

Effect of Different Types of Herbal Infusions on Functional Properties of Kombucha Fermented Beverages: Antibacterial, Antifungal and Antioxidant Characteristics

Dina Shahrapour*¹, Sara Naji-Tabasi²

1. Department of Food Safety and Quality Control, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

2. Department of Food Nanotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

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* Corresponding authors:

Dina Shahrapour

Tell: 051-35425351

E-mail:

d.shahrapour@rifst.ac.ir

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Abstract

Background and Objective: The type of substrate, fermentation conditions and microbial flora can affect the functional properties of fermented beverages. Therefore, this study aimed to compare the microbiological, chemical, antioxidant and antimicrobial properties of kombucha beverages produced from four herbal infusions (Green tea, Black tea, Lemon verbena and Shirazi thyme) and investigate the effects of fermentation on altering herbal infusions characteristics.

Material and Methods: Various herbal teas were fermented for 12 days after inoculation with the kombucha starter culture at 28°C. Total phenolic compounds and antioxidant activity were measured by Folin–Ciocalteu assay and DPPH free radical scavenging activity before and after fermentation of herbal infusions, respectively. Also, antibacterial activity against pathogenic gram-positive and gram-negative bacteria was evaluated by the well-diffusion method. Antifungal activity of various herbal infusions and Kombucha beverages against *Aspergillus niger* and *Aspergillus flavus* was analyzed by agar dilution and well-diffusion methods.

Results and Conclusion: The pH of different herbal infusions decreased significantly after fermentation. Total phenolic compounds increased markedly during fermentation, reaching 65.82–153.38 mg GAE.L⁻¹ in the Kombucha samples, and green tea Kombucha indicated the highest value. Microbial analysis revealed that the highest microbial population in all Kombucha samples belonged to acetic acid bacteria (10⁶–10⁷ CFU.mL⁻¹), followed by lactic acid bacteria (10⁴–10⁵ CFU.mL⁻¹), and yeasts (10⁵ CFU.mL⁻¹). Green tea infusion and its Kombucha beverage showed the strongest DPPH scavenging capacity. Unlike herbal infusions, all Kombucha beverages revealed antifungal and antibacterial activity. According to these findings, fermentation significantly enhanced the functional, antioxidant, and antimicrobial properties of herbal infusions and green tea Kombucha was introduced as a considerably promising functional food preservative.

Keywords: Herbal infusion, Functional beverage, Fermentation, Antioxidant, Antimicrobial activity

What is “already known” on this topic:

- Fermentation with Kombucha starters is an effective strategy to alter herbal infusions into functional beverages with bioactive properties.
- The type of substrate, fermentation conditions and microbial flora can affect the functional properties of Kombucha.

What this article adds:

- Comparison of antibacterial, antifungal, and antioxidant activities of various herbal infusions (green tea, black tea, lemon verbena, & thyme) with Kombucha in this study.
- All Kombucha beverages indicated antifungal activity against *A. niger* and *A. flavus*.
- Unlike herbal infusions, Kombucha beverages revealed robust antibacterial activity.
- Kombucha samples demonstrated more than 8.22 -11.09% DPPH scavenging activity compared to herbal infusions.
- The highest phenolic compounds, antioxidant and antimicrobial activities belonged to the green tea kombucha beverage.

1. Introduction

Kombucha is a traditional fermented, acidic drink with a relatively sweet taste, which has been popular in countries such as China, Russia, Japan, and Germany in the past due to its health-promoting properties. Today, the desire to consume this functional drink has increased in many countries, due to its antioxidant, antimicrobial, anticancer, antidiabetic, liver detoxification, cholesterol and blood pressure-lowering properties, and in the treatment of many heart diseases and insufficiency. Kombucha is the result of the fermentation of sweetened herbal tea for 7-14 days at 28-30 °C by acetic acid bacteria (AAB) (Acetobacter, Gluconobacter), lactic acid bacteria (LAB) and yeasts (Candida, Pichia, Saccharomyces, Schizosaccharomyces, Zygosaccharomyces, Brettanomyces, and Torula) that exist symbiotically in the SCOBY (symbiotic culture of bacteria and yeast). The type of tea, sweetener, microorganisms, and fermentation conditions can affect Kombucha drink characteristics, including its chemical composition, color, flavor, and microbial flora [1]. In a recent study, Li et al. comprehensively compared microbial community and quality of kombucha from four different regions of China. This study identified 197 indigenous yeast and bacterial strains and demonstrated that fermentation time strongly influenced color, phenolic composition, volatile profiles, and antioxidant activity [2].

Although black tea (*Camellia sinensis*) is the most common substrate, interest in using alternative herbal infusions is growing, driven by the potential to improve bioactivity and diversify sensory profiles [3]. In a study, the antibacterial and antioxidant activities, as well as the chemical composition of kombucha beverages prepared from linden (lime tree), chamomile, nettle, St. John's wort, rockrose (*Cistus* spp.), and green tea were comparatively evaluated [4]. SCOBY mediated fermentation significantly alters the chemical composition of herbal Kombucha, leading to notable changes in sugars, organic acids, and an increase in essential minerals such as iron, magnesium, and calcium. However, the type of herbal or plant waste infusion, such as mint, nettle, blackcurrant leaf, and banana peel, was identified as a key factor influencing the antioxidant potential and sensory attributes of the resulting beverages [5, 6].

Plant infusions such as green tea (*Camellia sinensis*), lemon verbena (*Aloysia citrodora*), and thyme (*Thymus vulgaris*) contain high levels of polyphenolic compounds and essential oils with well-documented antibacterial, antifungal, and antioxidant effects [7, 8]. The integration of these herbal extracts into Kombucha fermentation may influence both the microbial dynamics of SCOBY and the biochemical composition of the final product [9]. Several studies have investigated the antimicrobial and antioxidant

potential of Kombucha brewed from black and green tea [10, 11], and some have examined chemical and biological characteristics of Kombucha derived from herbal infusions such as peppermint, oregano, sage, winter savory, stinging nettle, elderberry, quince and yarrow [12-14]. All of these studies have emphasized the effect of substrate type on the formation of beneficial and health-promoting compounds during Kombucha fermentation. Also, various antimicrobial activities of Kombucha beverages were reported in some studies due to the presence of organic acids, phenolic compounds, microbial protein and enzymes [15, 16].

Kombucha fermentation is a combination of three processes: lactic, alcoholic, and acetic acid fermentation. During tea fermentation, yeasts in the medium first convert sucrose, which can be consumed as a sweetener, into glucose and fructose, and then, by consuming glucose, ethanol and carbon dioxide are produced in the drink. The concentration of ethanol produced is usually less than 1%, which is oxidized by acetobacteria and leads to the production of acetic acid and acetaldehyde. In addition, glucose is also converted to gluconic acid by acetobacteria. Gluconic acid has been of interest in various studies in recent years due to its detoxification properties. This acid can remove various types of toxic substances, such as pollutants, chemicals, excess steroid hormones, and bilirubin from the human body through the urinary system. Gluconic acid is considered a precursor to vitamin C. In addition, gluconic acid can be converted into glucosamine, which is a useful substance related to the synthesis of cartilage, collagen, and interarticular fluids that are effective in the treatment of osteoarthritis. Lactic acid is another metabolite produced by LAB during the fermentation of glucose and sucrose [17]. As mentioned before, Kombucha contains a variety of compounds, including organic acids, amino acids, simple sugars, caffeine, tannins, phenolic compounds, folic acid, minerals, and vitamins C and B. Therefore, despite the active and health-promoting compounds mentioned in Kombucha, the use of this fermented beverage and its starters in the formulation of various foods can have a positive effect on their nutritional, quality, and shelf life as a novel biopreservation [18-22].

