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Original Research

Six Herbal Kombucha Varieties: Physicochemical Composition, Antioxidant Activity, and Antimicrobial Properties

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Abstract

Background: Currently, Kombucha is one of the most popular fermented beverages, produced by fermenting sweetened tea with yeast and acetic acid bacteria, which generate a variety of bioactive compounds. Researchers continue to look for new infusion bases for kombucha production. Thus, this study aimed to test six herbs rich in health-promoting substances: field horsetail, linden, chamomile, nettle, St. John's wort, and rockrose. The aim was to characterize the qualitative and quantitative composition and to assess the antioxidant and antimicrobial properties of kombuchas prepared from these herbs. **Methods:** The study included measurements of pH, sugar content, biomass growth, microbial counts, antimicrobial activity, Fourier transform infrared (FTIR) spectra, ethanol and organic acid concentrations, antioxidant activity, and hydroxyl radical scavenging capacity. **Results:** All six herbs were suitable substrates for kombucha fermentation, yielding beverages enriched with antimicrobial and antioxidant compounds. Rockrose kombucha exhibited the strongest hydroxyl radical scavenging capacity (87.5% on day 1), high antioxidant activity (>80% on day 7), and antimicrobial effects. Moreover, this drink had the highest succinic acid concentration. Each herb created a distinct environment for the bacterial–fungal consortium, resulting in marked differences in the properties and composition of the beverages.

Keywords: antibiotics; antioxidant activity; herbal; fermentation; beverages

1. Introduction

Kombucha is produced by fermenting sweetened tea with a consortium of acetic acid bacteria and osmophilic yeasts, resulting in the formation of various bioactive compounds. The fermenting microorganisms, suspended in a cellulose matrix, create a symbiotic culture of bacteria and yeast (SCOBY) that forms on the surface of the drink and resembles a transparent gel sheet [1,2].

The products of the activity, both bacteria and yeasts, contribute to the aroma, taste, and health-promoting properties of kombucha. During fermentation, sucrose serves as the primary substrate for yeasts and is hydrolyzed by invertase into glucose and fructose. In subsequent stages of the process, yeasts produce ethanol, which is metabolized by acetic acid bacteria to acetic acid and numerous other organic acids and vitamins, including compounds with antioxidant properties [3,4].

The primary compounds produced by microorganisms during kombucha fermentation are organic acids. Acetic acid imparts the characteristic sour taste and aroma of kombucha. Meanwhile, glucuronic acid is considered an important antioxidant that neutralizes free radicals in the body by participating in glucuronidation processes [3,5]. Another organic acid, gluconic acid, produced via a pathway similar to that of glucuronic acid in glucose oxidation, contributes to the acidity and pleasant aftertaste of kombucha [6]. Succinic acid, another byproduct of sugar fermentation, imparts sour, bitter, and even salty flavors to the fermented beverages [7].

Polyphenols are important antioxidant compounds present in kombucha. In response to oxidative stress, polyphenols support the activity of enzymes and cell receptors. During kombucha fermentation, the levels of polyphenols naturally occurring in tea increase, enhancing the free-radical scavenging capacity [3].

Because this beverage contains antimicrobial compounds, kombucha is also considered a potential alternative to antibiotics. These properties are largely associated with the lowered pH of the beverage, resulting from the presence of acetic acid. Previous research on antimicrobial properties has shown that kombucha prepared from green or black tea infusions exhibits a broad-spectrum activity against bacteria and fungi. Considerably fewer studies have investigated kombucha produced from other infusions, although antimicrobial effects have been reported for kombucha made with infusions of yarrow, lemon balm, fennel, thyme, peppermint, and rosemary [8,9].

Recent research has shown that incorporating medicinal plant infusions into the fermentation process may increase the levels of compounds that alleviate oxidative stress. Elevated concentrations of antioxidant compounds have been observed in kombucha produced from yarrow,

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lemon balm, and mint. Thus, the analysis of additional herbs with well-documented positive effects on human health represents an interesting alternative to research focused solely on commonly used plant infusions [10,11].

The kombuchas analyzed in the present study were produced from six herbs whose health-promoting properties have been demonstrated in previous studies. For example, horsetail, rich in phenolic acids, flavonoids, tannins, and alkaloids, is considered to possess free radical-scavenging, antimicrobial, anti-inflammatory, and anticancer activities [12]. Meanwhile, linden flowers possess antioxidant properties due to a high phenolic content [13]. Chamomile is known for possessing anti-inflammatory, anti-allergic, and antioxidant properties [14]. Nettle also exhibits antioxidant and antimicrobial properties; indeed, nettle leaves contain numerous bioactive substances, particularly high concentrations of phenolic compounds [15]. St. John's wort is another herb with a chemical composition suggesting potential anti-inflammatory, antimicrobial, and antioxidant effects that may help prevent neurodegenerative diseases [16]. Rockrose, which contains bioactive compounds such as polyphenols and flavonoids, is known for its antimicrobial, antiviral, and antioxidant properties [17].

Therefore, this study was designed to identify beverages characterized by high antioxidant activity, pronounced antibacterial properties, and elevated levels of compounds considered beneficial to human health. Products obtained by fermenting herbal infusions may help mitigate oxidative stress in the human body or serve as an alternative to antibiotics. Thus, the study aimed to analyze the qualitative and quantitative composition, as well as the antioxidant and antibacterial properties, of kombucha prepared from herbs rarely used for this purpose, field horsetail, linden, chamomile, nettle, St. John's wort, and rockrose (*Cistus* × *incanus*), using green tea kombucha as a comparison.

2. Materials and Methods

2.1 Kombucha Preparations

Step I.

A green tea infusion (Milton Original, 8 bags, 1.75 g each) was prepared using 900 mL of water at 90 °C and sweetened with 100 g of sucrose p.a. (Chempur, Poland). A commercially available kombucha sheet with a diameter of 9 cm was introduced into the infusion along with 100 mL of liquid kombucha from which the SCOBY originated (Daveros, Poland). Fermentation was performed for 20 days in an incubator (BINDER, Germany) at 28 °C in four replicates. The incubation was conducted for 20 days to produce a sufficient amount of SCOBY for subsequent fermentation of the herbal infusions.

Step II.

Infusions of green tea (Milton Original, 4 bags of 1.75 g) and six herbs were prepared: field horsetail (Herbapol, 4 bags of 1.8 g); linden (Herbapol, 4 bags of 1.2 g); chamomile (Zielnik, 4 bags of 1 g); nettle (Zielnik 4 bags of 1.8 g); St. John's wort (Biofix 4 bags of 1.5 g); rockrose (*Cistus* × *incanus*; FitoApteka 6 g of loose product) in accordance with the infusion preparation method recommended by the manufacturers. Each tea and herb was steeped in 450 mL of boiling water (100 °C), except for green tea, which was steeped in water at 90 °C according to the packaging instructions. Each infusion was supplemented with 50 g of sucrose, 50 mL of 20-day liquid kombucha, and 30 g of cultured kombucha sheet.

Step III.

The entire process was repeated from the beginning, following the same procedure as in Step II, using the liquid and SCOBY obtained in that step as the inoculum.

Step IV.

The kombucha sheets and liquid starter from Step III were used to inoculate fresh infusions based on the same herb or green tea and fermented for 14 days. The fermentation duration was selected based on preliminary optimization and literature data indicating peak bioactivity between 7 and 14 days. The proportions of the beverage components were the same as in Steps II and III. Each kombucha beverage was prepared in triplicate.

2.2 Sampling Kombucha for Analysis

From each kombucha, a 20 mL sample was taken for analysis on the following fermentation days: 0 (infusion only, before inoculation with SCOBY), 1 (after inoculation), 3, 7, 10, and 14. The collected samples were centrifuged (MPW-260R laboratory centrifuge, Poland) for 10 minutes, at 6300 rpm at 4 °C to separate the microbial biomass, and the supernatants were subjected to further analyses.

2.3 Measurement of Kombucha pH

The pH of the kombuchas was measured on days 0, 1, 3, 7, 10, and 14 using a pH meter (HANNA Instruments, Cluj-Napoca, Romania). The final value for each day was the mean of three measurements.

