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Evaluating the Effect of Adding Selected Herbs, Spices, and Fruits to Fermented Olympus Mountain Tea (*Sideritis scardica*) Kombucha Sweetened with Thyme Honey: Assessment of Physicochemical and Functional Properties

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Abstract: This study examined the effects of adding herbs, spices, and fruits into fermented Olympus Mountain tea (*Sideritis scardica*) kombucha using thyme honey as a sweetener. This study evaluated how these additions affected the tea's physical, chemical, and functional characteristics. Two different enrichments were proposed: a "Golden Mountain tea and honey Kombucha" (KG) with fresh ginger, turmeric powder, and lemon zest and juice and a "Red Mountain tea and honey Kombucha" (KR) with dried hibiscus calyces, rose petals, and lavender blossoms. In KR, the levels of vitamin C increased from 33.2 ± 2.7 to 48.4 ± 4.5. Additionally, the levels of calcium increased from 31.0 ± 1.2 to 55.7 ± 1.2, while the levels of potassium practically doubled from 64.7 ± 0.6 to 115.7 ± 2.5. An increased potassium concentration was observed in KG, and ionic iron was found for the first time after both enrichments. The total phenolic and flavonoid contents, along with antioxidant capacity, as assessed by the ABTS and DPPH methods, were found to be substantially enhanced in KR. In KG, the total phenolic content increased, together with antioxidant activity, as assessed by ABTS. Enrichment with hibiscus calyces, rose petals, and lavender blossoms significantly increased inhibitory effects against α-amylase, α-glucosidase, acetylcholinesterase, and butyrylcholinesterase. On the other hand, enrichment with ginger, turmeric, and lemon zest and juice decreased inhibitory effects against α-glucosidase and increased those against α-amylase, acetylcholinesterase, and butyrylcholinesterase. KR had the strongest enzyme-inhibiting activity, with its α-glucosidase-inhibiting activity increased by approximately 18 times. Therefore, enrichment with selected herbs, spices, and fruits can transform fermented Olympus Mountain tea kombucha sweetened with honey into a novel beverage with enhanced functional properties.

Keywords: kombucha; *Sideritis scardica*; honey; functional beverages; hibiscus; rose petals; enzyme inhibition; vitamin C; antioxidants; medicinal plants

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1. Introduction

The functional beverage market has increased steadily over the past decade. Although functional beverages are readily accessible, there remains a necessity to investigate novel mixtures of ingredients and techniques to augment bioactivity in consumer goods. Studies have characterized kombucha as a functional beverage with numerous bioactive properties [1]. Herbs, spices, and fruits make an excellent choice for producing beverages with functional properties. According to a recent study [2], the use of medicinal herbs may enhance the recognition of kombucha as a functional beverage product. Herbs and spices are exceptional sources of phenolic compounds, ascorbic acid, and carotenoids, which have shown high antioxidant activity [3–6].

Hibiscus sabdariffa is used in foods, herbal drinks, and medicine. In vitro [7] and in vivo [8] studies revealed hibiscus calyces' antioxidant, hepatoprotective, and anti-diabetic activities [9]. Among 30 medicinal plant teas examined, tea made from rose blossoms was shown to have one of the most potent antioxidant activities [10], but also anti-inflammatory, analgesic [11], antibacterial [12,13], antiviral [14], and antifungal effects [12]. Lavender blossoms are a good source of phenolic compounds and anthocyanins [15]. Ginger has traditionally been ingested as a seasoning and botanical remedy for an extended period [16]. It has been discovered that ginger exhibits a variety of biological activities, including antioxidant properties [17] and anti-inflammatory [18], antimicrobial [19], and anticancer activities [20]. In addition, studies have indicated that ginger may have beneficial properties for preventing and treating various illnesses, such as neurodegenerative diseases [21], cardiovascular diseases [22], obesity [23], diabetes mellitus [24], chemotherapy-induced nausea and emesis [25], and respiratory disorders [26]. Lemon zest contains a high concentration of phenolic compounds [27,28]. Turmeric has been used as a dye and spice, and its good antioxidant activity has previously been demonstrated [29].

Based on the above, we aimed to enrich fermented Olympus Mountain tea kombucha with honey with herbs, spices, and fruits to produce two novel beverages with upgraded functional properties. Two proposals, designated with colors, were given for the enrichment of fermented Olympus Mountain tea kombucha sweetened with honey: a "Golden Mountain tea and honey Kombucha" (KG), seeking to impart a fruity aroma and taste from lemon and ginger and a deeper shade of yellow from turmeric, and a "Red Mountain tea with honey Kombucha" (KR), aiming for a floral character by enriching it with rose petals and lavender blossoms and for a red color from hibiscus calyces.

2. Materials and Methods

2.1. Materials

Greek organic *Sideritis scardica* and hibiscus calyces originating from Egypt were purchased from a local market in Athens (www.evripidou.gr, accessed on 15 September 2023, Athens, Greece). Greek organic lavender blossoms were provided by a local market in Crete (www.bachari.gr, accessed on 15 September 2023, Heraklion, Greece). Greek red rose petals were purchased from a local market in Veria (www.Herbstore.gr, accessed on 15 September 2023, Veria, Greece). Thyme honey (Lemnian Land, 2022) was willingly provided by a local beekeeping cooperative (Myrina, Greece). An organic kombucha culture was acquired from a company located in Cumbria (UK). Tap water underwent filtration using an AQUA-PURE filter (3M Hellas MEPE, Maroussi, Greece). All chemicals and enzymes used in this study were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of the Beverages

After the preparation of a fermented Olympus Mountain tea kombucha with honey (K) in three replications (A, B, C) as previously described [30], herbs, spices, and fruit were added (Figure 1). The aim was not to overpower the aroma of kombucha with Mountain tea and honey completely, so the maximum quantity of herbs, spices, and fruits for enrichment was set at 0.5% *w*/*w* (equal to the amount of Mountain tea added).

The proposed recipe for KG was 0.3% fresh ginger, 0.15% lemon zest, 0.04% lemon juice, and 0.01% ground turmeric, and for KR, it was 0.25% ground hibiscus calyces, 0.2% rose petals, and 0.05% lavender blossoms. After the addition of these ingredients, the drinks were bottled in the refrigerator for 48 h. Then, filtration and re-bottling were performed (AG, BG, and CG samples for KG; AR, BR, and CR samples for KR).

2.3. Sampling

After preparing the beverage samples K, KG, and KR, three replicates of each sample (A, B, C, AG, BG, CG, AR, BR, CR) were carried out (Table 1).

An analysis was conducted on the beverages before and after enrichment to determine the pH value; titratable acidity; color; content of sugars, ethanol, organic acids, minerals,

vitamin C, total phenolics, and total flavonoids; α-amylase and α-glucosidase inhibition activities; anticholinesterase activity; and antioxidant activity. Before analysis, the kombucha was centrifuged at 5000× *g* for 10 min, and the resultant supernatant was kept at −40 °C until further examination.

Figure 1. Fermented Olympus Mountain tea kombucha and honey after the addition of herbs, spices, and fruit.

Table 1. A sampling of studied kombucha beverages.