To produce Kombucha traditionally, black or green tea waste is usually used. Recently, Kombucha drinks based on various medicinal plants such as Thyme, Peppermint, and Lemon verbena, etc., are also been produced and marketed in Iran. However, there is limited information about functional properties such as phenolic components, antioxidants, antibacterial, and antifungal activities of various types of Iranian Kombucha beverages. Shirazi thyme (*Zataria multiflora* Boiss.) is one of the best-known medicinal plants in Iran from the mint family; most of its



therapeutic, antimicrobial, and antioxidant properties are attributed to the presence of phenolic compounds such as thymol and carvacrol in it [23]. Lemon verbena (*Aloysia citrodora*) is also an aromatic plant from the Verbenaceae family that can be cultivated in different regions of Iran. The leaves and flowering tops of this plant have medicinal properties, and its essential oils are rich in flavonoid compounds [24].

According to our current knowledge, there is limited information to compare the microbiology, antioxidant, and antimicrobial activity of herbal infusions before and after fermentation with Kombucha starters. Given the importance of the type of herbal tea on the properties of Kombucha, research in this area is necessary and continues. Therefore, this study aims to compare the microbiology, antioxidant, antibacterial, and antifungal properties of different types of Kombucha fermented beverage produced from four herbal infusions (green tea, black tea, lemon verbena, and Shirazi thyme). In addition, the total phenolic content, antioxidant, and antimicrobial activity of four unfermented herbal infusions are compared before the production of kombucha beverages.

2. Materials and Methods

2.1. Materials

Black Tea, green tea, lemon verbena, and thyme were purchased from Mashhad local market. Chemical material and microbial culture medium were obtained from Merck company.

2.2. Various herbal infusions and Kombucha production

In this study, four different types of Kombucha were prepared based on the formulation proposed by Cardoso et al., with slight modifications [25]. First, 80 g of sugar was dissolved in 1 L of water (95 °C) and 5 g of each dried herbal leaf, including black tea, green tea, Lemon verbena, or Shirazi thyme, was added, and the infusion was filtered through a strainer filter after 10 min. After cooling the infusion to 25 °C, the SCOBY Kombucha (4% w.v⁻¹) and 40 mL of previously produced Kombucha drink as a starter were inoculated and placed in a dark place at ambient temperature (28°C) for 12 days to ferment. Finally, a newly formed scoby was removed from the surface, and all Kombucha beverages were stored at 4 °C after passing through a strainer until the tests were performed. It should be noted that unfermented herbal infusions without sugar were used as controls in various tests in this study.

2.3. Microbiological characterization

To determine the microbial flora of the produced Kombucha samples, after preparing serial dilutions, enumeration of LAB, AAB, and yeasts were performed on MRS agar (Man Rogosa Sharpe, Liofilchem, Italy) (37°C for 48 h), glucose yeast extract agar (glucose 50 g.L⁻¹, yeast

extract 10 g.L⁻¹, and agar 20 g.L⁻¹) (30°C for 48 h), and yeast glucose chloramphenicol agar (YGC agar)(Condalab, Spain) (25°C for 72 h), respectively. The population of each group of microorganisms in the Kombucha beverage was reported as Log CFU.mL⁻¹.

2.4. pH and total acidity

The total acidity of each Kombucha beverage was determined by titration with standardized 0.01N NaOH and phenolphthalein as an indicator. The titrant was added dropwise until the pH reached 8.0, which was selected as the endpoint. The total acidity was calculated and expressed as g of acid / per L of sample (g.L⁻¹). All titrations were performed at room temperature (~25 °C). The pH was determined by a previously calibrated pH meter and in triplicate to ensure reproducibility.

2.5. Total phenolic content

The Folin-Ciocalteu method was used to measure the total phenolic content of the produced Kombucha and herbal infusions samples. For this purpose, 0.5 mL of each sample was added to 2.5 mL of Folin-Ciocalteu reaction solution (0.2 N) in a test tube. After 5 min at room temperature, 2 mL of sodium carbonate (7.5% w.v⁻¹) was mixed with the previous solution. After incubation for 30 min and the color change of the solution, the absorbance was measured at 760 nm. Total phenolic content was determined in terms of gallic acid equivalents using a gallic acid standard curve. The results were expressed as mg of gallic acid equivalents per mL of each Kombucha beverage (mg GAE.mL⁻¹) [25].

2.6. Antioxidant activity

First, 0.2 ml of filtered Kombucha sample or each herbal infusion was added to 2.8 ml of 0.1 mM ethanolic DPPH solution and after mixing with a shaker at room temperature, it was kept in a dark place for 30 minutes. Distilled water was used as a control in this test. Then, the absorbance of each of the tested solutions was measured at 517 nm and the percentage of DPPH radical scavenging activity was calculated with Eq. 1 [26].

$$\text{DPPH scavenging activity (\%)} = \frac{[A \text{ control} - A \text{ sample} / A \text{ control}] \times 100}{\text{Eq. 1}}$$

2.7. Antimicrobial activity

2.7.1. Antifungal activity

The antifungal properties of the Kombucha samples and herbal infusions against two common spoilage molds, *Aspergillus niger* (PTCC 5010) and *Aspergillus flavus* (PTCC 5004), were investigated by well diffusion and agar dilution methods. In the well diffusion method, after inoculating the spore suspension (100 µL) of each of the molds (10⁴ spores.mL⁻¹) on the surface of the YGC agar medium, wells were created on its surface with a sterile cork borer and 100 µL of each sample was inoculated into them. After the plates were incubated for 72 h at 25 °C, the



diameter of the inhibition zone was measured with a ruler and reported in mm [27].

Also, in the agar dilution method, various concentrations of Kombucha or herbal infusion samples (10 – 40 % (v.v⁻¹)) were added to molten YGC agar and poured in plates to solidify. Then, a blank disk was placed in the center of the plate and 10 µl of spore suspension of each mold (10⁴ spores.mL⁻¹) was inoculated into it. In this test, a YGC agar plate without Kombucha was used as a control. After 96 h of incubation at 25°C, the diameter of mold growth in the plates was measured and the percentage of growth inhibition in the presence of Kombucha or herbal infusion samples was calculated with Eq. 2 [28].