2.4 Determination of Sugar Content

Sugar content was measured using a refractometer (ABBE Digital Refractometer, MRC Laboratory-Instruments, UK). The reported values are the mean of three measurements. The results are expressed in sucrose units (°Brix).

2.5 Measurement of Kombucha Biomass Growth

Biomass growth was determined on day 14 of fermentation by weighing the SCOBY matrix filtered from the kombuchas on a laboratory scale (RADWAG PS 2100x2, Poland) and subtracting the initial mass (30 g). The result was expressed in grams as the mean of three measurements.

2.6 Microbiological Analysis of Cultures

Kombucha samples taken on day 10 of fermentation and diluted with 0.9% NaCl were used for inoculation. From each suspension, 200 µL was spread on plates. The number of lactic acid bacteria was assessed using the plate technique on de Man, Rogosa and Sharpe medium (MRS agar) (10 g/L meat extract, 5 g/L yeast extract, 10 g/L peptone, 2 g/L K_2HPO_4, 2 g/L dibasic ammonium citrate, 5 g/L sodium acetate × $3H_2O$, 5 mL salt solution A, 20 g/L glucose, 1 mL Tween 80, 15 g/L agar; pH 6.5) after incubation at 37 °C for 48 h. Yeast counts were performed using Yeast Extract-Glucose-Chloramphenicol (YGC) medium (5 g/L yeast extract, 20 g/L glucose, 0.1 g/L chloramphenicol, 15 g/L agar; pH 6.5). Cultures were incubated at 25 °C for 5 days. The number of acetic acid bacteria was determined after 72 h of incubation at 28 °C on plates with a medium selective for these bacteria (50 mg/L actidione, 0.3 g/L casein peptone, 0.3 g/L yeast extract, 0.7 g/L calcium carbonate, 2 g/L glucose, 20 g/L agar, 20 mL ethanol 96%; pH 5.0). Reagents for media preparation were purchased from ChemPur, Poland; POCH, Poland; CALBIOCHEM, USA; Sigma-Aldrich, USA; Merck, Germany. All reagents were of chemical purity (p.a.). The number of colonies on each plate was counted using a bacterial counter (ChemLand, Poland) and expressed as bacteria per 1 mL of kombucha. Each count was performed in triplicate.

2.7 Assessment of Antimicrobial Activity

The antimicrobial activity of herbal and green tea kombucha was evaluated by the diffusion method against two standard bacterial species: *Escherichia coli* (*E. coli*) (PCM 431) and *Staphylococcus aureus* (*S. aureus*) (PCM 2602). For this purpose, 24-hour cultures of the selected bacterial strains were adjusted to match a 0.5 McFarland turbidity standard using a Grant DEN-1 densitometer. Then, 100 µL of the target-strain suspension was transferred onto Mueller–Hinton medium. Sterile wells/cylinders with a diameter of 8 mm were placed on Petri dishes inoculated with the selected strains. Then, 100 µL of the prepared kombucha was added to each well; the sterile kombucha supernatant was obtained by filtration through a Millex-GV microfilter (0.22 µm pore size; cat. no. SLGV033RB, Merck Millipore, Darmstadt, Germany) using a sterile filtration apparatus. Two variants were tested: kombucha adjusted to pH 7.0 (using 1 M NaOH) and kombucha at the native acidic pH. Distilled water was used as the negative control. Plates were incubated at 37 °C for 18 h. After incubation, the diameter of the growth inhibition zone around each cylinder was measured. The measurements were compared with previously obtained antibiograms for the reference strains, which were the positive control. The drug susceptibility of *S. aureus* was tested against penicillin (P, 1 unit), cefoxitin (FOX, 30 µg), gentamicin (CN, 10 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), and levofloxacin (LEV, 5 µg). For the *E. coli* strain, the following drugs were used: imipenem (IMP, 10 µg), levofloxacin (LEV, 5 µg), tigecycline (TGC, 15 µg), piperacillin (PRL, 30 µg), cefoxitin (FOX, 30 µg), and gentamicin (CN, 10 µg). Antibiograms were performed using the disk diffusion method in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for one representative antibiotic from each antibiotic class.

2.8 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Kombucha samples were filtered through 0.45 µm syringe filters and qualitatively analyzed at 25 °C using a Thermo Scientific Nicolet iS5 FTIR spectrometer with an iD5 ATR accessory (Thermo Fisher Scientific, Waltham, MA, USA). The presence of the main functional groups in kombucha samples was analyzed over the range 400–4000 cm^{-1} at a resolution of 2 cm^{-1} (36 scans per sample).

2.9 Determination of Ethanol and Organic Acid Concentrations by High-Performance Liquid Chromatography (HPLC)

The concentrations of ethanol, acetic acid, lactic acid, succinic acid, and glucuronic acid were determined by HPLC. Analyses were performed using an Agilent Technologies model 1260 system (Santa Clara, CA, USA) with a refractometric detector and a Hi-Plex H column (Agilent Technologies, Santa Clara, CA, USA) at 60 °C, with a mobile-phase flow rate of 0.6 mL/min. Quantification was performed using external standards and calibration curves. The concentrations of gluconic acid and ascorbic acid were determined by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) with spectrophotometric detection on an Agilent Technologies 1220 liquid chromatograph equipped with a DAD detector, following Li et al. [18] with modifications. A Poroshell 120 EC-C18 column with a guard column (Agilent Technologies) was used at 30 °C with an isocratic mobile phase of 6 mM H_3PO_4 (pH 2.1): methanol (97:3) at a flow rate of 0.5 mL/min. Detection was performed at λ = 220 nm.

2.10 Antioxidant Activity Analysis by DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

Antioxidant activity was determined using the DPPH assay (Sigma-Aldrich, USA) as described by Xu et al. [19], with modifications. A total of 1 mL of the tested solution was pipetted into glass test tubes, and 1 mL of 0.02 mM DPPH in methanol (p.a., ChemPur, Poland) was added to each sample. Aliquots of 200 µL were then transferred to a 96-well plate. After a 30-minute incubation, absorbance was measured using a multi-detection reader (Synergy HTX multi-mode reader, Biotek, Winooski, VT, USA) at λ = 517 nm. Ascorbic acid (1 mg/mL) was used as a positive control in antioxidant assays, and sterile distilled water served as a negative control.

The percentage antioxidant activity was calculated from the following formula:

Antioxidant activity (%) = [1 – (A517 sample / A517 control)] × 100.

A517 sample: sample absorbance at λ = 517 nm.

A517 control: control absorbance at λ = 517 nm.

Each measurement was performed in triplicate.

2.11 Assessment of Hydroxyl Radical Scavenging Ability

The ability to scavenge free hydroxyl radicals was analyzed using a method based on the reaction of Fe^{3+} ions with 1,10-phenanthroline. Diluted samples were mixed in a test tube with 1 mL of 2.5 mM $FeSO_4$ (ChemPur, Poland), followed by the addition of 1 mL of 20 mM H_2O_2 (ChemPur, Piekary Slaskie, Poland). The samples were then incubated at room temperature for 10 minutes. After incubation, 1 mL of 2.5 mM 1,10-phenanthroline (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample. The control sample contained 1 mL of water, 1 mL of $FeSO_4$, 1 mL of water, and 1 mL of 1,10-phenanthroline (incubated as in the tested samples). A blank sample was prepared by mixing 3 mL of distilled water with 1 mL of 2.5 mM 1,10-phenanthroline. Aliquots of 200 µL were transferred to a 96-well plate, and absorbance was measured with a multi-detection reader at λ = 510 nm [20]. Measurements were performed in triplicate. The hydroxyl radical scavenging ability was calculated as follows:

$$\text{Hydroxyl radical scavenging ability (\%)} = \frac{\text{As}}{\text{Ac}} \times 100\%$$

As: sample absorbance at λ = 510 nm.

Ac: control sample absorbance at λ = 510 nm.