Replication	K	KG	KR
1	A	AG	AR
2	B	BG	BR
3	C	BG	CR

2.4. Analysis of Color

The color of the beverages was determined using a Lovibond LC 100 spectropolarimeter with the SV 100 Kit (Tintometer® Group, Amesbury, England). The device recorded L (lightness), a (redness), and b (yellowness) values. The total color difference was calculated using the equation $\Delta E = \sqrt{(\Delta L)^2 + (\Delta \alpha)^2 + (\Delta b)^2}$. The distinction in color between the samples pre- and post-enrichment can be categorized as not obvious (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), clearly visible (3.0–6.0), or great (>6.0) based on the value of ΔE [31].

2.5. Determination of Active and Titratable Acidities

The pH values were determined using an electronic pH meter calibrated at pH 4.0 and 7.0, (Consort C931, Consort bvba, Turnhout, Belgium). CO_2 was removed from the fermentation broth to determine the titratable acidity [32]. Then, a 20-mL sample was taken and titrated with NaOH 0.1 mol/L. The resulting titratable acidity levels were expressed in g/L of acetic acid in each sample.

2.6. Determination of Sugars, Ethanol, Organic Acids, and Minerals

Ethanol was quantified using the K-ETOH enzymatic kit (Megazyme, International Ireland Limited, Ayr, Scotland, U.K.), which has been validated for use in kombucha fermented drinks. In summary, ethanol undergoes oxidation to become acetaldehyde by the action of alcohol dehydrogenase. Subsequently, acetaldehyde is further oxidized by acetaldehyde dehydrogenase, resulting in the production of NADH, which is ultimately measured.

The contents of sugars, organic acids, and minerals were determined using enzymatic kits and an enzymatic analyzer (Miura One, TDI, Barcelona, Spain).

2.7. Vitamin C

Vitamin C was assessed using a photometric technique, as previously described [33], with some modifications. Concisely, a total of 1.0 mL of the beverage was sampled both before and after the enrichment process in 3.0% metaphosphoric acid, and these samples were then pooled with 9.0 mL of oxalic acid in EDTA, 2.0 mL of 50% sulfuric acid, and 4.0 mL of 5.0% ammonium molybdate and mixed thoroughly. Absorbance was measured at λ = 705 nm at room temperature for 3.0 min using a spectrophotometer (Lambda 25, Perkin Elmer, Norwalk, CT, USA). Vitamin C was used as a calibration standard; the results were expressed in mg/L.

2.8. Content of Total Phenolics

The Folin–Ciocalteu technique [34] was employed to determine the total phenolic content. Gallic acid served as a calibration standard, and the outcomes were quantified as micrograms of gallic acid equivalents (GAE) per milliliter.

2.9. Content of Total Flavonoids

Flavonoid content was estimated using a colorimetric assay [35]. Rutin was employed as a reference standard for calibration purposes, and the outcomes were quantified in terms of micrograms of rutin equivalents (RE) per milliliter.

2.10. Determination of Antioxidant Activity

The antioxidant capacity of kombucha beverages was evaluated by ABTS and DPPH assays. The DPPH assay was conducted according to the methodology described by Kwon et al. [36], while the ABTS assay followed Xia et al.'s [37] protocol. The inhibition of $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ was evaluated using the formula % inhibition activity = [(Abs control − Abs sample)/Abs control] × 100, followed by plotting the percentage of inhibition against the sample amount in µL to determine the amount for 50% inhibition (IC_{50}).

2.11. α-Glucosidase and α-Amylase Inhibition Assays

2.11.1. α-Glucosidase Inhibition Assay

The α-glucosidase inhibition evaluation was carried out using the methodology described by Kwon et al. [36] with some modifications. At first, 15.0 and 20.0 µL of the samples were mixed with 0.1 M phosphate buffer (pH 6.9, 700 µL) containing 10 µL of α-glucosidase solution (33.3 U/mL). The mixture was then incubated at 37 °C for 10 min. Next, 50.0 µL of a 5 mM solution of p-nitrophenyl-α-D-glucopyranoside in 0.1 M phosphate buffer (pH 6.9) was added to each well. The mixtures were finally incubated at 25 °C for 5 min, followed by absorbance measurement at 405 nm (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). The % inhibition was evaluated using the following equation: % inhibition = [(Abs reference − Abs sample)/Abs reference] × 100. The IC_{50} was estimated for each sample using a linear regression method with the data from the % inhibition results.

2.11.2. α-Amylase Inhibition Assay

The Caraway–Somogyi iodine/potassium iodide (IKI) method was used to determine the α-amylase inhibitory activity, as previously described [38], with some modifications. Firstly, a 200–250 µL sample solution was mixed with 1000 µL α-amylase solution, made in phosphate buffer with a pH value of 6.9 in 6.0 mM sodium chloride. The mixture was then subjected to an incubation period of 10 min at 37 °C. After this, the reaction was initiated by adding 500 µL of starch solution, 0.5% *w*/*v* in concentration. A blank was also prepared by adding a sample solution to all the reaction reagents except for the enzyme (α-amylase) solution. The reaction mixture was incubated for an additional 10.0 min at 37 °C, and then 250 µL of 1.0 M HCl was added to terminate the reaction. Next, an iodine–potassium iodide solution of 1000 µL was added. The sample and the blank absorbances were read at 630 nm (Lambda 25, Perkin Elmer, Norwalk, CT, USA). First, the α-amylase inhibitory activity was

expressed as a percentage of inhibition by dividing the sample absorbance by the blank absorbance. % inhibition = [(Abs reference − Abs sample)/Abs reference] × 100. The IC_{50} values were determined by fitting a straight line (linear regression) to the data obtained from the percentage inhibition values.

2.12. Anticholinesterase Assays: Ache and Bche Inhibition

A spectrophotometric method [39] with some modifications was used to determine acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities. In summary, a total of 1500 µL of 0.1 mM sodium phosphate buffer with a pH of 8.0, 50 µL of DTNB, 200 µL of the sample, and 25.0 µL of enzyme solution were combined and allowed to react for 15.0 min at a temperature of 25.0 degrees Celsius. Subsequently, 10.0 µL of either ACTHI or BCTHI was introduced. The final blend was incubated at a temperature of 25 °C for 25.0 min, and the absorbance was recorded at a wavelength of 412 nm. A blank was prepared and measured without extract. The enzyme activity inhibition percentage was calculated as follows: % inhibition = 100 × (Abs blank − Abs sample)/Abs blank. The final cholinesterase activity was presented in terms of the IC_{50} values, expressed as the mean ± standard deviation of three replicate values.

2.13. Statistical Analysis

All results were presented as the mean ± standard deviation (SD) of three replicates. The level of statistical significance among the means was analyzed using one-way ANOVA, an independent sample test, using SPSS. The correlation coefficients developed by Pearson and Spearman were used to assess correlations. The confidence limits were established with a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Macroscopic Changes Due to the Addition of Herbs, Spices, and Fruits: Color Changes

Minutes after the addition of the hibiscus calyces, the rose petals, and the lavender blossoms, a bright red color began to spread into the broth. Adding ginger, curcumin, lemon zest, and juice made the color difference less apparent macroscopically but noticeable with deeper shades of yellow. The outcomes of the alterations in the chromatic parameter validated the visual assessment and are shown in Table 2. The ΔE values of fermented Olympus Mountain tea kombucha with honey (K) and KR exceeded 6, indicating a significant degree of color variation. Moreover, the chromatic parameter a (redness) significantly increased ($p < 0.05$). The red color is attributed to delphinidin, an anthocyanidin abundant in hibiscus calyces that appears red in an acidic environment [40]. As pH increases, this primary plant pigment changes from red to purple, blue, and bluish green. The ΔE values of K and KG were 1.84, indicating a "noticeable" color difference. Furthermore, the chromatic parameter b (yellowness) showed an increase.