Inhibition of mold growth (%) = $1 - (\text{mold growth diameter in treatment plate} / \text{mold growth diameter in control plate}) \times 100$ Eq. 2

2.7.2. Antibacterial activity

The antibacterial activity of various Kombucha and herbal infusion samples against gram-negative (*Escherichia coli* (PTCC 1399), *Salmonella enteritidis* (PTCC 1709)) and gram-positive (*Listeria monocytogenes* (PTCC 1298), *Staphylococcus aureus* (PTCC 1431)) foodborne pathogenic bacteria was evaluated by the well diffusion method. First, each stock culture of the pathogenic indicator bacteria was activated in Muller-Hinton broth (MHB) medium for 24 h at 37 °C. Then, 100 µL of overnight bacterial culture at a concentration 1.5 × 10⁸ CFU.mL⁻¹ (0.5 McFarland) was inoculated onto the surface of Muller-Hinton agar (MHA) medium and spread well using a sterile swab. Then, wells with a diameter of 6 mm were created in the culture medium using a cork borer and 100 µL of filtered Kombucha was injected into them. All plates were transferred to an incubator at 37 °C. After 24 h, the inhibition zone around the wells was measured using a ruler and reported in mm [16].

2.8. Statistical analysis

In the present study, all tests were performed with three replications. Data analysis was performed using a completely randomized design using SPSS version 23. Analysis of variance (ANOVA) was used to determine significant differences at a 95% confidence level, and Duncan's test was used to compare means. Graphs and tables were drawn using Microsoft Excel version 2020. Moreover, Pearson's correlation coefficients (r) were calculated to assess the relationships among chemical parameters, bioactive compounds, functional properties, and microbial populations in herbal infusions before fermentation and kombucha beverages after fermentation. Two-tailed tests were applied, and statistical significance was set at $p < 0.05$ and $p < 0.01$.

3. Results and Discussion

3.1. Microbiological characterization

The microbial evaluation of four types of Kombucha demonstrated that the population of LABs varied between approximately 10⁴-10⁵ CFU.mL⁻¹, and the highest number of LABs was observed in BTK (4.89 ± 0.24 Log CFU.mL⁻¹) and GTK (4.85 ± 0.31 Log CFU.mL⁻¹), with no significant difference ($P > 0.05$), respectively. Moreover, there was no significant difference among the yeast population (5.29 ± 0.36 – 5.43 ± 0.35 Log CFU.mL⁻¹) in the four types of Kombucha ($P > 0.05$). The highest frequency belonged to the AAB and varied between 6.59 ± 0.47-7.35 ± 0.35 Log CFU.mL⁻¹ in the different studied Kombucha samples (Fig. 1). In the study by Cardoso et al., the population of LAB, AAB, and yeasts in two types of Kombucha (black tea and green tea) after 10 days of fermentation at 25 °C was reported to be about 10⁵-10⁶ CFU.mL⁻¹ [25]. Also, Zhao et al. calculated the number of mesophilic bacteria as 10⁶ CFU.mL⁻¹ and yeasts as 10⁵ CFU.mL⁻¹ in Kombucha after 10 days of fermentation [29]. This is while higher populations of AAB and LABs, as well as yeasts (10⁷ CFU.mL⁻¹) were obtained in the study by Neffe-Skocińska et al. [30] under similar fermentation conditions. This difference observed in different studies is likely due to differences in the amount of sweetener, type of herbal infusion, initial inoculum (amount of SCOBY and previously produced Kombucha as starter), and fermentation conditions and time. The study of Jafari et al. confirmed the presence of 3 species of yeasts, 2 species of AABs and 3 species of LABs in black tea kombucha after 14 days of fermentation [31].

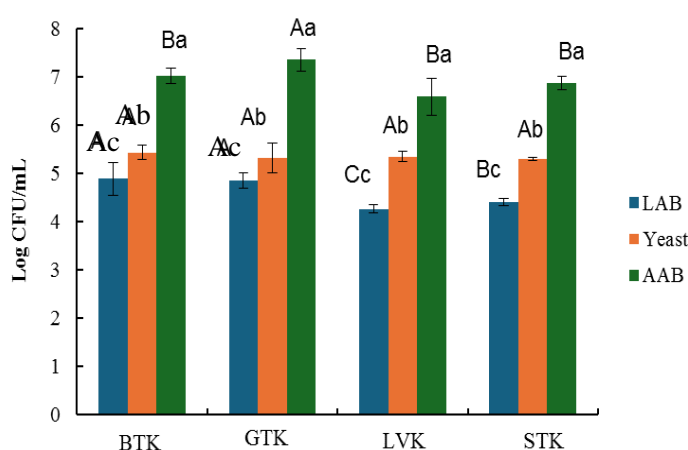


Fig. 1. Microbial population of various Kombucha samples (GTK: green tea Kombucha, BTK: black tea Kombucha, LVK: Lemon verbena Kombucha, STK: Shirazi thyme Kombucha). (Different lowercase letters on each column indicate a significant difference among various microbial counts in each kombucha sample at $p < 0.05$ and different capital letters on each column indicate a significant difference among various kombucha treatments at $p < 0.05$).



3.2. pH and acidity

According to Table 1, the herbal tea infusion samples exhibited pH values ranging from 6.54 to 7.60. As shown in Table 2, the pH of the Kombucha samples varied between 2.63-2.82, and there was no significant difference in this regard among the different treatments ($P > 0.05$). The lowest and highest pH belonged to the GTK and STK Kombucha samples, respectively. Also, the pH of all Kombucha samples was suitable for human consumption of fermented beverages ($\text{pH} > 2.5$). At pH values < 2.5 , the health of consumers will be at risk due to the high concentration of acetic acid. Similarly, at $\text{pH} > 4.4$, the microbiological safety of the beverage will be at risk [32]. The highest and lowest acidity were also related to GTK and STK Kombucha, respectively. Overall, there was a significant difference between the acidity of GTK and other Kombucha samples ($P < 0.05$). One of the factors that determines the end of the fermentation time of a fermented Kombucha beverage is the titratable acid level reaching about 4 to 6 $\text{g}\cdot\text{L}^{-1}$ [33]. During the preparation of Kombucha, sugars are consumed by microorganisms such as AAB and LAB as well as yeasts and are converted into organic acids such as acetic acid, lactic acid, gluconic acid, etc. Therefore, following the fermentation of sweet tea, a decrease in pH and an increase in acidity occur. Instead of the yeast population, the largest population of AAB and LABs was observed in GTK and BTK samples, which could be the reason for producing more acid. Similarly, in the study by Cardoso et al. [25], green tea Kombucha had a lower pH and higher acidity compared to black tea Kombucha. They attributed the difference in the acidity of the Kombuchas to the difference in the abundance of AAB and LAB populations in these two types of beverages and their ability to produce organic acids. According to their report, the populations of AAB, LAB, and yeasts in green tea Kombucha were higher than those of black tea, but no significant difference was observed between them. In fact, the microbial population of the Kombucha sample at the end of fermentation depends on the type and content of sugar, herbal tea, and the amount of inoculation of SCOBY and abundance of various strains of AAB, LAB, and yeasts in it, as well as the fermentation conditions (time and temperature).