2.12 Statistical Analysis

Statistical analysis was performed using Statistica 13.1 (StatSoft, Tulsa, OK, USA) and Excel version 2301 (2023, Microsoft Corporation, Redmond, WA, USA). Data in the figures are presented as mean ± standard deviation. The following quantitative variables were analyzed at each time point of the fermentation of green tea and herbal infusions: antioxidant activity according to DPPH (%); hydroxyl radical scavenging ability (%); concentrations of ethanol, acetic acid, succinic acid, glucuronic acid, gluconic acid, and ascorbic acid (g/L); yeast count, acetic acid bacteria count, and increase in kombucha biomass (g). The normality of the variables was assessed using the Shapiro–Wilk W test. One-way analysis of variance (ANOVA) was applied to assess main effects, followed by Tukey's honestly significant difference (HSD) test. Statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1 Fermentation Dynamics (pH, Sugar Consumption, and Metabolite Production)

Kombucha contains compounds that exert beneficial effects on human health. Researchers are continuously seeking new beverages that can serve as alternative substrates for kombucha production. Thus, this study aimed to determine whether infusions prepared from six selected herbs would exhibit increased contents of antioxidant compounds and enhanced antimicrobial activity after inoculation with SCOBY. The successful formation of SCOBY indicates that the tested herbal infusions can support the development of a microbial consortium comparable to that obtained with traditional tea substrates. The use of non-traditional base beverages for kombucha production is currently the subject of numerous studies. In addition to fruit juices and herbal infusions, ingredients such as seeds, microalgae, and mushrooms are being investigated as fermentation substrates to enhance health-promoting properties [21].

The initial pH of the green tea and herbal infusions ranged from approximately 6.9 to 7.2 (Fig. 1). However, already on the first day of fermentation, *i.e.*, after inoculation with kombucha, a significant increase in acidity (pH ~3) was observed in all beverages. Finally, by day 14 of the process, the pH had decreased further, reaching 2.5 (kombucha based on horsetail, chamomile, nettle, St. John's wort), 2.6 (kombucha based on green tea and linden), and 2.7 (rockrose kombucha).

The pH is a key parameter that describes the fermentation process. Our pH measurements are consistent with those reported by other authors, who also found values around 7.0 for unfermented teas and herbs, decreasing to ~3.0 in finished kombucha [22,23]. Naturally, minor differences exist, as in the study by Kayisoglu and Coskun [23]. These deviations may be due to differences in fermentation methods or the composition of active microorganisms.

The sugar content in beverages without kombucha was above 11 (°Brix) and was similar in all drinks. From day 3 to 14 of fermentation, a significantly lower sugar content was observed in kombucha prepared with nettle, St. John's wort, and rockrose compared with the other fermented beverages. The largest reductions in sugar content were observed in nettle kombucha (~20%), rockrose kombucha (>10%), and St. John's wort kombucha (~8%). Conversely, an increase in sugar content was observed in fermented drinks based on green tea, horsetail, linden, and chamomile (Fig. 2). This trend contradicts the expected course of fermentation and is not clearly explained or systematically compared.

Sugars are the primary carbon source for the microorganisms that form the SCOBY [24]. In our study, sugar content decreased in kombucha prepared from nettle, rockrose, and St. John's wort. After 14 days of black tea kombucha fermentation, Jakubczyk et al. [2] observed a 32%

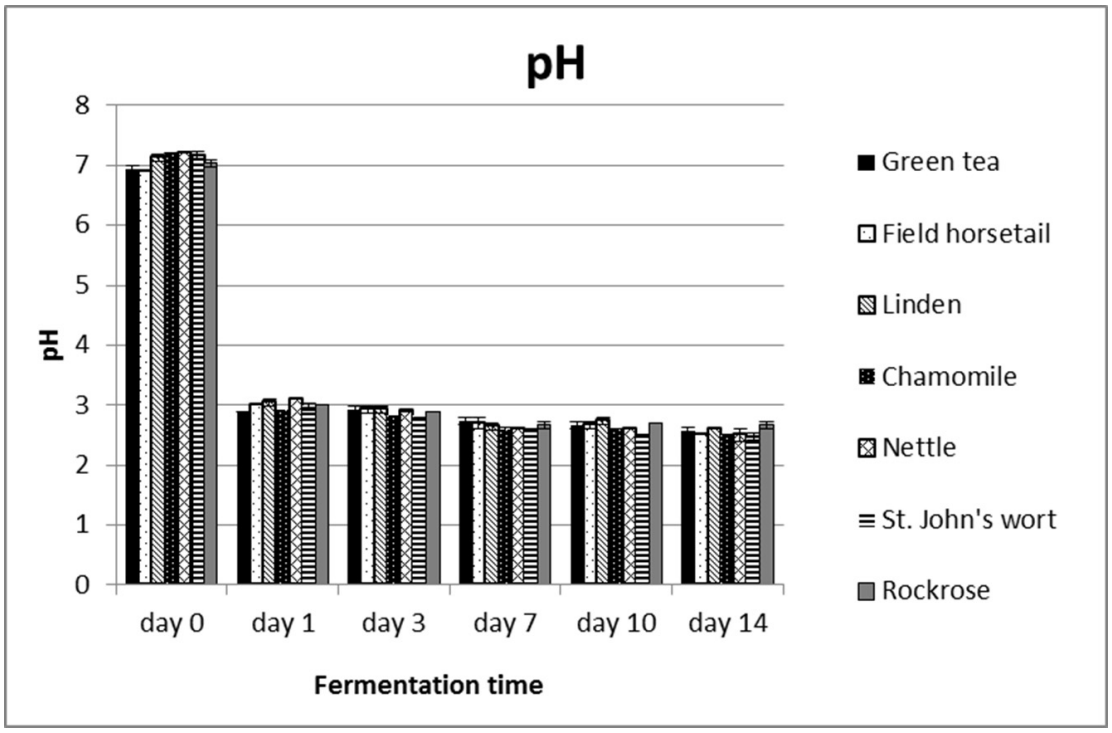


Fig. 1. Changes in the pH of the infusions and the subsequent prepared kombucha over 14 days of fermentation.

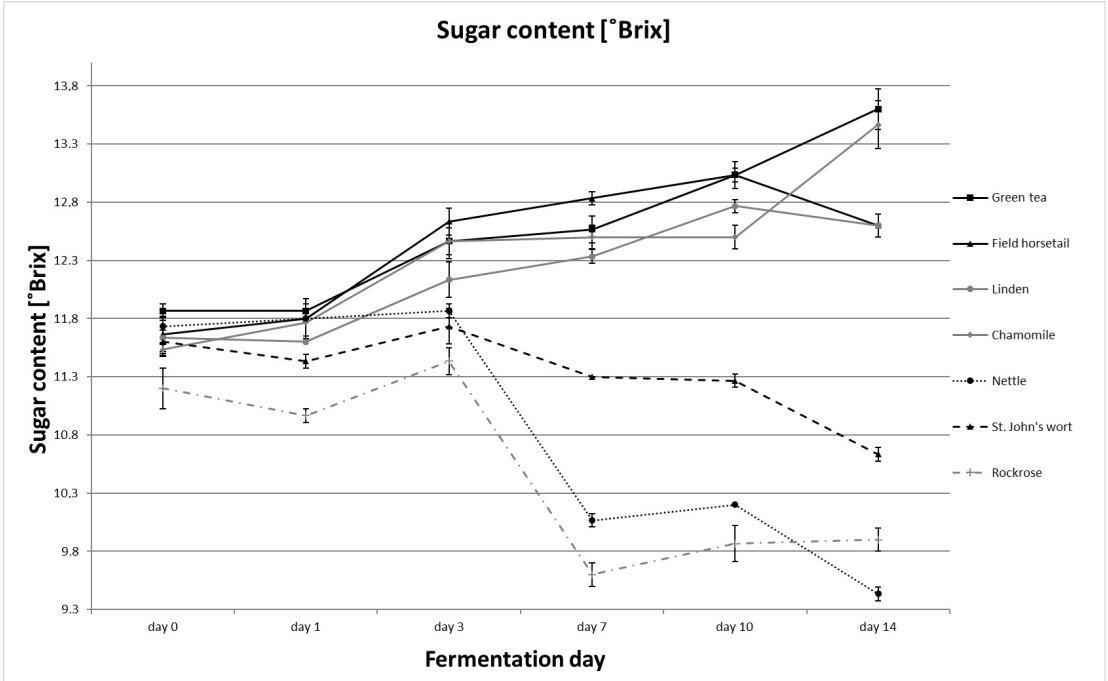


Fig. 2. Sugar content in infusions and the resulting kombucha prepared during a 14-day fermentation.

decrease in sugar content. However, the same authors reported that in green and red tea kombucha, after an initial decrease, the reducing sugar content had increased by day 7. Our study showed that the sugar content in kombucha made from green tea, horsetail, linden, and chamomile increased during fermentation up to day 10. Cohen et al. [25] also did not observe the expected decreases in sugar content during black tea fermentation and concluded that the sucrose concentrations used in their study may have exceeded the requirements of the microorganisms.

