th day of fermentation; in KJ samples, the ethanol concentration peaked at 1.4% on day 21. According to the study by Neffe-Skocińska et al., the ethanol concentration reached 0.78% on the seventh day and the highest value of 1.10% on day 10 [50]. Similar results were observed for kombucha enriched by the addition of fruit juices such as apples, pomegranates, red grapes, and sour cherry juices, in which the final ethanol concentrations were 0.82%, 0.84%, 0.59%, and 0.67% (v/v), respectively [51]. As the fermentation progressed, ethanol was converted to acetic acid, resulting in a decrease in ethanol by the end of the process. For commercial sale as a non-alcoholic beverage, the alcohol content of kombucha must remain below 0.5% alcohol by volume, but jujube-enriched kombucha at the end of the 45-day fermentation period was found to contain slightly more alcohol (0.6% v/v) and should therefore be labelled with a warning, as required by the Alcoholic Beverage Labelling Act of 1988 [52]. According to Food and Drug Administration (FDA) regulations, "hard kombucha" is known to have an alcohol content of around 3.5–5.5% v/v or higher [46].

Regarding the vitamin content, it is important to underline that kombucha beverage is naturally rich in water-soluble vitamins such as vitamin C and vitamins B1, B2, B6, and B12 [53]. In this study, vitamin C content was 0.25 ± 0.07 mg/100 mL in the control T, and during fermentation it increased to 1.32 ± 0.03 mg/100 mL on day 1 in the unsupplemented sample. Similarly, Lonar and others found the vitamin C content in kombucha was 1 mg per 100 mL [54]. Vitamin C content in the TJ control was equal to 2.9 ± 0.4 mg/100 mL due to the presence of vitamin C in jujube fruits. During fermentation, the vitamin C content of the KJ samples increased, achieving a maximum concentration of 7.11 ± 0.30 mg/100 mL on day 45. This content was higher than that obtained from kombucha fermentation of Olympus Mountain tea (*Sideritis scardica*) sweetened with honey, studied by Kartelias et al. [45]. They found the vitamin C concentration to be 3.6 ± 0.9 mg/100 mL on fourth day of fermentation. Malbaša et al. [55] studied the fermentation of black and green tea sweetened

with 7% of sucrose using a mixed culture of acetic bacteria and yeast as starter cultures. They reported that the maximum content of vitamin C was 2.99 mg/100 mL. It has been suggested that the vitamin C in kombucha derived from glucose metabolism is a result of the work of bacterial strains belonging to the *Gluconobacter* [56], and that gluconic acid, as a metabolite of AAB, is a precursor in the biosynthesis of natural vitamin C. Therefore, the vitamin C content from jujube powder added to black tea cannot be correlated with the increased production of vitamin C in the KJ samples compared to the K ones. Our results can be explained by the different substrates used. It is likely that jujube powder provided more nutrients to the acetic acid bacterium *Gluconobacter oxydans* for further biosynthesis of vitamin C [57]. Furthermore, the presence of the jujube biocompounds may protect vitamin C from the action of oxygen, which caused the degradation of ascorbic acid in the traditional kombucha. Similar trends were reported by Leonarski et al. for kombucha supplemented with acerola byproduct [58].

Previous studies [45,59] reported that the microbial consortium of SCOBY is able to synthesize not only vitamin C but also small amounts of B-complex vitamins, including vitamin B12. To date, there is no reliable source of vitamin B12 in the category of unfortified plant foods consumed by humans. So, kombucha represents the only source of vitamin B12 in the plant world to date. The intake of B12 in the plant-based diet known as veganism that is currently being adopted by more individuals is inadequate, and the consequences are very serious. That is why all individuals who consume a plant-based diet are advised to supplement, so kombucha could be a good natural vitamin B12 supplement for them.

Our study demonstrated that vitamin B12 was present in K samples on the eighth day of fermentation at a maximum concentration of 2.30 ± 0.05 mg/100 mL. In the KJ samples, vitamin B12 was present during the whole fermentation process in the same concentration as that of the K samples, which is a very good result since the acceptable daily intake of vitamin B12 is 2.4 micrograms. Thus, it should be noted that the addition of jujube powder to tea did not favor the increase in vitamin B12 content compared to that of the K samples, but it did guarantee its presence for long periods, contrary to what happened in the K samples. The mineral cobalt was present in tea in a concentration of 0.4 µg/100 mL, which would explain its probable inclusion in the biosynthesis of vitamin B12 [7]. On the other hand, the jujubes did not contain cobalt; therefore, the presence of vitamin B12 in the KJ samples up to day 45 of fermentation could be attributable to the protective action of vitamin C, which preserved its degradation.