Table 2. Chromatic parameters of K before and after enrichment (KR, KG).

Chromatic Parameter	K	KR	KG
L	66.5 ± 0.1^{a}	41.8 ± 0.3^{b}	67.3 ± 0.1^{c}
a	-4.1 ± 0.2^{d}	39.1 ± 0.4^{c}	-4.8 ± 0.1^{d}
b	22.0 ± 0.3^{e}	23.7 ± 0.1^{f}	23.5 ± 0.1^{g}

Different letters in each row indicate a significant difference ($p < 0.05$).

3.2. PH and TA

The changes in pH and titratable acidity between K (samples A, B, and C), KR (samples AR, BR, and CR), and KG (AG, BG, and CG) are shown in Table 3. Each value was obtained from three observations. The pH values decreased significantly ($p < 0.05$) after the enrichment with herbs, roots, and fruits.

Table 3. Values of pH and titratable acidity of K, KG, and KR.

Sample	pH	TA (Acetic Acid g/L)
A	3.06 ± 0.02	4.45 ± 0.02
AG	3.00 ± 0.01	4.68 ± 0.01
AR	2.96 ± 0.01	4.71 ± 0.02
B	3.02 ± 0.01	4.69 ± 0.04
BG	2.96 ± 0.02	4.86 ± 0.02
BR	2.94 ± 0.01	5.10 ± 0.04
C	2.96 ± 0.03	4.24 ± 0.05
CG	2.87 ± 0.01	4.35 ± 0.02
CR	2.85 ± 0.01	4.42 ± 0.01

Data for each sample represents the mean value ± standard deviation of three measurements.

The pH readings fall within the acceptable range of 2.5 to 4.2 for human consumption [41].

Following the enrichment, a statistically significant rise in the titratable acidity ($p < 0.05$) was detected in both KG (AG, BG, CG) and KR (AR, BR, CR). TA average values expressed in acetic acid (g/L). The decline in pH levels and the rise in acidity are assumed to be more due to the enrichment than the fermentation because the enrichment occurred in the fridge (temperature < 6.0 °C) and the fermentation slows down or stops at low temperatures. More specifically, organic and phenolic acids in herbs, spices, and fruits are infused into the broth after their addition. The hibiscus calyx extract contains many organic acids, including hibiscus acid as a major compound and oxalic and ascorbic acid as minor compounds [40]. The ascorbic acid content in calyces varies greatly between fresh (6.7–14 mg/100 g) and dried calyces (260–280 mg/100 g) [42]. Major organic acids in hibiscus extract are hydroxycitric acid, hibiscus acid, and their derivatives found in leaves and calyces [9,43–47]. In addition, hibiscus calyces contain chlorogenic acid, protocatechuic acid, and pelargonic acid, amongst others [40,48]. Free gallic acid was found in teas prepared from various rose cultivars [49]. In the study presented in [50], large amounts of phenolic acids were demonstrated in rose petal extracts. More specifically, large amounts of gallic acid (9.55 mg to 1.00 $\times 10^3$ mg of dry extract) were followed by smaller quantities of protocatechuic, gentisic, and coumaric acids. Organic acids are present in ginger. In addition, lemon fruits contain several nutrients, such as citric acid and ascorbic acid [51].

3.3. Changes in Sugars, Ethanol, Organic Acids, Minerals, and Vitamin C

3.3.1. Sugars

The total sugar content remained unchanged at 42 ± 0.0 g/L in all samples as the enrichment occurred at fridge temperatures, indicating that the fermentation stopped or slowed down (Table 4).

Table 4. Content of total sugars (glucose and fructose) in beverage samples.

Sample	Glucose (g/L)	Fructose (g/L)
A	20.4 ± 0.7	17.1 ± 0.8
AG	19.8 ± 0.9	16.5 ± 0.9
AR	20.1 ± 0.6	16.8 ± 0.9
B	20.1 ± 0.8	18.6 ± 1
BG	20.1 ± 0.6	18.3 ± 0.9
BR	20.1 ± 0.7	18.6 ± 0.8
C	18.6 ± 0.7	21 ± 0.9
CG	18 ± 0.9	19.5 ± 0.9
CR	18.3 ± 0.8	19.4 ± 1

The glucose values were 20.4 ± 0.7, 20.1 ± 0.8, and 18.6 ± 0.7 g/L for samples A, B, and C, respectively. After the enrichment with hibiscus calyces, rose petals, and lavender blossoms, no significant changes were observed in the values (20.1 ± 0.6, 20.1 ± 0.7, and

18.3 ± 0.8 for AR, BR, and CR, respectively). No significant changes were observed in the values after the enrichment with ginger, turmeric, lemon zest, and juice (19.8 ± 0.9, 20.1 ± 0.6, and 18 ± 0.9 for AG, BG, and CG, respectively).

The fructose values were 17.1 ± 0.8 and 18.6 ± 1 g/L for A and B and 21.0 ± 0.9 g/L for C. After the enrichment with hibiscus calyces, rose petals, and lavender blossoms, no significant changes were observed in the values (16.8 ± 0.9, 18.6 ± 0.8, and 19.4 ± 1 for AR, BR, and CR, respectively). No significant changes were observed in the values after the enrichment with ginger, turmeric, lemon zest, and juice (16.5 ± 0.9, 18.3 ± 0.9, and 19.5 ± 0.9 for AG, BG, and CG, respectively).

3.3.2. Acids

No significant differences ($p > 0.05$) were observed in the acetic and gluconic acid values after enrichment. The concentration of citric acid significantly increased in KG, from an average of 0.04 ± 0 g/L in K to 0.66 ± 0.05 g/L. The presence of lemon zest and juice in KG explains the above. Citric acid is one of the nutrients in lemon fruits, as noted by [51].

3.3.3. Ethanol

The ethanol content showed no statistically significant changes ($p > 0.05$). The ethanol values of the beverage after the fermentation and enrichment were less than 1.0% *v*/*v*.

3.3.4. Minerals

The analysis of trace elements in K detected the presence of calcium and potassium quantities of 31.0 ± 1.2 g/L and 64.0 ± 2.2 g/L, respectively, which almost doubled (55.7 ± 1.2 g/L and 115.7 ± 2.5 g/L) after the enrichment with hibiscus, rose petals, and lavender blossoms. An increase in potassium concentration (72.3 ± 1.5 g/L) was also observed after the enrichment with ginger, turmeric, lemon zest, and juice. Finally, iron was found for the first time after the enrichments in KG (0.13 ± 0.05 g/L) and KR (0.10 ± 0.04 g/L).

The above can be explained by the concentration of minerals in the herbs, spices, and fruits added to the kombucha fermented beverage. For example, hibiscus calyces are rich in calcium, potassium, and iron [42,52]. Moreover, rose petals contain potassium, calcium, and iron, amongst other minerals [53]. In another study [54], potassium and iron were present in appreciable amounts in ginger and turmeric. Potassium has also been detected in lemon peels [55].