In kombucha based on linden, chamomile, nettle, rockrose, St. John's wort, and green tea, ethanol concentrations increased as fermentation progressed (Fig. 3A). The highest ethanol concentration was found in nettle kom-

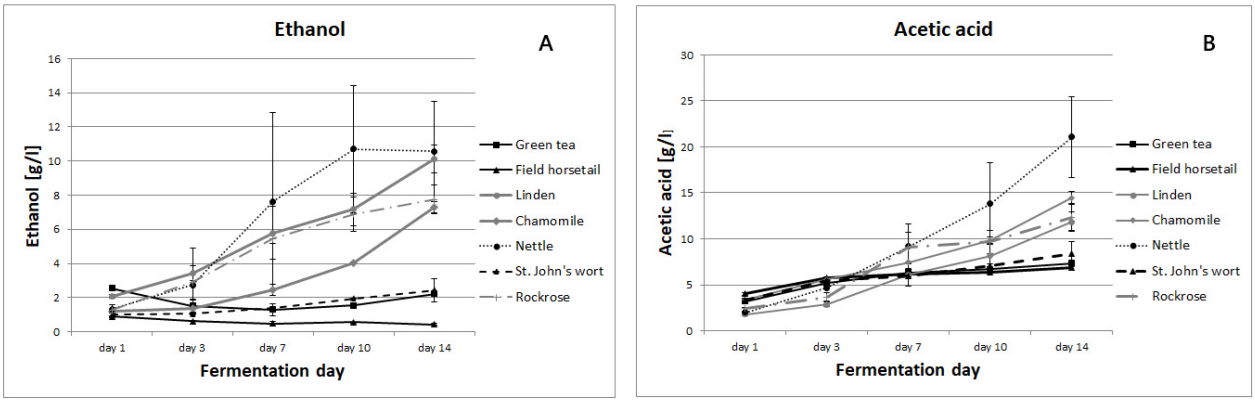


Fig. 3. Ethanol (A) and acetic acid concentrations (B) in infusions and in the kombucha prepared from them during a 14-day fermentation.

bucha, and the lowest in horsetail kombucha, with a ~20-fold difference on days 10 and 14 of fermentation ($p < 0.05$; **Supplementary Tables 1,2**).

Fermentation of the analyzed beverages generally increased the ethanol concentration. Some of the herbal infusions showed relatively high alcohol concentrations (>6 g/L) compared with green tea and with values reported for this beverage by other authors [26]. However, in those studies, the fermentation was conducted for a shorter period; therefore, it is reasonable to assume that similar final ethanol concentrations would eventually have been reached. Thus, the ethanol concentrations observed in the herbal teas in our study may indicate high yeast activity, possibly supported by compounds present in the infusions.

No acetic acid was found in green tea or in herbal infusions that were not inoculated with kombucha. The acetic acid concentration increased as fermentation progressed (Fig. 3B). The highest concentrations of this compound were observed on day 14 of the process in kombucha based on nettle (21.09 ± 4.403), chamomile (14.46 ± 0.712), rockrose (12.36 ± 1.469), linden (11.87 ± 1.095), St. John's wort (8.34 ± 1.328), green tea (7.34 ± 0.148) and horsetail (6.90 ± 0.367) ($p < 0.05$; **Supplementary Tables 1,2**). Lactic acid was not detected in any of the kombucha samples analyzed. Almost all fermented herbal infusions showed higher acetic acid production than green tea. This result is consistent with the data presented by Vitas et al. [10], who reported elevated acetic acid content in infusions of elderberry, winter savory, and peppermint compared with a tea infusion. Therefore, it can be concluded that the substrates used to produce kombucha clearly influence the course and dynamics of the fermentation process.

Analysis of organic acid concentrations, which are considered to contribute to the antioxidant properties of kombucha, showed that their profiles differed among infusions. Succinic acid was found in the infusions and fermented beverages based on linden, horsetail, St. John's wort, green tea, and rockrose (Fig. 4A). The concentration of this compound increased as fermentation progressed. On day 7 of fermentation, the highest succinic acid concentration was found in rockrose kombucha (10.57 ± 0.58) and the lowest in green tea kombucha (1.11 ± 0.03) ($p < 0.05$; **Supplementary Tables 1,2**). The highest succinic acid concentration observed in rockrose kombucha was almost ten times higher than that found in green tea kombucha. Therefore, rockrose kombucha can be considered the best source of succinic acid among the infusions analyzed. Succinic acid is believed to have antimicrobial and antiallergic properties [27]. Kaewkod et al. [5] examined kombucha based on green, black, and oolong tea, and, similar to our study, did not detect succinic acid in all beverages, reporting the presence of succinic acid only in green tea kombucha. The succinic acid concentration (3.05 g/L) reported by the authors for black tea kombucha was much lower than that observed in rockrose kombucha in our study (>10 g/L).

In both non-inoculated infusions and kombucha produced from all analyzed substrates, no glucuronic acid was detected until day 3 of the experiment (Fig. 4B). By day 10, there was a significant increase in glucuronic acid production compared to day 3 (**Supplementary Tables 1,2**), especially for rockrose kombucha (52%). The highest concentration of glucuronic acid was recorded in St. John's wort kombucha on day 14 of fermentation (1.213 ± 0.027 g/L); in horsetail, green tea, chamomile, nettle, and rockrose beverages, the concentrations were 1.056 ± 0.012, 0.9 ± 0.030, 0.876 ± 0.006, 0.560 ± 0.000, and 0.5 ± 0.006 g/L, respectively. Glucuronic acid was detected in almost all fermented beverages, except linden kombucha. The highest concentration was found in St. John's wort kombucha (1.21 g/L), which is lower than the values reported by Kaewkod et al. [5] for green and black tea kombucha. However, the mere presence of glucuronic acid in most of the fermented herbal drinks analyzed is noteworthy, given the health effects attributed to this acid, including detoxification, par-