The phenolic content in the K samples increased as the fermentation progressed, although with a fluctuating and non-linear trend, reaching the highest concentration values on days 1 and 21. A similar trend was observed by a study conducted by Zhao et al. [60] that revealed an increase in phenolics up to the 5th day of fermentation and a decreasing trend from day 5 to day 14, probably due to the oxidation of tea phenolics by oxygen free radicals released by microorganisms in low-pH conditions. The fluctuations trend of the phenolic content during fermentation may be associated with the metabolic activity of the microbial consortium of the SCOBY. The increase in the TPC value might be attributable to the enzymatic degradation in the acidic environment of polyphenol complexes from raw materials into smaller polyphenol monomers in the tea broth [60,61]. In addition, the study by Blanc suggested that enzymes such as phytases, released by the kombucha microbial culture, could break down into phenolic compounds the floating cellulose produced by a member of AAB, which is *Komagataeibacter xylinus* [62]. The microbial community of the kombucha SCOBY also released hydrolytic enzymes that degraded the polyphenols into other metabolites, and they used them as nutrient sources for metabolic activities, leading to a reduction in the TPC [63]. The total phenolic content in the KJ samples was significantly higher in comparison to the K ones (**** $p < 0.0001$). Thirteen flavonols, 10 flavan-3-ols, 1 flavanone, and 1 dihydrochalcone were identified in previous studies [64–66]. The phenolic content in TJ before the beginning of fermentation was 1.7 times higher than that of T due to the high phenolic content in the jujube fruits. Then, the contribution of phenolics from the jujubes to the KJ samples would explain the significant increases in TPC.

Similar results were reported in a study carried out on sweetened lemon balm (*Melissa officinalis* L.) infusion as the medium for kombucha fermentation; total phenols in lemon balm fermentation broth were higher by about 200 μg of GAE per mL than in the traditional beverage during the whole fermentation process [67].

Total flavonoid content achieved the maximum concentration after the first day of fermentation in both the K and the KJ samples. These data confirm the results obtained by Wang et al. [38] in a study where the flavonoid content largely increased in kombucha after 24 h of fermentation [38]. However, the flavonoid concentration decreased during fermentation in both the K and the KJ samples, showing significantly higher values for the unfortified samples (** $p < 0.01$). Similar results were found by Gaggia et al. [68] in a recent study, where total flavonoid content was 20% lower in K samples prepared using tea and an infusion of rooibos leaves (*Aspalathus linearis*) than in the corresponding kombucha beverages [68]. It was hypothesized that flavonoids undergo degradation by enzymes produced by kombucha microorganisms, which cause the C-ring of flavonoids to break down, leading to phenolic acids [69]. The result of these metabolic processes in flavonoids led to an increase in phenolic content and a concomitant decrease in flavonoids, which were found to be slightly lower in the KJ samples. Major biocompounds of all samples, identified and quantified by HPLC analysis, were ECGC, chlorogenic and ferulic acids, quercetin, and caffein. The four polyphenolic compounds belong to the different classes of polyphenols. Epigallocatechin-3-gallate is the most abundant catechin in tea, particularly in green tea. Chlorogenic acid is the ester obtained from the combination of caffeic acid, which is a hydroxycinnamic acid, with (L)-quinic acid. Ferulic acid belongs to the hydroxycinnamic acid group. Quercetin is a flavonoid belonging to the flavonol group. Caffeine is a methylxanthine alkaloid found in the seeds, fruits, nuts, and leaves of a great variety of plants, such as tea leaves, and helps to protect them against herbivores. The initial content of caffein in both tea and jujube infusion significantly decreased (**** $p < 0.0001$) during fermentation in both the K and the KJ samples due to the bacterial cellulose synthesis stimulated by caffein moieties. A significant decrease in caffeine content in kombucha tea was also observed in previous studies [61,70].

It was found that catechins in the tea during kombucha fermentation were degraded. In the K samples, the ECGC was stable up to the 14th day of fermentation and showed an increase on the 21st day, probably due to a release of catechins from the acid-sensitive microbial cells. Javalan et al. reported a marked increase of kombucha catechins on day 12 [71]. Degradation of EGCG was more evident in the KJ samples than in the K samples from day one up to day nine. The high number of hydroxyl groups made them very effective in scavenging free radicals. The decrease in the amount of ferulic and chlorogenic acids from the absolute highest values reached after 24 h of fermentation in both the K and the KJ samples did not correlate with the antioxidant capacities exhibited by the fermented samples.

It has been reported that phenolics, flavonoids, and beneficial metabolites such as organic acids, vitamins, minerals, and enzymes generated during fermentation through the metabolic activity of the microbial community in a SCOBY make vital contributions to the antioxidant properties of kombucha beverages [72].