Table 1. pH and total phenol content of various herbal infusion samples.

Herbal infusion	pH	Total phenol content (mg GAE. L^{-1})
BT	6.71 \pm 0.03 ^c	50.71 \pm 0.03 ^b
GT	6.54 \pm 0.03 ^d	70.53 \pm 1.07 ^a
LV	7.60 \pm 0.05 ^a	27.71 \pm 0.53 ^d
ST	7.16 \pm 0.11 ^b	47.96 \pm 1.35 ^c

*Results are reported as mean \pm standard deviation. Different lowercase letters in each column indicate a significant difference between treatments at $p < 0.05$. (GT: green tea, BT: black tea, LV: Lemon verbena, ST: Shirazi thyme).

3.3. Total phenol content

Chemical compounds consisting of at least one aromatic ring with at least one hydroxyl group constitute phenolic compounds. They belong to secondary metabolites synthesized in plants and can be divided into different subgroups, including simple phenols, phenolic acids, flavonoids, coumarins, lignins, and tannins. The total phenol content (TPC) of herbal tea infusions and the four Kombucha samples was determined by the Folin-Ciocalteu method and a gallic acid standard curve. There was a significant difference between the TPC of herbal infusion samples and also Kombucha samples ($P < 0.05$). The TPC of herbal tea infusion samples varied considerably, with the lowest value observed in LV (27.71 \pm 0.53 mg GAE. L^{-1}) and the highest in GT (70.53 \pm 1.07 mg GAE. L^{-1}) (Table 1). Following fermentation and Kombucha production, TPC increased markedly. The TPC results revealed a significant difference among Kombucha beverages that varied between 65.82 \pm 0.93 to 153.38 \pm 1.07 mg GAE. L^{-1} (Table 2). The highest and lowest TPC were assigned to GTK and LVK, respectively. The TPC of GTK was 18, 57.8, and 32.87 % higher than that of BTK, LVK, and STK, respectively (Table 2). In addition to increasing acidity, the fermentation led to an increase in the TPC of herbal tea infusions. In this study, TPC of GTK, BTK, LVK, and STK was 2.47, 2.17, 2.37, and 2.14 higher than GT, BT, LV and ZM infusions, respectively. Similarly, Kim et al. stated that the TPC of Kombucha samples (70.87- 250.52 μg GAE. mL^{-1}) prepared by inoculation of various AAB and yeasts was 1.27–3.53-fold higher compared with black tea infusion [33]. The TPC of studied herbal infusions is related to the inherent presence of phenolic and flavonoid compounds in these medicinal plants.

Table 2. pH, acidity and total phenol content of various Kombucha beverages.

Kombucha beverage	pH	Acidity (g/L)	Total phenol content(mg GAE. L^{-1})
BTK	2.65 \pm 0.06 ^a	4.68 \pm 0.26 ^b	125.73 \pm 0.01 ^b
GTK	2.61 \pm 0.03 ^a	6.01 \pm 0.93 ^a	153.38 \pm 1.07 ^a
LVK	2.69 \pm 0.07 ^a	4.54 \pm 0.83 ^b	65.82 \pm 0.93 ^d
STK	2.82 \pm 0.19 ^a	4.11 \pm 0.35 ^b	102.96 \pm 3.38 ^c

*Results are reported as mean \pm standard deviation. Different lowercase letters in each column indicate a significant difference between treatments at $p < 0.05$. (GTK: green tea Kombucha, BTK: black tea Kombucha, LVK: Lemon verbena Kombucha, STK: Shirazi thyme Kombucha).



The increase in TPC during Kombucha fermentation could be attributed to several microbial activities. Microbial enzymes hydrolyze complex polyphenols into simpler and more soluble phenolic components, while microbial metabolism generates additional phenolic compounds such as gallic and caffeic acids. The results of all these changes during fermentation are an enhancement in TPC [35-37].

3.4. Antioxidant activity

According to Fig. 2, the antioxidant activity of the four types of Kombucha samples was varied (47.13 – 92.11 %). The GTK had the highest DPPH free radical scavenging capacity (92.11%). This feature can be attributed to the presence of more phenolic compounds in green tea compared to other herbal teas, as confirmed in the previous section. There was no significant difference between the antioxidant properties of BTK and GTK, unlike LVK and STK. Similar results were observed for herbal diffusions (Fig. 3). The highest and lowest DPPH scavenging activity belonged to GT (81.01 %) and LV (38.91 %), respectively. Due to the fermentation of sweetened herbal infusions during Kombucha preparation by fermenting microorganisms such as AABs, LABs and yeasts, production of various organic acids and phenolic compounds had occurred, which could increase the antioxidant capacity of the beverage. Generally, these components are active as electron donors and could scavenge the DPPH free radicals. In this regard, Jakubczyk et al. also reported various DPPH radical scavenging capacity of different Kombuchas, including green tea (88.23%), black tea (61.04%), white tea (70.42%), and red tea (74.78%) after 14 days of fermentation [38]. Malbaša et al. observed that green tea and black tea Kombucha may have different antioxidant activity depending on the type of initial culture of yeast and AABs used in Kombucha production at 28°C for 10 days [39]. The highest DPPH radical scavenging capacity of black tea and green tea Kombucha in the presence of different starter cultures was determined to be about 50 %. In addition to the concentration of phenolic compounds in Kombucha, other metabolites produced during fermentation, such as ascorbic acid and other organic acids, may also modify the antioxidant capacity of Kombucha. In addition to starter cultures and herbal infusion types, the antioxidant capacity of Kombucha is also affected by the temperature and fermentation time. In Jafari et al. research, the invertase activity was enhanced in black tea kombucha by increasing time, then the content of polyphenolic compounds and subsequently the antioxidant capacity rose to 285.44 mg GAE. L⁻¹ and 89.32%, respectively [40].

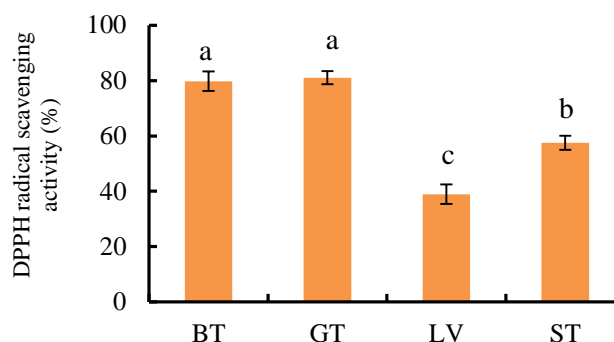


Fig 2. Antioxidant activity of various types of herbal infusion (GT: green tea, BT: black tea, LV: Lemon verbena, ST: Shirazi thyme). (Different lowercase letters on each column indicate a significant difference between treatments at $p < 0.05$.)