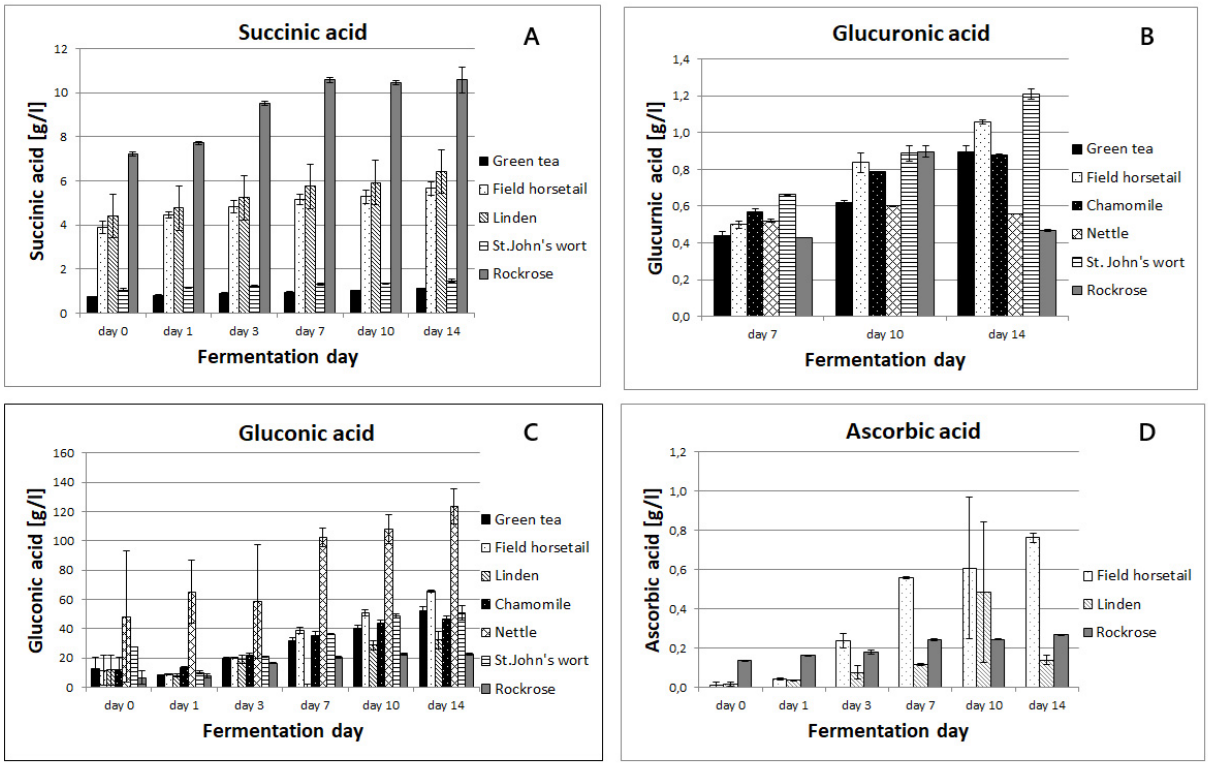


Fig. 4. Concentrations of succinic (A), glucuronic (B), gluconic (C), and ascorbic (D) acids in infusions and the kombucha prepared from them during a 14-day fermentation.

ticipation in the formation of glycosaminoglycans, and involvement in vitamin C synthesis [18].

The highest final concentration of gluconic acid on day 14 of fermentation was found in nettle kombucha (123.348 ± 11.877), followed by horsetail (65.620 ± 0.638), green tea (52.45 ± 2.274), St. John's wort (50.95 ± 4.765), chamomile (46.34 ± 2.660), linden (32.164 ± 5.914), and rockrose kombucha (22.659 ± 5.914). At the end of fermentation, the concentration of gluconic acid was similar in beverages made from green tea, chamomile, and St. John's wort ($p > 0.05$). Similarly, no differences in gluconic acid concentration were observed among linden, rockrose, and chamomile kombuchas ($p > 0.05$; **Supplementary Tables 1,2**; Fig. 4C). Gluconic acid was found at high concentrations (over 120 g/L) in the nettle kombucha samples on the last day of fermentation. These values are more than twice those observed in green tea kombucha. Kaewkod et al. [5] reported lower concentrations of this compound (with the highest value of 70 g/L for black tea kombucha). Therefore, we conclude that nettle infusion is the best substrate for gluconic acid production in a bacterial–fungal consortium fermentation. The presence of this acid may enhance the health benefits of kombucha, as gluconic acid is associated with prebiotic, antimicrobial, and antioxidant properties [28].

Ascorbic acid was present only in kombucha prepared from horsetail, linden, and rockrose, and in infusions of these herbs not inoculated with SCOBY (day 0) (Fig. 4D). In horsetail kombucha, a significant increase in ascorbic acid concentration was observed between days 7 and 14 of fermentation. On day 14 of fermentation, the ascorbic acid concentration was significantly higher in this beverage than in the kombuchas made from rockrose or linden ($p < 0.05$; **Supplementary Tables 1,2**). In the horsetail infusion, the initial ascorbic acid concentration was 0.013 ± 0.011, whereas on day 14 of fermentation, the concentration was 0.763 ± 0.108, an increase of almost 60-fold. Thus, ascorbic acid was found only in beverages based on horsetail, linden, and rockrose. Other authors [5,26] have reported low concentrations of vitamin C in black and green tea kombucha, as well as in fermented beverages based on other plants (fake kinkeliba, lemongrass); however, those values ranged from 0.006 g/L to 6 g/L. In our study, the highest concentration of ascorbic acid was found in horsetail kombucha (0.7 g/L), which is similar to the results reported by Kaewkod et al. [5] for black, green, and oolong teas (0.6–0.7 g/L). We did not detect vitamin C in the fermented drinks based on green tea, chamomile, nettle, or St. John's wort, which is surprising in view of previous reports on, for example, green tea [5,26]. Therefore, it is possible that the concentrations were below the detection limit of the analytical methods used.

To summarize, the observed decrease in pH was associated with intensified acetic acid production and enhanced

Table 1. Number of yeast cells and acetic acid bacteria, and kombucha biomass growth in infusions and kombucha prepared from them during 14 days of fermentation.

	Number of yeast (CFU/mL)				Number of acetic acid bacteria (CFU/mL)				Kombucha biomass growth (g)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Green tea	3450a	± 2100	1350	5550	28,250a	± 16,750	11,500	45,000	65.21a	± 1.47	63.74	66.67
Field horsetail	250a	± 100	150	350	28,000a	± 2500	25,500	30,500	62.60a	± 4.22	58.38	66.82
Linden	28,325b	± 3475	24,850	31,800	41,250ab	± 4250	37,000	45,500	26.52bcd	± 1.22	25.30	27.73
Chamomile	66,250c	± 1700	64,550	67,950	51,500ab	± 21,500	30,000	73,000	30.47c	± 2.47	28.00	32.93
Nettle	37,050b	± 7500	29,550	44,550	68,750bc	± 5250	63,500	74,000	50.76d	± 7.26	43.50	58.02
St. John's wort	4900a	± 2000	2900	6900	102,250c	± 10,250	92,000	112,500	68.89a	± 0.57	68.32	69.46
Rockrose	9300a	± 7000	2300	16,300	96,750c	± 17,250	79,500	114,000	18.82bd	± 3.85	14.97	22.66

CFU, Colony-Forming Unit; Mean, average value; SD, standard deviation; Min, minimum; Max, maximum. Different letters (a, b, c, d) indicate statistically significant differences between mean values within the same column ($p \leq 0.05$). Values sharing at least one letter are not significantly different.

sugar metabolism. However, the unexpected increase in sugar content in certain samples may indicate polysaccharide hydrolysis or reflect variability in analytical measurements. Furthermore, the highest concentrations of most of the analyzed organic acids in the respective kombucha samples were recorded on day 14 of fermentation. This finding suggests a time-dependent accumulation of these compounds and indicates that their production might increase further if the fermentation process were extended.

3.2 Kombucha Biomass Growth

A significantly greater increase in kombucha biomass (g) was observed in green tea (65.21 ± 1.47 g), horsetail (62.60 ± 4.22 g), and St. John's wort (68.89 ± 0.57 g) compared to the other herbs ($p < 0.05$). In contrast, the lowest increases were found in kombucha based on linden (26.52 ± 1.22 g) and rockrose (18.82 ± 3.85 g) (Table 1). In this study, kombucha biomass growth was measured to determine which tea would provide the best medium for bacterial and fungal cultures in terms of nutritional conditions. The best results were obtained for St. John's wort infusion, followed by green tea. Dogan et al. [29] analyzed the mass of the tea mushroom after fermentation of green and black tea, as well as rose and licorice infusions, and found that green tea was the most suitable medium for SCOBY growth. Therefore, based on our results, we can conclude that St. John's wort infusion is a promising source of compounds necessary for kombucha biomass production. This is an interesting result, especially in the context of emerging applications that utilize cellulose produced during beverage fermentation.