3.3.5. Vitamin C

An increase in the vitamin C content that is statistically significant ($p < 0.05$) was detected after the addition of hibiscus calyces, rose petals, and lavender blossoms. The average vitamin C content increased from 33.22 ± 2.67 mg/L in K to 48.39 ± 4.55 mg/L in KR. On the other hand, after the addition of ginger, curcumin, lemon zest, and juice, the vitamin C concentration did not exhibit a statistically significant variation ($p > 0.05$). The richness of vitamin C in the infused hibiscus calyces can explain the significant increase in vitamin C content in KR. Hibiscus calyces contain ascorbic acid, with concentrations ranging significantly between fresh (6.7–14 mg/100 g) and dried calyces (260–280 mg/100 g) [42]. On the contrary, the insignificant change in the vitamin C content in KG is due to the very small quantity of fresh lemon juice added (0.04% *w*/*w*), combined with the average concentration of vitamin C in lemon juice, 32.7 mg in 100 mL [56].

3.4. Changes in Total Phenolic Compounds, Flavonoids, and Phenolic Profile

The total phenolic compounds increased statistically significantly ($p < 0.05$) after the addition of herbs, spices, and fruits. As shown in Figure 2, the total phenolic content in K (samples A, B, and C) increased after the enrichment from 235.8 ± 14.25 μg GAE/mL to 301.7 ± 20.2 and 542 ± 38.6 μg GAE/mL for KG (samples AG, BG, and CG) and KR (samples AR, BR, and CR), respectively.

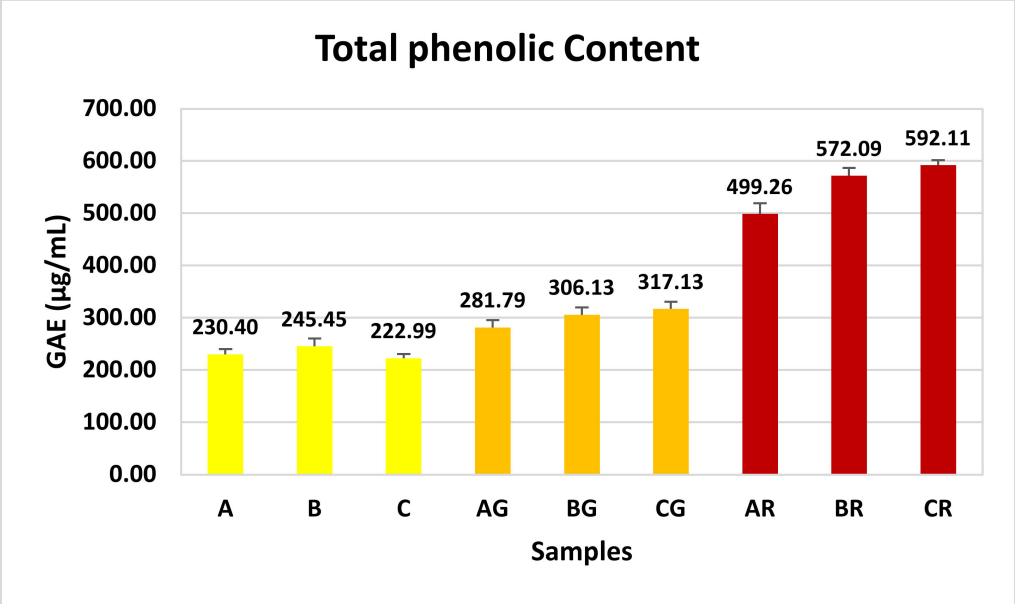

Figure 2. Total phenolic content in kombucha samples before (A, B, C) and after enrichment (AG, BG, CG, AR, BR, CR). Results are reported as mean ± standard deviation in gallic acid equivalents (GAE) of three independent measurements.

The explanation lies in the infused phenolic compounds from the herbs, spices, lemon zest, and juice in the fermented Olympus Mountain tea kombucha sweetened with thyme honey. In a previous study [50], a high phenolic content was found in rose petals, with tannins making up a substantial part. Other writers have previously reported significant quantities of tannins [57–59]. Most of the rose teas examined had a total phenolic component level that was either equivalent to or greater than that seen in green tea [49]. In addition, lavender blooms serve as a valuable reservoir of phenolic chemicals and anthocyanins [15]. Turmeric and ginger, when dried, contain high levels of phenolic curcuminoids such as curcumin, dimethoxycurcumin, and bisdemethoxycurcumin, as well as spicy phenolic compounds including gingerol and shogaol [60]. Likewise, lemon zest contains a high concentration of phenolic compounds [27,28].

After the addition of hibiscus, rose petals, and lavender (samples AR, BR, and CR), a statistically significant increase in the average values of the flavonoid content ($p < 0.05$) was recorded, from 123.4 ± 8.3 to 213.8 ± 5.8 μg RE/mL (Figure 3). On the other hand, the average value of total flavonoids decreased from 123.4 ± 8.3 to 118.5 ± 5 μg RE/mL. However, this difference was not statistically significant ($p > 0.05$) after the addition of ginger, turmeric, lemon zest, and juice (samples AG, BG, and CG).

The rich flavonoid content of the herbs infused can explain the significant increase in flavonoids in KR. Large amounts of flavonoids in rose petals have been previously demonstrated [50], where LC-ESI-MS/MS analysis revealed the presence of nine flavonoid glycosides, primarily quercetin and kaempferol derivatives. Hibiscus calyces contain flavonoids like hibiscitrin, sabdaritrin, gossypitrin, gossytrin, other gossypetin glucosides, quercetin, and luteolin [40,48]. The hibiscus calyces contain pigments called anthocyanins, which are flavonoid derivatives that change color depending on the pH [40]. Several studies have identified delphinidin-3-sambubioside (delphinidin-3-O-(2-O-b-D-xylopyranosyl)-b-D-glucopyranose) and cyanidin-3-sambubioside (cyanidin-3-O-(2-O-b-D-xylopyranosyl)-b-D-glucopyranoside) as the major anthocyanins present in extracts from hibiscus calyces [9,43,46,61–63]. The statistically insignificant change in the flavonoid content after the enrichment with ginger, turmeric, lemon zest, and juice could be explained by the small quantities added, the fact that ginger and lemon are in a fresh state, the considerably low flavonoid content of the above, and the acidic environment of K. Previous research indicated that the acidic nature of the fermented kombucha beverage led to the breakdown of

flavonoids [64]. Also, a decreased flavonoid content of Olympus Mountain tea sweetened with honey after kombucha fermentation has been previously reported [30].