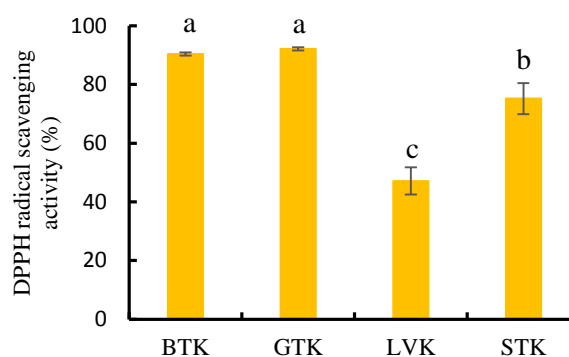


Fig 3. Antioxidant activity of various types of Kombucha beverage (GTK: green tea Kombucha, BTK: black tea Kombucha, LVK: Lemon verbena Kombucha, STK: Shirazi thyme Kombucha). (Different lowercase letters on each column indicate a significant difference between treatments at $p < 0.05$.)

3.5. Antimicrobial properties

3.5.1. Antifungal activity

In this study, the antifungal activity of Kombucha samples against two spoilage molds was evaluated by the well diffusion method. According to Fig. 4, *A. flavus* was more sensitive to the compounds of fermented Kombucha beverages compared to *A. niger*. The largest inhibition zone against *A. flavus* (20.33 mm) and *A. niger* (10.33 mm) belonged to GTK sample. Also, no significant difference was observed among other Kombucha samples against *A. niger* ($P > 0.05$). Moreover, no inhibition zone was observed for herbal infusions against both studied molds in the well diffusion test. These results are likely due to the low concentrations of phenolic compounds in the herbal infusions, indicating the need to use a greater amount of dry leaves when preparing these infusions in order to enhance their antifungal activity. In another antifungal activity evaluation method, unfermented herbal tea infusions also showed no antifungal activity. *A. niger* exhibited greater resistance to Kombucha compounds in the agar dilution method (Fig. 5).



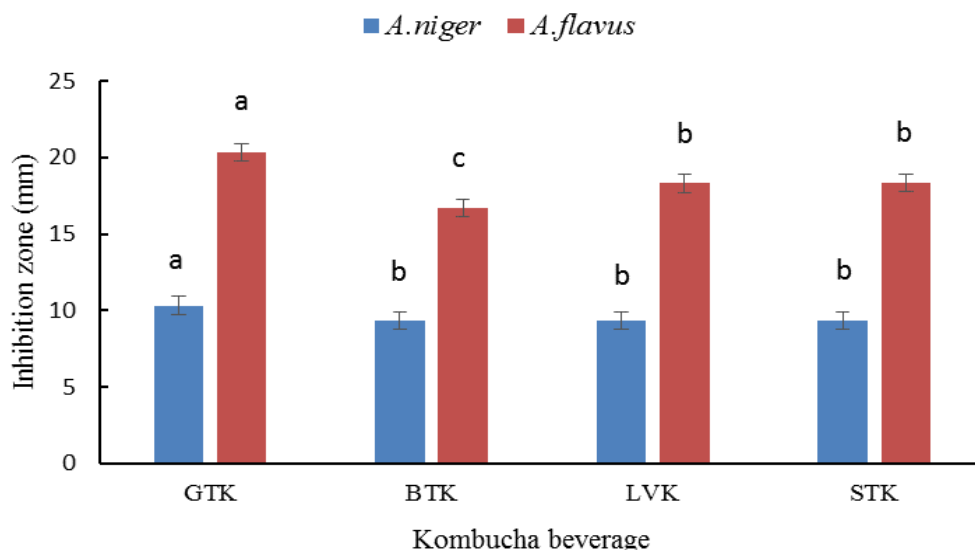


Fig 4. Antifungal activity of various types of Kombucha beverage in well diffusion method (GTK: green tea Kombucha, BTK: black tea Kombucha, LVK: Lemon verbena Kombucha, STK: Shirazi thyme Kombucha). (Different lowercase letters on each column indicate a significant difference between treatments at $p < 0.05$.)

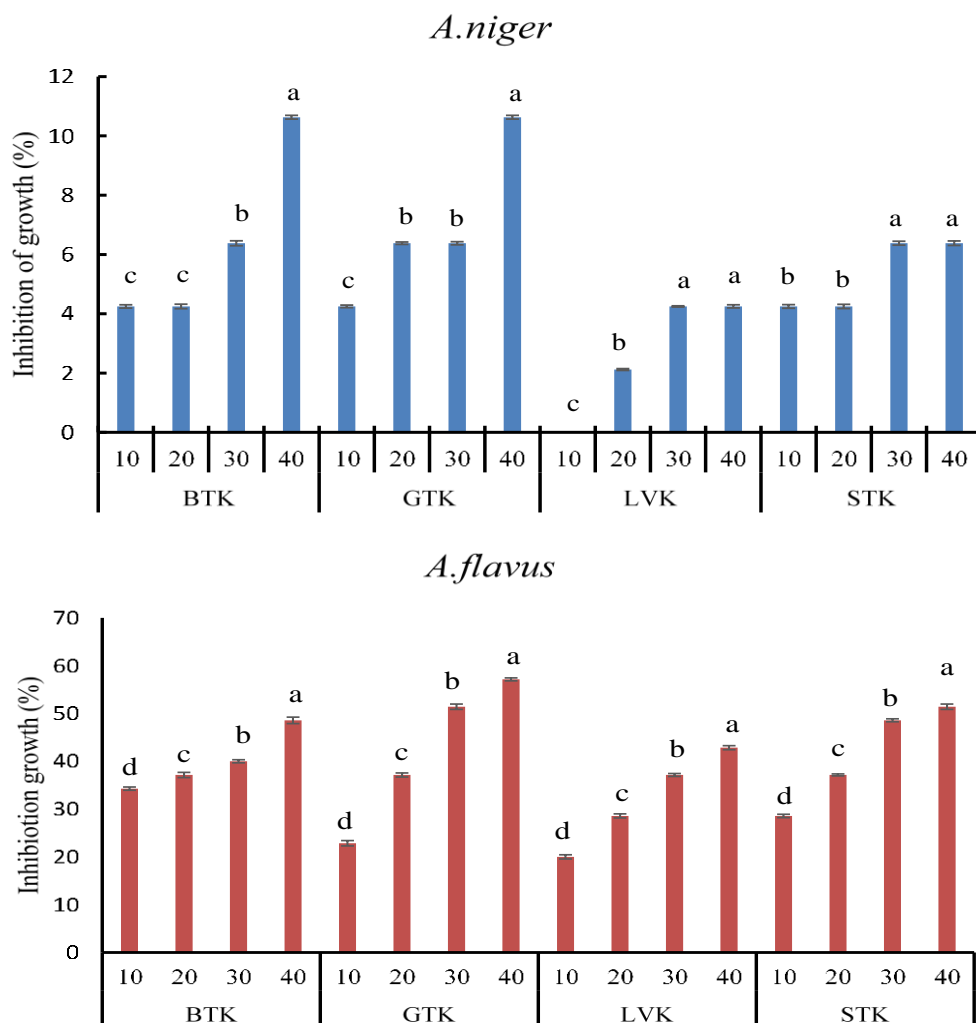


Fig 5. Antifungal activity of various types of Kombucha beverage in agar dilution method (GTK: green tea Kombucha, BTK: black tea Kombucha, LVK: Lemon verbena Kombucha, STK: Shirazi thyme Kombucha). (Different lowercase letters on each column indicate a significant difference among antifungal activity of various concentrations of each kombucha sample in medium at $p < 0.05$.)