3.3 Yeast, Acetic Acid Bacteria, and Lactic Acid Bacteria Counts

A statistically significantly higher number of yeast cells was found in chamomile kombucha (66,250 ± 1700 Colony-Forming Units (CFU)/mL) compared to other fermented beverages ($p < 0.05$). Similar numbers of yeast cells were observed in the nettle and linden kombuchas: 37,050 ± 7500 and 28,325 ± 3475 CFU/mL, respectively ($p > 0.05$). The remaining fermented beverages had lower yeast counts (Table 1). There were no statistically significant differences ($p > 0.05$) in the numbers of acetic acid bacteria among kombuchas based on nettle (68,750 ± 5250 CFU/mL), St. John's wort (102,250 ± 10,250 CFU/mL), and rockrose (96,750 ± 17,250 CFU/mL). The numbers of acetic acid bacteria in these beverages were significantly higher than in green tea kombucha (28,250 ± 16,750 CFU/mL) and horsetail kombucha (28,000 ± 2500 CFU/mL). Lower numbers of acetic acid bacteria were also found in fermented drinks made from linden and chamomile (Table 1). No bacterial colonies were detected on MRS agar in any of the samples analyzed. Interestingly, the mass of the tea mushrooms analyzed was consistent with the number of bacteria counted in plate cultures, with acetic acid bacteria being the most abundant in St. John's wort infusion. In contrast, the highest yeast count was found in chamomile kombucha. The absence of bacterial colonies on MRS agar across all kombucha samples, consistent with the negative HPLC analysis for lactic acid, aligns with the results of Gaggia et al. [1].

3.4 Analysis of the Antimicrobial Activity of Kombucha

The reference strains were susceptible to the antibiotics tested (Table 2) as well as to the different types of kombucha (Table 3). The only exception was Staphylococcus aureus, which showed intermediate susceptibility to levofloxacin. The inhibition zones produced by kombucha were comparable to those obtained in the antibiograms, confirming the antimicrobial activity of each kombucha tested. Additionally, in most cases, lower kombucha pH was associated with higher antimicrobial activity. The only exception was nettle kombucha against the *S. aureus* strain, for which no pH-related differences were found (Table 3). The effects on the tested bacterial strains varied by kombucha type. Green tea kombucha showed the greatest activity against the Gram-positive *S. aureus*, followed by horsetail and St. John's wort kombucha. Chamomile and nettle kombucha produced slightly smaller inhibition zones,

Table 2. Antibiotic resistance of the reference strains was determined using the disk diffusion method in accordance with EUCAST recommendations (version 13.0, effective from January 1, 2023).

Staphylococcus aureus					*Escherichia coli*				
Class of antibiotics	Antibiotic name (symbol, dose)	Zone of inhibition reference range (mm)		Inhibition zone of the tested strain (mm)	Class of antibiotics	Antibiotic name (symbol, dose)	Zone of inhibition reference range (mm)		Inhibition zone of the tested strain (mm)
		S≥	R<				S≥	R<	
Penicillins	Penicillin (P, 1 unit)	26	26	26	Carbapenems	Imipenem (IMP, 10 µg)	22	19	32
Cephalosporins	Cefoxitin (FOX, 30 µg)	22	22	33	Fluoroquinolones	Levofloxacin (LEV, 5 µg)	23	19	33
Fluoroquinolones	Levofloxacin (LEV, 5 µg)	50	22	30	Tetracyclines	Tigecycline (TGC, 15 µg)	18	18	19
Aminoglycosides	Gentamicin (CN, 10 µg)	18	18	20	Penicillins	Piperacillin (PRL, 30 µg)	20	20	27
Macrolides	Erythromycin (E, 15 µg)	21	21	29	Cephalosporins	Cefoxitin (FOX, 30 µg)	19	19	26
Tetracyclines	Tetracycline (TE, 30 µg)	22	22	29	Aminoglycosides	Gentamicin (CN, 10 µg)	17	17	22

Abbreviations: S, sensitive strain; R, resistant strain.

Table 3. Antimicrobial activity of green tea and herbal kombucha beverages.

Microorganism	pH of kombucha	Kombucha type						
		Green tea	Linden	Field horsetail	Chamomile	Nettle	St. John's wort	Rockrose
		Zone of growth inhibition (mm)						
Staphylococcus aureus	pH <7.0	34	25	33	30	30	31	28
	pH 7.0	30	22	30	28	30	28	24
Escherichia coli	pH <7.0	33	31	32	33	31	26	31
	pH 7.0	23	28	30	26	28	22	25

and rockrose and linden kombucha produced the smallest zones (Table 3). The Gram-negative *E. coli* strain was most susceptible to green tea and chamomile kombucha and slightly less susceptible to horsetail kombucha. Kombucha made from linden, nettle, and rockrose showed similar activity, whereas St. John's wort kombucha had the weakest effect on this strain (Table 3).

Analysis of the antimicrobial activity of kombucha produced from green tea and selected herbs showed that the reference strains of *E. coli* and *S. aureus* were sensitive to all applied infusions. Moreover, the low pH of most kombucha samples (with the exception of nettle kombucha) enhanced the antimicrobial activity of the beverages. Our findings are consistent with those of Al-Mohammadi et al. [27], who examined black tea kombucha and reported antimicrobial activity against many bacterial and fungal species, with *E. coli* and *S. aureus* being the most sensitive microorganisms. Additionally, these authors, consistent with our results, reported a stronger antimicrobial effect of low pH kombucha compared with kombucha neutralized to pH 7.0. Our study also showed that kombucha could have different effects on specific strains, which may be related to differences in cell wall structure among the bacterial species analyzed. The antimicrobial properties of herbal kombuchas were also demonstrated by Valiyan et al. [30], who analyzed fermented infusions (including lemon verbena and peppermint). Thus, herbs appear to be a promising alternative to commonly used teas, enhancing the antioxidant properties that develop during fermentation.

3.5 FTIR (Fourier Transform Infrared Spectroscopy) Analysis of Kombucha Samples

Analysis of the spectra of the tested fermented beverages showed increased absorbance across all kombucha samples at 1000 cm^{-1}, with slight differences in peak heights, indicating variations in the number and distribution of –OH functional groups among the samples. Vibrations in the 1000–1100 cm^{-1} region were also prominent, indicating the presence of C–O groups. Changes in the peak heights indicated differences in the amount and/or distribution of aromatic ester groups and primary and secondary alcohols (Fig. 5).

The results of the FTIR analysis are consistent with those reported by Kaashyap et al. [28], who analyzed com-