Different antioxidants react with free radicals through different mechanisms, such as binding pro-oxidant metals, scavenging free radicals, and inhibiting pro-oxidant enzymes. Therefore, various methods have been used to evaluate antioxidants, depending on their reaction mechanisms [73].

In this study, DPPH, ABTS, and FRAP tests were used to evaluate antioxidant activity. Data recovered from the three assays highlight that the fermentation process caused a significant increase in the antioxidant properties of the samples [68]. Also, the addition of jujube powder to the black teas improved the antioxidant activity of the resulting kombucha at all concentrations. Since the jujube contained several potential antioxidants, including polyphenols, minerals, and vitamins, as well as other beneficial substances that promoted the growth and metabolic activity of the microbial community in the kombucha SCOBY,

those compounds and the microbial metabolites generated during fermentation may have contributed to the total antioxidant activity of the samples. The minimum value of EC_{50} for both ABTS and DPPH was found for both the KJ14 and the K14 samples, indicating that both the K and the KJ samples reached maximum antioxidant capacity after 14 days of fermentation. These results are in disagreement with the total phenolic content, which exhibited the highest value on day 21. However, it is evident that antioxidants in all samples were less efficient against the DPPH radical than against the $ABTS^+$ one. In any case, it is well known that the DDPH assay was sensitive to the acidic environment, unlike the ABTS method, which showed wider sensitivity [74].

Also, the results of the FRAP assay indicate that the K and the KJ samples reached maximum antioxidant capacity after 14 days, but with higher FRAP values for the supplemented samples.

According to our results, Zubaidah et al. showed that sugared snake fruit juices fermented with a SCOBY reached the maximum antioxidant activity after 14 days of fermentation [75].

However, there was no correlation between total phenolic content of the K and the KJ samples and their antioxidant capacity, meaning that the samples with the highest radical scavenging capacity (K14 and KJ14) did not coincide with those with the highest TPC values (K1 and KJ1), as would be expected. Our results are in accordance with the study by Gamboa-Gomez and colleagues, who similarly did not find any correlation between the phenolic content and DPPH values [76].

A similar trend was found in a recent study, where there was no correspondence between the high phenolic content and antioxidant activity, which was lower than expected [76]. However, the total phenol content did not always determine the antioxidant activity of kombucha, whereas the different types of metabolites produced during fermentation might have produced the key effect. It is noteworthy that the KJ samples exhibited significantly lower EC_{50} values for both DPPH and ABTS than non-fortified ones, especially on days 14 and 21, which also corresponded with the highest values of vitamin C content in the KJ samples. These findings could explain the greater antioxidant efficacy of the fortified samples even after 21 days of fermentation, although there was a decrease in total phenolic content.

5. Conclusions

In this study, simultaneous comparison of traditional and alternative kombucha enriched with jujube powder (*Ziziphus jujuba* Mill.) after a fermentation time of 45 days was performed. Changes in antioxidant capacity, pH, protein, sugar, and ethanol, phenolic, flavonoid, and vitamins C and B12 content were investigated. The results show no significant differences in pH values of supplemented and non-supplemented samples (KJ and K, respectively) and a slight decrease in flavonoid content in jujube-enriched samples. Nevertheless, it was possible to observe how the addition of jujube powder led to a novel beverage characterized by significantly higher values of protein, phenols, and especially vitamin C, compared with traditional kombucha. The protein content in the KJ samples was 1.3-fold higher than its non-supplemented counterpart already after 1 day of fermentation as well after 21 days. The concentration of phenols in the KJ samples was 30% higher than in the K samples on days 1 and 21 and 35% higher on day 14. The DPPH, ABTS, and FRAP assays showed higher antioxidant capacity than the unsupplemented samples, with the highest activity on day 14. Supplemented samples were significantly richer in vitamin C than the unsupplemented samples throughout the whole process, reaching the maximum value (7.11 ± 0.30 mg/100 mL) on day 45. The most interesting result deriving from the supplementation was the data collected during the fermentation about the production of vitamin B12, which, although not significantly high, remained constant during the 45 days of fermentation, in contrast to the traditional kombucha, where vitamin B12 was present only from day 8 to day 14. This interesting result is probably due to the action of vitamin C, which increased progressively in the supplemented samples, preserving

vitamin B12 from degradation. These results offer the opportunity to explore jujube as a raw material for the supplementation of kombucha in order to prepare a novel beverage with an improved nutritional profile, depending on consumer needs. This study provides the basis for future investigations into the potential functional and biological properties of jujube-enriched kombucha.

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