By increasing the content of the four types of Kombucha in the solid medium from 10% to 40%, the percentage of growth inhibition was enhanced. The highest growth inhibition of *A. niger* (10.63%) occurred in the presence of 40% BTK or GTK, which is probably related to the higher organic acids and phenolic content of these types of Kombucha, as confirmed in the previous tests. In contrast, the highest growth inhibition (%) of *A. flavus* (57.14 %) occurred in the presence of 40 % GTK in the solid medium. Studies on the antifungal properties of Kombucha beverages against food-spoilage molds are limited. For example, black tea Kombucha produced in the study of Cetojevic-Simin et al. did not show any antifungal activity against *A. niger* and *A. Flavus* [41]. While in another study, the growth of *A. flavus* was inhibited in the presence of 5696 $\mu\text{g/mL}$ black tea Kombucha [42]. Also, Kombucha produced from supercritical water and yarrow extract in the microdilution method demonstrated a minimum inhibitory concentration of 1.39 $\mu\text{g/mL}$ against *A. niger* [13]. According to the report of Al-Mohammadi et al., fermented infusion, unfermented infusion, neutralized Kombucha, and heat-denatured Kombucha of black tea illustrated various antifungal activities. Black tea Kombucha showed higher antifungal activity against *A. flavus* and *A.niger* than heat-denatured Kombucha and neutralized Kombucha. While unfermented tea had no antifungal activity [43]. Generally, the antifungal mechanism of fermented Kombucha beverage is primarily attributed to its bioactive compounds, including organic acids (such as acetic acid), polyphenols, and enzymes produced during fermentation. These components collectively lower the pH, disrupt fungal cell membranes, and inhibit fungal growth through oxidative stress and metabolic interference. The synergistic effect of these metabolites creates an inhospitable environment for fungal proliferation, thereby conferring antifungal properties to Kombucha [1].

3.5.2. Antibacterial activity

According to Fig 6, in contrast to Gram-negative bacteria, no significant differences were observed in the antibacterial activity of the Kombucha samples against pathogenic Gram-positive bacteria ($p>0.05$). The highest and lowest antibacterial activities against *E. coli* and *S. enteritidis* in the well diffusion assay were associated with the GTK and LVK Kombucha samples, respectively. The Gram-positive *S. aureus*, as the most sensitive bacterium, exhibited an inhibition zone of around 22 mm in the presence of GKT components. This observation is related to the highest total phenolic content and acidity and the lowest pH of GTK sample. Moreover, no antimicrobial activity was observed against Gram-negative bacteria for non-fermented herbal tea infusions (Fig. 7).

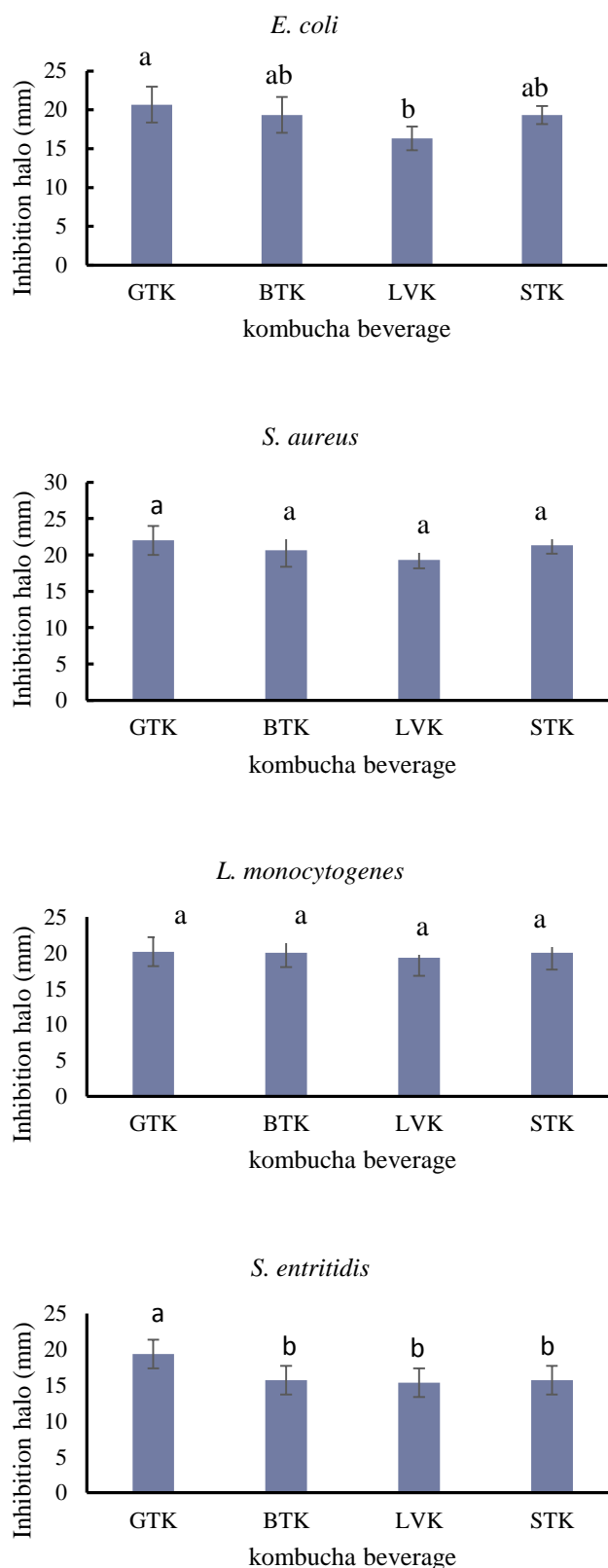


Fig 6. Antibacterial activity of various types of Kombucha beverage against pathogenic bacteria in the well diffusion method (GTK: green tea Kombucha, BTK: black tea Kombucha, LVK: Lemon verbena Kombucha, STK: Shirazi thyme Kombucha). (Different lowercase letters on each column indicate a significant difference between treatments at $p<0.05$.)



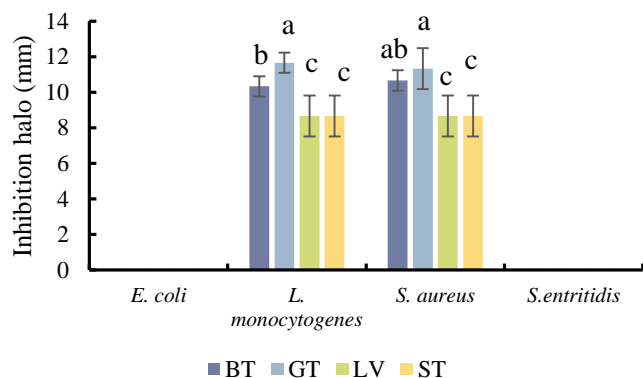


Fig 7. Antibacterial activity of various types of herbal infusion against pathogenic bacteria in well diffusion method (GT: green tea, BT: black tea, LV: Lemon verbena, ST: Shirazi thyme). (Different lowercase letters on each column indicate a significant difference between treatments at $p < 0.05$.)