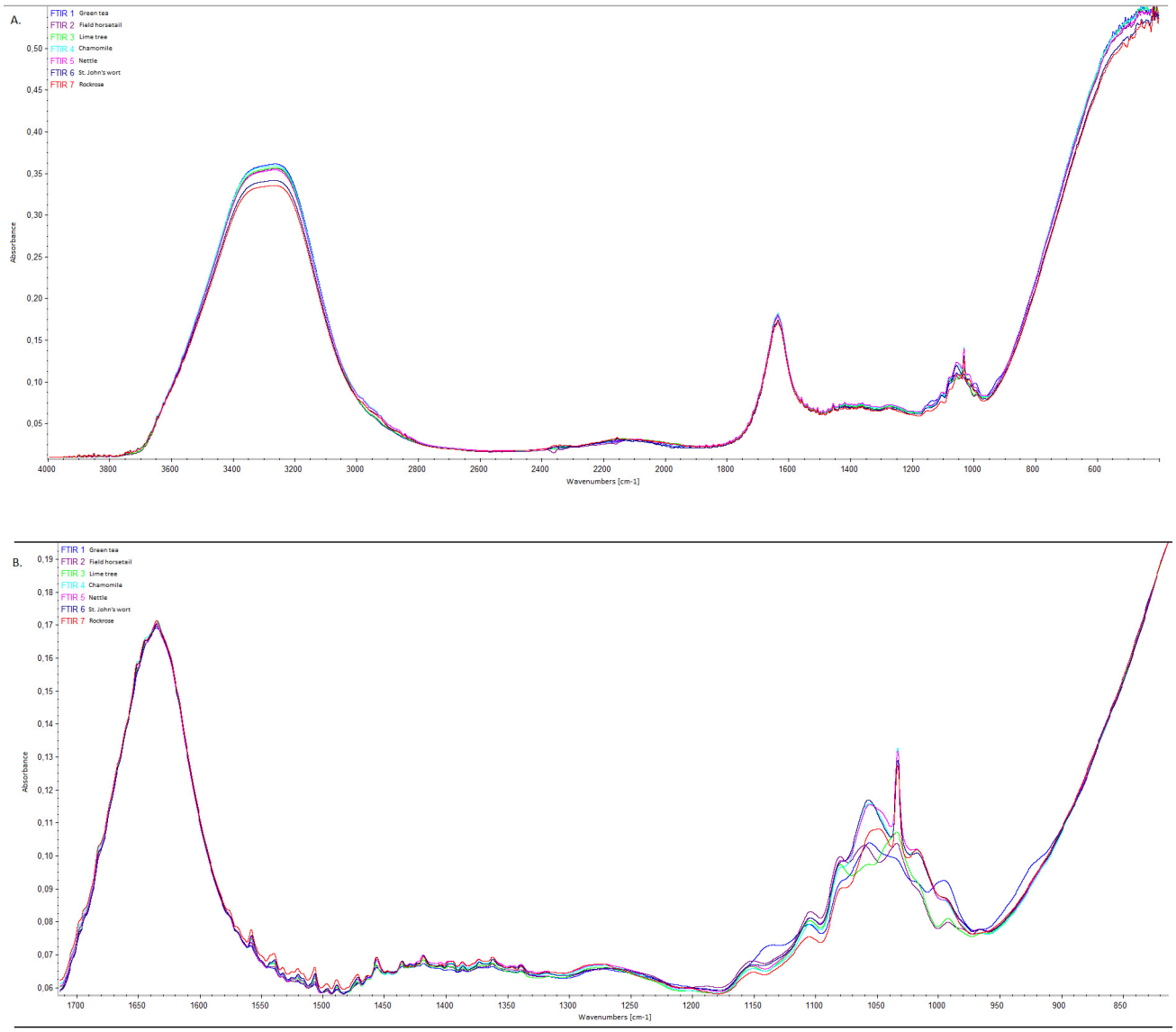


Fig. 5. Fourier transform infrared (FTIR) spectra of green tea kombucha and kombucha prepared from six herbal infusions in the 400–4000 cm^{-1} wavenumber range (A), with an enlarged view of the 800–1700 cm^{-1} range shown in (B).

mercial kombucha produced from a mixture of black and oolong tea. These authors emphasized the increased ester production induced by yeast. Ester groups are responsible for the taste, aroma, and shelf life, as well as the antimicrobial properties of kombucha. Our study showed different peak locations for these groups in herbal kombuchas, suggesting differences in the production of these compounds depending on the substrate used in the fermentation process.

3.6 Antioxidant Activity Assessed by DPPH

The antioxidant activity of kombucha prepared from the analyzed herbs and green tea varied as fermentation progressed (Fig. 6). The highest antioxidant activity in the analyzed beverages was observed on days 7–10 of fermentation. Statistically significantly higher activity was found in horsetail and chamomile kombucha compared with green tea kombucha. Finally, on day 14, the antioxidant activity of kombucha based on green tea and rockrose was statistically lower than that of the other beverages (**Supplementary Tables 1,2**). Collectively, 7–10-day kombuchas based on horsetail, linden, chamomile, nettle, St. John's wort, and rockrose exhibited high antioxidant activity (≥80%).

In our study, the DPPH assay was used to compare the antioxidant properties of the herbal kombuchas with those of the green tea kombucha. The results indicate that, on day 7 of fermentation, *i.e.*, when kombucha is considered a finished product and is usually consumed, the values of this parameter were markedly higher in the herbal kombuchas (>80%) than in the reference green tea kombucha (~60%). Moreover, on days 10 and 14, these values remained high

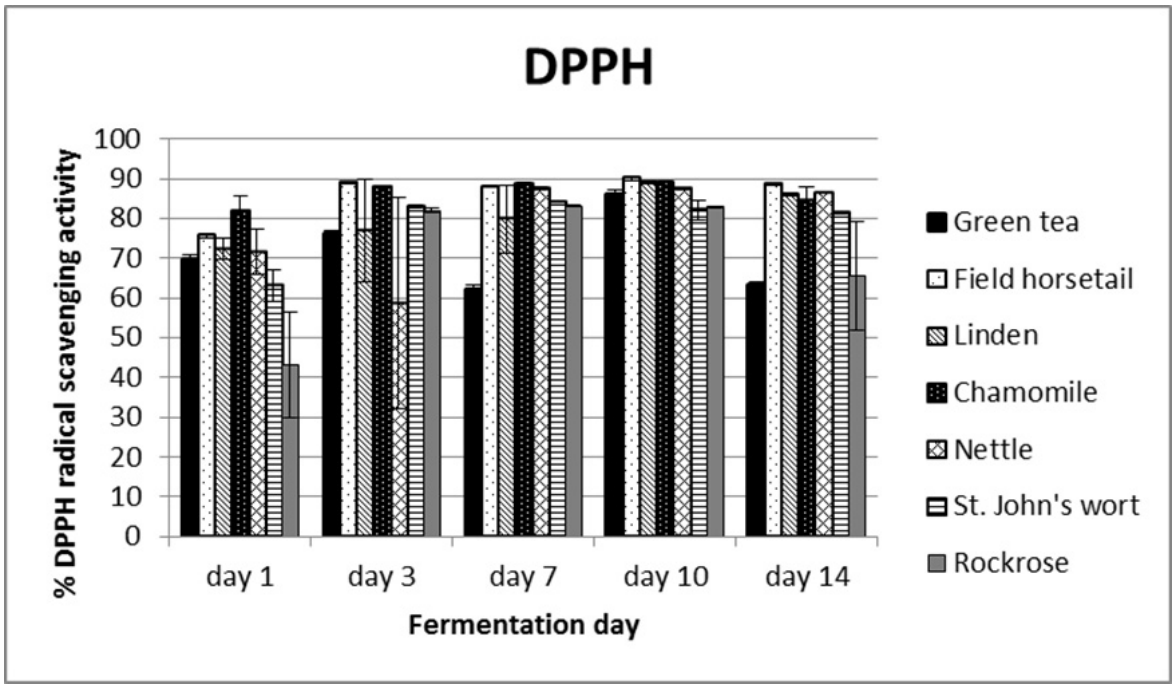


Fig. 6. Antioxidant activity of kombucha prepared from herbs and green tea during a 14-day fermentation.

in the herbal kombuchas, whereas for green tea, the antioxidant activity, after increasing by day 10, decreased significantly by the final day of fermentation (**Supplementary Tables 1,2**). Similar fluctuations in antioxidant activity during green tea fermentation were reported by Vitas et al. [10], who observed an increase on day 3 of the process, followed by a decrease through day 10. However, those authors found that nettle kombucha exhibited increased antioxidant activity up to day 3, followed by a marked decrease through the end of fermentation. In contrast, we observed a decrease on day 3 and an increase on subsequent days, with values remaining at relatively high levels. These differences in results may be attributed to differences in fermentation conditions or in the bacterial and fungal cultures used. Tarhan Kuzu et al. [31] reported that green tea kombucha exhibited higher antioxidant activity than the herbal infusions analyzed (sage and linden). Conversely, in our study, green tea kombucha exhibited high variability in this parameter over time and lower antioxidant activity than the other herbal infusions analyzed. Against this background, it is interesting that the antioxidant activity of the herbal kombuchas increased compared with the initial values (day 1). Therefore, it can be concluded that the kombucha culture used in the analysis increased the antioxidant activity of all tested herbal infusions, with the highest values observed for horsetail kombucha on days 3 and 10 of fermentation.