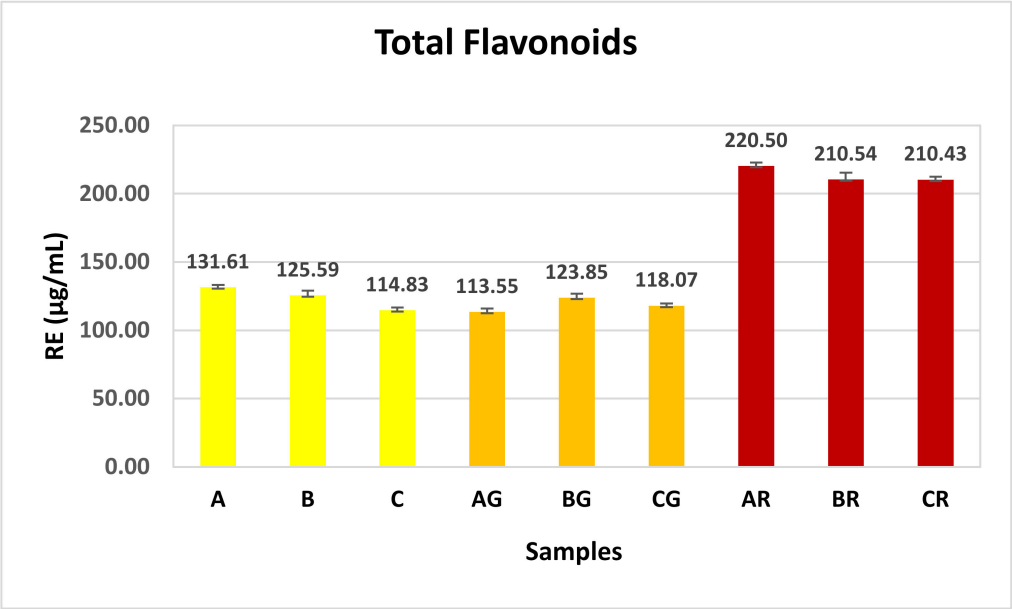

Figure 3. Total flavonoid content in kombucha samples before (A, B, C) and after enrichment (AG, BG, CG, AR, BR, CR). Results are reported as mean ± standard deviation in rutin equivalents (RE) of three independent measurements.

3.5. Evaluation of Antioxidant Activity

The antioxidant capacity of K was assessed using two distinct assays, namely the DPPH and ABTS assays. This assessment was conducted before and after the addition of herbs, spices, lemon zest, and juice. The antioxidant properties of all samples are given in Figures 4 and 5, and the results are expressed as IC_{50} values.

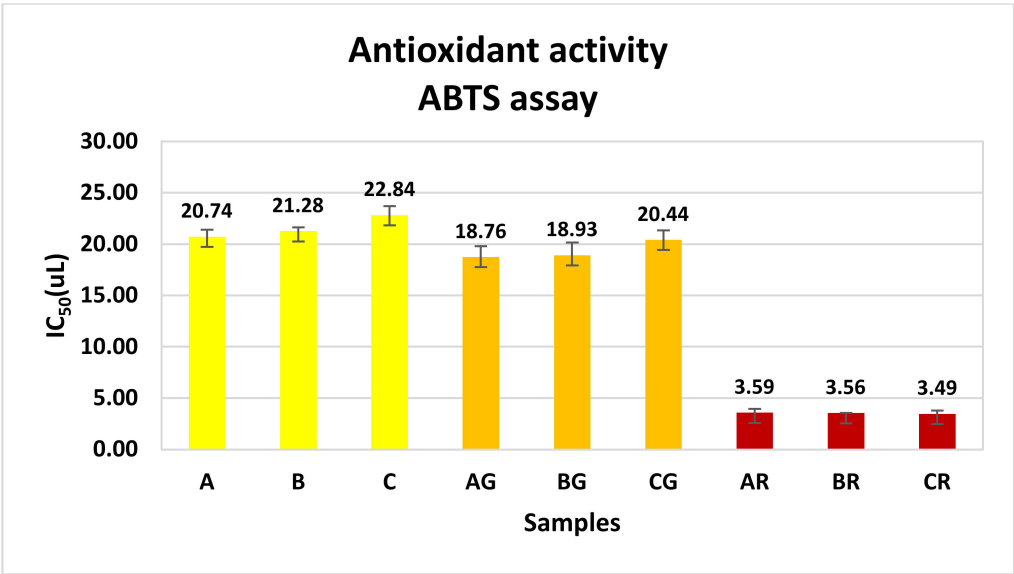

Figure 4. Antioxidant activity was evaluated by the ABTS assay before (A, B, C) and after enrichment (AG, BG, CG, AR, BR, CR). Data are presented as mean ± standard deviation in the amount (µL) required for 50% scavenging of $ABTS^{\bullet+}$ (IC_{50}) for three independent measurements.

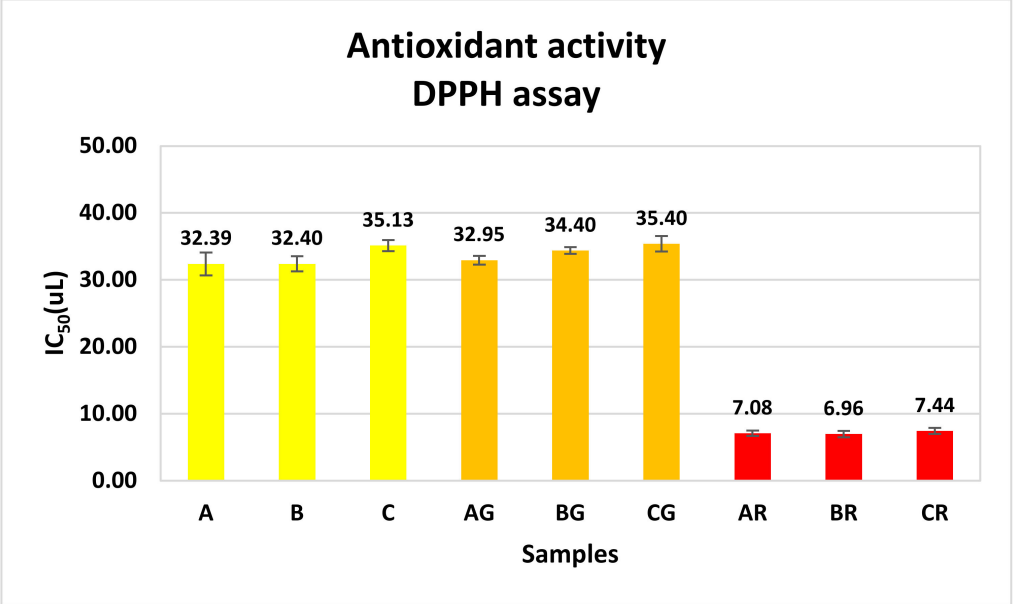

Figure 5. Antioxidant activity based on DPPH assay in samples before (A, B, C) and after enrichment (AG, BG, CG, AR, BR, CR). Data are presented as mean ± standard deviation in the amount (µL) required for 50% scavenging of DPPH• (IC_{50}) for three independent measurements.

The IC_{50} values of antioxidants for ABTS exhibited a substantial drop ($p < 0.05$) after the enrichment process for both KG and KR, indicating an increase in antioxidant activity. Furthermore, the addition of hibiscus, rose petals, and lavender flowers resulted in a statistically significant reduction in the IC_{50} values ($p < 0.05$) as determined by the DPPH test. In contrast, ginger, turmeric, lemon zest, and juice did not result in any substantial alteration ($p > 0.05$) in the antioxidant activity as determined by the DPPH test. The IC_{50} values for ABTS of K, KG, and KR were estimated to be 21.62 ± 1.11, 19.38 ± 1.22, and 3.55 ± 0.25 µL. For DPPH, the values were 33.30 ± 1.76, 34.25 ± 1.28, and 7.16 ± 0.44 µL, respectively.

Stratil et al. [65] observed that a high total phenolic content enhances antioxidant activity. Moreover, our study demonstrated a rise in total phenolic content (TPC) following the incorporation of herbs, spices, and fruits. This finding elucidates the augmentation of antioxidant activity.