The highest antibacterial activity of non-fermented herbal tea infusions against both Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) was obtained in the presence of GT and BT, with an inhibition zone ranging from 10.33 to 11.66 mm. According to these findings, fermentation of herbal infusions with Kombucha starters could improve antimicrobial characteristics. Similarly, Kombucha based on Black and green tea, lemon verbena (*Lippia citriodora*), and peppermint (*Mentha piperita*), demonstrated different antibacterial activity against Gram-positive and Gram-negative food-borne bacteria [16]. In another study, Green tea Kombucha showed higher antibacterial activity than black tea Kombucha in a microdilution assay due to the various contents of phenolic compounds and organic acids [25]. In contrast, no antibacterial activity was observed for Kombucha beverages prepared using banana peel, nettles, and black tea infusions in Ebrahimi Pure research [6]. The comparison of antimicrobial activity of fermented and non-fermented varieties of various herbal infusions (thyme, lemon verbena, rosemary, fennel, and peppermint) using the well-diffusion method against different bacterial pathogens, as well as the neutralization effect of Kombucha samples, revealed that the antibacterial activity varied depending on the type of Kombucha, and thyme Kombucha exhibited the strongest activity. Furthermore, neutralization resulted in a significant reduction in antimicrobial activity of the samples, indicating the role of organic acids in the antimicrobial properties. Also, a smaller inhibition zone was reported for unfermented herbal infusions compared to fermented Kombucha [44]. In this regard, unfermented tea and neutralized fermented tea derived from black, green, oolong, and mulberry teas showed no antibacterial activity [45]. In general, during the fermentation process of Kombucha, the levels of chemical components change rapidly, resulting in a rich concentration of organic acids, polyphenols, microbial enzymes, and bacteriocins, all of which contribute to its antimicrobial properties. Since an

aqueous extract of medicinal plants was used in the present study, the comparatively weaker antioxidant and antimicrobial performance of ST and LV infusions may be primarily attributed to the limited extraction efficiency of their key bioactive constituents under water-based conditions and also the short extraction time. Several studies have established that the major antimicrobial compounds of Thyme, particularly thymol and carvacrol, are lipophilic phenolic terpenoids that are poorly soluble in water but are extracted more effectively with organic or alcoholic solvents [46]. Similarly, for Lemon verbena, although water infusions contain some phenolic compounds, the extraction of key polyphenols such as verbascoside and more hydrophobic constituents like citral is enhanced with moderate polarity solvents (e.g., ethanol-water mixtures), yielding significantly higher total phenolic content and antioxidant capacity than water alone [47].

3.6. Correlation Analysis

Pearson's correlation analysis ($N = 4$) (Fig. 8) revealed distinct but complementary relationships among chemical, microbiological, and functional parameters in herbal infusions before fermentation and in the corresponding kombucha beverages. In herbal infusions, pH exhibited a very strong and statistically significant negative correlation with antioxidant activity ($r = -0.993$, $p = 0.007$). Strong negative correlations were also observed between pH and total phenolic content (TPC) ($r = -0.932$) as well as antibacterial activity ($r = -0.903$). These findings indicated that lower pH values were associated with higher antioxidant and antimicrobial capacity. In this regard, TPC showed positive correlation with both antioxidant ($r = 0.885$) and antibacterial activities ($r = 0.869$), suggesting that phenolic compounds are major contributors to the bioactive properties of the infusions. Following fermentation, the correlation structure became more complex due to microbial activity. In kombucha samples, TPC showed a strong and statistically significant positive correlation with antioxidant activity ($r = 0.953$, $p < 0.05$) and an exceptionally strong correlation with AAB counts ($r = 0.991$, $p < 0.01$), indicating a close link between microbial metabolism and phenolic compound availability. Total acidity was negatively correlated with pH ($r = -0.813$) and positively associated with both AAB ($r = 0.800$) and LAB ($r = 0.633$), highlighting the role of fermentation-driven acid production. Although several correlations involving antioxidant, antibacterial activity, and microbial populations were not statistically significant, their high correlation coefficients suggest biologically meaningful trends, likely constrained by the limited sample size. Overall, these results demonstrate that while the bioactivity of herbal infusions is primarily governed by inherent chemical composition, fermentation introduces microbial-driven transformations that strengthen and diversify the relationships among chemical composition, acidity, and functional properties in kombucha beverages.



A

Correlations

		pH	TPC	antioxidant	antibacterial
pH	Pearson Correlation	1	-.932	-.993**	-.903
	Sig. (2-tailed)		.068	.007	.097
	N	4	4	4	4
TPC	Pearson Correlation	-.932	1	.885	.869
	Sig. (2-tailed)	.068		.115	.131
	N	4	4	4	4
antioxidant	Pearson Correlation	-.993**	.885	1	.868
	Sig. (2-tailed)	.007	.115		.132
	N	4	4	4	4
antibacterial	Pearson Correlation	-.903	.869	.868	1
	Sig. (2-tailed)	.097	.131	.132	
	N	4	4	4	4

** . Correlation is significant at the 0.01 level (2-tailed).

B

Correlations

		pH	acidity	TPC	antioxidant	antibacterial	antifungal	yeast	AAB	LAB
pH	Pearson Correlation	1	-.813	-.498	-.347	-.098	.129	-.521	-.524	-.664
	Sig. (2-tailed)		.187	.502	.653	.902	.871	.479	.476	.336
	N	4	4	4	4	4	4	4	4	4
acidity	Pearson Correlation	-.813	1	.732	.504	.151	-.615	-.028	.800	.633
	Sig. (2-tailed)	.187		.268	.496	.849	.385	.972	.200	.367
	N	4	4	4	4	4	4	4	4	4
TPC	Pearson Correlation	-.498	.732	1	.953*	.770	-.250	.060	.991**	.903
	Sig. (2-tailed)	.502	.268		.047	.230	.750	.940	.009	.097
	N	4	4	4	4	4	4	4	4	4
antioxidant	Pearson Correlation	-.347	.504	.953*	1	.927	.020	.192	.905	.917
	Sig. (2-tailed)	.653	.496	.047		.073	.980	.808	.095	.083
	N	4	4	4	4	4	4	4	4	4
antibacterial	Pearson Correlation	-.098	.151	.770	.927	1	.339	.309	.681	.806
	Sig. (2-tailed)	.902	.849	.230	.073		.661	.691	.319	.194
	N	4	4	4	4	4	4	4	4	4
antifungal	Pearson Correlation	.129	-.615	-.250	.020	.339	1	.778	-.372	.114
	Sig. (2-tailed)	.871	.385	.750	.980	.661		.222	.628	.886
	N	4	4	4	4	4	4	4	4	4
yeast	Pearson Correlation	-.521	-.028	.060	.192	.309	.778	1	-.027	.483
	Sig. (2-tailed)	.479	.972	.940	.808	.691	.222		.973	.517
	N	4	4	4	4	4	4	4	4	4
AAB	Pearson Correlation	-.524	.800	.991**	.905	.681	-.372	-.027	1	.858
	Sig. (2-tailed)	.476	.200	.009	.095	.319	.628	.973		.142
	N	4	4	4	4	4	4	4	4	4
LAB	Pearson Correlation	-.664	.633	.903	.917	.806	.114	.483	.858	1
	Sig. (2-tailed)	.336	.367	.097	.083	.194	.886	.517	.142	
	N	4	4	4	4	4	4	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).
 **. Correlation is significant at the 0.01 level (2-tailed).