3.7 Hydroxyl Radical Scavenging Ability

All fermented beverages exhibited a pronounced decrease in hydroxyl radical scavenging ability on day 7 of fermentation, followed by an increase on day 10 (except for nettle kombucha, in which this parameter continued to decrease), and a further decrease on day 14 (excluding green tea kombucha, where this parameter was similar to that on day 10). Rockrose kombucha had the highest hydroxyl radical scavenging ability ($p < 0.05$), especially on days 1 (87.5%), 10 (85%), and 14 (77.5%) of fermentation. For comparison, kombucha prepared from green tea reached its highest free radical scavenging activity on the first day of fermentation (74%), showed a decrease on day 3, followed by a further decline to approximately 48% on day 7 and a subsequent recovery. The ability to scavenge hydroxyl radicals in nettle kombucha decreased significantly as fermentation progressed ($p < 0.05$; **Supplementary Tables 1,2**; Fig. 7).

The highest hydroxyl radical scavenging activity was observed for rockrose in both the infusion and the kombucha on days 1, 10, and 14 of fermentation. Slightly lower values were found in green tea kombucha, while the remaining fermented beverages exhibited even lower activity. Jayabalan et al. [32], focusing on black and green tea, demonstrated, similar to our study, that hydroxyl free radical scavenging varied throughout fermentation, with local decreases and increases. Moreover, the results in Jayabalan et al. [32] did not show a significant correlation with the antioxidant activity determined by the DPPH assay, consistent with the data obtained in the present study. Meanwhile, our study showed that rockrose was the herb with the best free hydroxyl radical-scavenging properties, outperforming green tea. These results are consistent with the data of Gaweł-Bęben et al. [33], who reported high antioxidant activity of rockrose. Enhancing the antioxidant prop-

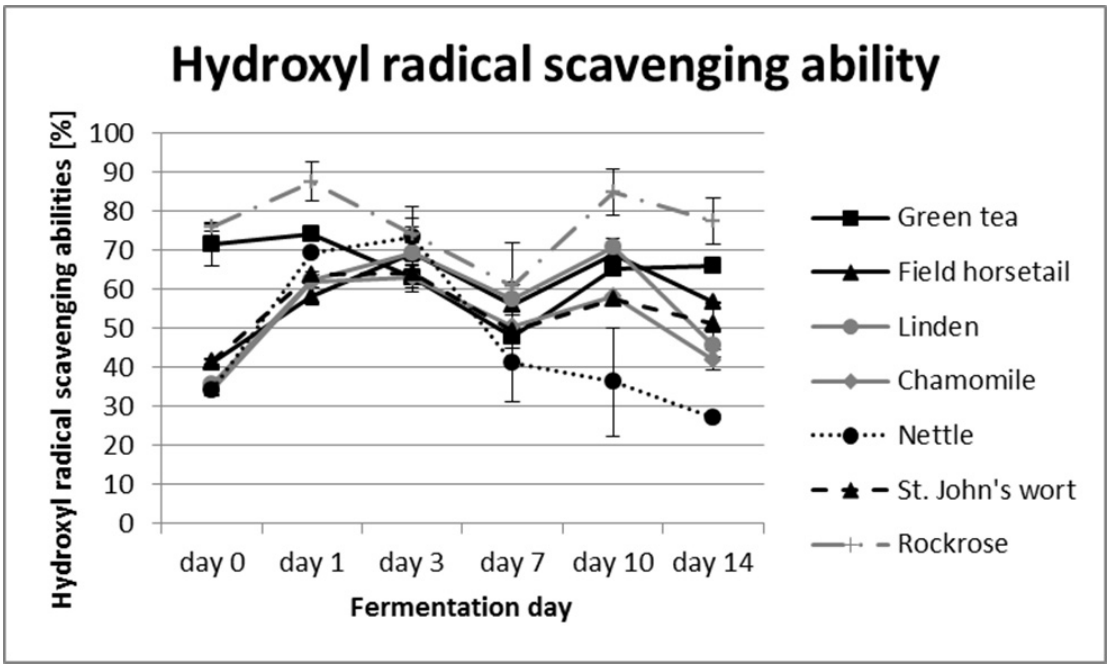


Fig. 7. Hydroxyl radical scavenging activity in infusions and the subsequent kombucha prepared from them during a 14-day fermentation.

erties of rockrose infusion through fermentation would be an interesting modification for use in medicine and cosmetics.

4. Limitations

Despite the broad scope of analyses performed and the numerous significant results obtained, the present study has certain limitations that also indicate directions for further research on fermented herbal beverages. One of the main limitations is that the fermentation process was conducted under laboratory conditions, which do not fully reflect the realities of industrial production, where technological parameters, process scale, and raw material variability may influence the final properties of the product. Another important factor limiting the generalizability of the results is the use of a specific SCOBY culture with a defined microbiological composition. Differences in the structure of microbial communities may significantly affect the course of fermentation, the rate of biochemical transformations, and the resulting metabolic profiles.

A further limitation concerns the selection of plant raw materials. The analysis included selected herb species originating from a specific source, whereas their chemical composition and biological properties may depend on many factors, such as geographical origin, harvest time, cultivation conditions, drying method, and storage conditions. These factors may affect the reproducibility and comparability of the results. Another limitation of the study is the scope of microbiological analyses, which included the assessment of antimicrobial activity against only two microbial strains. This does not allow for a comprehensive determination of the activity spectrum of the tested beverages, particularly against other bacteria, fungi, or clinically relevant microorganisms. Moreover, antioxidant activity was evaluated using *in vitro* methods, which are useful screening tools but do not always reflect actual biological effects after product consumption. Assessment of the bioavailability of bioactive compounds, their metabolism, and their effects on the human body would require studies conducted using more advanced experimental models or involving human participants.

Therefore, further studies addressing the above-mentioned aspects appear fully justified. Meanwhile, the results obtained in the present work constitute a valuable source of knowledge and provide a solid basis for further research on functional fermented beverages based on herbal raw materials.

5. Conclusion

Each of the analyzed herbs proved to be a suitable substrate for producing a fermented beverage containing various compounds with antimicrobial and antioxidant properties. Particularly noteworthy is the rockrose kombucha, which exhibited the strongest hydroxyl radical scavenging (87.5% on day 1 of fermentation) and high antioxidant activity. Moreover, this beverage contained the highest concentration of succinic acid (about 10.6 g/L, on day 7). Another kombucha with high antioxidant and antibacterial activity was the fermented St. John's wort infusion, which, in addition to succinic acid (>1.3 g/L on days 7–14), contained glucuronic acid at the highest concentration (1.2 g/L on day 14). This beverage also exhibited the highest

biomass growth and a high abundance of acetic acid bacteria, again suggesting that these microorganisms, also abundant in rockrose kombucha, may be responsible for the desired characteristics of fermented herbal beverages. Acetic acid bacteria are responsible for the production of many organic acids, including those with antioxidant, antifungal, and antibacterial properties [4,6]. It is also interesting to compare the fermentation of horsetail and nettle infusions. Horsetail kombucha produced the least ethanol and acetic acid; however, it had high antimicrobial activity against *S. aureus* and the highest antioxidant activity (90% on day 10) among the tested herbal kombuchas. Horsetail kombucha also contained a high concentration of gluconic acid (65.6 g/L on day 14) and the highest concentration of vitamin C (0.76 g/L on day 14) among the analyzed samples. Interestingly, this kombucha contained the lowest abundance of acetic acid bacteria. In contrast, nettle kombucha was characterized by the highest concentrations of ethanol (10.7 g/L on day 10) and acetic acid (21.1 g/L on day 14), as well as the highest concentration of gluconic acid (123.3 g/L on day 14) and, thus, high antioxidant activity (87% on days 7–10). Overall, the results suggest that herbal substrates may serve as a novel alternative to traditional tea-based beverages. Moreover, the pronounced differences observed in the metabolic profiles of fermentation clearly indicate the potential for their targeted exploitation, for example, in the production of beverages enriched in specific organic acids. This, in turn, provides a basis for the future design of functional beverages with tailored and desirable biological properties.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author, Joanna Maria Drozdz-Afelt, upon reasonable request.

Author Contributions

JMD-A, BK-P, MM-A and DM contributed to the study conception and design. Material preparation and data collection were performed by JMD-A and BK-P. Analyses were performed by JMD-A, BK-P, MM-A and DM. The first draft of the manuscript was written by JMD-A and BK-P and all authors commented on previous versions of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBE52335.

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