This study's robust negative correlation between total phenolic content (TPC) and antioxidant activity, as measured by the IC_{50} values obtained from the ABTS experiment (Spearman's correlation coefficient = −0.843, $p < 0.01$), further supports this claim. Furthermore, a moderate negative correlation was seen between the levels of total flavonoids and the IC_{50} values obtained from the ABTS experiment. This association was quantified using Spearman's connection coefficient, which was calculated at −0.574 ($p < 0.01$). In line with our previous study [30], we found a significant negative correlation between the total phenolic content and ABTS IC_{50} values (Pearson's correlation coefficient = −0.837, $p < 0.01$). Additionally, we observed a moderate negative correlation between the total flavonoids and ABTS IC_{50} values (Pearson's correlation coefficient = −0.370, $p \leq 0.05$) during the fermentation of Olympus Mountain tea sweetened with honey in the kombucha process.

On the other hand, DPPH IC_{50} values had a strong negative correlation with total flavonoids (Spearman's correlation coefficient = −0.811, $p < 0.01$) and a medium correlation with total phenolic content (Spearman's correlation coefficient = −0.683, $p < 0.01$).

Several studies have shown that extracts of hibiscus calyces have a potent antioxidant effect [66–69]. Moreover, rose petal teas exhibited high antioxidant activity, comparable to green and black teas [49]. Lavender blossoms also have a notable concentration of phenolic compounds and anthocyanins with antioxidant potential [15]. Ginger extracts have significant antioxidant activity equivalent to that of commercial antioxidant preservatives [70,71].

Both turmeric and ginger are rich sources of antioxidant compounds [54]. Lemon peels also have a considerable concentration of total phenols and antioxidant activity [27]. All the above indicates that herbs, spices, and fruits added to the beverage passed their antioxidant activities to the beverage.

Especially after the addition of hibiscus, rose petals, and lavender to K, there was an increase in TPC by 2.3 times, while the IC_{50} values decreased by 6.10 and 4.65 times for the ABTS and DPPH assays, respectively. This might suggest the existence of an additive or even a synergistic antioxidant activity between phenolic substances.

3.6. α-Glucosidase and α-Amylase Inhibition Assays

Previous scientific studies have demonstrated that extracts obtained from plants, including infusions and phytochemicals such as polyphenols, can substitute synthetic inhibitors for α-amylase and α-glucosidase [72,73]. Moreover, phenolic substances can control the metabolism of carbohydrates and lipids by suppressing the functions of α-glucosidase and α-amylase. These enzymes are inhibited by their ability to chelate, alter the structure, and restrict biological functions [74,75].

α-amylase and α-glucosidase play a crucial role in the metabolism of carbohydrates. Thus, by suppressing the activity of these enzymes, the rate at which glucose is absorbed into the bloodstream would decrease, ultimately leading to a reduction in glucose in the blood. The α-glucosidase IC_{50} values are presented in Figure 6. The IC_{50} values in the samples (AG, BG, and CG) after the addition of ginger, turmeric, lemon zest, and juice exhibited statistically significant increases ($p < 0.05$); therefore, the α-glucosidase inhibitory activity decreased after the addition. On the other hand, in the samples (AR, BR, CR) where hibiscus, rose petals, and lavender were added, a statistically significant decrease was observed, and the α-glucosidase inhibitory activity increased by approximately 18 times. The robust α-glucosidase inhibition is due to the herbs added.

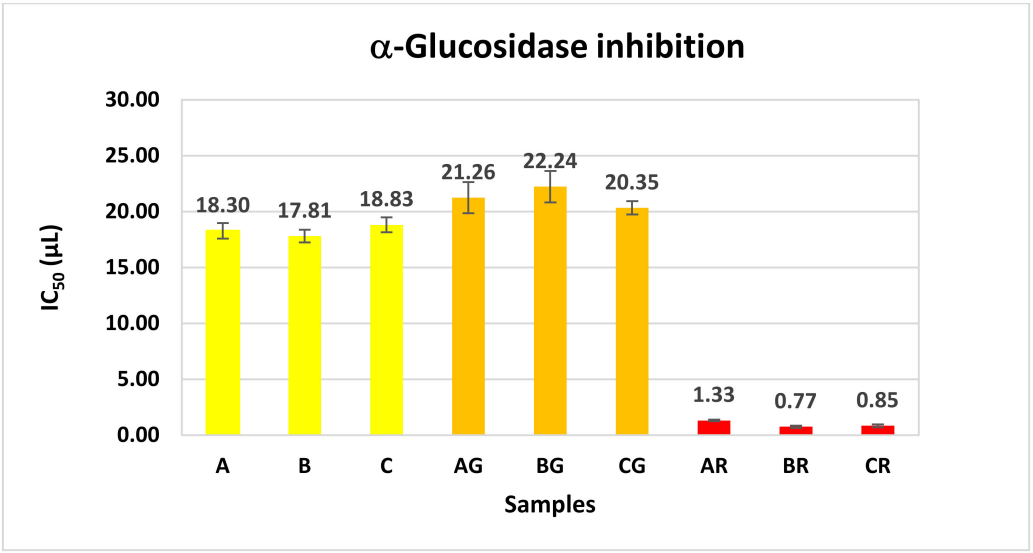

Figure 6. α-Glucosidase inhibition from samples before (A, B, C) and after (AG, BG, CG, AR, BR, CR) enrichment. Data are presented as mean ± standard deviation in the amounts (μL) required for 50% inhibition of α-glucosidase for three independent measurements.

Indeed, hibiscus calyces have a potent anti-diabetic effect [9]. Furthermore, all aqueous preparations of hibiscus demonstrated significant efficacy as inhibitors of α-glucosidase, with IC_{50} values lower than the positive control acarbose [76]. Also, Gholamhoseinian et al. [77] found that *Rosa damascena* extract had an intensive inhibitory effect on α-glucosidase.

The IC_{50} values of α-glucosidase inhibition showed a strong negative correlation (Spearman's correlation coefficient = -0.739, $p \leq 0.01$) with the values of total flavonoids

and a strong positive correlation (Spearman's correlation coefficient = 0.738, $p \leq 0.01$) with the IC_{50} values of the DPPH assay. That indicates an increase in the inhibition of α-glucosidase with the rise in the number of flavonoids and the DPPH antioxidant capacity of the samples. The IC_{50} α-glucosidase inhibition values exhibited a medium negative correlation with total phenolic compounds (Pearson's correlation coefficient = −0.505, $p < 0.01$), and a medium positive correlation with ABTS IC_{50} values (Pearson's correlation coefficient = 0.456, $p < 0.05$). Similarly, according to the Pearson correlation results, medicinal plants' total phenolic content and antioxidant activity were moderately correlated to the α-glucosidase inhibitory activity (r = 0.39 and r = 0.34, respectively) [78].

In a previous study, it was shown that cold maceration is optimal for preserving anthocyanins and that a strong correlation was observed between the IC_{50} values of a cold-macerated tea preparation and its content of delphinidin ($R^2 = 0.996$, $p < 0.05$) [76]. Those results demonstrated the advantage of cold extraction methods in preparing hibiscus tea as a hypoglycemic agent. So, it can be assumed that optimum results were achieved in our study regarding anthocyanins in the kombucha fermented beverage after the addition of herbs for 48 h at low temperatures. The α-amylase IC_{50} values are shown in Figure 7. The IC_{50} values in all the samples after the enrichment (AG, BG, CG, AR, BR, CR) exhibited a decrease ($p < 0.05$), and therefore, the α-amylase inhibitory activity increased.