Fig 8. Pearson's correlation analysis among chemical, microbiological and functional properties of herbal infusions (A) and kombucha beverages (B).



4. Conclusion

The findings of this study demonstrate that the type of herbal infusion used in Kombucha production plays a decisive role in determining its microbiological profile as well as its functional and antimicrobial properties. Across all formulations, AAB constituted the dominant microbial population, followed by LAB and yeasts, reflecting a stable and well-balanced fermentation ecosystem. The fermentation process markedly enhanced the biochemical characteristics of the beverages, resulting in increased total phenolic content, antioxidant activity, and acidity compared to the corresponding non-fermented herbal infusions. Among the evaluated samples, green tea Kombucha exhibited the most pronounced functional performance, particularly with respect to antioxidant capacity and antimicrobial activity against pathogenic bacteria and spoilage molds. In contrast, lemon verbena and thyme Kombuchas displayed relatively lower bioactive potential, although fermentation still led to notable improvements over their original infusions. These findings highlight Kombucha fermentation as an effective strategy for enhancing the nutritional and antimicrobial properties of herbal beverages. However, further studies are required to support their safe consumption and commercial development. Future research should prioritize cytotoxicity and safety evaluations, and minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC), as well as chemical and sensory analysis studies to determine palatability and market potential of these functional beverages.

5. Declaration

5.1. Acknowledgements

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5.2. Declaration of competing interest

The authors declare no competing interests.

5.3. Authors' Contributions

Dina Shahrapour: Conceptualization; methodology; software; validation and formal analysis; investigation; project administration; funding acquisition; writing (original draft preparation, Sara Naji-Tabasi: Conceptualization; methodology; data curation; supervision; writing) review and editing.

5.4. Using Artificial Intelligent Chatbots

Artificial Intelligence (AI) based tools were used solely for language editing and improving clarity during manuscript preparation; all scientific content and interpretations were provided by the authors. Also, some parts of the graphical abstract are designed by AI.

5.5. Ethical Consideration

This manuscript does not contain any studies involving human participants or animals performed by the authors.

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تأثیر انواع مختلف دمنوش‌های گیاهی بر ویژگی‌های عملکردی نوشیدنی تخمیری کامبوچا: خواص ضدباکتریایی، ضدکپکی و آنتی‌اکسیدان

دینا شهرام‌پور^{۱*}، سارا ناجی طبسی^۲

۱. گروه پژوهشی ایمنی و کنترل کیفیت مواد غذایی، موسسه پژوهشی علوم و صنایع غذایی، مشهد، ایران.

۲. گروه پژوهشی نانوفناوری مواد غذایی، موسسه پژوهشی علوم و صنایع غذایی، مشهد، ایران.

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نویسنده مسئول

دینا شهرام‌پور

تلفن: ۰۵۱-۳۵۴۲۵۳۵۱

پست الکترونیک:

d.shahrampour@rifst.ac.ir

چکیده

سابقه و هدف: براساس پیشینه پژوهش نوع بستره، شرایط تخمیر و فلورمیکروبی می‌تواند بر ویژگی‌های عملکردی نوشیدنی تخمیری موثر باشد. بنابراین، این مطالعه با هدف مقایسه خواص میکروبیولوژیکی، شیمیایی، آنتی‌اکسیدانی و ضد میکروبی نوشیدنی‌های کامبوچای تولید شده از چهار دمنوش گیاهی (چای سبز، چای سیاه، به لیمو و آویشن شیرازی) و بررسی تأثیر تخمیر بر تغییر ویژگی‌های دمنوش‌های گیاهی انجام شد.

مواد و روش‌ها: دمنوش‌های گیاهی مختلف پس از تلقیح کشت آغازگر کامبوچا در دمای ۲۸ درجه سانتیگراد، به مدت ۱۲ روز تخمیر شدند. ترکیبات فنلی کل و فعالیت آنتی‌اکسیدان به ترتیب به روش فولین سیکالتیو و مهارکنندگی رادیکال‌های آزاد DPPH قبل و بعد از تخمیر دمنوش‌های گیاهی اندازه‌گیری شدند. همچنین فعالیت ضدباکتریایی علیه باکتری‌های گرم مثبت و گرم منفی بیماری‌زا با روش انتشار چاهک ارزیابی شد. فعالیت ضدقارچی دمنوش‌های گیاهی مختلف و نوشیدنی‌های کامبوچا علیه *آسپرژیلوس نایجر* و *آسپرژیلوس فلاووس* با روش‌های رقت در آگار و انتشار چاهک مورد آزمون قرار گرفت.

یافته‌ها و نتیجه‌گیری: pH دمنوش‌های گیاهی مختلف پس از تخمیر به طور قابل توجهی کاهش یافت. ترکیبات فنلی کل دمنوش‌ها گیاهی به طور معناداری متفاوت بود و در طول تخمیر به طور قابل توجهی افزایش یافت و در نمونه‌های کامبوچا به ۱۵۳،۳۸-۶۵،۸۲ mg GAE/L رسید و کامبوچای چای سبز بالاترین مقدار را نشان داد. برطبق ارزیابی فلور میکروبی، بالاترین جمعیت میکروبی در تمام نمونه‌های کامبوچا متعلق به باکتری‌های اسید استیک (10^6-10^7 CFU/mL) و پس از آن باکتری‌های اسید لاکتیک (10^4-10^5) و مخمرها (10^5 CFU/mL) بودند. ارزیابی خواص آنتی‌اکسیدانی نشان داد که دمنوش چای سبز و کامبوچای حاصل از آن قوی‌ترین ظرفیت مهار رادیکال‌های آزاد DPPH را دارند. برخلاف دمنوش‌های گیاهی، نوشیدنی‌های کامبوچا فعالیت ضدکپکی و ضد باکتریایی نشان دادند. بر طبق یافته‌های این مطالعه، تخمیر به طور قابل توجهی خواص عملکردی، آنتی‌اکسیدانی و ضد میکروبی دمنوش‌های گیاهی را افزایش داد و کامبوچای چای سبز به عنوان یک نگهدارنده غذایی کاربردی بسیار امیدوارکننده معرفی شد.

واژگان کلیدی: دمنوش گیاهی، نوشیدنی عملگرا، تخمیر، آنتی‌اکسیدان، فعالیت ضد میکروبی