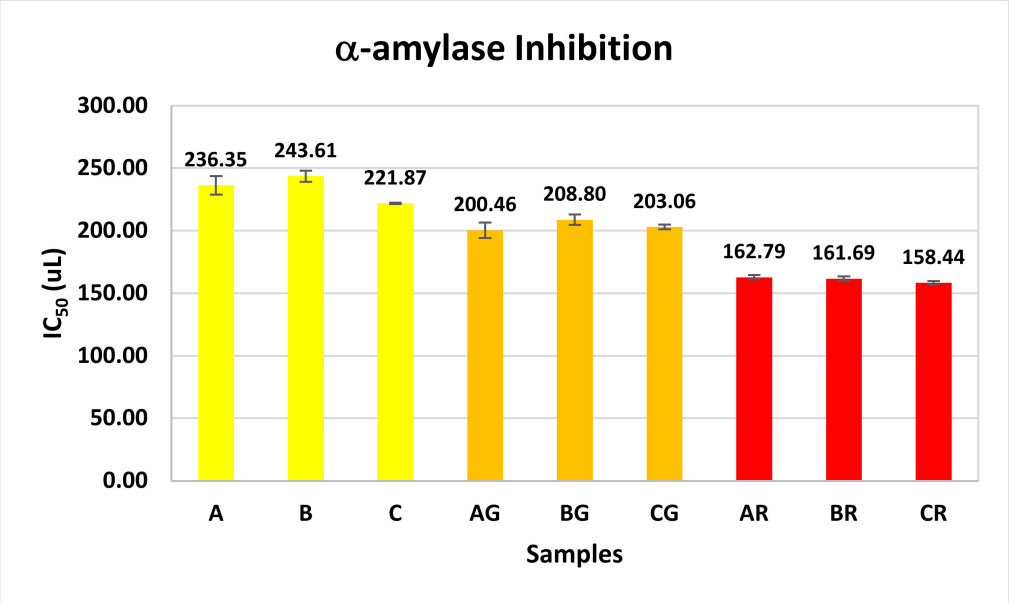

Figure 7. Inhibition of α-amylase from samples before (A, B, C) and after (AG, BG, CG, AR, BR, CR) enrichment. Results are presented as mean ± standard deviation in the amount (μL) required for 50% inhibition of α-amylase for three independent measurements.

The IC_{50} values of α-amylase inhibition showed a strong negative correlation (Spearman's correlation coefficient = −0.887, $p < 0.01$) with the content of total phenolics and a strong positive correlation (Spearman's correlation coefficient = 0.831, $p < 0.01$) with the IC_{50} values of the ABTS assay. That indicates an increase in the inhibition of α-amylase with the rise in the total phenolic content and the ABTS antioxidant capacity of the samples. The above agrees with previous studies [30,37,79].

Hibiscus calyces have a potent anti-diabetic effect [9]. Furthermore, research has shown that hibiscus extract is a very effective inhibitor of pancreatic α-amylase [80]. Comparable findings were seen for hibiscus acid (hibiscus-type (2S,3R)-hydroxy citric acid lactone) [81], which inhibited pancreatic α-amylase and intestinal α-glucosidase enzymes [82].

The IC_{50} α-amylase inhibition values exhibited a medium negative correlation with total flavonoids (Pearson's correlation coefficient = −0.467, $p < 0.01$) and a medium positive correlation with DDPH IC_{50} values (Pearson's correlation coefficient = 0.538, $p < 0.01$).

α-amylase inhibition, in contrast with α-glucosidase, correlates more with total phenolic content than flavonoids in the sample. Suppressing the activity of α-amylase hinders the release of maltose from starch, resulting in a delay in the conversion to glucose and a reduction in postprandial plasma glucose levels. α-glucosidase inhibition retards the liberation of D-glucose from oligosaccharides and disaccharides, resulting in a postponement of glucose absorption and a reduction in postprandial plasma glucose levels [83]. Therefore, adding herbs, spices, and fruits, especially hibiscus, rose petals, and lavender, to fermented Olympus Mountain tea kombucha creates a new functional beverage that could have an anti-diabetic effect.

Further studies are required to demonstrate therapeutic potential for human health.

3.7. Cholinesterase Inhibition Assays

The inhibition of cholinesterases is a common strategy for treating Alzheimer's disease.

The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity values (IC_{50}) are shown in Figures 8 and 9, respectively. The IC_{50} values in all the samples after the enrichment (AG, BG, CG, AR, BR, CR) exhibited a statistical decrease ($p < 0.05$). Consequently, the inhibitory activities of AChE and BchE were enhanced. The above can be attributed to the bioactive compounds of the spices and spices added, and more specifically to the hibiscus calyces, the rose petals, and the ginger root.

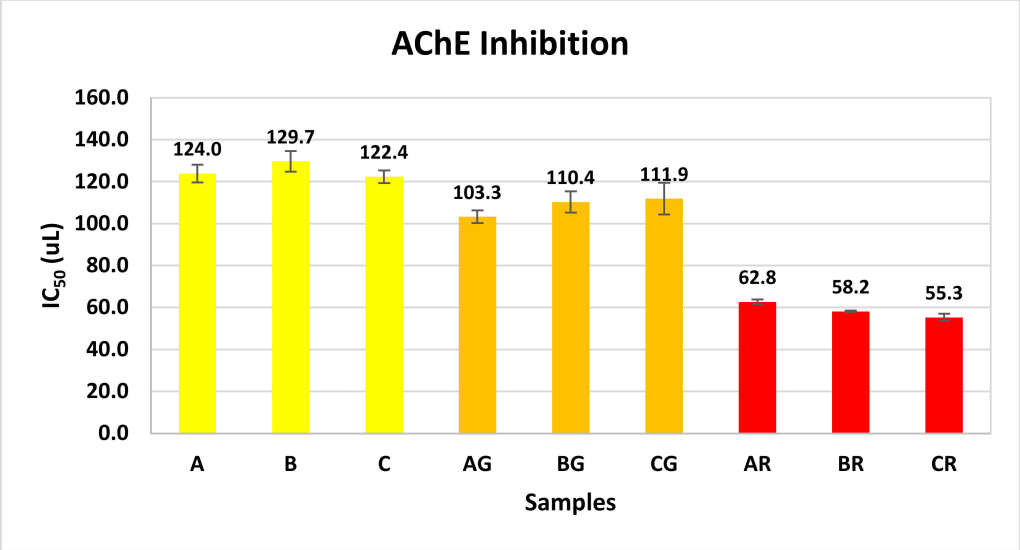

Figure 8. AChE inhibition from samples before (A, B, C) and after (AG, BG, CG, AR, BR, CR) enrichment. Data are presented as mean ± standard deviation in the amount (μL) required for 50% inhibition of acetylcholinesterase for three independent measurements.

The extract from hibiscus calyces has been shown to have inhibitory effects on the activities of AchE and BchE [84]. Furthermore, it was shown that phenolic chemicals in the extract of hibiscus calyces may be responsible for its inhibitory actions on AchE and BchE in a study indicating that hibiscus extracts have dual inhibitory effects, which might be more potent in treating and controlling Alzheimer's disease by inhibiting AchE and BchE, demonstrating symptomatic effectiveness [85]. As mentioned earlier, flavonoids, including quercetin, quercitrin, and rutin, function as competitive inhibitors of AchE and BchE by preventing their substrates from attaching to the active site of the enzyme [85,86]. Furthermore, these compounds have a structural resemblance to synthetic medicines like rivastigmine, enabling them to attach to the peripheral anionic site of the enzyme via their aromatic rings and hydroxyl groups. Furthermore, it was discovered that rose petal tea is a powerful inhibitor of acetylcholinesterase, with a 61.63% suppression of the enzyme [87].

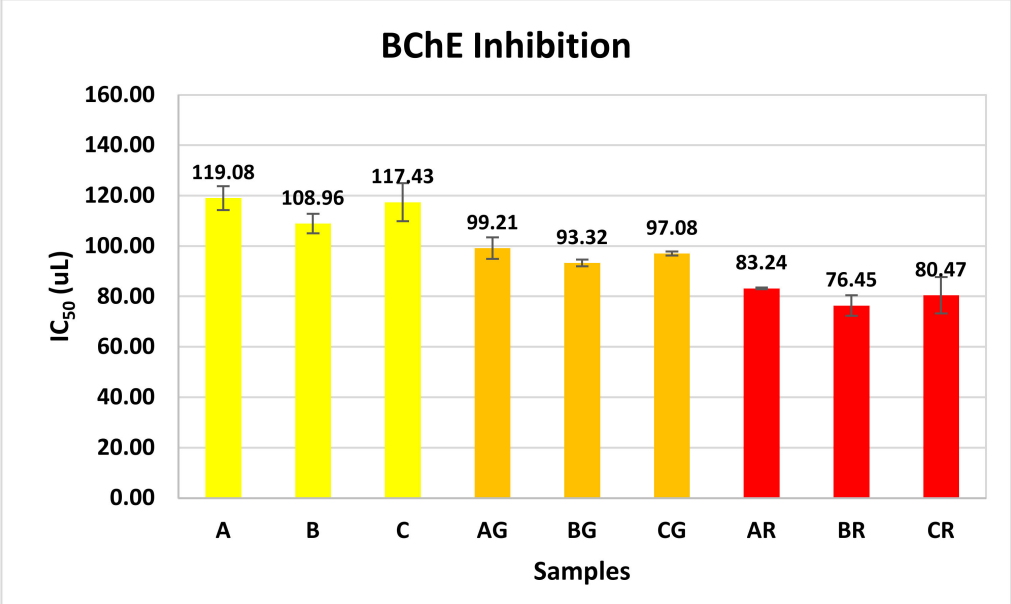

Figure 9. BChE inhibition from OMTWH kombucha samples before (A, B, C) and after (AG, BG, CG, AR, BR, CR) enrichment. Data are presented as mean ± standard deviation in the amount (μL) required for 50% inhibition of butyrylcholinesterase for three independent measurements.

The bioactive compounds present in ginger root have been shown to exert anti-AChE activity [88]. In addition, the total extract of dry ginger has been shown to have anti-AChE and anti-BChE activities [89]. Moreover, water extracts of red and white ginger root have been reported to inhibit AChE in a dose-dependent manner [90]. This protective effect may be attributed to ginger root flavonoids, tannins, alkaloids, and terpenoids. Separate research [91] showcased the AChE inhibitory properties of some active constituents found in ginger extracts, compared to donepezil, a pharmaceutical medicine used for treating Alzheimer's disease (AD) that also inhibits AChE. Additionally, ginger compounds have been observed to inhibit the activity of BChE [92].

The IC_{50} values of AChE and BChE inhibition showed a strong negative correlation (Spearman's correlation coefficient AChE = −0.873, Spearman's correlation coefficient BChE = −0.922, $p < 0.01$) with the values of total phenolic content and a strong positive correlation (Spearman's correlation coefficient AChE = 0.877, Spearman's correlation coefficient BChE = 0.872, $p < 0.01$) with the IC_{50} values of the ABTS assay. That indicates an increase in the inhibition of AChE and BChE with the rise in the samples' total phenolic content and the ABTS antioxidant capacity. The AChE and BChE IC_{50} inhibition values exhibited a medium negative correlation with total flavonoids (Spearman's correlation coefficient AChE = −0.502, Spearman's correlation coefficient BChE = −0.608, $p < 0.01$) and a medium positive correlation with DDPH IC_{50} values (Spearman's correlation coefficient AChE = 0.580, Spearman's correlation coefficient BChE = 0.596, $p < 0.01$). AChE and BChE inhibition correlate more with total phenolic content than flavonoids in the sample. The above observations are consistent with previous studies [93–99]. Furthermore, a prior investigation [100] established a correlation between the significant anti-AChE activity seen in the examined honey varieties and their substantial antioxidant activities, which subsequently resulted in high total phenolic content (TPC) in the analyzed types of honey.

4. Conclusions

This study aimed to evaluate the effects of adding herbs, spices, and fruits to fermented Olympus Mountain tea kombucha with honey and the changes that occur in its physicochemical and functional properties. Two different enrichments were proposed: a "Golden Mountain tea and honey Kombucha" (KG) with fresh ginger, turmeric powder, lemon zest, and juice and a "Red Mountain tea and honey Kombucha" (KR) with dried hibiscus

calyces, rose petals, and lavender blossoms. In KR, the levels of vitamin C increased from 33.2 ± 2.7 to 48.4 ± 4.5. Additionally, the levels of calcium increased from 31.0 ± 1.2 to 55.7 ± 1.2, while the levels of potassium practically doubled from 64.7 ± 0.6 to 115.7 ± 2.5. An increased potassium concentration was observed in KG, and ionic iron was found for the first time after both enrichments. The total phenolic and flavonoid contents, along with the antioxidant capacity, as assessed by the ABTS and DPPH methods, were substantially enhanced in KR. In KG, the total phenolic content and the antioxidant activity increased, as assessed by ABTS. The enrichment with hibiscus calyces, rose petals, and lavender blossoms significantly increased the inhibitory effect against α-amylase, α-glucosidase, acetylcholinesterase, and butyrylcholinesterase. On the other hand, the enrichment with ginger, turmeric, lemon zest, and juice decreased the inhibition effect against α-glucosidase and increased it against α-amylase, acetylcholinesterase, and butyrylcholinesterase. KR had the strongest inhibitory activity of all the enzymes studied, with the α-glucosidase inhibitory activity increasing by approximately 18 times. The results showed that enrichment with herbs, spices, and fruits could transform the fermented Olympus Mountain tea kombucha sweetened with honey into a unique beverage with improved functional characteristics. More research will investigate the role of each herb, spice, and fruit in the antioxidant and enzyme inhibitory activities and, particularly, the combination of hibiscus, rose petals, and lavender blossoms in greatly enhancing the beverage's functionality. Also, further in vivo studies are needed to investigate the anti-diabetic properties of the beverages and the potential role that they could play in Alzheimer's treatment as cholinesterase inhibitors.

Author Contributions: Conceptualization, I.G.K. and H.C.K.; methodology I.G.K., H.C.K. and C.N.; software, I.G.K.; validation, I.G.K., H.C.K. and C.N.; investigation, I.G.K., H.C.K. and I.P.; resources, H.C.K. and C.N.; data curation I.G.K., H.C.K. and C.N.; writing—original draft preparation, I.G.K.; writing—review and editing H.C.K. and C.N.; supervision, H.C.K.; project administration, H.C.K.; funding acquisition, H.C.K. and C.N. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

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